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Anti-inflammatory hydrogel enrichment for cell-based brain repair in Parkinson's disease

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Doctor of Philosophy

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Declaration

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

Calcium signalling recordings were performed by Dr. Niamh Moriarty and calcium signalling data analysis was performed by Dr. Leo Quinlan at National University of Ireland. Unseeded collagen hydrogel SEM images were taken by Ms. Jöelle Bizeau at National University of Ireland.

Signed: …… Date: ……

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Per tu Avi,

per haver-me ensenyat l'altra cara del Parkinson.

For you Grandpa,

For teaching me the other side of Parkinson's.

Abstract

Cell transplantation as a therapeutic approach for Parkinson's disease has been extensively studied over the past decades. The transplantation of primary dopaminergic neurons in the degenerated striatum has shown that these cells can survive the transplantation process, attach into the host tissue, provide dopamine and re-innervate the depleted striatum resulting in functional motor recovery. However, its broad use in the clinical field has been hampered by the poor survival of the transplanted cells. Biomaterials - such as injectable hydrogels - have the potential to address this issue by providing a supportive matrix enriched with neuroprotective growth factors that can preserve the transplanted cells from the traumatic transplantation process and the growth-factor deprived host environment. Nevertheless, cell grafting generates a host glial response – even when cells are encapsulated in a scaffold – that plays a key role in the survival of dopaminergic neuronal grafts. For this reason, the aim of this project was to assess the effects of an anti-inflammatory cytokine loaded collagen hydrogel on the host innate immune response and the long-term survival of primary dopaminergic cell grafts in the parkinsonian brain.

Firstly, several collagen hydrogel compositions were optimised for cell and cytokine encapsulation and intra-striatal delivery through a battery of tests. Through a series of preliminary *in vivo* studies, the best IL-10 loaded collagen hydrogel composition was chosen. Later, we assessed if the encapsulation of primary ventral mesencephalon cells in an IL-10 loaded collagen hydrogel could reduce the host immune response and ultimately improve the dopaminergic cell survival. Based on these results, we evaluated the effects of encapsulating primary ventral mesencephalon cells alongside IL-10 and GDNF in a collagen hydrogel on the dopaminergic cell survival and host immune response.

Collagen hydrogels proved to be biocompatible both *in vitro* and *in vivo*, and they successfully retained and released IL-10 over time in neuronal cultures and in the brain. More importantly, IL-10 was retained to a greater extent in the striatum when encapsulated in a collagen hydrogel 24h post-implantation. Furthermore, in an *in vivo* pilot study, the encapsulation of primary dopaminergic cells in an IL-10 collagen hydrogel reduced the striatal microgliosis at 4 weeks post-transplantation. However, the encapsulation of primary ventral mesencephalic cells in an IL-10 rich collagen hydrogel

did not reduce the host immune response or improve the dopaminergic cell survival of the resulting grafts at longer timepoints. Furthermore, we observed no amelioration of the host immune response nor an increase on cell survival when a multi-modal IL-10 and GDNF rich collagen hydrogel was used.

In conclusion, although functionalised collagen hydrogel scaffolds can be beneficial for the survival of the grafted cells, further studies are required to assess their potential in ameliorating the host immune response and ultimately enhancing the survival of the dopaminergic neurons.

Publications

Peer Reviewed Original Research Manuscripts and Reviews

- **S Cabre**, V Alamilla, R Kelly, E Dowd, (2019). Anti-inflammatory hydrogel enrichment for cell transplantation in Parkinson's disease. *Manuscript in preparation.*

- **S Cabre**, V Alamilla, R Kelly, E Dowd, (2019). The therapeutical potential of IL-10 in Parkinson's disease. *Manuscript in preparation*.

- L.K Olsen, A.G Cairns, J Ådén, N Moriarty, **S Cabre**, VR Alamilla, F Almqvist, E Dowd, D.P McKernan (2019). Viral-like neuroinflammatory priming exacerbates α-synuclein aggregate-induced neuroinflammation, nigrostriatal neurodegeneration and motor dysfunction in rats. *Brain Behav Immun., 2019;80:525-535*. https://doi.org/10.1016/j.bbi.2019.04.036

- N Moriarty**, S Cabre**, V Alamilla, A Pandit, E Dowd (2018). Encapsulation of young donor age dopaminergic grafts in a GDNF-loaded collagen hydrogel further increases their survival, re-innervation and functional efficacy after intra-striatal transplantation in hemi-Parkinsonian rats. *European Journal of Neuroscience, 2018;00:1–10. https://doi.org/10.1111/ejn.14090*

Other Research Dissemination

International Conferences

- **S Cabre**, V Alamilla, R Kelly, N Moriarty, E Dowd. Injectable IL-10 loaded collagen hydrogel for cell transplantation in Parkinson's disease. **Poster Presentation** at British Neuroscience Association (BNA) – Festival of Neuroscience 2019 - Dublin (Ireland), April 14th – 17th 2019.

- V Alamilla, **S Cabre**, N Moriarty, R Kelly, A Pandit, E Dowd. 2019. Assessment of the impact of GDF-5-loaded collagen hydrogels on ventral mesencephalic grafts in a rat model of Parkinson's disease. **Poster Presentation** at British Neuroscience Association (BNA) – Festival of Neuroscience 2019 - Dublin (Ireland), April 14th – 17th 2019.

- R Kelly, **S Cabre**, V Alamilla, A Cairns, J Ådén, F Almqvist, A Bemelmans, E Brouillet, D.P McKernan, E Dowd. Development of a novel rat model of Parkinson's disease induced using AAV-mediated α-synuclein overexpression combined with FN075-mediated α-synuclein aggregation. **Poster Presentation** at British Neuroscience Association (BNA) – Festival of Neuroscience 2019 - Dublin (Ireland), April 14th – 17th 2019

- LK Olsen, AG Cairns, J Ådén, N Moriarty, **S Cabre**, V Alamilla, F Almqvist, E Dowd E, D.P McKernan. Viral mimetic priming enhances α-synuclein-induced degeneration: Implications for Parkinson's disease. **Poster Presentation** at British Neuroscience Association (BNA) – Festival of Neuroscience 2019 - Dublin (Ireland), April 14th – 17th 2019.

- **S Cabre**, V Alamilla, N Moriarty, R Kelly, E Dowd. Potential of an injectable IL-10 rich collagen hydrogel for cell transplantation in Parkinson's disease. **Oral Presentation** at Network for CNS Transplantation & Restoration (NECTAR) 2018 – Paris (France), December 6th – 8th, 2018.

- V Alamilla, **S Cabre**, N Moriarty, R Kelly, A Pandit, E Dowd. Effects of GDF-5-loaded collagen hydrogels after intra-striatal transplantation of ventral mesencephalic cells in a rat model of Parkinson's disease. **Oral Presentation** at Network for CNS Transplantation & Restoration (NECTAR) 2018 – Paris (France), December 6th – 8th, 2018.

- **S Cabre**, V Alamilla, N Moriarty, E Dowd. Intra-striatal delivery of anti-inflammatory cytokines encapsulated in an injectable collagen hydrogel: potential for brain repair in Parkinson's disease. **Poster Presentation** at Federation of Neuroscience Societies (FENS) Forum 2018 – Berlin (Germany), July 7th – 11th 2018.

- V Alamilla, **S Cabre**, N Moriarty, LK Olsen, A Pandit, E Dowd. Utility of ventral mesencephalic tissue explants for assessment of neurotrophin-functionalised biomaterial hydrogels in the context of Parkinson's disease therapeutics. **Poster Presentation** at Federation of Neuroscience Societies (FENS) Forum 2018 – Berlin (Germany), July 7th – 11th 2018.

- LK Olsen, AG Cairns, J Ådén, N Moriarty, **S Cabre**, V Alamilla, F Almqvist, E Dowd, D.P McKernan. Viral mimetic priming enhances α-synuclein-induced degeneration: Implications for Parkinson's disease. **Poster Presentation** at Federation of Neuroscience Societies (FENS) Forum 2018 – Berlin (Germany), July 7th – 11th 2018.

- A Santaella Tortos-Salas, H Wessels, J Gloerich, BH Kuiperij, BR Bloem, AJ Van Goo, **S Cabre**, E Dowd, MM Verbeek. 2018. Proteomic Profiling of Striatal Tissue of a Rat Model of Parkinson's Disease after Implantation of Collagen-Encapsulated Mesenchymal Stem Cells. **Poster Presentation** at Federation of Neuroscience Societies (FENS) Forum 2018 – Berlin (Germany), July 7th – 11th 2018.

- F Gubinelli, **S Cabre**, V Alamilla, E Dowd, E Brouillet, E Flament. An injectable Collagen Hydrogel as a delivery system for Parkinson's disease. **Poster Presentation** at Physiopathology of Parkinson's disease, Lille (France), April 23rd-24th 2018.

- J Samal, N Moriarty, **S Cabre**, V Alamilla, R Saldova, PM Rudd, R O'Flaherty, E Dowd, A Pandit. Tuneable Fibrin-in-Fibrin system for improved survival of dopaminergic progenitors with a focus on spatial characterisation and modulation of brain N-glycans in response to 6-OHDA induced parkinsonism. **Poster Presentation** at Gordon Research Conference for Signal Transduction by Engineered Extracellular Matrices, Andover (United States), July 22nd – 27th 2018.

- LK Olsen, AG Cairns, J Aden, N Moriarty, **S Cabre**, V Alamilla, F Almqvist, E Dowd E, D.P McKernan. A novel Parkinson's disease model: Combined viral mediated neuroinflammation and α-synuclein aggregation. **Oral Presentation** at Network for CNS Transplantation & Restoration (NECTAR) 2017 – Dublin (Ireland), December 6th - 8th.

- LK Olsen, AG Cairns, J Aden, N Moriarty, **S Cabre**, V Alamilla, F Almqvist, E Dowd E, D.P McKernan. Pre-clinical Parkinson's model: Neuroinflammation and α-synuclein aggregation. **Poster Presentation** at British Psychological Society (BPS), Brighton (United Kingdom), May 3rd – 5th 2017.

National Conferences

- **S Cabre**, V Alamilla, R Kelly, N Moriarty, A Pandit, E Dowd. Potential of an injectable IL-10 rich collagen hydrogel for cell transplantation in Parkinson's disease. **Oral Presentation** at Galway Neuroscience Centre – Galway (Ireland), December 14th 2018.

- **S Cabre**, V Alamilla, R Kelly, N Moriarty, A Pandit, E Dowd. Injectable collagen hydrogel for intra-striatal delivery of anti-inflammatory cytokines in Parkinson's disease. **Poster Presentation** at Young Neuroscience Ireland Symposium – Dublin (Ireland), October 25th, 2018.

- V Alamilla, **S Cabre**, N Moriarty, LK Olsen, A Pandit, E Dowd. 2018. Assessment of neurotrophin-functionalised biomaterial hydrogels using ventral mesencephalic tissue explants in context of Parkinson's disease therapeutics. **Poster Presentation** at Young Neuroscience Ireland Symposium – Dublin (Ireland), October 25th, 2018.

- LK Olsen, AG Cairns, J Ådén, N Moriarty, **S Cabre**, V Alamilla, F Almqvist, E Dowd, D.P McKernan. Viral mimetic priming enhances α-synuclein-induced degeneration: Implications for Parkinson's disease. **Poster Presentation** at Young Neuroscience Ireland Symposium – Dublin (Ireland), October 25th, 2018.

- **S Cabre**, V Alamilla, N Moriarty, E Dowd. *In vivo* characterisation of an IL-10 rich collagen hydrogel scaffold for Parkinson's disease. **Poster Presentation** at Neuroscience Ireland Symposium, Galway (Ireland), August 29th, 2017.

- V Alamilla, **S Cabre**, N Moriarty, LK Olsen, A Pandit, E Dowd. Optimisation of a substantia nigra explant system for ex vivo Parkinson's disease modelling. **Poster Presentation** at Neuroscience Ireland Conference, Galway (Ireland), August 29th, 2017.

- LK Olsen, AG Cairns, J Ådén, N Moriarty, **S Cabre**, V Alamilla, F Almqvist, E Dowd E, D.P McKernan. A novel model of Parkinson's disease including viral mediated neuroinflammation. **Poster Presentation** at Neuroscience Ireland Conference, Galway (Ireland), August 29th, 2017.

List of commonly used abbreviations

ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
AP	Anterior-posterior
APCs	Antigen-Presenting Cells
ARTN	Arthemin
ATP	Adenosine triphosphate
BBB	Blood-Brain-Barrier
BDNF	Brain-Derived Neurotropic Factor
CD11b	Integrin alpha M/CR3
CED	Convection Enhanced Delivery
cm	Centimetre
CNS	Central Nervous System
COMT	Catechol-O-methyl-transferase
COX	Cyclooxygenase
DAB	Diaminobenzidine tetrahydrochloride
DAMPs	Damage-Associated Molecular Patterns
DAT	Dopamine transporter
DBS	Deep Brain Stimulation
DV	Dorso-ventral
E	Embryonic day
ECM	Extracellular Matrix
ESCs	Embryonic Stem Cells
FBS	Fetal Bovine Serum
Fig	Figure
fVM	Fetal Ventral Mesencephalon
g	Gram
G’	Storage modulus
G’’	Loss modulus
GABA	Gamma (γ)-aminobutyric acid
GDNF	Glial-Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein

GFRα	GDNF Family Receptor α
GID	Graft-induced dyskinesia
GPe	Globus Pallidus externa
GPi	Globus Pallidus interna
h	Hour
HBSS	Hank's Balanced Salt Solution
hESCs	Human Embryonic Stem Cells
hiPSCs	Human induced Pluripotent Stem Cells
HLA	Human Leukocyte Antigen
HMDS	Hexamethyldisilazane
H_2O_2	Hydrogen peroxide
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IL	Interleukin
IFN	Interferon
iNs	Induced Neurons
i.p	Intraperitoneal
iPSCs	Induced Pluripotent Stem Cells
iNOS	Induced Nitric Oxide Synthase
kg	Kilogram
L-Dopa	Levodopa and l-3,4-dihydroxyphenylalanine
LID	Levodopa-induced dyskinesia
LRRK2	Leucine-rich repeat kinase 2
LPS	Lipopolysaccharide
MAO	Monoamine oxidase
MCP	Monocyte Chemoattractant protein
MDS-UPDRS	Movement Disorder Society - Unified Parkinson's Disease Rating Scale
MFB	Medial Forebrain Bundle
MHC	Major Histocompatibility Complex
ML	Medio-lateral
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSCs	Mesenchymal Stem Cells

MSNs	Medium-Spiny Neurons
µg	Microgram
µl	Microlitre
µm	Micrometre
Mg	Milligram
Min	Minute
Mm	Millimetre
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NCAMs	Neural Cell Adhesion Molecules
Ng	Nanogram
NK	Natural Killer
Nm	Nanometre
NMDA	N-methyl-D-aspartate
NO	Nitrix oxide
NPCs	Neural Progenitor Cells
NRTN	Neurturin
OD	Optical Density
OX-42	Integrin alpha M
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PSCs	Pluripotent Stem Cells
PEG	Polyethylene glycol
PET	Positron Emission Tomography
PFA	Paraformaldehyde
PolyI:C	Polyinosinic:polycytidylic acid
PRRs	Pattern Recognition Receptors
PSPN	Persephin
rAAV	Recombinant Adeno-Associated Virus
rLV	Recombinant Lentivirus
RANTES	Chemokine ligand 5
RPM	Revolutions Per Minute
ROS	Reactive Oxygen Species
S	Seconds

SEM	Scanning Electron Microscopy
SEM	Standard Error of the Mean
SHH	Sonic HedgeHog
SN	Substantia Nigra
SNpc	Substantia Nigra pars compacta
SNpr	Substantia Nigra pars reticulata
STN	Subthalamic nucleus
TBS	Tris Buffered Saline
TGF-β	Transforming Growth Factor β
TH	Tyrosine Hydroxylase
T_H	T Helper
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
T_{REG}	T Regulatory
VTA	Ventral Tegmental Area
VM	Ventral Mesencephalon
4s-StarPEG	Poly(ethylene glycol) ether tetrasuccinimidyl glutarate
6-OHDA	6-hydroxydopamine
°C	Degrees Celcius

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

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CHAPTER 1: GENERAL INTRODUCTION

1.1 OVERALL RATIONALE

More than 200 years ago, James Parkinson described the clinical symptoms of a neurological disease in his "An Essay on the Shaking Palsy" (Parkinson, 1817, re-published in 2002). Later renamed in his name, Parkinson's disease is currently described as a complex motor and non-motor neurodegenerative disease. The cardinal motor symptoms of Parkinson's disease - bradykinesia, resting tremor, rigidity and postural instability - are mainly attributed to the loss of dopaminergic neurons in the nigrostriatal pathway. However, two centuries after James Parkinson's description, the cause of the disease is still unknown and its pathogenesis remains poorly understood (Schapira & Jenner, 2011). Consequently, most of the current treatment strategies for Parkinson's disease target the dopaminergic system. L-dopa (levodopa or 3,4-dihydroxy-L-phenylalanine) is currently the gold standard treatment for Parkinson's disease since it efficiently reduces the motor symptoms, especially in the first stages of the disease. However, L-dopa (and other dopaminomimetic drugs) does not replace dopamine specifically in the degenerated areas nor mimics the endogenous release of dopamine (Barker et al., 2017). As a result, a series of side effects such as dyskinesias[1] arise after prolonged administration. Moreover, L-dopa and the other current pharmacotherapies do not stop or cure the progression of the disease.

For this reason, there is an unmet clinical need for new therapies in Parkinson's disease that can treat, not only the motor symptoms, but modify the course of the disease. With no success in developing such drugs for neurological diseases in the recent decades, regenerative medicine has emerged as a promising new field that aims to heal and repair the diseased brain. Brain repair encompasses multiple approaches for Parkinson's disease like cell transplantation, gene therapy and neurotrophic factor therapy.

Parkinson's disease is an ideal candidate for cell replacement therapies due to the relatively selective loss of dopaminergic neurons in the nigrostriatal pathway. To date, cell replacement therapies in Parkinson's disease have focused on the transplantation

[1] Dyskinesia: impairment of voluntary movements resulting in fragmented motions (Phelps, 1930).

of dopaminergic cells. Many dopaminergic cell sources have been tested both preclinically and clinically, but so far, the transplantation of dopamine neuron-rich fetal ventral mesencephalon (VM) tissue is the one that has compiled most robust data. VM dopaminergic grafts have been shown to survive and re-innervate the striatum post-transplantation while restoring the dopamine levels and consequently dramatically improving motor function, both in animals and patients (Björklund & Stenevi, 1979; Perlow et al., 1979; Lindvall et al., 1994; Brundin et al., 2000b).

Despite long-term motor benefits in some patients, VM dopaminergic cell transplantation faces many limitations - such as the poor survival post-transplantation and the limited availability of tissue - that limit their translation into the clinic (Barker et al., 2013). While new, widely available, stem cell sources are being researched, the poor survival of the transplanted cells remains a major consideration.

Biomaterials – materials that have been engineered to interact with biological systems – have the potential to improve regenerative strategies for Parkinson's disease. Biomaterials are an attractive tool for brain repair due to their high variability and adaptability, such that that they can be tailored to any disease's needs. For instance, biomaterial scaffolds can be used to protect cells during the transplantation process while providing a supportive matrix where cells can attach to, and moreover, they can also be functionalised for the delivery of trophic factors to enhance the survival and re-innervation ability of transplanted cells (Orive et al., 2009). In particular, injectable collagen hydrogels spurred interest in the field of cell transplantation due to their easy administration intra-cranially, high biocompatibility and degradability profiles and collagen's nature of supporting cell adhesion (Parenteau-Bareil et al., 2010; Khan & Khan, 2013).

Injectable collagen hydrogels have the potential to improve the survival of transplanted cells by intervening in multiple steps of the transplantation process where cell death occurs. Thus, collagen scaffolds can provide structural support for cell adhesion before and during transplantation, deliver relevant growth factors in the local microenvironment upon transplantation as well as create a physical barrier between the transplanted cells and the host immune cells (Moriarty et al., 2019b).

While the combination of trophic factors and collagen hydrogels has been investigated for the enhancement of dopaminergic cell survival (Moriarty et al., 2017, 2019a), the

effects of loading collagen hydrogels with anti-inflammatory moieties for this same purpose has not been assessed.

Based on this, the work described in this thesis sought to assess the potential benefits of an injectable, anti-inflammatory loaded collagen hydrogel on the localised host immune response after transplantation, and the effect of this on the survival and integration of dopaminergic cell grafts in Parkinson's disease (**Figure 1.1**). This introductory chapter will provide a brief overview of Parkinson's disease followed by an evidence-based overview of cell replacement therapy in Parkinson's disease, as well as the potential of biomaterial scaffolds to overcome some of the current limitations of cell replacement therapies.

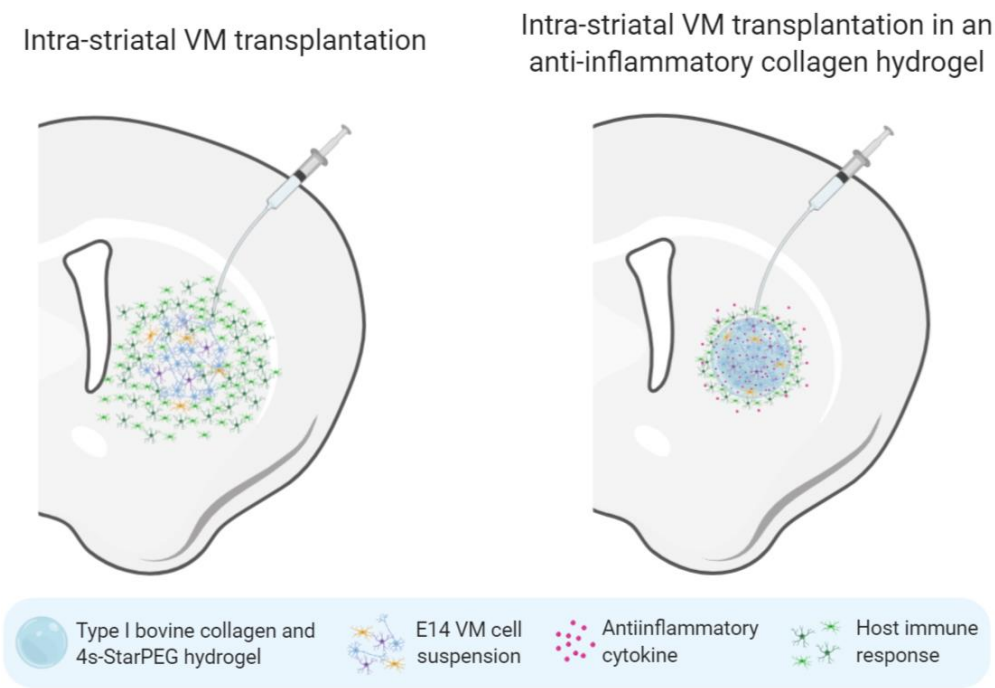

Figure 1.1 Therapeutic concept of an anti-inflammatory loaded biomaterial scaffold for brain repair in Parkinson's disease. The encapsulation and delivery of dopaminergic neurons in an IL-10 loaded collagen hydrogel could potentially improve cell survival by acting on multiple stages of the transplantation process. The encapsulation in a collagen scaffold provides protection and support for cell attachment whilst creating a physical barrier against the host immune cells upon transplantation (Moriarty et al., 2017, 2019a). Moreover, the release of anti-inflammatory cytokines at the implantation site has the potential to reduce the local host immune response and thus, indirectly benefit the viability of the grafted neurons.

1.2 PARKINSON'S DISEASE

Parkinson's disease is a chronic neurodegenerative disorder characterised by the loss of dopaminergic neurons from the substantia nigra and the accumulation of protein inclusions of α-synuclein, known as Lewy bodies. Parkinson's disease is the second most common neurodegenerative disease worldwide after Alzheimer's disease (Dorsey et al., 2007) and its incidence increases with age. The majority of Parkinson's disease cases are sporadic, while only 10% of them are found to have a familial form with genetic predisposition (De Lau & Breteler, 2006). It is believed that mitochondrial dysfunction, oxidative stress and protein misfolding play a key role in the pathogenesis of Parkinson's disease due to an interaction between non-genetic factors and susceptibility genes (Greenamyre & Hastings, 2004).

Parkinson's disease is a complex neurological disorder characterised by both motor and non-motor symptoms. The cardinal motor symptoms include resting tremor, rigidity, bradykinesia and postural instability (Gibb & Lees, 1988). Additionally, other motor symptoms can appear such as akinesia, freezing, speech impairment, shuffling of gait or dystonia. The non-motor symptoms associated with the disease include olfactory deficits, sleep disturbances, depression and anxiety, cognitive impairment and gastrointestinal dysfunction. Although there has been increasing awareness of the non-motor disturbances in Parkinson's disease, they are still not examined in as much detail as the motor symptoms.

The primary neuropathological feature of Parkinson's disease is the progressive degeneration of the dopaminergic neurons in the nigrostriatal system. It is estimated that the disease is clinically manifested when there is approximately 50-80% of dopaminergic neuronal loss, due to the brain's highly efficient compensatory mechanisms (Marsden, 1990; Fearnley & Lees, 1991; Lang & Lozano, 1998). The diagnosis of the disease is purely based on clinical criteria and confirmation of the disease can only be established *post-mortem*. The guidelines for a positive diagnostic of Parkinson's disease according to the UK Parkinson's Disease Society Brain Bank, include 1) the presence of at two of the four cardinal features, whereas one of these has to be bradykinesia or tremor, 2) the absence of several exclusion criteria that suggest an alternate diagnosis and 3) supportive positive criteria such as a response to levodopa or the progression of the disease (Gibb & Lees, 1988). The clinical diagnosis

can be straightforward in typical presentations of Parkinson's disease but, not as common forms of parkinsonism can be challenging to diagnose (Tolosa et al., 2006).

Currently, Parkinson's disease has no cure and the available treatments only address the symptomatology.

1.2.1 Pathophysiology

Since Parkinson's disease has been historically recognised as a motor disorder, the progressive degeneration of the nigrostriatal pathway is well characterised. As well, the formation of intracellular α-synuclein inclusions in the surviving neurons has also been investigated. Recently, substantial evidence suggested that neuroinflammation, mitochondrial dysfunction and oxidative stress play a role in the nigrostriatal degeneration.

1.2.1.1 Nigrostriatal degeneration

The dopaminergic cell loss observed in Parkinson's disease is a progressive neurodegeneration of the dopaminergic cells in the substantia nigra *pars compacta* that extends rostro-caudally to their terminals in the striatum (Damier et al., 1999a, 1999b). This neuronal loss results in an imbalance of dopamine in the striatum which impairs the normal circuitry of the basal ganglia, a system which is directly associated with the modulation of motor control.

1.2.1.1.1 The basal ganglia

The basal ganglia refers to the subcortical nuclei mainly responsible for motor control together with other roles such as motor learning and emotions. These nuclei include the caudate nucleus and putamen (striatum); the globus pallidus *pars interna* (GPi) and the globus pallidus *pars externa* (GPe); the substantia nigra *pars compacta* (SNc) and the substantia nigra *pars reticulata* (SNr); and the subthalamic nucleus (STN) (Lanciego et al., 2012). They can be categorised as; 1) input nuclei, which receive incoming information (caudate and putamen nuclei), 2) output nuclei, which send information to the thalamus (GPi, SNr nuclei) and 3) intrinsic nuclei, which pass on the information between input and output nuclei (GPe and SNc nuclei) (Lanciego et al., 2012).

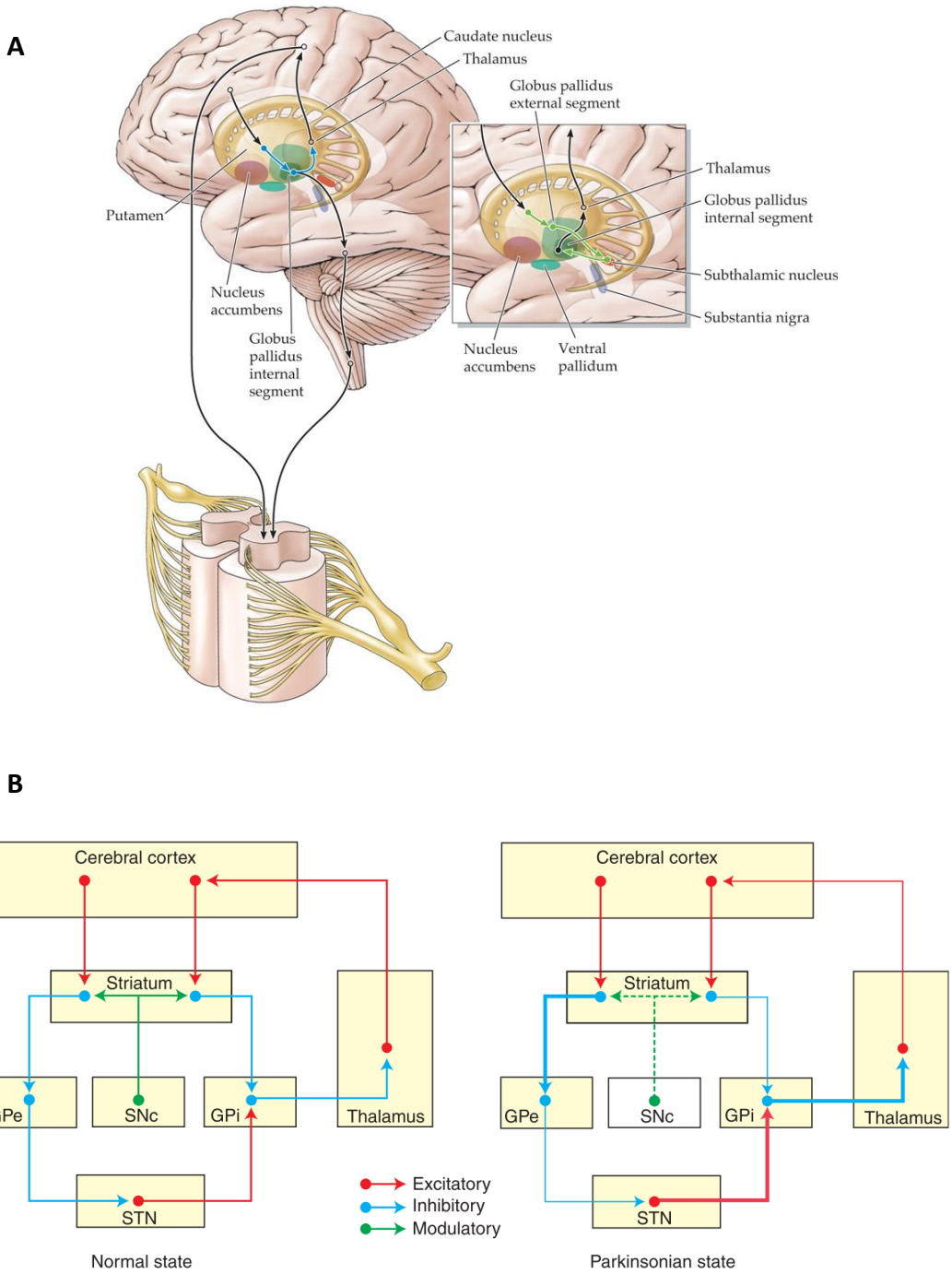

Figure 1.2 Neuroanatomy and circuitry of the basal ganglia. A) Neuroanatomy of the basal ganglia in the human brain. Taken from John H. Martin (2012). B) Nigrostriatal pathway circuitry in normal and parkinsonian conditions. Taken from Lanciego et al., (2012). GPi: Globus Pallidus interna, GPe: Globus Pallidus externa, SNc: Substantia Nigra pars compacta, STN: subthalamic nucleus.

The striatum contains two types of neurons; projection neurons, also known as medium-spiny neurons (MSNs), and interneurons (Kemp & Powell, 1971). Gamma-aminobutyric acid (GABA)-ergic MSNs are the most abundant neuronal type in the striatum (95%) and they express both D1 and D2 dopamine receptors (Gerfen et al., 1990).

The classic basal ganglia model is formed by the direct and the indirect pathway, which have opposite functional effects on the basal ganglia outputs (DeLong, 1990).

The direct pathway is an excitatory pathway that gets activated when dopamine interacts on the D1 receptors of the MSNs of the striatum. This output in the striatum results in an inhibition of the GPi and SNr. At the same time, this inhibitions blocks the thalamic inhibition from the GPi and SNr, increasing the thalamocortical activity and facilitating movement.

On the other side, the indirect pathway is an inhibitory pathway where dopamine activates D2 receptors on the GABAergic MSNs of the striatum and this results in the inhibition of GPe. Thus, there is an increase in the inhibitory actions of the GPe on the STN, resulting in activation of the GPi, which eventually ends in thalamic inhibition and consequently inhibiting movement (Lewis et al., 2003). Normal motor control requires a balance between both pathways (**Figure 1.2**).

In Parkinson's disease, there are depleted levels of dopamine (due to neuronal death), which results in a decreased activation of the direct pathway and increased activation of the indirect pathway (**Figure 1.2**). This imbalance in both pathways is followed by an over-activation of the GPi, an excessive thalamic inhibition, an under-activation of the cerebral cortex and ultimately a reduction in movement (DeLong, 1990; Obeso et al., 2000).

1.2.1.1.2 Dopaminergic cell loss

The main pathological feature of Parkinson's disease is the progressive loss of dopaminergic neurons in the substantia nigra. The neuronal death in the basal ganglia starts many years before the onset of motor symptoms and once they become apparent, more than half of the dopaminergic neurons are lost. This neuronal loss is not homogeneous as the ventral part of the substantia nigra seems to be more susceptible to degeneration (Damier et al., 1993, 1999b). Although dopaminergic neurons are the

main degenerating neurons, other neuronal cell types such as catecholaminergic and serotoninergic neurons are also affected (Lang & Lozano, 1998).

By the time motor symptoms appear, there is an 80% lower levels of dopamine in the striatum and 50% of the nigral neurons have been lost (Fearnley & Lees, 1991). This extraordinary compensatory ability of the dopaminergic system is attributed to an upregulation of dopamine release, a reduction of the dopamine uptake by its receptors and D2 receptor expression changes.

1.2.1.2 Lewy bodies and alpha-synuclein

A major hallmark of Parkinson's disease is the pathology linked to Lewy bodies. Lewy bodies, first discovered by Frederick Lewy in 1912, are proteinaceous eosinophilic inclusions in the surviving neurons of parkinsonian brains (Lewy, 1912). α-synuclein was identified as the main component of Lewy bodies along with ubiquitin and ubiquitinated proteins (Spillantini et al., 1997, 1998). These findings were supported with the finding that mutations in its gene *SNCA*, cause a familial form of the disease (Polymeropoulos et al., 1997). α-synuclein is present in the healthy brain, although its function is not well understood. In the Lewy pathology, misfolded α-synuclein becomes insoluble and forms intracellular aggregates - Lewy bodies - both in neurons bodies and neurites (Goedert et al., 2013).

Although it was known that Lewy bodies were found outside the substantia nigra in patients with Parkinson's disease, it was not until Braak's Parkinson's staging scheme that the temporal sequence was established (Braak et al., 2003). Braak and colleagues proposed six stages of the disease based on the spreading pattern of α-synuclein, suggesting that the disease started in the enteric system and the olfactory bulb, ascending caudo-rostrally to the lower brainstem and later to midbrain and neocortex (Braak et al., 2003). In early stages, before any motor symptoms are apparent, α-synuclein is found outside the nigrostriatal pathway, in locations such as the enteric nervous system and the olfactory bulb. In stage 3, α-synuclein is found in the substantia nigra and motor dysfunction appears. In later stages, the motor symptoms are severe with the appearance of cognitive impairment, which correlates with few surviving cells in the substantia nigra and α-synuclein in the neocortex (Braak et al., 2003; Halliday & McCann, 2010). All this evidence highlights the importance of abnormal α-synuclein in the progression of Parkinson's disease.

1.2.1.3 Neuroinflammation

Neuroinflammation was first linked to the pathogenesis of Parkinson's disease when the presence of activated microglia was detected in the substantia nigra pars compacta of a parkinsonian patient (Hirsch et al., 1987). Since then, many studies support the presence of neuroinflammation in Parkinson's disease pathogenesis in the form of activated microglia and increased levels of pro-inflammatory mediators. In Parkinson's disease there appears to be feedback loop cycle of neurodegeneration and neuroinflammation; dying neurons release factors that activate microglia and activated microglia release factors that kill neurons (Block et al., 2007).

1.2.1.4 Mitochondrial dysfunction and oxidative stress

Increasing evidence indicates that mitochondrial dysfunction and the consequent oxidative stress contribute to the pathogenesis of Parkinson's disease, although the specific mechanisms are not well understood. In particular, mitochondrial complex I activity deficits are found in brains from Parkinson's patients (Schapira et al., 1990). In line with these findings, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone, which are complex I inhibitors, can induce the irreversible degeneration of the dopaminergic nigrostriatal pathway in rodents. As well, Parkinson's disease-linked genes such as *PINK1* and *Parkin* are also related to mitochondrial maintenance and dysfunction (Gegg et al., 2009; Grünewald et al., 2010). The increased amount of mitochondria in neurons makes them more susceptible to mitochondrial dysfunction, which increases the production of reactive oxygen species (ROS). Furthermore, the oxidation of dopamine generates ROS, which can be neurotoxic (Hastings, 2009).

1.2.2 Etiology

The cause of Parkinson's disease is still unknown but the etiology of the disease is now widely seen as multifactorial; with ageing, genetic and environmental factors playing key roles in its manifestation.

1.2.2.1 Age

Parkinson's disease is the most common movement disorder and the second most common degenerative disease after Alzheimer's disease (Dorsey et al., 2007; Kalia &

Lang, 2015). The biggest risk factor for Parkinson's disease is aging as the incidence of the disease increases with age. In industrialised countries, the prevalence is estimated at 0.3% when considering the entire population and about 1% in people over 60 years old and it increases to 4% in populations over 85 years of age (Nussbaum & Ellis, 2003; De Lau & Breteler, 2006).

Although the mechanisms linking ageing and Parkinson's disease are not well understood, it has been observed that neuronal cells in the substantia nigra are particularly susceptible to the natural ageing process. This anatomical pattern correlated with the disease manifestations.

Gender is another risk factor since the male-to-female ratio is approximately 3:2 (De Lau & Breteler, 2006; Picillo et al., 2017). While some studies suggest a protective effect of oestrogen, it may also be due to differential environment exposures (since men are more likely to be exposed to occupational toxins in mine work or farming).

1.2.2.2 Environment

Numerous epidemiological studies have identified many environmental factors associated with the development of Parkinson's disease pathogenesis. The accidental intravenous injection of drugs contaminated with MPTP in 1983, resulted in several people developing typical signs of Parkinson's disease and the subsequent finding that MPTP selectively damages the dopaminergic neurons in the substantia nigra (Langston et al., 1983). Since then, many epidemiological studies have assessed the links between exposure to environmental toxins and the disease. Several meta-analyses and epidemiological studies have shown an increased risk of Parkinson's disease associated with pesticide and herbicide exposure (Van der Mark et al., 2012). Exposure to two pesticides, rotenone and paraquat, were associated with Parkinson's disease with an odds ratio of 2.5 (Tanner et al., 2011). Interestingly, these pesticides are selective mitochondrial complex-I inhibitors.

A number of studies conducted in the 1990s worldwide described the increased risk of Parkinson's due to lifestyle choices like rural living, farming and well-water drinking (Lai et al., 2002). Similarly, exposure to heavy metals - such as iron, manganese, lead, copper, mercury or zinc - have also been hypothesised to increase

the risk of the disease as the accumulation of heavy metals in the substantia nigra results in increased oxidative stress and disruptive neurotransmission (Lai et al., 2002).

Also, the observation that encephalitis lethargica often preceded Parkinson's disease during the pandemic of the early 1900s, led to propose that many viral infections could lead to parkinsonism such as HIV, Coxsackie, Herpes simplex, measles and influenza (Lai et al., 2002).

Between the environmental risk factors for Parkinson's disease, smoking is one of the most studied, and many studies have reported a significant inverse association between cigarette smoking and Parkinson's disease (Grandinetti et al., 1994; Hernán et al., 2001). Although many factors could contribute to bias in these association studies, the amount of studies reporting this inverse correlation suggests a consistent methodology. The biological basis under this association is not known but some investigations suggest that the neuroprotective effect of smoking could rely on nicotine, a modulator of dopamine release (Quik, 2004). Coffee consumption has also been linked with a decreased risk of developing Parkinson's disease and it has been suggested that caffeine may improve the motor aspects of the disease (Ross et al., 2000; Ascherio et al., 2001; Hernán et al., 2001). Recently, β2-adrenorceptor agonists - such as the asthma medication, salbutamol - were associated with a reduced risk of developing Parkinson's disease and conversely, the β2-adrenorceptor antagonist, propanolol correlated with an increased risk (Mittal et al., 2017).

Other miscellaneous factors investigated in relation to Parkinson's disease include dietary factors, oestrogens and head trauma.

1.2.2.3 Genetic predisposition

Although Parkinson's disease is predominantly an idiopathic disorder, a minority of cases (5-10%) are familial forms of the disease with a genetic predisposition, and of these, about 5% follow a mendelian inheritance (Lesage & Brice, 2009). To date, 23 loci and 19 disease-causing genes have been identified, of which 10 are autosomal dominant and 9 are autosomal recessive genes (Deng et al., 2018). However, in only 6 of those genes, there is conclusive evidence pointing out to be hereditable monogenic Parkinson's disease; *SNCA (PARK1)* and *LRRK2 (PARK8)* account for autosomal dominant forms of Parkinson's disease, while *Parkin (PARK2)*, *PINK1 (PARK6)*, *DJ-*

1 (PARK7) and *ATP13A2 (PARK9)* are responsible for the autosomal recessive forms of the disease. While some familial forms of the disease are indistinguishable from idiopathic Parkinson's, others are known to have atypical features such as young onset, dystonia or early dementia.

1.2.3 Current Pharmacological Treatments

Despite the advances in the understanding of the pathogenesis and etiology of Parkinson's disease, a cure is still not available. The current treatments for Parkinson's disease treat only the symptoms but not the underlying degeneration. As a consequence, although the treatments provide some symptomatic relief, the disease progresses over time and eventually, the quality of life decays significantly. The treatments for Parkinson's disease focus mainly on restoring dopamine in the brain but they do not ameliorate the progression of the disease nor try to repair the diseased brain.

1.2.3.1 Levodopa

Since its discovery, L-dopa has become the gold standard treatment for Parkinson's disease. In 1957, Carlsson and his colleagues (1957) published their experiments showing that this natural dopamine precursor could reverse a rodent model of muscle rigidity. Later in 1961, Hornykiewicz suggested the use of L-dopa for Parkinson's disease and Cotzias optimised the dosage of L-dopa to be effective in the brain (Fahn, 2008). L-Dopa reduces very efficiently the motor disturbances in patients, specifically, in the early stages of the disease, improving the patients' mobility dramatically (Fahn, 2008). Currently, levodopa is administered in combination with carbidopa, which inhibits DOPA decarboxylase and increases the availability of dopamine. However, the disease progression hampers the efficacy of L-dopa, as it becomes harder to manage therapeutic doses and consequently fluctuations appear (also known as the "on-off" states). Furthermore, the chronic administration of L-dopa results in the appearance of dyskinesias which are the main dose-limiting adverse effect (Tanner, 2000).

1.2.3.2 Dopamine agonists

Dopamine agonists - which mimic dopamine action - act on the intact post-synaptic dopamine receptors in the striatum. Dopamine agonists are classified as ergoline (such as cabergoline, bromocriptine) and non-ergoline agonists (such as pramipexole, ropinirole and rotigotine) and they all target the D_2-receptors (Brooks, 2000). Ergoline agonists are now rarely used since they are linked to cardiovascular abnormalities (Antonini & Poewe, 2007). Although not as effective as L-dopa in reducing the motor impairments, their use is associated with a lower risk of dyskinesias and thus, they are recommended as first line therapy for young patients with mild motor symptoms (Tanner, 2000). Furthermore, non-selective dopamine agonists like apomorphine are used to manage patients with major on-off fluctuations (Stibe et al., 1988; Manson et al., 2002).

1.2.3.3 MAO/COMT Inhibitors

Dopamine is metabolised by the enzymes monoamine oxidase (MAO), catechol-O-methyl transferase (COMT) and aldehyde dehydrogenase (ALDH). Thus, the use of inhibitors of these enzymes as pharmacotherapies for Parkinson's disease reduces the breakdown of dopamine and subsequently prolongs its availability in the striatum. Selective irreversible MAO-B inhibitors, selegiline and rasagiline, are used in monotherapy or as adjunctive therapy to levodopa to reduce the doses of L-dopa needed to provide symptomatic relief (Riederer & Laux, 2011). The COMT inhibitor, entacapone, is not effective as monotherapy but it is used in combination with levodopa to address levodopa-induced motor fluctuations (Brooks, 2008). However, these drugs have many adverse effects such as dyskinesias, dystonia, headaches and gastrointestinal problems (DeMaagd & Philip, 2015).

1.2.3.4 Non-dopaminergic treatments

Anticholinergics were used to treat Parkinson's disease before the introduction of levodopa. Anticholinergic treatment is still used in young patients with persistent tremors, although their use is very limited due to important side effects (DeMaagd & Philip, 2015).

Amantadine, an antiviral agent, is used in Parkinson's disease as a co-adjuvant therapy and it is believed that its action is mediated by a blockade of N-methyl-D-aspartate (NMDA) receptors (Schwab et al., 1969).

1.2.3.5 Deep Brain Stimulation

Alternative non-pharmacological strategies are also available for Parkinson's disease. Deep Brain Stimulation (DBS) is a neurosurgical approach to deliver electrical pulses to the brain through the implantation of an electrode. The electrodes are placed in the STN and the GPi and are thought to alleviate motor symptoms such as tremor, bradykinesia and rigidity through the modulation of glutamate and adenosine (Lee et al., 2011). DBS is the treatment of choice when the symptomatic relief cannot be controlled by dopaminergic therapy. However, the treatment implies a highly invasive procedure, incompatible with many comorbidities like dementia or atypical parkinsonism (DeMaagd & Philip, 2015).

1.2.3.6 Limitations of current treatments

Despite the efficacious symptomatic relief provided by levodopa, its chronic administration is hindered by disabling adverse effects such as motor fluctuations and dyskinesias (Brooks, 2008). Furthermore, these adverse reactions can occur as early as 6 months after the treatment initiation and can result in disabilities with high impact on the quality of life of the patient (Fahn & The Parkinson Study Group, 2005). Thus, levodopa and the other treatments for Parkinson's disease are associated with multiple side effects.

Moreover, current therapeutical strategies only provide symptomatic relief but they do not slow or stop the progression of the neurodegenerative disease. Therefore, the development of an effective, disease-modifying therapy remains a major need for the clinical treatment of Parkinson´s disease.

1.3 CELL REPLACEMENT THERAPIES FOR PARKINSON'S DISEASE

1.3.1 Rationale for cell replacement therapy

The absence of a curative or ameliorating therapy that targets directly the neurodegeneration of the nigrostriatal pathway, and the repeated failure of developing new pharmacological treatments that treat more than the motor symptoms, has led the scientific community to explore new approaches to try and heal the parkinsonian brain. As a result, there has been an interest in targeting the degeneration in the nigrostriatal pathway by using several non-pharmacological approaches like gene therapy or cell-based therapies.

It is the localised and relative selective loss of dopamine-producing neurons that makes Parkinson's disease the ideal candidate for cell transplantation. Yet, the rationale for cell replacement therapy relies on a very elementary concept; to replace those dying dopaminergic neurons with healthy and functional ones that will integrate and restore dopamine levels in the nigrostriatal system. Although the theoretical framework is simple, their practical application rises several complications like poor cell survival and a persistent immune response.

Nevertheless, cell transplantation has the potential to restore the nigrostriatal pathway, thus delaying the progression of the disease and offering patients an alternative to pharmacological treatments.

1.3.2 Historical Perspective

Cell transplantation holds great potential as an alternative therapy for Parkinson's disease that could ameliorate the motor symptoms while restoring dopamine levels in the striatum. Since its early conception, cell transplantation approaches have studied several dopamine rich cell sources (including fetal tissue or cells, embryonic or induced pluripotent stem cells) as well as the multiple implications of cell grafting, both in preclinical and clinical studies that will be covered extensively below. From the pioneering preclinical studies in the 1980s to the most recent launch of stem cell-based clinical trials, the cell transplantation field in Parkinson's disease has not stopped moving since its formation and has evolved with each barrier that it has come across (**Figure 1.3**).

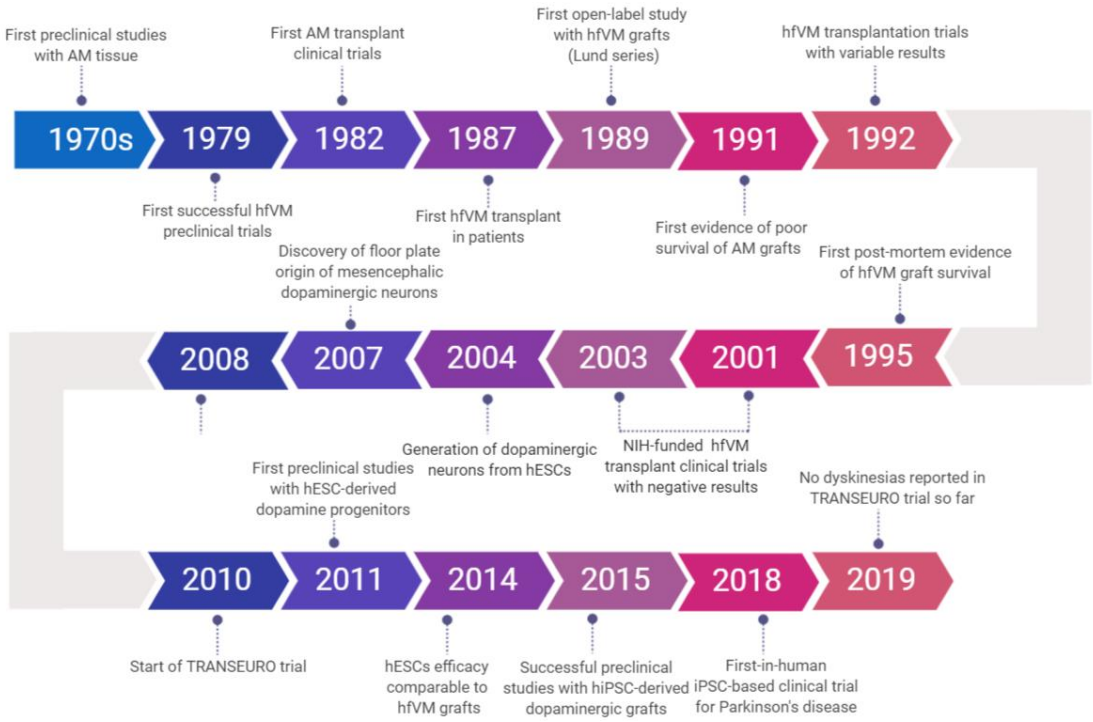

Figure 1.3 Timeline of cell-based therapy progression for Parkinson's disease. AM: Adrenal medullary tissue, hfVM: human fetal ventral mesencephalic tissue, hESCs: human embryonic stem cells, hiPSCs: human induced pluripotent stem cells.

1.3.2.1 The 1970s and 1980s - The beginnings of neural grafting in Parkinson's disease

In 1979, for the first time, two laboratories published that mesencephalic dopamine rich fetal nigral tissue transplanted into the depleted brain of parkinsonian rats were able to survive, integrate and re-innervate the surrounding area, while improving the animals' functional deficits (Björklund & Stenevi, 1979; Perlow et al., 1979). These findings became proof-of-concept that cell transplantation could reverse to a certain degree, the motor manifestations of Parkinson's disease. It has to be noted that cell transplantation was never thought as a curative therapy but an alternative method to restore the dopamine in the brain while avoiding the debilitating side-effects of dopaminomimetic drugs. Furthermore, these findings signified the possibility of a novel therapeutic approach that would treat one of the key pathological features of the disease instead of only treating the obvious motor symptoms.

These early preclinical studies opened a new broad research field to try to identify the best cell type for transplantation in Parkinson's disease. This area of research took

many directions; multiple cell types were studied, from autografts to xenografts and from fetal to adult sources, but all with a common dopamine rich background. In the 1980s, by using the recently developed the 6-hydroxydopamine (6-OHDA) model and the drug-induced rotational behaviour test described by Ungerstedt & Arbuthnott (1970), the efficacy of grafting dopaminergic cell sources was investigated by exploring different cell sources and transplant sites. The 6-OHDA-lesioned rat model of Parkinson's disease, although it does not fully represent the disease, it selectively and irreversibly lesions the nigrostriatal pathway. Hence, it is an invaluable tool to assess the ability of potential therapies to restore the dopaminergic tone in the nigrostriatal system. In parallel, the use of rotational behaviour model allows researchers to monitor any changes in the nigrostriatal dopaminergic function and as a result, both procedures were widely adopted in cell transplantation research.

1.3.2.1.1 Dopaminergic cell sources

Adrenal medullary tissue was explored during the 1980s as an alternative catecholamine-producing cell source to the fetal dopaminergic tissue. Adrenal medullary tissue could alleviate the rotational behaviour of unilateral dopaminergic denervation when implanted in the lateral ventricle close to the caudate nucleus in parkinsonian rats and monkeys (Freed et al., 1981; Morihisa et al., 1984). However, the number of long-term surviving chromaffin cells was very low in these preclinical studies (Morihisa et al., 1984; Bing et al., 1988).

At the same time, solid tissue of fetal ventral mesencephalon (fVM) tissue was grafted in several locations of the nigrostriatal pathway such as the caudate nucleus (Perlow et al., 1979; Freed et al., 1980) and the neostriatum (Björklund et al., 1980a; Björklund et al., 1981) and all studies found the grafting to be successful and reduce the symptomatology of dopaminergic denervation. Functional improvement was also observed when fVM tissue was implanted in the striatum of non-human primates in a MPTP degeneration model (Redmond et al., 1986). Nevertheless, solid pieces of embryonic tissue showed poor survival in the adult rat parenchyma due to low levels of vascularity (Stenevi et al., 1976). For this reason, a dissociated cell suspension from the substantia nigra tissue was developed, leading to a more widespread re-innervation area and the subsequent improvement in motor function (Gage et al., 1983). This technique was adopted later in the clinic.

1.3.2.1.2 Open-label clinical trials

Despite the promising data revealed by the preclinical studies in fetal VM tissue and the limited data on the adrenal medullary tissue, the first small open-label clinical transplant studies were conducted using a variety of non-VM tissues such as autografts of adrenal medulla (Olson & Malmfors, 1970).

In 1982, two patients were transplanted with adrenal medullary tissue in the striatum in Lund, Sweden with poor long-term benefits (Backlund et al., 1985). However, in Mexico, Madrazo et al., (1987) published preliminary data showing a marked improvement of parkinsonian symptomatology in two young patients after receiving an autograft of adrenal medullary tissue in the caudate nucleus. This publication resulted in many patients being treated with adrenal medullary tissue around the world although the *post-mortem* analysis showed very poor graft survival (Kordower et al., 1991). Consequently, many patients did not benefit from any therapeutic effect but also many experienced complications after surgery (Barker et al., 2015). This fact highlights the importance of a careful and critical analysis of all the preclinical data available before assessing a therapy in clinical trials.

In contrast to adrenal medullary tissue, fVM grafts did have a substantial amount of preclinical data supporting their potential benefits. The first open-label clinical trial involving fVM transplants was initiated in Lund, Sweden in 1987. The clinical benefits of these first transplants were heterogeneous, most likely due to changes in the tissue processing (amount of tissue implanted, age of fetal tissue and mode of delivery) (Barker et al., 2015).

1.3.2.2 1990s - Fetal ventral mesencephalic grafts

An extension of the fVM clinical trial – known as the Lund study – was carried out during the 1990s with standardised protocols and overall, patients showed clinical improvements after transplantation (Lindvall et al., 1994; Wenning et al., 1997; Brundin et al., 2000b). The clinical improvements were reinforced with positron emission tomography imaging data, which reported increased levels of [^{18}F]-fluorodopa uptake in the grafted putamen, thus proving the restoration of dopamine in the striatum (Lindvall et al., 1994; Wenning et al., 1997; Brundin et al., 2000b). A long-term follow up of these patients revealed that up to four patients from this cohort

were still experiencing a favourable clinical outcome 10 or more years post-transplantation - as stated with ^{18}F-DOPA PET scans - and were able to come off their dopaminergic medications (Piccini et al., 1999; Politis et al., 2011; Kefalopoulou et al., 2014). When fVM grafts were analysed in detail *post-mortem*, they were found to have extensively re-innervated the grafted area and even survived for up to 24 years (Li et al., 2016). At the same time, *post-mortem* analysis also reported a decline of dopaminergic function over time and the formation of Lewy bodies in the grafted cells after 14 years post-transplantation (Chu & Kordower, 2010).

This clinical trial in Lund led to a number of other similar studies around the world with variable outcomes. Once again, this could most likely be explained by the differences in patient selection, surgery logistics and clinical trial endpoints (Barker et al., 2013).

1.3.2.3 2000s – Double-blind placebo-controlled clinical trials

In the early 2000s, the field experienced a setback when the results of two NIH funded double-blind placebo-controlled clinical trials were published about the efficacy and safety of fVM transplantation. These studies – known as Colorado/Columbia and Tampa/Mount Sinai – reported no functional benefits in the transplanted patients when compared to controls, and also, reported for the first time, the appearance of side effects, a non-pharmacological dyskinesia (Freed et al., 2001; Olanow et al., 2003).

These graft-induced dyskinesias (GID) were firstly reported in the Colorado/Columbia study and they were described as different in nature to the L-dopa-induced dyskinesia (LID) since they were more dystonic and rhythmic and not related to the drug (Freed et al., 2001). Apart from being very common in these clinical trials, GIDs were in some cases very severe and needed further treatment with DBS (Cho et al., 2005). However, this could not be the solution to deal with GIDs, and many efforts were made to elucidate the origins and mechanism of GIDs. Thus, clinical data from all the previous clinical trials was analysed to find an explanation for this side effect but the huge variability between trials made it impossible to find a common cause of GIDs (Lane et al., 2010). Interestingly, that in this review of the data accumulated over the years, it was discovered that GIDs occurred in a subset of patients of the preliminary studies from Lund as well (Hagell et al., 2002).

Lane et al., (2010) classified the potential key factors that might play a major role in the development of GIDs as those related to the patients (i.e selection of patients, immunosuppression) or the transplantation procedure (i.e preparation, storage and surgical delivery of tissue) (**Figure 1.4**). In regards to the transplantation procedure, Freed et al., (2001) and others, hypothesised that the presence of GIDs could be explained by an excess of dopamine in the striatum or the presence of dopamine "hotspots" that led to an heterogeneous distribution of the neurotransmitter. However, the ^{18}F-DOPA PET scans of patients from the Lund series did not confirm this hypothesis (Hagell et al., 2002; Piccini et al., 2005). Besides, Soderstrom et al., (2008) determined that an elevated host immune response contributed to worsen these abnormal motor behaviours by increasing aberrant synaptic features of dopaminergic neurons.

Another hypothesis for the appearance of GIDs was the complex nature of the dopaminergic grafts, which include different dopaminergic neuronal types, commonly known as neurons from the A9 (future substantia nigra) and A10 (future ventral tegmental area) lineage. Intra-striatal VM grafts are comprised mostly of A9 neurons and a higher ratio of A9 neurons correlates with younger donor tissue (Bye et al., 2012). Interestingly, A9 neurons have been positively linked to reduce LIDs in rodents, but none of the subtypes has negatively been correlated with GIDs (Kuan et al., 2007). It has rather been the opposite, since A9 neurons have been positively correlated to improved functional recovery, due to their ability to target the dorsolateral striatum (Grealish et al., 2010).

Another aspect of graft composition that was analysed in detail was the role of serotonin neurons in fVM grafts. Serotonin neurons develop closely to dopaminergic neurons in the ventral mesencephalon, thus the transplantation of some serotoninergic neurons alongside the dopaminergic ones is unavoidable (Lane et al., 2010). However, there was no correlation found between the presence of serotonin neurons and the graft-induced dyskinesias in animal models (Lane et al., 2006; Carlsson et al., 2007).

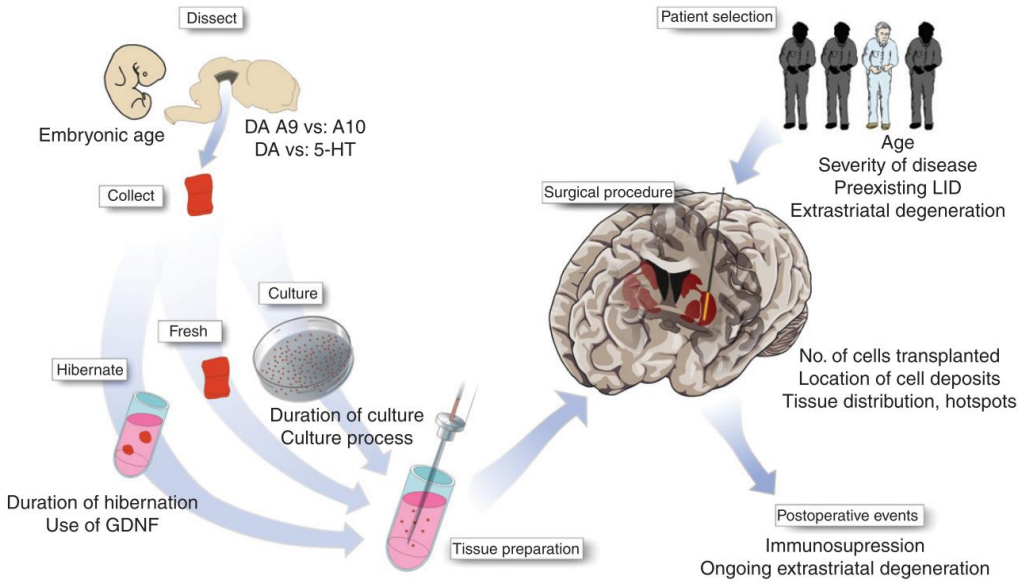

Figure 1.4 Factors hypothesized to contribute to GIDs. Several parameters identified in the numerous fVM clinical trials have been associated with the presence of GIDS including tissue preparation, surgical procedure, patient selection or post-operative events. DA: dopamine, GDNF: Glial-derived neurotrophic factor, LID: L-dopa induced dyskinesia, 5-HT: Serotonin. Image taken from Lane et al., (2010).

Although these trials failed to meet their endpoints, in the Colorado/Columbia trial, Freed et al., (2001) detected a positive correlation between a good L-dopa responders and a positive outcome. Patient selection in cell transplantation trials has proven to be crucial to obtain long-lasting clinical benefits as well as to avoid the manifestation of GIDs (Lane et al., 2010). Many correlation analyses performed with all the data gathered in clinical trials have suggested that elderly patients, advanced stages of the disease, bad-responders to L-dopa or extended dopaminergic degeneration further than the putamen, correlate with poor functional benefits from the grafts (Freed et al., 2001; Olanow et al., 2003; Piccini et al., 2005). Such correlations between patient and disease specifics, and therapeutic efficacy, emphasise that if cell transplantation ever reaches the clinical practice, it will only be suitable to a subset of Parkinson's disease patients.

1.3.2.4 Limitations of primary dopaminergic neuron transplantation

1.3.2.4.1 Poor survival

It is well established that only a small portion (1-20%) of the grafted dopaminergic neurons from fetal VM tissue survive the transplantation process (Brundin et al., 2000a). Although some cell death is attributable to the implantation process itself (cell death induced by the delay or the mechanical injection of cells), this fact does not explain the massive cell death experienced in primary dopaminergic neuron transplants. Thus, although some necrotic cell death occurs due to the dissection and implantation insult, the large extent of cell death in primary VM cells is attributed to apoptotic cell death (Mahalik et al., 1994). This extensive apoptotic cell death is considered to happen accross the entire transplantation process (Brundin et al., 2000a; Sortwell et al., 2000). First of all, the hypoxic and hypoglycemic insult caused by the removal of the embryo from the maternal blood supply and the mechanical stress produced by the axotomy and dissection of the ventral mesencephalon most likely contribute to neuronal damage (Brundin et al., 2000a). Secondly, the implantation of primary embryonic neurons into an adult host growth-factor deprived environment further contributes to this apoptotic cell death. Many studies have suggested that this period just after transplantation is critical for the survival of dopaminergic neurons, as around ~90% of dopaminergic neurons die in the first 1-4 days post-transplantation (Barker et al., 1996; Sortwell et al., 2000, 2001). Besides the lack of trophic factor support, neuroinflammation caused by the host immune response early on after transplantation is also linked to the poor survival of dopaminergic cells (Sortwell et al., 2001).

1.3.2.4.2 Graft-induced dyskinesias

As mentioned above, the manifestation of severe side effects in the form of GIDs in approximately 15% and 56% of the patients of the double-blind placebo-controlled clinical trials (Freed et al., 2001; Olanow et al., 2003). Although many efforts have been made to elucidate the origin of these adverse effects, the reasons are still not fully clear. The severity of these complications hampers the use of cell transplantation approaches. Thus, careful patient selection and tissue processing is needed to ensure the safety of cell transplantation strategies.

1.3.2.4.3 Availability

Clinical improvement after transplantation correlates with the number of surviving dopamine neurons, as stated by PET imaging (Hagell & Brundin, 2001). This fact in combination with the extreme poor survival of the transplanted cells results in the need to use several fetal donors per patient. In the Lund series, patients were transplanted with three to six fetuses per side grafted (Lindvall et al., 1994; Wenning et al., 1997; Brundin et al., 2000b) and *post-mortem* analysis of the open label trials have shown that the greatest clinical outcome was obtained when patients were transplanted with three to five fetuses per hemisphere (Hagell & Brundin, 2001). The elevated number of donors entails some logistical issues, such as collecting the required amount of tissue without compromising the viability of cells (Kirkeby et al., 2017a).

1.3.2.4.4 Standardisation

Although many efforts have been made to standardise the fetal VM tissue transplantation procedures, the use of donor tissue entails limited standardisation that has often led to wide variability in the functional outcomes.

1.3.2.4.5 Ethical concerns

Human fetal VM tissue is obtained from elective abortions. Tissue from spontaneous abortions is considered unsatisfactory because of high rate of chromosomal abnromalities and other fetal complications (Strong, 1991). Consequently the opposition to use fetal tissue is based on the ethics of abortion. Leaving the abortion issue behind, it has to be noted that obtaining and using fetal tissue did follow the usual ethical guidelines of tissue and organ donations (Strong, 1991).

1.3.2.5 TRANSEURO

In 2006, since the data collected from all the clinical studies using fVM grafts was inconsistent regarding the clinical benefit and safety of dopamine-producing cell therapy for Parkinson´s disease, it was decided amongst the top scientists in the field to re-evaluate the situation of this therapeutic approach, to ultimately decide if more clinical trials on the matter were justified, or on the contrary, if this approach reached a dead-end for the treatment of Parkinson’s disease.

In these meetings, a meticulous analysis of the raw data collected from the main hfVM clinical trials was carried out and the main factors that could explain why the data was so inconclusive were listed (Barker & TRANSEURO consortium, 2019). Amongst these factors, one can find those directly related to the patient and the disease (i.e disease stage or inaccurate diagnosis), those related to the surgery (i.e tissue obtainment and storage, tissue preparation protocol, and amount and location of tissue injected) and those related to the clinical trial design (i.e short-time endpoints, immunosuppression regime) (Barker & TRANSEURO consortium, 2019).

In light of this, TRANSEURO, a new European-Union funded hfVM trial, was launched in 2010 (NCT01898390) to determine 1) the clinical efficiency and consistency of hfVM grafting, 2) the safety of the procedure, meaning the absence of GIDs and 3) to serve as a guideline for future stem cell clinical trials (Barker & TRANSEURO consortium, 2019).

The TRANSEURO design was carefully planned to address all the identified factors that resulted in misleading results in the clinical trials done previously. For example, the stage and duration of the disease were specified as well as the patient's age. Also, the presence of LIDs was considered an exclusion criteria. The clinical trial was designed to have two treatment arms; the first group underwent a bilateral neural allotransplantation with fVM tissue, and the second non-surgical control group, which underwent the same observational assessments but no surgical intervention (Barker & TRANSEURO consortium, 2019).

The primary outcome in the TRANSEURO study is the change in the Movement Disorder Society - Unified Parkinson's Disease Rating Scale (MDS-UPDRS)[2] Part III motor score OFF medication at 36 months after transplantation when compared to their baseline score. The result of TRANSEURO will not be published until 2021. Nevertheless, the most recent review published in July 2019 revealed that patients are progressing in respect to their scores on the rating scale and no significant dyskinesias have been observed so far (Barker & TRANSEURO consortium, 2019). Although the trial is still ongoing, many lessons have already been learned from this study

[2] The MDS-UPDRS is a revision of the original Unified Parkinson's Disease Rating Scale developed in the 1980s that includes some clinically relevant features of Parkinson's disease poorly reported in the previous version (Goetz et al., 2008).

concerning the design of long observational and international clinical trials, the transplantation process and the immunosuppressive regimen.

Firstly, one of the main obstacles that the TRANSEURO consortium encountered was the difficulty in retaining patients in the study due to long assessment protocols with patients in the OFF medication state. Secondly and scientifically more relevant, another burden for the study was the limited availability of fetuses aged between 6-8 weeks post-conception (Barker & TRANSEURO consortium, 2019). This scarcity of viable tissue across the centres limited the extent of the study and only 11 patients received a graft over a 3-year period (2015-2018) although 20 patients were supposed to undergo surgery initially (Barker & TRANSEURO consortium, 2019). This fact highlights that fVM will never be a suitable dopaminergic cell source for the general clinical practice. It also indicates the importance of having a fully available source of dopaminergic neurons to ensure that cell transplantation can be used in broad clinical practice. Thirdly, although the best immunosuppressive regimen is still under debate, 12 months of triple therapy with cyclosporin A, azathioprine and steroids seemed to be the best cautious measure to adopt.

In short, to this date, the transplantation of human fVM cells is the only cell source that has proven to be efficacious in humans by surviving a long time and making connections to the host brain, releasing dopamine and ultimately ameliorating the motor symptoms. However, the limited availability of the tissue hampers its leap from clinical trials to large-scale clinical practice. Henceforward, all efforts in the cell replacement field are now put on the generation of dopaminergic neurons from stem cells.

1.3.2.6 2010s – The stem cell era

Fetal VM tissue has brought promising results both in preclinical studies and in patients, thus providing substantial proof-of-concept for cell replacement in Parkinson's disease. However its widespread use in patients is hindered by the poor survival of grafted cells and the ethical and logistical matters associated with their use. Hence, for cell transplantation to become a reality in the clinic, new dopaminergic cell sources are needed.

In this search for a highly available and low variable source of dopaminergic neurons for cell replacement therapies in Parkinson's disease, many attempts have been made to generate human midbrain dopaminergic neurons from different cells, ranging from neural progenitor cells (NPCs) (Maciaczyk et al., 2008), pluripotent stem cells (PSCs) (Fasano et al., 2010; Kriks et al., 2011; Kirkeby et al., 2012; Doi et al., 2014) and from somatic cells such as fibroblasts by direct reprogramming (Torper et al., 2013) (**Figure 1.5**).

Between all pluripotent stem cells sources, human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs) have been broadly investigated for this purpose. Mesenchymal stem cells (MSCs) have also been studied, but they will not be covered here because they do not act as dopaminergic grafts (Barker, 2014).

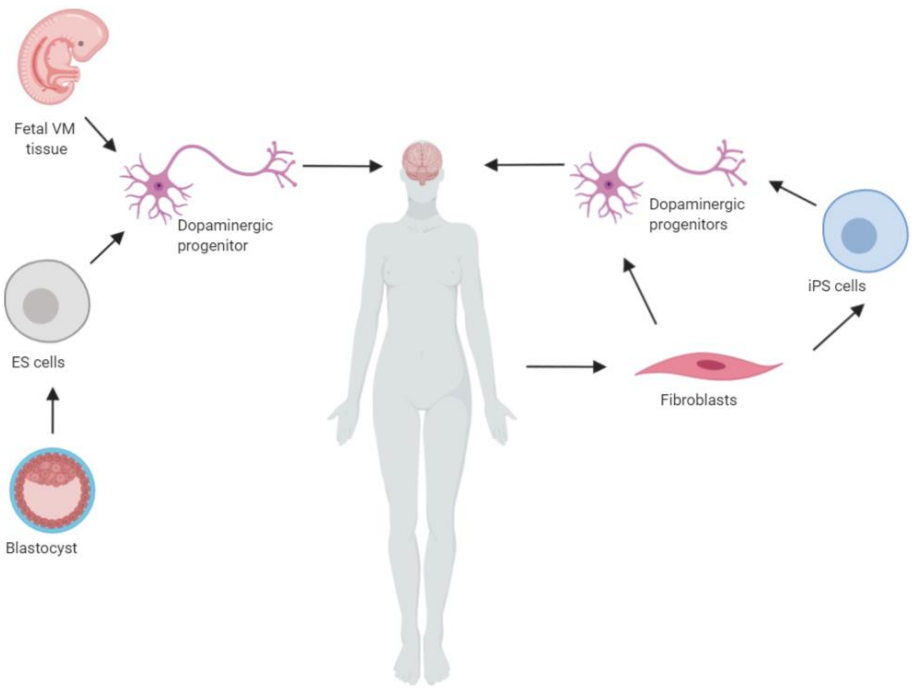

Figure 1.5 Cells under current investigation for use in cell transplantation therapies for Parkinson's disease. Fetal tissue has been traditionally used for cell transplantation in Parkinson's disease but it entails many ethical and logistical restrictions that limit their use. New dopaminergic cell sources are being investigated; hES and hIPS cells have both provided stable midbrain dopaminergic neurons. Direct reprogramming of somatic cells is also being investigated.

1.3.2.6.1 Embryonic Stem Cells

ESCs are self-renewing undifferentiated cells extracted from the blastocyst[3] that have the potential to differentiate into any cell type of an organism. They were firstly reported by Thomson et al., in (1998) and since then, many efforts have been made to generate stable dopaminergic neurons from ESCs using feeder cells as co-culture, adding soluble growth factors that naturally occur in the differentiation process, using genetic manipulation or a combination of these factors (Wijeyekoon & Barker, 2009; Barker et al., 2015).

The initial differentiation protocols to derive hESCs to dopaminergic neurons were based on previous experience with mouse ESCs and did indeed generate high numbers of dopaminergic cells, yet the midbrain properties of these neurons were not clear and their performance *in vivo* was limited (Parmar, 2018). Later on, a ground-breaking finding reported that midbrain dopaminergic neurons are not derived from neuroepithelial cells – like all the other neurons – but are specifically derived from floor-plate cells (Ono et al., 2007; Bonilla et al., 2008). Thus, the discovery of the unique cellular origin of midbrain dopaminergic neurons generated a new series of improved hESC differentiation protocols that used specific mesencephalic floor plate markers such as LMX1A, FOXA2, OTX2 and SHH (Fasano et al., 2010; Kriks et al., 2011; Kirkeby et al., 2013). These floor-plate-derived neurons did express specific midbrain dopaminergic markers and furthermore, they reported to release dopamine and improve the functional deficits in rodent models of Parkinson's disease (Kriks et al., 2011; Kirkeby et al., 2012; Steinbeck et al., 2015). Moreover, the *in vivo* capacity of ESC-derived dopaminergic neurons has been found to match those of fetal tissue (Grealish et al., 2014). Nevertheless, the routine differentiation of hESCs experienced a significant batch-to-batch variability in terms of how many dopaminergic neurons were ultimately produced and subsequently affected the efficiency of the dopaminergic grafts (Parmar, 2018). The subsequent identification of a set of markers - such as FGF8 – associated with the caudal midbrain region correlated with a higher dopaminergic neuron yield after transplantation (Kee et al., 2017; Kirkeby et al.,

[3] Blastocyst - from the greek, *blastos* which means sprout and *cystos* which means cavity - is an embryonic cellular mass that forms in early development. It consists of an inner cell mass that will develop into the embryonic tissues and an outer layer called trophoectoderm that will give rise to the placenta (Gilbert, 2011).

2017a). With all this compelling evidence, the generation of GMP-compliant floor-plate-derived dopaminergic neurons is being pursued (Barker et al., 2017; Kirkeby et al., 2017b), in order to be tested in patients.

In spite of the extensive improvements in the differentiation protocols of hESCs, tumorigenicity is always a major concern. However, some hESC-derived transplants have been performed in retinal degeneration and so far, no adverse effects regarding self-renewal or pluripotency have been reported (Seiler & Aramant, 2012; Tang et al., 2017). Furthermore, cell sorting allows the specific selection of markers of the floor plate, thus reducing the probability of transplanting pluripotent cells.

As means to evaluate the efficacy of hESCs in clinical trials, pre-clinical assessments have to be made with respect to the manufacturing process (ensuring sufficient quality and quantity of stem cell-derived dopaminergic neurons), their safety (guarantee that these cells are non-tumorigenic or teratoma-forming) and lastly ensuring that cells can be as efficient as the fetal-origin dopaminergic cells (Parmar, 2018). To date, these requirements have been met and many first-in-human clinical trials using hESCs are on the horizon (Barker et al., 2017; Kirkeby et al., 2017).

1.3.2.6.2 Induced Pluripotent Stem Cells

In 2012, Shinya Yamanaka won the Nobel Prize in Medicine for his discovery that mature cells could be reprogrammed and become pluripotent by retrovirally introducing gene transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) (Takahashi & Yamanaka, 2006). Thus, iPSCs are pluripotent stem cells reprogrammed from adult somatic cells such as skin fibroblasts. Soon after this ground-breaking finding, midbrain dopaminergic neurons were generated from hiPSCs and successfully transplanted in parkinsonian models (Hallett et al., 2015; Kikuchi et al., 2017a). When generating midbrain dopaminergic neurons, hiPSCs respond to the same differentiation cues and mature similarly upon transplantation as hESCs (Doi et al., 2014; Kikuchi et al., 2017a). The major difference between these stem cell sources relies on how iPSCs are derived (via reprogramming somatic cells) which makes them a highly available source. Therefore, these do not have the ethical restrictions of an embryonic-derived cell line and they open the door to patient-specific technologies. iPSCs would allow for Human Leukocyte Antigen (HLA)-matched donor transplants which have been reported to reduce the immune responses and increase the iPSCs-

derived dopaminergic neuron survival in non-human primates (Hallett et al., 2015; Morizane et al., 2017). This would avoid the use of immunosuppressant drugs upon transplantation, a major concern in fVM clinical trials (Takahashi, 2017),

Nevertheless, in addition to the existing concerns about using pluripotent cells, iPSCs entail major concerns about the reprogramming process itself (Liang & Zhang, 2013). Although this fact increases the pre-clinical assessments needed, it does not exclude iPSCs from clinical use (Kirkeby et al., 2017b). In fact, after ensuring safety, genomic stability and efficacy in models of Parkinson's, Jun Takahashi and colleagues at the University of Kyoto in Japan announced that they were to start the first iPSCs transplant clinical trial in Parkinson's patients in August 2018 (Takahashi, 2019). The objectives of the Kyoto trial are to evaluate the safety and efficacy of transplanting human iPSC-derived dopaminergic progenitors into the putamen of Parkinson's disease patients as well as to evaluate the safety and efficacy of using tacrolimus as an immunosuppressant therapy in this iPSC-based allogeneic[4] transplantation clinical trial (Takahashi, 2017, 2019).

1.3.2.6.3 Induced Neurons

Another interesting dopaminergic cell source is induced Neurons (iNs), which are neurons directly reprogrammed from skin cells without passing through the pluripotent state (Vierbuchen et al., 2010). These cells could also allow HLA-matched donor transplants with the advantage of avoiding pluripotency, providing a safer, less demanding preclinical assessment. Nevertheless, this reprogramming technique is very new and such cells need to be extensively investigated for their stability, safety and clinical use.

The future of cell replacement therapies for Parkinson's disease is focused on stem cells due to their potential to generate large numbers of dopaminergic cells in a standardised manner; essential requirements for clinical application. The use of dopaminergic progenitors derived from hESCs has been broadly studied but the ethical issues about their origin may limit their clinical application. On the other hand, dopaminergic progenitors derived from hiPSCs do not have this limitations and moreover they could be used in patient-specific therapies. In all likelihood in the

[4] Allogeneic transplantation: The donor and the recipient are different individuals but belong to the same species.

upcoming years many stem cell trials evaluating the efficacy of these new dopaminergic sources for cell transplantation in Parkinson's disease will take place.

In parallel, newly developed tissue-engineering strategies are being investigated to address the poor graft survival upon transplantation.

1.4 BIOMATERIALS FOR NEURAL REPAIR

1.4.1 Rationale for the use of biomaterials in cell therapy

Cell replacement therapies have shown their potential to be an alternative treatment to target nigrostriatal degeneration in Parkinson's disease, although their broad clinical success faces many obstacles. Cell-based therapies have encountered poor cell survival and integration in the host tissue after transplantation. Therefore it appears necessary to refine the delivery of the transplanted cells to enhance their survival and maximise the efficiency of such therapies.

Advances in cell replacement therapies, regenerative medicine and tissue engineering offer multiple new approaches for brain repair. Biomaterials - natural or synthetic materials tailored to provide a beneficial effect in a targeted biological system - have the potential to overcome some of the current hurdles of cell replacement therapies and ultimately improve brain repair and regeneration (Orive et al., 2009; Tuladhar et al., 2018). In particular, biomaterial scaffolds have the potential to improve the survival of dopaminergic cell grafts.

As mentioned earlier, the vast majority of embryonic mesencephalic dopaminergic neurons - estimated around 90% - die upon transplantation in the striatum and it is estimated that roughly only 5-20% of the dopaminergic neurons survive after grafting (Sortwell et al., 2001). This broad cell death is multifactorial and occurs at multiple times during the transplantation process. Sortwell and colleagues (2000) hypothesised that the triggers of apoptotic cell death during transplantation could be divided into three stages; pre-implantation, immediately post-grafting and long after transplantation (established graft).

Before transplantation, in the tissue dissection and preparation stage (stage 1), cells undergo a dissociation process from their original tissue into a single cell suspension, thus eliminating all pre-existing cell-matrix interactions. This detachment from the extracellular matrix (ECM) is enough to trigger apoptotic processes, known as

anoikis[5]. Consequently, some cells start apoptotic procedures during this stage, even before transplantation.

During the transplantation process, cells are delivered into a diseased and growth-factor deprived adult striatum. Most of the cell death occurs at this point immediately after transplantation (stage 2) and this is believed to be influenced by the trophic support withdrawal, oxidative stress and hypoxia (Sortwell et al., 2000, 2001). These circumstances are particularly determining in primary VM cells that are removed from an embryonic growth-factor rich microenvironment and transplanted into a growth-factor depleted microenvironment. Some investigations point out that the majority of VM cells die in the first 4 to 7 days post-transplantation, however apoptosis during the first 4 days seems to be the one limiting dopaminergic neuron survival (Sortwell et al., 2000). It is well established that the exposure of dopaminergic cells to a number of trophic factors before or during transplantation can significantly increase the survival of the grafted dopaminergic neurons (Sinclair et al., 1996; Apostolides et al., 1998; Zawada et al., 1998; Espejo et al., 2000; Chaturvedi et al., 2003). Hence, the lack of trophic factor support seems to be linked, at least in part, to the poor cell survival.

Once the cells have been implanted into the host tissue (stage 3), they are subjected to a hostile microenvironment where immune cells trigger pro-inflammatory cues that promote further cell death in the grafted cells (Duan et al., 1995, 1997; Barker et al., 1996).

Hence, targeting each of these stages constitutes a window of opportunity to improve the survival of the grafted dopaminergic neurons. Biomaterials can be designed to intervene in these key stages of transplantation to ultimately improve the survival of grafted cells. For example, the *anoikis* could potentially be reduced by using an ECM-like scaffold where cells could attach. Secondly, this same scaffold could be loaded with trophic factors of interest to enrich the cells' microenvironment upon transplantation and consequently reduce the apoptosis associated with a trophic-factor depleted microenvironment. Lastly, the scaffold could also protect from the host immune response by providing a physical barrier between the transplanted cells and

[5] Anoikis: Apoptotic signals after the disruption of cell-matrix interactions, the etymology of which comes from the Greek ἀν- (without) and -οικ- (home) and means "the state of being without a home" (Frisch & Francis, 1994).

the host immune cells (**Figure 1.6**).

Therefore, biomaterial scaffolds offer a multi-strategy approach through a single material to target several key aspects known to be involved in apoptotic cell death and consequently increase the chances of improved survival of dopaminergic cell death.

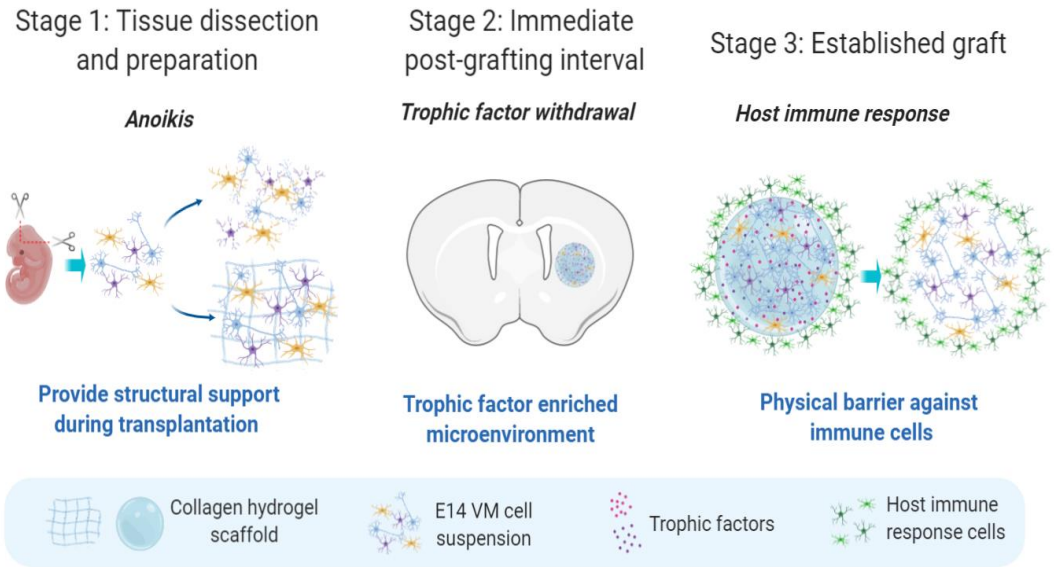

Figure 1.6 Biomaterial intervention during cell transplantation stages. The biomaterial scaffold should be able to provide structural support to transplanted cells during the transplantation process, deliver growth factors to the close microenvironment and create a physical barrier between transplanted cells and host immune cells.

1.4.2 Designing biomaterials for brain repair

The brain can be damaged by a wide range of neurodegenerative conditions, all of them with a complex pathophysiology and some extent of neuronal loss. This neuronal loss has devastating consequences due to the central nervous system's restricted regenerative ability after an injury or a disease. Furthermore, pharmacological treatments are currently unable to address the complexity of these neurodegenerative diseases and they cannot stop the neurodegeneration. At the same time, tissue regeneration strategies - such as cell and/or drug delivery - have shown promising results in preclinical studies but have found many difficulties in clinical translation.

For example, cell replacement therapies are limited by the poor cell survival and integration in the host upon transplantation and drug delivery is limited by the limited diffusion across the blood-brain barrier (BBB). Hence, there is a need to improve these delivery methods to the brain. Consequently, the use of biomaterials in promoting cell survival as well as a localised and sustained delivery of biomolecules is being investigated.

Biomaterials constitute an attractive strategy for brain protection, repair and regeneration due to their broad diversity and high adaptability to the organ, disease and intended use (Orive et al., 2019). However, designing biomaterials for brain repair has its own unique challenges due to the brain's restricted accessibility and capacity of self-repair, the existence of the BBB and the cellular and functional complexity of the brain.

First of all, due to the difficulty in accessing the brain, biomaterials designed for neural repair should ideally be delivered intra-cranially in a minimally invasive manner. Furthermore, such materials should be 1) biocompatible and not toxic to the host tissue (including its by-products after degradation), 2) chemically and physically stable long enough to perform its desired biological function but 3) fully biodegradable with no remaining residues once it has served its purpose, 4) adaptable to functionalisation, modifiable to cell functions such attachment or differentiation, 5) capable of controlled and sustained release of trophic factors and 6) processable in many shapes and scalable to the clinical practice (Tuladhar et al., 2018; Orive et al., 2019).

1.4.3 Injectable hydrogels as a cell scaffold

As already mentioned, the wide range of biomaterials available and their capability to be tuned to each application and disease needs makes them a very attractive tool for regenerative therapies (Orive et al., 2009). In cell transplantation therapies, combination strategies involving cells, biomolecules and biomaterials have been studied in order to enhance cell survival and integration after transplantation (Pakulska et al., 2012).

Biomaterial-based scaffolds can be fabricated to deliver cells to the desired site and to provide controlled drug release of biomolecules while serving as a physical scaffold for cell adhesion. Although early investigations used implantable scaffolds, the

enclosed location of the brain has promoted the development of injectable scaffolds, also known as hydrogels, that can be implanted with minimally invasive surgery (Nih et al., 2016). Hydrogels are a class of biomaterials with hydrophilic three-dimensional polymeric networks that can absorb large amounts of water, and thus have the potential to be used as cell matrices, drug delivery vectors and in many other medical applications (Chai et al., 2017).

Hydrogels have multiple properties that make them excellent scaffolds to be used in cell transplantation therapies. First of all, injectable hydrogels can form gels *in situ* after a pH or temperature change, which allows their intra-cranial delivery (Pakulska et al., 2012). Thus, injectable hydrogels can protect the transplanted cells from the mechanical forces exerted during the injection process, which is known to contribute to cell death (Mitrousis et al., 2018). Moreover, hydrogels can provide protection to the transplanted cells upon transplantation against the host immune response by physically isolating them from the host microenvironment. As demonstrated by Hoban et al., (2013), the encapsulation of MSCs in an injectable collagen hydrogel diminished the host immune response at the site of delivery.

As well, hydrogels can naturally promote cell survival by providing structural support to the dissociated cells, but more interestingly they can be modified to enhance cell adhesion. For example, the ECM-derived RGD[6] motif is incorporated into synthetic hydrogels to improve cell adhesion (Chien et al., 2012; Zustiak et al., 2013). Similarly, the addition of ECM-derived synthetic peptides, from common components of ECM such as fibronectin or laminin, to hydrogels, have been identified as promoters of cell adhesion and viability (Tam et al., 2014). Additionally, hydrogels can mimic the properties of the ECM to promote cell adhesion, proliferation and differentiation by using natural ECM factors as their main component – such as collagen or hyaluronic acid (Hinderer et al., 2016).

The inherent properties of the hydrogels also have many advantages over other scaffold materials. For example, the mechanical properties of hydrogels are similar to those of the body, which enhances cell survival. Interestingly, modifying the

[6] The RGD (Arg-Gly-Asp) motif - common in several ECM proteins such as laminin or fibronectin - is considered an integrin recognition site, through which cells attach to the ECM proteins. Collagen type I contains some inaccessible RGD motifs, thus the cell adhesion to collagen is thought to be mediated through other integrin motifs (Taubenberger et al., 2010).

mechanical and physical properties of the hydrogels has also been reported to influence cell fate (Aurand et al., 2014). As well, the viability of cells is enhanced by the high oxygen and nutrient permeability of hydrogels (Nisbet et al., 2008). Even though the natural properties of hydrogels are advantageous for cell transplantation, hydrogels can also be modulated by the crosslinking agent in order to modify relevant properties such as the biomaterial's stiffness or porosity. Thus, hydrogels can be fabricated out of natural or synthetic polymers, and these can be chemically or physically crosslinked to ensure structural stability. Certainly, the chemical crosslinking can also be used to modulate many factors that interfere with cell deployment and the properties of the hydrogel itself like its gelation process, porosity and its degradation pattern (Hennink & Van Nostrum, 2012). Due to their high content in water and their use of natural polymers, natural hydrogels have usually good biocompatibility and biodegradability, thus allowing the transplanted cells to grow and establish interactions with the host tissue.

Beyond cell carriers, hydrogels can also constitute a reservoir for the controlled release of growth factors, exposing cells to an enriched growth-factor microenvironment. The ideal duration of growth factor release is determined by a balance between the maximum benefits in cell survival and the minimum side effects caused by unnecessary long exposure (Mitrousis et al., 2018). As an example, Fon et al., (2014) used a gelatin-based hydrogel to release glial cell line-derived neurotrophic factor (GDNF) and attract neural progenitor cells towards the implant.

Finding the right biomaterial with all the desired properties is challenging and as described above, the intended use of the material will determine the importance of each of its properties (Tuladhar et al., 2018).

1.4.4 Injectable collagen hydrogels

Collagen-based biomaterials have been used both in research environments and medical applications in many medical areas such as wound closure, burn treatments, dentistry and cartilage and bone repair (Patino et al., 2002; Chajra et al., 2008; Parenteau-Bareil et al., 2010; Chattopadhyay & Raines, 2014; Ramshaw, 2016). Collagen is one of the most widely-used biomaterials because it is the major component of skin and bone and it is present in all connective tissue (Meyer, 2019).

In general, ECM is a 3D network where cells attach, migrate and differentiate to, thus acting as structural and biochemical support for the cytoarchitecture of tissues. The ECM is constituted by many macromolecules such as collagens, glycoproteins and proteoglycans. The composition of these varies immensely on the tissue function and the physiological conditions. Collagen is an abundant matrix protein in many ECMs, however its presence in the adult brain ECM is relatively low (Rutka et al., 1988). The brain ECM has a unique composition; abundant in lecticans and proteoglicans and a low content of fibrous matrix proteins such as collagens or fibronectins (Rutka et al., 1988; Ruoslahti, 1996).

The tropocollagen molecules are comprised of three α chains, tightly assembled together because of their high content of glycine, proline and hydroxyproline (Prockop & Kivirikko, 1995). Although there are 29 types of collagen, Type I collagen is the most abundant collagen (Henriksen & Karsdal, 2016) and in the field of tissue-engineering it is the gold standard biomaterial due to its high biocompatibility (Silvipriya et al., 2015). Collagen can be extracted from almost every living animal, however common sources of collagen used in tissue engineering applications include bovine skin and tendons, and rat tails (Parenteau-Bareil et al., 2010). Despite concerns regarding the potential of animal-origin collagen to evoke immune responses, collagen-biomaterials are accepted as safe materials and have been widely used in cosmetics and medical industry, with rare adverse reactions documented (Lynn et al., 2004). The immunogenicity of collagen mainly resides on the telopeptide regions (Parenteau-Bareil et al., 2010), thus the use of atelocollagen[7] in biomaterial platforms ensures the high biocompatibility of the product. Another valuable aspect of collagen-based biomaterials is their biodegradability, due to collagenases present in the host tissue (Khan & Khan, 2013).

In the context of cell transplantation, collagen's ability to naturally form a gel *in situ* makes collagen hydrogels an ideal candidate as cellular and trophic delivery systems. Since collagen hydrogels are usually weak, in an attempt to stabilise the collagen hydrogels and slow the degradation process, collagen hydrogels are usually chemically crosslinked with synthetic polymers. One of the most used chemical crosslinking methods consists of the covalent attachment of poly(ethylene glycol)

[7] Collagen formed by the removal of telopeptides, broadly used in cosmetics and cell culture.

(PEG) to collagen (Alconcel et al., 2011; Hennink & Van Nostrum, 2012). In particular, this covalent reaction occurs between the succinimidyl groups from the PEG molecule and the free amine groups present in the molecules of type I collagen (**Figure 1.7**).

PEG is a non-toxic, non-immunogenic polymer that is highly soluble in water and already approved by the FDA, which makes it the most commonly used synthetic polymer in tissue engineering (Veronese & Pasut, 2005; Peppas et al., 2006).

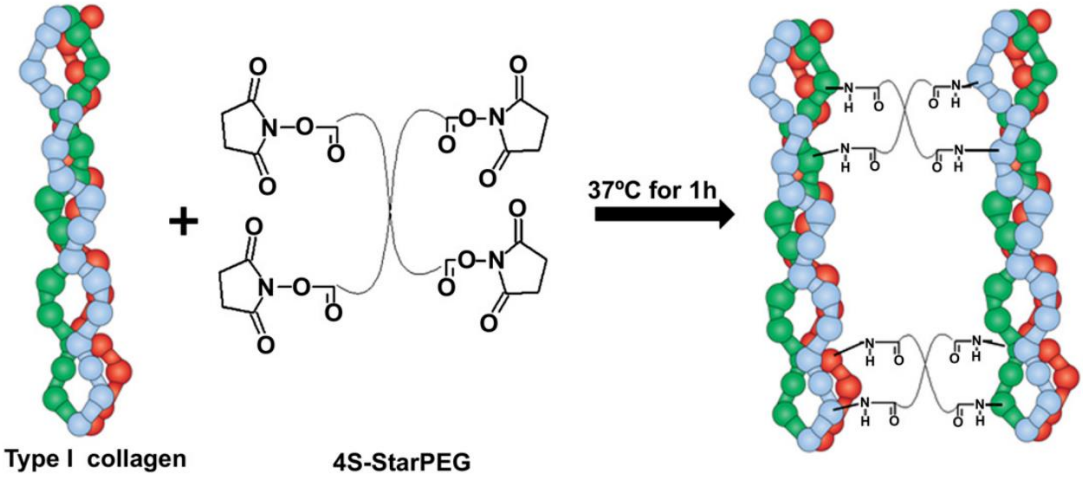

Figure 1.7 Type I collagen and 4s-StarPEG reaction. The succinimidyl groups from the PEG molecule react with the amine groups present in the collagen molecule at 37 °C. Adapted from Collin et al., (2011).

The use of collagen hydrogels for cell and trophic factor delivery has been gaining more interest in recent years. As mentioned before, Hoban and colleagues (2013) demonstrated that the transplantation of GDNF-secreting MSCs in a collagen hydrogel significantly reduced the host immune response around the delivery site in hemi-parkinsonian rats. Similarly, Moriarty and colleagues (2017) also reported a substantial decrease of the host immune response when E14 VM cells[8] were delivered in a collagen hydrogel to the striatum of 6-OHDA-lesioned rats. Most importantly, in this study and a similar one with E12 VM cells, Moriarty and colleagues showed that the encapsulation of VM cells - E12 and E14- in a GDNF loaded collagen hydrogel resulted in an improved cell survival and re-innervation of dopaminergic neurons as

[8] Ventral mesencephalon cells dissected from embryos at embryonic day 14.

well as a greater functional recovery (Moriarty et al., 2017, 2019a). Similarly, a functionalised hyaluronic acid-based collagen hydrogel dramatically improved the survival of neural progenitors derived from stem cells in the rat striatum (Adil et al., 2017).

All these recent experimental studies have demonstrated the potential of collagen hydrogels to enhance graft survival in cell transplantation therapies for Parkinson's disease. Moreover, these biomaterial-based scaffolds can also offer many possibilities as drug or trophic factor delivery systems.

1.5 NEUROTROPHIC THERAPY FOR PRIMARY DOPAMINERGIC NEURONS

1.5.1 Rationale for neurotrophic therapy

As discussed above, cell replacement therapies show promising potential as a therapeutic approach for Parkinson's disease. There is robust evidence supporting that transplanted dopaminergic neurons can survive, integrate with the host tissue and re-innervate the striatum while producing dopamine and improving motor deficits both in animals and humans. Nevertheless, their broad use in the clinic is hindered by the poor survival of the grafted cells. Dopaminergic neurons face many obstacles during and after transplantation that hamper their survival. One of the causes attributed to this cell death is the trophic-factor depleted microenvironment that cells are transplanted into. Dopaminergic neurons – either dissected from embryonic tissue or differentiated from stem cells - are removed from a rich trophic factor microenvironments and transplanted into the adult depleted and diseased host striatum. Thus, these cells could benefit from neurotrophic factor support to improve their viability upon transplantation.

In an attempt to improve dopaminergic cell survival, the addition of trophic factors to cell transplantation procedures has been investigated. Neurotrophic factors play essential roles in the development, survival and differentiation of cells. Many studies have demonstrated the benefits of neurotrophic therapy on the survival of dopaminergic neurons. Although many trophic factors have been investigated, GDNF has shown the greatest neuroprotective effects in dopaminergic neurons.

1.5.2 Discovery and classification of GDNF

The neurotrophic factor, GDNF, was first identified in neurons and glial cells in a rat glial culture, by Schubert et al. in 1974. Later, Lin et al., in 1993 characterised the protein, both papers highlighting the ability of the factor to exert positive effects on midbrain dopaminergic cultures. Lin and colleagues purified, cloned and named GDNF which they described as a glycosylated disulphide-bonded homodimer (Lin et al., 1993). Thanks largely to their work, we know now that human GDNF is a 211 amino-acid long protein (134 amino-acid long after splicing) with a 93% homology to

the rat protein (Lin et al., 1993). The GDNF gene is located on chromosome 5p12-p13.1 and it contains 5 exons and several isoforms (Schindelhauer et al., 1995). The GDNF ligand family is comprised of the structurally related neurotrophic factors; GDNF, neurturin (NRTN), arthemin (ARTN) and persephin (PSPN) (Kotzbauer et al., 1996; Ibáñez & Andressoo, 2017). They belong to the transforming growth factor-β (TGF-β) superfamily since they have similar conformations to other members of the TGF-β superfamily but low amino-acid homology (Airaksinen & Saarma, 2002). These neurotrophic factors bind to GDNF Family Receptors α (GFRα) - identified as GFRα1-4 - each of which is selective for a specific neurotrophic factor. GDNF in particular binds to GFRα1 and the GDNF/GFRα1 complex signals through the receptor tyrosine kinase RET (Takahashi, 2001) (**Figure 1.8**). The GDNF/GFRα1/RET complex then signals through Ras/MAP kinase and PI3K/AKT pathways (Ibáñez, 2013). Additionally, RET-independent signalling pathways are known to be associated with neural cell adhesion molecules (NCAM) (Sariola & Saarma, 2003).

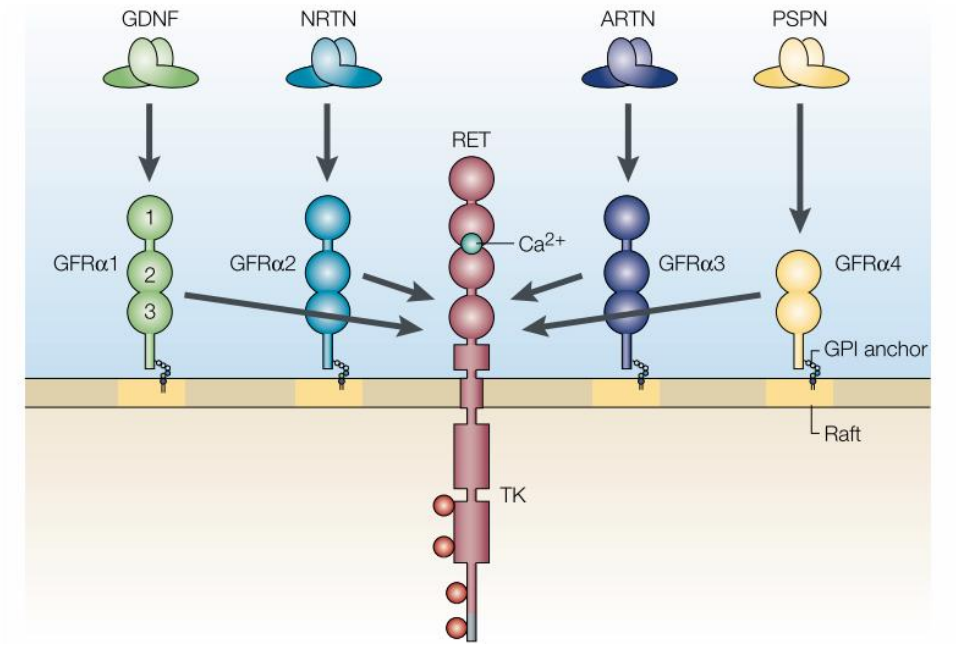

Figure 1.8 GDNF Family Receptors α 1-4 and their corresponding GDNF-family ligands. Homodimers of GDNF-family ligands first bind to GFRα the resulting complex binds to the receptor tyrosine kinase RET. GDNF: Glial cell line-derived neurotrophic factor, NRTN: neurturin, ARTN: arthemin, PSPN: persephin, GFRα: GDNF Family Receptors α. Image taken from Airaksinen & Saarma, (2002).

1.5.3 The role of GDNF in the Central Nervous System

GDNF is thought to have a significant role in the development of the nervous system, promoting growth and differentiation of many neuronal and non-neuronal populations in both central and peripheral nervous system. Thus, its wide expression over tissues indicates GDNF may have multiple roles. Although GDNF expression is not limited to neuronal populations or even the CNS, GDNF expression – as well as its receptor and its receptor components – is highly abundant in the developing striatum and ventral mesencephalon, including the substantia nigra (Nosrat et al., 1997; Choi-Lundberg et al., 1998; Glazner et al., 1998).

During the early stages of development of the nigrostriatal system, GDNF mRNA expression levels are moderate (Strömberg et al., 1993; Blum & Weickert, 1995). GDNF expression is at its highest levels in the striatum during early postnatal development, when the maturation of the dopaminergic system takes place (Scharr et al., 1993; Strömberg et al., 1993; Blum & Weickert, 1995). In fact, as part of the maturation process, two waves of natural programmed cell death occur in the dopaminergic neurons of the substantia nigra during the first two postnatal weeks, and GDNF selectively supports the viability of these neurons by preventing apoptosis, thus suggesting it may act as a target-factor for mesencephalic dopaminergic neurons (Burke et al., 1998; Oo et al., 2005). The fact that GDNF may be a limiting factor for the developing dopaminergic neurons of the substantia nigra is supported by the observation that a striatal injection of GDNF during the ontogenetic cell death period suppressed the apoptosis of these cells (Oo et al., 2003). Nonetheless, GDNF-deficient mice die soon after birth due to renal and enteric problems but show an intact dopaminergic system (Moore et al., 1996). Yet, RET-deficient mice exhibit a specific and progressive loss of dopaminergic neurons in the substantia nigra (Kramer et al., 2007). This data suggests that although GDNF is not essential for the early development of midbrain dopaminergic neurons it plays a key role in their maturation and maintenance.

In the adult brain, GDNF is present in many areas – striatum, hippocampus, thalamus and cortex – but in the nigrostriatal system, levels are low and steady, yet the GDNF levels are higher in the striatum than in the substantia nigra (Choi-Lundberg & Bohn, 1995; Oo et al., 2005). The source of striatal GDNF is not well characterised, although

GDNF is found in striatal neurons rather than in glial cells both in normal conditions and in injury (Hidalgo-Figueroa et al., 2012). Moreover, GDNF receptor expression was not detected in the adult striatum in physiological conditions but GDNF mRNA was highly expressed in nigral cells (Trupp et al., 1997). Furthermore, Tomac et al., (1995) reported that intra-striatal injected GDNF is carried via axonal transport from the dopamine nerve terminals to the cell bodies in the substantia nigra. These findings together suggest that GDNF has a specific action on nigral dopaminergic neurons.

Additionally, it has been suggested that dopamine could be a candidate to regulate GDNF expression in the nigrostriatal pathway since mice lacking D2 receptors exhibit lower levels of GDNF in the striatum, (Bozzi & Borrelli, 1999) and conversely, D1 and D2 agonists increase the synthesis of GDNF in mesencephalic cultures (Guo et al., 2002).

1.5.4 GDNF as a neuroprotective strategy

After the connecting link between GDNF and the nigrostriatal system was made, the scientific community grew interest in the neurotrophic factor, due to potential interest as a treatment for Parkinson's disease. Since then, many preclinical studies have reported the neuroprotective effects of GDNF on the survival of midbrain dopaminergic neurons in many lesioning models in rodents such as axotomy (Beck et al., 1995), 6-OHDA unilateral lesion model, (Kearns & Gash, 1995; Sauer et al., 1995; Shults et al., 1996; Björklund et al., 1997) and in non-human primates such as MPTP injection model (Gash et al., 1996). The 6-OHDA injection is a convenient model to investigate the neuroprotective and the neuroregenerative effects of GDNF due to the retrograde degeneration, and the slow and selective cell death of the dopaminergic neurons, consequently allowing the study of the neuroprotective effects of GDNF in the degenerating neurons in early stages, as well as the rescue effects on the remaining neurons during the chronic phase. It is well known that GDNF can protect neurons quite effectively against an acute insult such as 6-OHDA when the factor is given concurrently with the toxin and in close vicinity to it (Kirik et al., 2004). Interestingly, the intra-striatal delivery of GDNF but not the intra-nigral delivery results in functional recovery in rodent models of Parkinson's disease (Kirik et al., 2000, 2004). In spite of the well-established neuroprotective effects in nigrostriatal degeneration models, it is worth mentioning that GDNF failed to protect dopaminergic neurons

against an α-synuclein model of Parkinson's disease (Decressac et al., 2011). The neuroregenerative effects of GDNF have also been proven with different administration regimes (Winkler et al., 1996; Rosenblad et al., 1999). Long-term delivery of GDNF through viral vectors have also shown that GDNF provided beneficial functional effects in rodent models of Parkinson´s disease (Bilang-Bleuel et al., 1997; Mandel et al., 1997; Choi-Lundberg et al., 1998; Dowd et al., 2005b).

The experimental data from both rodent and non-human primate models provided support for the use of GDNF as a protective therapy for the degenerating dopaminergic neurons. Neurotrophic factors cannot cross the BBB and are quickly metabolised *in vivo*. Thus, the assessment of the potential benefits of GDNF required intracerebral application of the neurotrophic factor. In the early 2000s, the first GDNF clinical trial – a randomised trial using intraventricular delivery of GDNF - reported no significant improvement in the UPDRS score in patients treated with intracerebroventricular GDNF, and besides some patients experienced side effects of varied severity (Kordower et al., 1999; Nutt et al., 2003). More positive results were achieved in two open-label studies when a continuous infusion of GDNF was delivered into the putamen, where significant improvement was detected and no relevant side-effects were reported (Gill et al., 2003; Patel et al., 2005). Nevertheless, the favourable outcomes could not be replicated in a randomised, placebo-controlled study with a similar design (Lang et al., 2006). The latest GDNF clinical trial –which started in 2012 - presented several discrepancies in study design in comparison to previous GDNF clinical trials; this placebo-controlled, randomised double-blind clinical study delivered GDNF in the putamen intermittently every four weeks using a convection-enhanced delivery (CED) at a much lower dose than the Lang clinical trial (Whone et al., 2019b). Despite high expectations, this UK-based clinical trial did not observe an improvement in the OFF state of UPDRS score in comparison to placebo nor did they find any significant improvements in the secondary motor and non-motor endpoints (Whone et al., 2019b). In an extension of the study, all patients were infused with GDNF for an extra 40 weeks - including those that were originally administered placebo in the first 40 weeks – and no differences between the groups were seen in the primary or secondary outcome measures (Whone, et al., 2019a). The conflicting results acquired with the several GDNF clinical trials have raised questions about the growth factor hypothesis as well as the limitations of clinical studies. To date, the lack

of efficacy and drug delivery complications remain the main obstacles for GDNF therapy to become a reality in the clinic. Although the use of trophic factors such as GDNF for Parkinson's disease is a promising approach, its clinical use will depend on the ability to provide long term neuroprotection safely and efficiently.

1.5.5 GDNF for dopaminergic neuron transplantation

The use of GDNF for a more localised purpose, such in cell replacement therapies, has been more successful. The neuroprotective effects of GDNF on the survival of midbrain dopaminergic neurons aroused interest in the scientific community investigating cell replacement strategies for Parkinson´s disease. Since GDNF is unable to cross the BBB, the administration of GDNF has to be applied to the transplanted cells either *in vitro* before transplantation or concurrently with cells during the intracranial delivery. *In vitro* pre-treatment of VM cells with GDNF prior to transplantation into the striatum increased the dopaminergic cell survival and nerve outgrowth of the dopaminergic grafts (Apostolides et al., 1998; Espejo et al., 2000). This discovery was very valuable to the clinical field since fetal tissue was usually stored for days until there was enough tissue to transplant. Subsequently, Mendez et al., (2000, 2002) demonstrated that cells pre-treated with GDNF during the hibernation period resulted in improved graft survival and a reduction of the UPDRS scores in patients.

The once-off administration of GDNF during transplantation also generated positive outcomes with improved graft survival, re-innervation and reduced motor impairments in models of Parkinson's disease (Sinclair et al., 1996; Apostolides et al., 1998; Chaturvedi et al., 2003). Hence, considering the impressive effects that a single short-term administration of GDNF had on the dopaminergic transplanted cells, the effects of a prolonged administration of GDNF were also investigated. For instance, the co-transplantation of VM cells and GDNF-producing cells also promoted motor functional recovery in hemi-parkinsonian rats (Espejo et al., 2000; Deng et al., 2013; Perez-Bouza et al., 2017). Furthermore, the long-term delivery of GDNF using osmotic mini-pumps also improved the rate of dopaminergic cell survival and the density of fibre outgrowth (Sinclair et al., 1996; Yurek, 1998).

Another approach used to deliver GDNF intra-cranially for an extended period is the encapsulation of GDNF. The co-transplantation of VM cells with pharmacologically active microcarriers loaded with GDNF substantially improved the dopaminergic cell survival and fibre outgrowth in a parkinsonian rodent model (Tatard et al., 2007). Similar positive results were also achieved when GDNF was encapsulated in biodegradable capsules (Clavreul et al., 2006). Moreover, Moriarty et al., (2017, 2019a) reported a dramatic increase in dopaminergic cell survival when VM cells were delivered encapsulated in a GDNF-loaded collagen hydrogel.

The continuous delivery of GDNF has also been pursued using the overexpression of GDNF with recombinant adenovirus, recombinant adeno-associated virus (rAAV), and recombinant lentivirus (rLV) vectors (Torres et al., 2005; Redmond et al., 2013). However, the continuous long-term delivery of GDNF downregulated the tyrosine hydroxylase (TH) levels and produced aberrant sprouting of TH+ fibres in healthy animals, which could affect functional recovery in the compromised nigrostriatal pathway (Georgievska et al., 2004).

Briefly, in cell replacement therapies, the greatest effects are reported when the delivery of GDNF is given locally, before or during transplantation, and for short periods of time. Altogether, these investigations demonstrate the potent effects of GDNF on the survival of dopaminergic cell grafts and the importance of neuroprotection strategies for dopaminergic cell replacement therapy. Nevertheless, the use of neuroprotective strategies in combination with other approaches could benefit even further the dopaminergic cell survival upon transplantation.

1.6 ANTI-INFLAMMATORY THERAPY FOR PRIMARY DOPAMINERGIC NEURONS

1.6.1 Rationale for anti-inflammatory therapy

The brain has historically been considered as immunologically privileged in comparison to the peripheral organs. The brain is in fact a relative immunologically privileged transplantation site since allografts are tolerated (Head & Griffin, 1985). However, this does not mean that immune reactions do not occur as xenotransplants[9] are quickly rejected in immunocompetent subjects (Duan et al., 1995). In fact, the transplantation of exogenous cells *per se* generates a host immune response. But certainly, the intensity of the immunological response depends on the type of graft transplanted, the immunological disparity between donor and host and where and how the tissue is transplanted (Duan et al., 1995, 1997; Shinoda et al., 1995). Hence, the intense host immune response triggered by the transplantation itself is believed to interfere with dopaminergic cell survival. However, at this point it is essential to distinguish between the effect of the innate immune response and the adaptive immune response on the transplanted cells.

When cells are transplanted into the brain, a gliotic reaction is initiated, with the recruitment of astrocytes surrounding the lesion site to seal off the traumatic injury and the recruitment of microglia to the vicinity of the graft. This host innate immune response is very quick – present within hours after injection – and fades greatly over time (Barker et al., 1996). Thus, the host innate immune response is characterised by a recruitment of microglia, macrophages and astrocytes to the grafted site and surroundings. For instance, in an allogeneic VM graft, at 4 days after transplantation, increased levels of major histocompatibility complex (MHC) class II antigens expressed on Antigen-Presenting Cells (APCs) were reported, indicative of the presence of activated microglial cells and macrophages around the grafted area (Duan et al., 1995). In the same situation, the presence of CD4+ or CD8+ T cells was very low, indicative that there was no adaptive response at that time against the allogeneic grafts (Duan et al., 1995; Shinoda et al., 1995).

[9] Xenotransplantation: The donor and the recipient belong to different species.

Thus, it seems probable that the intense host innate immune response in particular is in part responsible of the massive dopaminergic cell death in the first stages of transplantation.

On the other hand, the adaptive immune response also takes effect in allogeneic grafting at longer timepoints, as CD4+ and CD8+ T cells can be detected after 6 weeks post-transplantation (Shinoda et al., 1995). However, this increase in lymphocytes has not been correlated with dopaminergic cell death because dopaminergic cell survival is stabilised at these stages post-grafting. However, it has to be noted that interventions in the adaptive immune response can also have beneficial effects in transplant rejection. For example, the depletion of T lymphocytes using monoclonal antibody treatments prolonged the xenograft survival dramatically (Wood et al., 1996; Okura et al., 1997). Furthermore, many efforts have been put into investigating the best immunological regime to avoid transplant rejection in the long-term both in preclinical and clinical studies. Moreover, the variable success of hfVM grafts in patients has usually been attributed to the different immunological regimes received. Therefore, the modulation of the host adaptive immune response is important to fight against chronic transplant rejection.

Thus, there is no question that by modulating the overall host immune response, the graft outcome can be modified. However, the effects of targeting the host innate immune response locally in the transplantation site to enhance cell survival during the early transplantation phase has been poorly investigated. There is a window to explore the potential benefits of targeting the host immune response during transplantation at the grafted area in order to challenge the extensive cell death of dopaminergic neurons in the first stages after transplantation. As mentioned earlier, great benefits have been obtained using this site-specific approach in neuroprotective strategies, where the co-administration of a neuroprotective factor with the dopaminergic cells resulted in improved survival.

Furthermore, the astrocytic response is associated with the secretion of axonal-growth inhibitors (like vimentin and tenascin) and thus it is possible that the reduction of the local host immune response could result in widespread axonal growth from the embryonic mesencephalic neurons (Barker et al., 1996). In conclusion, targeting the host innate immune response could have beneficial effects both in cell survival and re-

innervation. Thus, the work in this thesis will focus on targeting the host innate immune response using an anti-inflammatory cytokine loaded collagen hydrogel for dopamine cell replacement strategies. IL-10 was the anti-inflammatory cytokine chosen for this purpose due to its neuroprotective and anti-inflammatory role in the CNS.

1.6.2 Discovery and classification of IL-10

Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine first described by Mosmann and colleagues in 1989 as a cytokine synthesis inhibitory factor secreted by T helper 2 (T_H2) cells, due to its capacity to inhibit the production of pro-inflammatory cytokines produced by T helper 1 (T_H1) cells (Fiorentino et al., 1989). IL-10 belongs to a cytokine family that includes IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-20 (Commins et al., 2008). All these cytokines share a similar genomic and receptor structure as well as signalling pathways but entail very different biological functions (Commins et al., 2008). The gene encoding for human IL-10 is 4.7 kb pairs long, comprises five exons and is located on chromosome 1q21–32 (Mosser, 2008). Several polymorphisms of the human IL-10 gene have been studied (in particular five in the human IL-10 gene promoter region) which may be linked to a range of diseases such as graft-versus-host disease (Mosser, 2008). The human IL-10 gene encodes for a 178 amino acids long protein and has approximately a 75% homology to murine IL-10 (Sabat et al., 2010). IL-10 acts through an heterodimeric complex of two receptors; IL-10R1 and IL-10R2. The binding of IL-10 to its receptor complex is a two-step process where IL-10 first binds to IL-10R1 and this is followed by a conformational change that allows the association of IL-10R2 to the IL-10/IL-10R1 complex (Yoon et al., 2006).

IL-10R1 subunit expression is very specific to immune cells (Carson et al., 1995; Jurlander et al., 1997) whereas IL-10R2 is very common throughout non-immune cell types such as blood cells or keratinocytes (Wolk et al., 2004; Kunz et al., 2006). The binding of IL-10 to the receptor complex activates the JAK tyrosine kinases - JAK1 binds to IL-10R1 and Tyk2 binds to IL-10R2 - which results in the recruitment of STAT3, its phosphorylation and dimerisation (Finbloom & Winestock, 1995). Lastly, the dimer of STAT3 is translocated to the nucleus where transcription of targeted genes occurs (Rutz & Ouyang, 2016) (**Figure** 1.9). Contrary to what was first thought,

IL-10 is produced by a wide range of innate immune cells such as dendritic cells, macrophages, natural killer (NK) cells, mast cells and adaptive immune cells such as T_H1, T_H2, T_H17, regulatory T cells (T_{REGS}) and B cells (Moore et al., 2001; Wolk et al., 2002; O'Garra & Vieira, 2007). In the CNS, IL-10 is expressed by microglia, astrocytes and neurons (Hulshof et al., 2002; Helmut et al., 2011). Thus, IL-10 will be produced by one or another cell type depending on the stimulus, tissue and timepoint in the immune response.

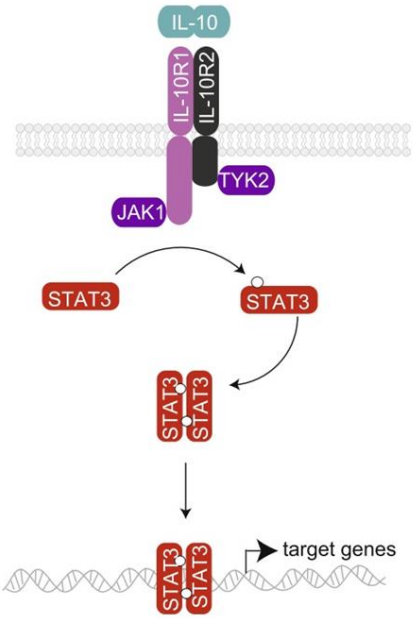

Figure 1.9 IL-10 receptor binding and signalling. IL-10 binds to its IL-10 receptor complex - which is comprised of IL-10R1 and IL-10R1 subunits – and this binding leads to the phosphorylation of tyrosine kinases Jak1 and Tyk2 which also phosphorylate STAT3. The subsequent dimerisation and nuclear translocation of STAT3 allows the transcription of IL-10 dependent genes Image taken from Rutz & Ouyang, (2016).

1.6.3 The role of IL-10 in immunity

The immune response is activated after injury or infection. The innate immune system - which represents a quick but non-specific defence for the body - starts with the activation of Pattern Recognition Receptors (PRRs) by damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs). This activation in the innate immune cells triggers the expression of cytokines and chemokines (DiSabato et al., 2016).

On the other hand, the adaptive immune system is a highly specialised response that will develop immunological memory. This strategy can be divided into cell-mediated responses and antibody responses executed by T and B lymphocytes respectively. Both sterile and non-sterile inflammation[10] can lead to the activation of T lymphocytes, which will secrete cytokines and differentiate into either cytotoxic T cells, T_H or T_{REG} cells (Zelenay & Reis e Sousa, 2013). Cytotoxic T cells will destroy virally-infected cells and T helper cells will activate macrophages and B cells. B cells will then differentiate to become antibody-secreting cells against specific antigens.

IL-10 is an immunoregulatory cytokine with a crucial role in limiting the immune response and protecting the host from excessive damage. This potent anti-inflammatory cytokine is involved in both the innate and adaptive immune system at multiple levels. Understanding IL-10 regulation is challenging, because IL-10 is involved in many processes and cell types. However the main biological functions of IL-10 are thought to be the inhibition of 1) antigen presentation, 2) the production of pro-inflammatory cytokines – like IL-1, IL-6, IL-12, tumor necrosis factor (TNF) – and 3) phagocytosis in dendritic cells and macrophages (Mosser et al., 2008; Sabat et al., 2010).

Both macrophages and dendritic cells have been shown to release IL-10 after PRRs activation *in vitro* (Saraiva & O'Garra, 2010). More specifically, the activation of PRRs like Toll-like receptors 4 (TLR4) by PAMPs like LPS induced the expression of IL-10 in macrophages (Agrawal et al., 2003). However, it has to be noted that endogenous IL-10 can also be induced by TLR-independent stimuli (Saraiva & O'Garra, 2010). Thus, an innate pro-inflammatory response by the macrophages and

[10] Non-sterile inflammation is the term used to describe inflammation triggered by causes other than infectious pathogens, such as trauma or ischemia.

dendritic cells will promote the autocrine release of IL-10, which will inhibit antigen presentation by blocking MHC II molecule expression (Mosser, 2008). Furthermore, IL-10 also limits the production of pro-inflammatory cytokines (IL-1α and β, IL-6, IL-12, IL-18, and TNF-α and many chemokines (MCP1, MCP5, RANTES, IL-8, IP-10 and MIP-2) (Couper et al., 2008). So, IL-10 acts as a negative feedback regulator, by limiting the pro-inflammatory responses in autocrine and paracrine signalling. IL-10 exerts its negative feedback mechanisms by inhibiting dendritic cell differentiation and maturation as well as modulating the production of T_H1 and T_H2-related cytokines. IL-10 can also immunoregulate the adaptive immune response by ameliorating excessive T_H1 and CD8+ and later, it was found to be produced by T_H1 and T_H17, but only under certain conditions (Saraiva et al., 2009). However, and unlike innate immune cells, CD4+ T cells need to differentiate into T_H1, T_H2 and T_H17 before producing IL-10 (Zhu et al., 2010). At the same time, these T-helpers are dependent on macrophage and dendritic cell-derived factors downregulated by IL-10, thus establishing a negative feedback loop to avoid unnecessary immunopathology (Saraiva & O'Garra, 2010).

However, not all IL-10 effects are immunosuppressive since IL-10 promotes B cell differentiation and proliferation (O'Garra, 1992) and it can co-stimulate the proliferation of NK cells (Cai et al., 1999). In conclusion, this knowledge suggests that the successful resolution of an immune process requires a coordinated answer between the initial pro-inflammatory response and the limitation of this response by IL-10 (amongst other proteins) to avoid immune-related pathology. Hence, IL-10 belongs to an intricate regulatory network that is involved in multiple processes in the immune system.

1.6.4 The role of IL-10 in the Central Nervous System

For decades, the brain was considered an immune-privileged site due to the believed absence of an immune response. Nowadays, it is known that active immune surveillance occurs in the CNS and that the resident innate immune cells act as the first line of defence, similarly as in the periphery (Lobo-Silva et al., 2016). Several cells in the CNS - including neurons, microglia, astrocytes and endothelial cells – express PRRs, thus contributing to the innate immune response (Lampron et al., 2013). Consequently, the CNS is also provided with a regulatory network to limit the acute

inflammatory phase and the potential neurological damage. IL-10 is a known regulator of immune cells in the CNS as IL-10, as well as other suppressive cytokines like IL-4 and IL-13, is expressed by glial cells in order to reduce the level of pro-inflammatory cytokines after an inflammatory response (Ledeboer et al., 2002). For example, IL-10 production by microglia and astrocytes has been detected *in vitro* after TLR activation as well as after induction in a PRR-independent manner (Burmeister & Marriott, 2018). Moreover, IL-10 also acts on the microglia-astrocyte crosstalk, since the cytokine enhanced the production of TFG-β on astrocytes, which in turn, can modulate the activation of microglia (Norden et al., 2014). More importantly, IL-10 production by resident CNS cells has also been documented *in vivo*. For example, an intra-nigral LPS injection in wild-type mice, led to IL-10 production via TLR4 stimulation (Arimoto et al., 2007; Henry et al., 2009). In another preclinical study, IL-10 was upregulated in microglia in a traumatic brain injury model (Izzy et al., 2019). Moreover, the presence of IL-10 can prompt microglia and astrocytes to differentiate into their neuroprotective phenotypes (Olson, 2004; Fernandes et al., 2014).

On another note, pro-inflammatory cytokines such as IL-6 and TNF-α have demonstrated to stimulate IL-10 production in microglia *in vitro*, which may seem contradictory but in fact is a mechanism to self-regulate inflammation (Sheng et al., 1995). All this data taken together indicates that IL-10 also plays a critical role in the resolution of the glial inflammatory responses in the CNS, thus limiting inflammation in the brain.

1.6.5 IL-10 role as a neuroprotective and anti-inflammatory strategy

Since IL-10 has often been seen as the quintessential anti-inflammatory cytokine, multiple attempts to investigate its potential as a therapeutic have been made, specifically in autoimmune diseases. As well, IL-10 treatments have been investigated for a plethora of neurological diseases such as neuropathic pain, multiple sclerosis, stroke or spinal cord injury (Saxena et al., 2015). IL-10 therapy - in the form of administration of recombinant IL-10, enhancement through agonists or continuous delivery through viral vectors - has been used in an attempt to treat almost any immune-related disease, usually with poor results (Lobo-Silva et al., 2016). Although the sole delivery of IL-10 might not be sufficient to address such complex pathologies,

these investigations have brought extensive knowledge on the neuroprotective and anti-inflammatory properties of IL-10.

While the best characterised effects of IL-10 are anti-inflammatory, IL-10 can also have neuroprotective effects directly on neurons. For instance, IL-10 treatments in primary cortical neuron cultures resulted in increased neuronal survival after exposure to oxygen-glucose deprivation or glutamate toxicity (Sharma et al., 2011). Furthermore, IL-10 induced neuroprotection was reported in a rodent model of ischemic stroke (Spera et al., 1998). Similarly, IL-10 acted as a neuroprotective agent against glutamate-induced apoptosis in primary cultures of embryonic spinal cord (Zhou et al., 2009) and increased tissue sparing and functional recovery was concurrently observed in spinal cord injuries (Thompson et al., 2013).

1.6.5.1 IL-10 for Parkinson's disease

Neuroinflammatory processes have been linked to Parkinson's disease, mainly identified as a driver of Parkinson's disease neurodegeneration (Yan et al., 2014). However, the information available about the relationship between IL-10 and Parkinson's disease is quite limited. Many studies have investigated the association between IL-10 polymorphisms and the risk of developing Parkinson's disease, but most of them found no correlation (Bialecka et al., 2007, 2008; Li et al., 2012; Liu et al., 2016). In parallel, some studies reported that Parkinson's disease patients had increased IL-10 levels in serum (Brodacki et al., 2008; Rentzos et al., 2009). More recently, Garcia-Esparcia et al., (2014) found out that IL-10 was downregulated in the substantia nigra of Parkinson´s patients. Thus it may be likely that some immunological disturbances related to IL-10 exist in Parkinson's disease but to date, these immunological disturbance characteristics remain unclear.

Since neuroinflammation is an important contributor to the pathogenesis of Parkinson's disease, anti-inflammatory agents are being studied as potential therapeutics (Hirsch & Hunot, 2009). The majority of experimental studies evaluating the therapeutic potential of IL-10 for Parkinson's disease have been conducted in animals. Due to the neuroinflammatory component of the neurodegeneration in

Parkinson's disease, the LPS model[11] has been extensively used in Parkinson's research. As already mentioned, IL-10 has proven to be protective against LPS-induced dopaminergic cell toxicity in rat primary mesencephalic neuron-glia co-cultures, since dopaminergic neuron degeneration was preserved through the inhibition of microglial activation (Qian et al., 2006a; Zhu et al., 2015). This inhibition of microglia exerted by IL-10 was stated to be due to downregulated pro-inflammatory factors typically activated or released by these cells such as induced nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), IL-1β and TNF-α (Zhu et al., 2015). More experiments support this neuroprotective effect dependent on microglia, as IL-10 failed to protect dopaminergic neurons in cultures lacking NADPH oxidase, a key enzyme for immune cells (Qian et al., 2006b). In an additional study, Qian et al., (2006a) attributed the neuroprotective effect of IL-10 on dopaminergic neuron degeneration to the reduction of LPS-stimulated microglial activation (again IL-10 inhibited the microglial production of TNF-α, NO and ROS). Nevertheless, in another *in vitro* study using neuron-enriched cultures from VM tissue, IL-10 did reduce the LPS-associated neuronal cell death in the absence of glial cells (Zhu et al., 2015).

Thus, the mechanisms underlying the neuroprotective effects of IL-10 are thought to be due, in part, to the suppression of microglial activation, since IL-10 has been reported to inhibit pro-inflammatory cytokines released by microglia such as TNF-α, IL-1β, IL-6, ROS and NO both *in vitro* and *in vivo* (Sheng et al., 1995; Ledeboer et al., 2000; Molina-Holgado et al., 2001; Sawada et al., 2001) (**Figure 1.10**). Contrastingly, some data suggests that IL-10 could directly protect dopaminergic neurons from LPS toxicity, but the underlying mechanism has not been elucidated. For example, in rodent models, the infusion of 50 ng/day of IL-10 using an osmotic mini-pump for 14 days into the substantia nigra after an LPS intra-nigral injection attenuated the degeneration of dopaminergic neurons by 50% and decreased the number of activated microglia (Arimoto et al., 2007). In contrast to the previous study, a single injection of recombinant IL-10 (5 – 50 μg) in the substantia nigra prior to LPS injection did not confer neuroprotection to the dopaminergic neurons (Arimoto et al.,

[11] LPS is the major component of the outer membrane of Gram-negative bacteria and it is used as an *in vitro* and *in vivo* inflammation model since it induces a potent stimulation of immune and glial cells causing the release of pro-inflammatory cytokines and free radicals.

2007). Thus, it seems that the repeated administration of IL-10 can have neuroprotective effects on dopaminergic cells *in vivo* as well.

The anti-inflammatory action of IL-10 has also been investigated in nigrostriatal degeneration models using 6-OHDA and MPTP. Similarly to GDNF, IL-10 cannot cross the BBB and has a short half-life *in vivo* (Kastin et al., 2003), therefore a sustained release of IL-10 is sought using viral vectors.

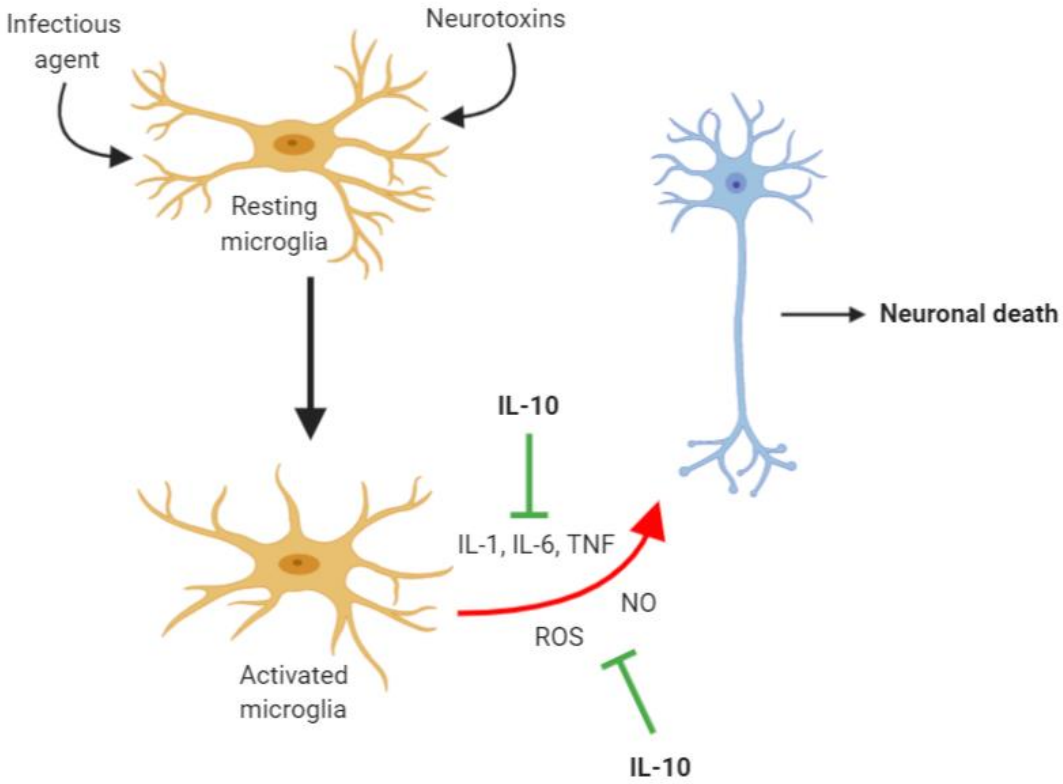

Figure 1.10 Effects of IL-10 on microglial activation. IL-10 can inhibit the release of pro-inflammatory cytokines and factors released by activated microglia. IL: interleukin, TNF: tumor necrosis factor, ROS: reactive oxygen species, NO: nitric oxide.

In the 6-OHDA lesion model, human IL-10 delivered to the striatum by an AAV2 vector reduced the nigral dopaminergic neuronal loss and the dopamine striatal deficiency by 50 and 20% respectively, after a 6-OHDA intra-striatal injection (Johnston et al., 2008). Moreover, in this study, IL-10 ameliorated the motor deficits produced by 6-OHDA assessed by rotational and forelimb akinesia tests (Johnston et al., 2008). Surprisingly enough, in this study IL-10 administration resulted in increased levels of IL-1β in the striatum (even in the absence of 6-OHDA), suggesting that IL-10 action in the substantia nigra is mediated through other mechanisms than suppressing pro-inflammatory cytokines.

The MPTP model induces glial activation and an inflammatory reaction followed by the degeneration of dopaminergic neurons. In an experiment using the rodent MPTP model, the preventive delivery of AAV2-hIL-10 reduced the neurodegenerative effects of this toxin (Joniec-Maciejak et al., 2014). Some data suggests that this protective effect of IL-10 in the MPTP model might be mediated through the inhibition of iNOS, highly involved in neuroinflammation processes (Schwenkgrub et al., 2013).

Hence, IL-10 is mostly reported to provide neuroprotection to dopaminergic cells by reducing the microglial activation and other inflammatory processes. However, fewer reports also indicated that IL-10 can protect dopaminergic and non-dopaminergic neurons directly. In summary, IL-10 has shown to have neuroprotective effects in the most relevant neurodegeneration models. Nevertheless, we must bear in mind that these toxins (6-OHDA, MPTP and LPS) induce microglial activation preceding the dopaminergic neurodegeneration and that this assumption is still controversial in the human pathogenesis of Parkinson's disease. To date, no studies evaluating the efficacy of IL-10 related therapies in Parkinson's patients have been carried out.

1.6.5.2 IL-10 for primary dopaminergic neuron transplantation

As abovementioned, IL-10 has been proven to protect dopaminergic neurons as well as to reduce the microglial activation both in *in vitro* and in *vivo* models of Parkinson's disease when administered locally in neuronal cultures or in the nigrostriatal pathway. However, the potential of a localised delivery of IL-10 to protect dopaminergic cells after transplantation has not been studied before.

Consequently, we hypothesise here that if IL-10 can protect the dopaminergic neurons by modifying the host innate immune response in neurodegeneration models, the administration of IL-10 could also be beneficial for dopaminergic cells undergoing cell transplantation processes. As discussed previously, dopaminergic cell survival is hampered by multiple factors but it is believed that the neuroinflammation produced by the host innate immune response is a central aspect of the massive cell death experienced upon transplantation. Therefore, in view of the ability of IL-10 to directly modify the host innate immune response in the CNS, we believe that this anti-inflammatory cytokine is a promising therapeutic factor that could potentially reduce the host innate immune response and eventually enhance the dopaminergic cell survival of ventral mesencephalic grafts.

1.7 HYPOTHESIS

Based on the evidence that biomaterials and growth factor therapy can improve the delivery and survival of the dopaminergic transplanted cells, we hypothesise that the use of an anti-inflammatory loaded collagen hydrogel will improve the survival of the transplanted cells, reduce the motor functional deficits and ameliorate the host innate immune response associated with the transplantation process. Furthermore, taking advantage of the adaptability of biomaterials, a multi-modal collagen hydrogel loaded with both an anti-inflammatory cytokine and a neuroprotective factor would be developed. We hypothesise that the combination of both factors will have a synergistic effect that will improve the survival of the transplanted cells, reduce the motor functional deficits and ameliorate the host innate immune response.

1.8 THESIS OBJECTIVES

The main aim of the work described here was to develop and evaluate an anti-inflammatory loaded collagen hydrogel for intra-striatal cell delivery in Parkinson's disease. Furthermore the efficacy of a multi-modal collagen hydrogel enriched with neuroprotective and anti-inflammatory moieties for intra-striatal cell delivery for Parkinson's disease was also evaluated.

Specifically, our goals were to:

- Characterise the best collagen hydrogel composition for its intra-striatal use in cell and protein delivery strategies.
- Optimise the best collagen hydrogel composition for its use in intra-striatal dopaminergic cell delivery in *in vivo* parkinsonian rodent models.
- Assess the effects of an IL-10 rich collagen hydrogel on the survival of the transplanted VM cells and the host immune response in hemi-parkinsonian rats.
- Assess the effects of an IL-10 and GDNF rich collagen hydrogel on the survival of the transplanted VM cells and the host immune response in hemi-parkinsonian rats.

CHAPTER 2: MATERIALS AND METHODS

2.1 ETHICAL STATEMENT

All procedures were approved by the Animal Care and Research Ethics Committee (ACREC) of the National University of Ireland, Galway, and carried out under the project licences issued to Dr. Eilís Dowd by the Irish Department of Health and Children (B100/3827) and the Irish Health Products Regulatory Authority (AE19125/P063), and under an Individual Authorisation issued to Ms. Sílvia Cabré (AE12125/I157), and carried out in compliance with the European Union Directive 2010/63/EU and S.I No. 543 of 2012.

2.2 GLOBAL EXPERIMENTAL DESIGN

The overall aim of this thesis was to develop and assess a multi-modal collagen hydrogel scaffold for the delivery of dopaminergic neurons to the hemi-parkinsonian brain (**Figure** 2.1). Initially, the properties of a biomaterial relevant to cell scaffolding and protein delivery were studied *in vitro* (Chapter 3) and further *in vivo* optimisation was carried out to select the best IL-10 collagen hydrogel for intra-striatal delivery (Chapter 4). After we proceeded to assess the effect of an IL-10 loaded collagen hydrogel on the long-term survival of VM cells and in the mitigation of the host immune response (Chapter 5). Later, the effects of an IL-10 and GDNF loaded collagen hydrogels were assessed for their efficacy on cell survival and reduction of the host immune response (Chapter 6).

In Chapter 3, we sought to characterise the mechanical and chemical properties of several collagen hydrogel compositions in order to identify the optimal biomaterial for intra-striatal delivery of dopaminergic cells. Once this was established, the cytocompatibility of the selected collagen hydrogels was determined as well as their suitability for anti-inflammatory cytokine delivery. These studies allowed us to proceed with *in vivo* studies with the optimised collagen hydrogel compositions.

In Chapter 4, we sought to observe how the collagen hydrogels performed *in vivo,* evaluating the biomaterial´s ability to deliver and retain IL-10 in the striatum.

Once this was established, the effects of the IL-10 loaded collagen hydrogel were assessed on dopaminergic neuron survival and reduction of the host immune response and are described in Chapter 5.

In Chapter 6, we sought to determine the effects of a multi-modal collagen hydrogel – loaded with both IL-10 and GDNF – on the long-term survival of dopaminergic neurons and on the reduction of the host immune response.

This chapter will provide details of all the different methodologies used throughout this thesis, although detailed experimental designs of each study will be provided in the relevant results chapters.

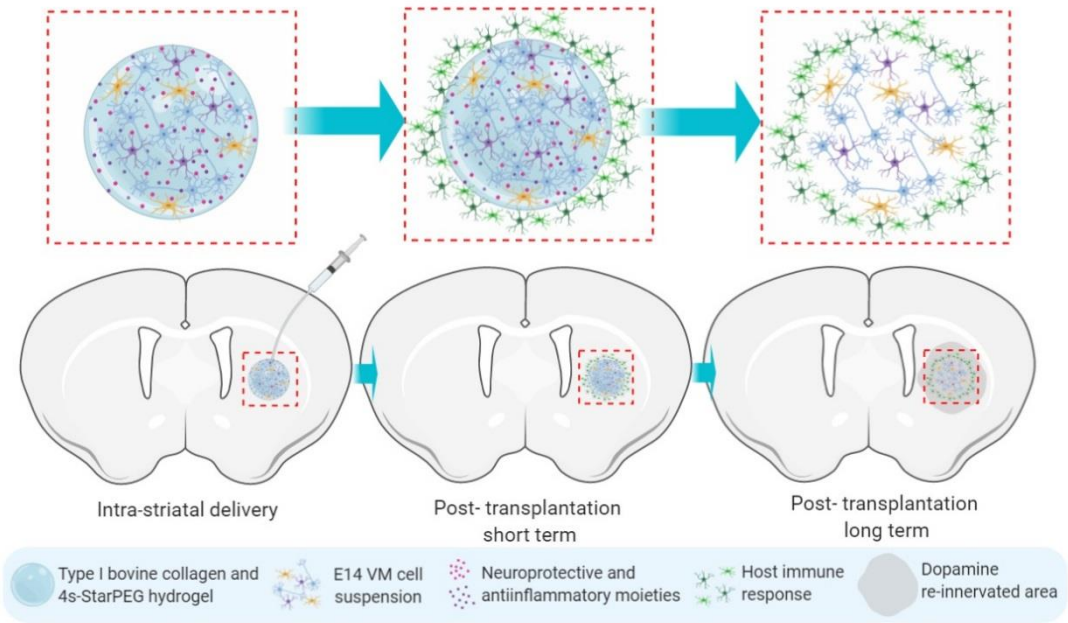

Figure 2.1 Primary thesis aim. This schematic overview illustrates the overall aim of the thesis; to develop a multi-modal collagen hydrogel scaffold for the delivery of dopaminergic neurons to the Parkinsonian brain. Primary dopaminergic cells were encapsulated with neuroprotective and anti-inflammatory factors in a collagen hydrogel to be implanted in the striatum. The collagen hydrogel retained the factors around the cells and was proposed to act as a barrier against the host immune response. This could facilitate the survival of the transplanted cells at long-term, even when the collagen hydrogel has been degraded.

2.3 ANIMAL HUSBANDRY

A total of 207 Sprague Dawley rats were used to complete this research. All animals were sourced from Charles River, UK. All animals were housed in groups of two or four per cage (depending on their body weight to ensure enough free moving space), in plastic bottom cages (50.50 x 13 x 24 cm) with a wire grid lid and 3R lab basic bedding. Each cage contained sizzle-nest and circular hollow plastic tunnels as environmental enrichment. Animals were kept on a 12:12 light/dark cycle (lights on at 8:00), at 20-24ºC, with relative humidity levels at 40-70%. For the duration of the experiments, animals were allowed food and water *ad libitum*, unless specifically stated for behavioural tests. In these cases, rats were food restricted for 24h maximum, with enough food to maintain their weight at 90% of their free-feeding weight as assessed by comparison to Charles River animal growth curve[12].

All behavioural testing and *ex vivo* analyses were performed blind to the treatment of the animals.

2.4 CHARACTERISATION OF COLLAGEN HYDROGELS

2.4.1 Fabrication of bovine type I collagen hydrogels

Before fabricating collagen hydrogels, all components needed (1x Phosphate Buffered Saline, PBS, 10x PBS, 0.1 M NaOH) were autoclaved to sterilise them. During the preparation of collagen hydrogels, all components were maintained on ice to prevent premature gelation. For a final volume of 100 µl of collagen hydrogel, 40 µl of 5 mg/ml bovine type I atelocollagen (Vornia Biomaterials, now Blafar Ltd), neutralised with 0.1 M NaOH until pH 7 reached, was added to 20 µl of 1x PBS containing 0.1, 0.2, 0.4, 0.6 or 1.2 mg of poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4s-StarPEG). 40 µl of either 10x PBS (standard hydrogels), cell suspension (seeded hydrogels), IL-10 solution (IL-10 loaded hydrogels) or IL-10 alone or IL-10/GDNF enriched cell suspension (IL-10 or IL-10/GDNF loaded hydrogels) was then added to the collagen-crosslinker in PBS solution and mixed thoroughly.

[12] Charles River website - Sprague Dawley Rat Growth Chart: https://www.criver.com/products-services/find-model/cd-sd-igs-rat?region=3671

For a final volume of 100 µl of collagen hydrogel using bovine type I atelocollagen from Collagen Solutions (Collagen Solutions), 31.7 µl of 6.3 mg/ml bovine type I atelocollagen (Collagen Solutions), neutralised with 0.1 M NaOH until pH 7 reached, was added to 28.3 µl of 1x PBS containing 0.1, 0.2, 0.4, 0.6 or 1.2 mg of 4s-StarPEG. 40 µl of either 10x PBS (standard hydrogels), cell suspension (seeded hydrogels), IL-10 solution (IL-10 loaded hydrogels) or IL-10 alone or IL-10/GDNF enriched cell suspension (IL-10 or IL-10/GDNF loaded hydrogels) was then added to the collagen-crosslinker in PBS solution and mixed thoroughly.

For *in vitro* experiments, 6 µl or 50 µl drops of the collagen-crosslinker mixture were transferred to a previously UV-sterilised hydrophobic surface (Teflon® tape) and placed at 37ºC to polymerise (**Figure** 2.2). For *in vivo* experiments, collagen hydrogels were kept on ice until transplantation to prevent gelation.

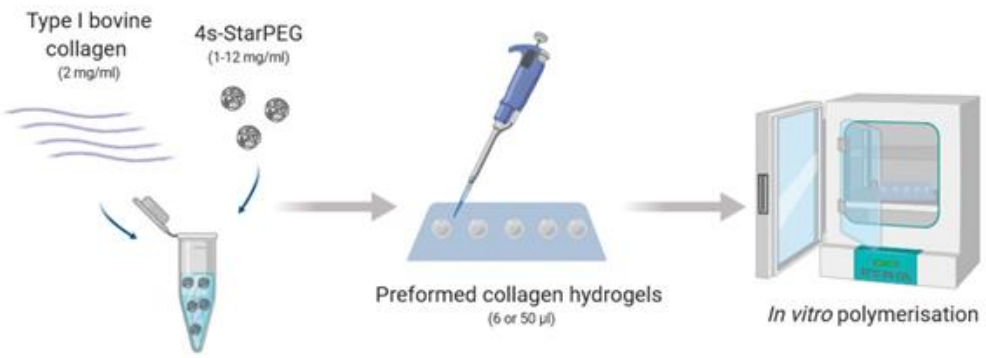

Figure 2.2 Collagen hydrogel fabrication for *in vitro* use. For a total volume of 100 µl of collagen hydrogel solution, 40 µl of type I bovine collagen (5 mg/ml) were mixed with 20 µl of 4s-StarPEG (diluted in PBS at the desired concentration) and added to 40 µl of PBS 10X. After thoroughly mixing the solution, 6 or 50 µl drops of the collagen-crosslinker solution were placed on a hydrophobic surface and placed at 37°C to polymerise.

2.4.2 SEM imaging

Prior to SEM imaging, seeded collagen hydrogel samples were fixed with a 2% glutaraldehyde buffer (2% glutaraldehyde + 2% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.2) for 2h at room temperature to preserve the biomaterial and

cellular structures. After, seeded collagen hydrogel samples were secondary fixed with osmium tetroxide buffer (1% osmium tetroxide in 0.1M sodium cacodylate buffer pH 7.2). Unseeded collagen hydrogels did not undergo fixation since they were immediately frozen in liquid nitrogen.

To visualise the differently crosslinked collagen hydrogels, 200 µl of unseeded collagen hydrogels of increasing concentrations of crosslinker (2-12 mg/ml) were fabricated and frozen immediately in liquid nitrogen. Once frozen, the collagen hydrogels were broken into small pieces in order to be able to visualise the inside of the sample. The small pieces were then frozen at -80°C for an hour and next the samples were freeze-dried overnight. Afterwards, samples were placed into aluminium stubs and gold-coated in a gold sputter coater Emitech K550 (Quorumtech, UK) or Emscope SC500 (Quorumtech, UK). Images from the sample's structure were taken using a Hitachi S-4700N Pressure Scanning Electron Microscope (Hitachi, UK).

For data analysis, for pore diameter measurements of at least three randomly-selected images were taken per collagen hydrogel composition and twenty pores were analysed per composition using ImageJ software.

To observe the distribution of the cells in the biomaterial, 200 µl of unseeded collagen hydrogels of increasing concentrations of crosslinker (4-12 mg/ml) were fabricated with either a low ($5x10^5$ cells/ml) or high ($5x10^6$ cells/ml) concentration of SH-SY5Y cells. The seeded collagen hydrogels were fixed either immediately after fabrication or after a 48h incubation period in plating media at 37ºC. To visualise the cell-seeded collagen hydrogels, three alternative protocols compatible with cell structure were carried out once the samples were fixed. Chemical drying, critical point drying and variable pressure protocols were undertaken to select the best one for cell-seeded collagen scaffold visualisation.

Chemical drying consisted of dehydrating the samples in ascending ethanol concentrations (50%, 70%, and 100%), in two rounds of 15 minutes per concentration. After dehydrating the samples, samples were put in Hexamethyldisilazane (HMDS) for 2x15 minutes. After, HMDS was removed and the samples were placed in filter paper in a petri dish and left to air dry overnight in a fume hood. The next day, samples were mounted onto aluminium stubs and samples were gold sputter-coated as mentioned earlier.

For samples processed via critical point drying, samples were also dehydrated in a series of ethanol solutions (50%, 70%, 100%), in two rounds of 15 min for each concentration. Samples were then transferred into a Critical Point Dryer (Emitech K850, Quorumtech, UK), which dehydrated the samples with liquid CO_2. CO_2 is brought to its critical point[13], thus drying the sample but preserving the sample´s structure to a higher extent than the chemical drying protocol. The next day, samples were mounted onto aluminium stubs and samples were gold sputter coated as mentioned earlier.

For variable pressure protocol, samples were also dehydrated in a series of ethanol solutions up to 70% (50%, 70%) in two rounds of 15 min for each concentration. Afterwards, samples were mounted onto aluminium stubs and samples were gold sputter coated as mentioned earlier. These samples were observed under low vacuum using variable pressure in a Hitachi S-2600N Pressure Scanning Electron Microscope (Hitachi, UK).

Structural analysis of the collagen hydrogels was observed using a Hitachi S-2600N Pressure Scanning Electron Microscope (Hitachi, UK). The SEM images were taken using an accelerating voltage of 15 kV and a high vacuum mode.

2.4.3 *In vitro* gelation and degradation assays

The polymerisation profile of injectable scaffolds is of utmost importance for a successful *in vivo* delivery. To study the effects of crosslinker concentration for the collagen hydrogel gelation profile *in vitro*, 150 µl of collagen hydrogel (with 1-12 mg/ml of crosslinker) were fabricated as stated before in section 2.4 and 50 µl drops of the collagen-crosslinker mixture were placed on a hydrophobic surface (Teflon® tape) and put in the incubator at 37°C. Samples were regularly checked until full polymerisation was detected. To evaluate if a sample was completely polymerised, samples were taken out of the incubator and each spherical hydrogel was rolled alongside the hydrophobic surface with a pipette tip. If the samples are fully

[13] The critical point of an element is the temperature and pressure where no distinction can be made between liquid and gas states. The critical point of CO_2 is 31°C and 72.9 atm (Atkins & De Paula, 2010).

polymerised, they can be moved around the hydrophobic surface without losing their shape or leaking any liquid, thus behaving as solid gels.

Similarly, studying both *in vitro* and *in vivo* degradation profile is important for a satisfactory release of any protein or drug of interest from the biomaterial. For *in vitro* degradation experiments, to study the crosslinker concentration's effects in the collagen hydrogel gelation profile *in vitro*, 150 µl of collagen hydrogel (with 2-12 mg/ml of crosslinker) were fabricated as stated before in section 2.4.1 and 50 µl drops of the collagen-crosslinker mixture were placed on an hydrophobic surface (Teflon tape®) and put in the incubator at 37 °C. Collagen hydrogels with 1 mg/ml of crosslinker were not tested since they take a very long time to fully polymerise and sometimes the polymerisation only occurs partially. Once the samples were fully polymerised, the collagen hydrogels were placed in a filtered solution of Coomassie brilliant blue (0.1 % w/v of Coomassie Brilliant Blue R, B0770, Sigma, Ireland in 50 % methanol + 5 % acetic acid glacial) for 6h at room temperature. Excess of staining solution was removed and Coomassie de-staining solution was added (20 % methanol + 5 % acetic acid) overnight with 60 rpm shaking at room temperature to remove the excess of staining. Next, de-staining solution was removed and samples were washed three times in water. Then, samples were put in water or plating media made of Dulbecco's modified Eagle's Medium/F-12 (DMEM/F-12, D6421, Sigma, Ireland). The Coomassie blue release from the hydrogels was measured as an indirect measurement of collagen hydrogel degradation over time by spectrophotometric readings of the supernatant at 595 nm. Blank readings of the diluting agent (water or media) were also performed and were used to calculate the % of Coomassie blue release from the collagen hydrogels. Data was shown as mean % of dye release ± SEM.

2.4.4 Rheology

Mechanical properties of biomaterials are also relevant when delivering a hydrogel *in vivo*. Amplitude sweeps are useful to analyse the behaviour of hydrogels since they can determine if a sample is behaving as a liquid or as a solid. To evaluate the mechanical properties of the collagen hydrogels, 600 µl of collagen hydrogel (with 2-12 mg/ml of crosslinker) were fabricated as stated before in section 2.4 and macro drops of 200 µl of the collagen-crosslinker mixture were placed on an hydrophobic

surface (Teflon® tape) and put in the incubator at 37 °C. Once the samples were fully polymerised, samples were placed into a Rheometer (MCR 302, Anton Paar GmbH, Austria) with a parallel plate of 9.975 mm (PP10 Anton Paar GmbH, Austria), ensuring the sample covered the entire base of the plate. An amplitude-sweep protocol was run; the frequency was fixed at 1 Hz and the amplitude of the shear stress range was set from 0.01% to 100%, meaning that the sample was deformed at several intensities and the samples' mechanical behaviour was recorded.

Frequency sweeps can determine the gel character, thus the degree of crosslinking. Frequency sweeps were also run on freshly made collagen hydrogels (with 2-12 mg/ml of crosslinker) using a frequency range of 0.01 to 10 Hz and a fixed shear strain of 1%. Data was shown as mean of storage or loss moduli ± SEM.

2.5 CELL CULTURE & ASSAYS

2.5.1 Culture of SH-SY5Y cells

The neuroblastoma SH-SY5Y cell line is a subline from the SK-N-SH cell line established in culture from a bone marrow biopsy of a 4-year-old female in 1970 (Biedler et al., 1978). The SH-SY5Y cell line expresses tyrosine hydroxylase – the enzyme that converts tyrosine to L-dopa – and dopamine-β-hydroxylase – which converts L-dopa to noradrenaline (Biedler et al., 1978; Ross & Biedler, 1985). Despite SH-SY5Y displaying a catecholaminergic phenotype and not being a purely dopaminergic cell line, its human origin and its ease of maintenance resulted in one of the main cell lines to study Parkinson's disease *in vitro* (Xicoy et al., 2017). SH-SY5Y cells were purchased from ATCC (CRL-2266^{TM})(**Figure** 2.3).

In this study, the SH-SY5Y cell line was used mainly for optimisation purposes, since high amounts of cells are easier and quicker to obtain than primary dopaminergic cells.

SH-SY5Y cells were cultured in feeding media made of DMEM/F-12 (D6421, Sigma, Ireland) supplemented with 10% Fetal Bovine Serum (FBS) (10270-106, Gibco, USA), 1% L-glutamine (G7513, Sigma, Ireland), 1% Pen-Strep (P4333, Sigma, Ireland). They were expanded in a T-75 flask (83.3911.002, Starstedt, Germany) in a sterile incubator at 37°C and 5% CO_2 and feeding media was changed every 2 days.

Cells were passaged when 80% confluency was reached. For maintenance, preservation or experiments, cells were sub-cultured. In a sterile flow-hood, media was removed from the flask and discarded. Then, cells were washed once with warmed sterile PBS (D8537, Sigma, Ireland) followed by a 3 min incubation in warmed trypsin (T4174, Sigma, Ireland) solution (10% trypsin in HBSS, H9394, Sigma, Ireland) at 37°C. After checking that cells were detached from the flask, trypsin was neutralised by adding an equal volume of feeding media. The resulting cell suspension was centrifuged at 1400 rpm for 5 min at room temperature. Afterwards, the trypsin-media supernatant was discarded and the cell pellet was re-suspended in feeding media. For culture maintenance, the cell pellet was re-suspended in fresh warmed media and cells were placed in a T-75 back in the incubator. For experiments or preservation, the cell pellet was re-suspended in 1 ml of media for counting.

For counting, a small amount of sample was taken from the cell solution and was diluted 1:10 in trypan blue (T8154, Sigma, Ireland). 10 µl of the corresponding solution were pipetted into a Neubauer-improved Haemocytometer chamber (AC1000 Improved Neubauer, Hawksley, UK). The trypan blue allows the visualisation of the cells under a microscope, while allowing to discern viable from dead cells. The grid of the haemocytometer is used as a reference when counting cells in an optical microscope.

Living cells in the four larger squares of the chamber were counted (**Figure** 2.4**a**). Briefly, living cells inside the squares were counted and the cells touching the upper and left limits were also included in the cell counts, unlike cells touching the lower and right limits, which were not taken into account (**Figure** 2.4**b**). This methodology provides an accurate estimation of cells. Furthermore, the counting procedure was performed in a zig-zag technique to ensure for accurate counting (**Figure** 2.4**c**). Once the four squares were counted, the average cell count was calculated and the dilution factor and the chamber volume were taken into account as depicted in **Figure** 2.4.

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Figure 2.3 SH-SY5Y neuroblastoma cell line. SH-SY5Y cells purchased from ATCC were used for all the experiments (Image extracted from ATCC).

After counting, SH-SY5Y cells were seeded at 100,000 cells/well of a 24 well-plate (50,000 cells/cm^2) and left for 24h to attach.

For storage, attached cells were lifted from the T-75 using trypsin as stated above, counted, re-suspended in cryopreservation media (DMEM/F-12 with 5% DMSO, D8418, Sigma Ireland) and placed in a cryopreservation vial (72.380, Starstedt, Germany). Vials were stored in a -80°C freezer using a Mr. Frosty Freezing Container (5100-0001, Thermo Fisher, Ireland) which regulates the cooling rate (approximate 1°C/min). Later, cells were transferred to a Liquid Nitrogen Tank.

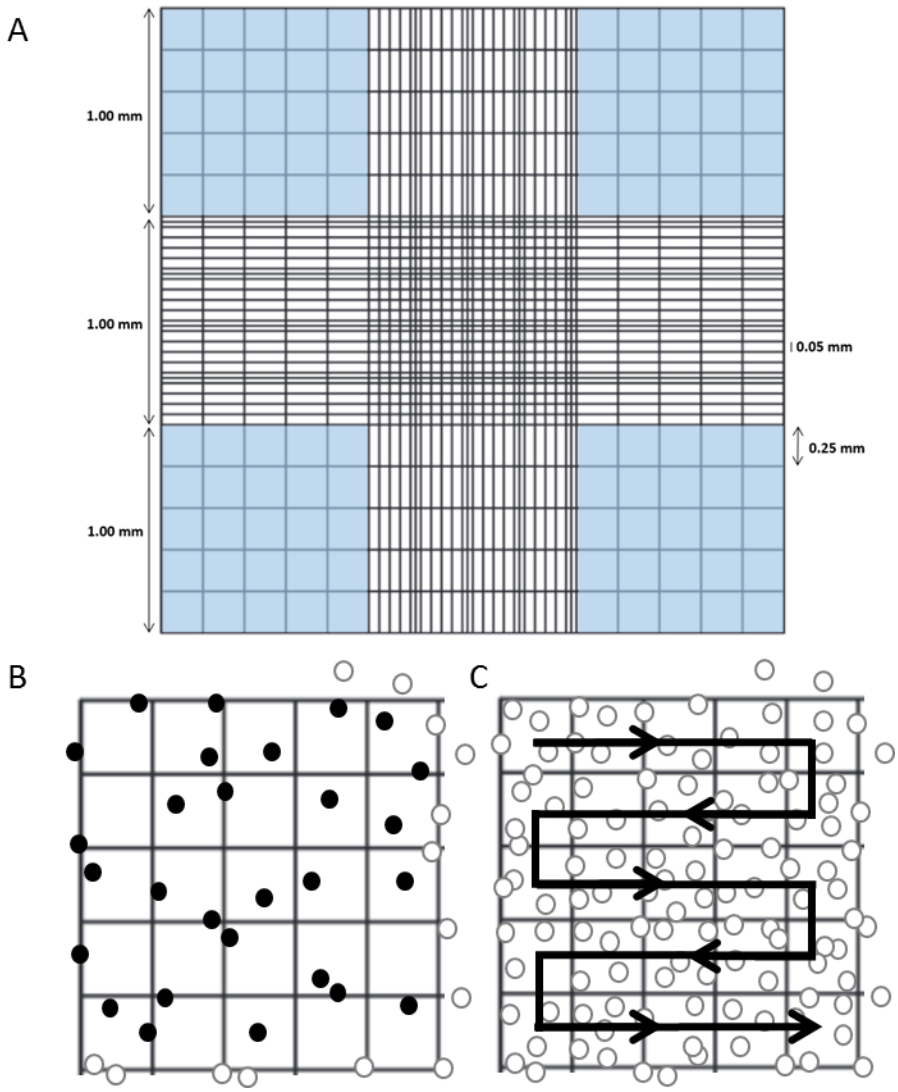

Figure 2.4 Cell counting in an improved-Neubauer Chamber. A) Cell counts were carried out in the corner squares shown in blue. B) To ensure for an accurate cell count, cells on the borders of the squared were only counted for two out of the four borders and each sub-square was counted in a zig-zag mode (C), to avoid double counting.

2.5.2 Ventral Mesencephalon dissection, preparation and culture

VM cultures or VM dissociated cells for transplantation were obtained from embryonic E14 rat tissue as described in Dunnett & Björklund, (1997). Briefly, 14 day time-mated female Sprague-Dawley rats (sourced from Charles River, UK) were anesthetised with isoflurane (5% O_2) in an induction chamber and were quickly decapitated using a guillotine. E14 embryos were quickly removed from the pregnant female rat by separating the horn by a laparotomy, using a tooth grasping forceps and scissors. The horn with the embryos was put in a petri dish filled with clear Hank's Balanced Salt Solution (HBSS, H8264, Sigma, Ireland) and kept on ice. All the embryos were removed from the uterine horn and their respective uterine yolk sac by using small surgical scissors and a curved forceps and also put in ice cold HBSS. All the embryos underwent three washes of ice cold HBSS, to ensure the complete removal of blood. Then, further dissection was carried out in an optical microscope (**Figure 2.5**). First, an incision was made just behind the eye and another one in the base of the brain with curved forceps and dissecting micro-scissors to remove the mesencephalon from the embryo. Then, a longitudinal cut alongside the neural tube in the mesencephalic tissue was made to unfold the ventral mesencephalon. This step results in a butterfly-shape piece of tissue (**Figure 2.5**). Following this, the meningeal tissue was removed carefully by "peeling" the meningeal layer from the tissue layer. Then, two longitudinal cuts through the lateral sides were performed to discard the outer parts of the mesencephalon. E14 VM dissected tissue was kept in HBSS on ice until dissociation.

Dissected VM tissue was centrifuged at 1400 rpm for 5 min at room temperature. The resulting pellet was diluted in 40% trypsin-HBSS solution for 4 min at 37°C with 5% CO_2. After, FBS was added to neutralise the trypsin and then it was centrifuged at 1400 rpm for 5 min. Then, the cell pellet was re-suspended in 1 ml of plating media (Dulbecco's modified Eagle's Medium/F-12 supplemented with 0.6% glucose, 1% L-glutamine, 1% Pen-Strep, 1% FBS and 2% B-27) using a P1000 Gilson pipette, followed by a sterile 25 gauge needle syringe to ensure tissue was completely dissociated. Once a single cell suspension was achieved, 10 µl of the cell suspension were taken and mixed with 90 µl of trypan blue. Cells were then manually counted using a Neubauer Haemocytometer as mentioned in section 2.5.1. Later, cell suspension was centrifuged at 1400 rpm for 5 min at room temperature and the cell

pellet was re-suspended in the appropriate volume of plating media (for *in vitro* cell culture) or transplantation media (for *in vivo* transplantation).

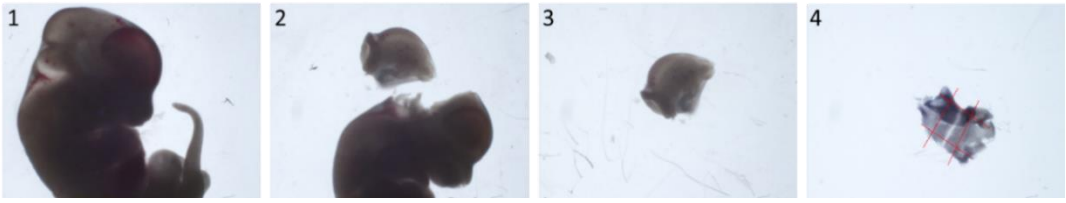

Figure 2.5 E14 rat embryo ventral mesencephalon dissection. First, (1) the embryo is removed from its yolk sac. Then (2) the whole mesencephalon is removed from the embryo by making two incisions in the forebrain-midbrain and in the midbrain-hindbrain boundaries followed by (3) a longitudinal cut through the neural tube. Finally (4) the outer parts of the mesencephalon are removed by making rostral and caudal cuts to ensure that the final tissue fragment only contains the ventral mesencephalon.

For *in vitro* studies, 100,000 cells/well of a 24-well plate (50,000 cells/cm^2) were plated down, from an initial solution of 2000 cells/µl into a 24-well plate previously coated with Poly-L-lysine (P4707, Sigma, Ireland). For *in vivo* transplantation surgeries, cells were re-suspended at 166,666 cells/µl or 222,222 cells/µl.

2.5.3 IL-10 release

SH-SY5Y cells were seeded at a density of 100,000 cells per well of a 24 well-plate (50,000 cells/cm^2) and left overnight to attach. The next day, SH-SY5Y cultures were incubated with unseeded preformed IL-10 collagen hydrogels (1 x 50 µl gels per well) with 4 mg/ml of 4s-StarPEG and 50 ng/ml of human IL-10 or left untreated. Media was removed at regular timepoints to assess the IL-10 release from the collagen hydrogels. IL-10 levels in the media were analysed using a human IL-10 ELISA kit. Data was shown as mean ± SEM of IL-10 release.

2.5.4 Poly I:C challenge and IL-10 loaded collagen hydrogel treatments

Polyinosinic:polycytidylic acid (Poly I:C) is a synthetic analog of double-stranded RNA. Its administration will produce an inflammatory response similar to what is evoked by a viral infection. VM cultures were seeded at a density of 100,000 cells per well of a 24 well-plate (50,000 cells/cm^2) and left overnight to attach. VM cultures were incubated with unseeded preformed IL-10 collagen hydrogels (1 x 50 µl gels per well) with 4 mg/ml of 4s-StarPEG and 50 ng/ml of human IL-10 or left untreated. After 1h incubation with collagen hydrogels, Poly I:C (20 or 50 µg/ml) was added to the wells or left untreated. After 24h incubation with Poly I:C, media was removed to analyse the release of pro-inflammatory cytokine IL-1β. Data was shown as mean ± SEM of IL-1β release.

2.5.5 Cell viability assays

VM cultures were seeded at a density of 100,000 cells per well of a 24 well-plate (50,000 cells/cm^2) and left overnight to attach. VM cultures were incubated with unseeded preformed collagen hydrogels (2 x 50 µl gels per well) of various 4s-StarPEG concentrations (1-4 mg/ml) for 48 h or left untreated. The metabolic activity of the cultures was measured as in indicator of cell viability using the alamarBlue® assay as previously described (Newland, 2013). In summary, 100 µl of a 10% solution of alamarBlue® (Invitrogen, Ireland) in HBSS was added to each well and incubated for 4h. Absorbance of sampled supernatant was read at 570 nm and 600 nm using a plate reader (Biotek Instruments, UK). Viability of VM cultures was assessed by normalisation of all results to controls following the manufacture's indications. Data was shown as mean ± SEM.

2.5.6 Calcium signalling

Briefly, VM cultures were loaded with the calcium-sensitive fluorescent probe Fluo-4 AM (Life Technologies) for 30 min at 37 °C, then washed and followed by 30 min incubation at 37 °C in the dark. Stimulation was achieved by the delivery of biphasic voltage pulses via platinum contacts on the stage of a Zeiss Axiovert 200 inverted microscope. The fluorescence was collected with an Orca 285 camera (Hamamatsu, Japan) and the software Openlab system. Changes in Ca^{2+} due to external stimuli were

measured via Adenosine triphosphate (ATP) and ATP was expressed as ΔF/F0, where F0 is the baseline fluorescence level and ΔF is the fluorescence when stimulated (Vallejo-Giraldo et al., 2016). Data was plotted as mean ATP response ± SEM and maximum ATP response ± SEM.

2.5.7 ELISA

Peprotech ELISA kit was used for rat IL-1β (900-K91) and R&D systems ELISA kit was used for human IL-10 (DuoSet DY217B). Briefly, capture antibody diluted in PBS was incubated in nunc MaxiSorp 96-well microplates (Thermo Scientific, UK) overnight. Plates were aspirated and washed three times with wash buffer (PBS with 0.05% Tween-20) in an automatic plate washer (ELx50 Biotek Instruments, UK). Then, the microplate was incubated with blocking solution (PBS with 1% bovine serum albumin) for 1h. After blocking, plates were aspirated and washed three times, and standard curve and experimental samples were incubated for 2h. Standard curve and experimental samples were diluted if needed in diluent solution (1% BSA in PBS). Plates were then aspirated and washed three times and followed by 1h detection antibody incubation. After three washes, samples were incubated in Avidin-HRP Conjugate for 20-30 min, followed by another three washes and incubation with ABTS (Peprotech) or TMB (R&D Systems) until colour development. Readings were collected using a colorimetric plate reader (μ-Quant, Biotek Instruments, UK) at 405 nm (Peprotech) or 450 nm (R&D Systems) and 570 nm wavelength (for correction). The standards were plotted and analysed using a spline fit line and unknowns were determined from this equation using GraphPad Prism.

2.5.8 Immunocytochemistry

VM cells were fixed in 4% PFA for 30 min before washing (3x 10 min) with PBS. To allow for permeabilisation, fixed cells were washed (3x 10 min) with tris-buffered saline (TBS) prior to incubation with blocking solution (TBS + 1% BSA, 5% normal goat serum, 0.3% Triton X-100, 0.01% Sodium azide) for 1h at room temperature. Afterwards cells were incubated overnight at room temperature with primary antibody (Mouse anti-TH, 1:1000, Millipore; Mouse anti-beta III tubulin, 1:333, Millipore, Rabbit anti-GFAP, 1:2000, DAKO Agilent) (see **Table 2.1**) prepared in TBS + 1% BSA and 0.01% Sodium Azide. After washing with TBS (3x 10 min), cells were

incubated with secondary antibodies (Rabbit anti-mouse Alexa Fluor 488 conjugated secondary antibody 1:1000 dilution, Biosciences or goat anti-rabbit Alexa Fluor 546 conjugated antibody 1:1000 dilution, Biosciences) (see **Table 2.2**) in TBS + 1% normal goat serum for 3h at room temperature. After secondary antibody incubation, cells were washed in TBS again (3x 10 min) and incubated in DAPI (1µg/ml in TBS) counterstaining for 5 min, followed by TBS washes (3x 10 min). Cell cultures were stored in 0.1% TBS azide at 4°C until imaging.

2.5.9 Image analysis

Fluorescent images were taken using a confocal microscope Olympus IX2-UCB (Olympus, Ireland) and Volocity software (Quorumtech, UK). Immunocytochemistry quantification was performed using ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA). Cell counts were measured as number of cell bodies (TH+ or GFAP+) in five randomly selected areas from the images. Data was presented as mean % of TH or GFAP cell bodies versus total cell counts.

To quantify immunofluorescent staining, the level of β-III tubulin florescence was quantified by measuring the threshold area of three randomly selected sample sites per well (**Figure 2.6**). Data was presented as mean ± SEM fluorescent area.

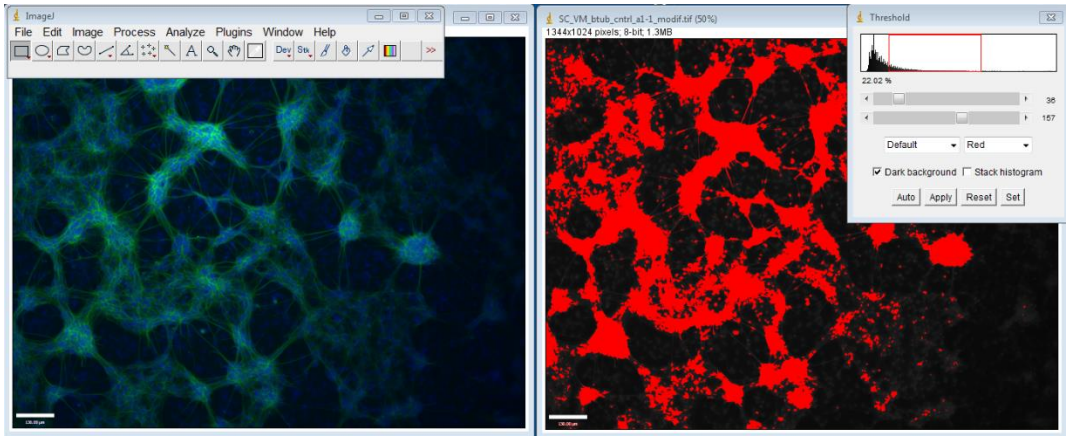

Figure 2.6 Threshold set up for β-III tubulin area measurements. Images taken from VM cultures were converted into grey-scale (8-bit) first, and then the threshold was set up in order to measure the fluorescent area of interest.

2.6 SURGERY

2.6.1 Stereotaxic surgery

All procedures were performed by trained and licenced personnel following aseptic techniques. All surgeries were performed by a surgeon – in charge of performing the surgery – and a non-sterile assistant – in charge of monitoring the animal breathing and temperature at all times. An analgesic drug (buprenorphine 0.01 mg/kg) was administered pre-operatively and post-operatively every 8-12 hours for 24h. All surgeries were performed under isoflurane anaesthesia (5% in O_2 for induction in an isoflurane chamber and 2% in O_2 for maintenance) in a stereotaxic frame with the nose bar set at -4.5 (for intra-MFB surgeries) or -2.3 (for intra-striatal surgeries). EMLA™ cream (lidocaine 2.5% w/w & prilocaine 2.5% w/w cream) was applied to the ears to reduce the potential pain caused by the stereotaxic ear bars. Similarly, Visidic® Eye gel (carbomer 0.2% w/w) was applied to the eyes to reduce potential discomfort caused by dry eyes.

The surgery area was shaved and the animal was secured to the stereotaxic frame using non-rupture ear bars by the non-sterile assistant. Animals were placed on a heating mat to keep their body temperature stable and temperature was monitored frequently. Then the surgeon disinfected the area with iodine and after, an incision was made through the skin on the head to expose the skull. An anaesthetic combination (lidocaine (0.5% w/v) with adrenaline (5 mg/ml) and bupivacaine (0.25% w/v)) was placed on the area prior to drilling. The coordinates for Bregma were established and used to calculate the site for cannula insertion. The injection cannula (30 gauge) was connected to a 50 µl Hamilton syringe via polyethene tubing (0.28 mm inner diameter) and filled with sterile saline. To expose dura mater, an electric drill was used. The cannula was lowered to dura mater to adjust the dorso-ventral coordinates. The specific toxin or cell/hydrogel suspension was slowly and carefully delivered to the desired coordinates through the monitoring of movement of a deliberately made air bubble in the polyethene tubing between the saline and the required suspension. The plunger on the Hamilton syringe was depressed at a steady rate of 1 µl/min using an automated pump (Harvard Apparatus, USA). Following injection, the cannula was left in place for a set diffusion time (2 min). After diffusion, the incision was sutured.

Animals were monitored throughout recovery before being placed back in their home cage.

2.6.2 Intra-MFB lesion surgeries

Lesion induction was performed using 6-OHDA in the Medial Forebrain Bundle (MFB). 6-OHDA was weighed out and dissolved in 0.01% sterile ascorbate saline at a concentration of 7.6 mg/ml. Dissolved 6-OHDA solution was kept on ice and in the dark. All lesion surgeries were performed under isoflurane anaesthesia (5% in O_2 for induction in an isoflurane chamber and 2% in O_2 for maintenance) in a stereotaxic frame with the nose bar set at -4.5. A total volume of 3 µl of 6-OHDA solution - at an infusion rate of 1 µl/min - was unilaterally infused in the MFB coordinates AP -4.0, ML -1.3 (from Bregma) and DV -7.0 below dura mater (**Figure** 2.7). After infusion, a further 2 min were allowed for diffusion.

Fig. removed due to copyright

Figure 2.7 Coordinates for intra-MFB lesion surgery. 6-OHDA was delivered to the MFB, corresponding to the following coordinates AP -4.0, ML -1.3, DV -7.0 and shown in the pictures by a red dot. Images extracted from the online version of Paxinos Atlas.

2.6.3 Intra-striatal surgeries

For the delivery of cells (VM cells), trophic factors (GDNF), anti-inflammatory proteins (IL-10) and collagen hydrogels (with or without cells and therapeutic moieties), all surgeries were performed under isoflurane anaesthesia (5% in O_2 for induction in an isoflurane chamber and 2% in O_2 for maintenance) in a stereotaxic

frame with the nose bar set at -2.3. The striatum was infused unilaterally or bilaterally at coordinates AP 0.00, ML ±3.7 (from Bregma) and DV -5.0 below dura mater (**Figure** 2.8). A total volume of 6 µl were infused at a rate of 1 µl/min with a 2 min diffusion time.

Fig. removed due to copyright

Figure 2.8 Coordinates for intra-striatal biomaterial delivery surgery. Collagen hydrogel was delivered to the striatum, corresponding to the following coordinates AP 0.00, ML ±3.7, DV -5.0 and shown in the pictures by a red dot. Images extracted from the online version of Paxinos Atlas.

2.7 BEHAVIOURAL TESTS OF MOTOR IMPAIRMENT

2.7.1 Methamphetamine-induced rotations

Methamphetamine-induced rotational behaviour was carried out as described previously (Ungerstedt & Arbuthnott, 1970). Rats were removed from their home cage and placed into plastic bowls containing standard bedding and allowed to habituate to their new environment for 10 minutes. After habituation, rats were injected intraperitoneally (i.p) with 2.5 mg/kg of methamphetamine. Full body rotations were manually counted on both sides – ipsilateral and contralateral from lesion site – for 1 minute in intervals of 10 minutes for a total of 60 minutes. Data was expressed as net ipsilateral turns/min.

2.7.2 Corridor test

The Corridor test evaluates the lateralised neglect in rats with unilateral dopamine-degenerating lesions, such as the ones developed after 6-OHDA injections (Dowd et

al. 2005a). In this test, 6-OHDA lesioned rats ignore the food presented in the contralateral side of their lesioned striatum and they mostly retrieve the food presented in their ipsilateral side.

The Corridor apparatus is a wooden box (150 cm long, 7 cm wide and 24.5 cm high) with two identical chambers, an empty one for habituation and another with lids separated every 10 cm where the food is placed (**Figure** 2.9).

Prior to the task, rats were food restricted overnight and their weight was monitored as specified before. On the test day, rats were placed in the habituation side of the corridor for 5 min. After habituation, rats were placed in the chamber containing CoCoPops®. CoCoPops® retrievals were counted in both ipsilateral and contralateral sides. The task was finalised when 20 retrievals were measured or 5 min were reached. Rats were habituated to the task for two weeks before a test day. On a test day, the task was carried out once per rat. The task was carried out for three consecutive days for each time point. Data shown in this thesis represents the mean ± SEM of all the attempts.

2.7.3 Stepping test

The Stepping test (Olsson et al., 1995) is a task for assessing forelimb kinesis. The rats' hindlimbs and one forelimb were restrained by the experimenter just allowing the rat to move the other forelimb. Rats were guided through a 90 cm long table at a steady pace, and were allowed to make steps with the free moving forelimb alongside the table in the forehand and backhand directions. The task was repeated with the other forelimb. The steps with the free moving forelimb were scored in each direction and paw. The number of steps made by each rat from the ipsilateral and/or contralateral sides were expressed as a sum of both directions (**Figure** 2.9).

Rats were habituated to the task for two weeks before a test day. On a test day, the task was carried out three consecutive times per rat. The task was carried out for three consecutive days for each time point. Data shown in this thesis represents the mean ± SEM of all the attempts.

2.7.4 Whisker test

The Whisker test (Schallert et al., 2000) measures for sensorimotor integration. This task harnesses the reflex response of the rat of placing the free moving forelimb on a table when its whiskers are brushed against it. The rats' hindlimbs and one forelimb were restrained by the experimenter just allowing the rat to freely move the other forelimb. The rats´ whiskers were rubbed against the edge of a table and the innate response of placing the free-moving forepaw against the table were measured. On a test day, the test was performed 10 times for both ipsilateral and contralateral sides (**Figure** 2.9). The task was carried out for three consecutive days for each time point. Data was shown as the mean ± SEM of all the attempts. As with the Stepping and Corridor test, the animals were habituated to the task for two weeks.

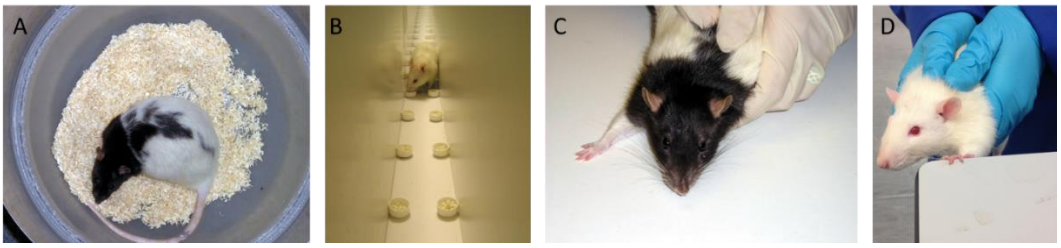

Figure 2.9 Behavioural motor tests used. A) Rotational behaviour to ipsi and contralateral sides are measured for 60 min after a single injection of methamphetamine (i.p 2.5 mg/kg). B) In the Corridor test, CoCoPops® retrievals are counted in both sides of the corridor. C) In the Stepping test, the use of ipsi and contralateral forepaws are assessed by restraining the animal and only leaving one free-moving forepaw to make steps alongside a table. D) In the Whisker test, vibrissae-elicited forepaw placings in the table are scored for both sides.

2.8 IMMUNOHISTOCHEMISTRY

2.8.1 Tissue processing

Rats were sacrificed by terminal anaesthesia (50 mg/kg pentobarbital i.p) and transcardially perfused with heparinised saline (5000 units/litre) followed by 4% paraformaldehyde (PFA) solution (pH=7.4) using an automatic pump. Brains were rapidly removed and post-fixed in 4% paraformaldehyde for 24h and then moved to a 30% sucrose solution with 0.1% sodium azide for cryopreservation. Serial coronal sections (30 µm) were cut using a freezing stage sledge microtome (Bright, Cambridgeshire, UK) and collected in a series of 12.

2.8.2 Immunohistochemistry

Immunohistochemistry (IHC) was performed using the streptavidin-biotin-peroxidase method as previously described (Hoban *et* al., 2013; Naughton et al., 2015) in free floating sections. First, endogenous peroxidase activity was quenched by placing the sectioned tissue in a 10% hydrogen peroxidase and 10% methanol solution for 5 minutes. Next, nonspecific binding was blocked using 3% normal serum (horse or goat depending on secondary antibody host) in TBS with 0.2% TritonX100 for 1 hour. Primary antibody (**Table 2.1**) was diluted in TBS with 0.2% Triton X-100 and 1:100 normal serum and incubated with the tissue at room temperature overnight. Corresponding secondary antibody (**Table 2.2**) was diluted in TBS and 1:100 normal serum and added to sections for 3 hours at room temperature. A streptavidin-biotin--horseradish peroxidase solution (Vector, UK) was added to sections for 2 hours at room temperature and covered from light. Staining development was performed using a 0.5% solution of diaminobenzidine tetrahydrochloride (DAB, Sigma, Ireland) in TNS with 0.3 µl/ml oh hydrogen peroxide. Sections were left in DAB solution until staining intensity was satisfactory. Sections were mounted into gelatin-coated slides and dehydrated in a series of ascending alcohols (50%, 70% and 100%), cleared in xylene and finally coverslipped using DPX mountant for DAB stained sections (Sigma, Ireland).

Table 2.1. Primary antibodies used in this thesis.

ICC: immunocytochemistry, IHC: immunohistochemistry

Target	Primary Antibody	Company	Catalogue number	Host	Dilution	Application
Catecholaminergic neurons	TH	Merck Millipore	MAB318	Mouse	1:1000	IHC/ICC
Astrocytes	GFAP	DAKO - Agilent	Z0334	Rabbit	1:2000	IHC/ICC
Microglia	CD11b	Merck Millipore	CBL1512	Mouse	1:400	IHC
Human IL10	Human IL10	Peprotech	500-P20	Rabbit	1/200	IHC
Collagen type I	Collagen type I	Abcam	ab117119	Rabbit	1/1000	IHC
Neural outgrowth	βIII-tubulin	Merck Millipore	MAB1637	Mouse	1:200	ICC

Table 2.2 Secondary antibodies used in this thesis.

ICC: immunocytochemistry, IHC: immunohistochemistry.

Secondary antibody	Company	Catalogue number	Host	Reactivity	Dilution	Application
Biotinylated	Vector	BA-2001	Horse	Mouse	1:200	IHC
Biotinylated	Jackson	111-065-144	Goat	Rabbit	1:200	IHC
Alexa Fluor 488	Biosciences	A32723	Goat	Mouse	1:1000	ICC
Alexa Fluor 546	Biosciences	A11035	Goat	Rabbit	1:1000	ICC

2.9 HISTOLOGICAL QUANTIFICATION

2.9.1 Cavalieri's principle

Cavalieri's principle was used to perform all volumetric analysis throughout this thesis. It states that the volume of an object can be estimated from the product of the distance between planes (Tt) and the sum of the areas on systemic random parallel sections through the object.

$$V = Tt \times \sum_{i=1}^{i} Ai$$

$$Tt = NS \times D$$

Where

- V is the total volume
- Tt is the overall distance of the object
- Ai is the area (determined using ImageJ software as described in section 2.9.2)
- NS is the number of sections in the series (i.e. 6 series)
- D is the known distance between tissue sections (i.e. 30 µm)

2.9.2 Quantification of Area/Volume

Image J software was used to measure area of relevant sections throughout this thesis. In order to determine volume, photographs of striatal sections were taken using an Olympus IX81 fluorescent microscope (Olympus UK, London, United Kingdom) or a VS120 Virtual Slide Microscope (Olympus UK, London, United Kingdom). ImageJ software was calibrated using a known distance provided by a graticule or the imaging software with the number of pixels of that known distance. Area was outlined with the freehand selection tool of ImageJ software from all striatal sections. For each animal, the area was measured in each striatal section of a 1:6 series. These measurements of area were converted to volume by using Cavalieri's Principle equation (section 2.9.1). This method was used to measure volume of cell graft, re-innervation, microgliosis, astrocytosis as well as volume of IL-10 and collagen.

2.9.3 Abercrombie's principle

Abercrombie's principle was used to normalise cell body counts in histological sections throughout this thesis. Apparent mean cell counts from a histological section are not accurate since one cell body can be present in multiple sections. For this reason, Abercrombie's principle takes the thickness and frequency of the sections alongside the average size of cell bodies into account in order to provide a more accurate estimation of cell body counts.

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

Where:

- T is the total number of cells
- F is the frequency of sections (i.e. 6)
- A is the total cell counts
- M is the thickness of sections (i.e. 30 µm)
- D is average cell diameter

2.9.4 Quantification of tyrosine hydroxylase-positive cell bodies

ImageJ multi-point tool was used to count tyrosine hydroxylase-positive cell bodies present in the striatum (**Figure** 2.10). Photographs of TH^+ stained striatal sections were taken using an Olympus microscope BX40 and Olympus C5060 digital camera (Olympus UK, London, United Kingdom) or a VS120 Virtual Slide Microscope (Olympus UK, London, United Kingdom). For each animal, the number of TH^+ cell bodies were counted in each striatal section of a 1 in 6 series. Also, the average diameter of the cell bodies was measured using ImageJ to correct the final value using Abercrombie's Principle equation (section 2.9.3).

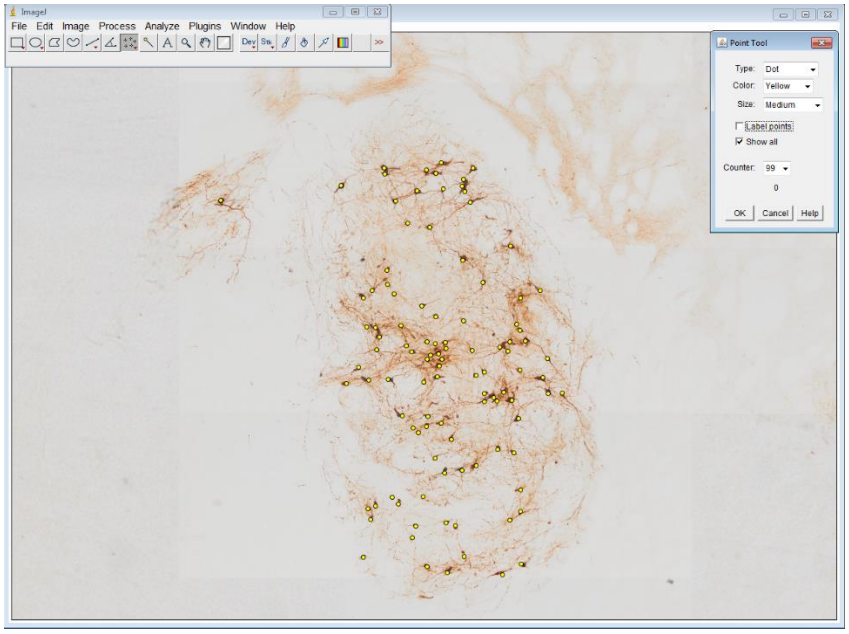

Figure 2.10 Screen shot of the ImageJ software used to determine the number of TH$^+$ cell bodies in the transplanted striatum. Yellow counter dots on the image represent the location of TH$^+$ cell bodies. Cell bodies were counted with the ImageJ plug-in "Cell counter".

2.9.5 Quantification of striatal density

Optical density measurements were taken throughout this thesis to analyse the response of immune cells – astrocytes and microglial cells – to the grafted cells. Photographs of GFAP or OX-42 immunostained sections were taken using an Olympus microscope BX40 and Olympus C5060 digital camera (Olympus UK, London, United Kingdom) or a VS120 Virtual Slide Microscope (Olympus UK, London, United Kingdom). For optical density analyses, three representative regions of the transplanted area in the striatum were chosen per animal. These areas were chosen based on the visible transplant in TH$^+$ immunostained sections. As well, three representative regions in the contralateral striatum were selected per animal as a control. Then, a circular shape was drawn in the transplantation or control area and the mean grey value of the selected region was measured (**Figure** 2.11).

Mean grey values were converted to optical density vs control side by applying the following formula:

$$OD = Log10\ (255/mean\ grey\ value)$$

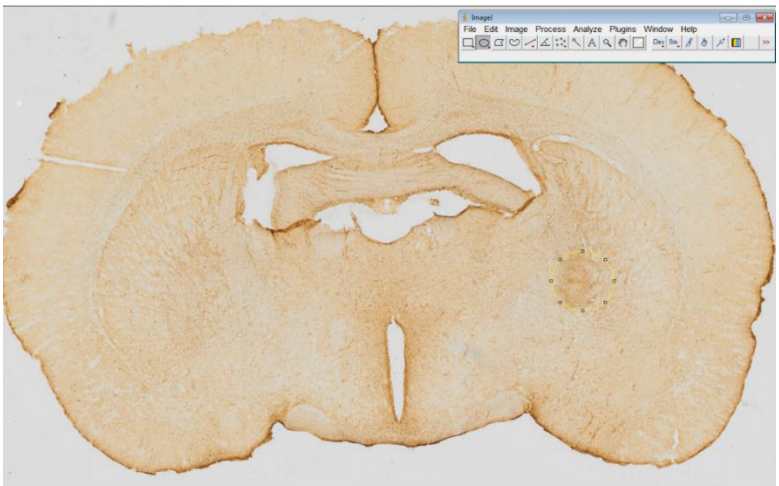

Figure 2.11 Screenshot of ImageJ software used to measure optical density in the striatum. The yellow circle around the graft site was used to analyse the density of the immunostaining. The same circular shape was used on the contralateral striatum as a control measurement.

2.10 STATISTICAL ANALYSIS

Statistical analyses were carried out with SPSS Statistics 23 software package. Data was analysed for normality (Shapiro-Wilk test) and sphericity (Mauchly's sphericity test in repeated measures analysis of variance) after confirming normality. Data are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to compare the mean of more than two groups on one factor, two-way ANOVA was used to compare more than two groups on two factors. A two-way repeated measures ANOVA was used to compare with within subject factor of time and between subject factor of group in behavioural data. Bonferroni *post-hoc* test was carried out when required. In some cases, to keep consistency amongst studies, not normal data was normalised using transformations such as square-root or natural logarithm transformation of raw data for data analysis. Data was deemed to be significant at $p < 0.05$ at all cases. Throughout the results chapters, the main effects of the ANOVA analyses will be indicated in the text, while the outcome of the *post-hoc* test will be shown on the corresponding figure. Biological and technical replicates are also indicated in the figure.

2.11 GRAPHIC SCHEMATIC STUDY DESIGNS

All schematic design figures were drawn using the free online version of Biorender (www.biorender.com).

CHAPTER 3: CHARACTERISATION OF A COLLAGEN HYDROGEL SCAFFOLD FOR INTRA-STRIATAL CELL TRANSPLANTATION

3.1 INTRODUCTION

Parkinson's disease pathology is associated with a relative loss of dopaminergic cells from the substantia nigra pars compacta (Braak & Braak, 2000). This cell specificity makes this neurodegenerative disease an ideal candidate for cell transplantation (Schapira, 2011). In the last decades, transplantation of dopaminergic cell bodies into the nigrostriatal pathway has been broadly studied both in animals and patients. The transplantation of VM cells into the striatum has proven that VM cells can survive, attach and re-innervate the host striatum while restoring dopamine levels in the nigrostriatal pathway and ameliorating the motor symptoms (Lindvall et al., 1990; Kordower et al., 1998). However, cell transplantation therapy in Parkinson´s disease has encountered many limitations, one of these being the poor survival of the grafted cells (Orive et al., 2009). As summarised by Brundin et al. (2000a) it is believed that only 1-20% of dopaminergic neurons from VM tissue survive the transplantation process. If we take into account that dopaminergic neurons account for only 30-40% of all the VM cells, the total amount of VM cells needed for transplantation is very high. Henceforth, there is a demand to look for new approaches to improve the survival of transplanted cells.

Biomaterials – natural or synthetic materials designed to interact with biological systems – have the potential to address the poor survival of the transplanted cells by offering neuroprotection during and after the transplantation process. Many biomaterials exist, all of them in varying sizes, shapes and forms, but what makes them an extraordinary tool for brain repair is their biocompatibility and stability within the human body and their adaptability to any disease´s needs (Orive et al., 2009).

In particular, collagen-based biomaterials have broadly been used in the tissue engineering field (for cartilage and bone reconstruction) due to collagen's low immunogenicity, its biodegradability, and its high availability and versatility (Parenteau-Bareil et al., 2010). In particular, collagen type I is the most used collagen in this field thanks to its high biocompatibility (Silvipriya et al., 2015). Although

collagen can polymerise on its own, the mechanical properties of the resulting collagen hydrogels are weak, and consequently chemical modifications are used to improve their stability (Peppas et al., 2006).

PEGylation – the covalent attachment of PEG to proteins (more generally known as chemical crosslinking – can be used to increase the stiffness and decrease the degradability of collagen hydrogels (Alconcel et al., 2011; Hennink & Van Nostrum, 2012). Chemical crosslinking of naturally-derived hydrogels with PEGs has been widely utilised since it is very straightforward procedure. Furthermore, PEG is the most used synthetic polymer for tissue engineering because it is a non-toxic, non-immunogenic polymer that is highly soluble in water and already approved by the FDA (Veronese & Pasut, 2005; Peppas et al., 2006).

In the field of cell transplantation for Parkinson's disease, special attention has been given to injectable naturally-derived scaffolds since they hold a series of advantages with respect to other biomaterials; 1) they are easy to deliver intra-cranially in the brain, 2) they show low immunogenic properties, 3) they provide structural support to transplanted cells and 4) they can be tuned to release therapeutic molecules (Orive et al., 2009).

Most importantly, collagen hydrogels have been proven to improve survival and re-innervation of intra-striatal transplanted VM cells when used alone or in conjunction with GDNF in a rodent model of Parkinson´s disease (Moriarty et al., 2017, 2019a).

The overall aim of this thesis was to evaluate the efficacy of an anti-inflammatory collagen hydrogel on improving cell survival and reducing the host immune response in cell replacement therapies for Parkinson’s disease. To successfully achieve that goal, the biomaterial first had to be optimised for its delivery in the brain.

All of the abovementioned being considered, the aim of the research in this chapter was to characterise and optimise the properties of the crosslinked collagen hydrogels for their use in *in vivo* cell transplantation processes. Thus, an initial characterisation of the collagen hydrogels was done – including polymerisation, cytokine release and cytotoxicity studies - in order to choose the optimal collagen hydrogel composition for *in vivo* intra-striatal delivery of VM cells and therapeutic molecules - especially IL-10 - in a dopamine-depleted model of Parkinson´s disease.

3.2 METHODS

3.2.1 Experimental design

The studies presented in this chapter were designed to characterise several collagen hydrogel compositions in order to find the best composition for intra-striatal transplantation of primary dopaminergic cells and IL-10. Collagen and 4s-StarPEG crosslinker are the main components of collagen hydrogels, hence their properties and concentrations may alter many of the resulting collagen hydrogel features. Therefore, in this chapter we completed a characterisation of several collagen hydrogel compositions (with varying crosslinker concentrations) for their future use *in vivo* transplantation studies in hemi-parkinsonian rats (**Figure** 3.1).

Initially, the structural, mechanical and chemical properties of the collagen hydrogels crosslinked with 2-12 mg/ml of 4s-StarPEG were described. Of particular interest was the analysis of the gelation profile of these compositions, since this property is very important when optimising an injectable and biodegradable collagen hydrogel for cell transplantation. Once the basic intrinsic features of the collagen hydrogels were outlined, their ability to retain and release IL-10 (while keeping IL-10 functionality) was assessed *in vitro*. After, their *in vitro* biocompatibility was measured. Detailed experimental designs of the studies carried out are outlined below.

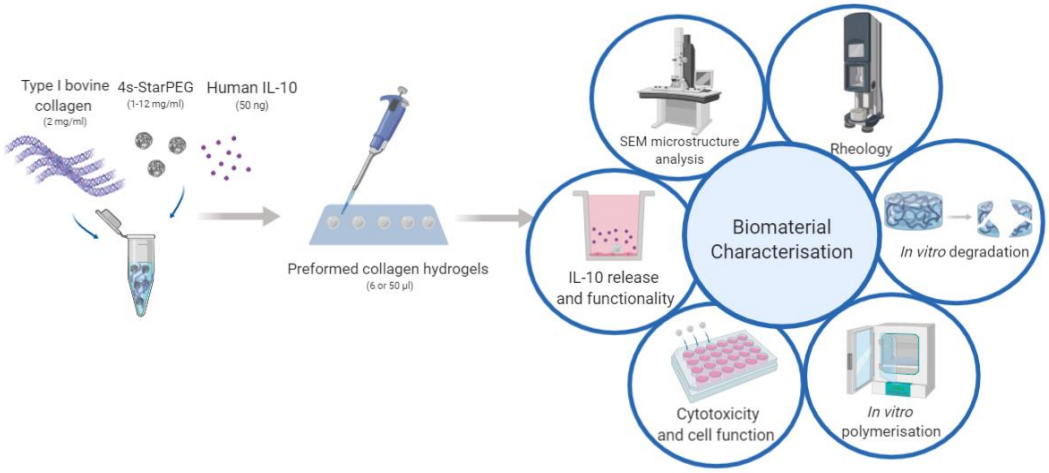

Figure 3.1 Collagen hydrogel characterisation experiments. Schematic view of the experimental design of the characterisation process. Bovine type I collagen (2 mg/ml) was neutralised and mixed with 4s-StarPEG crosslinker (1-12 mg/ml) and the mixture was pipetted into a hydrophobic surface and left to polymerise at 37°C. Once the collagen hydrogels were polymerised, they were used for structural, rheological, chemical and cytotoxicity characterisation. IL-10 collagen hydrogels were used to measure IL-10 release and functionality.

3.2.1.1 Effects of crosslinker concentration on collagen hydrogel polymerisation time

Since the concentration of crosslinker used in the collagen hydrogels will affect the speed and intensity of polymerisation, the gelation time of the multiple collagen hydrogels was assessed *in vitro*. Once the collagen/4s-StarPEG/PBS solution was mixed, 50 µl drops were placed on top of a hydrophobic surface (Teflon® tape) and put in the incubator at 37°C and 5% CO_2. The drops were checked regularly to evaluate their polymerisation until they became solid.

3.2.1.2 Effects of crosslinker concentration on collagen hydrogel microstructure

To visualise the effects of chemically crosslinking the collagen hydrogels with several concentrations of 4s-StarPEG, the microstructure of fully polymerised collagen hydrogels was examined. In short, 200 µl of fresh and fully polymerised collagen hydrogels – crosslinked with 2, 4, 6 or 12 mg/ml - were processed for scanning electron microscopy as detailed in Chapter 2 (section 2.4.2). Pore diameter

measurements were taken from SEM micrographs a total of twenty times in at least 2 different and randomly selected regions of the collagen hydrogel sample.

To further visualise how encapsulated dopaminergic cells are distributed in the collagen hydrogel, seeded collagen hydrogels with either 6 or 12 mg/ml of crosslinker were observed under scanning electron microscopy. In this case, 50 µl collagen hydrogels were seeded with SH-SY5Y cells at a low or high concentration (500,000 or 5,000,000 cells/ml) and they were fixed after polymerisation or 48h after incubation to allow the cells to attach. Later, samples were processed with cell-compatible protocols for SEM visualisation (detailed in section 2.4.2). Three processing protocols – chemical dehydration, critical point drying or variable pressure - were tested since collagen scaffolds can be destroyed easily during processing. The optimisation process was performed with collagen hydrogels with 4 mg/ml of crosslinker.

3.2.1.3 Effects of crosslinker concentration on collagen hydrogel degradation

In vitro degradation profile of collagen hydrogels was studied using a spectrophotometric degradation assay. In brief, 6 and 50 µl spherical collagen hydrogels (with 2, 4, 6 and 12 mg/ml of crosslinker) were fabricated and left to polymerise. Polymerised hydrogels were immersed in a Coomassie Brilliant Blue solution for 6h at room temperature. After, the blue-stained hydrogels were submerged in a de-staining solution and left in a shaker overnight at room temperature. Collagen hydrogels were washed with water and put in DMEM/F-12 media or water. The release of the blue dye from the collagen hydrogel was measured as an indirect measurement of collagen hydrogel degradation using an absorbance plate reader at 570 nm.

3.2.1.4 Effects of crosslinker concentration on collagen hydrogel rheological properties

As abovementioned, to determine the mechanical properties of the several compositions and to identify any difference between the various collagen hydrogels, their rheological behaviour was analysed. Again, 200 µl of fresh and fully polymerised collagen hydrogels – crosslinked with 2, 4, 6 or 12 mg/ml - were fabricated and assessed in a rheometer, as mentioned in detail in Chapter 2 (section 2.4.4). Amplitude

and a frequency sweep protocols (detailed in section 2.4.4) were run to determine the storage (G') and loss (G'') modulus.

3.2.1.5 In vitro IL-10 release from collagen hydrogels

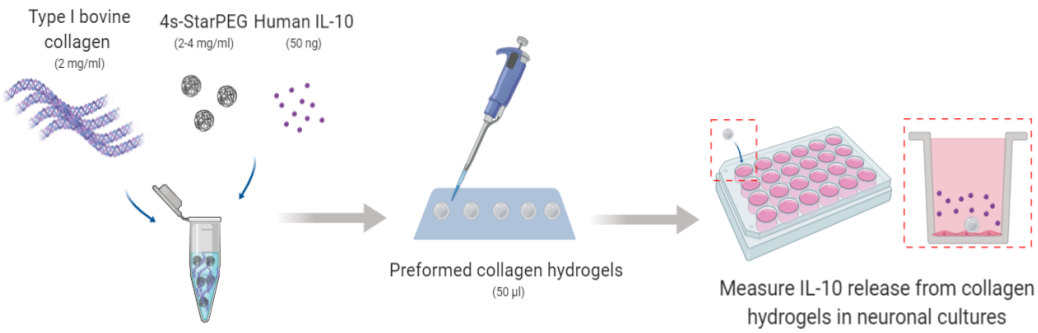

Figure 3.2 *In vitro* IL-10 release from collagen hydrogels. Schematic representation of the *in vitro* IL-10 release experiments. 50 ng of human IL-10 were encapsulated in collagen hydrogels with 2 or 4 mg/ml of crosslinker. Once polymerised, collagen hydrogels were put in neuronal cultures to assess the *in vitro* IL-10 release from the collagen hydrogels. The release of IL-10 was measured using an ELISA kit.

The *in vitro* release of IL-10 from the collagen hydrogels was evaluated via human IL-10 ELISA as stated in Chapter 2 (section 2.5.2). In short, 50 ng of human IL-10 were encapsulated in 50 µl of collagen hydrogels with either 2 or 4 mg/ml of crosslinker and the collagen hydrogels were left to polymerise at 37°C. After, each 50 µl collagen hydrogel was incubated in a well of a 24-well plate with SH-SY5Y cells (50,000 cells/cm^2). IL-10 containing supernatant was collected at several timepoints until the collagen hydrogels were fully degraded (not detectable when media was removed). The amount of IL-10 present in the media was analysed with a human IL-10 ELISA kit and IL-10 release rate from the collagen hydrogels was calculated (**Figure** 3.2).

3.2.1.6 In vitro functionality assessment of IL-10 released from collagen hydrogels

Moreover, to prove that IL-10 released from the collagen hydrogel was fully functional, a Poly I:C challenge was used. *In vitro* Poly I:C administration generates

a viral-like inflammatory response which involves the release of pro-inflammatory cytokines like IL-1β. IL-10 can reduce this release through its anti-inflammatory effect. Briefly, E14 VM cultures were obtained from the dissection of E14 rat embryos and were plated at 100,000 cells per well of a 24 well plate (50,000 cells/cm^2) and left for 48h to attach. After that, VM cultures (50,000 cells/cm^2) were pre-treated with IL-10 (50 ng/ml) either as a single bolus or inside a collagen hydrogel and an hour later Poly I:C (20 and 50 µg/ml) was administered. After 24h of incubation with Poly I:C, media was collected for pro-inflammatory cytokine levels evaluation as a measure of inflammation. IL-1β levels were measured using an ELISA kit to confirm that IL-10 was functional (**Figure** 3.3).

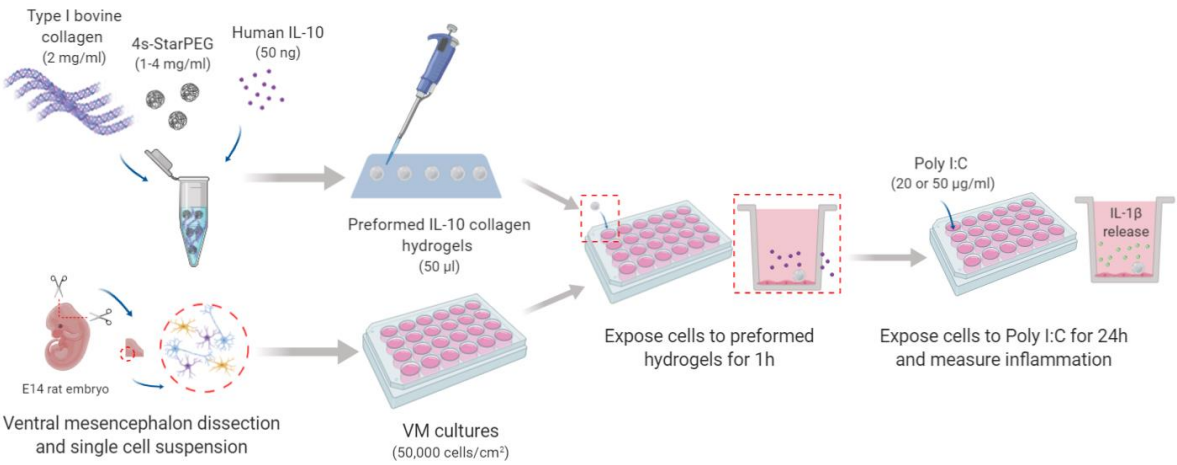

Figure 3.3 *In vitro* functionality assessment of IL-10 released from collagen hydrogels. Schematic representation of the *in vitro* assessment of IL-10 functionality after a Poly I:C challenge. VM cultures were exposed to IL-10 as a bolus or encapsulated in a collagen hydrogels for 1h prior to Poly I:C administration. After 24h incubation, IL-1β levels were measured using an ELISA kit.

3.2.1.7 In vitro assessment of the impact of crosslinker concentration on cell viability and cell function

Before using collagen hydrogels *in vivo*, their biocompatibility with 2D VM cultures was assessed. Briefly, the toxicity of the collagen hydrogel was measured using AlamarBlue® cell viability assay as well as immunocytochemistry on VM cultures as specified in Chapter 2 (section 2.5.5). In short, E14 VM cultures were obtained from the dissection of E14 rat embryos and were plated at 100,000 cells per well of a 24 well plate (50,000 cells/cm^2) and left for 48h to attach. The VM cultures were then

incubated with unseeded preformed collagen hydrogels crosslinked with 1, 2 or 4 mg/ml (2 x 50 µl) for 24h or left untreated. Then, media was removed and AlamarBlue® cell viability solution was added or cells were fixed for future ICC staining (**Figure** 3.4).

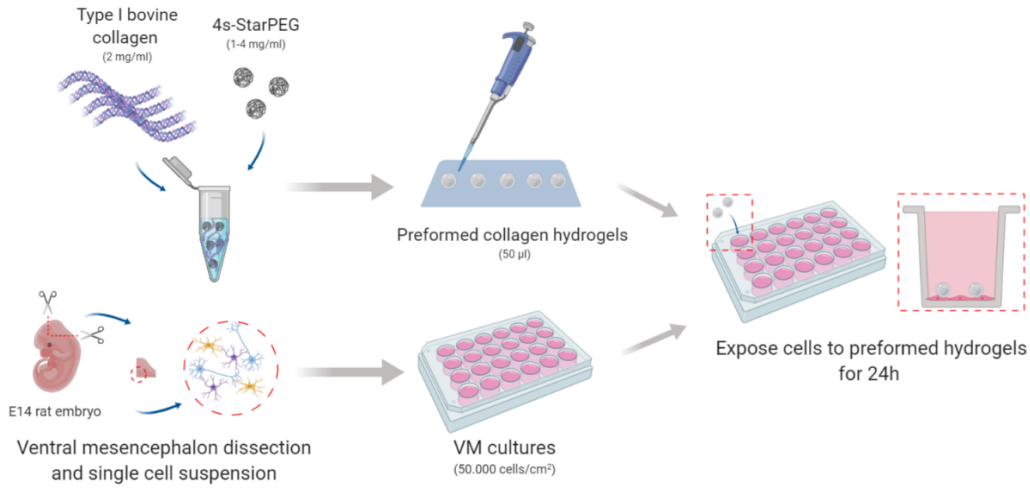

Figure 3.4 *In vitro* cytocompatibility studies of collagen hydrogels. Schematic representation of the *in vitro* experimental design. E14 VM cultures obtained from the mesencephalon of E14 rat embryos were seeded (50,000 cells/cm^2) and left for 24h to attach. Then VM cultures were exposed to unseeded preformed collagen hydrogels (2 x 50 µl) with increasing concentrations of crosslinker. The cytocompatibility of the biomaterial was assessed with AlamarBlue® cell viability assay, immunocytochemistry or calcium signalling.

To further analyse if the collagen hydrogels had any detrimental effects for cell culture, calcium signalling was used on E14 VM cultures to test their cell function when in contact with the collagen hydrogels. As mentioned earlier, E14 VM cultures were obtained from the dissection of E14 rat embryos and were plated at 100,000 cells per well of a 24 well plate (50,000 cells/cm^2) and left for 48h to attach. The VM cultures were then incubated with unseeded preformed collagen hydrogels crosslinked with 1, 2 or 4 mg/ml (2 x 50 µl) for 24h or left untreated. Changes in intracellular calcium (Ca^{2+}) signalling of primary VM cultures were measured as described previously in Chapter 2 (section 2.5.6).

3.3 RESULTS

3.3.1 *In vitro* assessment of the impact of crosslinker concentration on the biomaterial properties

Before assessing the effects of the several collagen hydrogel compositions on cell viability, collagen hydrogels with increasing concentrations of 4s-StarPEG were formulated on top of a hydrophobic Teflon® tape and were placed at 37°C. Once the hydrogels were polymerised, their structural, rheological, mechanical and chemical properties were studied.

3.3.1.1 Effects of crosslinker concentration on collagen hydrogel polymerisation time

Gelation time is a crucial feature of injectable scaffolds used for cell delivery; a rapid polymerisation can hamper the biomaterial delivery into the brain and a slow polymerisation will not protect the injected cells from the environment. The rate of gelation can be tailored by the type of crosslinker, its molecular weight or its concentration. In this thesis, the latter was assessed.

Several crosslinked collagen hydrogels with increasing concentrations of 4s-StarPEG ranging from 1 to 12 mg/ml were formulated. As expected, higher concentrations of 4s-StarPEG shortened the time to polymerisation (**Figure** 3.5; Crosslinker concentration, $F_{(4, 20)} = 819.9$, $P<0.001$).

Two brands of bovine type I atelocollagen (Vornia Biomaterials and Collagen Solutions) were used to produce collagen hydrogels. Collagen hydrogels made out from Collagen Solutions Collagen polymerised quicker than collagen hydrogels made out from Vornia Biomaterials when 1 mg/ml concentration was used (**Figure** 3.5; Collagen brand, $F_{(1, 20)} = 18.11$, $P<0.01$).

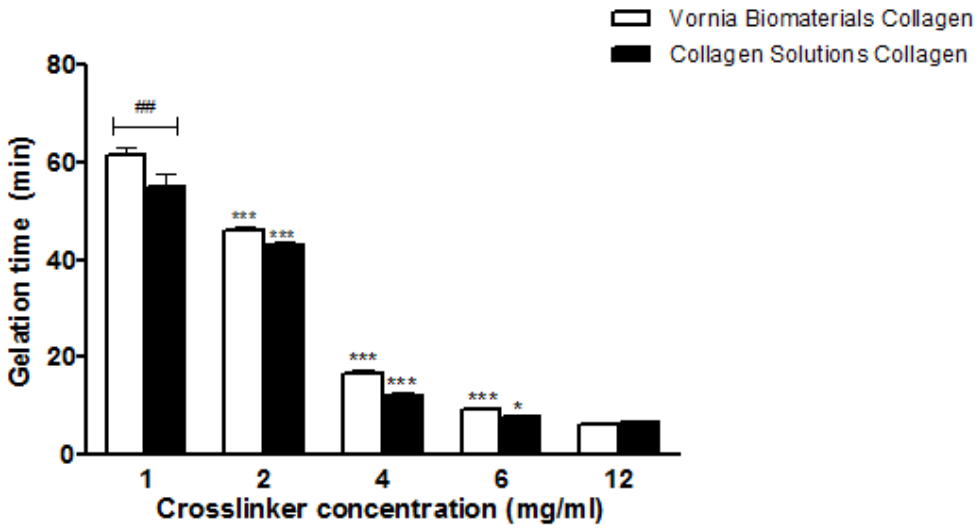

Figure 3.5 Effects of 4s-StarPEG crosslinker concentration on collagen hydrogel polymerisation time *in vitro*. The time required for the collagen hydrogels to gel decreased at increasing concentrations of 4s-StarPEG at 37°C. n = 3 biological replicates, generated in triplicate. Data are represented as mean ± SEM and were analysed by two-way ANOVA with *post-hoc* Bonferroni test. *** $P < 0.001$, * $P < 0.05$ with respect to previous concentration. ## $P < 0.01$ with respect to Vornia Biomaterials Collagen vs Collagen Solutions.

3.3.1.2 Effects of crosslinker concentration on collagen hydrogel microstructure

Collagen hydrogels are highly porous scaffolds made out of neutralised bovine type I collagen, 4s-StarPEG crosslinker and phosphate saline. Unlike synthetic hydrogels, collagen hydrogels present an irregular but highly porous matrix (**Figure 3.6**). The crosslinker concentration influences the structure of the hydrogel's matrix: the more crosslinker molecules in the hydrogel solution, the more bonds these make with the collagen strands resulting in a tighter matrix with smaller pores (**Figure 3.6** and **Figure 3.7**). As seen in **Figure 3.7**, when the concentration of crosslinker was increased, the pore diameter of the hydrogel matrix decreased.

The porosity of the hydrogels – and at the same time their high water content - is what makes them an ideal candidate for cell encapsulation. Thus, the effects of crosslinker

concentration on the hydrogel microstructure have to be investigated also when encapsulating cells.

Since the usual processing methods for SEM samples do not preserve the structure of cells, alternative protocols like chemical dehydration, critical point drying and variable pressure were used to visualise the freshly-made encapsulated cells in collagen hydrogels with 4 mg/ml of crosslinker. Chemical dehydration and critical point drying enabled the visualisation of encapsulated cells (**Figure 3.8**) even at high magnification (**Figure 3.9**). On the contrary, the variable pressure protocol, even though is the protocol that kept the original collagen hydrogel structure less modified, did not produce good quality visualisation of the encapsulated cells (**Figure 3.8**). Since chemical dehydration is a quicker and simpler processing method than critical point drying, the first was chosen as the most adequate processing protocol for the visualisation of seeded collagen hydrogels.

For this reason, a low and a high concentration of encapsulated cells were studied in combination with multiple collagen hydrogel compositions used for cell encapsulation (4, 6 and 12 mg/ml). Collagen hydrogel compositions with low crosslinker concentration (1, 2 mg/ml) were discarded as suitable compositions for cell encapsulation since they provide very weak hydrogels, with long polymerisation times and quick degradation times. Encapsulated cells were visualised either in freshly-made collagen hydrogels (fixed upon fabrication) or in collagen hydrogels incubated in media at 37°C for 48h (fixed after incubation). Interestingly, the seeded collagen hydrogel composition with 4 mg/ml of 4s-StarPEG did not hold its shape and appeared to be partially degraded after 48h in culture and could not be processed for SEM imaging (data not shown).

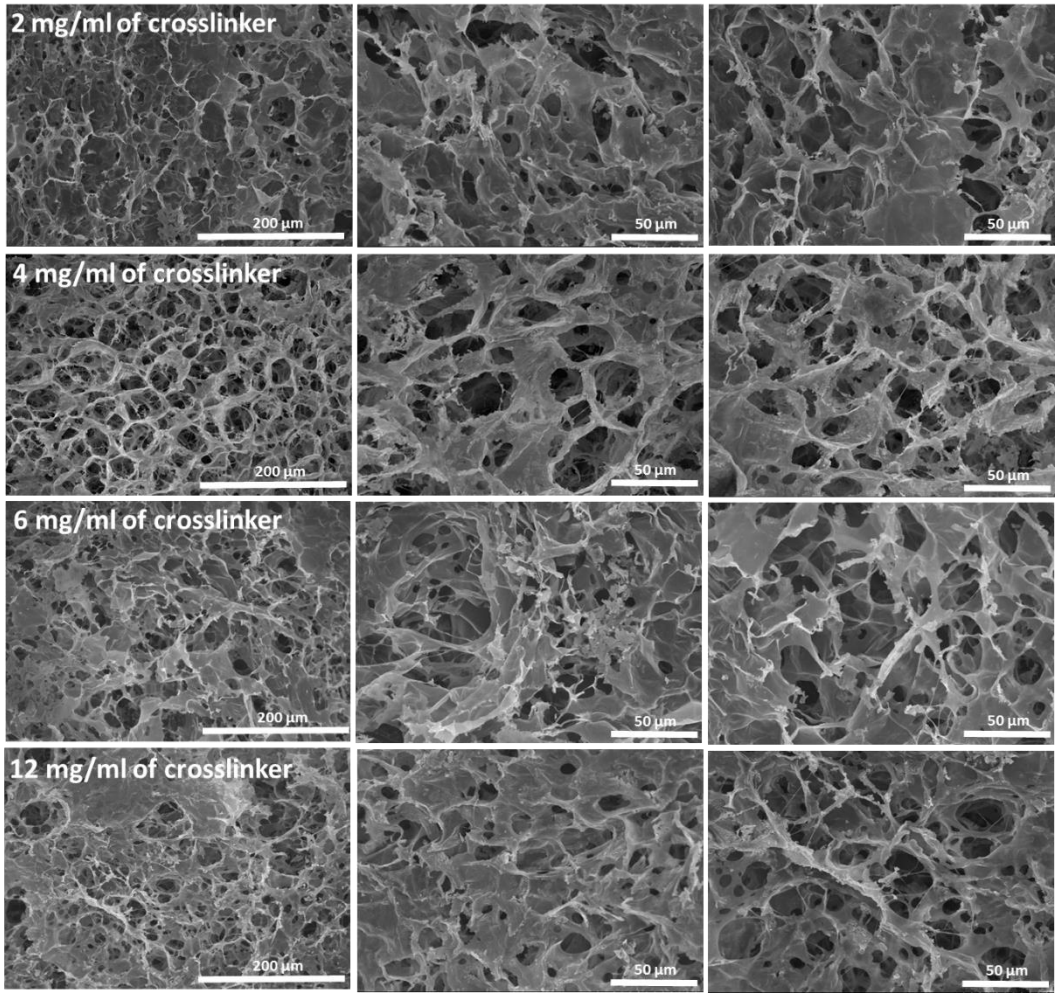

Figure 3.6 Collagen hydrogel structure under SEM imaging. Photomicrographs of freshly made collagen hydrogels – composed of 2 mg/ml of type I bovine collagen and different concentrations of 4s-StarPEG - were taken under an SEM microscope**.**

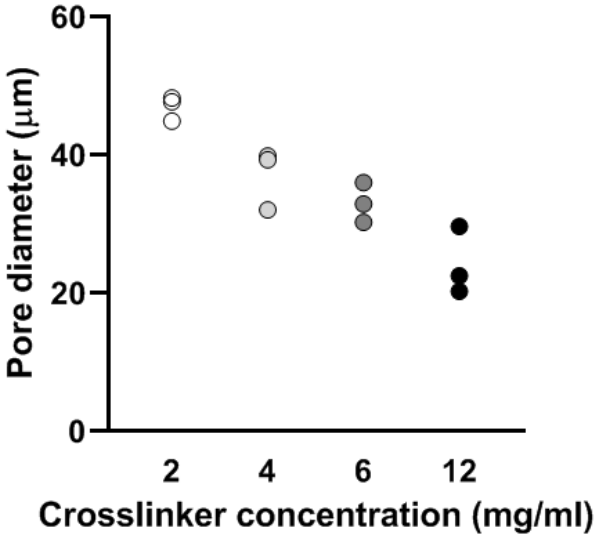

Figure 3.7 Effect of crosslinker concentration on the hydrogel pore matrix. The pore diameter of the collagen hydrogel network decreased at increasing concentrations of 4s-StarPEG. n = 1 biological replicate, generated in triplicate. Data are represented as technical replicates. As biological replicates were not produced, this data was not analysed.

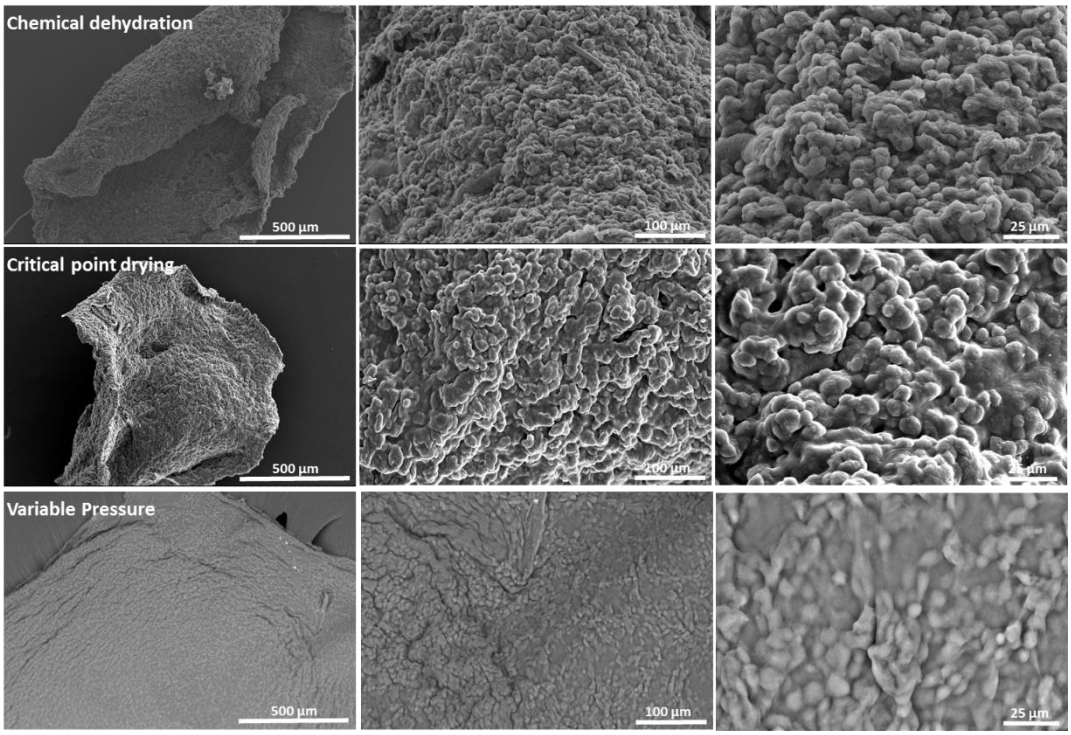

Figure 3.8 Processing of seeded collagen hydrogels for SEM visualisation. SH-SY5Y cells were encapsulated in collagen hydrogels with 4 mg/ml of crosslinker and fixed upon fabrication. Several dehydration protocols were investigated to ensure cell structure was preserved. Both chemical dehydration and critical point drying protocols enabled visualization of encapsulated cells.

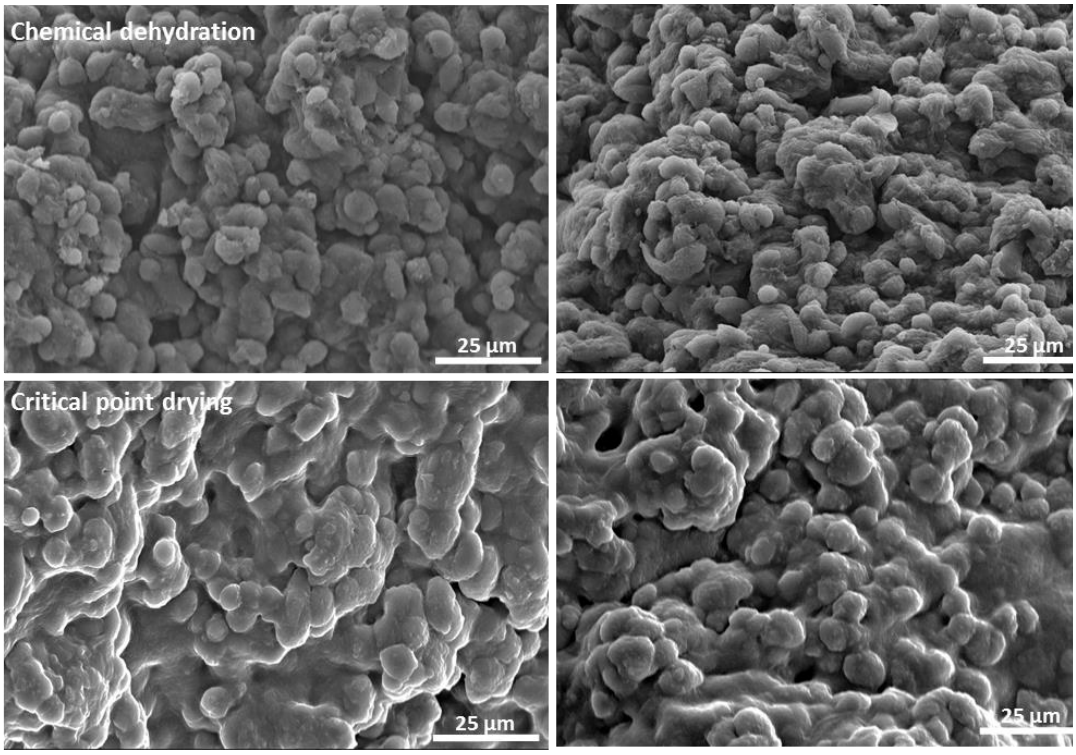

Figure 3.9 Microstructure of seeded collagen hydrogels. SH-SY5Y cells were encapsulated in collagen hydrogels with 4 mg/ml of crosslinker and fixed immediately after to test the best sample processing for SEM visualisation. Both chemical dehydration and critical point drying protocols enabled visualization of encapsulated cells.

The effects of the crosslinking concentration on cell encapsulated collagen hydrogels was obvious even upon fabrication. When seeded collagen hydrogels with 4 mg/ml of crosslinker were cultured for 48h, the collagen hydrogels lost their shape and they appeared to be partially degraded, particularly at the lowest cell concentration (data not shown). These partially degraded hydrogels disintegrated during the manipulation involved in the fixation process. On the other hand, collagen hydrogels with high concentrations of crosslinker (6 and 12 mg/ml) kept their shape and could be fixed and observed under SEM. As expected, collagen hydrogel samples with the highest crosslinker concentration (12 mg/ml) were found to be stiffer and less degraded at 48h, especially when high amounts of cells were encapsulated. Despite the differential processing of these samples, there were not many considerable differences between compositions at 48h post-encapsulation in the microstructure of the biomaterial when low or high concentrations were encapsulated in it (**Figure** 3.10 and **Figure** 3.11).

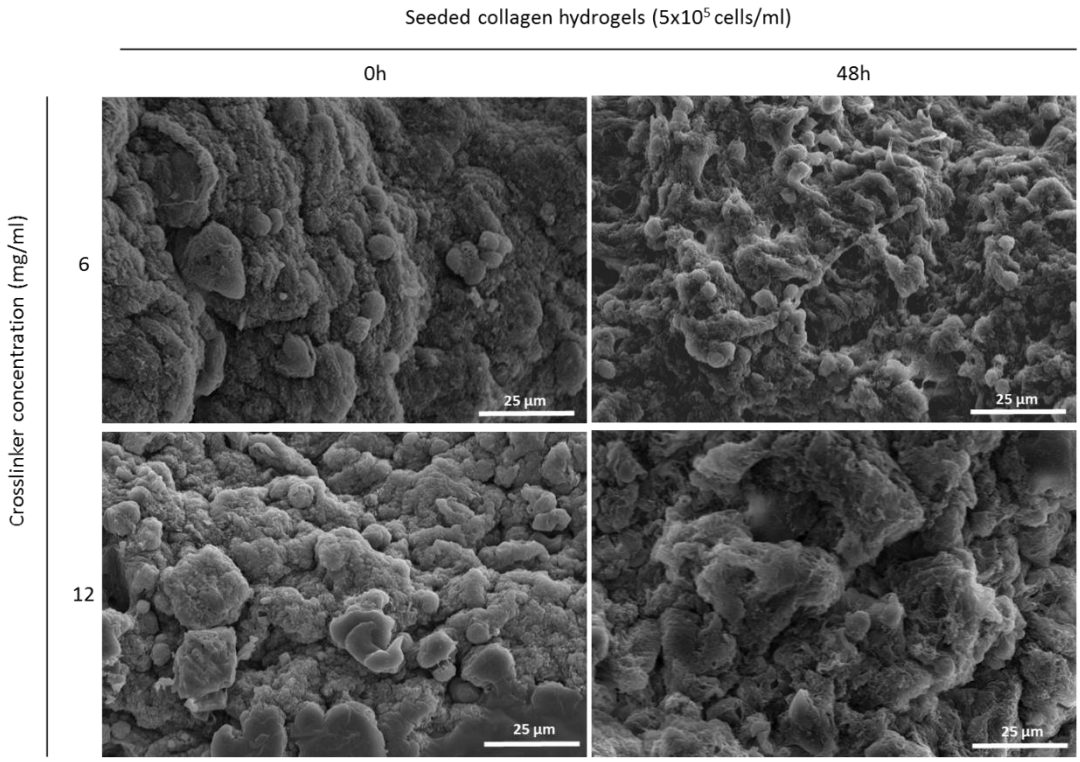

Figure 3.10 Microstructure of seeded collagen hydrogels. A low concentration of SH-SY5Y cells ($5x10^5$ cells/ml) was encapsulated in collagen hydrogels with 6 or 12 mg/ml of crosslinker and fixed upon fabrication or after 48h incubation in media.

SEM visualisation allowed to confirm a homogeneous distribution of the cells in the collagen hydrogels both at low and high concentrations (**Figure** 3.10 and **Figure** 3.11). In samples that were frozen immediately after fabrication, cells were laying on the biomaterial, covering its entire surface and exhibited a round shape, typical of non-attached cells (**Figure** 3.10 and **Figure** 3.11). Yet, after 48h the whole structure of the biomaterial changed, since more collagen fibrils and pores were visible and cells seemed to be attached into the fibrils. Nonetheless, cells still exhibit a round shape after 48h of culture (**Figure 3.12**).

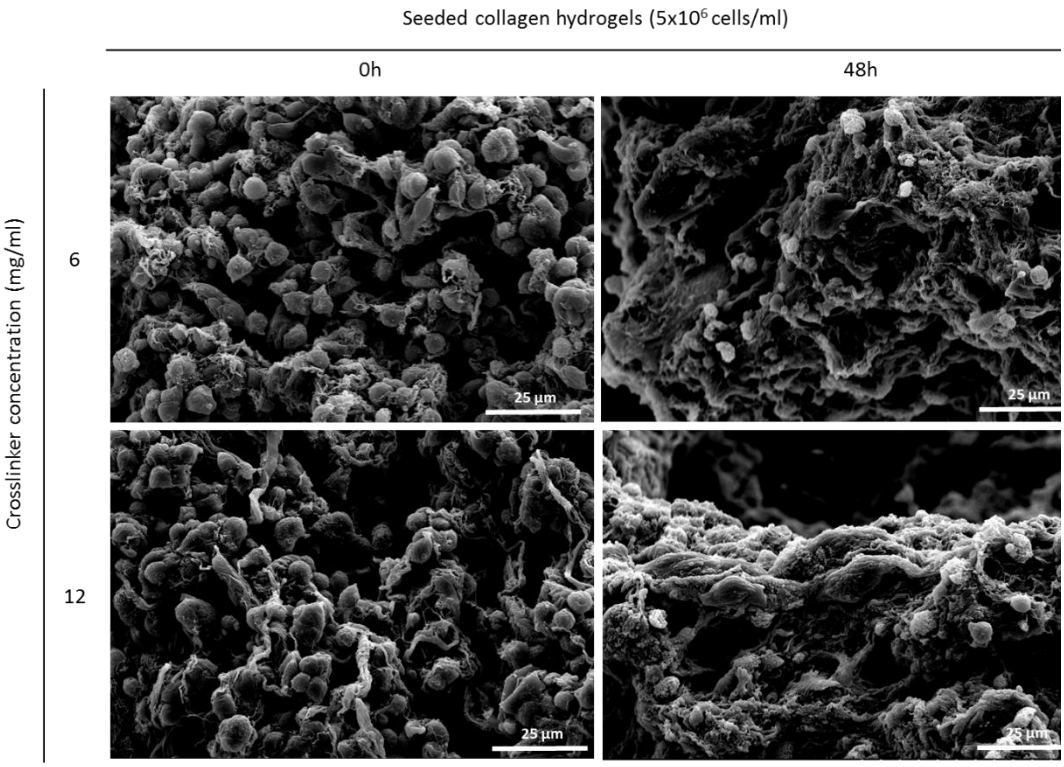

Figure 3.11 Microstructure of seeded collagen hydrogels. A high concentration of SH-SY5Y cells ($5x10^6$ cells/ml) was encapsulated in collagen hydrogels with 6 or 12 mg/ml of crosslinker and fixed upon fabrication or after 48h incubation in media.

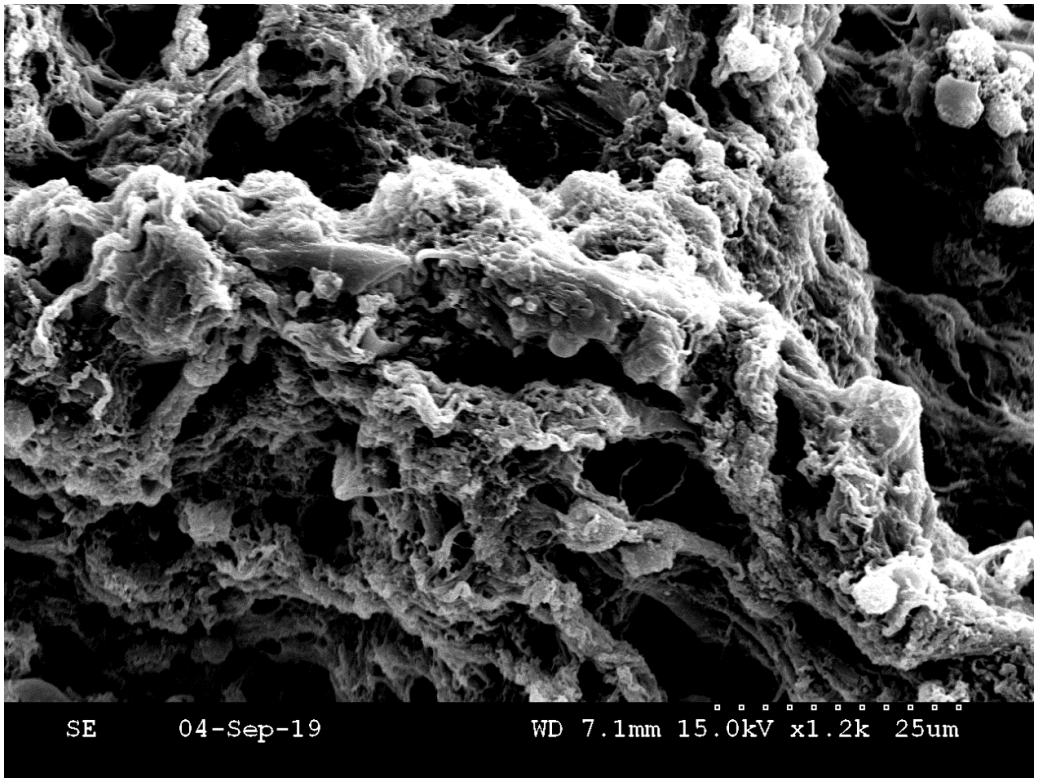

Figure 3.12 Detailed view of the microstructure of seeded collagen hydrogels. SH-SY5Y cells were encapsulated in collagen hydrogels with 6 or 12 mg/ml of crosslinker. After 48h of culture, more collagen fibrils and pores are visible and cells appeared to be attached to the collagen fibrils.

3.3.1.3 Effects of crosslinker concentration on collagen hydrogel degradation

In an attempt to evaluate the degradation of the collagen hydrogels, they were stained with Coomassie Blue and the dye's release from the collagen hydrogel was measured as an indirect measurement of degradation. The dye was released from the collagen hydrogels over time (**Figure** 3.13; Time, $F_{(6, 18)} = 1725$, $P<0.0001$). The dye's release pattern from the differently crosslinked collagen hydrogels was very similar (**Figure** 3.13; Crosslinker concentration, $F_{(3, 18)} = 6.1149$, $P>0.05$). Both macro- (50µl) and micro- (6µl) collagen hydrogels followed the same pattern of Coomassie blue release when submerged in media; with a prominent release of Coomassie blue dye in the first 24h (**Figure** 3.13**)**. At the same time, the macrogels submerged in media also exhibited evident signs of swelling that were not observable when they were immersed in water (**Figure** 3.14). Moreover, when macrogels were immersed in water, the release of the dye was low and slow since at 31 days after polymerisation the macrogels were still stained dark blue and showed little signs of degradation over time (data not quantified).

Although macrogels submerged in media did release Coomassie blue into the media this did not relate to the hydrogel's degradation as Coomassie blue was completely released by day 5 but transparent hydrogels were still visible by that time (**Figure** 3.14**)**. Thus, this experiment might be more representative of a dye release than a measurement of degradation of the collagen hydrogel.

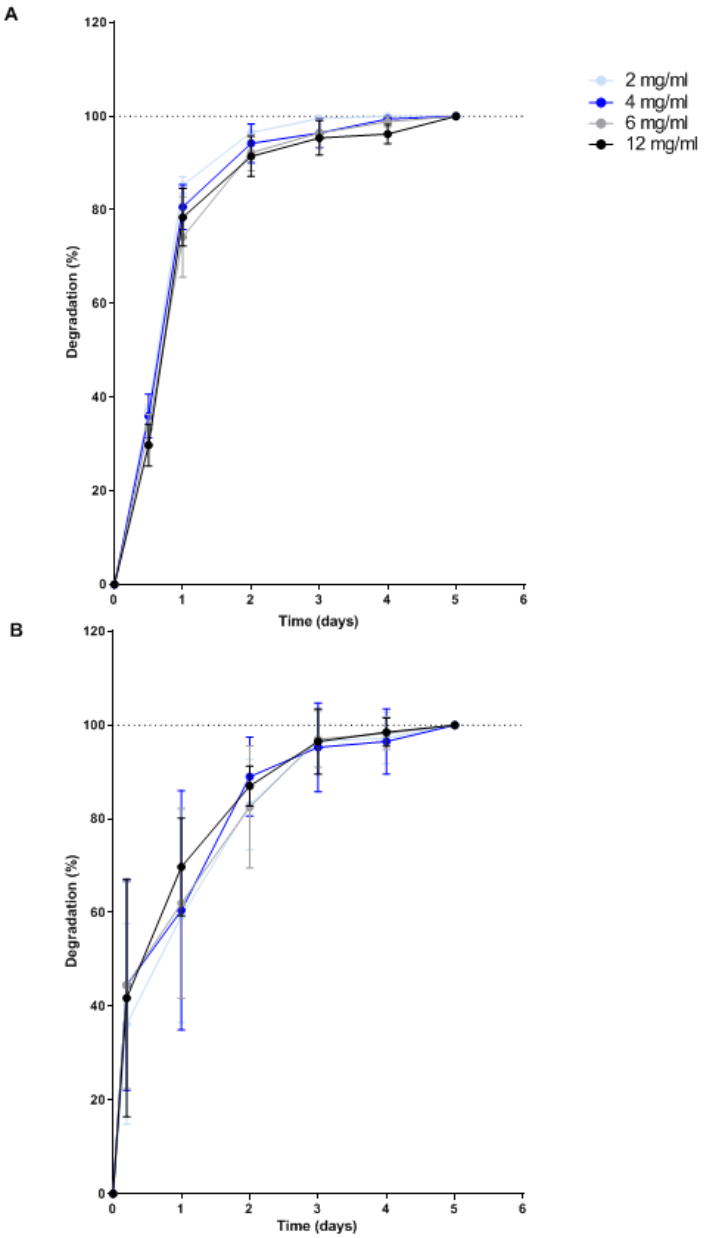

Figure 3.13 Coomassie Blue release from collagen hydrogels *in vitro*. The dye release pattern of the several collagen hydrogels compositions – with increasing concentrations of 4s-StarPEG - was very similar in the Coomassie Brilliant Blue assay for A) micro- collagen hydrogels (6 µl) and B) macro- collagen hydrogels (50 µl). n = 3 biological replicates, generated in triplicate. Data are expressed as mean ± SEM and were analysed by repeated-measures one-way ANOVA with Bonferroni *post-hoc* test.

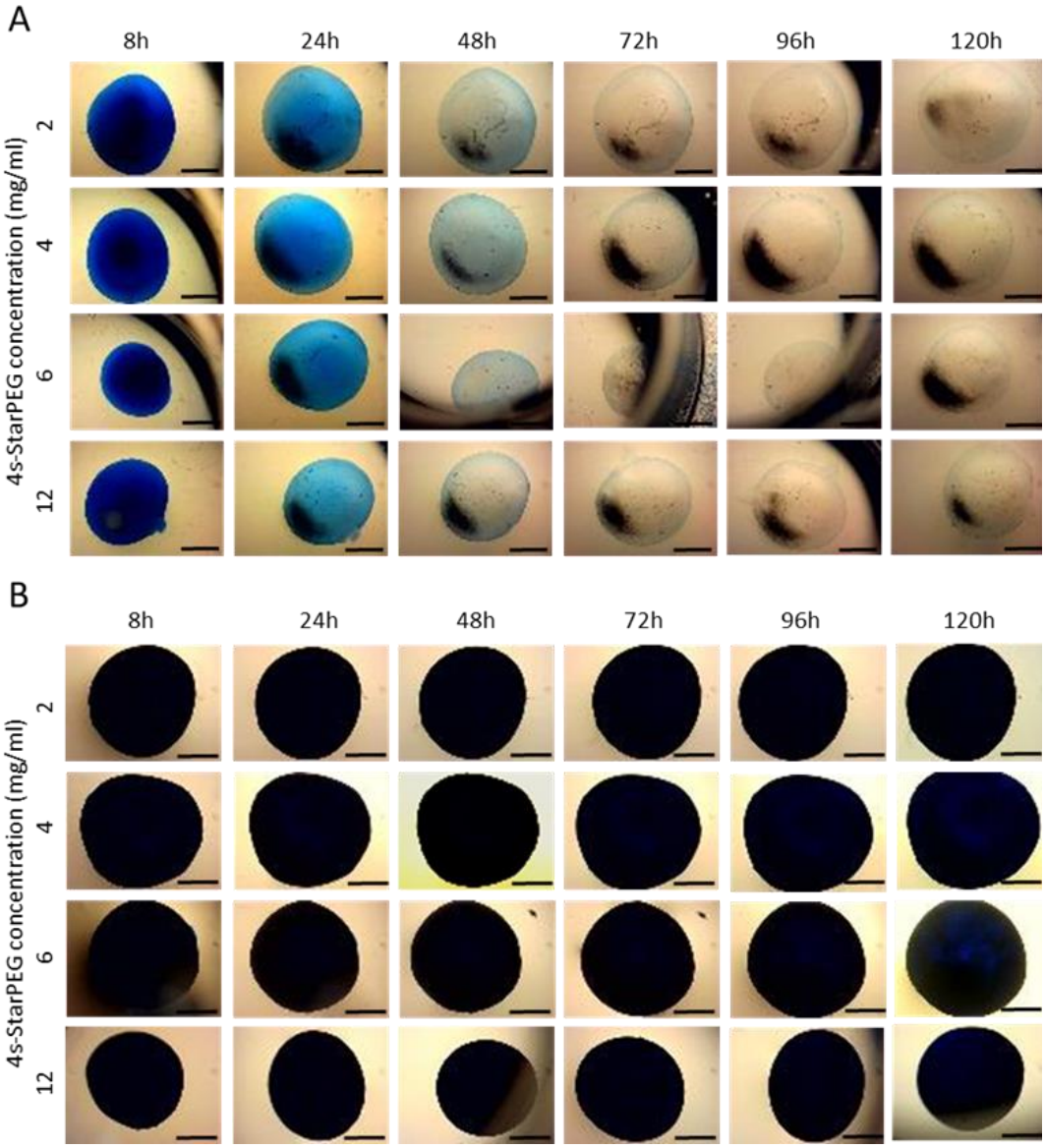

Figure 3.14 Representative photographs of collagen hydrogels over time. Photographs display the Coomassie Brilliant Blue stained collagen hydrogels over time when they were kept in A) DMEM media or B) water at 37ºC. Collagen hydrogels immersed in plating media released the dye but this did not equate with collagen hydrogel degradation. Scale bar is A) 2 mm and B) 1 mm.

3.3.1.4 Effects of crosslinker concentration on collagen hydrogel rheological properties

When developing a hydrogel material for a specific application in regenerative medicine, it is important to accurately describe its mechanical properties since the biomaterial will be interacting with the host. For a successful implantation of a biomaterial in the brain, the mechanical properties of the former should match the ones from the latter.

Thus, a rheological characterisation comprised of both amplitude and frequency sweeps was carried out to analyse the stiffness and elasticity of the collagen hydrogels as well as the effects of crosslinker concentration on the collagen hydrogels.

An amplitude sweep test was run on freshly-made, fully polymerised and differently crosslinked collagen hydrogels. For these measurements, full polymerisation of the samples was guaranteed since gelation times were previously studied (section 3.1.1). For all the collagen hydrogel compositions tested, the storage modulus (G') was almost a magnitude higher than the loss modulus (G'') in the amplitude sweep test, indicating of a predominant elastic behaviour of the biomaterial **(Figure** 3.15).

Frequency sweeps were performed to further understand if the crosslinker concentration modified the hydrogel stiffness. In the frequency sweep test, there was no apparent differences due to the crosslinker concentration in any of the points assessed (**Figure** 3.16**)**.

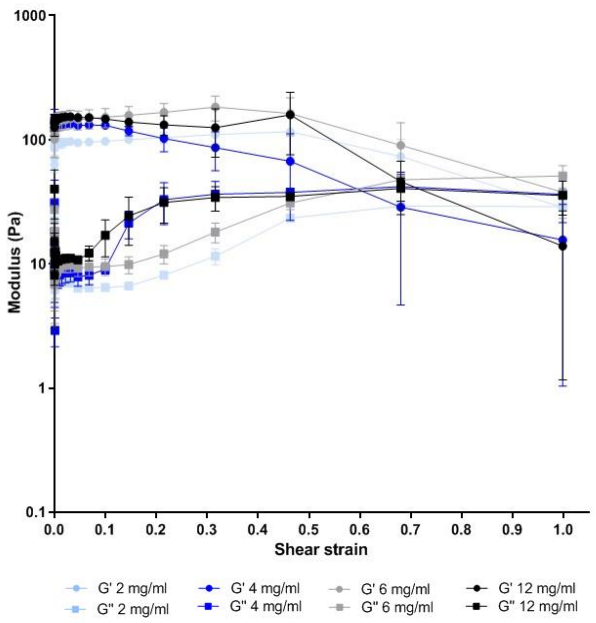

Figure 3.15 Amplitude sweep of freshly made collagen hydrogels. All pre-formed collagen hydrogel compositions tested behaved as elastic solids at 37°C. n = 1 biological replicate, generated in triplicate. Data are represented as mean ± SEM of technical replicates. As biological replicates were not produced, this data was not analysed.

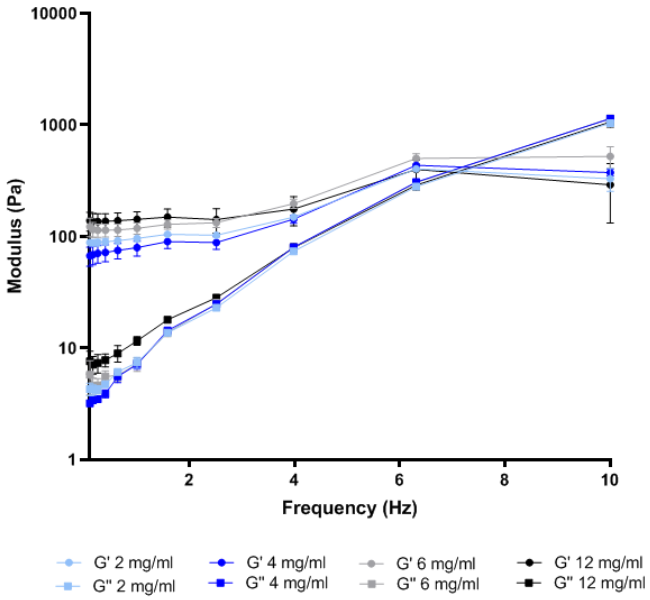

Figure 3.16 Frequency sweep of freshly made collagen hydrogels. Crosslinker concentration did not alter the mechanical properties of the collagen hydrogels. n = 1 biological replicate, generated in triplicate. Data are represented as mean ± SEM of technical replicates. As biological replicates were not produced, this data was not analysed.

3.3.1.5 In vitro IL-10 release from collagen hydrogels

The *in vitro* release of IL-10 from collagen hydrogels incubated with SH-SY5Y cultures was assessed using a human IL-10 ELISA kit. Instead of assessing the IL-10 release from the collagen hydrogels alone, we studied the release kinetics of the cytokine in a neuronal culture since this condition resembles more to the *in vivo* environment. Both collagen hydrogel compositions successfully retained and released IL-10 into the media over time until they were fully degraded (collagen hydrogels were not visible when media was removed) (**Figure** 3.17**;** Time, $F_{(6, 28)} = 25.39$, $P<0.0001$). Furthermore, the collagen hydrogel with lower concentration of crosslinker degraded quicker, consequently releasing IL-10 into the media faster (**Figure** 3.17**;** Crosslinker concentration, $F_{(1, 28)} = 57.34$, $P<0.0001$).

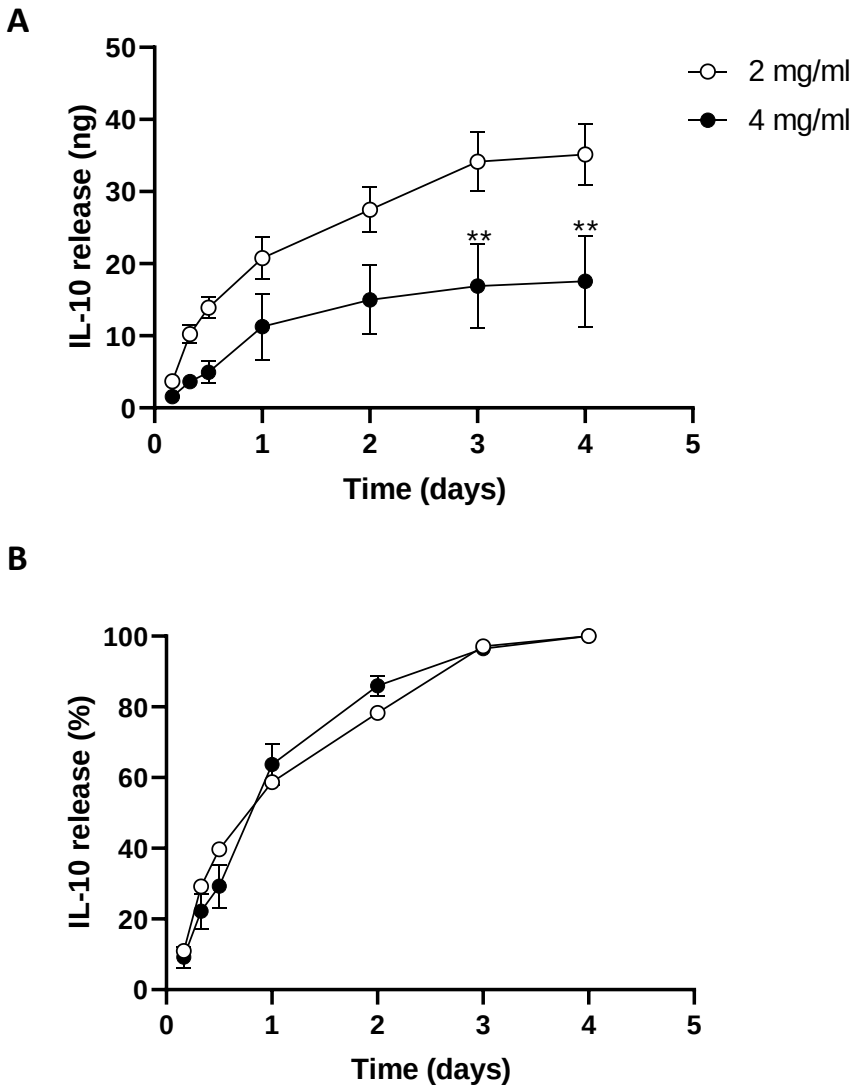

Figure 3.17 *In vivo* cumulative IL-10 release from collagen hydrogels in neuronal cultures. Collagen hydrogels with 2 and 4 mg/ml of crosslinker successfully retained IL-10 and they provided a sustained release of IL-10 over time in SH-SY5Y cultures. Cumulative release of IL-10 from collagen hydrogels depicted in A) nanograms or B) percentage. n = 3 biological replicates, generated in triplicate. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test. ** $P < 0.01$ vs 2 mg/ml crosslinker.

3.3.1.6 In vitro functionality assessment of IL-10 released from collagen hydrogels

Furthermore, to ensure IL-10 released from the collagen hydrogels was functional, its effects were assessed using a Poly I:C challenge. The administration of Poly I:C to VM cell cultures generated an inflammatory response as observed by the release of the pro-inflammatory factor IL-1β into the media (**Figure** 3.18**;** Poly I:C, $F_{(2, 18)} = 11.84$, $P<0.001$). Pre-treatment of VM cultures with IL-10 – either as a bolus or released from a collagen hydrogel – attenuated this response (**Figure** 3.18**;** IL-10, $F_{(2, 18)} = 9344$, $P<0.01$).

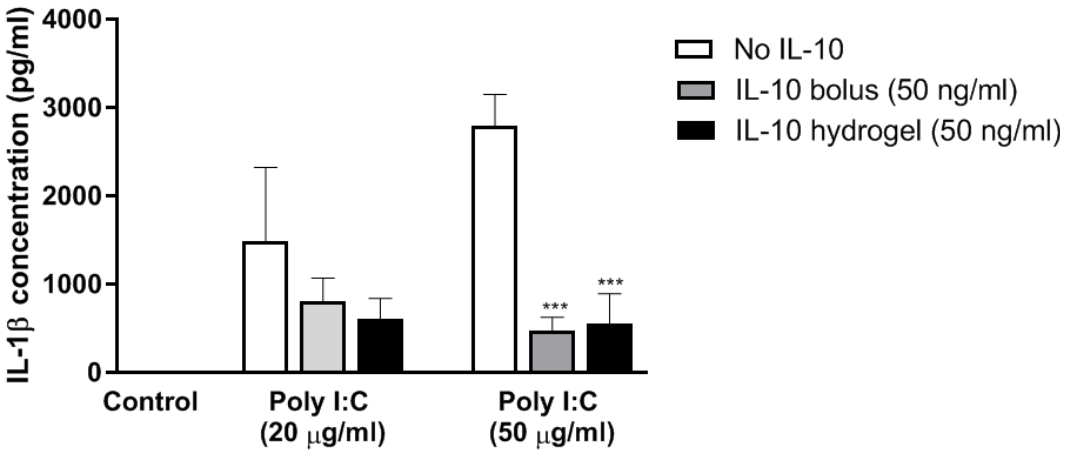

Figure 3.18 Poly I:C neuroinflammatory challenge and an IL-10 treatment in primary VM cultures. IL-10 either in a bolus or released from collagen hydrogels reduced the production of IL-1β in the media after Poly I:C (50 μg/ml). n = 3 biological replicates, generated in triplicate. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test. *** $P < 0.001$ vs Poly I:C treatment alone.

3.3.2 *In vitro* assessment of the impact of crosslinker concentration on cell viability and function

Once the collagen hydrogels with increasing concentrations of crosslinker were characterised, their biocompatibility was assessed. The collagen hydrogels with highest crosslinker concentrations (6 and 12 mg/ml) were not used for biocompatibility assays since Moriarty et al., (2018) reported these doses of 4s-StarPEG being harmful to mesenchymal stem cell transplants and to the host tissue due to strong gelation.

In vitro biocompatibility of collagen hydrogels (with 1-4 mg/ml of crosslinker) was assessed by exposing VM cultures to preformed collagen hydrogels of increasing concentrations of crosslinker for 24h. The morphology of neuronal cells, dopaminergic neurons and astrocytes was not modified by any of the tested collagen hydrogels (**Figure** 3.19). Likewise, none of the collagen hydrogel compositions with increasing concentrations of crosslinker modified the ratios of VM subpopulations; neuronal cells (**Figure** 3.20**a**; Crosslinker concentration, $F_{(3,\ 8)} = 0.245$, $P>0.05$), dopaminergic neurons (**Figure** 3.20**b**; Crosslinker concentration, $F_{(3,\ 8)} = 0{,}208$ $P>0.05$) and astrocytes (**Figure** 3.20**c**; Crosslinker concentration, $F_{(3,\ 8)} = 0.389$, $P>0.05$).

Most importantly, the overall viability of VM cultures was not compromised by the exposure to any of the collagen hydrogels used (**Figure** 3.20**d**; Crosslinker concentration $F_{(3,\ 8)} = 1.412$, $P>0.05$).

To further assess if the collagen hydrogels had any detrimental effects on the VM cultures, the primary mixed cultures were exposed to collagen hydrogels for 24h and their calcium responses to several stimuli were measured by calcium imaging techniques (**Figure** 3.21 and **Figure** 3.22). The mean and maximum ATP responses from VM cultures were to be unaffected by the exposure to preformed unseeded collagen hydrogels.

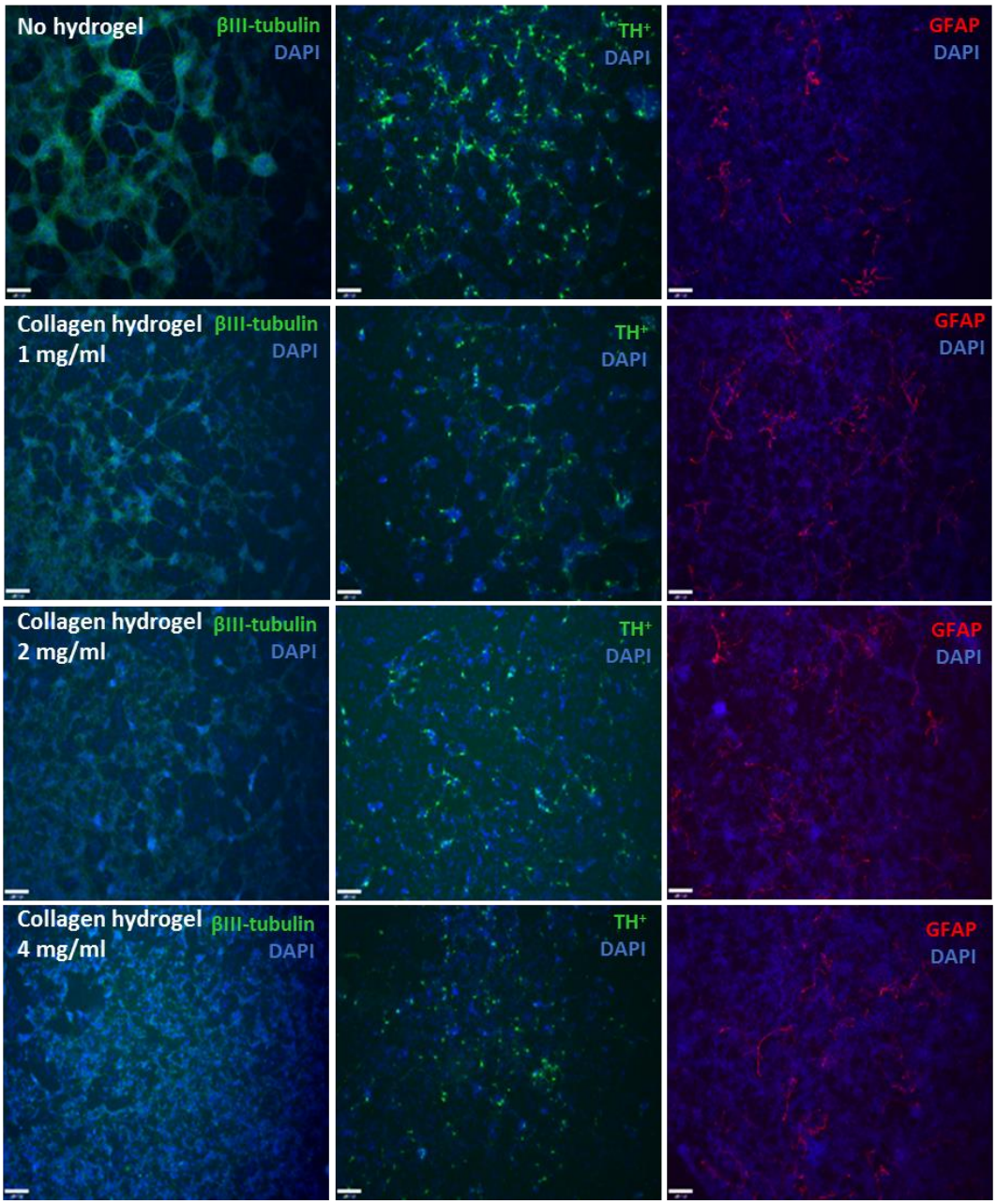

Figure 3.19 Collagen hydrogel exposure on several sub-populations of VM cultures. The incubation of collagen hydrogels crosslinked with various concentrations of 4s-StarPEG had no negative impact on neuronal (βIII-tubulin), dopaminergic (TH+) or astrocytic (GFAP) cell morphologies. Photomicrographs show VM cultures incubated with collagen hydrogels containing 1-4 mg/ml of 4s-StarPEG or left untreated. Scale bar represents 100 µm.

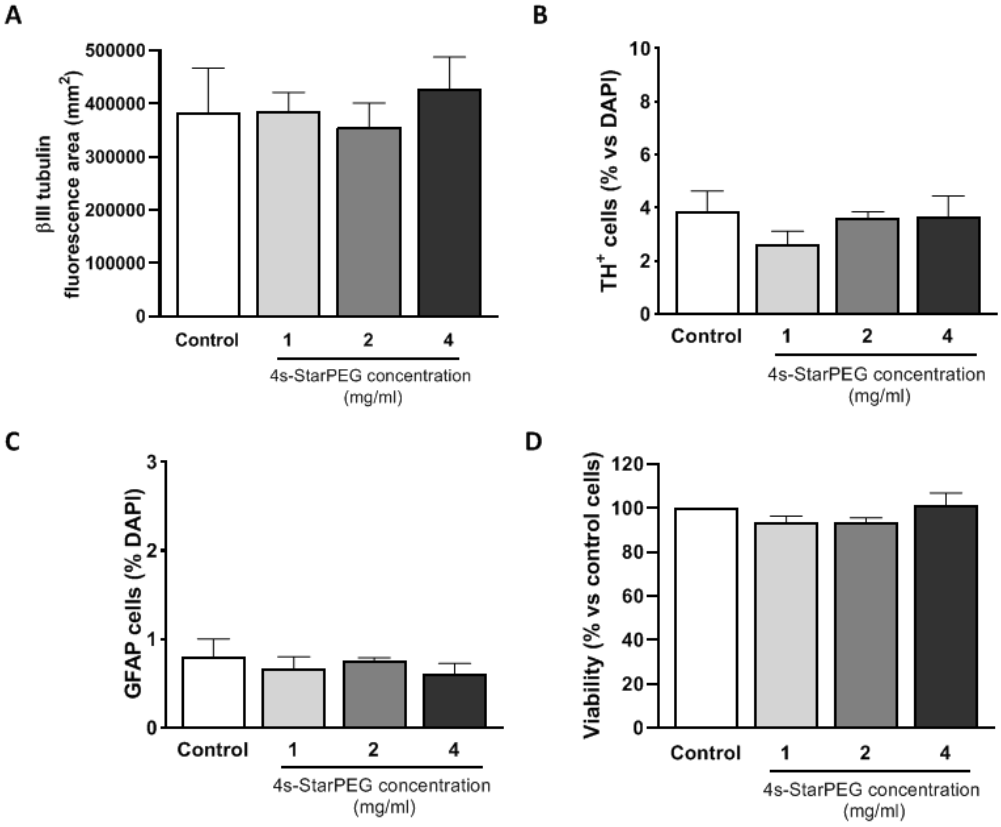

Figure 3.20 Effects of collagen hydrogel exposure on the viability of sub-populations of VM cultures. The incubation of collagen hydrogels crosslinked with various concentrations of 4s-StarPEG had no negative impact on the cell counts of A) neuronal, B) dopaminergic and C) astrocytic populations present in a VM culture as well as in D) the overall viability of the culture. n = 3 biological replicates, generated in triplicate. Data are expressed as mean ± SEM and were analysed by one-way ANOVA with Bonferroni *post-hoc* test.

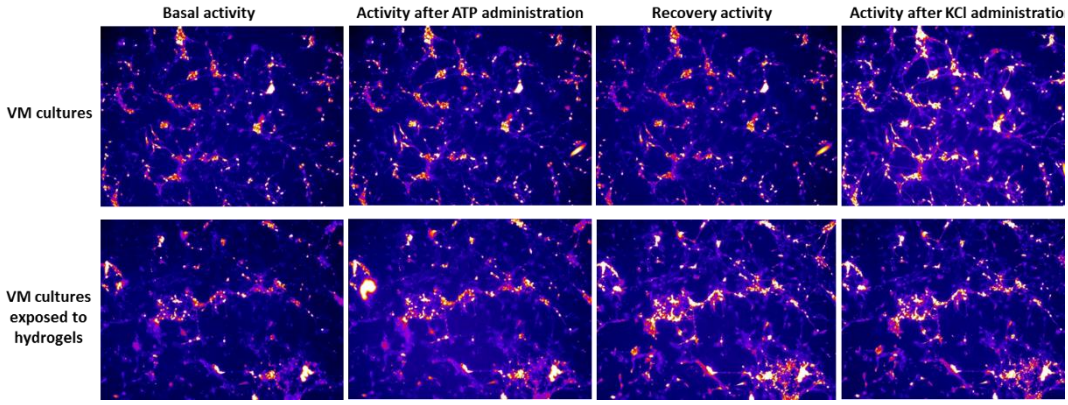

Figure 3.21 Representative snapshots during calcium imaging recordings of primary dopaminergic VM cultures. Photomicrographs show similar calcium response of VM cultures when they were previously exposed and not exposed to collagen hydrogels.

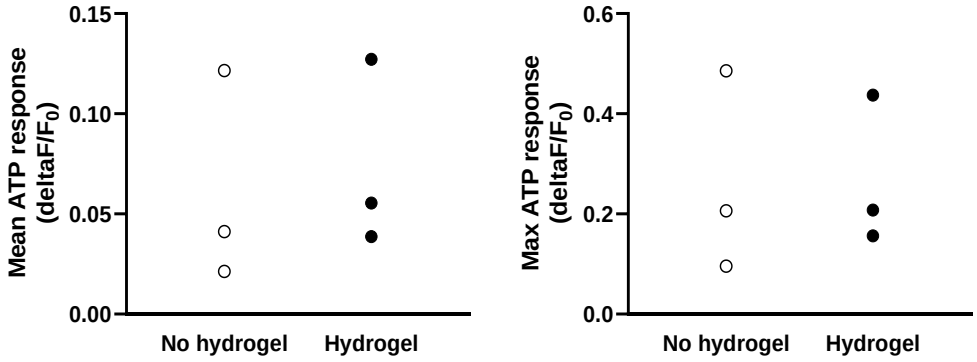

Figure 3.22 Crosslinked collagen hydrogels effect on ATP response in collagen hydrogels. The incubation of collagen hydrogels crosslinked with 4 mg/ml of 4s-StarPEG alongside VM cell cultures had no effect on the ATP response in calcium stimulation experiments. n = 1 biological replicate, generated in triplicate. Data are represented as technical replicates. As biological replicates were not produced, this data was not analysed.

3.4 DISCUSSION

Cell transplantation is a promising disease-modifying therapy that could develop into an alternative treatment for Parkinson's disease. However, the main limitation of this therapy that has prevented its progression into the clinic is the poor survival of the dopaminergic cells after transplantation (Brundin et al., 2000a). Thus, there is a demand to improve the survival of the grafted cells. Nevertheless, new approaches in tissue engineering have brought some light into this matter. Biomaterials, especially injectable scaffolds, have the potential to protect the dopaminergic neurons during the transplantation process and after by providing a structural matrix that creates a favourable microenvironment and protects dopaminergic cells from the host immune response, consequently increasing their chances of survival.

Hydrogels have been extensively used for cell encapsulation as a result of their high content of water and porosity, which facilitate 3D cell distribution and nutrient diffusion (Peppas et al., 2006). In particular, injectable hydrogels can be used in cell replacement therapies in the CNS as a scaffold thanks to their similar softness to the human body, their ability to form 3D networks and their adjustability to degrade over time (Khan & Khan, 2013). Furthermore, natural polymer based hydrogels - such as collagen or hyaluronic acid hydrogels - have broadly been used for cell or drug delivery in the brain, since they hold a series of advantages in respect to synthetic hydrogels like biocompatibility and biodegradability (Orive et al., 2009). Particularly, naturally-derived hydrogels have shown great potential in improving cell survival in cell replacement therapies for Parkinson's disease. The encapsulation and intra-striatal delivery of primary E12 VM cells in a collagen hydrogel resulted in a greater functional recovery in hemi-parkinsonian rats (Moriarty et al., 2019a). Similarly, the encapsulation of hESC-derived midbrain dopaminergic neurons in an engineered hyaluronic acid hydrogel increased cell viability *in vitro* and enhanced dopaminergic cell survival in the striatum by 5-fold compared to unencapsulated cells (Adil et al., 2017). Hence, these studies reflect that biomaterial based scaffolds can be an advantageous and easily adaptable tool to improve dopaminergic neuron survival in Parkinson's disease, independently of the cells' origin.

Likewise, hydrogels have also been used as biomaterial drug-delivery systems to deliver neurotrophins (such as Brain-Derived Neurotropic Factor (BDNF) or

Neurotrophin-3) in models of many brain diseases, like stroke (Clarkson et al., 2015; Cook et al., 2017) or spinal cord (Houweling et al., 1998; Piantino et al., 2006). In Parkinson's disease, Senthilkumar et al. (2007) showed that the implantation of a DA-embedded hydrogel in the striatum of 6-OHDA lesioned rats significantly reduced apomorphine-induced contralateral rotations for up to 26 days, proving soluble hydrogels directly injected into the striatum of parkinsonian rats could deliver functional molecules.

Yet an even more interesting strategy is to combine the encapsulation and delivery of dopaminergic cells with neurotrophic factors to create a suitable microenvironment upon transplantation that ultimately improves cell survival. In this direction, Moriarty and colleagues reported that a GDNF-enriched collagen hydrogel significantly improved the survival of dopaminergic neurons by 5-fold and their striatal re-innervation was dramatically increased by 3-fold after intra-striatal transplantation into the degenerated striatum (Moriarty et al., 2017).

Therefore, biomaterials can improve the regenerative potential of cells and biomolecules by providing an adhesion matrix to transplanted cells and a localised and sustained delivery of neutrophins. The framework in this thesis was to evaluate the potential benefits of an anti-inflammatory loaded collagen hydrogel for cell transplantation in Parkinson's disease.

For this reason, the aim of the work summarised this chapter was to design, assess and adapt an injectable collagen hydrogel that could be loaded with anti-inflammatory cytokines to be used in cell transplantation for Parkinson's disease. Through a battery of tests, the main properties of the collagen hydrogels that could affect the correct delivery of the biomaterial, the molecules of interest and/or the encapsulated cells were studied.

Although collagen *per se* is able to polymerise and form an *in situ* hydrogel (Peppas et al., 2006), natural and synthetic polymers – like collagen – are crosslinked to avoid the dissolution of these chains in an aqueous environment. By crosslinking these polymers, the structure of the hydrogel is stabilised and its polymerisation and degradation can be regulated (Hennink & Van Nostrum, 2012). Chemical crosslinking is broadly used since it is a simple and versatile method, although some crosslinkers are associated with toxicity. 4S-StarPEG is a PEG that is covalently bound to the

collagen strands to provide stiffness and stability to the collagen hydrogel and it has the advantage of being non-toxic, low immunogenic and already approved by the FDA (Veronese & Pasut, 2005; Alconcel et al., 2011).

In this chapter we have used collagen hydrogels fabricated with 2 mg/ml of bovine type I collagen and multiple concentrations of 4s-StarPEG (1-12 mg/ml) as main components of the biomaterial. Type I collagen was chosen since it has been widely used for cell encapsulation due to its properties of mimicking the extracellular matrix (Grinnell & Petroll, 2010; Streeter & Cheema, 2011). Moreover, collagen hydrogels with 2 mg/ml of bovine type I collagen have been successfully used for cell transplantation previously (Moriarty et al., 2019a). Thus, collagen concentration was decided to be fixed at 2 mg/ml based on previous publications from the group (Hoban et al., 2013; Moriarty et al., 2017, 2019a) and also to reduce the amount of collagen hydrogel compositions to be assessed. Hence, in these initial studies, we analysed several collagen hydrogel compositions (with increasing concentration of crosslinker) to determine how the concentration of 4s-StarPEG crosslinker could modify its structural, mechanical and chemical properties. Later we assessed the ability of the biomaterial to retain and release anti-inflammatory cytokines *in vitro* and lastly we assessed their cytocompatibility with neural cultures.

Before using an injectable collagen hydrogel for cell transplantation, the biomaterial's polymerisation and degradation profiles have to be determined and tailored for its use *in vivo*. The idea behind a thermoresponsive injectable hydrogel is that its components are mixed before injection, the liquid solution is then delivered *in vivo* and the hydrogel polymerises *in situ* at body temperature. Ergo, the polymerisation time is of crucial importance for a successful delivery to the brain, since too quick of a gelation will impede the proper delivery of the hydrogel to the brain and a too slow polymerisation will cause the hydrogel to spread away from the injection point, reducing its functionality. Hence, we showed that the higher the concentration of crosslinker, the quicker the polymerisation occurred *in vitro* at 37°C (**Figure** 3.5). Our polymerisation data revealed that collagen hydrogels with too low concentration of crosslinker might not be suitable candidates for *in vivo* use since they took a long time to polymerise. Similarly, the collagen hydrogel compositions with high concentrations of crosslinker polymerised in a few minutes, indicating their delivery could be troublesome. These results are in line with previous work, where Moriarty and

colleagues used a collagen hydrogel with 4 mg/ml of 4s-StarPEG for cell transplantation in hemi-parkinsonian rats (Moriarty et al., 2017, 2019a).

The degree of crosslinking is a key component for many of the hydrogel's properties like elasticity or swelling behaviour (Maitra & Shukla, 2014). It is known that chemical crosslinking of collagen changes the rigidity of the collagen hydrogel matrix and this impacts on its polymerisation profile and microstructure. Here, we have shown that the crosslinker concentration did modify the fibrous network of the collagen hydrogel, reducing the pore size of the matrix (**Figure 3.6** and **Figure 3.7**) and affecting its ability to encapsulate cells *in vitro* (since collagen hydrogels with low concentrations of crosslinker could not hold encapsulated cells).

In an attempt to analyse the degradation of collagen hydrogels *in vitro*, we stained several compositions of collagen hydrogels with Coomassie blue dye in order to measure degradation of the hydrogels indirectly. However we found out that this release assay did not correlate with the degradation of the collagen hydrogels (**Figure** 3.14). Nevertheless, this experiment highlighted how the environment can modify the hydrogel's properties since collagen hydrogels behaved very differently when they were immersed in media or water.

On a different note, rheology is an appropriate method to characterise the hydrogel mechanical properties since it is quick, sensitive and provides with substantial information about the differences in structural architecture of the different collagen hydrogels (Zuidema et al., 2014). Biomaterial characterisation has to take into account the mechanical properties of the tissue or organ where the biomaterial will be implanted. Matching the mechanical properties of a biomaterial with the ones of the tissue microenvironment can result in favourable outcomes. For example, Georges et al. (2006) reported that softer gels promoted neurite outgrowth in mixed cortical cultures better than stiffer gels. Furthermore, biomaterials with a similar stiffness as the brain can result in a favourable neuronal differentiation (Seidlits et al., 2010). Similarly, astrocytes grown in stiff polyacrylamide gels tended to spread and exhibit a more complex morphology than astrocytes grown in softer gels (Moshayedi et al., 2010).

It is well known that the brain is one of the softest tissues in the body, although there is some discrepancies in the storage moduli values of the rat brain. Buxboim et al.,

(2010) estimated the storage moduli ranging between 0.2-1 KPa in the rat brain. In parallel, Niemczyk et al., (2018) pointed out that biomaterials with a storage moduli of approximately 0.5 KPa were a good indicator for brain regeneration. This variability in the storage moduli measurements can be explained depending on the region of the brain measured, the age of the subjects or quality and processing of the tissue. Moreover, Gefen et al., (2003) demonstrated that the stiffness of the brain diminished with age and storage moduli of adult rat brain were found around 0.1-1 KPa, which constituted similar values than porcine, primate and human brain specimens.

Here we have shown that collagen hydrogels behave as elastic solids once they have polymerised (**Figure** 3.15 and **Figure** 3.16). The collagen hydrogels assessed were slightly softer than the modulus of the rodent brain. Notwithstanding, collagen hydrogels presented similar storage moduli values (~100-170 Pa) and loss moduli values (~25 Pa) as other collagen hydrogels (Zuidema et al., 2014; Vázquez-Portalati et al., 2016).

However, the slightly lower stiffness of the collagen hydrogels in comparison to the brain could be a positive event; Aurand et al., (2014) hypothesised that the survival and differentiation of fetal-derived neural progenitor cells would be improved when encapsulated into softer hydrogels since they more closely match the originating fetal tissue (which is known to be softer than the adult brain). Thus the same could prevail for primary VM cells.

Regarding the crosslinker concentration, we did not find any differences due to the concentration of crosslinker in their rheological properties. Although this is unusual, this could be explained by the fact that the collagen concentration – which was unchanged in this characterisation - has a higher impact on the stiffness of the biomaterial in comparison to the crosslinker concentration. In our observations, even the collagen hydrogels with the highest crosslinker concentration generated weak hydrogels. Higher concentrations of collagen have correlated with higher stiffness (Montalbano et al., 2018), thus the collagen concentration could be increased in case that stiffer hydrogels were necessary for optimal cell and drug encapsulation.

Although the collagen hydrogels seem to be compatible for intra-striatal delivery, the complex environment of the brain can modify the mechanical properties of the collagen hydrogels (Niemczyk et al., 2018). Furthermore, the encapsulation of cells

will most likely modify the mechanical properties of the hydrogels. Thus, although the stiffness and elasticity of the collagen hydrogels *in vitro* was similar to the one of the host tissue, they will have to be assessed in further *in vivo* studies.

Next, we evaluated the ability of the collagen hydrogels to retain and release IL-10. We have shown here that IL-10 is released over time in *in vitro* neuronal cultures until the collagen hydrogel is fully degraded (**Figure** 3.17). More importantly, we have demonstrated by using a Poly I:C challenge that the released IL-10 is able to exert its biological functions such as reducing the pro-inflammatory levels of certain cytokines (**Figure** 3.18). These results show that collagen hydrogels can be used as drug-delivery systems *in vitro*.

Chemical crosslinked hydrogels are characterised by a high stability and mechanical strength, however the use of chemical molecules can result in toxicity. Thus before using the collagen hydrogels in the brain, their biocompatibility must be assessed *in vitro*. Our preliminary *in vitro* assessments showed that the incubation of collagen hydrogels along with VM cultures did not have any negative impact on the cell culture survival at any of the crosslinker concentrations assessed (**Figure** 3.19 and **Figure** 3.20). This fact is in agreement with data reported previously (Hoban et al., 2013; Moriarty et al., 2017). Furthermore, we observed that VM cultures respond normally to calcium signalling stimulus after being exposed to collagen hydrogels (**Figure** 3.21 and **Figure** 3.22). Collagen hydrogels with high concentrations of crosslinker (6 and 12 mg/ml) were not initially tested for *in vitro* biocompatibility since they were found detrimental for MSC graft survival *in vivo* (Moriarty, 2018) and consequently were discarded as good candidates for *in vivo* experiments.

Dopaminergic cell replacement therapies have been shown to bring clinical benefits in the treatment of the cardinal symptoms of Parkinson's disease. However, the extremely poor survival of the transplanted cells hampers their broad use in the clinical practice. Yet, new approaches in tissue engineering like using biomaterial-based scaffolds to protect cells upon transplantation have the potential to improve the current limitations of cell replacement therapies. Thus, before evaluating the functionality of the IL-10 loaded collagen hydrogels as a cell scaffold for dopaminergic cell transplantation in Parkinson's disease, we have presented here an initial characterisation of the inherent properties of the collagen hydrogels that could affect

this application. This data altogether suggests that collagen hydrogels with lower concentrations of crosslinker may be the best candidates for *in vivo* delivery since they had cytocompatible, slow gelling properties and were able to retain and release IL-10.

This biomaterial optimisation constituted the first and essential step to further optimise and assess the potential of an IL-10 loaded collagen hydrogel to improve the survival of dopaminergic cell grafts in Parkinson's disease.

CHAPTER 4: *IN VIVO* OPTIMISATION OF AN IL-10 LOADED COLLAGEN HYDROGEL FOR INTRA-STRIATAL CELL TRANSPLANTATION

4.1 INTRODUCTION

Dopaminergic cell-based therapies for Parkinson´s disease – although very promising – face many challenges including poor cell viability that impede the field achieving clinical efficacy. Nevertheless, the emergence of tissue engineering has brought new insights in the field of cell replacement therapies in the context of neural repair. Biomaterials offer prospects to overcome or reduce many of the current challenges in cell transplantation processes. Biomaterials can be tailored to provide desired benefits to the targeted biological system. Consequently, biomaterial scaffolds can be designed to deliver cells to the targeted tissue, as well as to deliver relevant biomolecules locally to protect them from the host microenvironment. Thus, the characteristics of a biomaterial will depend on its final use, however some common features are found when targeting a specific organ (Tuladhar et al., 2018).

When designing biomaterials for application in the brain, special attention has to be paid to the biomaterial's biocompatibility since the brain is more sensitive to environmental stresses than other organs (Saxena & Caroni, 2011). As well, the mechanical properties of the material should match the ones from brain tissue to maximise biocompatibility (Spencer et al., 2017). The isolation of the brain makes the implantation of a biomaterial more complex than in other organs, thus injectable and flexible materials are preferred as they can be implanted with less invasive surgery (Tuladhar et al., 2018). Moreover, biomaterials for brain repair have to be chemically and mechanically stable long enough in the brain to perform their desired biological function but be biodegradable once their function has been fulfilled. Last but not least, the by-products of the biomaterial degradation also need to be non-toxic (Orive et al., 2009).

Biomaterials for cell transplantation therapies in Parkinson´s disease need to protect dopaminergic cells during the transplantation process and integration in the host tissue, while also retaining biomolecules that promote cell survival and engraftment (Burdick et al., 2016). Hydrogel scaffolds can be fabricated to deliver cells into the targeted

tissue as well as to release relevant biomolecules in the transplantation site in a controlled and local manner. Hydrogels are an excellent scaffold for cell transplantation due to their highly hydrated and porous nature which enables encapsulated cells to attach and develop in the host tissue while permitting nutrient exchange with the surrounding microenvironment (Burdick et al., 2016; Talebian et al., 2019). Furthermore, thermoresponsive hydrogels are liquid solutions upon fabrication that polymerise *in situ* in the brain, making them injectable and consequently easier to implant.

In an attempt to mimic the ECM, natural polymers like collagen are used in hydrogel scaffolds to mimic some of the roles of natural ECMs, such as providing structural support or controlling the function of cells and allowing the diffusion of nutrients and metabolites (Orive et al., 2009). Moreover, the use of collagen in biomedicine has been approved by many regulatory agencies, thus representing an advantage versus synthetic materials (Berthiaume et al., 2011).

Therefore, since collagen hydrogels hold all these properties and advantages summarised above, they are being investigated as a scaffold for cell transplantation in many disciplines. In Parkinson's disease research, when these naturally-derived injectable scaffolds have been used with primary dopaminergic cells, cell survival and nerve outgrowth were dramatically improved upon transplantation (Moriarty et al., 2017, 2019a). Similarly, the survival of neural stem cells has also been improved using collagen hydrogels functionalised with several growth factors (Egawa et al., 2011; Nakaji-Hirabayashi et al., 2012, 2013). However, the use of hydrogel scaffolds to reduce the host immune response and to provide neuroprotection has been poorly investigated.

In the previous chapter we described the properties of collagen hydrogels relevant to cell and drug encapsulation *in vitro*. Collagen hydrogels were chemically and mechanically stable, cytocompatible and could retain and release IL-10 *in vitro*. Hence, the aim of the following chapter was to test the crosslinked IL-10 loaded collagen hydrogels *in vivo* to choose the most adequate composition for cell transplantation in Parkinson´s disease.

4.2 METHODS

4.1.1 Experimental design

The aim of this chapter was to optimise the collagen hydrogels for the *in vivo* intra-striatal delivery of primary dopaminergic cells and IL-10. In Chapter 3, we reported the characterisation of collagen hydrogels *in vitro,* establishing their polymerisation patterns, cytocompatibility and their ability to retain and release IL-10 *in vitro*. However, the host tissue environment is more complex than the *in vitro* setting, requiring for an *in vivo* optimisation of the biomaterial.

Hence, in this chapter, through a series of pilot studies, we address the biocompatibility, polymerisation and degradation of collagen hydrogels when delivered in the striatum, as well as their ability to retain and release IL-10 *in vivo*. Lastly, we report here the effects of cell encapsulation in an IL-10 collagen hydrogel.

4.2.1.1 Preliminary in vivo intra-striatal delivery of collagen hydrogels

At the beginning of this project, two brands of bovine type I atellocollagen were used to fabricate collagen hydrogels. To assess if the brand of bovine type I collagen had any impact on the *in vivo* delivery and *in situ* polymerisation and degradation of collagen hydrogels, a small pilot study was designed. 6 female Sprague-Dawley rats received a bilateral intra-striatal injection of 6 µl of collagen hydrogel crosslinked with 4 mg/ml of 4-StarPEG fabricated either with neutralised bovine type I collagen from Vornia Biomaterials or Collagen Solutions. The animals were sacrificed at days 1 or 4 post-transplantation (**Table 4.1**). A schematic of the study design is shown below in **Figure 4.1**.

Table 4.1 Experimental groups used in collagen hydrogel optimisation study. COS = Collagen Solutions Collagen, Vornia = Vornia Biomaterials Collagen. Collagen hydrogels with 4 mg/ml 4s-StarPEG

Experimental Group	Sample size	Sample size
	Sacrificed 24h after injection	Sacrificed 96h after injection
COS collagen hydrogel	3	3
Vornia collagen hydrogel	3	3

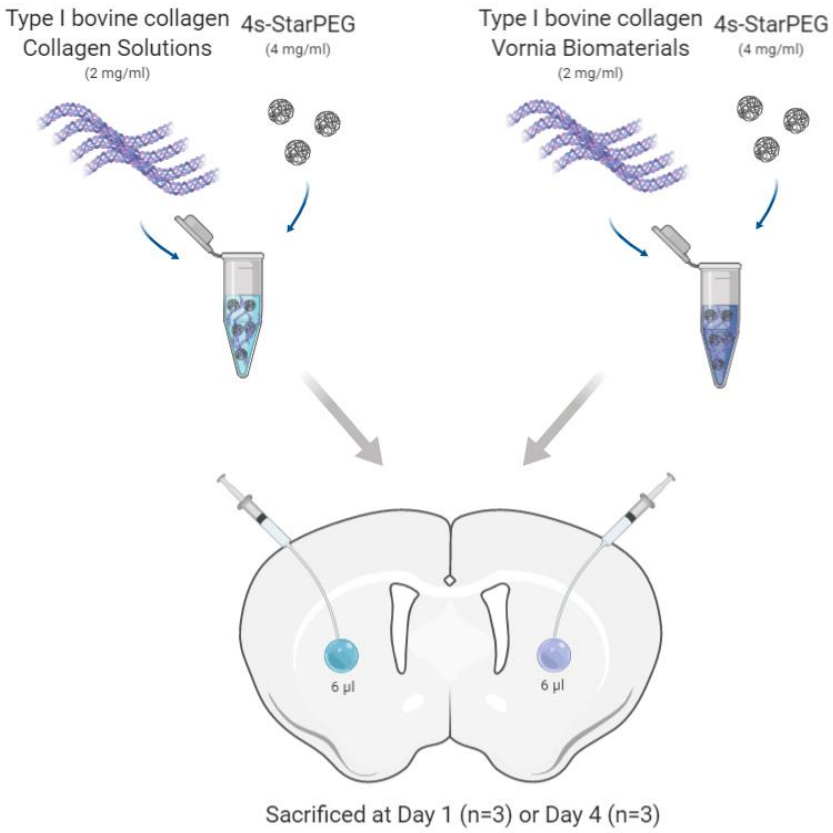

Figure 4.1 Preliminary *in vivo* study to assess the impact of collagen brand on collagen hydrogel polymerisation and immunogenicity. Schematic representation of the fabrication and *in vivo* delivery of collagen hydrogel with 4 mg/ml of crosslinker, made out of either Collagen Solutions or Vornia Biomaterials bovine type I collagen. *In situ* polymerisation, degradation and host immune response were assessed at 1 and 4 days post-injection.

4.2.1.2 In vivo assessment of IL-10 release from collagen hydrogels

To evaluate the effects of crosslinker concentration *in vivo*, 24 adult male Sprague-Dawley rats received a bilateral intra-striatal infusion of 1000 ng (in 6 µl) of human IL-10 as a bolus or encapsulated in 6 µl of collagen hydrogel crosslinked with either 1, 2 or 4 mg/ml of 4s-StarPEG. The animals were then sacrificed for *post-mortem* analysis at days 1, 2 or 4 post-surgery (**Table 4.2**). *In situ* polymerisation and degradation, i*n vivo* biocompatibility, and IL-10 release were assessed using immunohistochemistry analysis. A schematic view of the experimental design is shown in **Figure 4.2**.

Table 4.2 Experimental groups used in an IL-10 release study. Human IL-10: 1000 ng/6 µl

Experimental Group	Sample size	Sample size	Sample size
	Sacrificed 24h after injection	Sacrificed 48h after injection	Sacrificed 96h after injection
Human IL-10 bolus	4	4	4
Collagen hydrogel with 1 mg/ml of 4s-StarPEG and IL-10	4	4	4
Collagen hydrogel with 2 mg/ml of 4s-StarPEG and IL-10	4	4	4
Collagen hydrogel with 4 mg/ml of 4s-StarPEG and IL-10	4	4	4

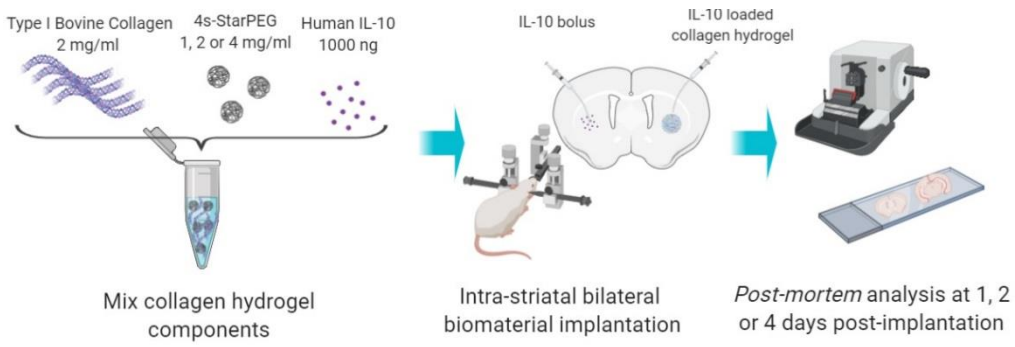

Figure 4.2 *In vivo* IL-10 release from collagen hydrogels. Schematic view of the delivery of human IL-10 (1000 ng/6µl) as a bolus or encapsulated in an IL-10 collagen hydrogel. *Post-mortem* analysis was performed at 1, 2 or 4 days after-injection.

4.2.2 *In vivo* assessment of the early effects of VM transplantation

This study aimed to study the host immune response elicited just after the implantation of VM cells encapsulated in an IL-10 collagen hydrogel in the parkinsonian brain. 40 adult male Sprague-Dawley rats received a unilateral injection of 6-OHDA in the MFB. Two weeks later, the extent of their lesion was assessed using drug-induced rotations and animals were equally distributed into the experimental groups through performance-matching. After lesion assessment, animals received a unilateral intra-striatal infusion of VM cells (400,000 cells) alone or encapsulated in a collagen hydrogel with 4 mg/ml of crosslinker. Cells alone or encapsulated in the biomaterial were administered in conjunction with 1000 ng of human IL-10 as a bolus or encapsulated in the collagen hydrogel. The animals were then sacrificed for *post-mortem* analysis at days 1 or 4 post-transplantation (**Table 4.3**). Dopaminergic cell graft survival and host immune response were assessed using immunohistochemistry analysis. A schematic view of the experimental design is shown in **Figure 4.3**.

Table 4.3 Experimental groups used in a transplantation study using encapsulated VM cells on an IL-10 collagen hydrogel. VM cells: 400,000 cells/6 µl, human IL-10: 1000 ng/6 µl.

Experimental Group	Sample size	Sample size
	Sacrificed 24h after injection	Sacrificed 96h after injection
VM cells alone	5	5
VM cells + IL-10	5	5
VM cells in collagen hydrogel	5	5
VM cells in an IL-10 collagen hydrogel	5	5

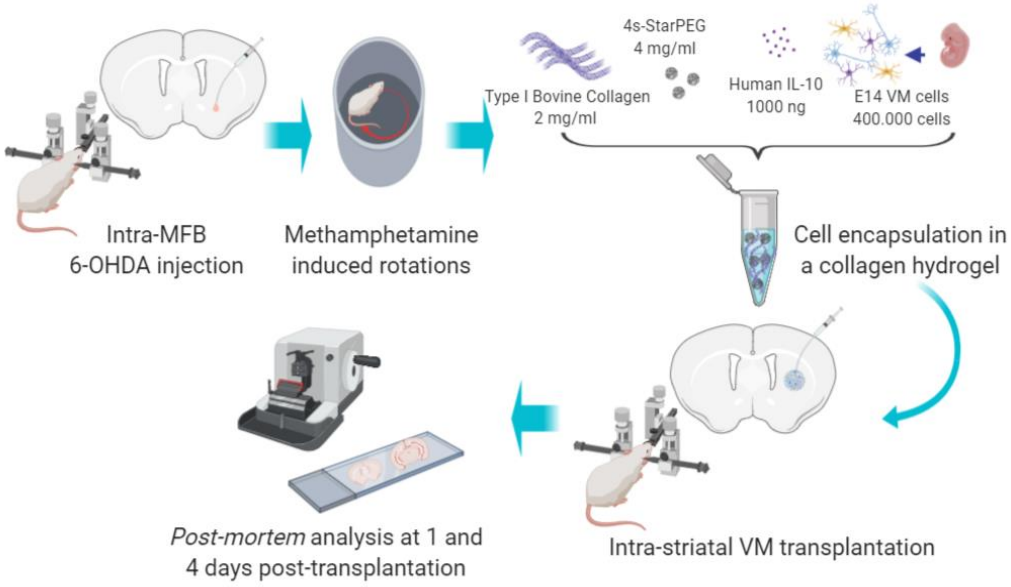

Figure 4.3 *In vivo* early effects of VM encapsulation in an IL-10 collagen hydrogel. Schematic view of the delivery of human IL-10 (1000 ng/6µl) as a bolus or encapsulated in an IL- collagen hydrogel. Animals were sacrificed at 1, 2 or 4 days after-injection.

4.3 RESULTS

4.3.1 Preliminary *In vivo* intra-striatal delivery of collagen hydrogels

4.3.1.1 In situ collagen hydrogel polymerisation

As mentioned in Chapter 3, two sources of bovine type I collagen (Vornia Biomaterials and Collagen Solutions) were used to fabricate collagen hydrogels *in vitro*. Thus, a preliminary *in vivo* study was carried out to assess if both collagen hydrogels polymerised *in situ* in the same manner and generated a comparable host immune response. Both collagen hydrogels - formulated with Vornia Biomaterials Collagen or Collagen Solutions collagen - successfully polymerised *in situ* in the striatum as shown by bovine collagen immunostaining (**Figure 4.4a** and **Figure 4.4b**). The collagen immunostaining showed a consistent and uniform staining of collagen for both collagen hydrogels and the collagen volume was similar for both collagen hydrogels at 1 and 4 days after injection (**Figure 4.4c**; Collagen brand, $F_{(1, 8)} = 0.089$, $P>0.05$). As expected, a decrease in collagen volume was observed over time in both collagen hydrogels (**Figure 4.4c**; Time, $F_{(1, 8)} = 21.44$, $P<0.05$).

Although the collagen volume was similar in both collagen hydrogels assessed, depicting a comparable polymerisation and degradation pattern, the Collagen Solutions collagen hydrogel seemed to produce more damage in the injected area than the Vornia Biomaterials collagen hydrogel (**Figure 4.4d**).

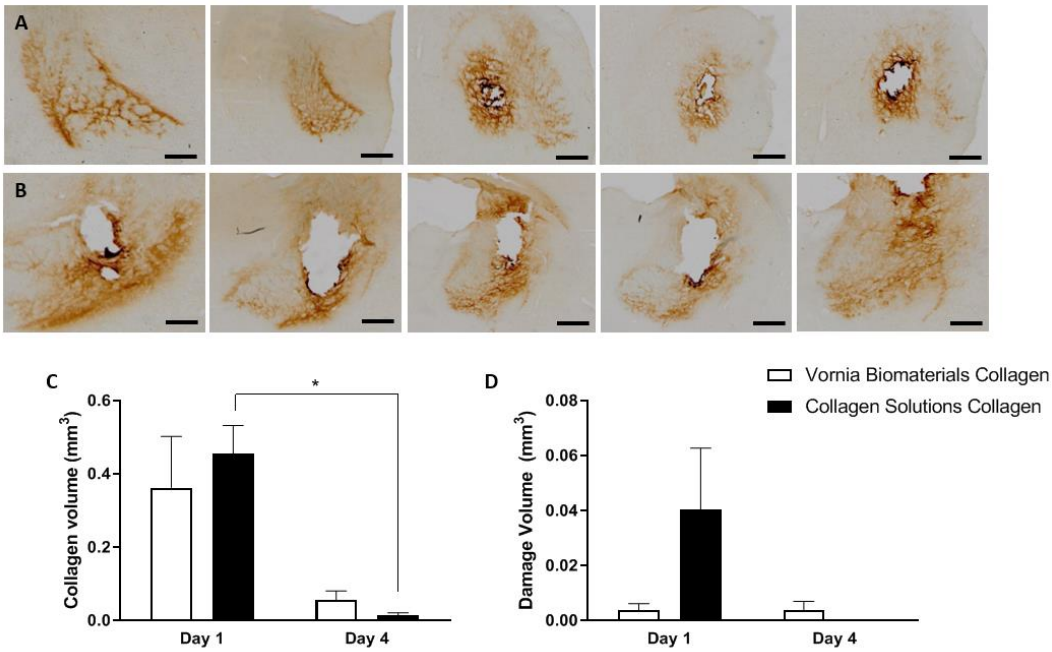

Figure 4.4 Collagen hydrogel polymerisation *in situ*. Representative photomicrographs show collagen immunostaining and damage caused in the striatum 24h after collagen polymerisation *in situ* with A) Vornia Biomaterials Collagen and B) Collagen Solutions Collagen. C) Collagen and D) Damage volumes. Although both collagen hydrogels showed similar content of collagen, the damage extent was highly variable between subjects in the Collagen Solutions Collagen hydrogel group. Collagen volume decreased with time as expected, in particular Collagen Solutions collagen hydrogel (* $P < 0.05$) vs day 1. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test. Scale bar = 100 µm.

4.3.1.2 Host immune response to collagen hydrogel injection

An injection into the brain's parenchyma leads to some degree of tissue damage which is usually followed by a reaction of the innate immune cells. Immunostaining for both microglial and astrocytic markers confirmed that the collagen hydrogel itself generated an immune response by the host (**Figure 4.5** and **Figure 4.6**).

Both collagen hydrogels assessed generated a comparable response in terms of density of astrocytes (**Figure 4.5b**; Collagen brand, $F_{(1, 8)} = 0.203$, $P>0.05$) and microglia

(**Figure 4.6b**; Brand, $F_{(1, 8)} = 0.367$, $P>0.05$). Similarly, there was no differences in the volume of astrocytosis (**Figure 4.5d**; Collagen brand, $F_{(1, 8)} = 1.638$, $P>0.05$) or microgliosis (**Figure 4.6d**; Collagen brand, $F_{(1, 8)} = 0.813$, $P>0.05$) between the two collagen hydrogels.

While the astrocytosis response was very quick and the astrocytosis volume did not change from day 1 to 4 post-injection (**Figure 4.5c**; Time, $F_{(1, 8)} = 0.2479$, $P>0.05$), the microgliosis volume was markedly increased 4 days after injection (**Figure 4.6c**; Time, $F_{(1, 8)} = 16.55$, $P<0.01$).

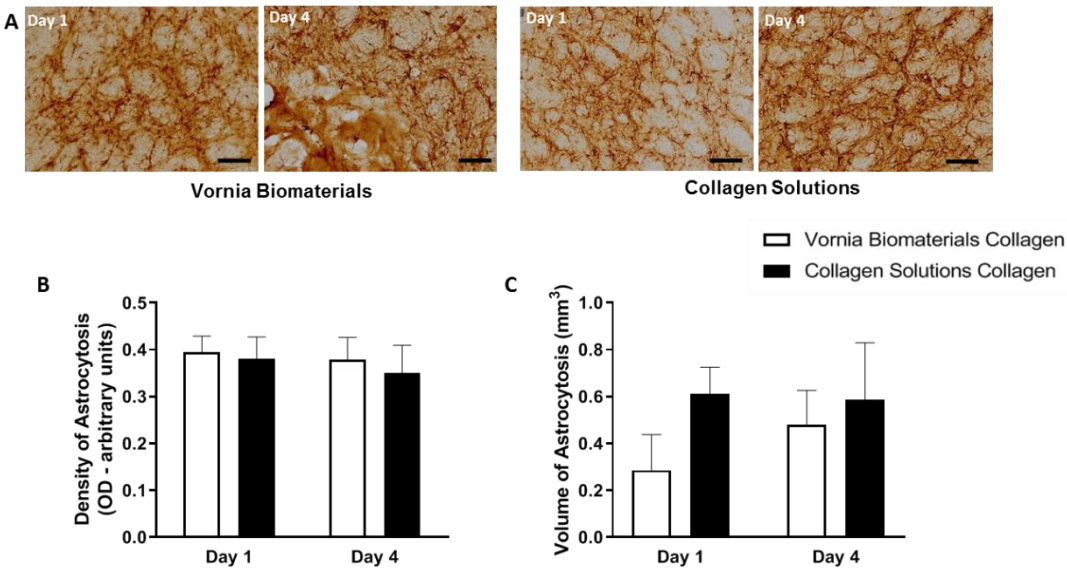

Figure 4.5 Astrocytic response to a collagen hydrogel injection in the striatum. A) GFAP immunostaining for astrocytes showed no significant differences in B) optical density or C) volume at the site of injection between which brand of collagen – Vornia Biomaterials or Collagen Solutions - was used to make the collagen hydrogels. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test. Scale bar = 100 µm

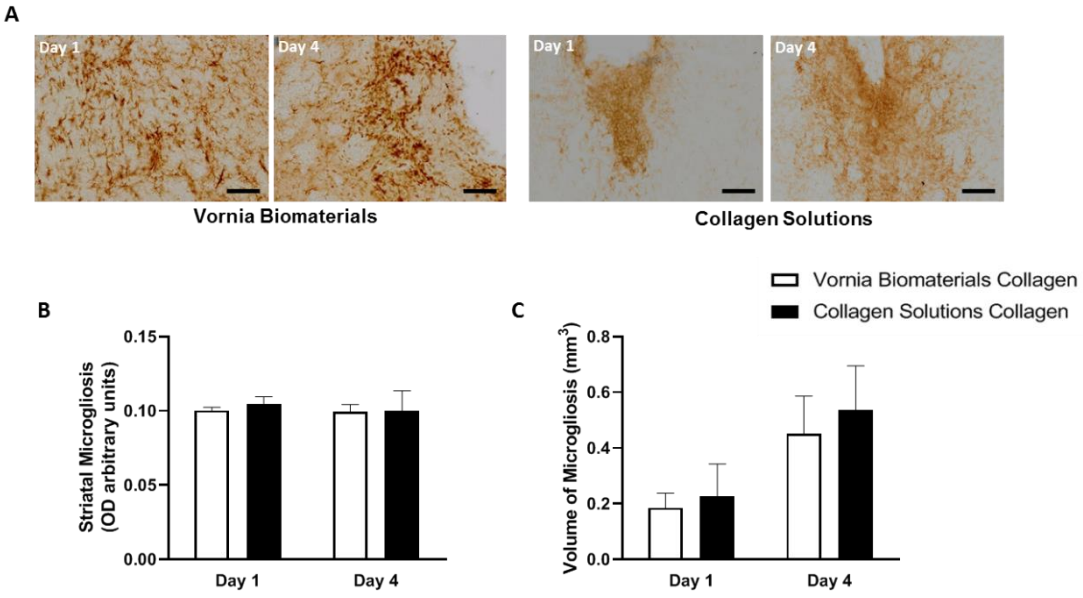

Figure 4.6 Microglial response to a collagen hydrogel injection in the striatum. A) OX-42 immunostaining for microglia injection showed no significant differences in B) optical density or C) volume at the site of injection between which brand of collagen – Vornia Biomaterials or Collagen Solutions - was used to make the collagen hydrogels. Microglial volume increased over time. Data are represented as mean ± SEM and were analysed by two-way ANOVA and Bonferroni *post-hoc* test. Scale bar = 100 µm.

4.3.2 *In vivo* assessment of IL-10 release from collagen hydrogels

Due to the damage observed in the Collagen Solutions collagen hydrogels, we decided to use Vornia Biomaterials collagen for future experiments. Thus, after selecting the best collagen source for collagen hydrogels, we moved on to assess the effects of the crosslinker concentration *in vivo*.

4.3.2.1 In situ polymerisation and degradation of collagen hydrogels

Type I bovine collagen immunostaining confirmed the presence of collagen hydrogel in the striatum throughout all experimental groups (**Figure 4.7**). A strong and defined staining of collagen was present in the striatum, indicating that all collagen hydrogels polymerised *in situ*. The volume of collagen was similar between all collagen

hydrogel compositions, although the collagen hydrogel with 4 mg/ml of crosslinker tended to show greater collagen staining, although it was not statistically significant (**Figure 4.7**; Crosslinker, $F_{(2, 27)} = 1.703$, $P>0.05$). As well, the collagen staining tended to decrease with time although it was not statistically significant (**Figure 4.7**; Time, $F_{(2, 27)} = 1.622$, $P>0.05$).

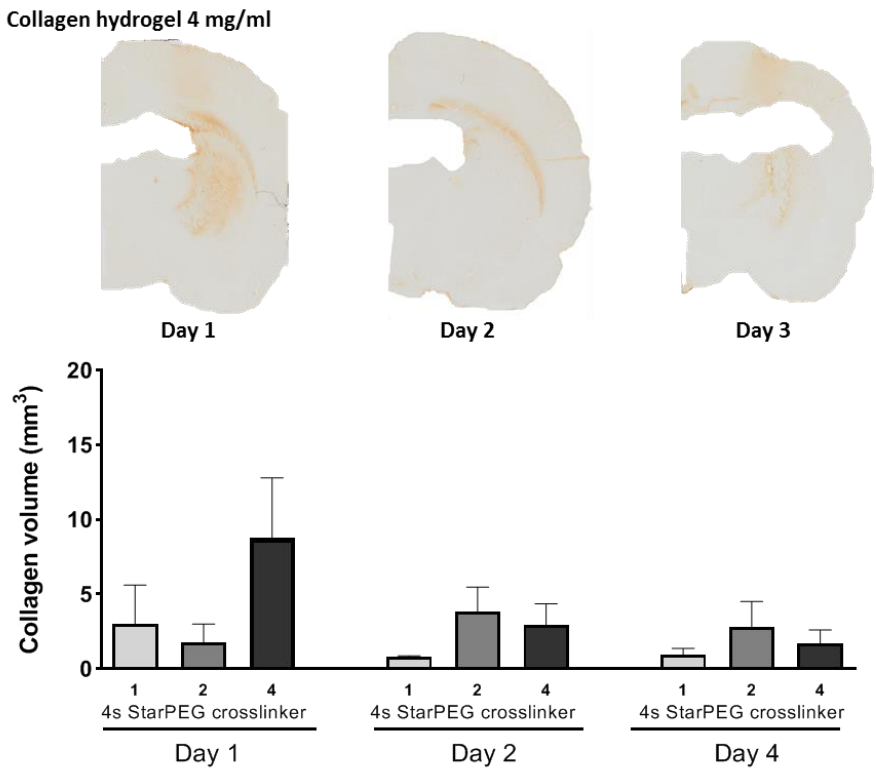

Figure 4.7 Collagen hydrogel gelation *in situ*. Type I bovine collagen immunostained photomicrographs show the successful *in situ* polymerisation of collagen hydrogels crosslinked with 1, 2 or 4 mg/ml of 4s-StarPEG and its biodegradability over time. Data are expressed as mean ± SEM and were analysed by two-way ANOVA and Bonferroni *post-hoc* test. Scale bar = 100 µm.

4.3.2.2 Host immune response to collagen hydrogels

Determining if the use of 4s-StarPEG as a crosslinker was biocompatible with the host brain and ensuring that it did not cause an exaggerated host immune response was crucial before moving on to more complex transplantation studies. Although, immunostaining for astrocyte and microglial markers confirmed an astrocytic and microglial response, this response was similar to the one elicited by the IL-10 bolus both in astrocytes (**Figure 4.8**; IL-10 delivery, $F_{(3, 34)} = 1.824$, $P>0.05$) and microglia (**Figure 4.9**; IL-10 delivery, $F_{(3, 34)} = 3.137$, $P<0.05$). This result suggests that collagen hydrogels crosslinked with 4s-StarPEG at a concentration range of 1-4 mg/ml are biocompatible with its intra-striatal delivery.

Although the host immune response elicited by astrocytes and microglia after an IL-10 collagen hydrogel injection was comparable to the one elicited by an IL-10 bolus, we did notice an increase of this response over time in the density of astrocytes (**Figure 4.8**; Time, $F_{(2, 34)} = 13.971$, $P<0.001$) and microglia (**Figure 4.9**; Time, $F_{(2, 34)} = 13.775$, $P<0.001$) in the surrounding striatum.

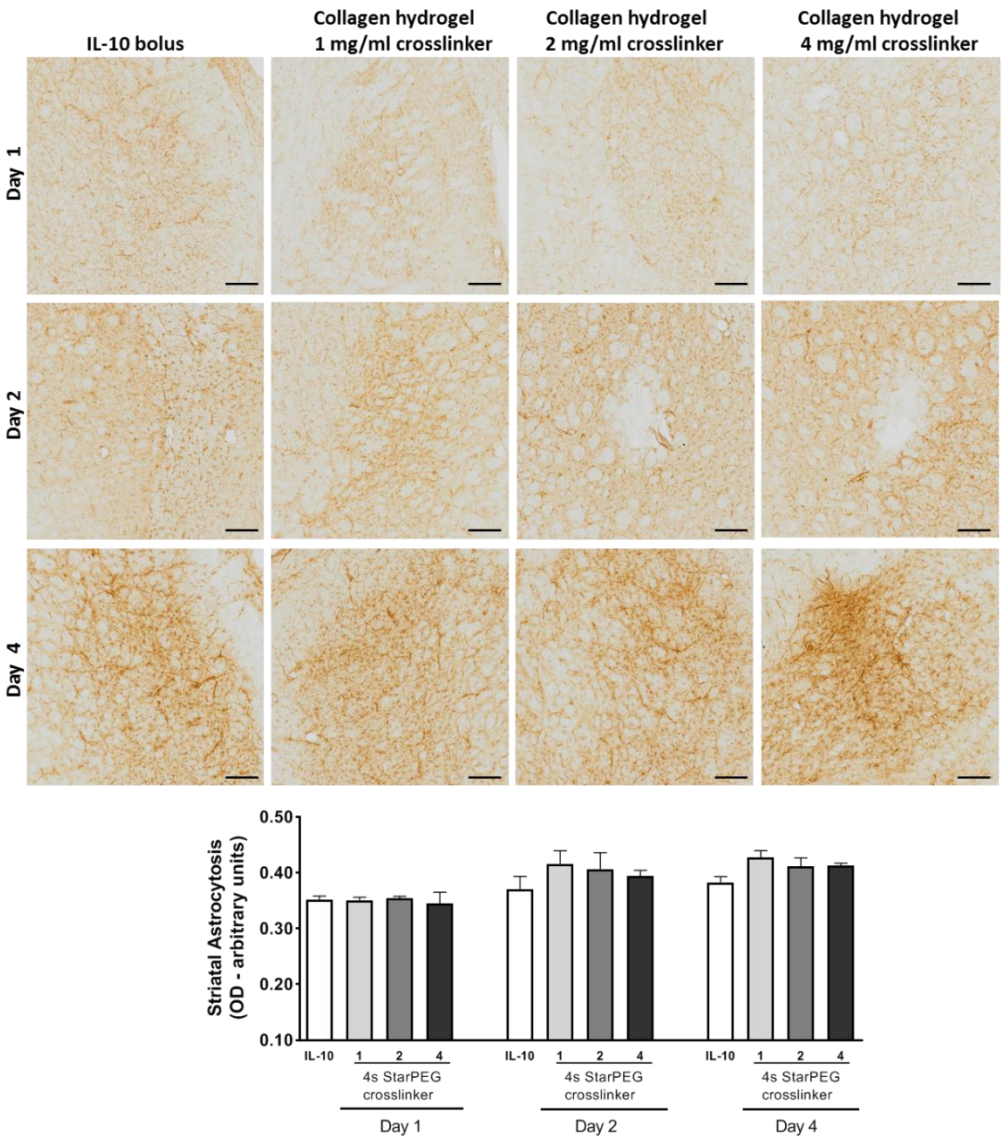

Figure 4.8 Striatal astrocytic response to an injection of an IL-10 loaded collagen hydrogel. GFAP immunostained photomicrographs show the astrocytic response around the injected area. The delivery of an IL-10 loaded collagen hydrogel to the striatum did not exacerbate the astrocytic response around the injected area. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test. Scale bar = 200 µm.

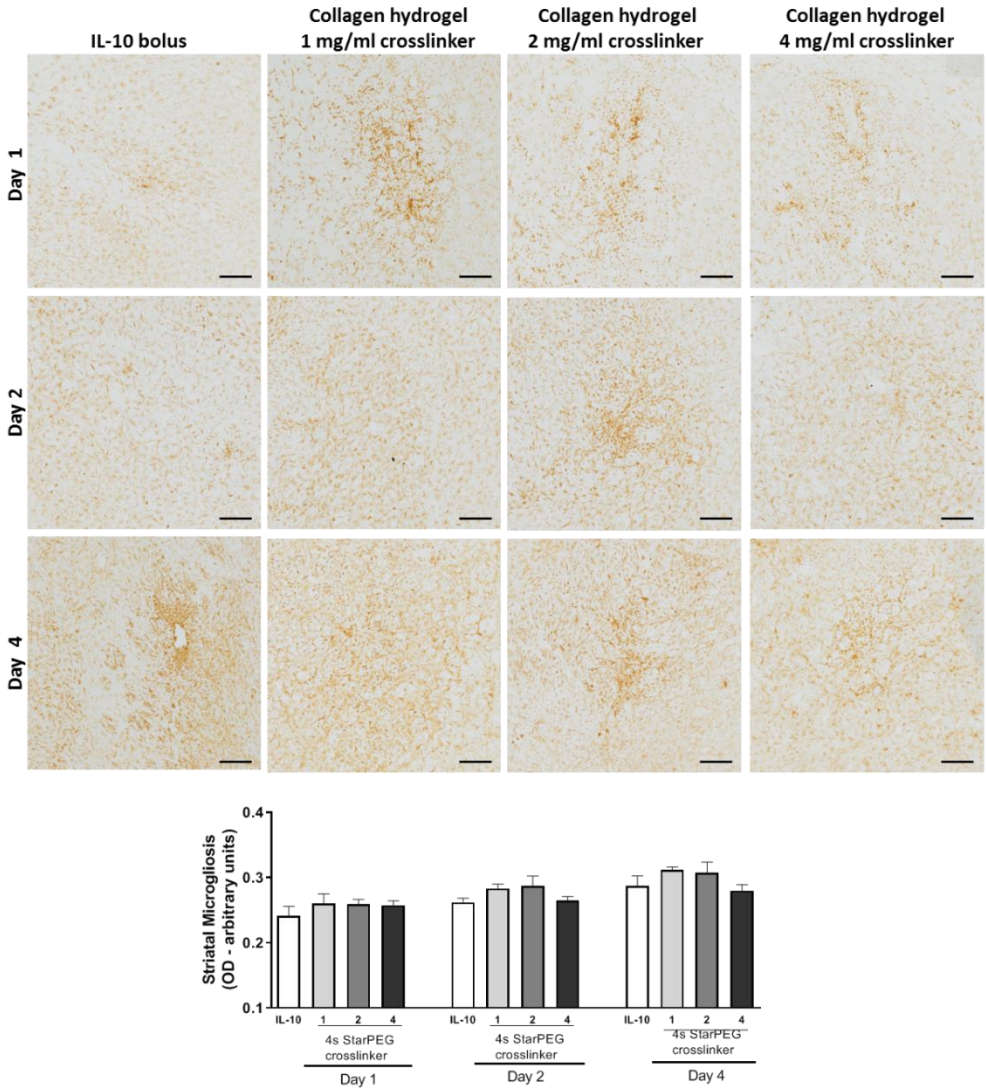

Figure 4.9 Striatal microglial response to an injection of an IL-10 loaded collagen hydrogel. OX-42 immunostained photomicrographs show the microglial response around the injected area. The delivery of an IL-10 loaded collagen hydrogel to the striatum did not exacerbate the microglial response around the injected area. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test. Scale bar = 200 µm.

4.3.2.3 In vivo IL-10 release from collagen hydrogels

Once the *in situ* polymerisation and the *in vivo* biocompatibility of collagen hydrogel compositions were verified, the ability of the collagen hydrogels to retain IL-10 in the surrounding striatum was assessed using IL-10 immunostained photomicrographs. Despite the fact that the staining of IL-10 in the striatum decreased quickly over time (**Figure 4.10**; Time, $F_{(2, 36)} = 9.034$, $P<0.001$), the collagen hydrogels significantly retained more IL-10 in the striatum than the IL-10 bolus at 24h post-implantation (**Figure 4.10**; IL-10 delivery, $F_{(3, 36)} = 3.824$, $P<0.05$).

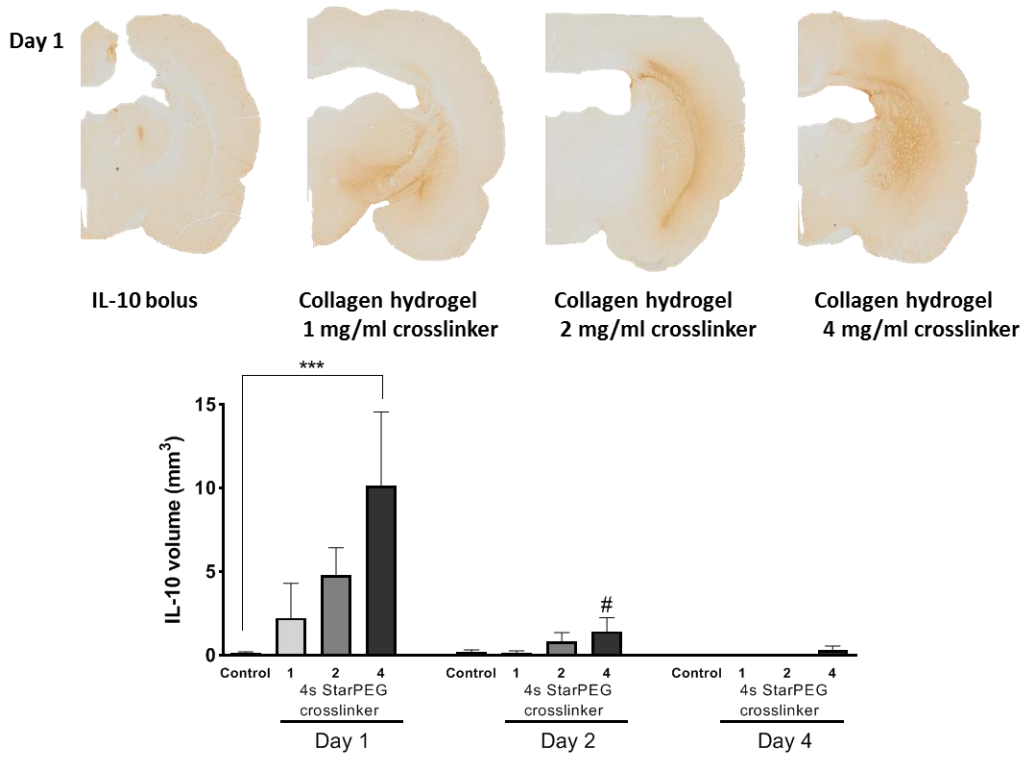

Figure 4.10 *In vivo* assessment of IL-10 retention in the striatum from collagen hydrogels. Human IL-10 immunostaining showed IL-10 was degraded quickly over time. IL-10 immunostaining revealed that IL-10 retention in the striatum was greater when IL-10 was delivered in a collagen hydrogel 24 hours after administration. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test. *** P < 0.001 vs IL-10 bolus, # P < 0.05 vs previous timepoint.

4.3.3 *In vivo* assessment of the encapsulation of primary dopaminergic cells in an IL-10 collagen hydrogel

4.3.3.1 The early effects of VM cell encapsulation in an IL-10 rich collagen hydrogel on dopaminergic cell grafts

Since the goal of this study was to analyse the effects of VM cell encapsulation in an IL-10 collagen hydrogel, the dopaminergic grafts were analysed. TH+ staining revealed dopaminergic cells around the injected area, although not surprisingly, dopaminergic grafts were atypical and there was not fibre outgrowth due to the immediate timepoints used in this study (**Figure 4.11**). Dopaminergic cell counts were analysed to account for any potential differences between experimental groups and none were found (**Figure 4.12**; Hydrogel x factor, $F_{(1, 16)} = 0.490$, $P>0.05$).

Collagen hydrogel polymerisation and IL-10 delivery were confirmed with immunostaining (**Figure 4.13** and **Figure 4.14**).

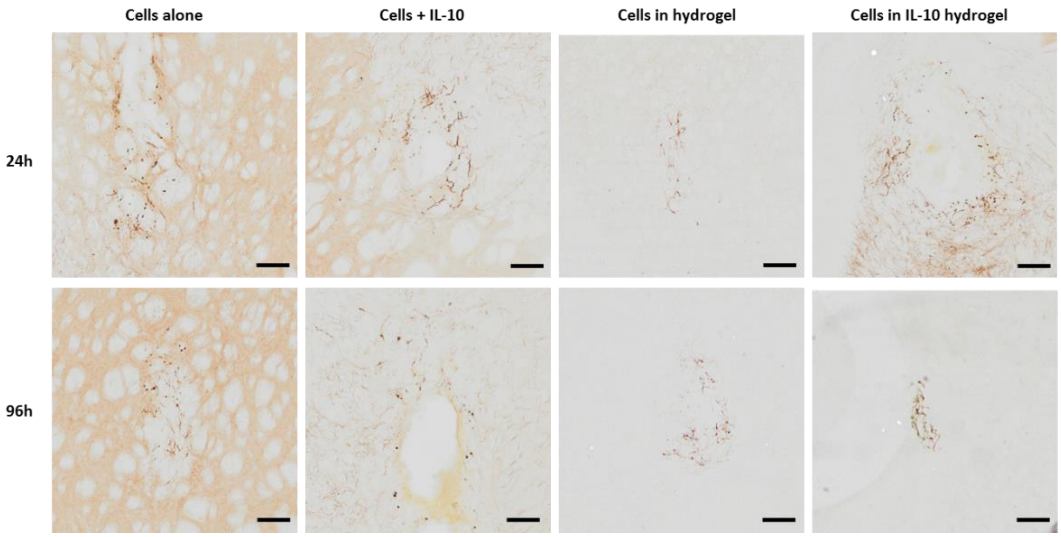

Figure 4.11 Dopaminergic cell grafts at early stages after transplantation. TH+ immunostaining was used to determine the number of surviving dopaminergic cells through the striatum. Dopaminergic cells were atypical and there was no evidence of re-innervation area due to early timepoints after transplantation. Scale bar is 200 µm.

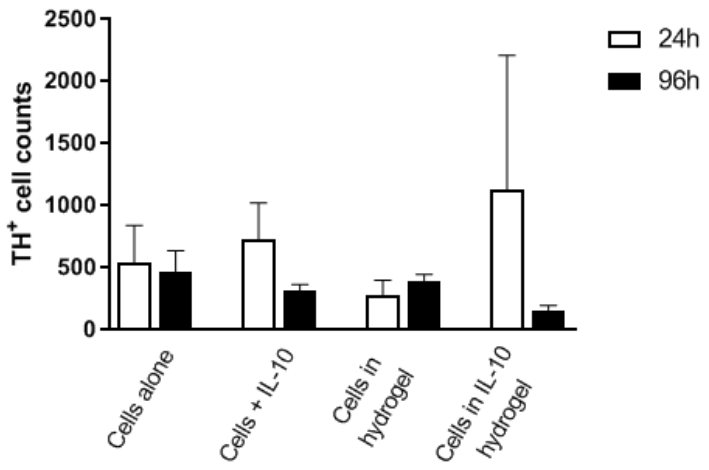

Figure 4.12 Dopaminergic cell survival at early stages after transplantation. Not surprisingly, the encapsulation in a collagen hydrogel or the addition of IL-10 did not alter the dopaminergic cell counts at the early timepoints assessed. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test.

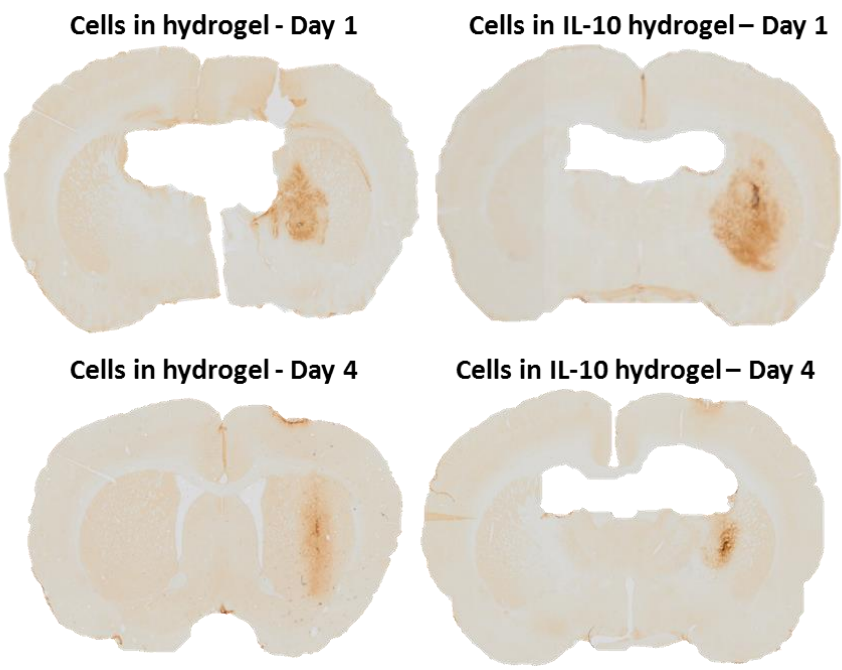

Figure 4.13 Confirmation of collagen staining Sections were stained for collagen type I to ensure the delivery of the IL-10 collagen hydrogels. As observed previously, the collagen staining tends to reduce over time.

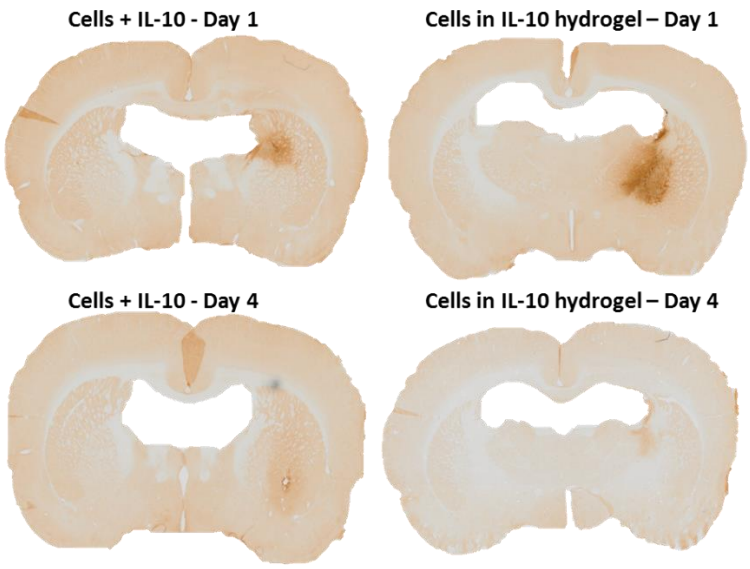

Figure 4.14 Confirmation of IL-10 staining Sections were stained for human IL-10 to ensure the delivery of the IL-10 collagen hydrogels. As observed previously, IL-10 is almost undetectable at day 4 post-injection.

4.3.3.2 The early effects of VM encapsulation in an IL-10 collagen hydrogel on the host immune response

After establishing that collagen hydrogels did not elicit an exaggerated host immune response after injection, we sought to determine if the encapsulation of VM cells in collagen hydrogels could reduce the host immune response in the striatum in the first stages after transplantation when the collagen hydrogel is still present.

As seen in **Figure 4.15** and **Figure 4.16**, in these initial stages after transplantation, there was an astrocytic response surrounding the graft site and extending through the striatum.

The qualitative analysis of astrocyte morphology illustrated reactive astrocytes with prolongations around the grafted area in all experimental groups and in a steady manner over time (**Figure 4.17**). As previously seen, we did not see an exacerbation or amelioration of the density of astrocytes (**Figure 4.21a**; Group, $F_{(3, 28)} = 0.775$, $P>0.05$) or the volume of astrocytes (**Figure 4.21b**; Group, $F_{(3, 28)} = 1.963$, $P>0.05$) due to the IL-10 loaded collagen hydrogel.

Similarly, at these early stages after transplantation, there was a microglial response in the immediate surroundings the graft site. The recruitment of microglia could be observed radially around the grafted area in a more confined area in comparison to the astrocyte response (**Figure 4.18** and **Figure 4.19**). After a closer visual examination to the microglial response, microglial cells appeared reactive, with thicker bodies but a ramified shape, particularly around the grafted area in all experimental groups (**Figure 4.20**).

In line with our observations of the astrocytosis response, the encapsulation of VM cells in an IL-10 collagen hydrogel did not modify the microglial response at early stages of transplantation in terms of density (**Figure 4.22a**; Group, $F_{(3, 29)} = 0.565$, $P>0.05$) or volume of microglia (**Figure 4.22b**; Group, $F_{(3, 29)} = 0.777$, $P>0.05$).

In line with our previous observations, the microglial response remarkably increased over time (**Figure 4.22a**; Time, $F_{(1, 28)} = 5.492$, $P<0.05$, **Figure 4.22b**; Time, $F_{(1, 28)} = 2.230$, $P<0.05$).

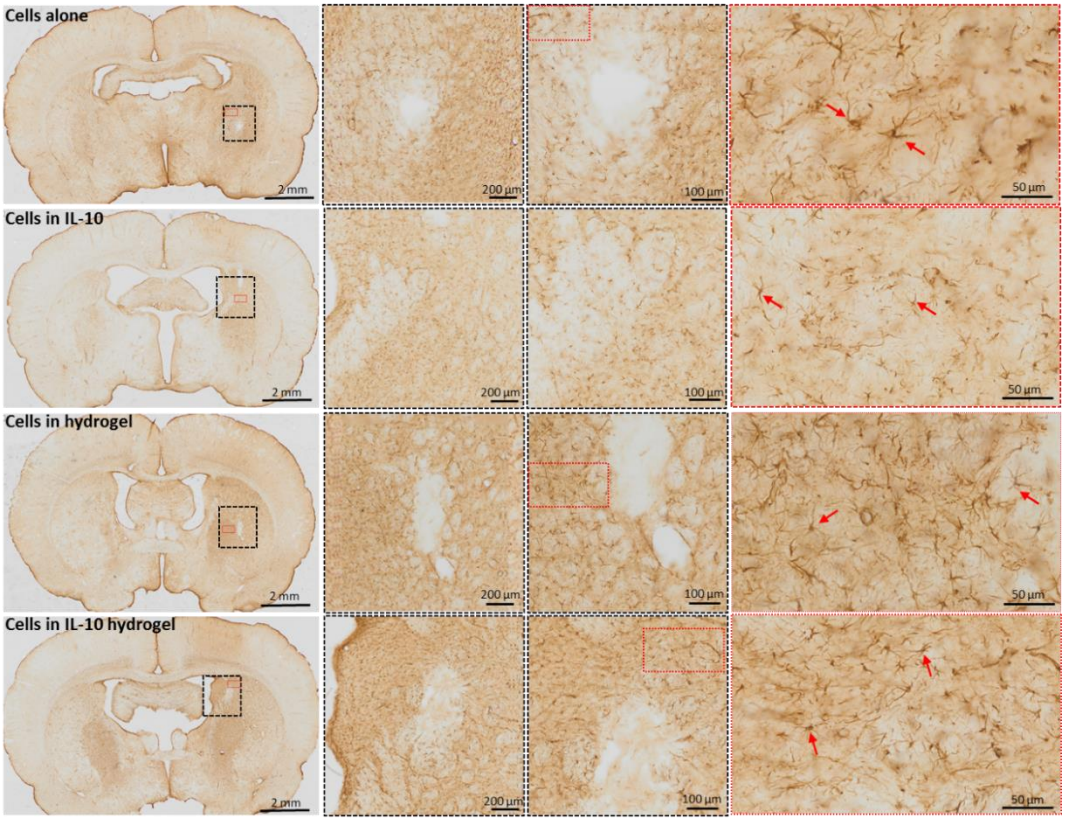

Figure 4.15 Striatal astrocytic response 24h after a dopaminergic cell transplant in an IL-10 loaded collagen hydrogel. Representative micrographs show GFAP immunostaining at the injection site 24h post-transplantation. Red arrows show reactive astrocytes.

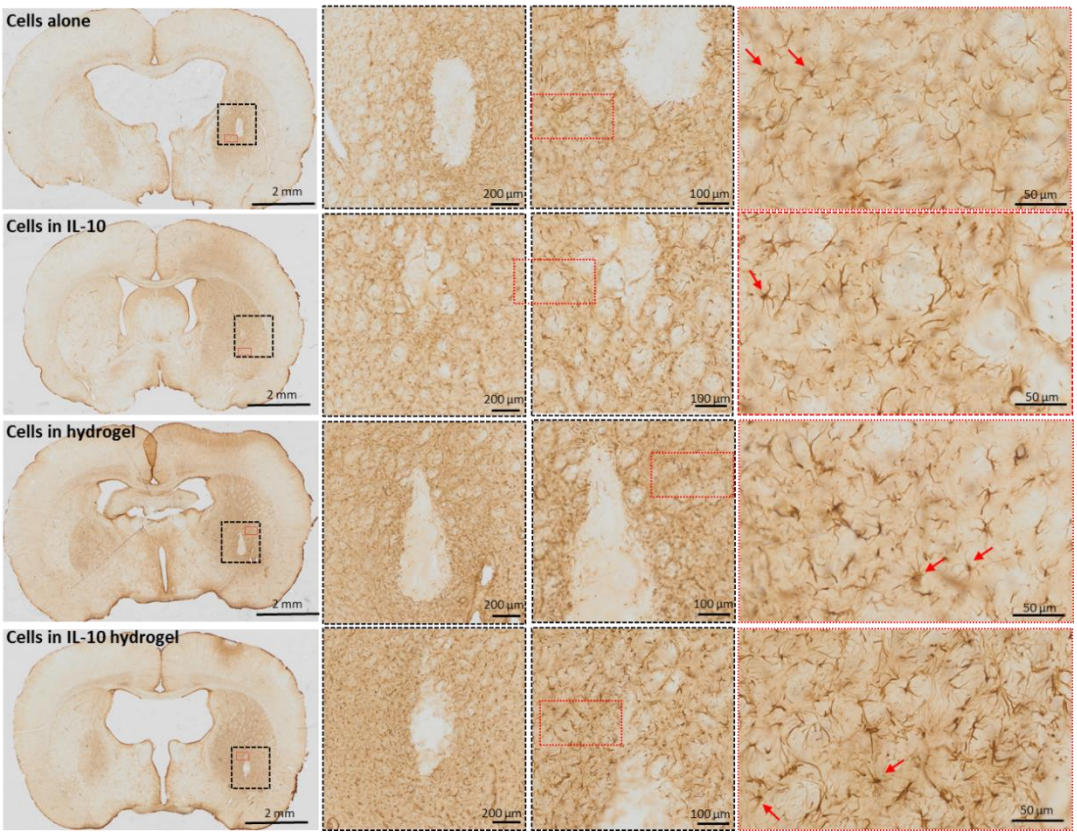

Figure 4.16 Striatal astrocytic response 96h after a dopaminergic cell transplant in an IL-10 loaded collagen hydrogel. Representative micrographs show GFAP immunostaining at the injection site 96h post-transplantation**.** Red arrows show reactive astrocytes.

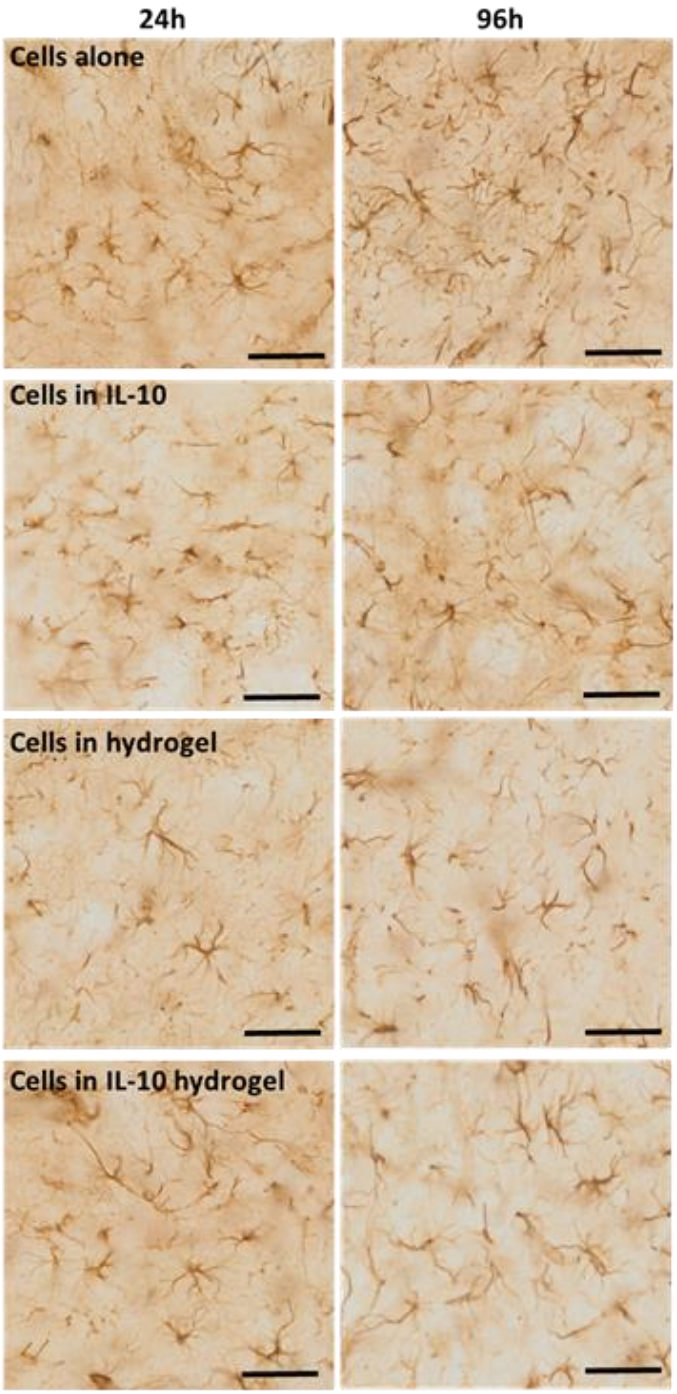

Figure 4.17 Detailed photomicrographs of striatal astrocytosis after a dopaminergic cell transplant in an IL-10 loaded collagen hydrogel. GFAP immunostaining revealed reactive astrocytes surrounding the grafted area in all experimental groups. Scale bar is 50 µm.

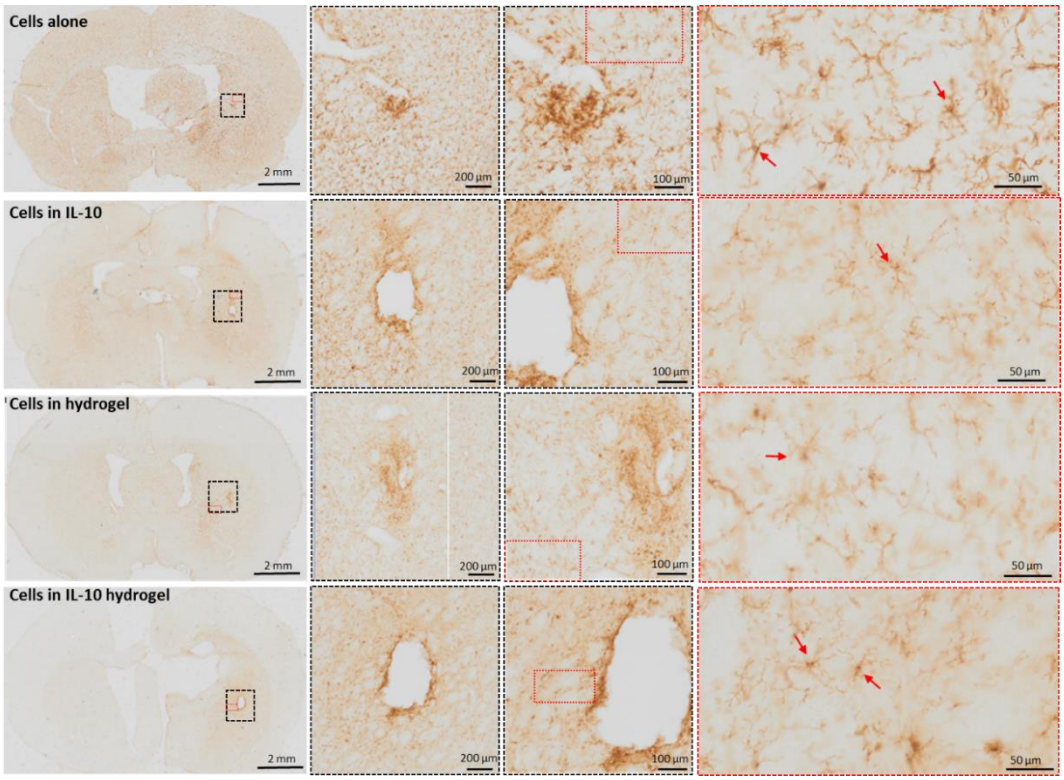

Figure 4.18 Striatal microglial response 24h after a dopaminergic cell transplant in an IL-10 loaded collagen hydrogel. Representative micrographs show OX-42 immunostaining at the injection site 24h post-transplantation. Red arrows show reactive microglia.

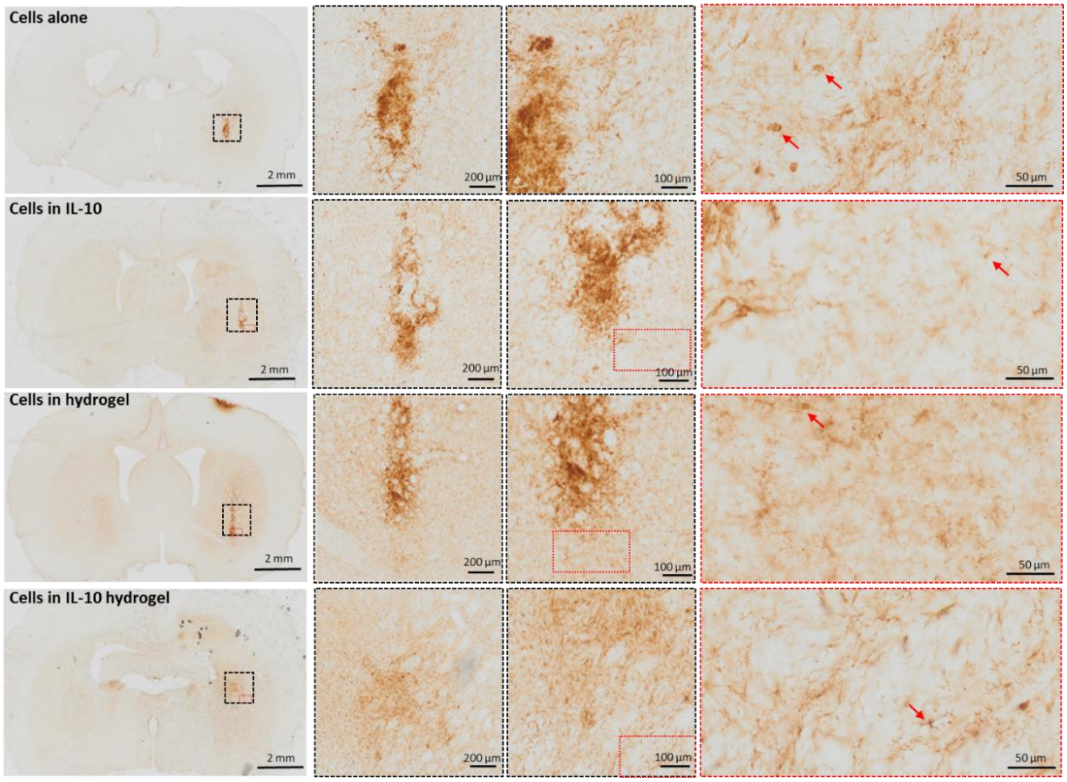

Figure 4.19 Striatal microglial response 96h after a dopaminergic cell transplant in an IL-10 loaded collagen hydrogel. Representative micrographs show OX-42 immunostaining at the injection site 96h post-transplantation. Red arrows show reactive microglia.

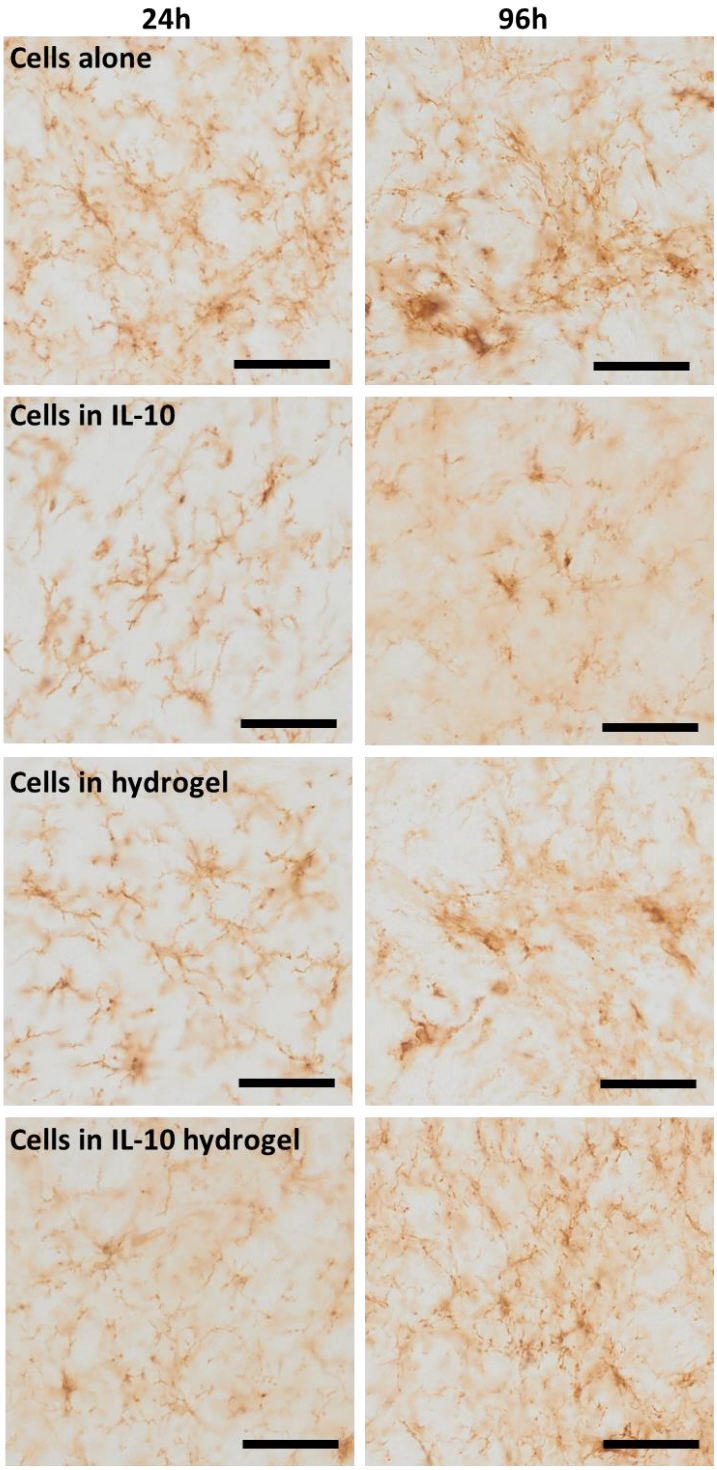

Figure 4.20 Detailed photomicrographs of striatal microgliosis after a dopaminergic cell transplant in an IL-10 loaded collagen hydrogel. OX-42 immunostaining revealed reactive microglia surrounding the grafted area in all experimental groups. Scale bar is 50 µm.

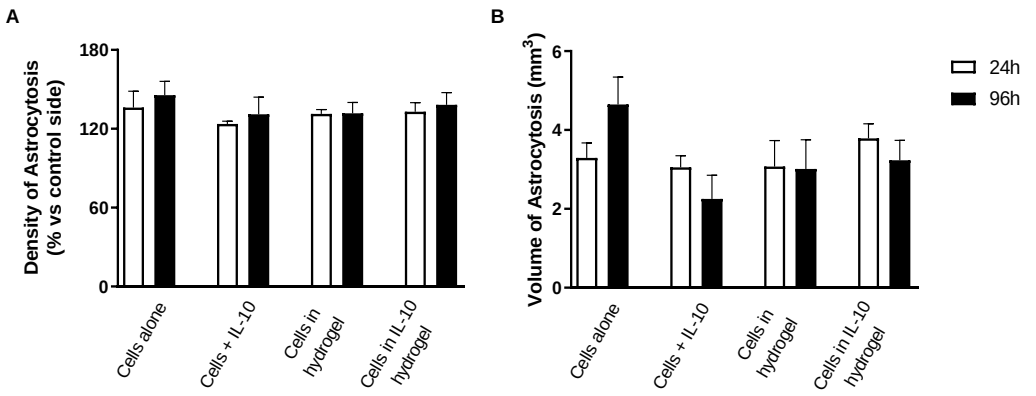

Figure 4.21 Impact of VM encapsulation in an IL-10 rich collagen hydrogel on the host astrocytic response. GFAP immunostaining revealed that A) density and B) volume of striatal astrocytosis were not modified by the encapsulation of cells in an IL-10 collagen hydrogel. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test.

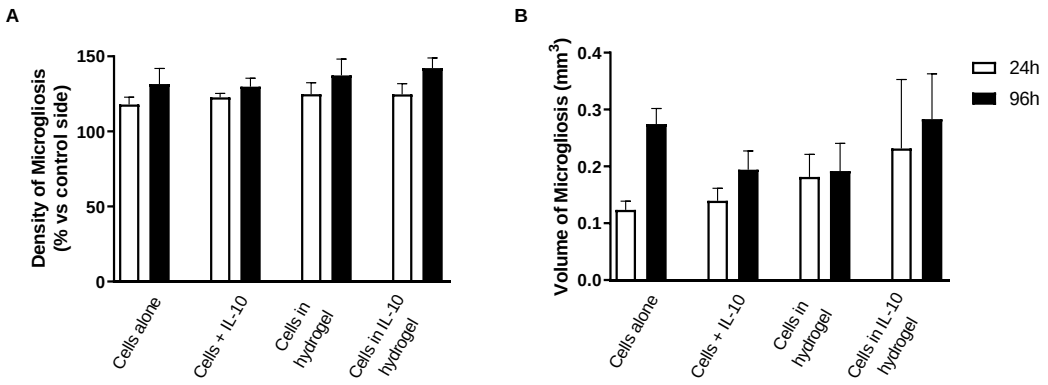

Figure 4.22 Impact of VM encapsulation in an IL-10 rich collagen hydrogel 0n the host microglial response. OX-42 immunostaining revealed that density and volume of striatal microgliosis were not modified by the encapsulation of cells in an IL-10 collagen hydrogels at any of the investigated timepoints. Time significantly increased density and volume of striatal microgliosis. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test.

4.4 DISCUSSION

After decades of investigation, dopaminergic cell replacement therapies have proven to be a promising reparative therapy for Parkinson´s disease yet the poor survival of the dopaminergic cells has not been solved. The use of biomaterials offers a great opportunity to tackle this issue. Biomaterial scaffolds can help to provide a microenvironment that supports and enhances cell survival (Burdick et al., 2016). In fact, many biomaterials – in particular injectable collagen hydrogels – have been successfully applied in preclinical studies of cell transplantation for Parkinson´s disease resulting in improved survival, neurite outgrowth and improved motor function (Nakaji-Hirabayashi et al., 2012, 2013; Moriarty et al., 2017, 2019a).

Previously we have shown that collagen hydrogels are stable, cytocompatible and can act as drug-delivery systems *in vitro*. Through a series of pilot studies, the aim of this chapter was to optimise collagen hydrogels to be used as cell and protein scaffolds for intra-striatal delivery in hemi-parkinsonian rats. The specific goals were to gather more information on polymerisation, degradation and biocompatibility of the collagen hydrogels *in vivo* as well as to assess the *in vivo* release of IL-10 to ultimately choose the best IL-10 loaded collagen hydrogel composition for future efficacy testing in rodent models of Parkinson´s disease.

Here we have shown the importance of testing each component of collagen hydrogels in the body. In our initial pilot study, we injected into the striatum collagen hydrogels with a fixed concentration of collagen (2 mg/ml) and crosslinker (4 mg/ml) but using two different brands of bovine type I atellocollagen that were routinely used. Both collagen hydrogels polymerised *in situ* and degraded quickly over time independently of the supplier (**Figure 4.4**). In line with our previous *in vitro* cytocompatibility studies shown in Chapter 3, the injection of collagen hydrogels with 4 mg/ml of crosslinker did not generate an exacerbated immune response due to the biomaterial implantation (**Figure 4.5** and **Figure 4.6**).

Despite the fact that the resulting collagen hydrogels are technically the same, we identified that collagen hydrogels made from Collagen Solutions collagen produced a greater damage volume than collagen hydrogels made from Vornia Biomaterials collagen (**Figure 4.4**). Furthermore, collagen from Collagen Solutions was prone to sudden polymerisation after neutralisation, even when kept on ice. Thus, due to the

damage observed upon delivery of collagen hydrogels fabricated from Collagen Solutions collagen, it was decided to proceed using Vornia Biomaterials collagen for future experiments.

Once the best collagen source was determined, we moved on to assess the effects of the crosslinker concentration in the host tissue. As shown in Chapter 3, the crosslinker concentration determines many of the resulting properties of the collagen hydrogels. As described in Chapter 3, we determined the *in vitro* polymerisation time of the collagen hydrogels crosslinked with 1-12 mg/ml of 4s-StarPEG. The results of that study showed that increasing the concentration of crosslinker reduced the polymerisation time, as expected. We hypothesised that collagen hydrogels with high concentrations of crosslinker (6 and 12 mg/ml) could be troublesome in terms of a suitable intra-cranial delivery of the biomaterial due to their quick polymerisation times. As well, Moriarty and colleagues reported that higher levels of 4s-StarPEG crosslinker were detrimental to MSC graft survival due to intense gelation of the collagen hydrogels (Moriarty, 2018). Considering all that, these collagen hydrogel compositions were dismissed as good candidates for intra-striatal delivery and we chose to test *in vivo* those compositions with lower crosslinker concentrations (1-4 mg/ml of 4s-StarPEG).

In this chapter we have shown that all collagen hydrogel compositions (with 1-4 mg/ml of crosslinker) injected into the striatum successfully polymerised *in situ* and that the collagen hydrogel with the greater crosslinker concentration (4 mg/ml) showed the strongest and most defined collagen staining at 24h post-transplantation (**Figure 4.7**). As expected, collagen hydrogels degraded quickly over time as they were mostly degraded by day 4 post-injection as seen previously (**Figure 4.4** and **Figure 4.7**). However, protein enriched collagen hydrogels are used to deliver the encapsulated protein of interest during the initial and critical stages of transplantation since most of the cell death occurs in the first 4 days post-transplantation (Barker et al., 1996; Sortwell et al., 2000). Consequently, the collagen hydrogel degradation pattern aligned with the objective of protecting the cells during the first critical hours post-transplantation.

Similarly to other type I collagen hydrogels injected into the brain (Hoban et al., 2013; Moriarty et al., 2017, 2019a), we determined once again that collagen hydrogels were

biocompatible with the host striatum considering the host immune response generated by microglia and astrocytes was comparable to an IL-10 bolus injection (**Figure 4.8** and **Figure 4.9**).

After establishing that injectable thermoresponsive collagen hydrogels can in fact polymerise *in situ* and that they are biocompatible with their administration into the brain we sought to assess their ability to retain and release proteins of interest. We have reported here that collagen hydrogel with 4 mg/ml of crosslinker successfully retained more IL-10 in the striatum in the first 24h after-transplantation (**Figure 4.10**). Based on these findings, we chose the collagen hydrogel composition with 4 mg/ml of crosslinker as our candidate for future cell transplantation studies. This fact is in line with the literature, where Moriarty et al. also used a collagen hydrogel with 4 mg/ml of crosslinker to be used in primary dopaminergic cell transplantation in hemi-parkinsonian rats (Moriarty et al., 2017, 2019a).

Taking into account the data summarised in this chapter, collagen hydrogels could be used as a drug-delivery system to locally release relevant biomolecules to the injection area. Although the retention time of IL-10 was short, others have reported benefits from retaining trophic factors for short periods of time alongside transplanted cells. For example, a GDNF enriched collagen hydrogel successfully improved cell survival of primary dopaminergic cells even though GDNF was fully degraded by day 4 post-injection (Moriarty et al., 2017).

Knowing that collagen hydrogels were well tolerated in the brain and that they could retain IL-10 in the striatum, we sought to assess if the collagen hydrogels could work as a dual system to encapsulate dopaminergic cells and retain relevant biomolecules in the grafted area. Particularly, we wanted to evaluate if an IL-10 loaded collagen hydrogel could ameliorate the host innate immune response produced immediately after the transplantation of exogenous dopaminergic cells.

In line with other investigations where collagen hydrogels have been used for cell transplantation processes, the delivery of primary dopaminergic cells encapsulated in an IL-10 loaded collagen did not exhibit any detrimental effects on the dopaminergic cells at early timepoints of transplantation (**Figure 4.12**). Although the delivery of cells in collagen scaffolds for neural repair has been investigated (Hoban et al., 2013; Nakaji-Hirabayashi et al., 2013; Moriarty et al., 2017, 2019a), this is the first time that

cells have been delivered in an anti-inflammatory enriched collagen scaffold to the hemi-parkinsonian brain. The injection of the biomaterial – with and without cells – did elicit an innate immune response by the host tissue with the presence of astrocytes and microglia but this response was comparable to an injection of a bolus or cells alone. As reported here, the IL-10 loaded collagen hydrogel did not exacerbate the host immune response but it did not reduce the density or volume of immune cells around the grafts or in the striatum at early timepoints after transplantation. (**Figure 4.21** and **Figure 4.22**).

All these *in vivo* data altogether confirms that collagen scaffolds are biocompatible with the encapsulation and intra-striatal delivery of primary dopaminergic cells and IL-10. Although there were no observed effects on the host immune response, the timepoints used in this initial study were very early after transplantation implying very immature dopaminergic cell grafts and a much damaged area around the injection site. Thus a further assessment of the efficacy of an IL-10 loaded collagen hydrogel for the encapsulation of primary dopaminergic cells will be assessed in long-term studies to ultimately evaluate if an anti-inflammatory loaded scaffold can improve the survival of the transplanted cells, enhance neurite outgrowth and ameliorate the host immune response elicited by the cell transplantation process.

CHAPTER 5: AN IL-10 LOADED COLLAGEN HYDROGEL SCAFFOLD FOR INTRA-STRIATAL CELL TRANSPLANTATION

5.1 INTRODUCTION

Cell transplantation in Parkinson's disease relies upon a quite simple framework; to replace those dying dopaminergic neurons with healthy new ones. Although an elementary theoretical concept, in practice, the therapy is quite more complex. Poor cell survival upon transplantation is a fundamental obstacle for this therapy to gain broad clinical success. Thus, an improvement of long-term viability of the grafted dopaminergic cells is urgently needed. Currently, it is estimated that approximately 90% of the embryonic mesencephalic dopaminergic neurons die upon transplantation in the first 4 days post-transplantation (Sortwell et al., 2000). Contrary to what was first believed, this massive cell death can be explained by apoptosis during the first 24 hours and not by a necrotic death due to the injection trauma or poor vascularisation (Mahalik et al., 1994; Sortwell et al., 2000; Hemshekhar et al., 2016). Several issues contribute to apoptotic death in each stage of grafting dopaminergic neurons; 1) the dissection and preparation stage is characterised by the detachment from the extracellular matrix, which triggers pro-apoptotic cascades, 2) the early transplantation stage comes with the deprivation of growth factors in the host tissue and 3) the latter transplantation stage is presented with migration of immune cells surrounding the graft that create an inflammatory environment (Sortwell et al., 2000). Targeting the factors believed to be involved in this apoptotic cell death could potentially promote cell survival, making cell transplantation a more efficient process.

Injectable collagen hydrogels can be used as scaffolds to provide a support matrix resembling the ECM for the dissociated cells to adhere to, both during and after transplantation, thus potentially reducing the anoikis and subsequent cell death (Béduer et al., 2015; Wang et al., 2016; Moriarty et al., 2017, 2019a).

Furthermore, survival of grafted dopaminergic neurons can be enhanced by exposing cells to different trophic factors such as BDNF, GDNF or neurotrophin-4/5 (Collier & Sortwell, 1999; Huang & Reichardt, 2001). Injectable collagen hydrogels can target several of the complex causes of apoptosis since they can also be used as a reservoir

of trophic factors that can be released into the depleted host striatum. Recently, Moriarty and colleagues reported that the encapsulation of VM cells in a GDNF-loaded collagen hydrogel dramatically improved the survival and neurite outgrowth of dopaminergic grafts (Moriarty et al., 2017).

However, the effects of reducing the early inflammatory response on dopaminergic cell survival have not been broadly investigated. The brain is commonly described as an immunologically privileged transplantation site, often explained by the existence of the BBB, the low levels of the MHC antigens on brain cells and the sparse lymphatic drainage from the CNS (Duan et al., 1993; Barker & Widner, 2004). Despite this low immunologic response and the subsequent long-term graft survival observed in rat VM allografts, the injection of cells to the brain does elicit a host immune response (Duan et al., 1993, 1995; Hakan Widner, 1993; Barker et al., 1996). In experimental data, both syngeneic and allogeneic VM grafts on Sprague-Dawley rats recruited MHC class I and II cells, activated microglia and macrophages as early as day 4 post-transplantation and this recruitment persisted for 18 weeks post-transplantation (Duan et al., 1995). Furthermore, microglial recruitment was detected around the grafted site in the *post-mortem* analysis of some patients from the double-blind, placebo-controlled clinical trials (Freed et al., 2001; Olanow et al., 2003). Thus, it is clear that understanding and modulating the host innate and adaptive neuro-immune response both is a key factor for the successful implantation of dopaminergic neurons in the brain.

Here we propose that injectable collagen hydrogels could also be employed to address the early stages of the host innate immune response after cell transplantation. The addition of anti-inflammatory cytokines in a collagen hydrogel could potentially reduce the host immune response surrounding the grafted area and eventually improve the survival of grafted cells. Therefore, the aim of this chapter was to determine whether the encapsulation and delivery of VM cells in an anti-inflammatory cytokine-loaded collagen hydrogel could ameliorate the host immune response and enhance the long-term survival of dopaminergic neurons.

5.2 METHODS

5.2.1 Experimental design

After establishing that collagen hydrogels were well tolerated *in vitro* and *in vivo* and that collagen hydrogels successfully polymerised *in situ* and retained IL-10 in the striatum, in this chapter we assessed the efficacy of an IL-10 loaded collagen hydrogels on improving the survival of the grafted cells as well as ameliorating the host immune response.

The studies presented in this chapter were designed to determine the best IL-10 loaded collagen hydrogel composition for intra-striatal delivery as well as to assess the effects of an IL-10 collagen hydrogel for the encapsulation and delivery of primary dopaminergic neurons to the hemi-parkinsonian brain.

Based on the results found in Chapter 4, we chose the collagen hydrogel with 4 mg/ml of crosslinker as the best collagen hydrogel composition for intra-striatal delivery of VM cells. Here, we studied the effects of several IL-10 concentrations on VM cell survival and host immune response. Then we moved on to assess the effects of an IL-10 loaded collagen hydrogel on VM dopaminergic neuron transplantation by analysing the effects of the biomaterial on dopaminergic cell survival and neurite outgrowth, and on the host immune response.

5.2.1.1 In vivo assessment of IL-10 concentration encapsulated in a collagen hydrogel for primary dopaminergic cell transplantation

Once the best collagen hydrogel composition for IL-10 release was chosen (see Chapter 4), we wanted to assess the best IL-10 concentration to be loaded in a collagen hydrogel for primary dopaminergic cell transplantation.

To achieve this aim, 13 male Sprague-Dawley rats received a unilateral intra-MFB injection of 6-OHDA (12 µg in 3 µl) (**Table 5.1**). Three weeks later, motor function was assessed via methamphetamine-induced rotations (2.5 mg/kg) and based on these results, rats were performance matched into four groups (n=4-5 per group). In the same week, rats underwent unilateral intra-striatal transplants of E14 VM cells alone (300,000 cells in 6 µl), or E14 VM cells encapsulated in an IL-10 loaded collagen

hydrogel (crosslinked with 4 mg/ml 4s-StarPEG) with either 500 or 1000 ng of human IL-10 (**Table 5.1**). Animals were then sacrificed for p*ost-mortem* assessment at 4 weeks post-transplantation. A schematic view of the experimental design is shown in **Figure 5.1**. The dopaminergic cell survival and re-innervation volume, and the host immune response were assessed using immunohistochemistry analysis.

Table 5.1 Experimental groups used in an intra-striatal VM transplant study in hemi-Parkinsonian rats. VM cells: 300,000 cells/6 µl, human IL-10: 1000 ng/6 µl.

Experimental Group	Sample size
VM cells in hydrogel	4
VM cells in IL-10 hydrogel (500 ng)	4
VM cells in IL-10 hydrogel (1000 ng)	5

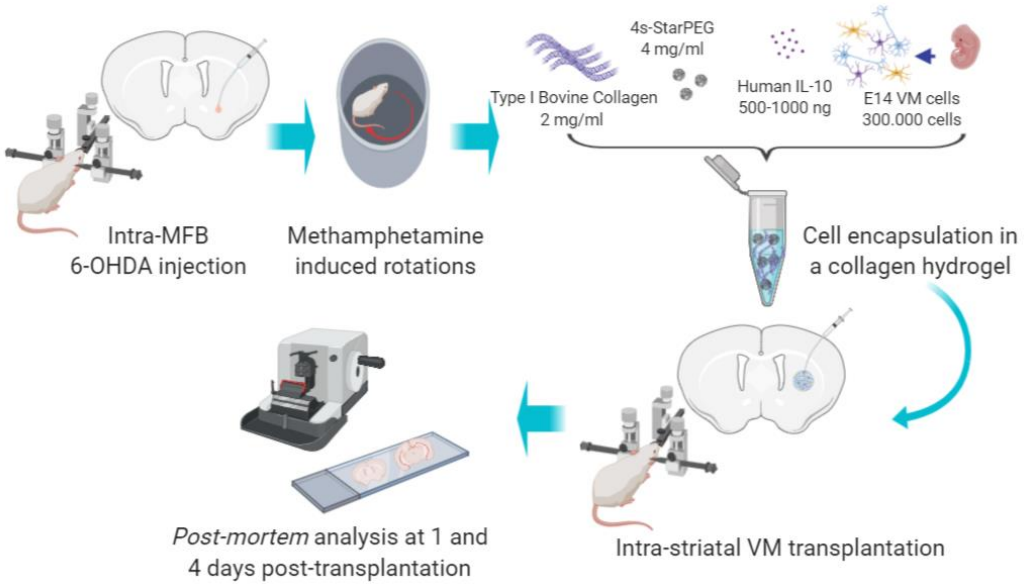

Figure 5.1 IL-10 collagen hydrogel for dopaminergic cell transplantation in Parkinson´s disease. Schematic view of the delivery of E14 VM cells (300,000 cells/6µl) encapsulated in an IL-10 (500 or 1000 ng) collagen hydrogel in unilaterally lesioned hemi-parkinsonian rats. Dopaminergic cell survival and re-innervation ability, and host immune response to the transplant were measured at 4 weeks post-transplantation.

5.2.1.2 In vivo assessment of the effects of an IL-10 collagen hydrogel on primary dopaminergic cell transplantation

After selecting the best IL-10 concentration to encapsulate in a collagen hydrogel for cell transplantation purposes, we sought to assess if encapsulating VM cells in an IL-10 loaded collagen hydrogel could benefit the dopaminergic cell survival and/or reduce the host immune response to the transplanted cells.

To do this, 24 male Sprague-Dawley rats received a unilateral intra-MFB injection of 6-OHDA (12 µg in 3 µl). Three weeks later, methamphetamine-induced rotations (2.5 mg/kg) were conducted to assess the animals' motor function and based on these results, rats were performance matched into four groups (n=6 per group). In the same week, rats underwent unilateral intra-striatal transplants of E14 VM cells alone (400,000 cells in 6 µl), E14 VM cells with IL-10 (1000 ng) or E14 VM cells encapsulated in an IL-10 loaded collagen hydrogel (crosslinked with 4 mg/ml 4s-StarPEG) (**Table 5.2**). Then, the animals were sacrificed for *post-mortem* assessment. The dopaminergic cell survival and re-innervation volume, and the host immune response were assessed using immunohistochemistry analysis. A schematic view of the experimental design is shown in **Figure 5.2**.

Table 5.2 Experimental groups used in an intra-striatal VM transplant study in hemi-Parkinsonian rats. VM cells injected: 400,000 cells/6 µl, IL-10: 1000 ng/6 µl.

Experimental Group	Sample size
VM cells	6
VM cells + IL-10	6
VM cells in hydrogel	6
VM cells in IL-10 collagen	6

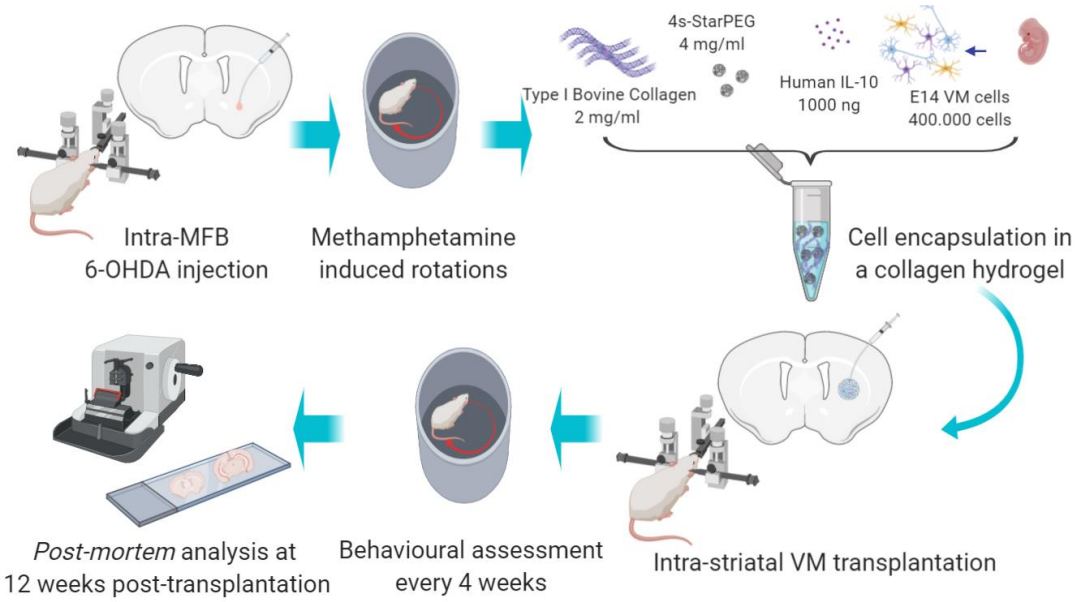

Figure 5.2 IL-10 collagen hydrogel for dopaminergic cell transplantation in Parkinson´s disease. Schematic view of the delivery of E14 VM cells (400,000 cells/6µl) encapsulated in an IL-10 (1000 ng/6µl) collagen hydrogel in unilaterally lesioned hemi-parkinsonian rats. Dopaminergic cell survival and re-innervation volume, and host immune response to the transplant were measured at 12 weeks post-transplantation.

5.3 RESULTS

5.3.1 *In vivo* assessment of IL-10 concentration in a collagen hydrogel for primary dopaminergic cell transplantation

5.3.1.1 The effects of cell encapsulation in an IL-10 rich collagen hydrogel for primary dopaminergic cell survival

In order to evaluate the best IL-10 concentration to load into a collagen hydrogel to be used as a cell scaffold for neural grafting, the survival of dopaminergic grafts was assessed using TH^+ immunostained photomicrographs. Dopaminergic grafts were observed throughout all the experimental groups (**Figure 5.3**).

In this study, the encapsulation of IL-10 alongside VM cells in the collagen hydrogel did not improve the survival of the dopaminergic neurons at any of the concentrations tested (**Figure 5.3**; IL-10 concentration, $F_{(2, 5)} = 0.3038$, $P<0.05$).

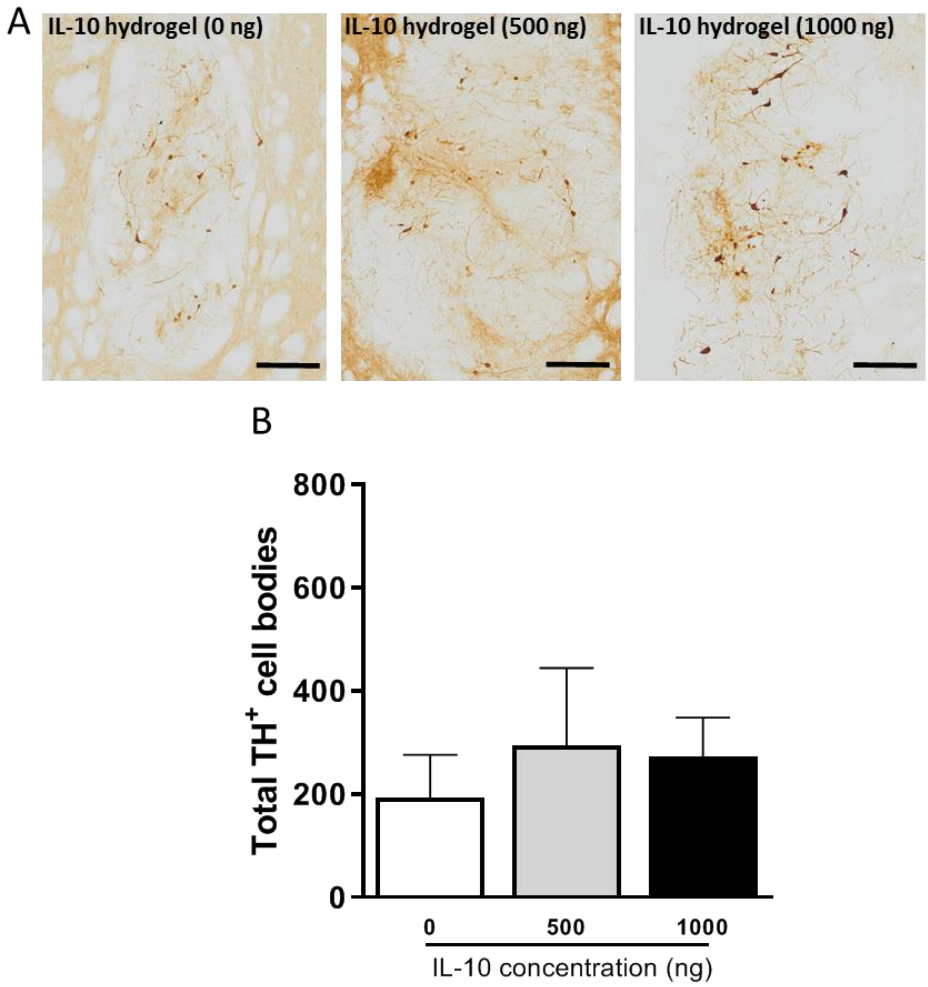

Figure 5.3 Effects of two IL-10 loaded collagen hydrogels on dopaminergic cell survival. A) Representative photomicrographs of primary dopaminergic grafts at 4 weeks post-transplantation and B) Dopaminergic cell counts. IL-10 concentration in the collagen hydrogel did not alter the number of surviving cells. Data are represented as mean ± SEM and were analysed by one-way ANOVA with Bonferroni *post-hoc* test. Scale bar = 200 µm.

5.3.1.2 Biodegradability of collagen hydrogels in vivo

Type I bovine collagen immunostaining revealed some minimal traces of collagen at 4 weeks post-transplantation in some brains throughout the experimental groups, indicating that the collagen hydrogel is mostly degraded at that stage (**Figure 5.4**; Crosslinker, $F_{(2,3)} = 0.2432$, $P>0.05$).

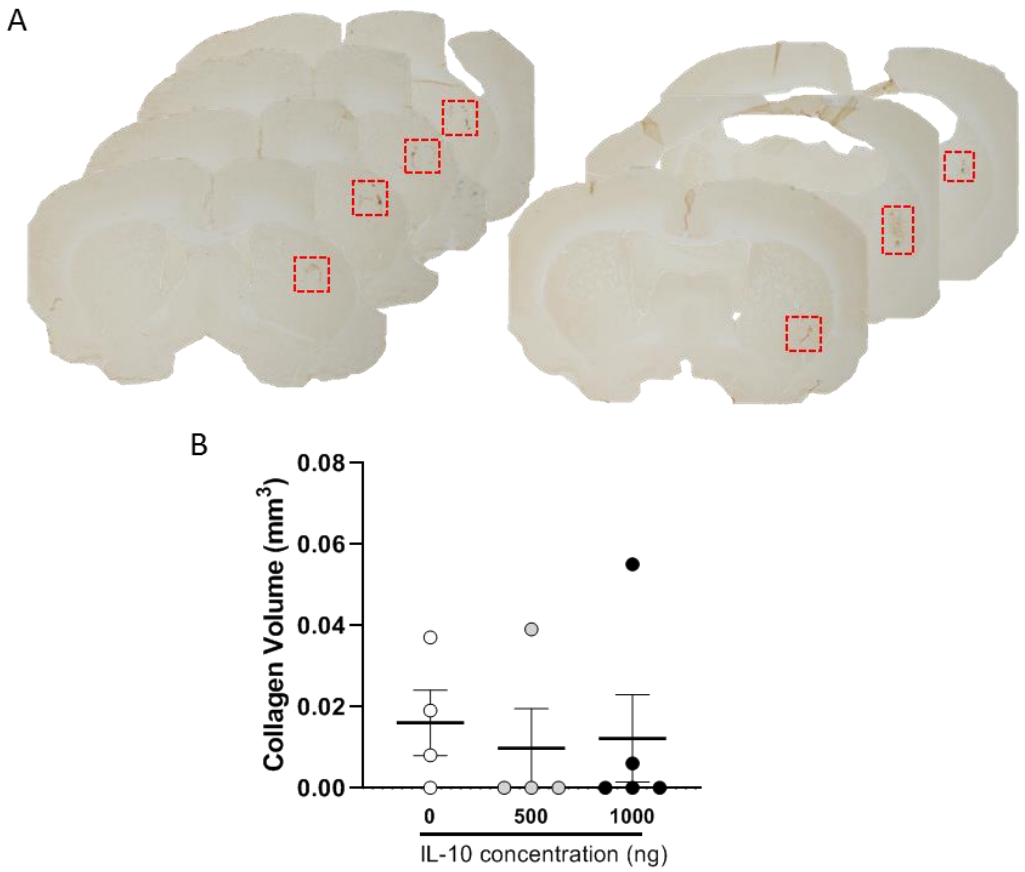

Figure 5.4 Remaining traces of collagen in the striatum at four weeks post-transplantation. A) Representative images showing traces of collagen immunostaining four weeks post-transplantation B and C) Collagen volume after 4 weeks post-injection. Collagen hydrogels are mostly degraded 4 weeks after injection but some traces of collagen staining were still visible in some animals. Data are represented as mean ± SEM and were analysed by one-way ANOVA with Bonferroni *post-hoc* test.

5.3.1.3 Host immune response to primary dopaminergic cells encapsulated in an IL-10 collagen hydrogel

In line with our previous studies, the delivery of the collagen hydrogels (in this case containing VM cells) did elicit a host immune response in the surroundings of the injection site (**Figure 5.5** and **Figure 5.6**). The striatal astrocytosis response elicited by the implantation of VM cells encapsulated in a collagen hydrogel did not differ due to the IL-10 dose administered in the density of astrocytosis (**Figure 5.5b**; IL-10 concentration, $F_{(2,10)} = 1.038$, $P>0.05$) and in the volume of astrocytosis (**Figure 5.5c**; IL-10 concentration, $F_{(2,10)} = 0.187$, $P>0.05$).

The collagen hydrogel with the highest IL-10 dose attenuated the density of microglia surrounding the grafted area (**Figure 5.6b**; IL-10 concentration, $F_{(2,10)} = 6.243$, $P<0.05$) but not the overall volume of microgliosis (**Figure 5.6c**; IL-10 conncetration, $F_{(2,10)} = 0.381$, $P>0.05$)

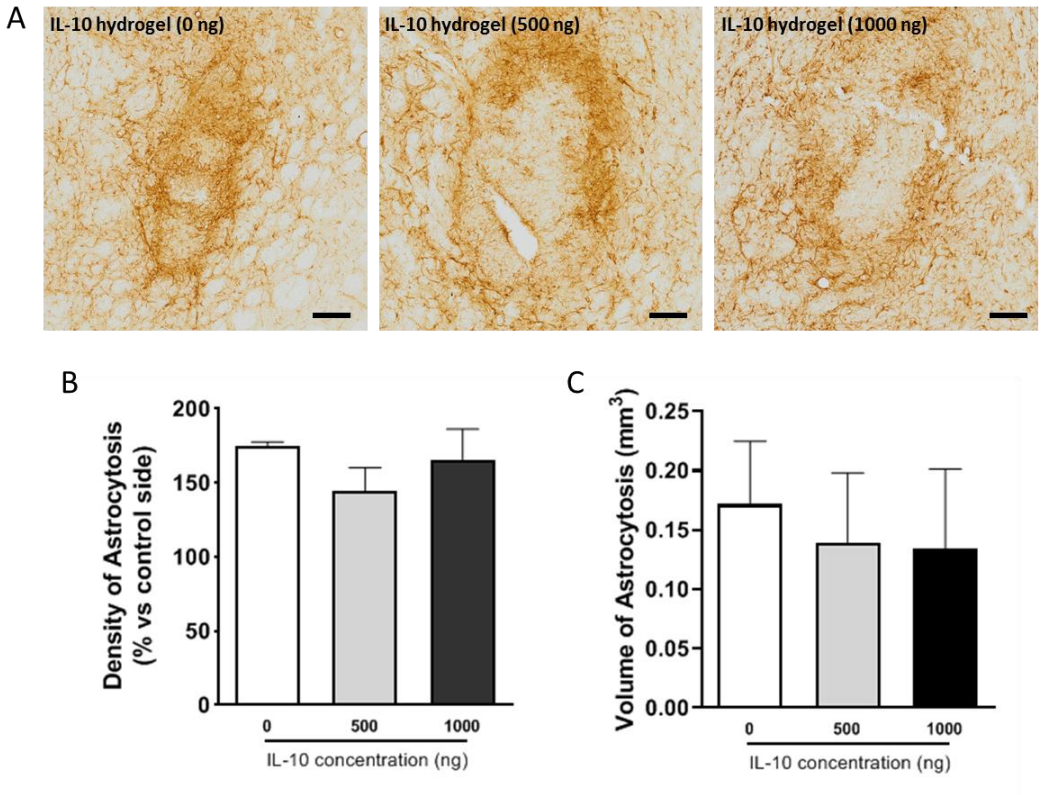

Figure 5.5 Effects of two IL-10 loaded collagen hydrogels on the astrocytic response. A) Representative photographs of striatal astrocytosis around the grafted site and B) density and C) volume of astrocytosis. The astrocyte response to a VM cell transplantation in a collagen hydrogel was not modified by the addition of IL-10 in any of the tested concentrations at 4 weeks post-transplantation. Data are represented as mean ± SEM and were analysed by one-way ANOVA with Bonferroni *post-hoc* test. Scale bar = 200 µm.

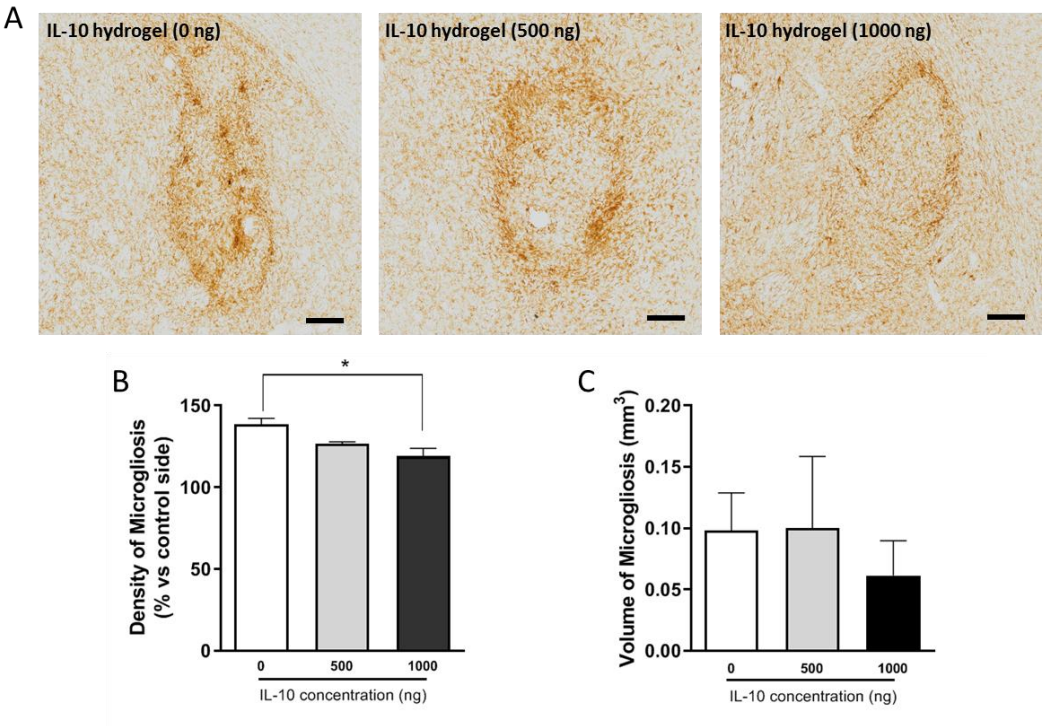

Figure 5.6 Effect two IL-10 loaded collagen hydrogel on the microglial response. A) Representative photographs of striatal microgliosis around the grafted site and B) density and C) volume of microgliosis An IL-10 loaded collagen hydrogel reduced the striatal microglial density around the graft site when compared to a collagen hydrogel alone at 4 weeks post-transplantation. Data are represented as mean ± SEM and were analysed by one-way ANOVA with Bonferroni *post-hoc* test. *$P < 0.05$ vs collagen hydrogel alone (0 ng of IL-10). Scale bar = 200 µm.

5.3.2 *In vivo* assessment of effects of IL-10 collagen hydrogel on primary dopaminergic cell transplantation

Since the encapsulation of primary dopaminergic cells in an IL-10 loaded collagen hydrogel (with 1000 ng of human IL-10) had a positive impact on the reduction of microgliosis at four weeks post-transplantation, the effects of VM encapsulation in an IL-10 loaded collagen hydrogel (with 1000 ng of human IL-10) were studied at 12 weeks post-transplantation. At this stage after transplantation, VM cells are adjusted into the host tissue, produce dopamine and the resulting grafts exhibit significant fibre outgrowth.

5.3.2.1 The effect of encapsulation in an IL-10 rich collagen hydrogel on primary dopaminergic cell survival

In order to evaluate the effects of an IL-10 collagen hydrogel on the survival of transplanted primary dopaminergic cells, the number of TH-immunoreactive cell bodies in the striatum was counted using TH immunostained photographs. The addition of IL-10 did not improve the survival of the grafted cells (**Figure 5.7a**; Factor, $F_{(1,20)} = 0.051$, $P>0.050$) nor did it the encapsulation of cells in a collagen hydrogel (**Figure 5.7a**; Hydrogel, $F_{(1,20)} = 0.787$, $P>0.050$). Consequently, the encapsulation of VM cells in an IL-10 loaded collagen hydrogel did not enhance the survival of dopaminergic cells (**Figure 5.7a**; Hydrogel x Factor, $F_{(1,20)} = 0.115$, $P>0.050$).

Correspondingly, the graft size was not modified by the addition of IL-10 (**Figure 5.7b**; Factor, $F_{(1,20)} = 0.129$, $P>0.05$), by the encapsulation of VM cells (**Figure 5.7b**; Hydrogel, $F_{(1,20)} = 0.232$, $P>0.05$) or by the encapsulation of VM cells in an IL-10 collagen hydrogel (**Figure 5.7b**; Hydrogel x Factor, $F_{(1,20)} = 0.240$, $P>0.05$).

Not surprisingly, the nerve outgrowth measured by the re-innervation volume was unchanged in the different experimental groups and no effects were observed due to the addition of IL-10 (**Figure 5.7c**; Factor, $F_{(1,20)} = 0.083$, $P>0.05$), the VM cell encapsulation in a collagen hydrogel (**Figure 5.7c**; Hydrogel, $F_{(1,20)} = 0.019$, $P>0.05$) or the encapsulation of VM cells in an IL-10 collagen hydrogel (**Figure 5.7c**; Hydrogel x Factor, $F_{(1,20)} = 0.066$, $P>0.05$).

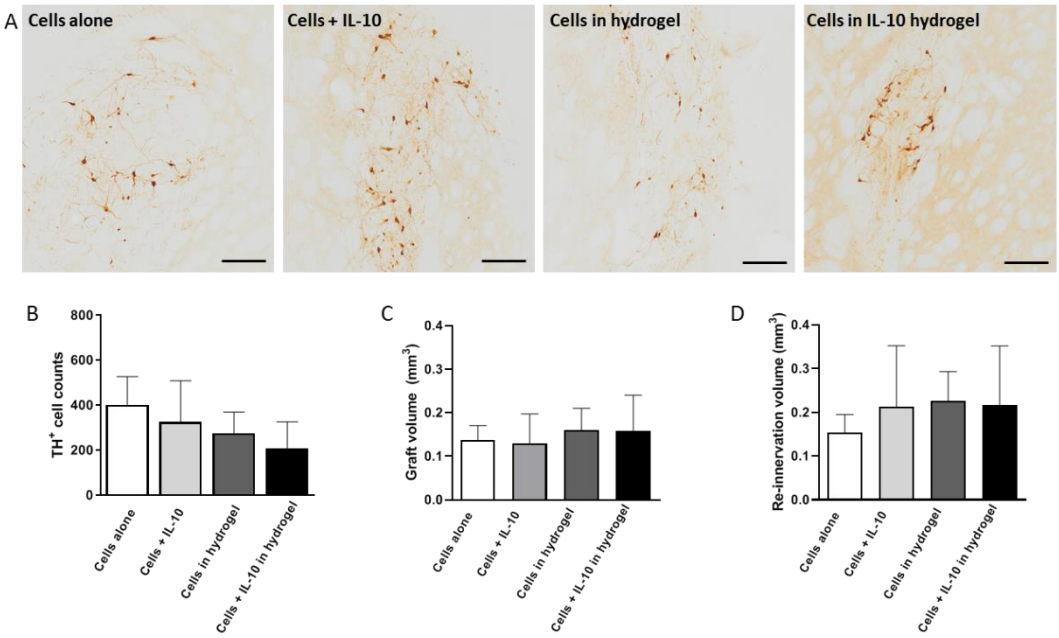

Figure 5.7 Impact of an IL-10 loaded collagen hydrogel on dopaminergic cell survival. Representative photomicrographs of primary dopaminergic cell grafts at 12 weeks post-transplantation. The addition of IL-10, the encapsulation in a collagen hydrogel or the combination of both factors did not improve the dopaminergic cell survival, graft size or re-innervation volume at 12 weeks post-transplantation. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test. Scale bar = 200 µm.

5.3.2.2 Host immune response to primary dopaminergic cells encapsulated in an IL-10 collagen hydrogel

In line with our previous studies (see Chapter 4), the transplantation of VM cells evoked a neuro-inflammatory response from the host brain observable through the recruitment of astrocytes and microglial cells to the transplantation site (**Figure 5.8** and **Figure 5.9**). The delivery of VM cells encapsulated in a collagen hydrogel did not reduce the striatal astrocytosis in terms of density (**Figure 5.8b**; Hydrogel, $F_{(1,20)} = 0.105$, $P>0.05$) or volume (**Figure 5.8c**; Hydrogel, $F_{(1,20)} = 0.277$, $P>0.05$) around the grafted area. Likewise, the encapsulation of the grafted VM cells did not reduce the striatal microgliosis in terms of density (**Figure 5.9b**; Hydrogel, $F_{(1,20)} = 0.644$, $P>0.05$) or volume (**Figure 5.9c**; Hydrogel, $F_{(1,20)} = 0.378$, $P>0.05$) of microgliosis around the grafted area.

Unlike our expectations, the addition of IL-10 – alone or encapsulated in a collagen hydrogel – did not ameliorate astrocytosis density (**Figure 5.8b**; Factors, $F_{(1,20)} = 0.086$, $P>0.05$) or volume (**Figure 5.8c**; Factors, $F_{(1,20)} = 0.692$, $P>0.05$). Again, the microglial density (**Figure 5.9b**; Factors, $F_{(1,20)} = 0.787$, $P>0.05$) or volume (**Figure 5.9c**; Factors, $F_{(1,20)} = 0.616$, $P>0.05$) was not modified by the addition of IL-10 either as a bolus or encapsulated in a collagen hydrogel. However, there was a trend towards a reduction of microglial volume when cells were delivered with IL-10 (**Figure 5.9**).

Correspondingly, the VM encapsulation in an IL-10 loaded collagen did not mitigate the density of astrocytes (**Figure 5.8b**; Hydrogel x Factors, $F_{(1,20)} = 0.619$, $P>0.05$) nor the volume of astrocytosis (**Figure 5.8c**; Hydrogel x Factors, $F_{(1,20)} = 0.145$, $P>0.05$). Similarly, the combination of IL-10 and the biomaterial did not translate into a reduction of the density of microglia around the graft (**Figure 5.9b**; Hydrogel x Factors, $F_{(1,20)} = 0.491$, $P>0.05$) nor the volume of microgliosis in the striatum (**Figure 5.9c**; Hydrogel x Factors, $F_{(1,20)} = 0.904$, $P>0.05$).

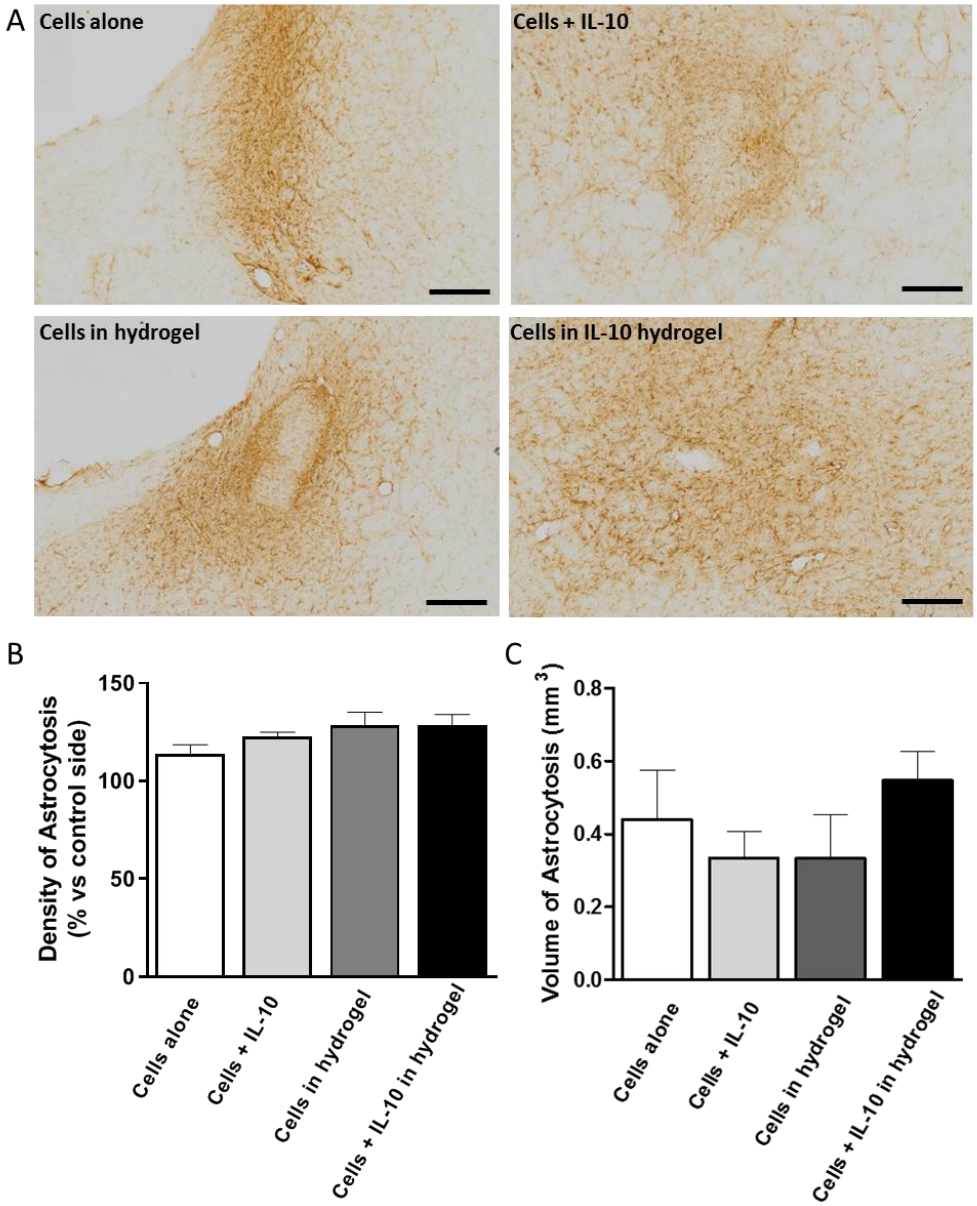

Figure 5.8 Effect of the IL-10 loaded collagen hydrogel on the astrocytic response. A) GFAP immunostaining revealed the appearance of striatal astrocytosis measured via B) density and C) volume of astrocytes around the grafted site at 12 weeks post-transplantation. The loading of IL-10 in a collagen hydrogel did not reduce the striatal astrocytosis in the surrounding graft tissue. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test. Scale bar = 200 µm.

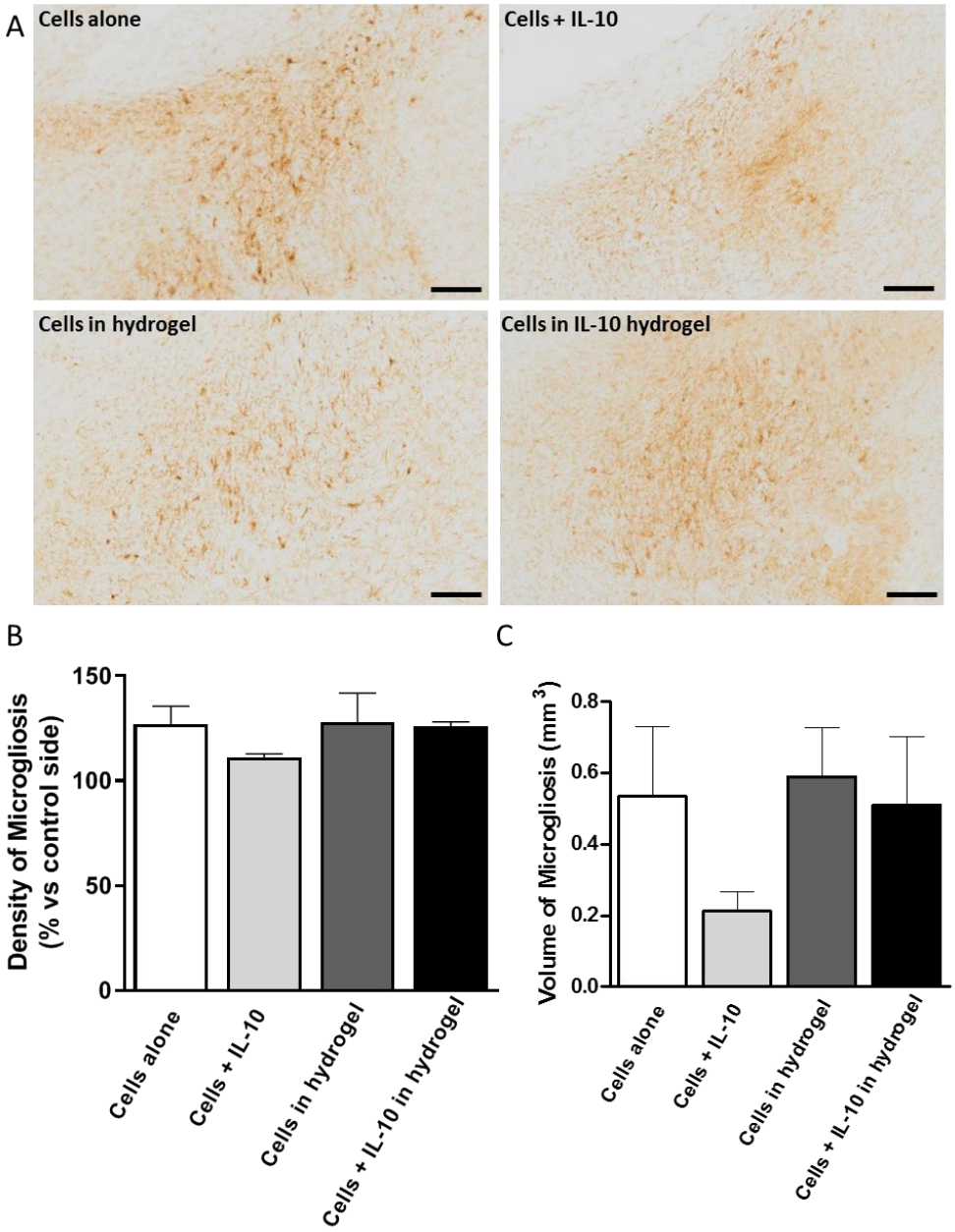

Figure 5.9 Effect of the IL-10 loaded collagen hydrogel on the microglial response. A) OX-42 immunostaining revealed the appearance of striatal astrocytosis via B) density and C) volume of microglia around the grafted site at 12 weeks post-transplantation. The loading of IL-10 in a collagen hydrogel did not reduce the striatal microgliosis in the surrounding graft tissue. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test. Scale bar = 200 µm.

5.4 DISCUSSION

Primary dopaminergic neurons from the ventral mesencephalon have been used to provide proof-of-concept of the benefits of cell transplantation for Parkinson's disease. Primary VM cell grafts have proven to survive, attach and re-innervate in the striatum while restoring the motor function in rodent models of Parkinson's disease (Dunnett et al., 1981; Brundin et al., 1986). Later, several clinical trials outlined the reduction of the motor impairment in patients, even removing the need for pharmacological drug treatment in some patients (Kefalopoulou et al., 2014). However, these clinical trials also highlighted the logistical and ethical concerns that using a limited primary dopaminergic cell source entailed. One of the main logistic limitations of VM cell transplantation relies on the cell's own nature of being of primary embryonic origin, which cannot be solved. However, there are other current limitations, like the low poor survival of these primary dopaminergic neurons that can be addressed. Furthermore, this problem also applies to other dopaminergic cell sources like stem cells.

As mentioned earlier, primary dopaminergic neurons undergo a high extent of cell death during and after transplantation process due to a number of heterogeneous factors such as the absence of a support matrix during sample preparation and injection, a growth-factor depleted environment upon transplantation and an incursion of immune cells around the grafted cells.

In situ forming collagen hydrogels have the potential to protect these cells during and after the transplantation process by offering a matrix where the cells can attach to. It has already been reported that the encapsulation of VM cells in a GDNF-loaded collagen hydrogel resulted in a 5-fold increase in dopaminergic neuron survival and a 3-fold increase of striatal re-innervation ability (Moriarty et al., 2017). This highlights the benefits that trophic factor enriched biomaterials can have in cell survival upon transplantation. These results also prove the efficacy of targeting the apoptosis checkpoints where most of the cell death is thought to be happening. However, the potential advantages of these biomaterials in targeting the host immune response have not been investigated.

Suspensions of VM cells exhibit much better dopaminergic survival when they are grown in three-dimensional cultures than when they are transplanted *in vivo*. Thus, there are features – beyond cell preparation – in the host environment that are toxic to dopaminergic neurons (Barker & Widner, 2004). This environment is characterised by the gliotic reaction originated from the transplantation of exogenous cells as well as graft and host interactions (Barker & Widner, 2004).

Therefore, the aim of this chapter was to investigate the impact of an anti-inflammatory-loaded collagen hydrogel on reducing the host innate immune response in the graft surrounding area and ultimately assess if it could improve the dopaminergic cell survival and re-innervation of the primary dopaminergic neurons.

In Chapter 3, we established the cytocompatibility of collagen hydrogels as well as their ability to release functional anti-inflammatory factors *in vitro*. In Chapter 4 we reported that all collagen hydrogel compositions (with 1-4 mg/ml of crosslinker) injected into the striatum were found to be biocompatible. We have also shown that the encapsulation of IL-10 in a collagen hydrogel successfully retained more IL-10 in the striatum for the first 24h post-injection.

In neural grafting, the mere injection of cells in the striatum will evoke a host immune response characterised by the recruitment and activation of macrophages, microglia and astrocytes and the intensity of this immune response will be defined by multiple factors such as tissue origin, preparation and implantation site (Barker & Widner, 2004). However, this neuro-immune response can be ameliorated by encapsulating and delivering cells in a collagen hydrogel, which will act as a physical barrier between the transplanted cells and the host neuro-immune cells (Moriarty et al., 2017). Nevertheless, in the studies reported here we did not see a reduction of the host immune response due to the hydrogel (**Figure 5.8** and **Figure 5.9**).

In this chapter we have shown that the encapsulation of VM cells in an IL-10 loaded collagen reduced the density of microglial cells around the grafted site at four weeks post-transplantation when measured via optical density (**Figure 5.6**). However, when we analysed the volume of striatal microgliosis, no changes were observed in this parameter when cells were transplanted alongside IL-10 either as a bolus or inside a collagen hydrogel. This can be explained because the IL-10 will remain in the delivered area (surrounding the graft) whereas the volumetric measurement of striatal

microgliosis takes into account a far bigger volume than the one of the graft. It has to be noted that often the microglial response can be visible in half or more of the striatum. Despite this reduction of the optical density of striatal microgliosis at four weeks post-transplantation, we did not observe this effect at 12 weeks post-transplantation (**Figure 5.9**). In a time-framed study of the immunological reactions after a VM transplantation, Duan et al., (1995) reported that CD11b immunological response peaked at 1.5 weeks post-transplantation but markedly decreased around 5 to 8 weeks post-transplantation, and that there was a later increase of the response at 12 and 18 weeks post-transplantation. This pattern could explain why we observed a different result on the striatal microgliosis at the two timepoints assessed. On the other hand, although IL-10 reduced the activation of microglia after an LPS injection *in vivo*, several studies found IL-10 to be insufficient to re-direct activated microglia (Fenn et al., 2012; Norden et al., 2014).

Astrocytes are known to express high levels of IL-10R1 and be sensitive to the anti-inflammatory effects of IL-10. Moreover, IL-10 can re-direct astrocytes to a less reactive type, which in turn attenuate the activation of microglia (Norden et al., 2014). Nevertheless, in regards to the striatal astrocytosis response, we found no changes in the optical density or volume of striatal astrocytosis either at 4 (**Figure 5.5**) or 12 weeks post-transplantation (**Figure 5.8**). This fact could again be explained because the effects of IL-10 on astrocytes occur at a molecular level and thus are not distinguishable in gross measurements such as the optical density or volumetric analysis.

The analysis described in this thesis aimed to test if an IL-10 loaded collagen could ameliorate the host immune response and benefit the transplanted VM cells. To reach this goal, a general overview of the immune response was measured, but any potential effects of the cytokine on a molecular or cellular level were not analysed. However, it was not the scope of this study to extensively analyse the effects of IL-10 on the host immune response but to test if these effects could improve on the survival of transplanted dopaminergic neurons. At the end of these investigations, we did not find IL-10 to be helpful in the reduction of the host immune response after VM cell transplantation.

Regarding this issue, the encapsulation of VM cells in an IL-10 loaded collagen hydrogels did not improve the survival of dopaminergic grafts nor their outgrowth capacity neither at 4 (**Figure 5.3**) or 12 weeks post-transplantation (**Figure 5.7**). The hypothesis that an IL-10 loaded collagen hydrogel would improve dopaminergic neuron survival depended on reduction of the host immune response. Consequently, without relevant effects on the host immune response, it is not surprising the lack of improvement on cell survival of the grafted cells.

The data in this chapter suggests that IL-10 used in the conditions of the study was inefficacious in ameliorating the host immune response or enhancing the dopaminergic cell survival. However, the potential effects of IL-10 can still be investigated by, for example, modifying the concentration. Another interesting variable to consider apart from the dose would be to retain the IL-10 in the grafted area for longer periods of time. We have shown that the biomaterial retained the cytokine in the striatum for 24h (see Chapter 4) but initial microglial recruitment extends up to day 4 as stated in Chapter 4 (Duan et al., 1995). Also Barker et al. (1996) reported few OX-42 immuno-reactive cells at day 3 post-transplantation but an intense recruitment of these cells by 2 weeks post-transplantation. Thus, it might be necessary to retain IL-10 in the striatum for longer periods of time in order to have a broad anti-inflammatory effect on the host immune response.

Our data suggests that IL-10 is unable to promote the survival of the transplanted cells. Consequently, another interesting strategy could be the combination of IL-10 with neuroprotective factors that have shown to enhance dopaminergic cell survival. Taking advantage of the versatility of collagen hydrogels, IL-10 could be delivered together with GDNF to promote a synergistic effect of both factors on the grafted cells and the surrounding microenvironment.

CHAPTER 6: A MULTI-MODAL COLLAGEN HYDROGEL SCAFFOLD FOR INTRA-STRIATAL CELL TRANSPLANTATION

6.1 INTRODUCTION

Preclinical and clinical investigations over the years have shown that cell replacement stands as a potential therapy to repair the degenerating nigrostriatal pathway in Parkinson's disease. Transplanted fetal dopaminergic neurons can integrate with the host brain and produce dopamine, subsequently improving the motor function both in animals and Parkinson's disease patients. However, the efficacy of the fetal dopaminergic transplants is, in part, limited by the extreme poor survival of the transplanted cells. The causes behind this extensive cell death immediately after transplantation are multifactorial, but the removal of primary dopaminergic neurons from the developing fetal brain and subsequent transplantation into the host adult trophic factor-deprived brain is thought to be an essential key point for the lack of survival.

Many efforts have been put into investigating neuroprotective strategies in an attempt to improve the survival of dopaminergic grafts. The addition of neurotrophic factor therapy to support cell replacement therapies has been investigated, aiming to provide the dopaminergic cells with a trophic-factor enriched microenvironment pre- and post-transplantation. Several neurotrophic factors - known to be involved with dopaminergic neuron differentiation and maintenance - have been studied, such as GDNF or neurturin (Paul & Sullivan, 2019). Intracerebral administration of GDNF has consistently been shown to bring neuroprotection to midbrain neurons in *in vivo* models of Parkinson's disease when administered at the time of lesioning or after (Kordower & Bjorklund, 2013), thus highlighting the potential of neurotrophic therapy for cell replacement.

Furthermore, GDNF has been proven to have potently enhanced the survival of transplanted primary dopaminergic cells in hemi-parkinsonian rats (Rosenblad et al., 1996; Apostolides et al., 1998). Since GDNF is unable to cross the BBB, its administration in cell replacement therapies has to be done either previous to transplantation or intra-cranially at the same time as the transplanted cells. For

example, co-transplantation of human recombinant GDNF alongside VM cells resulted in an improvement of dopaminergic cell survival as well as an improved functional recovery at six weeks post-transplantation (Chaturvedi et al., 2003). Due to the short half-life of GDNF, others have investigated the effects of a long-term delivery of the trophic factor. For instance, Clavreul et al., (2006) showed that the implantation of GDNF-loaded microspheres close to a primary dopaminergic graft improved the dopaminergic graft survival and functional behaviour. Moreover, the co-encapsulation of primary dopaminergic VM cells and GDNF in a collagen hydrogel dramatically improved the survival of the transplanted cells upon transplantation, thanks to a combined effect of the collagen scaffold and the GDNF (Moriarty et al., 2017; Moriarty, 2019a).

It is well known that the transplantation of exogenous cells in the brain parenchyma generates a host innate immune response against the grafted cells. This quick immune reaction is thought to create an inflammatory microenvironment that contributes to the extensive cell death experienced early after transplantation.

The potent anti-inflammatory effects of IL-10 on microglia and astrocytes make IL-10 an interesting candidate to use it as an anti-inflammatory treatment against the host immune response associated to cell transplantation in the CNS. Nevertheless, as stated in previous chapters, the delivery of primary VM cells encapsulated in an IL-10 loaded collagen hydrogel did not ameliorate the host immune response around the grafted cells nor did it improve the survival of the grafted cells. However, the co-administration of IL-10 with other potent neuroprotective agents for cell transplantation therapies has not been assessed before.

Taking into account everything mentioned above, this chapter aimed to assess if the combination of a neuroprotective factor with an anti-inflammatory cytokine in a collagen scaffold could have a synergistic effect on improving the survival of dopaminergic cell grafts. Thus we assessed the efficacy of a collagen hydrogel loaded with IL-10 and GDNF on the dopaminergic cell survival and re-innervation, motor function recovery and host immune response.

6.2 METHODS

6.1.1 Experimental design

Since the encapsulation of VM cells in an IL-10 loaded collagen hydrogel did not improve the survival of dopaminergic grafts and re-innervation, we proceeded to investigate if the combination of IL-10 with GDNF – a known neuroprotective factor – could improve the dopaminergic cell survival and outgrowth of transplanted dopaminergic cells in the striatum of hemi-parkinsonian rats as well as to reduce the host immune response after transplantation.

To study this, 48 adult male Sprague-Dawley rats received a unilateral intra-MFB injection of 6-OHDA (12 µg in 3 µl). Three weeks later, the animals' motor function was assessed via methamphetamine-induced rotations (2.5 mg/kg) and based on these results, rats were performance matched into eight groups (n=6). In the same week, rats underwent unilateral intra-striatal transplants of E14 VM cells alone (400,000 cells in 6 µl), E14 VM cells with IL-10 (1000 ng), E14 VM cells with GDNF (1000 ng), E14 VM cells encapsulated in a collagen hydrogel or E14 VM cells encapsulated in an IL-10-loaded collagen hydrogel, E14 VM cells encapsulated in a GDNF-loaded collagen hydrogel or E14 VM cells encapsulated in an IL-10/GDNF-loaded collagen hydrogel (**Table 6.1**). Methamphetamine-induced rotations were carried out at 12 weeks post-transplantation. Other behavioural motor tests – Corridor test, Stepping test and Whisker test - were carried out at weeks 8 and 12 post-transplantation on habituated animals. Then, animals were sacrificed for p*ost-mortem* assessment. A schematic view of the experimental design is shown in **Figure** 6.1.

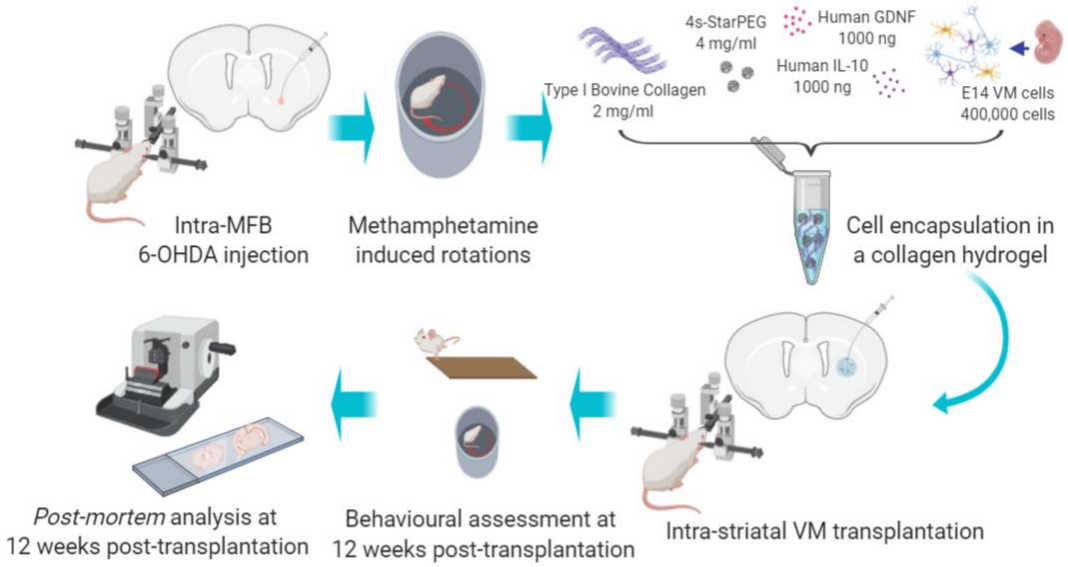

Figure 6.1 Multi-modal collagen hydrogel for dopaminergic cell transplantation in Parkinson´s disease. Schematic view of the delivery of E14 VM cells (400,000 cells/6µl) encapsulated in an IL-10/GDNF (1000 ng/6µl of each protein) collagen hydrogel in unilaterally lesioned hemi-parkinsonian rats. Motor function recovery, dopaminergic cell survival and re-innervation, and host immune response to the transplant were measured at 12 weeks post-transplantation.

Table 6.1 Experimental groups used in an intra-striatal VM transplant study in hemi-Parkinsonian rats. VM cells injected: 400,000 cells/6 µl, IL-10 and GDNF: 1000 ng/6 µl.

Experimental Group	Sample size	Experimental Group	Sample size
VM cells	6	VM cells in hydrogel	6
VM cells + IL-10	6	VM cells + IL-10 in hydrogel	6
VM cells + GDNF	6	VM cells + GDNF in hydrogel	6
VM cells + IL-10 + GDNF	6	VM cells + IL-10 + GDNF in hydrogel	6

6.3 RESULTS

6.3.1 Impact of an IL-10 and GDNF loaded collagen hydrogel on primary dopaminergic neuron survival

As means to evaluate the survival of dopaminergic grafts, the number of TH^+ cell bodies in the striatum were counted using TH^+ immunostained photomicrographs. As illustrated in the photographs, the presence of dopaminergic grafts was easily appreciable, meaning a successful transplantation of dopaminergic neurons into the striatum (**Figure** 6.2**a**). The encapsulation of VM cells in a collagen hydrogel did not improve the number of surviving cells (**Figure** 6.2**b**; Hydrogel, $F_{(1,30)} = 0.017$, P>0.05) and surprisingly, the addition of IL-10 or GDNF did not modify the survival of the grafted cells (**Figure** 6.2**b**; Factors, $F_{(3,30)} = 0.504$, P>0.05). Consequently, the combination of the biomaterial with the factors did not improve the outcome either (**Figure** 6.2**b**; Hydrogel x Factors, $F_{(3,30)} = 0.950$, P>0.05).

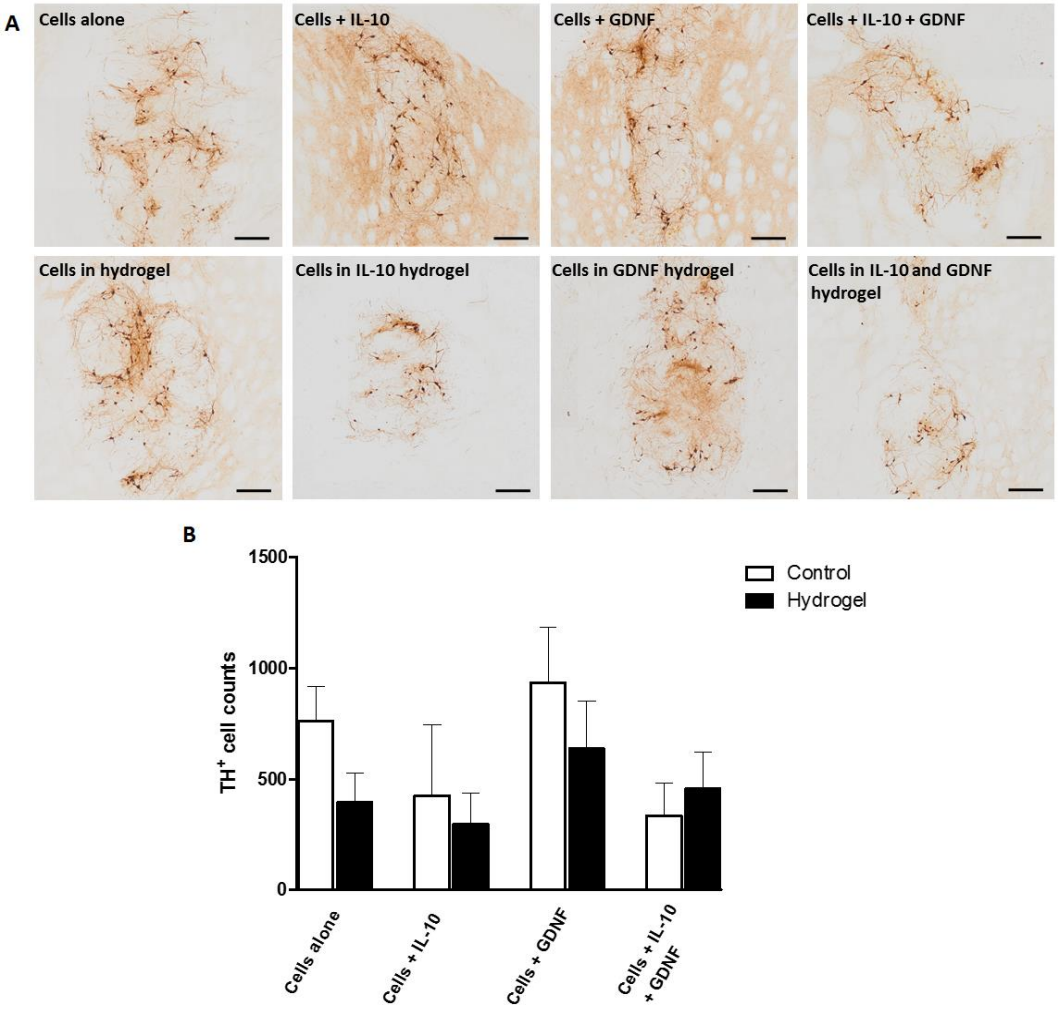

Figure 6.2 Impact of an IL-10 and GDNF collagen hydrogel on dopaminergic cell survival. A) Representative TH+ immunostained photomicrographs showing primary dopaminergic cell grafts. B) Dopaminergic cell bodies from the TH+ immunostained pictures were counted for each experimental group at 12 weeks post-transplantation. The addition of IL-10, GDNF, the encapsulation of VM cells in a collagen hydrogel or the combination of the factors and the collagen hydrogel did not modify the dopaminergic cell survival at 12 weeks post-transplantation. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test. Scale = 200 µm.

6.3.2 Impact of an IL-10 and GDNF loaded collagen hydrogel on primary dopaminergic neuron striatal re-innervation

We also sought to determine if the dual collagen hydrogel improved the ability of the surviving cells to grow neurites and re-innervate in the striatum. The area of the graft and of re-innervation was measured using TH^+ immunostained photomicrographs.

Consistent with the previous results, the dopaminergic graft size was also found to be similar between all experimental groups and the encapsulation of cells in the biomaterial did not increase the graft size (**Figure** 6.3**b**; Hydrogel, $F_{(1,30)} = 0.320$, $P>0.05$), nor did it the addition of IL-10 and/or GDNF (**Figure** 6.3**b**; Factors, $F_{(3,30)} = 1.278$, $P>0.05$) or the encapsulation in an IL-10 and GDNF collagen hydrogel (**Figure** 6.3**b**; Hydrogel x Factors, $F_{(3,30)} = 0.957$, $P>0.05$).

All VM grafts did re-innervate a portion of the striatum, but the encapsulation of primary VM cells in a collagen hydrogel did not increase the re-innervation volume (**Figure** 6.3**c**; Hydrogel, $F_{(1,30)} = 1.377$, $P>0.05$), nor did the addition of neuroprotective and/or anti-inflammatory factors (**Figure** 6.3**c**; Factors, $F_{(3,30)} = 0.803$, $P>0.05$) or its combination (**Figure** 6.3**c**; Hydrogel x Factors, $F_{(3,30)} = 1.126$, $P>0.05$).

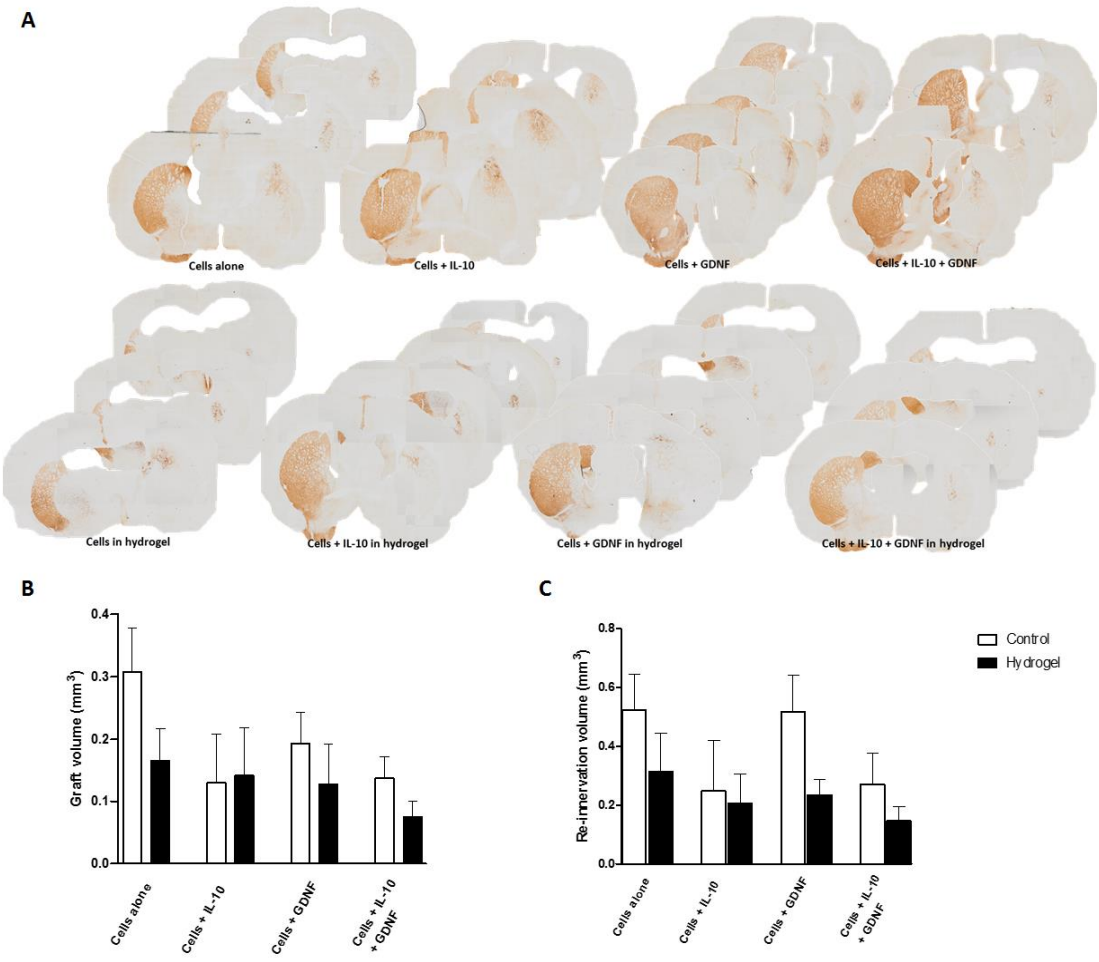

Figure 6.3 Impact of an IL-10 and GDNF collagen hydrogel on dopaminergic re-innervation. A) Representative photographs of primary dopaminergic grafts and re-innervated volume. B) Graft and C) re-innervation volumes of dopaminergic grafts at 12 weeks post-transplantation. The addition of IL-10, GDNF, the encapsulation of VM cells in a collagen hydrogel or the combination of the factors and the collagen hydrogel did not modify the dopaminergic cell survival at 12 weeks post-transplantation. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test.

6.3.3 Impact of an IL-10 and GDNF loaded collagen hydrogel on functional motor recovery

In addition to the histological analysis, we assessed the ability of VM cell transplants to reduce the motor impairments produced by the 6-OHDA lesion using methamphetamine-induced rotations at 12 weeks post-transplantation. The effects of VM encapsulation in an IL-10 and GDNF rich collagen hydrogel were also assessed in other behavioural tests such as the Corridor test of contralateral neglect, the Stepping test of forelimb akinesia and the Whisker test of sensorimotor integration.

The unilateral administration of 6-OHDA in the MFB, denervated the ipsilesional hemisphere, but not the contralesional hemisphere as it can be appreciated in TH^+ immunostained photographs (**Figure** 6.3). This was supported by the data acquired from the drug-induced rotation test before transplantation (**Figure** 6.4**a**). Correspondingly, the different behavioural tests used also exposed a profound impairment of the contralateral side of the body but not the ipsilateral side (Corridor test in **Figure** 6.5 vs **Figure** 6.6, Stepping test in **Figure** 6.8 vs **Figure** 6.9 and Whisker test in **Figure** 6.10 vs **Figure** 6.11).

Thus, the ipsilateral side of the body was used as a control measurement in the Corridor test, Stepping test and Whisker test.

6.3.3.1 Drug-induced rotational behaviour

The intra-MFB injection of 6-OHDA generated a unilateral depletion of dopamine on the ipsilesional side of the brain and this was manifested by a marked drug-induced rotational behaviour towards the ipsilateral side (**Figure** 6.4**a**). All experimental groups exhibited a pronounced rotational behaviour prior to transplantation, ensuring there was not a lesion bias in any of the experimental groups (**Figure** 6.4**a**; Hydrogel x Factors, $F_{(3,39)} = 0.168$, $P>0.05$).

In line with the literature, the delivery of primary dopaminergic VM cells in the striatum significantly reduced this behaviour in the methamphetamine-induced rotation test (**Figure** 6.4). However, the encapsulation of VM cells in a collagen hydrogel did not reduce the rotational behaviour to a greater extent than the sole injection of VM cells alone (**Figure** 6.4**b**; Hydrogel, $F_{(1,39)} = 3.395$, $P>0.05$). On the other hand, the addition of GDNF and IL-10 alongside the VM cells did reduce the rotational behaviour at 12 weeks post-transplantation according to the two-way ANOVA (**Figure** 6.4**b**; Factors, $F_{(3,39)} = 7.487$, $P<0.001$). Although the Bonferroni *post-hoc* test did not show differences between the relevant comparisons, there was a clear trend for GDNF to reduce the rotational behaviour at 12 weeks post-transplantation (**Figure** 6.4**b)**.

Thus, data extracted from the drug-induced rotations show that overall, the dopaminergic grafts were functional although the experimental groups showed no significant improvement versus the injection of VM cells alone.

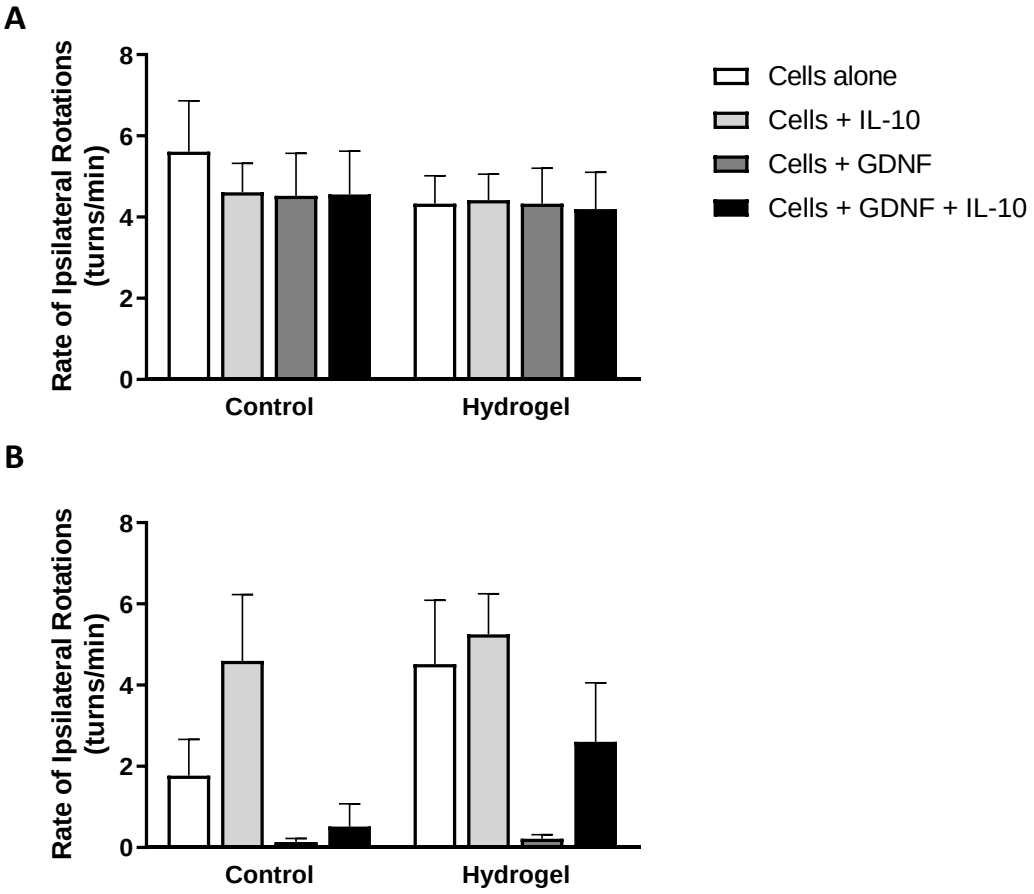

Figure 6.4 Impact of an IL-10 and GDNF collagen hydrogel on drug-induced rotational behaviour. A) Animals presented a marked ipsilateral rotational behaviour before transplantation. B) In general, the transplantation of VM grafts in the striatum decreased the rate of ipsilateral rotations at 12 weeks post-transplantation. The addition of IL-10, GDNF, the encapsulation of cells in a collagen hydrogel or the combination of the factors and the collagen hydrogel did not improve the level of functional recovery at 12 weeks post-transplantation. Data are represented as mean ± SEM and were analysed by a repeated measures two-way ANOVA with Bonferroni *post-hoc* test.

6.3.3.2 Corridor test of contralateral neglect

As expected, the intra-MFB administration of 6-OHDA on the ipsilesional hemisphere had no impact on ipsilateral performance in the Corridor test at 8 weeks post-transplantation (**Figure** 6.5**b**; Hydrogel x Factors, $F_{(3,27)} = 0.128$, P>0.05) and at 12 weeks post-transplantation (**Figure** 6.5**c**; Hydrogel x Factors, $F_{(3,40)} = 1.109$, P>0.05).

Predictably, the contralateral side of the body was substantially impaired due to the 6-OHDA injection as depicted by the contralateral performance in the Corridor test. The transplantation of VM cells encapsulated in an IL-10 and GDNF loaded collagen hydrogel on the denervated striatum did not rescue the impairment of the contralateral motor function in the Corridor test over time (**Figure** 6.6). Furthermore, this impairment was equally seen across all the experimental groups at 8 weeks post-transplantation (**Figure** 6.6**b**; Hydrogel x Factors, $F_{(3,27)} = 0.115$, P>0.05). At 12 weeks post-transplantation, a significant interaction due to the factors was detected by a two-way ANOVA (**Figure** 6.6**c**; Factors, $F_{(3,40)} = 4.144$, P<0.05) but the Bonferroni *post-hoc* test showed no statistical differences between relevant comparisons.

Thus, the Corridor test confirmed an impairment of the contralateral side to the 6-OHDA injection and this impairment was not overcome with the transplantation of VM cells, independently if they were transplanted encapsulated in a collagen hydrogel or not and alongside IL-10 and GDNF or not.

Additionally, we did notice an overall decrease in time to perform the task (**Figure** 6.7), indicating that the animals habituated to the task and learned over time.

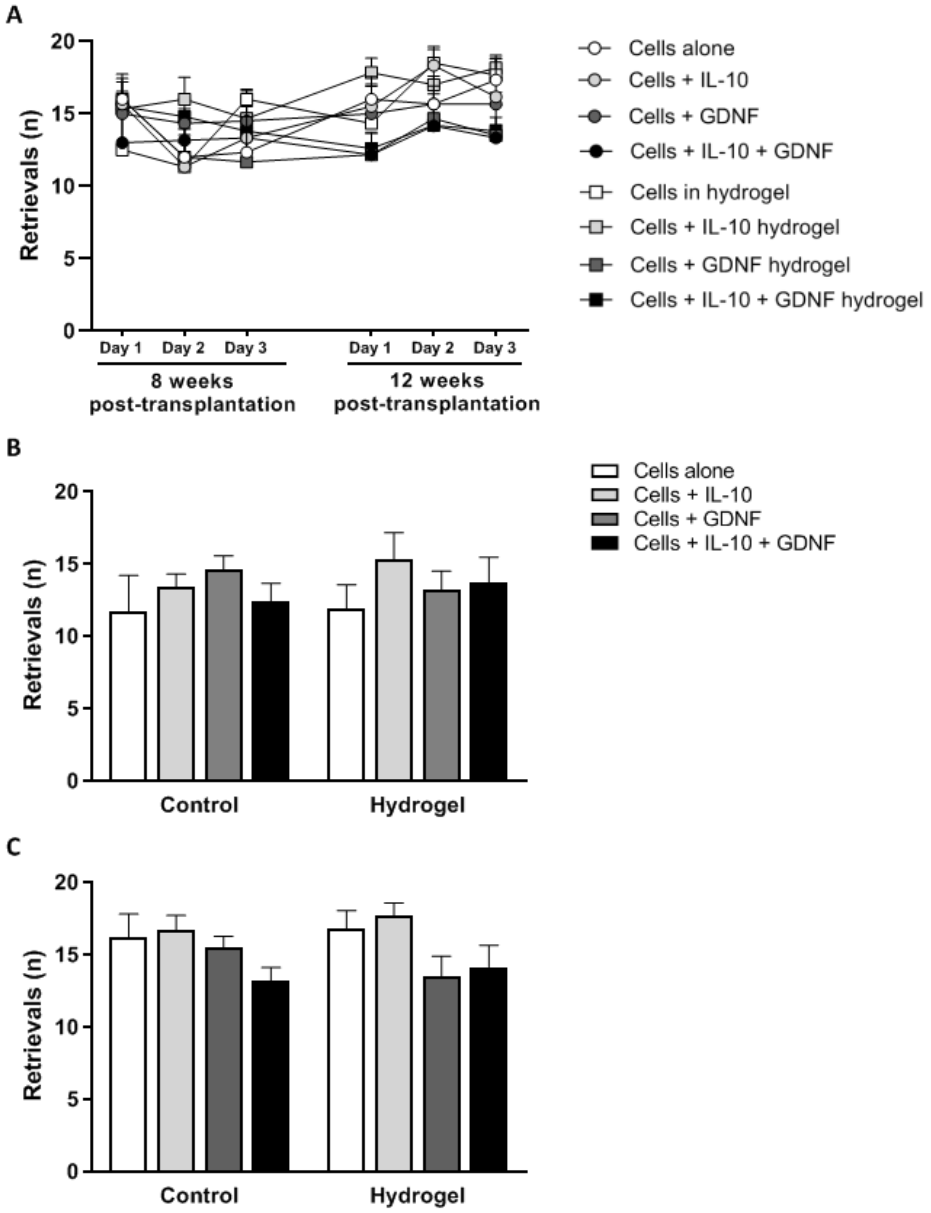

Figure 6.5 Impact of the encapsulation of VM cells on an IL-10 and GDNF collagen hydrogel on ipsilateral motor function. CoCoPops® retrievals from the ipsilateral side A) over time B) at 8 weeks post-transplantation and C) at 12 weeks post-transplantation. XY graph represents data at each single trial whereas bar charts represent collapsed data from the same timepoint. As expected, the 6-OHDA lesion had no effect on the ipsilateral behaviour in the Corridor test. Data are represented as mean ± SEM and were analysed by A) repeated measures two-way ANOVA with Bonferroni *post-hoc* test, B) and C) two-way ANOVA with Bonferroni *post-hoc* test.

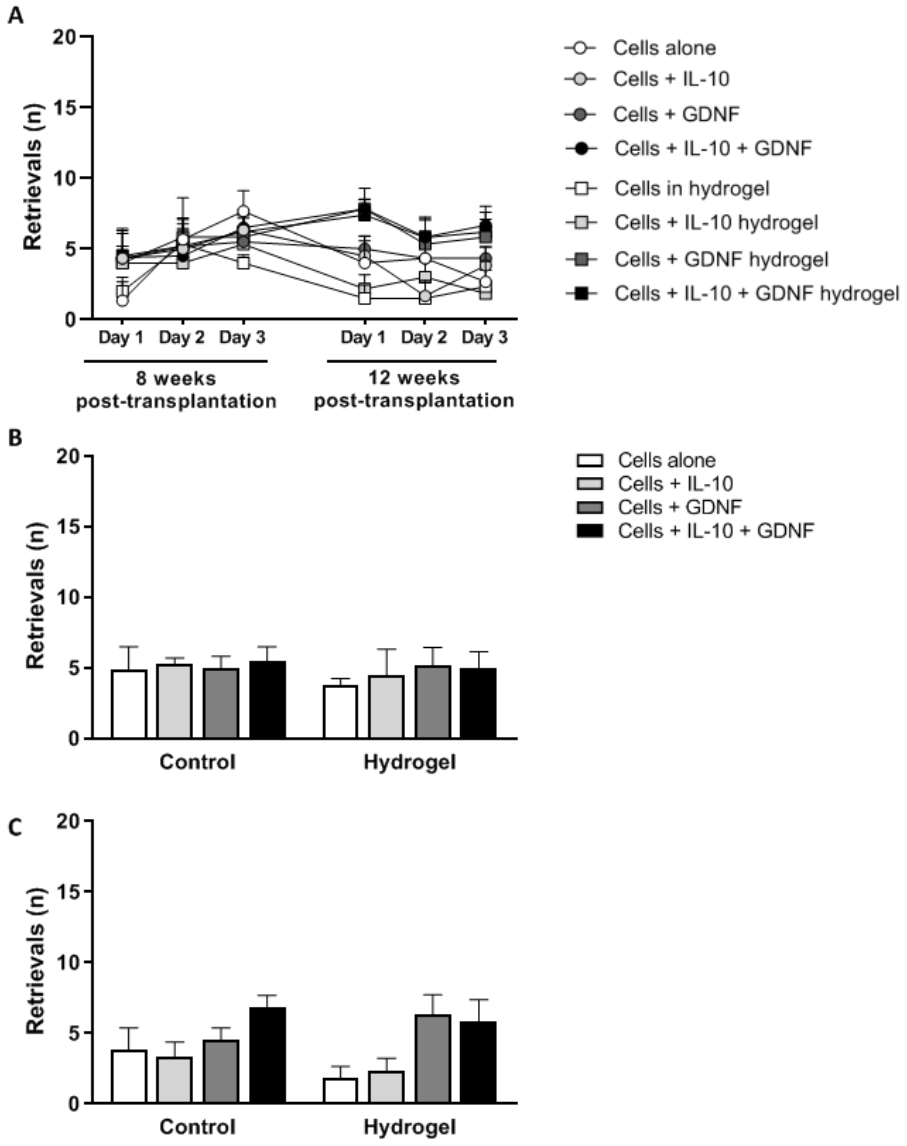

Figure 6.6. Impact of the encapsulation of VM cells on an IL-10 and GDNF collagen hydrogel on contralateral motor function. CoCoPops® retrievals from the contralateral side A) over time B) at 8 weeks post-transplantation and C) at 12 weeks post-transplantation. XY graph represents data at each single trial whereas bar charts represent collapsed data from the same timepoint. The 6-OHDA lesion exhibited a profound impairment on the contralateral behaviour in the Corridor test. The encapsulation of VM cells on an IL-10 and GDNF collagen hydrogel did not modify this behaviour. Data are represented as mean ± SEM and were analysed by A) repeated measures two-way ANOVA with Bonferroni *post-hoc* test, B) and C) two-way ANOVA with Bonferroni *post-hoc* test.

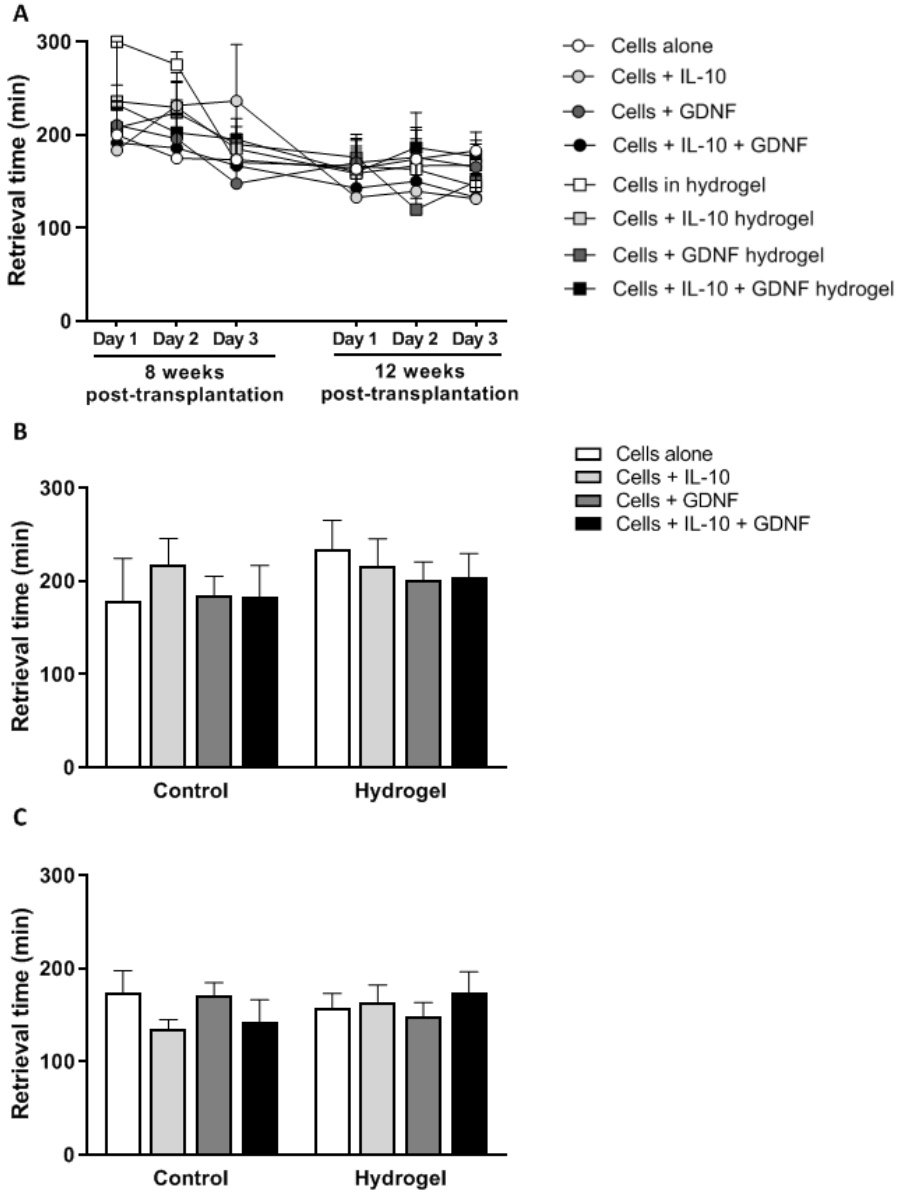

Figure 6.7 Impact of the encapsulation of VM cells on an IL-10 and GDNF collagen hydrogel on time to perform Corridor test. Time to perform the Corridor test A) over time B) at 8 weeks post-transplantation and C) at 12 weeks post-transplantation. XY graph represents data at each single trial whereas bar charts represent collapsed data from the same timepoint. The encapsulation of VM cells on an IL-10 and GDNF collagen hydrogel did not modify this behaviour. However, animals became quicker at performing the task. Data are represented as mean ± SEM and were analysed by A) repeated measures two-way ANOVA with Bonferroni *post-hoc* test, B) and C) two-way ANOVA with Bonferroni *post-hoc* test.

6.3.3.3 Stepping test of forelimb akinesia

As expected, the intra-MFB administration of 6-OHDA on the ipsilesional hemisphere had no impact on ipsilateral performance in the Stepping test at 8 weeks post-transplantation (**Figure** 6.8**b**; Hydrogel x Factors, $F_{(3,27)} = 0.507$, P>0.05) and at 12 weeks post-transplantation (**Figure** 6.8**c**; Hydrogel x Factors, $F_{(3,40)} = 1.101$, P>0.05).

In the Stepping test, the performance of the contralateral paw was substantially impaired because of the 6-OHDA injection. Similar results as in the Corridor test were obtained when the contralateral side was analysed in the Stepping test; the transplantation of VM cells either alone or in combination with a collagen hydrogel or GDNF or IL-10 did not rescue the contralateral performance on the task at 8 weeks post-transplantation (**Figure** 6.9**b**; Hydrogel x Factors, $F_{(3,27)} = 0.587$, P>0.05) or 12 weeks post-transplantation (**Figure** 6.9**c**; Hydrogel x Factors, $F_{(3,40)} = 0.909$, P>0.05).

In line with our results in the Corridor test, the Stepping test revealed a dramatic impairment to perform the task correctly on the contralateral side to the 6-OHDA injection. As seen previously with the Corridor test, this impairment was not overcome with the transplantation of VM cells alone or in combination with a collagen hydrogel or neuroprotective and/or anti-inflammatory factors.

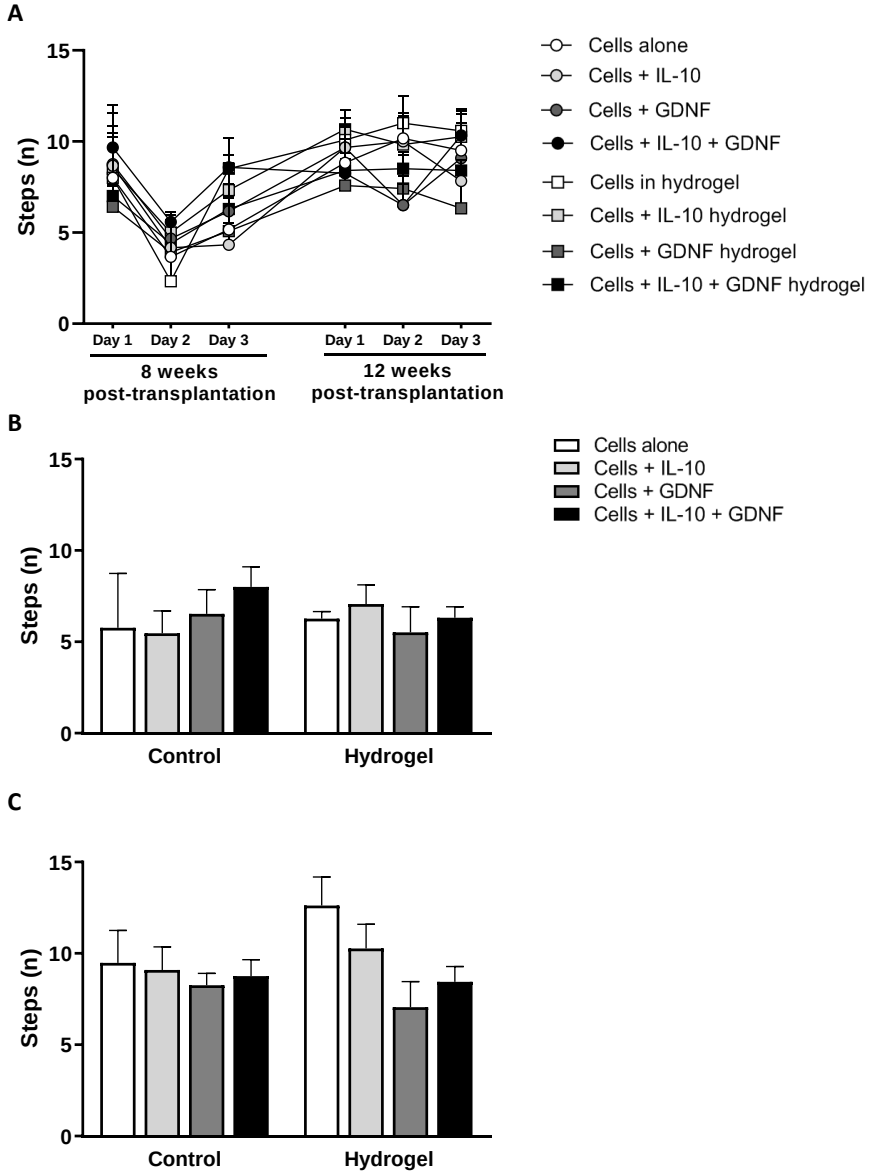

Figure 6.8. Impact of the encapsulation of VM cells on an IL-10 and GDNF collagen hydrogel on ipsilateral motor function. Step placings with the ipsilateral paw A) over time B) at 8 weeks post-transplantation and C) at 12 weeks post-transplantation. XY graph represents data at each single trial whereas bar charts represent collapsed data from the different trials on the same week. As expected, the 6-OHDA lesion had no effect on the ipsilateral behaviour in the Stepping test. Data are represented as mean ± SEM and were analysed by A) repeated measures two-way ANOVA with Bonferroni *post-hoc* test, B) and C) two-way ANOVA with Bonferroni *post-hoc* test.

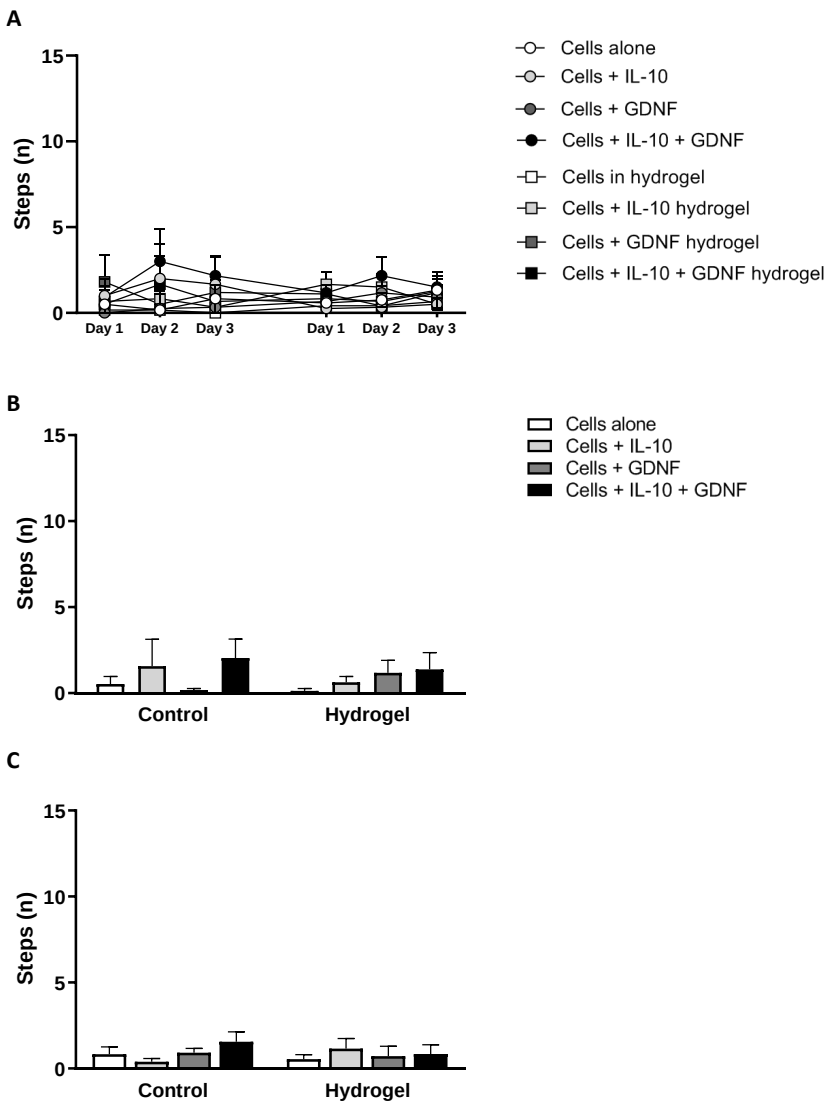

Figure 6.9 Impact of the encapsulation of VM cells on an IL-10 and GDNF collagen hydrogel on contralateral motor function. Step placings with the contralateral paw A) over time B) at 8 weeks post-transplantation and C) at 12 weeks post-transplantation. XY graph represents data at each single trial whereas bar charts represent collapsed data from the same timepoint. The 6-OHDA lesion exhibited a profound impairment on the contralateral behaviour in the Stepping test. The encapsulation of VM cells on an IL-10 and GDNF collagen hydrogel did not modify this behaviour. Data are represented as mean ± SEM and were analysed by A) repeated measures two-way ANOVA with Bonferroni *post-hoc* test, B) and C) two-way ANOVA with Bonferroni *post-hoc* test.

6.3.3.4 Whisker test of sensorimotor integration

Similarly to the Corridor and Stepping tests, the intra-MFB administration of 6-OHDA on the ipsilesional hemisphere had no impact on ipsilateral performance in the Whisker test at 8 weeks post-transplantation (**Figure** 6.10**b**; Hydrogel x Factors, $F_{(3,27)}$ = 0.100, P>0.05) or 12 weeks post-transplantation (**Figure** 6.10**c**; Hydrogel x Factors, $F_{(3,40)}$ = 0.542, P>0.05).

As well as in the other tests, the Whisker test depicted an impairment in the contralateral side of the body. Taking into account the results from the Corridor test and the Stepping test, not surprisingly, the transplantation of VM cells did not revert the impairment in the contralateral side of the body at any of the timepoints assessed (**Figure** 6.11). Similarly to the other tests, the transplantation of VM cells either alone or in combination with a collagen hydrogel or the factors did not improve the performance on the Whisker test at 8 weeks post-transplantation (**Figure** 6.11**b**; Hydrogel x Factors, $F_{(3,27)}$ = 2.357, P>0.05) and at 12 weeks post-transplantation (**Figure** 6.11**c**; Hydrogel x Factors, $F_{(3,40)}$ = 1.000, P>0.05).

In line with the previous behavioural tests, the Whisker test confirmed an impaired contralateral side of the body and this dysfunction was not overcome with the transplantation of VM cells alone or in combination with a collagen hydrogel or neuroprotective and/or anti-inflammatory factors.

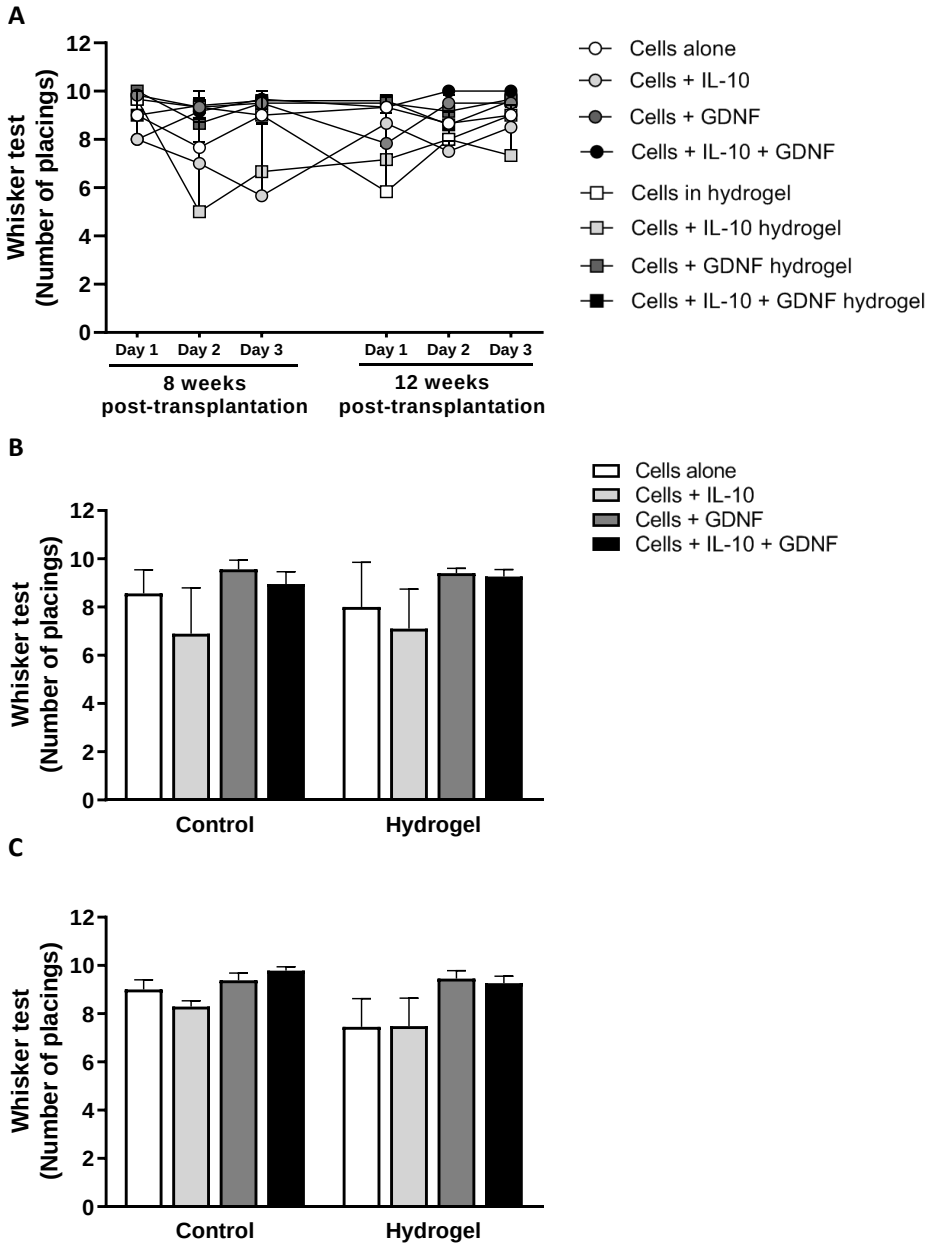

Figure 6.10 Impact of the encapsulation of VM cells on an IL-10 and GDNF collagen hydrogel on ipsilateral motor function. Ipsilateral paw placings A) over time B) at 8 weeks post-transplantation and C) at 12 weeks post-transplantation. XY graph represents data at each single trial whereas bar charts represent collapsed data from the same timepoint. As expected, the 6-OHDA lesion had no effect on the ipsilateral behaviour in the Whisker test. Data are represented as mean ± SEM and were analysed by A) repeated measures two-way ANOVA with Bonferroni *post-hoc* test, B) and C) two-way ANOVA with Bonferroni *post-hoc* test.

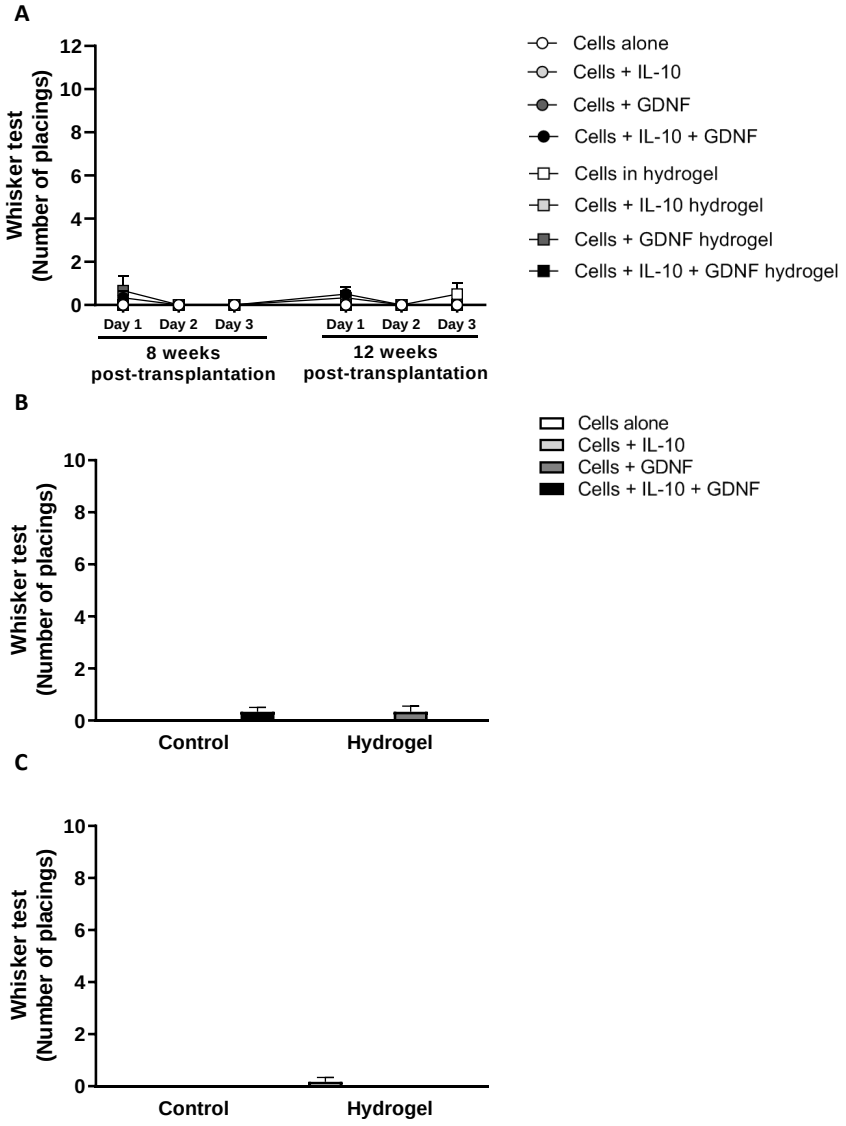

Figure 6.11 Impact of the encapsulation of VM cells on an IL-10 and GDNF collagen hydrogel on contralateral motor dysfunction. Contralateral paw placings on the contralateral side from lesion A) over time B) at 8 weeks post-transplantation and C) at 12 weeks post-transplantation. XY graph represents data at each single trial whereas bar charts represent collapsed data from the same timepoint. The 6-OHDA lesion exhibited a profound impairment on the contralateral behaviour in the Stepping test. The encapsulation of VM cells on an IL-10 and GDNF collagen hydrogel did not modify this behaviour. Data are represented as mean ± SEM and were analysed by A) repeated measures two-way ANOVA with Bonferroni *post-hoc* test, B) and C) two-way ANOVA with Bonferroni *post-hoc* test.

6.3.4 Impact of an IL-10 and GDNF loaded collagen hydrogel on the host immune response

We further assessed if the combination of GDNF and IL-10 encapsulated in a collagen hydrogel could reduce the host immune response elicited after the transplantation of VM cells.

In line with previous results from Chapter 3 and 4, the density of astrocytes around the grafted area after a VM transplant was not modified by the encapsulation of VM cells in a collagen hydrogel (**Figure 6.13b**; Hydrogel, $F_{(1,39)} = 0.023$, P>0.05), nor by the addition of GDNF or/and IL-10 (**Figure 6.13b**; Factors, $F_{(3,40)} = 2.375$, P>0.05) or the combination of the factors and the biomaterial (**Figure 6.13b**; Hydrogels x Factors, $F_{(3,39)} = 1.479$, P>0.05). As well, the volume of astrocytes recruited after the transplantation of VM cells was not modified by the cell encapsulation in a collagen hydrogel (**Figure** 6.12 and **Figure 6.13c**; Hydrogel, $F_{(1,39)} = 0.199$, P>0.05). Yet the volume of striatal astrocytosis was modified when IL-10 or/and GDNF were added to the VM cells (**Figure** 6.12 and **Figure 6.13c**; Factors, $F_{(3,39)} = 2.921$, P<0.05) and when the VM cells were encapsulated in an IL-10 and GDNF collagen hydrogel (**Figure** 6.12 and **Figure 6.13c**; Hydrogel x Factors, $F_{(3,39)} = 3.281$, P<0.05). However in these cases, the Bonferroni *post-hoc* test reported no significant differences between relevant comparisons.

The density of microglia around the grafted area was similar when cells were encapsulated in a collagen hydrogel (**Figure** 6.15**b**; Hydrogel, $F_{(1,39)} = 0.645$, P>0.05), injected alongside IL-10 and GDNF (**Figure** 6.15**b**; Factors, $F_{(3,39)} = 1.456$, P>0.05), or when they were injected in an IL-10 and GDNF collagen hydrogel (**Figure** 6.15**b**; IL-10, $F_{(3,39)} = 0.090$, P>0.05). Similar results were obtained when the volume of striatal microgliosis was assessed, since no modifications were seen in this output when the VM cells were encapsulated in a collagen hydrogel (**Figure 6.14** and **Figure** 6.15**c**; Hydrogel, $F_{(1,39)} = 1.859$, P>0.05), injected alongside both factors (**Figure 6.14** and **Figure** 6.15**c**; Factors, $F_{(3,39)} = 0.229$, P>0.05), or when they were injected in an IL-10 and GDNF collagen hydrogel (**Figure 6.14**and **Figure** 6.15**c**; Hydrogel x factors, $F_{(3,39)} = 0.453$, P>0.05).

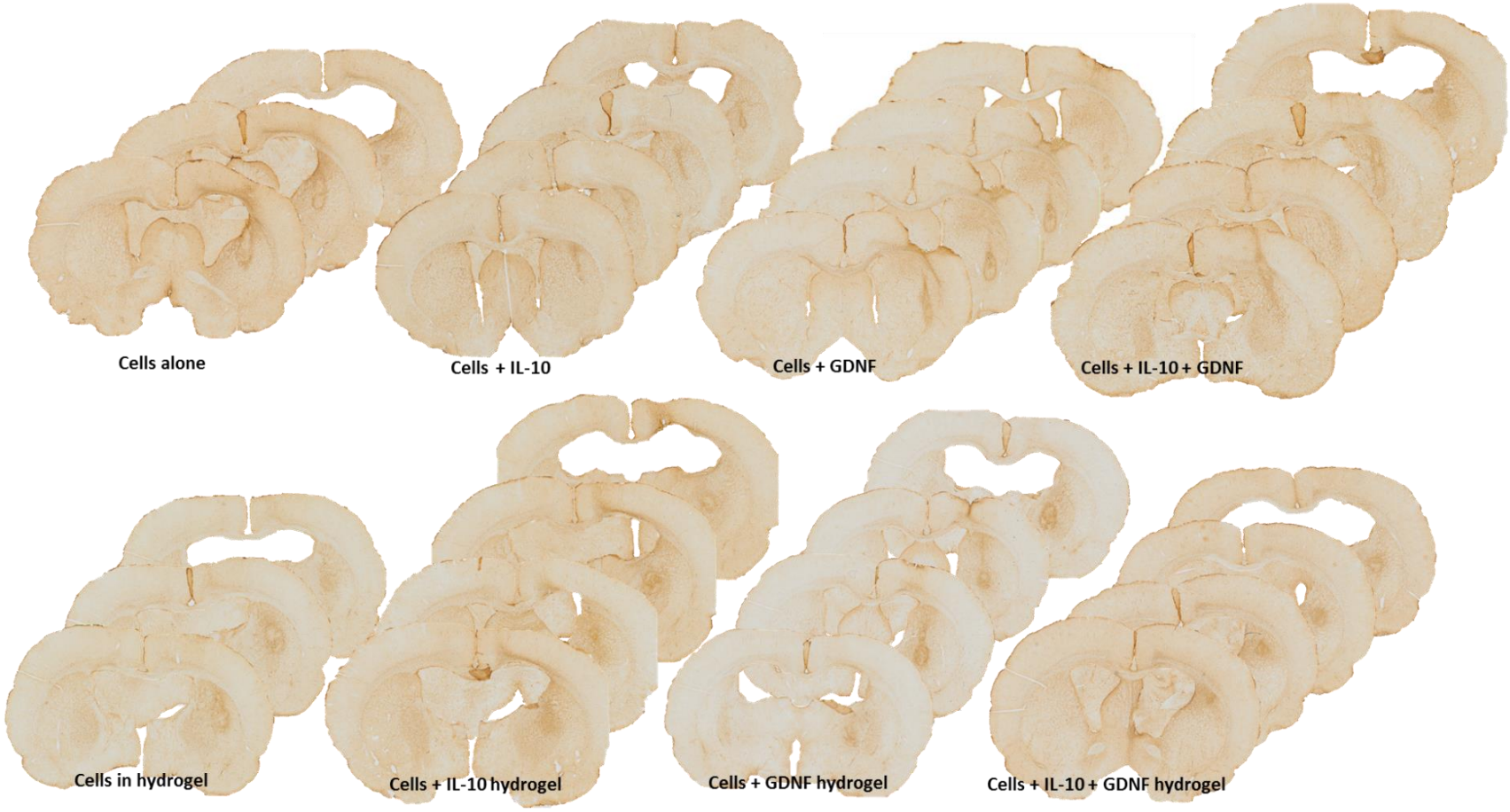

Figure 6.12 Striatal astrocytosis response to an IL-10 and GDNF collagen hydrogel at 12 weeks post-transplantation. GFAP immunostaining revealed the appearance of striatal astrocytosis around the grafted site at 12 weeks post-transplantation.

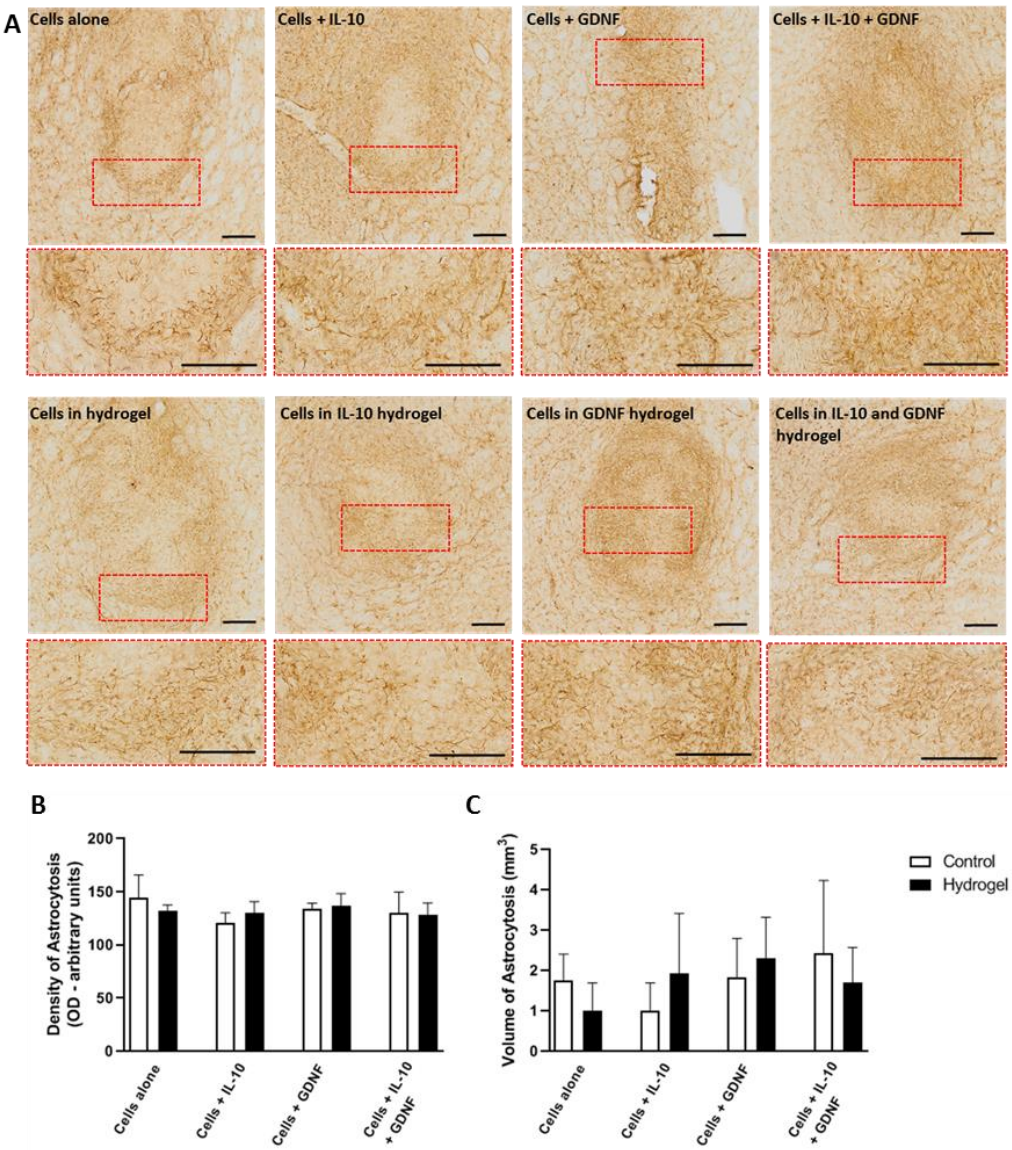

Figure 6.13 Impact of an IL-10 and GDNF collagen hydrogel on striatal astrocytosis at 12 weeks post-transplantation. A) Representative GFAP immunostained micrographs showing astrocytosis around the grafted area. B) Striatal density and C) volume of astrocytosis at 12 weeks post-transplantation. The encapsulation of VM cells in an IL-10 and GDNF collagen hydrogel did not modify the striatal astrocytosis at 12 weeks post-transplantation. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test. Scale = 200 µm.

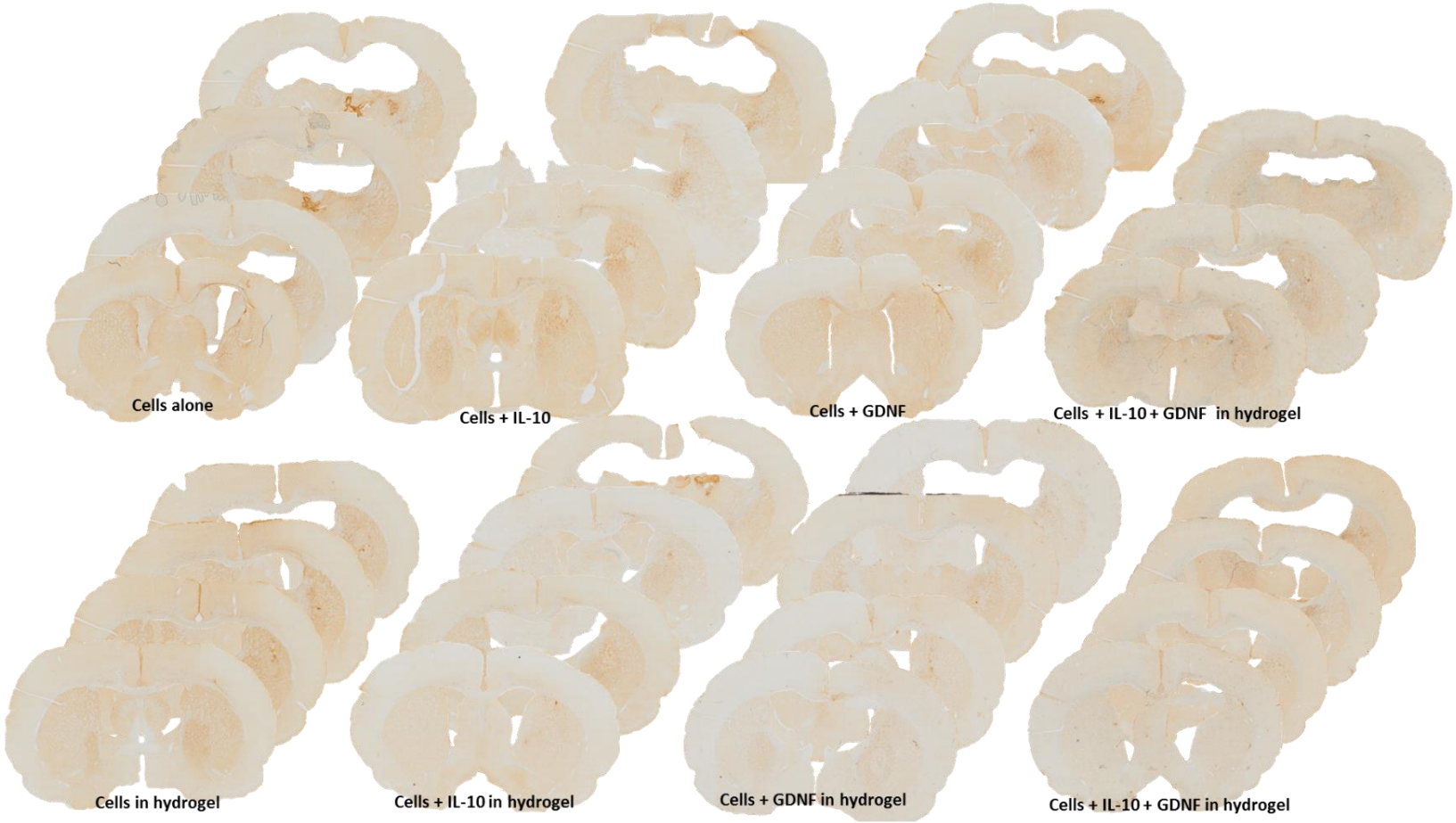

Figure 6.14 Striatal microgliosis response to an IL-10 and GDNF collagen hydrogel at 12 weeks post-transplantation. OX-42 immunostaining revealed the appearance of striatal microgliosis around the grafted site at 12 weeks post-transplantation.

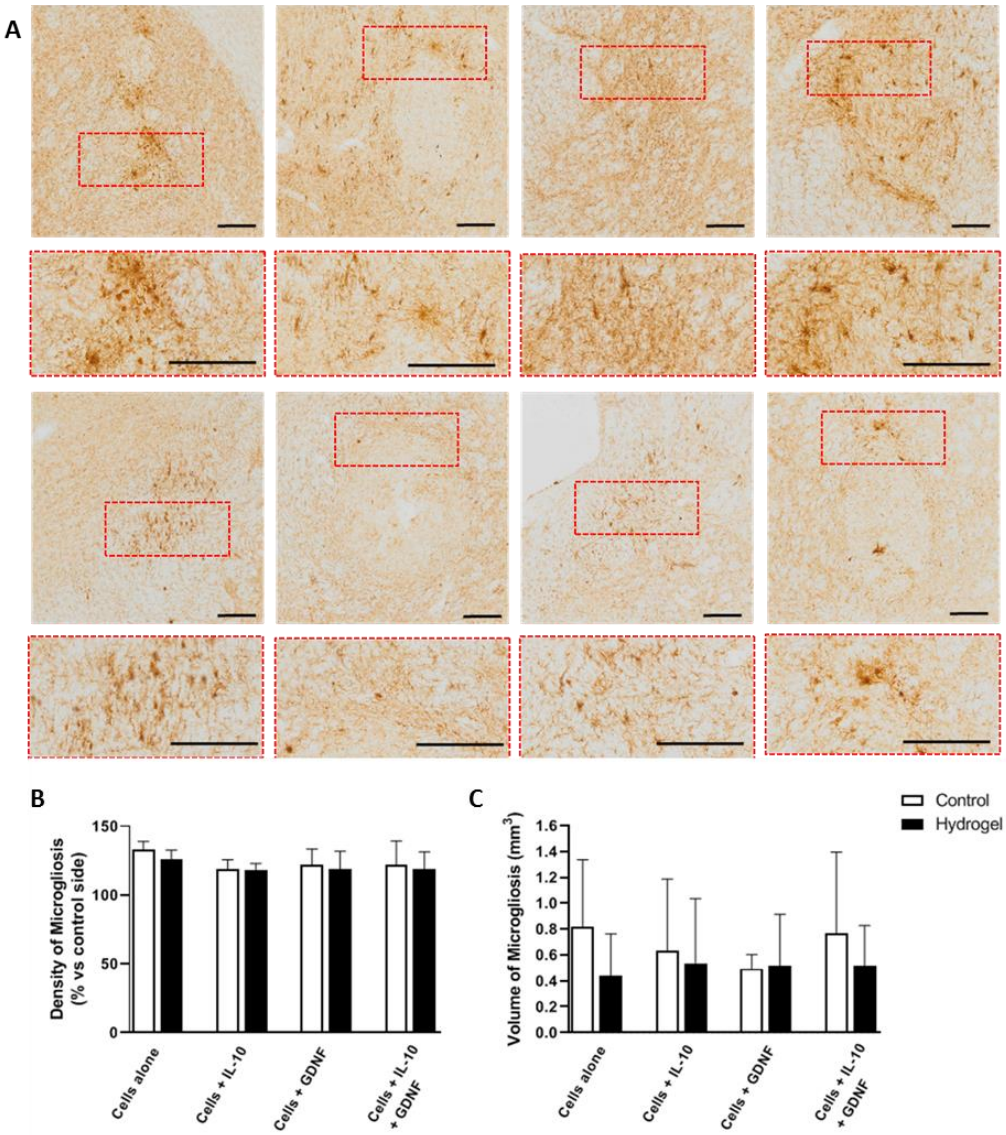

Figure 6.15 Impact of an IL-10 and GDNF collagen hydrogel on striatal microgliosis at 12 weeks post-transplantation. A) Representative OX-42 immunostained photomicrographs showing microgliosis around the grafted area. B) Striatal density and C) volume of microgliosis were calculated from OX-42 immunostained photographs at 12 weeks post-transplantation. The encapsulation of VM cells in an IL-10 and GDNF collagen hydrogel did not modify the striatal microgliosis at 12 weeks post-transplantation. Data are represented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni *post-hoc* test. Scale = 200 µm.

6.4 DISCUSSION

Primary dopaminergic VM tissue has demonstrated the potential for cell transplantation for Parkinson´s disease as another dopamine replacement therapy. Several publications over decades endorse the idea that dopaminergic neurons from the heterogeneous VM tissue can re-innervate the striatum and by doing so, they can reduce the motor impairments caused by a denervated nigrostriatal pathway both in animals and patients (Björklund et al., 1980b; Freed et al., 2001; Olanow et al., 2003). Despite clinical trials establishing the remarkable motor benefits of VM cell grafts in patients, they also highlighted the extensive cell death upon transplantation and the difficulty to obtain viable tissue. In an attempt to overcome the high amounts of cells needed, new approaches using biomaterials such as collagen hydrogels as scaffolds, are being developed. Even more interesting is the combination of these biomaterial scaffolds with neurotrophic factor therapy. Particularly relevant is the higher cell survival and extensive re-innervation obtained by Moriarty et al. when they used a GDNF loaded collagen hydrogel to encapsulate VM cells and deliver it to the striatum of hemi-parkinsonian rats (Moriarty et al., 2017, 2019a). GDNF is well known for its neuroprotective and neuro-restorative properties in midbrain dopaminergic neurons. Consequently, the simultaneous use of a biomaterial scaffold and a neuroprotective factor during cell transplantation can have a synergistic direct effect on the survival of primary dopaminergic neurons.

From this perspective, collagen hydrogels can be used to deliver any relevant biomolecules that could potentially benefit the survival of dopaminergic cells. In terms of direct neuroprotection to dopaminergic neurons, GDNF is by far the biomolecule that has shown the most potent effects. Nonetheless, the survival of dopaminergic neurons can be addressed by tackling other aspects known to be involved in the massive cell death of transplanted cells. For instance, the host immune response elicited by the transplantation process generates a recruitment of neuro-immune cells that creates an inflamed microenvironment. This hostile microenvironment is believed to challenge the survival of the transplanted cells, in particular during the first days after transplantation. Hence, the by tackling the host immune response – perhaps using anti-inflammatory biomolecules – the cell survival might be improved.

Henceforth, in this chapter, we aimed to assess if a dual loaded collagen hydrogel with both neurotrophic and anti-inflammatory factors could improve the dopaminergic cell survival and reduce the host immune response in VM cell transplants.

In the study presented here, the delivery of VM grafts into the striatum generated defined dopaminergic cell grafts that re-innervated a portion of the striatum (**Figure** 6.2). These grafts translated into an overall improvement of the functional motor recovery as stated with methamphetamine-induced rotations (**Figure** 6.4). Predictably, the encapsulation of VM cells in a collagen hydrogel did not improve the overall dopaminergic cell survival as we have not seen this effect in previous studies nor has it been reported by others (Moriarty et al., 2017). This being said, Moriarty et al., (2019) did report a positive trend in terms of primary dopaminergic cell survival when encapsulating E12 VM cells on a collagen hydrogel that translated to a significant recovery of the motor function. However, this positive effect of the collagen hydrogel was attributed to its ability to retain the released developmental factors from the meningeal tissue involved in E12 dissections (Moriarty et al., 2019a).

Not surprisingly, the co-transplantation of cells and IL-10 did not result in an improvement of cell survival even when they were encapsulated in a collagen hydrogel, as we have seen in previous experiments (Chapter 5). However, the aim of this study was to analyse the effects of IL-10 alongside GDNF. To our surprise, in this study the addition of GDNF alongside primary E14 VM cells did not enhance the survival of dopaminergic cells. Moreover, the encapsulation of primary VM cells in a GDNF-loaded collagen did not result in an improvement of cell survival. These results contrast with the numerous publications reporting outstanding improvements on dopaminergic neuron survival upon transplantation when GDNF is used before or during transplantation (Rosenblad et al., 1996; Apostolides et al., 1998). A possible explanation for this lack of neuroprotective effect of the GDNF observed in the study presented here might reside in the number of surviving neurons and the size of the dopaminergic grafts. The number of surviving neurons - and subsequently the size of the grafts in this study - was smaller than what has been reported by Moriarty et al. (2017) even though the same number of VM cells was transplanted. Thus, we hypothesise that, perhaps, the pool of dopaminergic neurons susceptible to survive was too small to benefit from the GDNF neuroprotective action.

Accordingly with the lack of effects on dopaminergic cell survival, we did not see an increase of graft size or re-innervation volume due to the addition of IL-10 or/and GDNF, the encapsulation in a collagen hydrogel or the combination of the factors in a collagen hydrogel. The methamphetamine-induced rotations also reiterated this lack of efficacy of the factors, the encapsulation in a collagen hydrogel or the combination the factors and the biomaterial since none of the experimental groups presented an improved motor behaviour in comparison to the control group (VM cells transplanted alone).

The unilateral dopamine depletion generated by the injection of 6-OHDA generates a well-described behavioural syndrome that goes beyond the most known and assessed symptom; the spontaneous and drug-induced rotational behaviour. This parkinsonian-like behavioural syndrome includes other manifestations such as contralateral sensorimotor neglect, contralateral akinesia and impaired contralateral forelimb use and they can be tested using non-pharmacological behavioural tasks (Dowd et al., 2005b).

The Corridor test of contralateral neglect was initially designed to assess 6-OHDA-induced unilateral dopamine depletion, and in the initial publication it was reported that the transplantation of primary embryonic dopamine neurons in the denervated striatum could not completely revert the ipsilateral bias in MFB-lesioned rats (Dowd et al., 2005b). As well, other E14 VM transplantation studies in MFB-lesioned rats did report a reduction of the ipsilateral bias in this task (Torres et al., 2008; Cordeiro et al., 2010). In the study reported here, we did not observe an improvement on this task due to the transplantation of primary dopaminergic neurons nor any differences due to the experimental treatments associated with them (**Figure** 6.6).

In parallel, the Stepping test of forelimb akinesia was designed as a drug-free test to monitor forelimb akinesia in unilaterally 6-OHDA lesioned rats as well as to assess the ability of dopaminergic neuron transplants to reverse this deficit (Olsson et al., 1995). Olsson et al. (1995) described a partial reversion of the 6-OHDA lesion in rats that received a VM transplant in several locations in both striatum and substantia nigra. Similarly, Torres et al. (2008) noticed an improvement on the Stepping task in E14 VM transplanted rats in comparison to 6-OHDA-lesioned rats. In line with our results from the Corridor test, we did not find any improvements on the contralateral side

performance over time in any of our experimental groups, nor any differences between the experimental groups (**Figure** 6.9).

As well, the Whisker test of sensorimotor integration measures the recovery of sensorimotor function and contrary to the Corridor and the Stepping tests it was not conceived specially and solely to assess 6-OHDA lesions but broader lesions in the CNS (Schallert et al., 2000). Consequently, there is less evidence that this task reflects the motor function recovery of dopaminergic grafts. However, similar tasks of sensorimotor integration (Schallert & Hall, 1988) have been used to test the efficiency of VM grafts on the reduction of sensorimotor integration impairments with moderate results (Mandel et al., 1990). Consistently with our other data from the non-pharmacological behavioural tests, the implantation of dopaminergic cell grafts in the striatum (and independently of the factors or the encapsulation in a collagen hydrogel) did not revert the impairment in the contralateral side of the body (**Figure** 6.11).

Taken together, in the study presented here, these sensorimotor behavioural tests – Corridor test, Stepping test and Whisker test – showed no differences between the experimental groups in regards to task performance in agreement with data extracted from the drug-induced methamphetamine rotation test.

Interestingly though, is the fact that methamphetamine-induced rotations did report an improvement on motor function due to the transplantation of primary VM cells (and independently of the factors or the encapsulation in a collagen hydrogel). Yet, the Corridor test, the Stepping test and the Whisker test did not manifest this improvement of the motor function due to the transplantation of primary dopaminergic neurons. As mentioned previously, although it is well documented that rotational behaviour can be fully reverted by the dopaminergic grafts and that this complete reversion is not seen with the other behavioural tasks, we did not see an improvement of the non-pharmacological tests due to the dopaminergic grafts. A plausible interpretation could be that the limited graft size and re-innervation volume seen in our study was insufficient to have a positive effect in these less sensitive behavioural tasks.

The transplantation of exogenous cells to the brain elicits a host response manifested by the recruitment and activation of astrocytes and microglia (Barker et al., 1996). However, the encapsulation of cells in a collagen hydrogel provides a physical barrier between the transplanted cells and the host immune cells that as demonstrated by

Moriarty et al. (2017) translated into an attenuated astrocytosis and microgliosis around the graft. In Chapter 4 we demonstrated that collagen hydrogels are immune-neutral upon intra-striatal implantation. However in further experiments, the sole encapsulation of cells in collagen hydrogels did not reduce the host immune response either at early timepoints after transplantation nor at 4 and 12 weeks post-transplantation.

As mentioned before, the transplantation of exogenous cells is associated with a host immune response that hampers the survival of the transplanted cells. In this study, we combined a known neuroprotective agent with an anti-inflammatory cytokine to try to reduce the host immune response associated with the transplantation process. Not surprisingly at this stage, the addition of IL-10 failed to ameliorate the host immune response at 12 weeks post-transplantation, as seen before in Chapter 5. Also in line with the results exposed in Chapter 5, the encapsulation of VM cells in an IL-10 loaded collagen hydrogel also did not modify the host immune response at late stages after transplantation. The results presented here contrast with the amelioration of microgliosis observed at four weeks post-transplantation when cells were delivered in an IL-10 loaded collagen (Chapter 4).

In parallel, GDNF acts relatively specifically on dopaminergic neurons as a neuroprotective factor. Thus it is no surprise that the addition of this neurotrophic factor did not alter the host immune response in this study. Moriarty et al. (2017) did report a reduction of both astrocytosis and microgliosis in the area surrounding VM grafts that were encapsulated in a GDNF collagen hydrogel but these effects were attributed to the physical barrier created by the collagen hydrogel and not the neurotrophic factor itself. The encapsulation of VM cells in an IL-10 and GDNF collagen hydrogel did not reduce the striatal astrocytosis or microgliosis at 12 weeks post-transplantation.

In conclusion, the use of a multi-modal collagen hydrogel with neuroprotective and anti-inflammatory factors did not have a synergistic effect in this study and the triple approach used here did not benefit the survival of dopaminergic neurons and did not ameliorate ameliorated the host immune response in the surrounding transplantation area.

It is unknown to us the reasons of the inefficacy of GDNF in this study since the effects of GDNF are well studied in midbrain dopaminergic neurons as well as its positive effects on the transplantation of these cells. After all, we hypothesise that the inefficacy of IL-10 can be interpreted in many ways. As discussed in Chapter 5, perhaps the IL-10 concentration used in this study – although it translated into a reduction of the striatal microgliosis at 4 weeks post-transplantation – was not high enough to provide substantial effects on the astrocytosis or microgliosis at a later stage. Even though dosage might be the most obvious explanation, other aspects have to be considered like factor retention in the striatum. We have shown in Chapter 4 that IL-10 is retained in the striatum for 24h but we have also observed that the host immune response keeps increasing after that timepoint. Hence, perhaps if the IL-10 was retained in the striatum for longer, it would have a greater impact on the host immune response.

Although the encapsulation of primary VM cells in a multi-modal collagen hydrogel loaded with a neuroprotective factor and an anti-inflammatory cytokine did not achieve the expected results in the study presented here, certain modifications in the concentration of the factors or the collagen hydrogel's capacity to retain those factors for longer periods of time could potentially ameliorate the host immune response and increase the survival of dopaminergic cells.

CHAPTER 7: GENERAL DISCUSSION

The work presented in this thesis sought to evaluate the potential benefits of an injectable anti-inflammatory loaded collagen hydrogel on cell transplantation strategies for Parkinson's disease. In particular, we wanted to assess if the encapsulation of dopaminergic neurons in an anti-inflammatory collagen scaffold could ameliorate the host innate immune response elicited by the transplantation process, and if this reduction of the host immune response could ultimately benefit the survival and integration of the grafted dopaminergic neurons. Since biomaterial scaffolds can be designed to target many aspects of the transplantation process we also sought to evaluate the efficacy of a multi-modal collagen scaffold loaded with both neuroprotective and anti-inflammatory factors on improving the dopaminergic cell survival and re-innervation ability as well as on reducing the host immune response.

The main findings from this body of work are: 1) collagen hydrogels successfully polymerise *in situ* in the brain and they are well tolerated both in cultured cells and in the striatum, 2) collagen hydrogels can retain and release IL-10 both *in vitro* and *in vivo*, 3) primary dopaminergic cells can survive encapsulated in collagen hydrogels, 4) encapsulation of primary dopaminergic cells in an IL-10 loaded collagen hydrogel can reduce the microgliosis around the grafted area at 4 weeks post-transplantation. However, 5) the encapsulation and delivery of primary dopaminergic cells in an IL-10 loaded collagen hydrogel did not ameliorate the host immune response or improve the survival of grafted cells at long-term, and 6) the encapsulation and delivery of primary dopaminergic cells in an IL-10 and GDNF loaded collagen hydrogel did not ameliorate the host immune response or enhance the survival of dopaminergic grafts.

As the worldwide life expectancy increases, the population living with Parkinson's disease is estimated to reach more than 9 million by 2030 (Dorsey et al., 2007). To date, the available pharmacological treatments for Parkinson's disease only ease the symptoms of the disease, and although they are very effective at providing symptomatic relief at early stages of the disease, they come with significant adverse effects. Moreover, these dopaminergic medications do not modify the course of the disease. Although developing strategies that can stop or reduce the progression of the disease is a daunting task, there is an unmet clinical need to develop new therapies that

target the underlying pathology of the disease. The lack of new pharmacological treatments for Parkinson's disease in recent decades and the advances in regenerative medicine have opened a new emerging therapeutic range beyond pharmacotherapies. Regenerative medicine is a relatively new translational discipline that aims to develop novel therapeutics to repair and regenerate the brain by using tissue engineering and cell-based therapies.

Cell replacement therapies in Parkinson's disease are a neuro-restorative approach that have the potential to deliver dopamine in a more physiologically relevant manner. Many decades of neural grafting studies have proven that cell replacement therapies could be a suitable alternative therapy for some Parkinson's disease patients. However, it has to be pointed out that these dopamine cell-based transplants will only treat the disabling motor aspects of the disease (Stoker & Barker, 2016),

Investigations on cell-based dopamine replacement strategies began over forty years ago, when the first pioneering studies reported that dopaminergic rich tissue from embryonic origin could be grafted in the rat brain and these could survive and function in the host brain (Björklund et al., 1980a; Dunnett et al., 1981; Gage et al., 1983; Lindvall & Björklund, 1989). Further preclinical studies reported that these dopaminergic transplants were responsible for the behavioural recovery seen in animals (Björklund et al., 1980b; Dunnett et al., 1981; Brundin et al., 1986). All this preclinical evidence led to the first small clinical trials soon after, where long-term graft survival and significant benefits in motor function were reported (Lindvall et al., 1990; Freed et al., 1992; Wenning et al., 1997; Brundin et al., 2000b). These promising results were challenged when two larger double-blind clinical trials failed to report benefits from transplantation and also they reported the appearance of GIDs in many patients (Freed et al., 2001; Olanow et al., 2003). A re-evaluation of the previous clinical data was conducted and this led to the identification of many technical issues between the designs of these clinical trials that were thought to have compromised the integrity and survival of the grafts (Barker et al., 2013). Based on that analysis, a new open-label clinical trial was established – the TRANSEURO trial - in 2010, where all those issues were addressed to ultimately demonstrate the efficacy and safety of fetal neural grafting for Parkinson's disease (Barker & TRANSEURO consortium, 2019). This on-going clinical trial has highlighted the issues around the scarcity of fetal-

derived tissue as this problem made impossible to transplant the initially planned number of patients. Therefore, the ethical and logistical issues surrounding the availability and usage of fetal tissue highlighted the imperative need to find a better source of dopaminergic cells. Stem cells – both embryonic and induced pluripotent – could solve the availability aspect but many other issues arise with their usage as dopaminergic progenitors.

On another note, the poor survival of the dopaminergic grafts upon transplantation (5-20%) (Brundin et al., 2000a) has also hampered the development of such cell replacement therapies. Thus, it is necessary to further investigate possible enhancements of dopaminergic cell survival to ensure the full potential of dopaminergic cell-based therapies.

Biomaterials platforms - devices or platforms manufactured with materials specifically engineered for therapeutic purposes - show great potential to substantially improve restorative strategies (Orive et al., 2009; Moriarty et al., 2019b). Namely, biomaterials can be used to serve as cell scaffolds, drug or protein delivery devices, reduce the negative impact of the hostile microenvironment, provide guidance cues for regeneration and all of this in a localised and targeted manner (Tuladhar et al., 2018). Their high versatility and diversity makes it possible to design and adapt a scaffold based on its desired application.

All biomaterials should ideally be adaptable, stable and non-toxic, but when designing biomaterial-based platforms for the brain, other properties are decisive for this organ such as its biodegradability and biocompatibility (Orive et al., 2009). Scaffolds for cell transplantation and delivery to the brain can be modified to improve transplanted cell survival, differentiation and integration in the host and moreover the host tissue can be manipulated by inducing mechanical, chemical or structural changes in the biomaterial (Mitrousis et al., 2018)(**Table 7.1**).

Although the sole encapsulation of primary cells in a collagen scaffold resulted in a reduction of the host immune response in some studies (Moriarty et al., 2017), a maximised enhancement of cell survival is achieved when the cell scaffold is also loaded with neuroprotective factors such as GDNF (Moriarty et al., 2017, 2019a). We have hypothesised here that the use of an anti-inflammatory cytokine in the collagen

scaffold could reduce the host innate immune response and by doing so enhancing the dopaminergic cell survival.

Table 7.1 Desirable properties of biomaterials for cell transplantation purposes. ECM: extracellular matrix. Based on Mitrousis et al., (2018).

Biomaterial design for cell transplantation	
Property	**Approach**
Injectability	Shear-thinning materials
Rapid gelation *in vivo*	Crosslinking in materials responsive to temperature, light or pH
Growth and differentiation factor support	Chemical or physical incorporation of factors in the material
Cell deployment	Porosity Modification with peptides that mimic ECM proteins Viscoelasticity
Mechanical cues	Crosslinking in materials responsive to temperature, light or pH
Vascularisation	Release of pro-angiogenic factors Porosity Pro-angiogenic materials
Inflammatory response	Backbone selection Hydrophilicity Shape and size

In Chapters 3 and 4, we characterised and optimised several collagen hydrogels – a mix of bovine type I collagen and PEG – to be used for the encapsulation and intra-striatal delivery of dopaminergic cells and therapeutic factors in parkinsonian rodent models. Collagen hydrogels have the ability to form *in situ* gels after a pH or temperature change. Here we have shown that collagen hydrogels can be prepared in solution alongside cells and proteins and the resulting solution can be easily injected intra-cranially and polymerise *in situ* in the delivery site. Furthermore, we have

demonstrated that changes in the concentration of crosslinker allow the modification of polymerisation and degradation rates, suggesting a very adaptable material for each application. More importantly, collagen hydrogels were well tolerated in neuronal cultures and in the brain since they did not elicit an extreme host immune response upon transplantation. As well, the collagen hydrogels reported here are suitable scaffolds to encapsulate and deliver primary dopaminergic cells into the striatum, since their viability was not compromised by the hydrogel. Furthermore, collagen hydrogels can be used as drug or protein delivery systems for a local release of proteins of interest. In this work, we demonstrated that collagen hydrogels can retain and release IL-10 over time both *in vitro* and *in vivo*. More importantly, we have shown that IL-10 can be retained for longer in the striatum when it is delivered encapsulated in a collagen hydrogel (Chapter 4). Thus in the initial characterisation and optimisation, we have shown that collagen hydrogels can be tuned and adapted to be used for cell and protein encapsulation and delivery in the brain. Furthermore, when primary dopaminergic cells were encapsulated in an IL-10 collagen hydrogel, there was a reduction of the host innate microglial response around the grafted area at four weeks post-transplantation (Chapter 4).

Once the best IL-10 loaded collagen hydrogel composition was chosen for cell transplantation studies, we studied the potential benefits of delivering primary dopaminergic cells encapsulated in an IL-10 collagen hydrogel (Chapter 5). Similarly as reported by Moriarty and colleagues (2017, 2019a), in this study the encapsulation of cells in a collagen hydrogel alone did not result in an improvement of dopaminergic cell survival or re-innervation. Surprisingly, the administration of IL 10 alongside with the primary dopaminergic cells (alone or encapsulated in a collagen hydrogel) did not ameliorate the host innate immune response around the grafted area at 12 weeks post-transplantation as it did at earlier timepoints. Not surprisingly then, the combined delivery of primary dopaminergic cells in an IL-10 collagen hydrogel did not modify the survival of the grafted cells nor the host innate inflammation around the grafted site.

Since IL-10 in a collagen hydrogel did not result in significant improvements on cell survival nor reduction of the host immune response, we hypothesised that its combination with the potent neuroprotective factor, GDNF, could have a synergistic

effect on those outcomes. Thus, in Chapter 6, we assessed the effects of encapsulating primary dopaminergic cells in an IL-10 and GDNF rich collagen hydrogel on cell survival and re-innervation as well as its effects on the host immune response. Against our expectations, this combination of an anti-inflammatory factor, a neuroprotective factor and a biomaterial scaffold did not benefit the survival of dopaminergic cells nor significantly reduced the host innate immune response. However the neuroprotective effects of GDNF on dopaminergic neuron undergoing transplantation are well established (Apostolides et al., 1998; Espejo et al., 2000; Mendez et al., 2000, 2002; Moriarty et al., 2017, 2019a). Therefore, the lack of GDNF effects in our study might be due to the size of the dopaminergic grafts; smaller than those seen before.

Overall, the data shown in this thesis summarises that the delivery of IL-10 with primary dopaminergic cells encapsulated in a collagen hydrogel did not result in an improvement of cell survival or in a reduction of the host innate immune response. However, previous *in vitro* and *in vivo* studies have reported IL-10 to be able to reduce microglial activation and protect neurons upon a pro-inflammatory and neurotoxic challenge (Qian et al., 2006a, 2006b; Arimoto et al., 2007; Zhu et al., 2015). Nevertheless, the molecular changes of the host tissue were not analysed and consequently, we cannot rule out that IL-10 might have had some effects on the targeted cells, although insufficient to reduce the overall immune response.

In an attempt to explain this lack of efficacy, we hypothesise that maybe IL-10 needs to be retained for longer in the striatum in order to have a significant effect. For instance, Arimoto et al., (2007) reported neuroprotection of dopaminergic neurons when IL-10 was continuously delivered for two weeks in an LPS neuroinflammation model, but this effect was not observed with a single acute injection of IL-10. A prolonged release of trophic factors and proteins can be easily achieved with many biomaterial-based drug delivery systems (Mohtaram et al., 2013). Hence, assessing the effects of a longer release and retention of IL-10 in the grafted area may shed some light into our proposal outlined here.

Future Directions

The scarce availability, and the ethical and logistical concerns, have prevented the use of fetal mesencephalic tissue for cell replacement therapies in Parkinson's disease from ever becoming a routine practice in the clinic. However, their study as a potential dopaminergic source for cell transplantation over decades has provided extremely valuable knowledge not only about transplantation and survival of dopaminergic cells but also about the underlying biology of the development of midbrain dopaminergic neurons. Moreover, their use in many clinical trials – with around a hundred patients transplanted with fetal mesencephalic cells - have provided robust evidence about the long-term survival and integration of dopaminergic cell grafts as well as their ability to restore the dopamine levels in the nigrostriatal pathway, thus bringing motor functional recovery. Today, the field of cell transplantation is fully focused on generating substantial amounts of safe and true dopaminergic progenitors from stem cells to be trialled in clinical studies.

Stem cells are undeniably the most promising dopaminergic cell source to date, as both hESCs and hiPSCs have demonstrated that they can generate dopaminergic progenitors that survive and integrate in the host striatum (Wernig et al., 2008; Sundberg et al., 2013; Doi et al., 2014; Grealish et al., 2014; Hallett et al., 2015; Kikuchi et al., 2017b). hiPSCs have many advantages over hESCs since they avoid many of the ethical and political problems associated embryonic tissue and moreover have the potential to allow for patient-specific therapies with minimal immunosuppression (Kirkeby et al., 2017b). Nevertheless, hiPSCs come up with additional risks associated with the reprogramming process, which increases the cost and the pre-clinical assessments needed. Thus, before a clinical trial can be planned, stem-cell-derived products must be deeply assessed in terms of manufacture, safety, authenticity and efficacy (Parmar, 2018). In particular, the dopaminergic progenitors to be used in transplants need to not contain pluripotency markers nor proliferate in an uncontrolled manner *in vitro*, produce dopaminergic-like neurons that express markers of dopaminergic neurons (TH, DAT), physiologically behave as nigral dopaminergic neurons and release dopamine (Barker, 2014). Furthermore, these cells need to survive long-term upon transplantation and have similar and comparable functional benefits to those seen in hfVM grafts (Arenas et al., 2015). While all these issues are being

addressed by the experts in the field, the first-in-human iPSCs clinical trial for cell transplantation in Parkinson's disease has been launched in Japan (Takahashi, 2017).

	EUROPEAN STEM-PD[a]	NYSTEM-PD	CiRA Trial	Summit for PD Trial
Cell source	ESCs	ESCs	allogeneic iPSCs	autologous iPSCs
Cryopreserved cell product?	yes	yes	no	yes
Genetic testing of cell product	TBD	karyotype / + TBD	sequencing for certain genes	full genome sequencing
Cell delivery method	"Rehncrona" instrument previously used in fetal VM trials	MRI/Clearpoint system	purpose made needle	MRI/Clearpoint system
Dosing?	low dose, high dose	low dose, high dose	one dose	low dose, high dose
Immunosuppressive regime	yes, at least 12 months; probably CiclosporinA; Azathioprine; steroids	yes, 12 months; FK506; Basiliximab; TBD ± mycophenolate	yes, 1–2 years; FK506	none
Patient characteristics				
Age	<70 years old	40–70 years old	50–70 years old	45–70 years
Disease duration	<12 years	5–12 years	>5 years	>5 years
Significant LIDs?	no	no	no	no
L-dopa response?	>30%	>50%	>30%	>20%
Pre-transplant run-in period	>1 year	>1 year	TBD	>1 year
Follow-up period	indefinitely	at least 2 years	at least 2 years	at least 1 year
PET imaging	F-dopa; PE2i	F-dopa; PE2i; DPA 713	F-dopa; DAT-SPECT; FLT GE180	F-dopa; DAT-SPECT FLT GE180
Primary end point	adverse events	adverse events	adverse events	adverse events
Secondary clinical end points (changes in)	UPDRS motor 3 in defined "off"; PDQ39; Addenbrooke's Cognitive Examination (Revised)	UPDRS motor 3 in defined "off"; PDQ39; Montreal Cognitive Assessment	UPDRS motor 3 in defined "off"; "off" time period PDQ39; Mini Mental State Examination score	UPDRS motor 3 in defined "off"; PDQ39; Mini Mental State Examination Score
Date for planned first-in-human study	2019–2020	2018	2018	2019

[a]The outcome of NeuroStemCellRepair and TRANSEURO.

Figure 7.1 Future human clinical trials of stem cell-derived dopaminergic progenitors for Parkinson's disease. Clinical trials assessing the efficacy of hES and hiPS cells for cell transplantation in Parkinson's disease will probably co-exist in the upcoming years. The CiRA trial (also known as the Kyoto trial) is the only stem-cell related clinical trial launched for Parkinson's disease so far. Taken from Barker et al., (2017).

In parallel, many lessons have already been learned from the TRANSEURO trial, especially in clinical trial design and patient selection, but its outcomes will also give more information about the patients that respond better to dopaminergic cell transplantation. This knowledge will surely positively impact in the following stem-cell-based clinical trials to come (**Figure 7.1**).

The long-term survival of these stem-cell derived dopaminergic grafts in Parkinson's patients is still unknown. Dopaminergic progenitors utilised for transplantation are not

fully matured at the time of injection and undergo terminal maturation *in situ* in the host adult depleted striatum. Thus dopaminergic progenitors may benefit from neurotrophic factor support upon transplantation just as their fetal counterparts do. Otherwise, these cells will surely encounter a hostile inflamed microenvironment after transplantation due to the host immune response. Strategies to ameliorate the surrounding host immune response could contribute to the long-term maximised survival of grafted cells. Consequently, our hypothesis about the use of a combination strategy using biomaterials and anti-inflammatory properties could benefit the survival of the dopaminergic progenitors. Once more, biomaterials could be adapted to suit these dopaminergic progenitors' needs and hence improve the overall efficacy of cell replacement therapies.

If these cell-replacement therapies will ultimately be competitive against the current dopaminergic-based treatments or DBS for some patients in Parkinson's disease, will not be revealed soon, and it will need many more complex clinical trials in the future decades before this decision can be taken.

Concluding remarks

As cell replacement therapy for Parkinson's disease enters a new era were stem-cell based therapies are being assessed in clinical trials, it is of utmost importance to ensure dopaminergic cell survival, integration and functional recovery while guaranteeing the maximal safety for patients. Although many advances in cell therapies have been achieved, the limited survival of the transplanted cells still limits the potential of such therapies. However, biomaterial scaffolds are a promising tool that have extensively proven to help overcome these hurdles by enhancing these neuro-restorative strategies by directly protecting the transplanted cells and creating a favourable microenvironment for their survival. Nevertheless, further work remains to be conducted to identify the scalability and efficacy of these biomaterials to be tested in clinical trials.

References

Adil, M. M., Vazin, T., Ananthanarayanan, B., Rodrigues, G. M. C., Rao, A. T., Kulkarni, R. U., Miller, E. W., Kumar, S., & Schaffer, D. V. (2017). Engineered hydrogels increase the post-transplantation survival of encapsulated hESC-derived midbrain dopaminergic neurons. *Biomaterials*, *136*, 1–11.

Agrawal, S., Agrawal, A., Doughty, B., Gerwitz, A., Blenis, J., Van Dyke, T., & Pulendran, B. (2003). Cutting Edge: Different Toll-Like Receptor Agonists Instruct Dendritic Cells to Induce Distinct Th Responses via Differential Modulation of Extracellular Signal-Regulated Kinase-Mitogen-Activated Protein Kinase and c-Fos. *The Journal of Immunology*, *171*(10), 4984–4989.

Airaksinen, M. S., & Saarma, M. (2002). The GDNF family: Signalling, biological functions and therapeutic value. *Nature Reviews Neuroscience*, *3*(5), 383–394.

Alconcel, S. N. S., Baas, A. S., & Maynard, H. D. (2011). FDA-approved poly(ethylene glycol)–protein conjugate drugs. *Polymer Chemistry*, *2*(7), 1442.

Antonini, A., & Poewe, W. (2007). Fibrotic heart-valve reactions to dopamine-agonist treatment in Parkinson's disease. *The Lancet Neurology*, *6*(9), 826–829.

Apostolides, C., Sanford, E., Hong, M., & Mendez, I. (1998). Glial cell line-derived neurotrophic factor improves intrastriatal graft survival of stored dopaminergic cells. *Neuroscience*, *83*(2), 363–372.

Arenas, E., Denham, M., & Villaescusa, J. C. (2015). How to make a midbrain dopaminergic neuron. *Development (Cambridge, England)*, *142*(11), 1918–1936.

Arimoto, T., Choi, D.-Y. Y., Lu, X., Liu, M., Nguyen, X. V., Zheng, N., Stewart, C. A., Kim, H.-C. C., & Bing, G. (2007). Interleukin-10 protects against inflammation-mediated degeneration of dopaminergic neurons in substantia nigra. *Neurobiology of Aging*, *28*(6), 894–906.

Ascherio, A., Zhang, S. M., Hernán, M. A., Kawachi, I., Colditz, G. A., Speizer, F. E., & Willett, W. C. (2001). Prospective study of caffeine consumption and risk of Parkinson's disease in men and women. *Annals of Neurology*, *50*(1), 56–63.

Atkins, P., & De Paula, J. (2010). Physical Chemistry. In *Oxford University Press*.

Aurand, E. R., Wagner, J. L., Shandas, R., & Bjugstad, K. B. (2014). Hydrogel formulation determines cell fate of fetal and adult neural progenitor cells. *Stem Cell Research*, *12*, 11–23.

Backlund, E.-O., Granberg, P.-O., Hamberger, B., Knutsson, E., Mårtensson, A., Sedvall, G., Seiger, Å., & Olson, L. (1985). Transplantation of adrenal medullary tissue to striatum in parkinsonism. *Journal of Neurosurgery*, *62*(2), 169–173.

Barker, R. A. (2014). Developing Stem Cell Therapies for Parkinson's Disease: Waiting Until the Time Is Right. *Stem Cell*, *15*, 539–542.

Barker, R. A., Barrett, J., Mason, S. L., & Björklund, A. (2013). Fetal dopaminergic transplantation trials and the future of neural grafting in Parkinson's disease. *The Lancet Neurology*, *12*(1), 84–91.

Barker, R. A., Drouin-Ouellet, J., & Parmar, M. (2015). Cell-based therapies for Parkinson disease-past insights and future potential. *Nature Reviews Neurology*, *11*(9), 492–503.

Barker, R. A., Dunnett, S. B., Faissner, A., & Fawcett, J. W. (1996). The Time Course of Loss of Dopaminergic Neurons and the Gliotic Reaction Surrounding Grafts of Embryonic Mesencephalon to the Striatum. *Experimental Neurology*, *141*(1), 79–93.

Barker, R. A., Parmar, M., Studer, L., & Takahashi, J. (2017). Human Trials of Stem Cell-Derived Dopamine Neurons for Parkinson's Disease: Dawn of a New Era. *Cell Stem Cell*, *21*(5), 569–573.

Barker, R. A., & TRANSEURO consortium. (2019). Designing stem-cell-based dopamine cell replacement trials for Parkinson's disease. *Nature Medicine*, *25*(7), 1045–1053.

Barker, R. A., & Widner, H. (2004). Immune Problems in Central Nervous System Cell Therapy. *NeuroRx*, *1*(4), 472–481.

Beck, K. D., Valverde, J., Alexi, T., Poulsen, K., Moffat, B., Vandlen, R. A., Rosenthal, A., & Hefti, F. (1995). Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. *Nature*, *373*(6512), 339–341.

Béduer, A., Braschler, T., Peric, O., Fantner, G. E., Mosser, S., Fraering, P. C., Benchérif, S., Mooney, D. J., & Renaud, P. (2015). A compressible scaffold for minimally invasive delivery of large intact neuronal networks. *Advanced Healthcare Materials*, *4*(2), 301–312.

Berthiaume, F., Maguire, T. J., & Yarmush, M. L. (2011). Tissue Engineering and Regenerative Medicine: History, Progress, and Challenges. *Annual Review of Chemical and Biomolecular Engineering*, *2*(1), 403–430.

Bialecka, M, Klodowska-Duda, G., Kurzawski, M., Slawek, J., Gorzkowska, A., Opala, G., Bialecki, P., Sagan, L., & Droździk, M. (2008). Interleukin-10 (IL-10) and tumor necrosis factor alpha (TNF) gene polymorphisms in Parkinson's disease patients. *Parkinsonism & Related Disorders*, *14*(8), 636–640.

Bialecka, M, Klodowska-Duda, G., Kurzawski, M., Slawek, J., Opala, G., Bialecki, P., Safranow, K., & Droździk, M. (2007). Interleukin-10 Gene Polymorphism in Parkinson's Disease Patients. *Archives of Medical Research*, *38*(8), 858–863.

Biedler, J. L., Roffler-Tarlov, S., Schachner, M., & Freedman, L. S. (1978). Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. *Cancer Research*, *38*(11 Pt 1), 3751–3757.

Bilang-Bleuel, A., Revah, F., Colin, P., Locquet, I., Robert, J. J., Mallet, J., & Horellou, P. (1997). Intrastriatal injection of an adenoviral vector expressing glial-cell-line-derived neurotrophic factor prevents dopaminergic neuron degeneration and behavioral impairment in a rat model of Parkinson disease. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(16), 8818–8823.

Bing, G., Notter, M. F. D., Hansen, J. T., & Gash, D. M. (1988). Comparison of adrenal medullary, carotid body and PC12 cell grafts in 6-OHDA lesioned rats. *Brain Research Bulletin*, *20*(3), 399–406.

Björklund, A., Dunnett, S. B., Stenevi, U., Lewis, M. E., & Iversen, S. D. (1980a). Reinnervation of the denervated striatum by substantia nigra transplants: Functional consequences as revealed by pharmacological and sensorimotor testing. *Brain Research*, *199*(2), 307–333.

Björklund, A, Rosenblad, C., Winkler, C., & Kirik, D. (1997). Studies on neuroprotective and regenerative effects of GDNF in a partial lesion model of Parkinson's disease. *Neurobiology of Disease*, *4*(3–4), 186–200.

Björklund, A, Stenevi, U., Dunnett, S. B., & Iversen, S. D. (1981). Functional reactivation of the deafferented neostriatum by nigral transplants. *Nature*, *289*(5797), 497–499.

Björklund, A, Schmidt, R. H., & Stenevi, U. (1980b). Functional reinnervation of the neostriatum in the adult rat by use of intraparenchymal grafting of dissociated cell suspensions from the substantia nigra. *Cell and Tissue Research*, *212*(1), 39–45.

Björklund, A, & Stenevi, U. (1979). Reconstruction of the nigrostriatal dopamine pathway by intracerebral nigral transplants. *Brain Research*, *177*(3), 555–560.

Block, M. L., Zecca, L., & Hong, J.-S. (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nature Reviews Neuroscience*, *8*(1), 57–69.

Blum, M., & Weickert, C. S. (1995). GDNF mRNA expression in normal postnatal development, aging, and in weaver mutant mice. *Neurobiology of Aging*, *16*(6), 925–929.

Bonilla, S., Hall, A. C., Pinto, L., Attardo, A., Götz, M., Huttner, W. B., & Arenas, E. (2008). Identification of midbrain floor plate radial glia-like cells as dopaminergic progenitors. *Glia*, *56*(8), 809–820.

Bozzi, Y., & Borrelli, E. (1999). Absence of the dopamine D2 receptor leads to a decreased expression of GDNF and NT-4 mRNAs in restricted brain areas. *The European Journal of Neuroscience*, *11*(4), 1275–1284.

Braak, H., & Braak, E. (2000). Pathoanatomy of Parkinson's disease. *Journal of Neurology, Supplement*, *247*(2), 3–10.

Braak, H., Del Tredici, K., Rüb, U., De Vos, R. A. I., Jansen Steur, E. N. H., & Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiology of Aging*, *24*(2), 197–211.

Brodacki, B., Staszewski, J., Toczyłowska, B., Kozłowska, E., Drela, N., Chalimoniuk, M., & Stępien, A. (2008). Serum interleukin (IL-2, IL-10, IL-6, IL-4), TNFα, and INFγ concentrations are elevated in patients with atypical and idiopathic parkinsonism. *Neuroscience Letters*, *441*(2), 158–162.

Brooks, D. J. (2000). Dopamine agonists: their role in the treatment of Parkinson's disease. *Journal of Neurology, Neurosurgery, and Psychiatry*, *68*(6), 685–689.

Brooks, D. J. (2008). Optimizing levodopa therapy for Parkinson's disease with levodopa/carbidopa/entacapone: implications from a clinical and patient perspective. *Neuropsychiatric Disease and Treatment*, *4*(1), 39–47.

Brundin, P., Nilsson, O. G., Strecker, R. E., Lindvall, O., Åstedt, B., & Björklund, A. (1986). Behavioural effects of human fetal dopamine neurons grafted in a rat model of Parkinson's disease. *Experimental Brain Research*, *65*(1), 235–240.

Brundin, P, Karlsson, J., Emgård, M., Kaminski Schierle, G. S., Hansson, O., Petersén, Å., & Castilho, R. F. (2000a). Improving the survival of grafted dopaminergic neurons: A review over current approaches. *Cell Transplantation*, *9*(2), 179–195.

Brundin, P, Pogarell, O., Hagell, P., Piccini, P., Widner, H., Schrag, A., Kupsch, A., Crabb, L., Odin, P., Gustavii, B., Björklund, A., Brooks, D. J., Marsden, C. D., Oertel, W. H., Quinn, N. P., Rehncrona, S., & Lindvall, O. (2000b). Bilateral caudate and putamen grafts of embryonic mesencephalic tissue treated with lazaroids in Parkinson's disease. *Brain : A Journal of Neurology*, *123*, 1380–1390.

Burdick, J. A., Mauck, R. L., & Gerecht, S. (2016). To Serve and Protect: Hydrogels to Improve Stem Cell-Based Therapies. *Cell Stem Cell*, *18*(1), 13–15.

Burke, R. E., Antonelli, M., & Sulzer, D. (1998). Glial cell line-derived neurotrophic growth factor inhibits apoptotic death of postnatal substantia nigra dopamine neurons in primary culture. *Journal of Neurochemistry*, *71*(2), 517–525.

Burmeister, A. R., & Marriott, I. (2018). The interleukin-10 family of cytokines and their role in the CNS. *Frontiers in Cellular Neuroscience*, *12*(November), 1–13.

Bye, C. R., Thompson, L. H., & Parish, C. L. (2012). Birth dating of midbrain dopamine neurons identifies A9 enriched tissue for transplantation into Parkinsonian mice. *Experimental Neurology*, *236*(1), 58–68.

Cai, G., Kastelein, R. A., & Hunter, C. A. (1999). IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-gamma when combined with IL-18. *European Journal of Immunology*, *29*(9), 2658–2665.

Carlsson, A., Lindqvist, M., & Magnusson, T. (1957). 3,4-Dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists. *Nature*, *180*(4596), 1200.

Carlsson, T., Carta, M., Winkler, C., Björklund, A., & Kirik, D. (2007). Serotonin neuron transplants exacerbate L-DOPA-induced dyskinesias in a rat model of Parkinson's disease. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *27*(30), 8011–8022.

Carson, W. E., Lindemann, M. J., Baiocchi, R., Linett, M., Tan, J. C., Chou, C. C., Narula, S., & Caligiuri, M. A. (1995). The functional characterization of interleukin-10 receptor expression on human natural killer cells. *Blood*, *85*(12), 3577–3585.

Chai, Q., Jiao, Y., & Yu, X. (2017). Hydrogels for Biomedical Applications: Their Characteristics and the Mechanisms behind Them. *Gels (Basel, Switzerland)*, *3*(1).

Chajra, H., Rousseau, C. F., Cortial, D., Ronzière, M. C., Herbage, D., Mallein-Gerin, F., & Freyria, A. M. (2008). Collagen-based biomaterials and cartilage engineering. Application to osteochondral defects. *Bio-Medical Materials and Engineering*, *18*(1 Suppl), S33-45.

Chattopadhyay, S., & Raines, R. T. (2014). Review collagen-based biomaterials for wound healing. *Biopolymers*, *101*(8), 821–833.

Chaturvedi, R., Agrawal, A., Seth, K., Shukla, S., Chauhan, S., Shukla, Y., Sinha, C., & Seth, P. (2003). Effect of glial cell line-derived neurotrophic factor (GDNF) co-transplantation with fetal ventral mesencephalic cells (VMC) on functional restoration in 6-hydroxydopamine (6-OHDA) lesioned rat model of Parkinson's disease: neurobehavioral, neurochemical. *International Journal of Developmental Neuroscience*, *21*(7), 391–400.

Chien, H.-W., Tsai, W.-B., & Jiang, S. (2012). Direct cell encapsulation in biodegradable and functionalizable carboxybetaine hydrogels. *Biomaterials*, *33*(23), 5706–5712.

Cho, C., Alterman, R., Miravite, J., Shils, J. & Tagliati, M. (2005). Subthalamic DBS for the treatment of "runaway" dyskinesias after embryonic or fetal tissue transplant. *Mov. Disord.*, *20*, 1237.

Choi-Lundberg, D. L., & Bohn, M. C. (1995). Ontogeny and distribution of glial cell line-derived neurotrophic factor (GDNF) mRNA in rat. *Developmental Brain Research*, *85*(1), 80–88.

Choi-Lundberg, D. L., Lin, Q., Schallert, T., Crippens, D., Davidson, B. L., Chang, Y. N., Chiang, Y. L., Qian, J., Bardwaj, L., & Bohn, M. C. (1998). Behavioral and cellular protection of rat dopaminergic neurons by an adenoviral vector encoding glial cell line-derived neurotrophic factor. *Experimental Neurology*, *154*(2), 261–275.

Chu, Y., & Kordower, J. H. (2010). Lewy body pathology in fetal grafts. *Annals of the New York Academy of Sciences*, *1184*(1), 55–67.

Clarkson, A. N., Parker, K., Nilsson, M., Walker, F. R., & Gowing, E. K. (2015). Combined ampakine and BDNF treatments enhance poststroke functional recovery in aged mice via AKT-CREB signaling. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *35*(8), 1272–1279.

Clavreul, A., Sindji, L., Aubert-Pouëssel, A., Benoît, J.-P., Menei, P., & Montero-Menei, C. N. (2006). Effect of GDNF-releasing biodegradable microspheres on the function and the survival of intrastriatal fetal ventral mesencephalic cell grafts. *European Journal of Pharmaceutics and Biopharmaceutics*, *63*(2), 221–228.

Collier, T. J., & Sortwell, C. E. (1999). Therapeutic Potential of Nerve Growth Factors in Parkinson??s Disease. *Drugs & Aging*, *14*(4), 261–287.

Collin, E. C., Grad, S., Zeugolis, D. I., Vinatier, C. S., Clouet, J. R., Guicheux, J. J., Weiss, P., Alini, M., & Pandit, A. S. (2011). An injectable vehicle for nucleus pulposus cell-based therapy. *Biomaterials*, *32*(11), 2862–2870.

Commins S, Steinke JW, B. L. (2008). The extended IL-10 superfamily: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, and IL-29. *J Allergy Clin Immunol*, *121*, 1108–1111.

Cook, D. J., Nguyen, C., Chun, H. N., L Llorente, I., Chiu, A. S., Machnicki, M., Zarembinski, T. I., & Carmichael, S. T. (2017). Hydrogel-delivered brain-derived neurotrophic factor promotes tissue repair and recovery after stroke. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *37*(3), 1030–1045.

Cordeiro, K. K., Jiang, W., Papazoglou, A., Tenório, S. B., Döbrössy, M., & Nikkhah, G. (2010). Graft-mediated functional recovery on a skilled forelimb use paradigm in a rodent model of Parkinson's disease is dependent on reward contingency. *Behavioural Brain Research*, *212*(2), 187–195.

Couper, K. N., Blount, D. G., & Riley, E. M. (2008). IL-10: the master regulator of immunity to infection. *Journal of Immunology (Baltimore, Md. : 1950)*, *180*(9), 5771–5777.

Damier, P., Hirsch, E. C., Agid, Y., & Graybiel, A. M. (1999a). The substantia nigra of the human brain I. Nigrosomes and the nigral matrix, a compartmental organization based on calbindin D28K immunohistochemistry. *Brain*, *122*(8), 1421–1436.

Damier, P., Hirsch, E. C., Agid, Y., & Graybiel, A. M. (1999b). The substantia nigra of the human brain II. Patterns of loss of dopamine-containing neurons in Parkinson's disease. *Brain*, *122*(8), 1437–1448.

Damier, P., Hirsch, E. C., Zhang, P., Agid, Y., & Javoy-Agid, F. (1993). Glutathione peroxidase, glial cells and Parkinson's disease. *Neuroscience*, *52*(1), 1–6.

De Lau, L. M., & Breteler, M. M. (2006). Epidemiology of Parkinson's disease. *The Lancet Neurology*, *5*(6), 525–535.

Decressac, M., Ulusoy, A., Mattsson, B., Georgievska, B., Romero-Ramos, M., Kirik, D., & Björklund, A. (2011). GDNF fails to exert neuroprotection in a rat α-synuclein model of Parkinson's disease. *Brain*, *134*(8), 2302–2311.

DeLong, M. R. (1990). Primate models of movement disorders of basal ganglia origin. *Trends in Neurosciences*, *13*(7), 281–285.

DeMaagd, G., & Philip, A. (2015). Parkinson's Disease and Its Management: Part 3: Nondopaminergic and Nonpharmacological Treatment Options. *P & T : A Peer-Reviewed Journal for Formulary Management*, *40*(10), 668–679.

Deng, H., Wang, P., & Jankovic, J. (2018). The genetics of Parkinson disease. *Ageing Research Reviews*, *42*, 72–85.

Deng, X., Liang, Y., Lu, H., Yang, Z., Liu, R., Wang, J., Song, X., Long, J., Li, Y., Lei, D., & Feng, Z. (2013). Co-transplantation of GDNF-overexpressing neural stem cells and fetal dopaminergic neurons mitigates motor symptoms in a rat model of Parkinson's disease. *PloS One*, *8*(12), e80880.

DiSabato, D. J., Quan, N., & Godbout, J. P. (2016). Neuroinflammation: the devil is in the details. *Journal of Neurochemistry*, *139 Suppl 2*(Suppl 2), 136–153.

Doi, D., Samata, B., Katsukawa, M., Kikuchi, T., Morizane, A., Ono, Y., Sekiguchi, K., Nakagawa, M., Parmar, M., & Takahashi, J. (2014). Isolation of Human Induced Pluripotent Stem Cell-Derived Dopaminergic Progenitors by Cell Sorting for Successful Transplantation. *Stem Cell Reports*, *2*(3), 337–350.

Dorsey, E. R., Constantinescu, R., Thompson, J. P., Biglan, K. M., Holloway, R. G., Kieburtz, K., Marshall, F. J., Ravina, B. M., Schifitto, G., Siderowf, A., & Tanner, C. M. (2007). Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. *Neurology*, *68*(5), 384–386.

Dowd, E., Monville, C., Torres, E. M., & Dunnett, S. B. (2005a). The Corridor Task: A simple test of lateralised response selection sensitive to unilateral dopamine deafferentation and graft-derived dopamine replacement in the striatum. *Brain Research Bulletin*, *68*(1–2), 24–30.

Dowd, E., Monville, C., Torres, E. M., Wong, L.-F., Azzouz, M., Mazarakis, N. D., & Dunnett, S. B. (2005b). Lentivector-mediated delivery of GDNF protects complex motor functions relevant to human Parkinsonism in a rat lesion model. *European Journal of Neuroscience*, *22*(10), 2587–2595.

Duan, W.-M., Cameron, R. M., Brundin, P., & Widner, H. (1997). Rat Intrastriatal Neural Allografts Challenged with Skin Allografts at Different Time Points. *Experimental Neurology*, *148*(1), 334–347.

Duan, W.-M., Widner, H., Björklund, A., & Brundin, P. (1993). Sequential intrastriatal grafting of allogeneic embryonic dopamine-rich neuronal tissue in adult rats: Will the second graft be rejected? *Neuroscience*, *57*(2), 261–274.

Duan, W.-M., Widner, H., & Brundin, P. (1995). Temporal pattern of host responses against intrastriatal grafts of syngeneic, allogeneic or xenogeneic embryonic neuronal tissue in rats. *Experimental Brain Research*, *104*(2), 227–242.

Dunnett, S. B., Björklund, A., Stenevi, U., & Iversen, S. D. (1981). Behavioural recovery following transplantation of substantia nigra in rats subjected to 6-OHDA lesions of the nigrostriatal pathway. II. Bilateral lesions. *Brain Research*, *229*(2), 457–470.

Egawa, E. Y., Kato, K., Hiraoka, M., Nakaji-Hirabayashi, T., & Iwata, H. (2011). *Enhanced proliferation of neural stem cells in a collagen hydrogel incorporating engineered epidermal growth factor*.

Espejo, M., Cutillas, B., Arenas, T. E., & Ambrosio, S. (2000). Increased survival of dopaminergic neurons in striatal grafts of fetal ventral mesencephalic cells exposed to neurotrophin-3 or glial cell line-derived neurotrophic factor. *Cell Transplantation*, *9*(1), 45–53.

Fahn, S. (2008). The history of dopamine and levodopa in the treatment of Parkinson's disease. *Movement Disorders*, *23*(S3), S497–S508.

Fahn, S., & The Parkinson Study Group. (2005). Does levodopa slow or hasten the rate of progression of Parkinson's disease? *Journal of Neurology*, *252*(S4), iv37–iv42.

Fasano, C. A., Chambers, S. M., Lee, G., Tomishima, M. J., & Studer, L. (2010). Efficient Derivation of Functional Floor Plate Tissue from Human Embryonic Stem Cells. *Cell Stem Cell*, *6*(4), 336–347.

Fearnley, J. M., & Lees, A. J. (1991). Ageing and parkinson's disease: Substantia nigra regional selectivity. *Brain*, *114*(5), 2283–2301.

Fenn, A. M., Henry, C. J., Huang, Y., Dugan, A., & Godbout, J. P. (2012). Lipopolysaccharide-induced interleukin (IL)-4 receptor-α expression and corresponding sensitivity to the M2 promoting effects of IL-4 are impaired in microglia of aged mice. *Brain, Behavior, and Immunity*, *26*(5), 766–777.

Fernandes, A., Miller-Fleming, L., & Pais, T. F. (2014). Microglia and inflammation: conspiracy, controversy or control? *Cellular and Molecular Life Sciences : CMLS*, *71*(20), 3969–3985.

Finbloom, D. S., & Winestock, K. D. (1995). IL-10 induces the tyrosine phosphorylation of tyk2 and Jak1 and the differential assembly of STAT1 alpha and STAT3 complexes in human T cells and monocytes. *Journal of Immunology (Baltimore, Md. : 1950)*, *155*(3), 1079–1090.

Fiorentino D. F., Bond M. W. (1989). Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med.*, *170*(6), 2081–95.

Fon, D., Al-Abboodi, A., Chan, P. Y., Zhou, K., Crack, P., Finkelstein, D. I., & Forsythe, J. S. (2014). Effects of GDNF-loaded injectable gelatin-based hydrogels on endogenous neural progenitor cell migration. *Advanced Healthcare Materials*, *3*(5), 761–774.

Freed, C. R., Breeze, R. E., Rosenberg, N. L., Schneck, S. A., Kriek, E., Qi, J. X., Lone, T., Zhang, Y. B., Snyder, J. A., Wells, T. H., Ramig, L. O., Thompson, L., Mazziotta, J. C., Huang, S. C., Grafton, S. T., Brooks, D., Sawle, G., Schroter G., Ansari, A. A. (1992). Survival of implanted fetal dopamine cells and neurologic improvement 12 to 46 months after transplantation for Parkinson's disease. *The New England Journal of Medicine*, *327*(22), 1549–1555.

Freed, C. R., Greene, P. E., Breeze, R. E., Tsai, W. Y., DuMouchel, W., Kao, R., Dillon, S., Winfield, H., Culver, S., Trojanowski, J. Q., Eidelberg, D., & Fahn, S. (2001). Transplantation of embryonic dopamine neurons for severe Parkinson's disease. *The New England Journal of Medicine*, *344*(10), 710–719.

Freed, W. J., Morihisa, J. M., Spoor, E., Hoffer, B. J., Olson, L., Seiger, A., & Wyatt, R. J. (1981). Transplanted adrenal chromaffin cells in rat brain reduce lesion-induced rotational behaviour. *Nature*, *292*, 351–352.

Freed, W. J., Perlow, M. J., Karoum, F., Seiger, A., Olson, L., Hoffer, B. J., & Wyatt, R. J. (1980). Restoration of dopaminergic function by grafting of fetal rat substantia nigra to the caudate nucleus: Long-term behavioral, biochemical, and histochemical studies. *Annals of Neurology*, *8*(5), 510–519.

Frisch, S. M., & Francis, H. (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. *Journal of Cell Biology*, *124*(4), 619–626.

Gage, F. H., Dunnett, S. B., Brundin, P., Isacson, O., & Björklund, A. (1983). Intracerebral grafting of embryonic neural cells into the adult host brain: an overview of the cell suspension method and its application. *Developmental Neuroscience*, *6*(3), 137–151.

Garcia-Esparcia, P., Llorens, F., Carmona, M., & Ferrer, I. (2014). Complex deregulation and expression of cytokines and mediators of the immune response in Parkinson's disease brain is region dependent. *Brain Pathology*, *24*(6), 584–598.

Gash, D. M., Zhang, Z., Ovadia, A., Cass, W. A., Yi, A., Simmerman, L., Russell, D., Martin, D., Lapchak, P. A., Collins, F., Hoffer, B. J., & Gerhard, G. A. (1996). Functional recovery in parkinsonian monkeys treated with GDNF. *Nature*, *380*(6571), 252–255.

Gefen, A., Gefen, N., Zhu, Q., Raghupathi, R., & Margulies, S. S. (2003). Age-Dependent Changes in Material Properties of the Brain and Braincase of the Rat. *Journal of Neurotrauma*, *20*(11), 1163–1177.

Gegg, M. E., Cooper, J. M., Schapira, A. H. V., & Taanman, J.-W. (2009). Silencing of PINK1 Expression Affects Mitochondrial DNA and Oxidative Phosphorylation in DOPAMINERGIC Cells. *PLoS ONE*, *4*(3), e4756.

Georgievska, B., Kirik, D., & Björklund, A. (2004). Overexpression of glial cell line-derived neurotrophic factor using a lentiviral vector induces time- and dose-dependent downregulation of tyrosine hydroxylase in the intact nigrostriatal dopamine system. *Journal of Neuroscience*, *24*(29), 6437–6445.

Gerfen, C., Engber, T., Mahan, L., Susel, Z., Chase, T., Monsma, F., & Sibley, D. (1990). D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science*, *250*(4986), 1429–1432.

Gibb, W. R., & Lees, A. J. (1988). The relevance of the Lewy body to the pathogenesis of idiopathic Parkinson's disease. *Journal of Neurology, Neurosurgery, and Psychiatry*, *51*(6), 745–752.

Gilbert, S. (2011). *Developmental Biology* (8th editio).

Gill, S. S., Patel, N. K., Hotton, G. R., O'Sullivan, K., McCarter, R., Bunnage, M., Brooks, D. J., Svendsen, C. N., & Heywood, P. (2003). Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nature Medicine*, *9*(5), 589–595.

Glazner, G. W., Mu, X., & Springer, J. E. (1998). Localization of glial cell line-derived neurotrophic factor receptor alpha and c-ret mRNA in rat central nervous system. *Journal of Comparative Neurology*, *391*(1), 42–49.

Goedert, M., Spillantini, M. G., Del Tredici, K., & Braak, H. (2013). 100 years of Lewy pathology. *Nature Reviews Neurology*, *9*(1), 13–24.

Goetz, C. G., Tilley, B. C., Shaftman, S. R., Stebbins, G. T., Fahn, S., Martinez-Martin, P., Poewe, W., Sampaio, C., Stern, M. B., Dodel, R., Dubois, B., Holloway, R., Jankovic, J., Kulisevsky, J., Lang, A. E., Lees, A., Leurgans, S., LeWitt P. A., Nyenhuis D, Olanow C. W., Rascol O, Schrag A, Teresi J. A., Van Hilten J. J., LaPelle N. (2008). Movement Disorder Society-Sponsored Revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS): Scale presentation and clinimetric testing results. *Movement Disorders*, *23*(15), 2129–2170.

Grandinetti, A., Morens, D. M., Reed, D., & MacEachern, D. (1994). Prospective study of cigarette smoking and the risk of developing idiopathic Parkinson's disease. *American Journal of Epidemiology*, *139*(12), 1129–1138.

Grealish, S., Diguet, E., Kirkeby, A., Mattsson, B., Heuer, A., Bramoulle, Y., Van Camp, N., Perrier, A. L., Hantraye, P., Björklund, A., & Parmar, M. (2014). Human ESC-derived dopamine neurons show similar preclinical efficacy and potency to fetal neurons when grafted in a rat model of Parkinson's disease. *Cell Stem Cell*, *15*(5), 653–665.

Grealish, S., Jönsson, M. E., Li, M., Kirik, D., Björklund, A., & Thompson, L. H. (2010). The A9 dopamine neuron component in grafts of ventral mesencephalon is an important determinant for recovery of motor function in a rat model of Parkinson's disease. *Brain : A Journal of Neurology*, *133*(Pt 2), 482–495.

Greenamyre, J Timothy. Hastings, T. G. (2004). Parkinson's - Divergent causes, convergent mechanisms. *Science*, *304*, 1120–1121.

Grinnell, F., & Petroll, W. M. (2010). Cell Motility and Mechanics in Three-Dimensional Collagen Matrices. *Annual Review of Cell and Developmental Biology*, *26*(1), 335–361.

Grünewald, A., Voges, L., Rakovic, A., Kasten, M., Vandebona, H., Hemmelmann, C., Lohmann, K., Orolicki, S., Ramirez, A., Schapira, A. H. V., Pramstaller, P. P., Sue, C. M., & Klein, C. (2010). Mutant Parkin Impairs Mitochondrial Function and Morphology in Human Fibroblasts. *PLoS ONE*, *5*(9), e12962.

Guo, H., Tang, Z., Yu, Y., Xu, L., Jin, G., & Zhou, J. (2002). Apomorphine induces trophic factors that support fetal rat mesencephalic dopaminergic neurons in cultures. *The European Journal of Neuroscience*, *16*(10), 1861–1870.

Hagell, P., & Brundin, P. (2001). Cell survival and clinical outcome following intrastriatal transplantation in Parkinson disease. *Journal of Neuropathology and Experimental Neurology*, *60*(8), 741–752.

Hagell, P., Piccini, P., Björklund, A., Brundin, P., Rehncrona, S., Widner, H., Crabb, L., Pavese, N., Oertel, W. H., Quinn, N., Brooks, D. J., & Lindvall, O. (2002). Dyskinesias following neural transplantation in Parkinson's disease. *Nature Neuroscience*, *5*(7), 627–628.

Hakan Widner, P. B. (1993). Sequential intracerebral transplantation of allogeneic and syngeneic fetal dopamine-rich neuronal tissue in adult rats: will the first graft be rejected? *Cell Transplantation*, *2*, 307–317.

Hallett, P. J., Deleidi, M., Astradsson, A., Smith, G. A., Cooper, O., Osborn, T., Sundberg, M., Moore, M. A., Perez-Torres, E., Brownell, A.-L., Schumacher, J., Spealman, R. D., & Isacson, O. (2015). Successful function of autologous iPSC-derived dopamine neurons following transplantation in a non-human primate model of Parkinson's disease. *Cell Stem Cell*, *16*(3), 269–274.

Halliday, G. M., & McCann, H. (2010). The progression of pathology in Parkinson's disease. *Annals of the New York Academy of Sciences*, *1184*, 188–195.

Hastings, T. G. (2009). The role of dopamine oxidation in mitochondrial dysfunction: implications for Parkinson's disease. *Journal of Bioenergetics and Biomembranes*, *41*(6), 469–472.

Head, J. R., & Griffin, W. S. (1985). Functional capacity of solid tissue transplants in the brain: evidence for immunological privilege. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, *224*(1236), 375–387.

Helmut, K., Hanisch, U. K., Noda, M., & Verkhratsky, A. (2011). Physiology of microglia. *Physiological Reviews*, *91*(2), 461–553.

Hemshekhar, M., Thushara, R. M., Chandranayaka, S., Sherman, L. S., Kemparaju, K., & Girish, K. S. (2016). Emerging Roles of Hyaluronic Acid Bioscaffolds in Tissue Engineering and Regenerative Medicine. In *International Journal of Biological Macromolecules*.

Hennink, W. E., & Van Nostrum, C. F. (2012). Novel crosslinking methods to design hydrogels. *Advanced Drug Delivery Reviews*, *64*, 223–236.

Henriksen, K., & Karsdal, M. A. (2016). Type I Collagen. *Biochemistry of Collagens, Laminins and Elastin*, 1–11.

Henry, C. J., Huang, Y., Wynne, A. M., & Godbout, J. P. (2009). Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1beta and anti-inflammatory IL-10 cytokines. *Brain, Behavior, and Immunity*, *23*(3), 309–317.

Hernán, M. A., Zhang, S. M., Rueda-DeCastro, A. M., Colditz, G. A., Speizer, F. E., & Ascherio, A. (2001). Cigarette smoking and the incidence of Parkinson's disease in two prospective studies. *Annals of Neurology*, *50*(6), 780–786.

Hidalgo-Figueroa, M., Bonilla, S., Gutiérrez, F., Pascual, A., & López-Barneo, J. (2012). GDNF is predominantly expressed in the PV+ neostriatal interneuronal ensemble in normal mouse and after injury of the nigrostriatal pathway. *Journal of Neuroscience*, *32*(3), 864–872.

Hinderer, S., Layland, S. L., & Schenke-Layland, K. (2016). ECM and ECM-like materials - Biomaterials for applications in regenerative medicine and cancer therapy. *Advanced Drug Delivery Reviews*, *97*, 260–269.

Hirsch, E. C., Graybiel, A. M., Duyckaerts, C., & Javoy-Agid, F. (1987). Neuronal loss in the pedunculopontine tegmental nucleus in Parkinson disease and in progressive supranuclear palsy. *Proceedings of the National Academy of Sciences of the United States of America*, *84*(16), 5976–5980.

Hirsch, Etienne C, & Hunot, S. (2009). Neuroinflammation in Parkinson's disease: a target for neuroprotection? *The Lancet Neurology*, *8*(4), 382–397.

Hoban, D. B., Newland, B., Moloney, T. C., Howard, L., Pandit, A., & Dowd, E. (2013). The reduction in immunogenicity of neurotrophin overexpressing stem cells after intra-striatal transplantation by encapsulation in an in situ gelling collagen hydrogel. *Biomaterials*, *34*(37), 9420–9429.

Houweling, D. A., Lankhorst, A. J., Gispen, W. H., Bär, P. R., & Joosten, E. A. (1998). Collagen containing neurotrophin-3 (NT-3) attracts regrowing injured corticospinal axons in the adult rat spinal cord and promotes partial functional recovery. *Experimental Neurology*, *153*(1), 49–59.

Huang, E. J., & Reichardt, L. F. (2001). Neurotrophins: roles in neuronal development and function. *Annual Review of Neuroscience*, *24*, 677–736.

Hulshof, S., Montagne, L., De Groot, C. J. A., & Van Valk, P. Der. (2002). Cellular localization and expression patterns of interleukin-10, interleukin-4, and their receptors in multiple sclerosis lesions. *Glia*, *38*(1), 24–35.

Ibáñez, C. F. (2013). Structure and physiology of the RET receptor tyrosine kinase. *Cold Spring Harbor Perspectives in Biology*, *5*(2), 1–10.

Ibáñez, C. F., & Andressoo, J.-O. (2017). Biology of GDNF and its receptors — Relevance for disorders of the central nervous system. *Neurobiology of Disease*, *97*, 80–89.

Izzy, S., Liu, Q., Fang, Z., Lule, S., Wu, L., Chung, J. Y., Sarro-Schwartz, A., Brown-Whalen, A., Perner, C., Hickman, S. E., Kaplan, D. L., Patsopoulos, N. A., El Khoury, J., & Whalen, M. J. (2019). Time-Dependent Changes in Microglia Transcriptional Networks Following Traumatic Brain Injury. *Frontiers in Cellular Neuroscience*, *13*, 307.

Johnston, L. C., Su, X., Maguire-Zeiss, K., Horovitz, K., Ankoudinova, I., Guschin, D., Hadaczek, P., Federoff, H. J., Bankiewicz, K., & Forsayeth, J. (2008). Human interleukin-10 gene transfer is protective in a rat model of Parkinson's disease. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *16*(8), 1392–1399.

Joniec-Maciejak, I., Ciesielska, A., Wawer, A., Sznejder-Pachołek, A., Schwenkgrub, J., Cudna, A., Hadaczek, P., Bankiewicz, K., Członkowska, A., & Członkowski, A. (2014). The influence of AAV2-mediated gene transfer of human IL-10 on neurodegeneration and immune response in a murine model of Parkinson's disease. *Pharmacological Reports*, *66*(4), 660–669.

Jurlander, J., Lai, C. F., Tan, J., Chou, C. C., Geisler, C. H., Schriber, J., Blumenson, L. E., Narula, S. K., Baumann, H., & Caligiuri, M. A. (1997). Characterization of interleukin-10 receptor expression on B-cell chronic lymphocytic leukemia cells. *Blood*, *89*(11), 4146–4152.

Kalia, L. V, & Lang, A. E. (2015). Parkinson's disease. *The Lancet*, *386*(9996), 896–912.

Kastin, A. J., Akerstrom, V., & Pan, W. (2003). Interleukin-10 as a CNS therapeutic: the obstacle of the blood-brain/blood-spinal cord barrier. *Brain Research. Molecular Brain Research*, *114*(2), 168–171.

Kearns, C. M., & Gash, D. M. (1995). GDNF protects nigral dopamine neurons against 6-hydroxydopamine in vivo. *Brain Research*, *672*(1–2), 104–111.

Kee, N., Volakakis, N., Kirkeby, A., Dahl, L., Storvall, H., Nolbrant, S., Lahti, L., Björklund, Å. K., Gillberg, L., Joodmardi, E., Sandberg, R., Parmar, M., & Perlmann, T. (2017). Single-Cell Analysis Reveals a Close Relationship between Differentiating Dopamine and Subthalamic Nucleus Neuronal Lineages. *Cell Stem Cell*, *20*(1), 29–40.

Kefalopoulou, Z., Politis, M., Piccini, P., Mencacci, N., Bhatia, K., Jahanshahi, M., Widner, H., Rehncrona, S., Brundin, P., Björklund, A., Lindvall, O., Limousin, P., Quinn, N., & Foltynie, T. (2014). Long-term clinical outcome of fetal cell transplantation for Parkinson disease: two case reports. *JAMA Neurology*, *71*(1), 83–87.

Kemp, J. M., & Powell, T. P. (1971). The connexions of the striatum and globus pallidus: synthesis and speculation. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *262*(845), 441–457.

Khan, R., & Khan, M. H. (2013). Use of collagen as a biomaterial: An update. *Journal of Indian Society of Periodontology*, *17*(4), 539–542.

Kikuchi, T., Morizane, A., Doi, D., Magotani, H., Onoe, H., Hayashi, T., Mizuma, H., Takara, S., Takahashi, R., Inoue, H., Morita, S., Yamamoto, M., Okita, K., Nakagawa, M., Parmar, M., & Takahashi, J. (2017a). Human iPS cell-derived dopaminergic neurons function in a primate Parkinson's disease model. *Nature Publishing Group*, *548*.

Kikuchi, T., Morizane, A., Doi, D., Magotani, H., Onoe, H., Hayashi, T., Mizuma, H., Takara, S., Takahashi, R., Inoue, H., Morita, S., Yamamoto, M., Okita, K., Nakagawa, M., Parmar, M., & Takahashi, J. (2017b). Human iPS cell-derived dopaminergic neurons function in a primate Parkinson's disease model. *Nature*, *548*(7669), 592–596.

Kirik, D, Rosenblad, C., Bjorklund, A., & Mandel, R. J. (2000). Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *20*(12), 4686–4700.

Kirik, D, Georgievska, B., & Björklund, A. (2004). Localized striatal delivery of GDNF as a treatment for Parkinson disease. *Nature Neuroscience*, *7*(2), 105–110.

Kirkeby, A., Grealish, S., Wolf, D. A., Nelander, J., Wood, J., Lundblad, M., Lindvall, O., & Parmar, M. (2012). Generation of Regionally Specified Neural Progenitors and Functional Neurons from Human Embryonic Stem Cells under Defined Conditions. *Cell Reports*, *1*(6), 703–714.

Kirkeby, A., Nelander, J., & Parmar, M. (2013). Generating regionalized neuronal cells from pluripotency, a step-by-stepprotocol. *Frontiers in Cellular Neuroscience*, 10–13.

Kirkeby, A., Nolbrant, S., Tiklova, K., Heuer, A., Kee, N., Cardoso, T., Ottosson, D. R., Lelos, M. J., Rifes, P., Dunnett, S. B., Grealish, S., Perlmann, T., & Parmar, M. (2017a). Predictive Markers Guide Differentiation to Improve Graft Outcome in Clinical Translation of hESC-Based Therapy for Parkinson's Disease. *Cell Stem Cell*, *20*(1), 135–148.

Kirkeby, A., Parmar, M., & Barker, R. A. (2017b). Strategies for bringing stem cell-derived dopamine neurons to the clinic: A European approach (STEM-PD). *Progress in Brain Research*, *230*, 165–190.

Kordower, J. H., & Bjorklund, A. (2013). Trophic Factor Gene Therapy for Parkinson's Disease. *Movement Disorders*, *28*(1), 96–109.

Kordower, J. H., Cochran, E., Penn, R. D., & Goetz, C. G. (1991). Putative chromaffin cell survival and enhanced host-derived TH-Fiber innervation following a functional adrenal medulla autograft for Parkinson's disease. *Annals of Neurology*, *29*(4), 405–412.

Kordower, J. H., Freeman, T. B., Chen, E. Y., Mufson, E. J., Sanberg, P. R., Hauser, R. A., Snow, B., & Olanow, C. W. (1998). Fetal nigral grafts survive and mediate clinical benefit in a patient with Parkinson's disease. *Movement Disorders*, *13*(3), 383–393.

Kordower, J. H., Palfi, S., Chen, E. Y., Ma, S. Y., Sendera, T., Cochran, E. J., Cochran, E. J., Mufson, E. J., Penn, R., Goetz, C. G., & Comella, C. D. (1999). Clinicopathological findings following intraventricular glial-derived neurotrophic factor treatment in a patient with Parkinson's disease. *Annals of Neurology*, *46*(3), 419–424.

Kotzbauer, P. T., Lampe, P. A., Heuckeroth, R. O., Golden, J. P., Creedon, D. J., Johnson, E. M., & Milbrandt, J. (1996). Neurturin, a relative of glial-cell-line-derived neurotrophic factor. *Nature*, *384*(6608), 467–470.

Kramer, E. R., Aron, L., Ramakers, G. M. J., Seitz, S., Zhuang, X., Beyer, K., Smidt, M. P., & Klein, R. (2007). Absence of Ret signaling in mice causes progressive and late degeneration of the nigrostriatal system. *PLoS Biology*, *5*(3), e39.

Kriks, S., Shim, J. W., Piao, J., Ganat, Y. M., Wakeman, D. R., Xie, Z., Carrillo-Reid, L., Auyeung, G., Antonacci, C., Buch, A., Yang, L., Beal, M. F., Surmeier, D. J., Kordower, J. H., Tabar, V., & Studer, L. (2011). Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature*, *480*(7378), 547–551.

Kuan, W.-L., Lin, R., Tyers, P., & Barker, R. A. (2007). The importance of A9 dopaminergic neurons in mediating the functional benefits of fetal ventral mesencephalon transplants and levodopa-induced dyskinesias. *Neurobiology of Disease*, *25*(3), 594–608.

Kunz, S., Wolk, K., Witte, E., Witte, K., Doecke, W.-D., Volk, H.-D., Sterry, W., Asadullah, K., & Sabat, R. (2006). Interleukin (IL)-19, IL-20 and IL-24 are produced by and act on keratinocytes and are distinct from classical ILs. *Experimental Dermatology*, *15*(12), 991–1004.

Lai, B. C., Marion, S., Teschke, K., & Tsui, J. K. (2002). Occupational and environmental risk factors for Parkinson's disease. *Parkinsonism & Related Disorders*, *8*(5), 297–309.

Lampron, A., Elali, A., & Rivest, S. (2013). Innate immunity in the CNS: redefining the relationship between the CNS and Its environment. *Neuron*, *78*(2), 214–232.

Lanciego, J. L., Luquin, N., & Obeso, J. A. (2012). Functional neuroanatomy of the basal ganglia. *Cold Spring Harbor Perspectives in Medicine*, *2*(12), a009621.

Lane, E.L., Winkler, C., Brundin, P., & Cenci, M. A. (2006). The impact of graft size on the development of dyskinesia following intrastriatal grafting of embryonic dopamine neurons in the rat. *Neurobiology of Disease*, *22*(2), 334–345.

Lane, E.L, Björklund, A., Dunnett, S. B., & Winkler, C. (2010). Neural grafting in Parkinson's disease. In *Progress In Brain Research- Volume 184* (Vol. 184).

Lang, A. E., Gill, S., Patel, N. K., Lozano, A., Nutt, J. G., Penn, R., Brooks, D. J., Hotton, G., Moro, E., Heywood, P., Brodsky, M. A., Burchiel, K., Kelly, P., Dalvi, A., Scott, B., Stacy, M., Turner, D., Wooten V. G. F., Elias W. J., Laws, E. R., Dhawan, V., Stoessl A. J., Matcham, J., Cooffey R. J., Traub, M. (2006). Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. *Annals of Neurology*, *59*(3), 459–466.

Lang, A. E., & Lozano, A. M. (1998). Parkinson's Disease. *New England Journal of Medicine*, *339*(15), 1044–1053.

Langston, J. W., Ballard, P., Tetrud, J. W., & Irwin, I. (1983). Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science (New York, N.Y.)*, *219*(4587), 979–980.

Ledeboer, A, Brevé, J. J., Poole, S., Tilders, F. J., & Van Dam, A. M. (2000). Interleukin-10, interleukin-4, and transforming growth factor-beta differentially regulate lipopolysaccharide-induced production of pro-inflammatory cytokines and nitric oxide in co-cultures of rat astroglial and microglial cells. *Glia*, *30*(2), 134–142.

Ledeboer, A, Brevé, J. J., Wierinckx, A., Van Der Jagt, S., Bristow, A. F., Leysen, J. E., Tilders, F. J., & Van Dam, A.-M. M. (2002). Expression and regulation of interleukin-10 and interleukin-10 receptor in rat astroglial and microglial cells. *European Journal of Neuroscience*, *16*(7), 1175–1185.

Lee, K. H., Hitti, F. L., Chang, S.-Y., Lee, D. C., Roberts, D. W., McIntyre, C. C., & Leiter, J. C. (2011). High frequency stimulation abolishes thalamic network oscillations: an electrophysiological and computational analysis. *Journal of Neural Engineering*, *8*(4), 046001.

Lesage, S., & Brice, A. (2009). Parkinson's disease: from monogenic forms to genetic susceptibility factors. *Human Molecular Genetics*, *18*(R1), R48–R59.

Lewis, S. J. G., Caldwell, M. A., & Barker, R. A. (2003). Modern therapeutic approaches in Parkinson's disease. *Expert Reviews in Molecular Medicine*, *5*(10), 1–20.

Lewy, F. (1912). Paralysis agitans. I. Pathologische anatomie. *Handbuch Der Neurologie*.

Li, D., He, Q., Li, R., Xu, X., Chen, B., & Xie, A. (2012). Interleukin-10 promoter polymorphisms in Chinese patients with Parkinson's disease. *Neuroscience Letters*, *513*(2), 183–186.

Li, W., Englund, E., Widner, H., Mattsson, B., van Westen, D., Lätt, J., Rehncrona, S., Brundin, P., Björklund, A., Lindvall, O., & Li, J.-Y. (2016). Extensive graft-derived dopaminergic innervation is maintained 24 years after transplantation in the degenerating parkinsonian brain. *Proceedings of the National Academy of Sciences*, *113*(23), 6544–6549.

Liang, G., & Zhang, Y. (2013). Genetic and Epigenetic Variations in iPSCs: Potential Causes and Implications for Application. *Cell Stem Cell*, *13*(2), 149–159.

Lin, L. H., Doherty, D. H., Lile, J. D., Bektesh, S., Lin, L. H., Doherty, D. H., Lile, J. D., Bektesh, S., & Collins, F. (1993). GDNF : A Glial Cell Line-Derived Neurotrophic Factor for Midbrain Dopaminergic Neurons. *Science*, *260*(5111), 1130–1132.

Lindvall, O., Brundin, P., Widner, H., Rehncrona, S., Gustavii, B., Frackowiak, R., Leenders, K. L., Sawle, G., Rothwell, J. C., Marsden, C. D., & Björklund, A. (1990). Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. *Science*, *247*(4942), 574–577.

Lindvall, O., Sawle, G., Widner, H., Rothwell, J. C., Björklund, A., Brooks, D., Brundin, P., Frackowiak, R., Marsden, C. D., Odin, P., & Rehncrona, S. (1994). Evidence for long-term survival and function of dopaminergic grafts in progressive Parkinson's disease. *Annals of Neurology*, *35*(2), 172–180.

Lindvall O, Björklund, A. (1989). Transplantation strategies in the treatment of Parkinson's disease: experimental basis and clinical trials. *Acta Neurologica Scandinavica*, *80*(126), 197–210.

Liu, Z., Guo, J., Wang, Y., Li, K., Kang, J., Wei, Y., Sun, Q., Xu, Q., Xu, C., Yan, X., & Tang, B. (2016). Lack of association between IL-10 and IL-18 gene promoter polymorphisms and Parkinson's disease with cognitive impairment in a Chinese population. *Scientific Reports*, *6*, 19021.

Lobo-Silva, D., Carriche, G. M., Castro, A. G., Roque, S., & Saraiva, M. (2016). Balancing the immune response in the brain: IL-10 and its regulation. *Journal of Neuroinflammation*, *13*(1), 297.

Lynn, A. K., Yannas, I. V., & Bonfield, W. (2004). Antigenicity and immunogenicity of collagen. *Journal of Biomedical Materials Research - Part B Applied Biomaterials*, *71*(2), 343–354.

Maciaczyk, J., Singec, I., Maciaczyk, D., & Nikkhah, G. (2008). Combined use of BDNF, ascorbic acid, low oxygen, and prolonged differentiation time generates tyrosine hydroxylase-expressing neurons after long-term in vitro expansion of human fetal midbrain precursor cells. *Experimental Neurology*, *213*(2), 354–362.

Madrazo, I., Drucker-Colín, R., Díaz, V., Martínez-Mata, J., Torres, C., & Becerril, J. J. (1987). Open microsurgical autograft of adrenal medulla to the right caudate nucleus in two patients with intractable Parkinson's disease. *The New England Journal of Medicine*, *316*(14), 831–834.

Mahalik, T. J., Hahn, W. E., Clayton, G. H., & Owens, G. P. (1994). Programmed Cell Death in Developing Grafts of Fetal Substantia Nigra. *Experimental Neurology*, *129*(1), 27–36.

Maitra, J., & Shukla, V. K. (2014). *Cross-linking in hydrogels - A review*. *4*(2), 25–31.

Mandel, R J, Spratt, S. K., Snyder, R. O., & Leff, S. E. (1997). Midbrain injection of recombinant adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protects nigral neurons in a progressive 6-hydroxydopamine-induced degeneration model of Parkinson's disease in rats. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(25), 14083–14088.

Mandel, Ronald J., Brundin, P., & Björklund, A. (1990). The Importance of Graft Placement and Task Complexity for Transplant-Induced Recovery of Simple and Complex Sensorimotor Deficits in Dopamine Denervated Rats. *European Journal of Neuroscience*, *2*(10), 888–894.

Manson, A. J., Turner, K., & Lees, A. J. (2002). Apomorphine monotherapy in the treatment of refractory motor complications of Parkinson's disease: Long-term follow-up study of 64 patients. *Movement Disorders*, *17*(6), 1235–1241.

Marsden, C. D. (1990). Parkinson's disease. *The Lancet*, *335*(8695), 948–949.

Martin J. H. (2012). *Neuroanatomy Text and Atlas* (4th Edition).

Mendez, I., Dagher, A., Murray, H., Gaudet, P., Law, A., Weerasinghe, S., King, D., Desrosiers, J., Darvesh, S., Acorn, T., Roberstson, H. (2002). Simultaneous intrastriatal and intranigral fetal dopaminergic grafts in patients with Parkinson disease: a pilot study. Report of three cases. *Journal of Neurosurgery*, *96*(3), 589–596.

Mendez, I., Dagher, A., Hong, M., Hebb, A., Gaudet, P., Law, A., Weerasinghe, S., King, D., Desrosiers, J., Darvesh, S., Acorn, T., & Robertson, H. (2000). Enhancement of survival of stored dopaminergic cells and promotion of graft survival by exposure of human fetal nigral tissue to glial cell line--derived neurotrophic factor in patients with Parkinson's disease. Report of two cases and technical considera. *Journal of Neurosurgery*, *92*(5), 863–869.

Meyer, M. (2019). Processing of collagen based biomaterials and the resulting materials properties. *Biomedical Engineering Online*, *18*(1), 24.

Mitrousis, N., Fokina, A., & Shoichet, M. S. (2018). Biomaterials for cell transplantation. *Nature Reviews Materials*, *3*(11), 441–456.

Mittal, S., Bjørnevik, K., Im, D. S., Flierl, A., Dong, X., Locascio, J. J., Abo, K. M., Long, E., Jin, M., Xu, B., Xiang, Y. K., Rochet, J.-C., Engeland, A., Rizzu, P., Heutink, P., Bartels, T., Selkoe, D. J., Caldarone, B. J., Glicksman, M. A., Khurana, V., Schüle, B., Park, D. S., Riise, T., Scherzer, C. R. (2017). β2-Adrenoreceptor is a regulator of the α-synuclein gene driving risk of Parkinson's disease. *Science (New York, N.Y.)*, *357*(6354), 891–898.

Mohtaram, N. K., Montgomery, A., & Willerth, S. M. (2013). Biomaterial-based drug delivery systems for the controlled release of neurotrophic factors. *Biomedical Materials (Bristol, England)*, *8*(2), 022001.

Molina-Holgado, F., Grencis, R., & Rothwell, N. J. (2001). Actions of exogenous and endogenous IL-10 on glial responses to bacterial LPS/cytokines. *Glia*, *33*(2), 97–106.

Montalbano, G., Toumpaniari, S., Popov, A., Duan, P., Chen, J., Dalgarno, K., Scott, W. E., & Ferreira, A. M. (2018). Synthesis of bioinspired collagen/alginate/fibrin based hydrogels for soft tissue engineering. *Materials Science and Engineering: C*, *91*, 236–246.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L., & O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. *Annual Review of Immunology*, *19*(1), 683–765.

Moore, M. W., Klein, R. D., Fariñas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A. M., Carver-Moore, K., & Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. *Nature*, *382*(6586), 76–79.

Moriarty, N. (2018). *The potential of injectable collagen hydrogels to enhance dopaminergic cell replacement therapies for Parkinson's disease*.

Moriarty, N., Cabré, S., Alamilla, V., Pandit, A., & Dowd, E. (2019a). 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. *The European Journal of Neuroscience*, *49*(4), 487–496.

Moriarty, N., Pandit, A., & Dowd, E. (2017). Encapsulation of primary dopaminergic neurons in a GDNF-loaded collagen hydrogel increases their survival, re-innervation and function after intra-striatal transplantation. *Scientific Reports*, *7*(1), 16033.

Moriarty, N., Parish, C. L., & Dowd, E. (2019b). Primary tissue for cellular brain repair in Parkinson's disease: Promise, problems and the potential of biomaterials. *European Journal of Neuroscience*, *49*(4), 472–486.

Morihisa, J. M., Nakamura, R. K., Freed, W. J., Mishkin, M., & Wyatt, R. J. (1984). Adrènal medulla grafts survice and exhibit catecholamine-specific fluorescence in the primate brain. *Experimental Neurology*, *84*(3), 643–653.

Morizane, A., Kikuchi, T., Hayashi, T., Mizuma, H., Takara, S., Doi, H., Mawatari, A., Glasser, M. F., Shiina, T., Ishigaki, H., Itoh, Y., Okita, K., Yamasaki, E., Doi, D., Onoe, H., Ogasawara, K., Yamanaka, S., & Takahashi, J. (2017). MHC matching improves engraftment of iPSC-derived neurons in non-human primates. *Nature Communications*, *8*(1), 385.

Moshayedi, P., da F Costa, L., Christ, A., Lacour, S. P., Fawcett, J., Guck, J., & Franze, K. (2010). Mechanosensitivity of astrocytes on optimized polyacrylamide gels analyzed by quantitative morphometry. *Journal of Physics: Condensed Matter*, *22*(19), 194114.

Mosser D M, Zhang X. (2008). New perspectives on an old cytokine. *Immunological Reviews*, *226*, 205–218.

Nakaji-Hirabayashi, T., Kato, K., & Iwata, H. (2012). Improvement of neural stem cell survival in collagen hydrogels by incorporating laminin-derived cell adhesive polypeptides. *Bioconjugate Chemistry*, *23*(2), 212–221.

Nakaji-Hirabayashi, T., Kato, K., & Iwata, H. (2013). In vivo study on the survival of neural stem cells transplanted into the rat brain with a collagen hydrogel that incorporates laminin-derived polypeptides. *Bioconjugate Chemistry*, *24*(11), 1798–1804.

Naughton, C., Moriarty, N., Feehan, J., O'Toole, D., & Dowd, E. (2015). Differential pattern of motor impairments in neurotoxic, environmental and inflammation-driven rat models of Parkinson's disease. *Behavioural Brain Research*, *296*, 451–458.

Niemczyk, B., Sajkiewicz, P., & Kolbuk, D. (2018). Injectable hydrogels as novel materials for central nervous system regeneration. *Journal of Neural Engineering*, *15*(5), 051002.

Nih, L. R., Carmichael, S. T., & Segura, T. (2016). Hydrogels for brain repair after stroke: an emerging treatment option. *Current Opinion in Biotechnology*, *40*, 155–163.

Nisbet, D. R., Crompton, K. E., Horne, M. K., Finkelstein, D. I., & Forsythe, J. S. (2008). Neural tissue engineering of the CNS using hydrogels. *Journal of Biomedical Materials Research. Part B, Applied Biomaterials*, *87*(1), 251–263.

Norden, D. M., Fenn, A. M., Dugan, A., & Godbout, J. P. (2014). TGFβ produced by IL-10 redirected astrocytes attenuates microglial activation. *Glia*, *62*(6), 881–895.

Nosrat, C. A., Tomac, A., Hoffer, B. J., & Olson, L. (1997). Cellular and developmental patterns of expression of Ret and glial cell line-derived neurotrophic factor receptor alpha mRNAs. *Experimental Brain Research*, *115*(3), 410–422.

Nussbaum, R. L., & Ellis, C. E. (2003). Alzheimer's Disease and Parkinson's Disease. *New England Journal of Medicine*, *348*(14), 1356–1364.

Nutt, J. G., Burchiel, K. J., Comella, C. L., Jankovic, J., Lang, A. E., Laws, E. R. R., Lozano, A. M., Penn, R. D., Simpson, R. K. K., Stacy, M., Wooten, G. F., & ICV GDNF Study Group. Implanted intracerebroventricular. Glial cell line-derived neurotrophic factor. (2003). Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. *Neurology*, *60*(1), 69–73.

O'Garra, A. et al. (1992). Ly‐1 B (B‐1) cells are the main source of B cell‐derived interleukin 10. *Eur. J. Immunol.*, *22*, 711–717.

O'Garra, A., & Vieira, P. (2007). T(H)1 cells control themselves by producing interleukin-10. *Nature Reviews. Immunology*, *7*(6), 425–428.

Obeso, J. A., Rodriguez-Oroz, M. C., Rodriguez, M., Lanciego, J. L., Artieda, J., Gonzalo, N., & Olanow, C. W. (2000). Pathophysiology of the basal ganglia in Parkinson's disease. *Trends in Neurosciences*, *23*, S8–S19.

Okura, Y., Tanaka, R., Ono, K., Yoshida, S., Tanuma, N., & Matsumoto, Y. (1997). Treatment of rat hemiparkinson model with xenogeneic neural transplantation: Tolerance induction by anti-T-cell antibodies. *Journal of Neuroscience Research*, *48*(5), 385–396.

Olanow, C. W., Goetz, C. G., Kordower, J. H., Stoessl, A. J., Sossi, V., Brin, M. F., Shannon, K. M., Nauert, G. M., Perl, D. P., Godbold, J., & Freeman, T. B. (2003). A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. *Annals of Neurology*, *54*(3), 403–414.

Olson JK, M. S. (2004). Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. *J Immunol.*, *173*, 3916–24.

Olson, L., & Malmfors, T. (1970). Growth characteristics of adrenergic nerves in the adult rat. Fluorescence histochemical and 3H-noradrenaline uptake studies using tissue transplantations to the anterior chamber of the eye. *Acta Physiologica Scandinavica. Supplementum*, *348*, 1–112.

Olsson, M., Nikkhah, G., Bentlage, C., & Bjorklund, A. (1995). Forelimb akinesia in the rat Parkinson model: Differential effects of dopamine agonists and nigral transplants as assessed by a new stepping test. *Journal of Neuroscience*, *15*(5 II), 3863–3875.

Ono, Y., Nakatani, T., Sakamoto, Y., Mizuhara, E., Minaki, Y., Kumai, M., Hamaguchi, A., Nishimura, M., Inoue, Y., Hayashi, H., Takahashi, J., & Imai, T. (2007). Differences in neurogenic potential in floor plate cells along an anteroposterior location: Midbrain dopaminergic neurons originate from mesencephalic floor plate cells. *Development*, *134*(17), 3213–3225.

Oo, T. F., Kholodilov, N., & Burke, R. E. (2003). Regulation of natural cell death in dopaminergic neurons of the substantia nigra by striatal glial cell line-derived neurotrophic factor in vivo. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *23*(12), 5141–5148.

Oo, T. F., Ries, V., Cho, J., Kholodilov, N., & Burke, R. E. (2005). Anatomical basis of glial cell line-derived neurotrophic factor expression in the striatum and related basal ganglia during postnatal development of the rat. *The Journal of Comparative Neurology*, *484*(1), 57–67.

Orive, G., Anitua, E., Pedraz, J. L., & Emerich, D. F. (2009). Biomaterials for promoting brain protection, repair and regeneration. *Nature Reviews Neuroscience*, *10*(9), 682–692.

Orive, G., Echave, M. C., Pedraz, J. L., Golafshan, N., Dolatshahi-Pirouz, A., Paolone, G., & Emerich, D. (2019). Advances in cell-laden hydrogels for delivering therapeutics. *Expert Opinion on Biological Therapy*, *00*(00), 1–4.

Pakulska, M. M., Ballios, B. G., & Shoichet, M. S. (2012). Injectable hydrogels for central nervous system therapy. *Biomedical Materials*, *7*(2).

Parenteau-Bareil, R., Gauvin, R., & Berthod, F. (2010). Collagen-based biomaterials for tissue engineering applications. *Materials*, *3*(3), 1863–1887.

Parkinson, J. (1817). An essay on the shaking palsy. Reprint (2002). *The Journal of Neuropsychiatry and Clinical Sciences*, *14*(2), 223–236.

Parmar, M. (2018). Towards stem cell based therapies for parkinson's disease. *Development (Cambridge)*, *145*(1).

Patel, N. K., Bunnage, M., Plaha, P., Svendsen, C. N., Heywood, P., & Gill, S. S. (2005). Intraputamenal infusion of glial cell line-derived neurotrophic factor in PD: a two-year outcome study. *Annals of Neurology*, *57*(2), 298–302.

Patino, M. G., Neiders, M. E., Andreana, S., Noble, B., & Cohen, R. E. (2002). Collagen as an Implantable Material in Medicine and Dentistry. *Journal of Oral Implantology*, *28*(5), 220–225.

Paul, G., & Sullivan, A. M. (2019). Trophic factors for Parkinson's disease: Where are we and where do we go from here? *European Journal of Neuroscience*, *49*(4), 440–452.

Peppas, N. A., Hilt, J. Z., Khademhosseini, A., & Langer, R. (2006). Hydrogels in biology and medicine: From molecular principles to bionanotechnology. *Advanced Materials*, *18*(11), 1345–1360.

Perez-Bouza, A., Di Santo, S., Seiler, S., Meyer, M., Andereggen, L., Huber, A., Guzman, R., & Widmer, H. R. (2017). Simultaneous Transplantation of Fetal Ventral Mesencephalic Tissue and Encapsulated Genetically Modified Cells Releasing GDNF in a Hemi-Parkinsonian Rat Model of Parkinson's Disease. *Cell Transplantation*, *26*(9), 1572–1581.

Perlow, M. J., Freed, W. J., Hoffer, B. J., Seiger, A., Olson, L., & Wyatt, R. J. (1979). Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system. *Science*, *204*(4393), 643–647.

Phelps, W. M. (1930). Dyskinesia. *The Yale Journal of Biology and Medicine*, *3*(1), 39–48.

Piantino, J., Burdick, J. A., Goldberg, D., Langer, R., & Benowitz, L. I. (2006). An injectable, biodegradable hydrogel for trophic factor delivery enhances axonal rewiring and improves performance after spinal cord injury. *Experimental Neurology*, *201*(2), 359–367.

Piccini, P., Brooks, D. J., Björklund, A., Gunn, R. N., Grasby, P. M., Rimoldi, O., Brundin, P., Hagell, P., Rehncrona, S., Widner, H., & Lindvall, O. (1999). Dopamine release from nigral transplants visualized in vivo in a Parkinson's patient. *Nature Neuroscience*, *2*(12), 1137–1140.

Piccini, P., Pavese, N., Hagell, P., Reimer, J., Björklund, A., Oertel, W. H., Quinn, N. P., Brooks, D. J., & Lindvall, O. (2005). Factors affecting the clinical outcome after neural transplantation in Parkinson's disease. *Brain*, *128*(12), 2977–2986.

Picillo, M., Nicoletti, A., Fetoni, V., Garavaglia, B., Barone, P., & Pellecchia, M. T. (2017). The relevance of gender in Parkinson's disease: a review. *Journal of Neurology*, *264*(8), 1583–1607.

Politis, M., Oertel, W. H., Wu, K., Quinn, N. P., Pogarell, O., Brooks, D. J., Bjorklund, A., Lindvall, O., & Piccini, P. (2011). Graft-induced dyskinesias in Parkinson's disease: High striatal serotonin/dopamine transporter ratio. *Movement Disorders*, *26*(11), 1997–2003.

Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di lorio, G., Golbe, LI., Nussbaum, R. L. (1997). Mutation in the -Synuclein Gene Identified in Families with Parkinson's Disease. *Science*, *276*(5321), 2045–2047.

Prockop, D. J., & Kivirikko, K. I. (1995). Collagens: molecular biology, diseases, and potentials for therapy. *Annual Review of Biochemistry*, *64*, 403–434.

Qian, L, Block M, Wei S, Lin C, Reece J, Pang H, Wilson B, Hong J, F. P. (2006a). Interleukin-10 Protects Lipopolysaccharide-Induced Neurotoxicity in Primary Midbrain Cultures by Inhibiting the Function of NADPH Oxidase. *Journal of Pharmacology and Experimental Therapeutics*, *319*(1), 44–52.

Qian, L., Hong, J. S., & Flood, P. M. (2006b). Role of microglia in inflammation-mediated degeneration of dopaminergic neurons: Neuroprotective effect of interleukin 10. *Journal of Neural Transmission, Supplement*, (70), 367–371.

Quik, M. (2004). Smoking, nicotine and Parkinson's disease. *Trends in Neurosciences*, *27*(9), 561–568.

Ramshaw, J. A. M. (2016). Biomedical applications of collagens. *Journal of Biomedical Materials Research - Part B Applied Biomaterials*, *104*(4), 665–675.

Redmond, D. E., McEntire, C. R. S., Kingsbery, J. P., Leranth, C., Elsworth, J. D., Bjugstad, K. B., Roth, R. H., Samulski, R. J., & Sladek, J. R. (2013). Comparison of fetal mesencephalic grafts, AAV-delivered GDNF, and both combined in an MPTP-induced nonhuman primate Parkinson's model. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *21*(12), 2160–2168.

Redmond, D. E., Roth, R. H., Elsworth, J. D., Sladek, J. R., Collier, T. J., Deutch, A. Y., & Haber, S. (1986). Fetal Neuronal Grafts in Monkeys Given Methylphenyltetrahydropyridine. *The Lancet*, *327*(8490), 1125–1127.

Rentzos, M., Nikolaou, C., Andreadou, E., Paraskevas, G. P., Rombos, A., Zoga, M., Tsoutsou, A., Boufidou, F., Kapaki, E., & Vassilopoulos, D. (2009). Circulating interleukin-10 and interleukin-12 in Parkinson's disease. *Acta Neurologica Scandinavica*, *119*(5), 332–337.

Riederer, P., & Laux, G. (2011). MAO-inhibitors in Parkinson's Disease. *Experimental Neurobiology*, *20*(1), 1.

Rosenblad, C., Kirik, D., Devaux, B., Moffat, B., Phillips, H. S., & Björklund, A. (1999). Protection and regeneration of nigral dopaminergic neurons by neurturin or GDNF in a partial lesion model of Parkinson's disease after administration into the striatum or the lateral ventricle. *The European Journal of Neuroscience*, *11*(5), 1554–1566.

Rosenblad, C., Martinez-Serrano, A., & Björklund, A. (1996). Glial cell line-derived neurotrophic factor increases survival, growth and function of intrastriatal fetal nigral dopaminergic grafts. *Neuroscience*, *75*(4), 979–985.

Ross, G. W., Abbott, R. D., Petrovitch, H., Morens, D. M., Grandinetti, A., Tung, K. H., Tanner, C. M., Masaki, K. H., Blanchette, P. L., Curb, J. D., Popper, J. S., & White, L. R. (2000). Association of Coffee and Caffeine Intake With the Risk of Parkinson Disease. *JAMA*, *283*(20), 2674.

Ross, R. A., & Biedler, J. L. (1985). Presence and regulation of tyrosinase activity in human neuroblastoma cell variants in vitro. *Cancer Research*, *45*(4), 1628–1632.

Ruoslahti, E. (1996). Brain extracellular matrix. *Glycobiology*, *6*(5), 489–492.

Rutka, J. T., Apodaca, G., Stern, R., & Rosenblum, M. (1988). The extracellular matrix of the central and peripheral nervous systems: Structure and function. *Journal of Neurosurgery*, *69*(2), 155–170.

Rutz, S., & Ouyang, W. (2016). Regulation of Cytokine Gene Expression in Immunity and Diseases. *Regulation of Cytokine Gene Expression in Immunity and Diseases, Advances in Experimental Medicine and Biology*, *941*(II), 89–116.

Sabat, R., Grutz, G., Warszawska, K., Kirsch, S., Witte, E., Wolk, K., & Geginat, J. (2010). Biology of interleukin-10. *Cytokine & Growth Factor Reviews Journal*, *21*, 331–344.

Saraiva, M., Christensen, J. R., Veldhoen, M., Murphy, T. L., Murphy, K. M., & O'Garra, A. (2009). Interleukin-10 production by Th1 cells requires interleukin-12-induced STAT4 transcription factor and ERK MAP kinase activation by high antigen dose. *Immunity*, *31*(2), 209–219.

Saraiva, M., & O'Garra, A. (2010). The regulation of IL-10 production by immune cells. *Nature Reviews Immunology*, *10*(3), 170–181.

Sariola, H., & Saarma, M. (2003). Novel functions and signalling pathways for GDNF. *Journal of Cell Science*, *116*(Pt 19), 3855–3862.

Sauer, H., Rosenblad, C., & Björklund, A. (1995). Glial cell line-derived neurotrophic factor but not transforming growth factor beta 3 prevents delayed degeneration of nigral dopaminergic neurons following striatal 6-hydroxydopamine lesion. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(19), 8935–8939.

Sawada, M., Suzumura, A., Hosoya, H., Marunouchi, T., & Nagatsu, T. (2001). Interleukin-10 Inhibits Both Production of Cytokines and Expression of Cytokine Receptors in Microglia. *Journal of Neurochemistry*, *72*(4), 1466–1471.

Saxena, A., Khosraviani, S., Noel, S., Mohan, D., Donner, T., & Hamad, A. R. A. (2015). Interleukin-10 paradox: A potent immunoregulatory cytokine that has been difficult to harness for immunotherapy. *Cytokine*, *74*(1), 27–34.

Saxena, S., & Caroni, P. (2011). Selective Neuronal Vulnerability in Neurodegenerative Diseases: from Stressor Thresholds to Degeneration. *Neuron*, *71*(1), 35–48.

Schallert, T, & Hall, S. (1988). "Disengage" sensorimotor deficit following apparent recovery from unilateral dopamine depletion. *Behavioural Brain Research*, *30*(1), 15–24.

Schallert, Timothy, Fleming, S. M., Leasure, J. L., Tillerson, J. L., & Bland, S. T. (2000). CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology*, *39*(5), 777–787.

Schapira, Anthony H., & Jenner, P. (2011). Etiology and pathogenesis of Parkinson's disease. *Movement Disorders*, *26*(6), 1049–1055.

Schapira, A. H. V., Cooper, J. M., Dexter, D., Clark, J. B., Jenner, P., & Marsden, C. D. (1990). Mitochondrial Complex I Deficiency in Parkinson's Disease. *Journal of Neurochemistry*, *54*(3), 823–827.

Schapira, A. H V. (2011). Challenges to the development of disease-modifying therapies in Parkinson's disease. *European Journal of Neurology*, *18*(SUPPL. 1), 16–21.

Scharr, D. G., Sieber, B.-A., Dreyfus, C. F., & Black, I. B. (1993). Regional and Cell-Specific Expression of GDNF in Rat Brain. *Experimental Neurology*, *124*(2), 368–371.

Schindelhauer, D., Schuffenhauer, S., Gasser, T., Steinkasserer, A., & Meitinger, T. (1995). The gene coding for glial cell line derived neurotrophic factor (GDNF) maps to chromosome 5p12-p13.1. *Genomics*, *28*(3), 605–607.

Schubert, D., Heinemann, S., Carlisle, W., Tarikas, H., Kimes, B., Patrick, J., Steinbach, J. H., Culp, W., & Brandt, B. L. (1974). Clonal cell lines from the rat central nervous system. *Nature*, *249*(454), 224–227.

Schwab, R. S., England, A. C., Poskanzer, D. C., & Young, R. R. (1969). Amantadine in the Treatment of Parkinson's Disease. *JAMA: The Journal of the American Medical Association*, *208*(7), 1168.

Schwenkgrub, J., Joniec-Maciejak, I., Sznejder-Pachołek, A., Wawer, A., Ciesielska, A., Bankiewicz, K., Członkowska, A., & Członkowski, A. (2013). Effect of human interleukin-10 on the expression of nitric oxide synthases in the MPTP-based model of Parkinson's disease. *Pharmacological Reports*, *65*(1), 44–49.

Seiler, M. J., & Aramant, R. B. (2012). Cell replacement and visual restoration by retinal sheet transplants. *Progress in Retinal and Eye Research*, *31*(6), 661–687.

Senthilkumar, K. S., Saravanan, K. S., Chandra, G., Sindhu, K. M., Jayakrishnan, A., & Mohanakumar, K. P. (2007). Unilateral implantation of dopamine-loaded biodegradable hydrogel in the striatum attenuates motor abnormalities in the 6-hydroxydopamine model of hemi-parkinsonism. *Behavioural Brain Research*, *184*(1), 11–18.

Sharma, S., Yang, B., Xi, X., Grotta, J. C., Aronowski, J., & Savitz, S. I. (2011). IL-10 directly protects cortical neurons by activating PI-3 kinase and STAT-3 pathways. *Brain Research*, *1373*, 189–194.

Sheng, W. S., Hu, S., Kravitz, F. H., Peterson, P. K., & Chao, C. C. (1995). Tumor necrosis factor alpha upregulates human microglial cell production of interleukin-10 in vitro. *Clinical and Diagnostic Laboratory Immunology*, *2*(5), 604–608.

Shinoda, M., Hudson, J. L., Strömberg, I., Hoffer, B. J., Moorhead, J. W., & Olson, L. (1995). Allogeneic grafts of fetal dopamine neurons: immunological reactions following active and adoptive immunizations. *Brain Research*, *680*(1–2), 180–195.

Shults, C. W., Kimber, T., & Martin, D. (1996). Intrastriatal injection of GDNF attenuates the effects of 6-hydroxydopamine. *Neuroreport*, *7*(2), 627–631.

Silvipriya, K. S., Krishna Kumar, K., Bhat, A. R., Dinesh Kumar, B., John, A., & Lakshmanan, P. (2015). Collagen: Animal sources and biomedical application. *Journal of Applied Pharmaceutical Science*, *5*(3), 123–127.

Sinclair, S. R., Svendsen, C. N., Torres, E. M., Martin, D., Fawcett, J. W., & Dunnett, S. B. (1996). GDNF enhances dopaminergic cell survival and fibre outgrowth in embryonic nigral grafts. *Neuroreport*, *7*(15–17), 2547–2552.

Sladek, J. R., Collier, T. J., Haber, S. N., Deutch, A. Y., Elsworth, J. D., Roth, R. H., & Redmond, D. E. (1987). Reversal of Parkinsonism by Fetal Nerve Cell Transplants in Primate Brain. *Annals of the New York Academy of Sciences*, *495*(1 Cell and Tiss), 641–657.

Soderstrom, K. E., Meredith, G., Freeman, T. B., McGuire, S. O., Collier, T. J., Sortwell, C. E., Wu, Q., & Steece-Collier, K. (2008). The synaptic impact of the host immune response in a parkinsonian allograft rat model: Influence on graft-derived aberrant behaviors. *Neurobiology of Disease*, *32*(2), 229–242.

Sortwell, C. E., Camargo, M. D., Pitzer, M. R., Gyawali, S., & Collier, T. J. (2001). Diminished Survival of Mesencephalic Dopamine Neurons Grafted into Aged Hosts Occurs during the Immediate Postgrafting Interval. *Experimental Neurology*, *169*(1), 23–29.

Sortwell, C. E., Pitzer, M. R., & Collier, T. J. (2000). Time course of apoptotic cell death within mesencephalic cell suspension grafts: implications for improving grafted dopamine neuron survival. *Experimental Neurology*, *165*(2), 268–277.

Spencer, K. C., Sy, J. C., Ramadi, K. B., Graybiel, A. M., Langer, R., & Cima, M. J. (2017). Characterization of Mechanically Matched Hydrogel Coatings to Improve the Biocompatibility of Neural Implants. *Scientific Reports*, *7*(1), 1952.

Spera, P. A., Ellison, J. A., Feuerstein, G. Z., & Barone, F. C. (1998). IL-10 reduces rat brain injury following focal stroke. *Neuroscience Letters*, *251*(3), 189–192.

Spillantini, M. G., Crowther, R. A., Jakes, R., Hasegawa, M., & Goedert, M. (1998). alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(11), 6469–6473.

Spillantini, M. G., Schmidt, M. L., Lee, V. M.-Y., Trojanowski, J. Q., Jakes, R., & Goedert, M. (1997). α-Synuclein in Lewy bodies. *Nature*, *388*(6645), 839–840.

Steinbeck, J. A., Choi, S. J., Mrejeru, A., Ganat, Y., Deisseroth, K., Sulzer, D., Mosharov, E. V, & Studer, L. (2015). Optogenetics enables functional analysis of human embryonic stem cell–derived grafts in a Parkinson's disease model. *Nature Biotechnology*, *33*(2), 204–209.

Stenevi, U., Björklund, A., & Svendgaard, N. A. (1976). Transplantation of central and peripheral monoamine neurons to the adult rat brain: Techniques and conditions for survival. *Brain Research*, *114*(1), 1–20.

Stibe, C. M. H., Kempster, P. A., Lees, A. J., & Stern, G. M. (1988). Subcutaneous apomorphine in parkinsonian on-off oscillations. *The Lancet*, *331*(8582), 403–406.

Stoker, T. B., Barker R. A.(2016). Cell therapies for Parkinson's disease: How far have we come? *Regen. Med*. 11(8), 777–786

Streeter, I., & Cheema, U. (2011). Oxygen consumption rate of cells in 3D culture: The use of experiment and simulation to measure kinetic parameters and optimise culture conditions. *The Analyst*, *136*(19), 4013.

Strömberg, I., Björklund, L., Johansson, M., Tomac, A., Collins, F., Olson, L., Hoffer, B., & Humpel, C. (1993). Glial cell line-derived neurotrophic factor is expressed in the developing but not adult striatum and stimulates developing dopamine neurons in vivo. *Experimental Neurology*, *124*(2), 401–412.

Strong, C. (1991). Fetal tissue transplantation: Can it be morally insulated from abortion? *Journal of Medical Ethics*, *17*(2), 70–76.

Sundberg, M., Bogetofte, H., Lawson, T., Jansson, J., Smith, G., Astradsson, A., Moore, M., Osborn, T., Cooper, O., Spealman, R., Hallett, P., & Isacson, O. (2013). Improved cell therapy protocols for Parkinson's disease based on differentiation efficiency and safety of hESC-, hiPSC-, and non-human primate iPSC-derived dopaminergic neurons. *Stem Cells (Dayton, Ohio)*, *31*(8), 1548–1562.

Takahashi, J. (2017). Strategies for bringing stem cell-derived dopamine neurons to the clinic: The Kyoto trial. In *Progress in Brain Research* (1st ed., Vol. 230).

Takahashi, J. (2019). Preparing for first human trial of induced pluripotent stem cell-derived cells for Parkinson's disease: an interview with Jun Takahashi. *Regenerative Medicine*, *14*(2), 93–95.

Takahashi, K., & Yamanaka, S. (2006). Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell*, *126*(4), 663–676.

Takahashi, M. (2001). The GDNF/RET signaling pathway and human diseases. *Cytokine & Growth Factor Reviews*, *12*(4), 361–373.

Talebian, S., Mehrali, M., Taebnia, N., Pennisi, C. P., Kadumudi, F. B., Foroughi, J., Hasany, M., Nikkhah, M., Akbari, M., Orive, G., & Dolatshahi-Pirouz, A. (2019). Self-Healing Hydrogels: The Next Paradigm Shift in Tissue Engineering? *Advanced Science*, *1801664*.

Tam, R. Y., Fuehrmann, T., Mitrousis, N., & Shoichet, M. S. (2014). Regenerative therapies for central nervous system diseases: a biomaterials approach. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *39*(1), 169–188.

Tang, Z., Zhang, Y., Wang, Y., Zhang, D., Shen, B., Luo, M., & Gu, P. (2017). Progress of stem/progenitor cell-based therapy for retinal degeneration. *Journal of Translational Medicine*, *15*(1), 99.

Tanner, C. M. (2000). Dopamine Agonists in Early Therapy for Parkinson Disease. *JAMA*, *284*(15), 1971.

Tanner, C. M., Kamel, F., Ross, G. W., Hoppin, J. A., Goldman, S. M., Korell, M., Marras, C., Bhudhikanok, G. S., Kasten, M., Chade, A. R., Comyns, K., Richards, M. B., Meng, C., Priestley, B., Fernandez, H. H., Cambi, F., Umbach, D. M., Blair, A., Sandler, D. P., Langston, J. W. (2011). Rotenone, paraquat, and Parkinson's disease. *Environmental Health Perspectives*, *119*(6), 866–872.

Tatard, V. M., Sindji, L., Branton, J. (Godbee), Aubert-Pouëssel, A., Colleau, J., Benoit, J. P., & Montero-Menei, C. N. (2007). Pharmacologically active microcarriers releasing glial cell line - derived neurotrophic factor: Survival and differentiation of embryonic dopaminergic neurons after grafting in hemiparkinsonian rats. *Biomaterials*, *28*(11), 1978–1988.

Taubenberger, A. V., Woodruff, M. A., Bai, H., Muller, D. J., & Hutmacher, D. W. (2010). The effect of unlocking RGD-motifs in collagen I on pre-osteoblast adhesion and differentiation. *Biomaterials*, *31*(10), 2827–2835.

Thompson, C. D., Zurko, J. C., Hanna, B. F., Hellenbrand, D. J., & Hanna, A. (2013). The therapeutic role of interleukin-10 after spinal cord injury. *Journal of Neurotrauma*, *30*(15), 1311–1324.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., & Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science (New York, N.Y.)*, *282*(5391), 1145–1147.

Tolosa, E., Wenning, G., & Poewe, W. (2006). The diagnosis of Parkinson's disease. *The Lancet Neurology*, *5*(1), 75–86.

Tomac, A., Widenfalk, J., Lin, L. F., Kohno, T., Ebendal, T., Hoffer, B. J., & Olson, L. (1995). Retrograde axonal transport of glial cell line-derived neurotrophic factor in the adult nigrostriatal system suggests a trophic role in the adult. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(18), 8274–8278.

Torper, O., Pfisterer, U., Wolf, D. A., Pereira, M., Lau, S., Jakobsson, J., Björklund, A., Grealish, S., & Parmar, M. (2013). Generation of induced neurons via direct conversion in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(17), 7038–7043.

Torres, E. M., Dowd, E., & Dunnett, S. B. (2008). Recovery of functional deficits following early donor age ventral mesencephalic grafts in a rat model of Parkinson's disease. *Neuroscience*, *154*(2), 631–640.

Torres, E. M., Monville, C., Lowenstein, P. R., Castro, M. G., & Dunnett, S. B. (2005). Delivery of sonic hedgehog or glial derived neurotrophic factor to dopamine-rich grafts in a rat model of Parkinson's disease using adenoviral vectors Increased yield of dopamine cells is dependent on embryonic donor age. *Brain Research Bulletin*, *68*(1–2), 31–41.

Trupp, M., Belluardo, N., Funakoshi, H., & Ibáñez, C. F. (1997). Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor-alpha indicates multiple mechanisms of trophic actions in the adult rat CNS. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *17*(10), 3554–3567.

Tuladhar, A., Payne, S. L., & Shoichet, M. S. (2018). Harnessing the Potential of Biomaterials for Brain Repair after Stroke. *Frontiers in Materials*, *5*(March), 1–25.

Ungerstedt, U., & Arbuthnott, G. W. (1970). Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. *Brain Research*, *24*(3), 485–493.

Vallejo-Giraldo, C., Pugliese, E., Larrañaga, A., Fernandez-Yague, M. A., Britton, J. J., Trotier, A., Tadayyon, G., Kelly, A., Rago, I., Sarasua, J. R., Dowd, E., Quinlan, L. R., Pandit, A., & Biggs, M. J. P. (2016). Polyhydroxyalkanoate/carbon nanotube nanocomposites: Flexible electrically conducting elastomers for neural applications. *Nanomedicine*, *11*(19), 2547–2563.

Van der Mark, M., Brouwer, M., Kromhout, H., Nijssen, P., Huss, A., & Vermeulen, R. (2012). Is pesticide use related to Parkinson disease? Some clues to heterogeneity in study results. *Environmental Health Perspectives*, *120*(3), 340–347.

Vázquez-Portalati N, N., Kilmer, C. E., Panitch, A., & Liu, J. C. (2016). Characterization of Collagen Type I and II Blended Hydrogels for Articular Cartilage Tissue Engineering. *Biomacromolecules*, *17*(10), 3145–3152.

Veronese, F. M., & Pasut, G. (2005). PEGylation, successful approach to drug delivery. *Drug Discovery Today*, *10*(21), 1451–1458.

Vierbuchen, T., Ostermeier, A., Pang, Z. P., Kokubu, Y., Südhof, T. C., & Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. *Nature*, *463*(7284), 1035–1041.

Wang, T.-Y., Bruggeman, K. F., Kauhausen, J. A., Rodriguez, A. L., Nisbet, D. R., & Parish, C. L. (2016). Functionalized composite scaffolds improve the engraftment of transplanted dopaminergic progenitors in a mouse model of Parkinson's disease. *Biomaterials*, *74*, 89–98.

Wenning, G. K., Odin, P., Morrish, P., Rehncrona, S., Widner, H., Brundin, P., Rothwell, J. C., Brown, R., Gustavii, B., Hagell, P., Jahanshahi, M., Sawle, G., Björklund, A., Brooks, D. J., Marsden, C. D., Quinn, N. P., & Lindvall, O. (1997). Short- and long-term survival and function of unilateral intrastriatal dopaminergic grafts in Parkinson's disease. *Annals of Neurology*, *42*(1), 95–107.

Wernig, M., Zhao, J. P., Pruszak, J., Hedlund, E., Fu, D., Soldner, F., Broccoli, V., Constantine-Paton, M., Isacson, O., & Jaenisch, R. (2008). Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(15), 5856–5861.

Whone, A., Boca, M., Luz, M., Woolley, M., Mooney, L., Dharia, S., Broadfoot, J., Cronin, D., Schroers, C., Barua, N. U., Longpre, L., Barclay, C. L., Boiko, C., Johnson, G. A., Fibiger, H. C., Harrison, R., Lewis, O., Pritchard, G., Howell, M., Irving, C., Johnson, D., Kinch, S., Marshall, C., Lawrence, A. D., Blinder S., Sossi, V., Stoessl J., Skinner P., Mohr, E., Gill, S. S. (2019a). Extended Treatment with Glial Cell Line-Derived Neurotrophic Factor in Parkinson's Disease. *Journal of Parkinson's Disease*, *9*(2), 301–313.

Whone, A., Luz, M., Boca, M., Woolley, M., Mooney, L., Dharia, S., Broadfoot, J., Cronin, D., Schroers, C., Barua, N. U., Longpre, L., Barclay, C. L., Boiko, C., Johnson, G. A., Fibiger, H. C., Harrison, R., Lewis, O., Pritchard, G., Howell, M., Irving, C., Johnson, D., Kinch, S., Marshall, C., Lawrence, A. D., Blinder S., Sossi, V., Stoessl J., Skinner P., Mohr, E., Gill, S. S. (2019b). Randomized trial of intermittent intraputamenal glial cell line-derived neurotrophic factor in Parkinson's disease. *Brain : A Journal of Neurology*, *142*(3), 512–525.

Wijeyekoon, R., & Barker, R. A. (2009). Cell replacement therapy for Parkinson's disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1792*(7), 688–702.

Winkler, C., Sauer, H., Lee, C. S., & Björklund, A. (1996). Short-term GDNF treatment provides long-term rescue of lesioned nigral dopaminergic neurons in a rat model of Parkinson's disease. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *16*(22), 7206–7215.

Wolk, K., Kunz, S., Asadullah, K., & Sabat, R. (2002). Cutting edge: immune cells as sources and targets of the IL-10 family members? *Journal of Immunology (Baltimore, Md. : 1950)*, *168*(11), 5397–5402.

Wolk, K., Kunz, S., Witte, E., Friedrich, M., Asadullah, K., & Sabat, R. (2004). IL-22 increases the innate immunity of tissues. *Immunity*, *21*(2), 241–254.

Wood, M. J. A., Sloan, D. J., Wood, K. J., & Charlton, H. M. (1996). Indefinite survival of neural xenografts induced with anti-CD4 monoclonal antibodies. *Neuroscience*, *70*(3), 775–789.

Xicoy, H., Wieringa, B., & Martens, G. J. M. (2017). The SH-SY5Y cell line in Parkinson's disease research: a systematic review. *Molecular Neurodegeneration*, *12*(1), 10.

Yan, J., Fu, Q., Cheng, L., Zhai, M., Wu, W., Huang, L., & Du, G. (2014). Inflammatory response in Parkinson's disease (Review). *Molecular Medicine Reports*, *10*(5), 2223–2233.

Yoon, S. Il, Logsdon, N. J., Sheikh, F., Donnelly, R. P., & Walter, M. R. (2006). Conformational changes mediate interleukin-10 receptor 2 (IL-10R2) binding to IL-10 and assembly of the signaling complex. *The Journal of Biological Chemistry*, *281*(46), 35088–35096.

Yurek, D. M. (1998). Glial cell line-derived neurotrophic factor improves survival of dopaminergic neurons in transplants of fetal ventral mesencephalic tissue. *Experimental Neurology*.

Zawada, W. M., Zastrow, D. J., Clarkson, E. D., Adams, F. S., Bell, K. P., & Freed, C. R. (1998). Growth factors improve immediate survival of embryonic dopamine neurons after transplantation into rats. *Brain Research*, *786*, 96–103.

Zelenay, S., & Reis e Sousa, C. (2013). Adaptive immunity after cell death. *Trends in Immunology*, *34*(7), 329–335.

Zhou, Z., Peng, X., Insolera, R., Fink, D. J., & Mata, M. (2009). Interleukin-10 provides direct trophic support to neurons. *Journal of Neurochemistry*, *110*(5), 1617–1627.

Zhu, J., Yamane, H., & Paul, W. E. (2010). Differentiation of effector CD4 T cell populations (*). *Annual Review of Immunology*, *28*, 445–489.

Zhu, Y., Chen, X., Liu, Z., Peng, Y.-P., & Qiu, Y.-H. (2015). Interleukin-10 Protection against Lipopolysaccharide-Induced Neuro-Inflammation and Neurotoxicity in Ventral Mesencephalic Cultures. *International Journal of Molecular Sciences*, *17*(1), 25.

Zuidema, J. M., Rivet, C. J., Gilbert, R. J., & Morrison, F. A. (2014). A protocol for rheological characterization of hydrogels for tissue engineering strategies. *Journal of Biomedical Materials Research - Part B Applied Biomaterials*, *102*(5), 1063–1073.

Zustiak, S. P., Wei, Y., & Leach, J. B. (2013). Protein-hydrogel interactions in tissue engineering: mechanisms and applications. *Tissue Eng Part B Rev*, *19*(2), 160–171.

Appendix I: Buffers for Perfusion –Fixation

To perfuse animals, PFA/PBS solution and heparinised saline are needed. To fixate and preserve samples, these are kept in a sucrose solution or TBS azide solutions.

1. 0.2M Phosphate buffer solution

Stock A
Sodium dihydrogen phosphate monohydrate (NaH_2PO4 x H_2O MW=137.99)

Stock B
Disodium hydrogen phosphate dehydrate (Na_2HPO_4 x $2H_2O$ MW=177.99)

→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 stocks in 2000 ml of d H_2O.
Note: To be used in a 1:1 dilution with PFA.

2. Fixative: 4% Paraformaldehyde (4L) (in fume hood)

1. Heat 1.5L of d H_2O to 60 °C.
2. Add 160g of PFA powder.
3. Add a few NaOH pellets in order to dissolve PFA.
4. Stir until clear.
5. Fill to 2L with d H_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.

2. <u>Heparinised Saline</u>

1 ml heparin is added per 1L of saline.

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

3. **<u>25% w/v Sucrose Solution (1L)</u>**

1. Dissolve 5 PBS tablets in ~500ml dH_2O (1 tablet per 200ml water) .
2. Add 250g of sucrose.
3. Stir until dissolved, apply heat if necessary.
4. Make up to 1L with dH_2O.

4. **<u>0.1% w/v TBS-Azide</u>**

1. Weigh out 12g Trizma Base, 9g NaCl, 1g Sodium Azide.
2. Add to 1L dH_2O and dissolve.
3. pH to 7.4.

Appendix II – Immunohystochemistry Protocol

Solutions for immunohistochemistry

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

General Immunohistochemistry Protocol

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

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

Step by step process

Day 1

1. Wash 1*5 min in TBS.
2. Quench for 5 min.
   - Methanol 5ml
   - 30% H_2O_2 5ml
   - Distilled H_2O 40ml
3. Wash 3*5 min with TBS.
4. Incubate sections in 3% NHS or NGS for 60min.
   - 30µl/ml NHS or NGS in TXTBS (freshly made-up)
5. Draw off excess and incubate in primary in 1% NHS or NGS at RT overnight.

Day 2

6. Wash 3*10 min with TBS.
7. Incubate in biotinylated secondary in 1% NHS or NGS for 3 hours.
8. Wash 3*10 min with TBS. Make ABC Complex when you start these washes. Must be made 30 mins before incubating the tissue in it. Cover the tubes with tin foil.

   - 5µl of solution A and 5µl of solution B per ml in 10µl/ml NHS or NGS in TBS.

9. Incubate in ABC Complex for 2 hours. Keep the pots covered with tin foil.
10. Wash 3*10 min with TBS.
11. Wash with TNS (freshly made-up) leave overnight @ 4 °C.
12. In the fume hood, incubate in H_2O_2/DAB solution until colour develops (No more than 5 mins). First, use only one pot per immuno to determine the time and darkness of staining. Check the tissue then proceed with the rest of the pots.

    - TNS 40ml
    - DAB 20mg (frozen in 2ml aliquots).
    - 30% H_2O_2 12µl

13. In the fume hood wash 3*5min with TNS.
14. Mount (in TBS with a little TXTBS) on gelatin-coated slides, air dry overnight.
15. Dehydrate slides in ascending series of alcohols:

    - 50% ethanol for 5 min
    - 70% ethanol for 5 min
    - 100% ethanol for 5 min
    - 100% ethanol for 5 min

16. Clear in xylene in the fume hood:

    - 100% xylene for 5 min
    - 100% xylene for 5 min

17. Coverslip using DPX mountant.

Slide Subbing

Materials

- Gelatin (10 g/L)
- Chromic potassium sulphate (500 mg/L)
- Distilled H_2O
- Slides

Method

1. Heat H_2O 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 ~30°C.

4. Slides are placed in holders and dipped into subbing medium for ~1 min.

5. Remove slides and allow to dry on aluminium foil for ~ 1 week.

Appendix III – 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.

Step by step process

Day 1

1. Wash wells 1 x 5 min in TBS.

2. Incubate cells in blocking solution for 1 hour at room temperature.

<u>40ml Blocking Solution</u>
0.4 g Bovine Serum Albumin
2 ml Serum* (*serum dependent on 2o host)
120 µl Triton X-100 (0.3% is final conc.)
40 µl Sodium Azide (10% Stock Solution (0.01% is final concentration))
37.84 ml TBS

3. Draw off blocking solution and incubate wells in primary antibody at room temperature overnight (i.e. 1:1000):

-1 µl/ml of 1° + 1 µl/ml sodium azide (from 10% stock solution)
in 998 µl/ml TBS containing 1% BSA

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 (i.e. 1:200):

-5 µl/ml of o + 10 µl/ml serum* (*serum dependent on 2o host)
in 985 µl/ml TBS

7. Wash wells 3 x 5 min with TBS.

8. Incubate sections in 1 µg/ml DAPI in TBS for ~5 min.

9. Wash wells 3 x 10 min TBS.

10. Store wells in 0.1% TBS Azide in the fridge.