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<th>The potential of injectable collagen hydrogels to enhance dopaminergic cell replacement therapies for Parkinson's disease</th>
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The Potential of Injectable Collagen Hydrogels to Enhance Dopaminergic Cell Replacement Therapies for Parkinson’s Disease

Niamh Moriarty

Supervisor: Dr. Eilís Dowd

Pharmacology & Therapeutics
National University of Ireland, Galway

Doctor of Philosophy

September 2018
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:

Generation, differentiation and characterisation of human iPSC-derived dopaminergic precursors was performed by Ms. Yixi Chen and Dr. Tilo Kunath, Centre for Regenerative Medicine, The University of Edinburgh.

Signed: ………………………………………………… Date: …………………
Acknowledgements

First and foremost, I would like to express my sincere gratitude to my supervisor Dr. Eilís Dowd for her endless support and encouragement, both personally and professionally. It has been a difficult, but extremely rewarding, 4 years and it goes without saying that I would not be in the position of writing these final words without your unwavering mentorship. We have come a long way from those early days of arguing over GDNF experimental designs (aren’t we glad I insisted on those pilot studies now 😊) and I consider it a pleasure to have had the opportunity to work with you. I will be eternally grateful for everything you have done for me, thank you wholeheartedly.

To the staff of the pharmacology department, it takes a special group of people to create such a supportive and enabling environment for students. Thank you all for your encouragement, words of advice and laughter over the last 8 years – I will be sure to come back as a guest judge for the next tiramisu-off! To the members of the CNS lab, past and present, I don’t have the words to describe you beautiful bunch of people. There is a sense of comradery in this lab, that goes unmatched in comparison to anywhere I have experienced before, and I hope that never changes. I will forever cherish the memories we have made, the stories we have shared, and the many moments of uncontrolled laughter. Let’s not become strangers - if you ever fancy a cheeky mineral, I’ll meet you in the Front Door! Dunkey Kerr, what can I say, you’re the glue that keeps our lab together. Over the last 4 years you have been my science guru, my mechanic, my GAA rival, and my friend. The man who found this little Dub a golden all-Ireland ticket will never be forgotten, thank you for everything. Amby, I will fondly remember all your words of wisdom, the place isn’t the same without you. To my fellow Dowds, and Dowd half-sister (Yes Laura, I’m talking about you) – you’re all exceptional. It has been one hell of a journey, full of laughter, sometimes tears, often chaos, but always banter. I could not have asked for a better group of girls to share these 4 years with and I will treasure your friendships forever.

To my original girls - Shauna, Teresa, Deirdre, Kim and Lauren – it’s rare to find a group of people who are equally as crazy as you, and I count myself extremely lucky. Thank you for always being so understanding, constantly listening to my complaints, keeping me sane, and most of all always being there for me. I will never have the words to express how grateful I am – love ye!

Carol and Ruth, are there even words? I knew this PhD was going to be a completely new adventure, little did I know the adventures that awaited. Out of all the things that I have gained
from this PhD, I hold our friendship the closest. You’re incredible women - my science inspirations, my role models, and most importantly my “fun as shit” best sister-friends. From gypsy costumes, to pizza, wine, nuggets and unforgettable trips, it’s always grade A banter and I wouldn’t change it for the world. I love you more than anything and I can’t wait to see what’s next for Three Doctors and Fetus ♥. And Kelly, the original K.McH in my life, it’s hard to tell if we’re bad influences on each other or the best influences. From walks on the prom, to gin fests, to 6 nation days, to trips to Croker and so many random nights, it has been crazy to say the least. Going for “one” just won’t be the same without you. Nat, I hope you keep her “day sesh” needs going in my absence! And don’t worry, “Zaz and friends” will be back for many more celebrations, we won’t abandon you both.

Kieran, from day one it has been the three of us and I think it’s time to finally let the thesis go. Although, I don’t know what I’ll blame all my crazy on now! You were always in my corner, to offer advice, encouragement, much needed perspective, endless cups of tea, and often just a wee hug when needed. I appreciate everything you have done for me – you’re the best person I know. I hate to admit it, but you were right, we have sooo got this!! Now for the next adventure... Xx

To my family - Dad, Sean, Ciara and Nana Betty, your eternal support, love and encouragement has made me the person I am today and none of this would have been possible without you. Papa MOR, the one and only, thank you for being our anchor and always pushing us to achieve our best. The MOR household can be a crazy place, but it’s the best kind of crazy, our crazy. Mam, I truly wish you were here to share this achievement with me. You always knew what I was capable of and instilled a sense of belief in me that I have carried with me to this day. I hope I have made you proud – this is for you.
“But you know, happiness can be found even in the darkest of times, if one only remembers to turn on the light.”

_J.K. Rowling, Harry Potter and the Prisoner of Azkaban_
Abstract

Extensive pre-clinical and clinical assessment has shown that, when transplanted into the Parkinsonian brain, primary dopaminergic neurons can survive, integrate with the host system, produce dopamine and provide functional recovery. However, their widespread use as a routine clinical procedure is hindered by their extremely poor survival after transplantation and the subsequent requirement to use multiple fetal donors for each Parkinson’s disease patient. Injectable biomaterial scaffolds, particularly collagen hydrogels, have the potential to improve the engraftment of encapsulated cells through the provision of a supportive and growth factor-rich environment that can shield cells from the external environment. Thus, the overarching aim of this project was to assess the effect of a glial-derived neurotrophic factor (GDNF)-loaded collagen hydrogel on the long-term survival and efficacy of dopaminergic neurons after transplantation into the Parkinsonian brain.

Through a series of preliminary in vitro and in vivo studies, we first optimised a collagen hydrogel for the intra-cranial delivery of dopaminergic neurons. Following this, we assessed whether the encapsulation of primary dopaminergic neurons (derived from the developing ventral mesencephalon (VM)) in a GDNF-loaded collagen hydrogel could enhance their survival, re-innervative capacity and function after transplantation. Based on these results, we investigated the potential of the GDNF-loaded collagen hydrogel to enhance the survival and efficacy of human induced pluripotent stem cell (iPSC)-derived dopaminergic neurons.

In brief, we found that crosslinked collagen hydrogels were well tolerated in the brain and supported the survival and neural outgrowth of encapsulated cells. Moreover, we demonstrated that the encapsulation of primary dopaminergic neurons within a collagen hydrogel attenuated the host immune response to the transplanted cells, and that the encapsulation of GDNF in our collagen hydrogel resulted in a significantly greater
retention of striatal GDNF immediately post-transplantation. Together, these preliminary findings demonstrated that crosslinked collagen hydrogels possessed attractive characteristics that warranted further investigation into their ability to enhance long-term dopaminergic cell transplantation strategies. Building on this, we found that the encapsulation of embryonic day (E) 14 VM cells in a GDNF-loaded collagen hydrogel could dramatically improve the survival (5-fold), re-innervation (3-fold) and functionality of primary dopaminergic neurons after transplantation into the Parkinsonian brain. Furthermore, the encapsulation of VM cells derived from younger embryonic donors (including their meningeal layer), namely E12, also resulted in a dramatic improvement in the survival (4-fold), re-innervation (5-fold) and functionality of dopaminergic neurons after transplantation. However, unfortunately the assessment of iPSC-derived dopaminergic cell survival and efficacy was impeded by widespread graft rejection that was seen across all groups.

In conclusion, GDNF-loaded collagen hydrogels can improve the efficacy of primary dopaminergic neuron cell replacement therapies in Parkinson’s disease by providing cells with a supportive environment throughout delivery, increased trophic factor support upon transplantation and the attenuation of the host immune response. While these collagen hydrogel scaffolds show great potential to enhance such neurorestorative approaches in Parkinson’s disease, further studies are required to assess their potential to enhance stem cell based cell replacement therapies.
Publications

*Peer Reviewed Original Research Manuscripts and Reviews*


- **N Moriarty**, A Pandit, E Dowd (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, Volume 7, Article number: 16033*

**Peer Reviewed Published Abstracts**


Other Research Dissemination

International Conferences

- **N Moriarty**, E Dowd. A GDNF-loaded collagen hydrogel for the delivery of dopaminergic neurons. **Oral presentation** at the 14th International meeting on Neural Transplantation and Repair (INTR) in Port Douglas, Australia. September 2017


- **LK Olsen**, AG Cairns, J Ádén, **N Moriarty**, S Cabre, V Alamilla, F Almqvist, E Dowd, DP McKernan. Viral-like neuroinflammatory priming exacerbates α-synuclein aggregation-induced Parkinsonism in rats: Implications for a viral etiology of Parkinson’s disease. Federation of Neuroscience Societies (FENS), Berlin, Germany. July 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. Federation of Neuroscience Societies (FENS), Berlin, Germany. July 2018


• J Samal, N Moriarty, D.B Hoban, C Naughton, R.M Concannon, E Dowd, A Pandit. Fibrin-based Hollow Microsphere Reservoirs for Controlled Delivery of Neurotrophic Factors to the Brain. 4th TERMIS World Congress, Boston, Massachusetts. September 2015

National Conferences

• N Moriarty, A Pandit, E Dowd. A GDNF-functionalised biomaterial matrix for the delivery of primary dopaminergic neurons. Oral presentation at Neuroscience Ireland Conference, National University of Ireland, Galway, Ireland. September 2017
• N Moriarty, A Pandit, E Dowd. The encapsulation of primary dopaminergic neurons in a GDNF-loaded collagen hydrogel dramatically increases their survival and re-innervation. Poster presentation at the Brain Research Ireland Conference, Trinity College Dublin, Ireland. March 2017

• N Moriarty, A Pandit, E Dowd. The delivery of ventral mesencephalon cells encapsulated in a collagen hydrogel reduced the host immune response to the transplanted graft. Poster presentation at the Young Neuroscientists Symposium Ireland, Trinity College Dublin, Ireland. September 2016

• N Moriarty, A Pandit, E Dowd. An injectable collagen hydrogel for the delivery of ventral mesencephalon cells into the Parkinsonian brain. Poster presentation at Neuroscience Ireland Conference, Dublin City University, Ireland. September 2015


**List of commonly used abbreviations**

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ALDH</td>
<td>aldehyde dehydrogenase</td>
</tr>
<tr>
<td>AP</td>
<td>anterio-posterior</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain-barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotropic factor</td>
</tr>
<tr>
<td>CD11b</td>
<td>integrin alpha M/ CR3</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>COMT</td>
<td>catechol-O-methyl transferase</td>
</tr>
<tr>
<td>D</td>
<td>differentiation stage</td>
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<td>DAB</td>
<td>diaminobenzidine tetra hydrochloride</td>
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<tr>
<td>DAT</td>
<td>dopamine transporter</td>
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<tr>
<td>DBS</td>
<td>deep brain stimulation</td>
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<tr>
<td>°C</td>
<td>degrees celsius</td>
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<tr>
<td>DOPAC</td>
<td>3,4-dihydroxyphenyl-acetaldehyde</td>
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<tr>
<td>DV</td>
<td>dorso-ventral</td>
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<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular membrane</td>
</tr>
<tr>
<td>ENS</td>
<td>enteric nervous system</td>
</tr>
<tr>
<td>ESC</td>
<td>embryonic stem cell</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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g  gram
GABA  gamma (γ)-aminobutyric acid
GDNF  glial-derived neurotrophic factor
GFAP  glial fibrillary acidic protein
GFP  green fluorescent protein
GFR  GDNF family receptor
GID  graft induced dyskinesia
Gpe  globus pallidus externa
Gpi  globus pallidus interna
GIT  gastrointestinal
h  hour
HBSS  Hank’s Balanced Salt Solution
HLA  Human leukocyte antigen
HuNu  Human Nuclear Antigen
HVA  homovanillic acid
H₂O₂  hydrogen peroxide
ICC  immunocytochemistry
i.p  intraperitoneal
iPSC  induced pluripotent stem cell
IHC  immunohistochemistry
IF  immunofluorescence
IL  interleukin
INF  interferon
kg  kilogram
LID  Levodopa-induced dyskinesia
<table>
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<tr>
<td>LRRK2</td>
<td>leucine-rich repeat kinase 2</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>MFB</td>
<td>medial forebrain bundle</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>ML</td>
<td>medial-lateral</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>MSN</td>
<td>medium spinal neurons</td>
</tr>
<tr>
<td>3-MT</td>
<td>methoxytyramine</td>
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<tr>
<td>µg</td>
<td>microgram</td>
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<td>µl</td>
<td>microliter</td>
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<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mg/kg</td>
<td>milligram per kilogram</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
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<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
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<td>MPP+</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
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</tr>
<tr>
<td>nm</td>
<td>nanometre</td>
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>OX42</td>
<td>integrin alpha M</td>
</tr>
<tr>
<td>PND</td>
<td>postnatal day</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
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<td>Abbreviation</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-induced putative kinase 1</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error ± mean</td>
</tr>
<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>SNpc</td>
<td>substantia nigra pars compacta</td>
</tr>
<tr>
<td>SNpr</td>
<td>substantia nigra pars reticulata</td>
</tr>
<tr>
<td>STR</td>
<td>striatum</td>
</tr>
<tr>
<td>STN</td>
<td>subthalamic nucleus</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>UPDRS</td>
<td>Unified Parkinson’s Disease Rating Scale</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
</tr>
<tr>
<td>VM</td>
<td>ventral mesencephalon</td>
</tr>
<tr>
<td>4s-StarPEG</td>
<td>poly(ethylene glycol) ether tetrasuccinimidyl glutarate</td>
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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

200 years after James Parkinson’s essay on “The Shaking Palsy” first described the clinical syndrome that would later bare his name, the treatment of Parkinson’s disease is still solely symptomatic and does not target the underlying process of neurodegeneration (Parkinson, 1817). Since its discovery over 60 years ago, the dopamine precursor, levodopa, remains the ‘gold-standard’ treatment (LeWitt et al., 2016). Despite its ability to provide superlative results in the early stages of the disease, its long-term use is limited by the generation of dyskinesias and a significant abating of therapeutic effect (Jankovic, 2002). Therefore, there is a major unmet clinical need for new and improved therapies that not only symptomatically treat the disease, but ultimately repair the diseased brain.

The relatively selective loss of dopaminergic neurons from the nigrostriatal pathway makes Parkinson’s disease an ideal candidate for cell replacement therapies. To date, the focus of cell replacement therapies in Parkinson’s disease has been on the transplantation of dopamine neuron-rich fetal ventral mesencephalon (VM) grafts which have shown to both survive and re-innervate the striatum post-transplantation, whilst also restoring motor function (Brundin et al., 1987; Freed et al., 1990; Kordower et al., 1995; Lindvall et al., 1989; Olanow et al., 2003). However, despite long-term symptomatic relief in some patients, significant limitations, particularly poor survival post-transplantation, prevent this therapy being utilised as a potential restorative approach for Parkinson’s disease (Barker et al., 2013).

Biomaterials - that is, materials that have been specifically engineered to interact with living systems for therapeutic purposes - have the potential to substantially improve brain repair approaches for Parkinson’s disease. Structural biomaterials can be used as scaffolds
to provide a supportive matrix for transplanted cells, and can also be functionalised for
delivery of therapeutic molecules that can enhance the survival, axonal outgrowth and
connectivity of transplanted cells (Orive et al., 2009). In particular, naturally-derived
biomaterials, such as collagen hydrogels, hold the advantage of being characteristically
similar to the body’s native tissue, making them highly biocompatible and biodegradable,
while also naturally supporting cell adhesion (Khan et al., 2013). Collagen is also capable
of forming in situ gelling (and therefore injectable) hydrogels, thus making it an attractive
candidate for improving cell replacement therapies in neurodegenerative disorders such as
Parkinson’s disease. Furthermore, collagen-derived biomaterials have the benefit of
already having clinical approval for a wide variety of applications. Thus, in theory,
collagen hydrogels have the potential to increase the engraftment of cells by intervening at
various points throughout the transplantation process where cell death occurs, such as, 1)
providing a supportive matrix environment for cell adhesion, 2) providing a reservoir for
localised growth factor delivery, and 3) creating a physical barrier between the
transplanted cells and the host neuro-immune cells (Fig 1.1).

With this in mind, the work described in this thesis sought to determine the impact of an
injectable, growth factor-loaded collagen hydrogel on the survival, integration and efficacy
of transplanted dopaminergic neurons and thus shed light on the potential of biomaterials
to improve such reparative therapies in Parkinson’s disease and other neurodegenerative
diseases.

This introductory chapter will provide an evidence-based overview of cell-replacement
therapy in Parkinson’s disease, the issues that have hampered its clinical translation and the
potential of biomaterial scaffolds to enhance its efficacy. For a general overview of
Parkinson’s disease, see Appendix 1.
Figure 1.1. Therapeutic concept of biomaterials for brain repair in Parkinson’s disease. Encapsulation of transplanted dopaminergic neurons in a glial-derived neurotrophic factor (GDNF)-loaded collagen hydrogel could improve brain repair in Parkinson’s disease through a number of different mechanisms. These include provision of 1) a physical scaffold for cell adhesion during intracerebral delivery and engraftment, 2) a local reservoir for GDNF at the implantation site, and 3) a protective barrier against the host immune response.
1.1 CELL REPLACEMENT THERAPY FOR PARKINSON’S DISEASE

1.1.1 RATIONALE FOR CELL REPLACEMENT THERAPY

The absence of a current therapeutic treatment that targets the underlying neurodegeneration of Parkinson’s disease has led to a surge of research into the search for novel therapies that can repair the diseased brain. Cell replacement therapy has emerged from a relatively simple conceptual framework - if the primary pathological feature of a disorder is the degeneration and subsequent loss of a specific neuronal subtype, then the replacement of such cells should in theory repair the brain and restore function to the patient. The relatively selective loss of dopaminergic neurons from the substantia nigra makes Parkinson’s disease an ideal candidate for cell replacement therapy (Fearnley et al., 1991). The transplantation of dopaminergic neurons to the nigrostriatal pathway of diseased brains holds enormous potential to restore lost dopamine transmission in the striatum and therefore ameliorate the motor complications experienced by patients and thus significantly improve their quality of life.

1.1.2 NON-VM SOURCES OF DOPAMINERGIC NEURONS FOR TRANSPLANTATION

Various cell based strategies have been explored as a treatment option for Parkinson’s disease (Behari et al., 2011). While all are based on the transplantation of dopamine rich cells, tissue source varies from autologous to allogeneic, and from fetal/embryonic to adult. This section will briefly cover some of the non-VM tissue sources of dopamine precursor cells that have been investigated for cell replacement therapy in Parkinson’s disease.
Chapter 1: Introduction

Adrenal medullary tissue

The rationale that autologous grafts would be immunologically compatible and thus reduce any donor-to-host disease risk factors and/or immune rejection led to the transplantation of autologous adrenal medullary tissue (Olson et al., 1970). While the transfer of catecholamine-secreting cells was successful, transplantation into the 6-hydroxydopamine (6-OHDA) rat model (Freed et al., 1981) and MPTP non-human primate model (Morihisa et al., 1984) showed limited cell survival. The first human trials of adrenal transplant showed significant clinical improvement (Backlund et al., 1985; Madrazo et al., 1987) and were subsequently followed by a larger multi-center study (Goetz et al., 1989). Here Goetz et al. (1989), found an increase in the mean “on” time and a decrease in the mean “off” time, although the dose of anti-Parkinsonian medications could not be lowered. Unfortunately, clinical improvement lasted only 18 months (Olanow et al., 1990) and post mortem analysis 16 years later showed poor transplant survival (Kompoliti et al., 2007).

Sympathetic ganglion cells

In a similar manner, transplantation of autologous sympathetic ganglion cells from the superior cervical ganglion showed a marked improvement in the rotational behavior of 6-OHDA lesioned rats (Nakao et al., 1995). When moved to the clinic, transplantation of autologous sympathetic ganglion to 35 patients displayed improvements in bradykinesia and gait disturbances in only half of patients, while tremor and rigidity remained unaffected (Itakura et al., 1997).

The carotid body

Another source of dopamine rich cells is the carotid body, and transplantation of such cells has shown significant motor improvement in both rat (Espejo et al., 1998) and non-human
primate models (Luquin et al., 1999). Bilateral transplantation of 12 patients with carotid body cell aggregates resulted in clinical improvement in 10 patients (Minguez-Castellanos et al., 2007). 6 months post-transplant there was a mean increase in UPDRS score of 23%, however after three years this improvement had dramatically declined and only 3 patients exhibited any improvements. The acute clinical improvements were thought to be as a result of trophic factor release, as opposed to an increase in dopamine transmission.

Despite the considerable effort to provide cell replacement strategies for Parkinson’s disease that do not involve the use of human embryonic tissue, the transplantation of primary dopaminergic cells derived from the fetal VM remains the gold standard in the field of cell replacement therapy. From here, focus will therein be placed on the preclinical and clinical data obtained thus far on the transplantation of fetal primary dopaminergic grafts. Subsequently, the limitations associated with the use of fetal VM tissue will be described as these underpin the need for advancements in fetal VM cell delivery and the investigation of stem cells as an alternative source for dopaminergic neurons.

1.1.3 TRANSPLANTATION OF PRIMARY DOPAMINERGIC NEURONS

1.1.3.1 Pioneering studies

The transplantation of primary dopaminergic neurons began in the 1970s when Bjorklund and colleagues transplanted small pieces of tissue (containing dopaminergic, serotonergic and noradrenergic neurons) dissected from the fetal rat brain into the cerebral cortex and hippocampus of unlesioned adult rats. These cells showed good survival after transplantation and substantial axonal outgrowth that could form extensive fiber patterns within the graft itself and with adjacent brain tissue (Bjorklund et al., 1976; Stenevi et al., 1976). During this time, Ungerstedt & Arbuthnott developed the 6-OHDA rat model of
Parkinson’s disease, which through the selective degeneration of dopaminergic neurons along the nigrostriatal pathway generates motor deficits that can be quantified by asymmetric rotational behavior following amphetamine administration (Ungerstedt et al., 1970). This model gave experimenters the opportunity to explore the transplantation of dopamine neuron-rich fetal tissue to the degenerated striatum. Perlow et al., (1979b) transplanted solid tissue pieces to the lateral ventricle in contact with the caudate-putamen, while Björklund et al., (1980a) transplanted solid tissue pieces to the dorsal cortical cavity overlying the caudate-putamen. Both studies noted improvement in amphetamine-induced rotational behavior and post mortem analysis revealed survival and re-innervation of the lesioned striatum (Björklund et al., 1980a; Perlow et al., 1979b). A setback to these original studies was the use of pieces of dissected brain tissue, necessitating the need to create a highly vascularised cavity within the brain, while also preventing the targeting of deep brain structures (Dunnett et al., 1981). This led to the generation and use of dissociated cell suspensions, which allowed for cell transplantation at multiple sites of the striatum, leading to more widespread innervation and better functional recovery in rotational behavior as well as sensorimotor tests (Björklund et al., 1980b; Björklund et al., 1983; Schmidt et al., 1983). Interestingly, it was shown that functional recovery caused by the transplantation of fetal tissue was specific to cells derived from the dopamine rich VM. This confirmed that the effects seen were dependent on dopamine replacement in the striatum and not non-specific stimulation by the fetal tissue (Dunnett et al., 1988a).

From here, the field of cell replacement therapy in Parkinson’s disease rapidly expanded. Numerous studies corroborated the efficacy of VM grafts to restore dopamine transmission and improve both motor and non-motor function in the 6-OHDA hemi-Parkinsonian rat model (Dowd et al., 2004; Dowd et al., 2005; Hahn et al., 2009; Nikkhah et al., 1993;
In parallel, the successful transplantation of human VM xenografts to the hemi-Parkinsonian rat with significant survival, maturation, fiber extension and innervation, alongside amelioration of motor deficits, highlighted the potential of this cell source to translate clinically and restore dopamine neurotransmission in Parkinson’s disease patients (Brundin et al., 1986; Rath et al., 2013; Strömberg et al., 1992; Strömberg et al., 2001). Another crucial step towards clinical application was the confirmation of successful grafting in non-human primate models of the disease (Annett et al., 1997; Bakay et al., 1985; Sladek et al., 1987; Taylor et al., 1991).

As a whole, these seminal studies have shed light on the vast potential of dopamine cell transplantation as a restorative approach to Parkinson’s disease and were crucial to the progression of the field towards clinical trials.

1.1.3.2 Restoration of dopamine neurotransmission

The efficacy of cell replacement strategies in Parkinson’s disease is dependent on 1) the survival and maturation of dopaminergic neurons in the host brain, 2) appropriate axonal outgrowth from the transplanted cells, 3) integration with the host system and 4) restoration of dopamine transmission. Numerous studies have shown that dopaminergic neurons from the developing VM can mature and function in the host adult striatum following transplantation (Annett et al., 1997; Brundin et al., 1987; Dowd et al., 2004; Hahn et al., 2009; Torres et al., 2008). Additionally, electrophysiological and neurochemical studies have shown that grafted dopaminergic neurons are capable of the synthesis, release and uptake of dopamine (Rose et al., 1985; Schmidt et al., 1982; Zetterström et al., 1986), exerting electrical firing patterns (Wuerthele et al., 1981), re-innervating the host striatum and developing graft-to-host synaptic connections (Bolam et
Importantly it must be noted that the ectopic placement of grafts in the striatum does not limit or impede dopamine neurotransmission, as normal physiological dopamine transmission is regulated by the release of dopamine from striatal terminals and not solely nigrostriatal input (Grace, 1991). Moreover, it has been shown that the control of movement is maintained through tonic dopamine release which maintains a sustained extracellular dopamine concentration (Schultz, 2007). Further evidence to support the efficacy of transplanted dopaminergic neurons is their ability to reduce drug-induced rotations, which are used to measure a graft’s ability to synthesise and release dopamine (Björklund et al., 1980b; Hahn et al., 2009; Torres et al., 2007a), and their ability to restore basal dopamine levels towards normal (Piccini et al., 1999). Overall, while transplanted dopaminergic neurons are capable of survival and extensive afferent and efferent connectivity with the host brain, it must be noted that there are a number of factors which can affect the efficacy of VM transplantation.

1.1.3.3 Graft placement

Preliminary in vivo studies have given significant insight into how the specific placement of fetal VM grafts in the brain can affect the efficacy of transplantation. In the normal physiological scenario, dopaminergic cell bodies reside in the substantia nigra, from which they extend long axonal projections along the trajectory tract of the medial forebrain (MFB) to the terminal striatum where dopamine transmission is required. To date, the ectopic placement of grafts in the striatum has been favored over intra-nigral delivery. Intra-nigral transplantation often results in poorer cell survival. Thompson and colleagues have reported a yield of 3.5% (Thompson et al., 2005) dopaminergic neurons from grafts placed in the striatum compared to only 1% following nigral grafting (Thompson et al., 2009). Intra-nigral transplantation is also associated with insufficient striatal innervation,
which is most likely caused by the absence of guidance cues along the already developed substantia nigra pathway (Bentlage et al., 1999) and the corresponding presence of inhibitory factors (Wictorin et al., 1990).

As described above, the ectopic transplantation of VM grafts to the target striatum of hemi-Parkinsonian animals successfully ameliorates motor deficiency in simple, spontaneous or drug-induced behavioral tests. However, the recovery of more skilled motor tasks, such as skilled paw usage appears to be unachievable with such grafting. Notably, this limitation can be somewhat overcome by the placement of multiple deposits throughout the striatum (Nikkhah et al., 1993), highlighting the importance of widespread striatal innervation in behavioral recovery. Nevertheless, even in the most impressive cases, certain aspects of motor function remain unrecoverable through intra-striatal grafting, even in the face of extensive striatal re-innervation (Winkler et al., 2000). Other factors that may contribute to this are sub-optimal dopamine release in the striatum due to a lack of adequate midbrain afferent input and/or the local loss of dopamine signaling in the midbrain that cannot be restored through striatal grafting. Therefore, in order to achieve more skilled motor recovery, reconstruction of the nigrostriatal pathway may be essential as opposed to the replacement of striatal dopamine alone.

Early studies showed that while dopaminergic neurons survive intra-nigral grafting, they failed to extend axons along the nigrostriatal pathway or produce any behavioral recovery (Bjorklund et al., 1983). These failings were understood to be due to the restrictive host environment and not a reflection of the cells capacity to grow. Proof-of-principle arose from the micro-transplantation of VM cells to the substantia nigra of 6-OHDA lesioned neonates, where nigro-striatal reconstruction was observed upon adulthood (Nikkhah et al., 1995). Moreover, the transplantation of “bridge” grafts of Schwann cells that stretch from
the transplantation site to the striatum alongside intra-nigral VM grafts showed that grafted dopamine neurons had the intrinsic potential to extend axons to the denervated striatum (Brecknell et al., 1996; Wilby et al., 1999). Xenografting studies have also shown that human VM cells placed into the substantia nigra of hemi-Parkinsonian adult rats could innervate regions of the host striatum, eluding to the concept that this was a result of the outgrowing axons’ failure to recognise species specific inhibitory factors (Isacson et al., 1995). While these studies suggest that the adult human brain is incapable of supporting long-distance axonal growth, recent studies using VM tissue from GFP transgenic mice have shown a notable pattern of axon growth towards the striatum, along with the normalisation of rotational behavior (Gaillard et al., 2009; Thompson et al., 2009). Further studies are required in order to determine the potential of such nigrostriatal reconstruction, particularly in respect to the recovery of skilled motor functions.

1.1.3.4 Neuronal subtype

Dopaminergic neuron subtype is another factor that holds influence over the efficacy of VM transplants. VM grafts are heterogeneous with respect to cell type, with dopaminergic neurons forming one component of the total cell population. Dopaminergic neurons can be further divided into three major cell groups, A8, A9 and A10, based on the classification of cerebral monoamine neurons by Dahlstrom and Fuxe (1964). A10 neurons are phenotypically small round cells that send projections to the cortical and limbic structures including the amygdala, nucleus accumbens, hippocampus and prefrontal cortex to form the mesocorticolimbic structure. A9 neurons are phenotypically larger angular cells that send projections predominantly to the dorsolateral striatum to form the nigrostriatal pathway. While, A8 neurons innervate limbic and striatal structures and provide local innervation to both A9 and A10 neurons (Bjorklund et al., 2007). The A9 neurons are the
most vulnerable to degeneration in Parkinson’s disease, while the A10 neurons are relatively resistant to disease pathology and are one of the last to degenerate. (Damier et al., 1999). Moreover, the A9 component of VM grafts have been found to be the most important for functional recovery due to their exceptional ability to target the dorsolateral striatum which is involved in movement (Grealish et al., 2010), highlighting the importance of a subtype ratio that is favorable to A9 neurons. Quantification of A9 and A10 marker proteins (G protein-gated inwardly rectifying potassium channel (Girk2) and calcium binding protein (calbindin), respectively) shows that dopaminergic neurons in intra-striatal VM grafts are comprised of 60-70% A9 neurons and 30-40% A10 neurons (Bye et al., 2012). Interestingly, it was found that A9 neurons precede the birth of A10 neurons and as a result the use of younger embryonic donor tissue generated grafts which were composed of >75% A9 neurons (Bye et al., 2012). Furthermore, a recent study has demonstrated that A9 neurons from younger embryonic donor tissue are more responsive to environmental cues at the transplantation site when adopting a dopaminergic phenotype during differentiation post-grafting (Fjodorova et al., 2017). All in all, these findings allude to the use of younger embryonic donor tissue in transplantation studies.

1.1.3.5 Embryonic donor age

As highlighted above, embryonic donor age is of particular importance in VM transplantation. The VM must be collected during a specific time frame of neurogenesis to allow for the level of maturity required for commitment to a dopaminergic neuron phenotype, while also avoiding over-differentiation prior to transplantation. Early experiments established the upper limits of donor age for VM cell suspensions to be embryonic day (E) 15-16 (rat) (Brundin et al., 1985) and in more recent years the conventional donor age has become E14 (rat) as it was perceived to coincide with peak
dopamine neurogenesis (Hegarty et al., 2013). More recently, the influence of donor age on graft efficacy has received heightened interest. Torres and colleagues looked at the yield of dopaminergic neurons from intra-striatal grafts of VM dissected at E11, E12, E13, E14 and E15 (estimated by crown-rump length). It was found that when grafting the same number of embryos, the E12 preparations yielded the highest dopaminergic neurons, 5-fold that of the E14 preparation (Torres et al., 2007b). The authors hypothesised that the enhanced survival with this younger donor age may be a result of trophic support from the attached meningeal layer, a component that is easily removed in E14 preparations. Moreover, a recent study showed that the addition of meningeal cells from young donors with VM preparations enhanced both the survival and axonal outgrowth of dopaminergic neurons (Somaa et al., 2015). While the exact mechanism through which meningeal cells support dopaminergic neurons remains to be elucidated, these studies highlight the potential benefits of using VM tissue from younger embryonic donors in future studies, both experimentally and clinically.

1.1.4 PROGRESSION TO CLINICAL TRIALS

The extremely positive results found pre-clinically led to a swift movement to clinical trials, with the first open label clinical trial taking place less than 10 years after dopaminergic neurons from VM grafts were first shown to be suitable for transplantation. It is important to note that even at the initial clinical trial stage, there was an understanding that it would be unlikely that striatal VM grafts would be capable of reversing all aspects of Parkinson’s disease in patients, but that instead the level of recovery seen would hopefully give patients an improved quality of life.
1.1.4.1 Open label trials

The first clinical trials were carried out in the 1980s, firstly by Madrazo and colleagues in Mexico (Madrazo et al., 1988) and then by Lindvall and colleagues in Sweden (Lindvall et al., 1989). In both studies, 2 patients received an intra-striatal transplantation of human VM tissue from 12-14 and 8-10 week old embryos, respectively. While Madrazo and colleagues reported dramatic motor improvements, particularly in the disappearance of rigidity and dyskinesia, Lindvall and colleagues noted minimal clinical improvement. This lead to refinements in surgical technique such as a reduction in the diameter of the implantation device and an increase in the number of implantation sites. As a result, Lindvall and colleagues reported a significant reduction in the rigidity and bradykinesia of two further patients, with a marked decrease in the patients’ "on-off" phenomenon (Lindvall et al., 1990). Moreover, long-term follow up showed that the grafted VM cells were capable of surviving and exerting functional benefit 3 years after transplantation (Lindvall et al., 1994). This led to several subsequent open label trials which reported significant changes in UPDRS scores, quality of life and levodopa requirements (Brundin et al., 2000a; Freed et al., 1992; Hauser et al., 1999; Peschanski et al., 1994; Spencer et al., 1992; Wenning et al., 1997). Importantly, these positive results were associated with an increase in flurodopa uptake, as measured by $^{18}$F-flurodopa positron emission tomography (PET) which is widely used as a measure of graft viability (Piccini et al., 2005). Likewise, the binding of $^{11}$C-raclopride, a D$_2$ receptor antagonist, showed that the transplanted graft restored D$_2$ receptor occupancy to normal levels. Encouragingly, the grafts’ capacity to release dopamine can be maintained for at least a decade, despite their exposure to the ongoing disease progression (Piccini et al., 1999). Furthermore, short-term (18 and 19 months) (Kordower et al., 1998; Kordower et al., 1995) and long-term (14
years) (Mendez et al., 2008) post-mortem analysis has shown that grafted dopaminergic neurons can survive the procurement and transplantation process, are capable of re-innervating the denervated striatum and can form graft-to-host synapses.

These initial open label clinical trials demonstrated that transplanted dopaminergic neurons could survive in the Parkinson’s disease brain without any major adverse effects, functionally integrate with the host system and provide sustained long-term clinical benefit. However, results were variable between studies and the concern over not yet fully optimised methodologies raised doubt over whether the data was sufficient to merit further investigation. Despite this, the National Institutes of Health (NIH), opted to fund two double-blind, placebo-controlled clinical trials.

1.1.4.2 Double-blind, placebo-controlled clinical trials

The first trial by Freed and colleagues (2001) included 40 patients between the age of 34 and 75 years with a mean disease duration of 14 years. They were randomly assigned to receive bilaterally either a fetal cell transplantation (tissue from two embryos/side, each between 7 and 8 weeks of age) or sham surgery. No immunosuppression was given pre or post-operatively and follow-up continued for 12 months post-transplantation. The primary outcome was a subjective self-report global rating of clinical improvement at 12 months post transplantation. Although \(^{[18]}\) F-flurodopa PET and post-mortem analysis confirmed the survival and growth of transplanted grafts, the study failed to reach its primary endpoint, while no significant difference was found in total UPDRS scores between the treatment and placebo groups. However, significant improvement in “off” state UPDRS and motor scores were found in transplant patients below 60 years of age. Concerningly, 5 of 33 patients who received a transplant (including those from the sham group who elected
to have the surgery after the study) developed dyskinesias within the first year. These dyskinesias persisted after the reduction or cessation of dopaminergic medication, and are now known as graft-induced dyskinesias (GID). Subsequent pre-clinical, as well as retrospective clinical assessments of patient transplants, indicated that the presence of serotonergic neurons within the donor preparations (a consequence of poor/broad tissue dissection), as well as uneven striatal reinnervation by the grafts were likely responsible for the observed GID (Carlsson et al., 2007; Carlsson et al., 2006; Hagell et al., 2002).

The second placebo-controlled trial included 34 patients between the ages of 30 and 75 years of age (Olanow et al., 2003). Patients were randomly assigned to receive bilaterally either a fetal cell transplantation (from one or four embryos/side, each between 6 to 9 weeks) or sham surgery. All patients received immunosuppression with cyclosporine 2 weeks pre-operatively and up to 6 months post-operatively. The primary outcome measure was a significant difference in the “off” state UPDRS score from baseline to the final 24 month visit. Again, the primary endpoint was not achieved, although there was a trend towards improved motor scores in patients who received a 4 embryo transplant. Stratification based on disease severity, also showed significant improvement in motor scores in less severe patients who received 4 embryo transplants. Patients also showed significant motor improvement at 6 and 9 months post-transplant with deterioration afterwards, which may be a result of the cessation of immunosuppression. Post-mortem analysis did show good survival of dopaminergic neurons and re-innervation of the striatum, while PET analysis revealed increases in flurodopa uptake. However, worryingly, within a year GIDs were found in 56.5% of grafted patients, similar to the previous findings of Freed and Colleagues (Freed et al., 2001).
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The results of these trials have raised serious concern and question over the efficacy and safety of fetal VM transplants in Parkinson’s disease, and while the reasons behind the negative outcomes and GIDs remain unclear, patient selection, tissue preparation, tissue placement, immunosuppression and follow-up time are all thought to be contributing factors. The best results of these placebo-controlled trials were found in patients with lower disease severity (rated by UPDRS scores) and good levodopa response pre-transplantation, suggesting that disease severity is a factor that could affect the efficacy of transplantation. Large variations in tissue preparations were seen between the studies. Freed et al. (2001) delivered a lower volume of tissue that had been stored for 4 weeks prior to transplant, while Olanow et al. (2003) delivered larger quantities of tissue that was only stored for 2 days prior to transplant. The long storage of tissue prior to transplant may have been deleterious to cell survival, which could be further affected by the lower quantity of tissue delivered. Further to this, donor age (range of 6-9 weeks) and tissue composition (strands vs. pieces) are other factors that could have affected graft efficacy. Freed et al. (2001) also implemented a new trajectory to the striatum which may have affected cell distribution. The breach of the blood-brain-barrier (BBB) during surgery and the subsequent addition of a cell transplant are both factors that can instigate a host immune response, therefore the decision to implement (Olanow et al., 2003) or not (Freed et al., 2001) an immunosuppressive regimen may have affected graft survival. The decision to stop immune suppression after 6 months in the Olanow et al. (2003) study is notable because, up until this point, grafted patients had improved at a rate similar to that seen in the open label studies. The deterioration after cessation of immunosuppression could be explained by a delayed immune response that compromised long-term survival. Concern also arises over the decision to base the primary outcome on results found at just one year. Open label
studies have shown that graft-induced recovery can take months or years to develop. A follow-up of the Olanow et al. (2003) study at 2 and 4 years post-transplant showed significant improvements in UPDRS motor scores and flurodopa uptake. Moreover, the increase in flurodopa uptake over the course of the study correlates with the clinical outcome (Ma et al., 2010).

Thus, despite the PET and post-mortem analysis which showed the survival and functionality of transplanted dopaminergic cells in the host brain, the failure to reach the primary outcomes and the appearance of GIDs in the double-blind, placebo-controlled trial, combined with the reports of similar dyskinesias in a parallel open label trial (Hagell et al., 2002) resulted in a heated argument over the safety and validity of fetal VM cell transplantation which resulted in the halting of all clinical trials in 2003 (Winkler et al., 2005a).

1.1.4.3 TRANSEURO

Despite the halting of clinical trials, experimental studies continued to further explore the efficacy of fetal VM transplants and to investigate the cause and mechanisms behind GIDs. In an effort to generate a hypothesis for the mechanism of GIDs, each study center has further analysed their transplant patients. However, comparing studies has highlighted the considerable variations in procedure from trial to trial (Winkler et al., 2005a). As a result, a number of possible clinical and technical parameters have been highlighted that may play a role in GID development (Fig. 1.3) (Lane et al., 2010). Moreover, Kefalopoulou et al. (2014) recently reported on the long term (18 years after transplantation) symptomatic relief in two patients, along with their discontinuation of any anti-Parkinsonian medication. This highlights the long-term success of effective fetal VM transplantation and stresses the
importance of having standardised clinical trials. With this in mind, the field of cell replacement therapy in Parkinson’s disease was reconsidered, leading to the commencement of a new open label multi-center trial, TRANSEURO, in 2012. The principal goals of TRANSEURO are to 1) show that the efficacy of fetal VM transplantation can be improved by paying special attention to tissue preparation, tissue delivery, patient selection and immunosuppression, 2) to show that fetal VM transplantation can be clinically efficacious in the absence of any GIDs and 3) to develop a template protocol that can serve future cell replacement approaches (www.transeuro.org.uk).

**Figure 1.3. Factors hypothesised to contribute to GIDs.** Variations between clinical studies have highlighted a number of parameters that may contribute to variations in graft efficacy and the generation of GIDs, including tissue dissection, dopaminergic cell content, tissue storage, patient selection, surgical procedure, immunosuppression and ongoing extrastriatal degeneration. DA: dopaminergic; LID: levodopa-induced dyskinesia; GDNF: glial-derived neurotrophic factor. Taken from Lane et al., (2010).
1.1.5 LIMITATIONS IN PRIMARY DOPAMINERGIC NEURON TRANSPLANTATION

1.1.5.1 Tissue source

The fetal origin of VM tissue for transplantation is a major hurdle for cell replacement strategies in Parkinson’s disease. The use of human fetal tissue raises ethical concerns regarding the use of donor tissue from elective abortions and logistical difficulties regarding the complexity of obtaining multiple donors for a single transplant. Open label trials have shown that the clinical efficacy is dependent on the transplantation of at least 3 to 5 VMs per hemisphere (Hagell et al., 2001). Moreover, studies have shown that the level of striatal re-innervation required for the amelioration of motor symptoms is dependent on the number of dopaminergic neurons in the VM transplant (Schierle et al., 1999). The clinical and pre-clinical experiences gained so far have all reported extremely poor survival of dopaminergic neurons after transplantation. This can partly be explained by the heterogeneous nature of VM transplants, with dopaminergic neurons forming only 30-40% of the population. Furthermore, it is well reported that only a small number of these dopaminergic neurons (1-20%) survive transplantation (Brundin et al., 2000a). This extremely poor survival results in the need for multiple fetal donors per transplant and it goes without saying that a significant improvement in cell survival would reduce the number of fetal donors required for a single transplant (Hagell et al., 2001).

1.1.5.2 Poor survival of dopaminergic neurons

It was previously assumed that cell death in VM grafts was predominantly necrotic occurring as a result of cell insult during the tissue dissection and transplantation process. However, while some necrotic cell death does occur, the large extent of cell death in VM grafts occurs post-transplantation through apoptosis and is predominantly driven by
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external factors in the cells’ environment rather than a physiological insult (Mahalik et al., 1994; Schierle et al., 1999; Sortwell et al., 2000; Zawada et al., 2001). Apoptotic cell death is triggered at various points of the transplantation process (Fig. 1.4) by factors such as 1) detachment from the extracellular matrix during tissue dissection, known as anoikis (Reddig et al., 2005), 2) immediate growth factor deprivation upon transplantation into the adult striatum (Collier et al., 1999), and 3) the recruitment of host neuro-immune cells to the established exogenous graft (Duan et al., 1995).

1.1.5.2.1 Cell death pre-transplantation

Prior to transplantation, embryonic tissue must be collected, dissected and dissociated into a cell suspension. Even with the clean and efficient dissection of VM tissue, mechanical destruction caused by the cells detachment from the extracellular matrix (known as anoikis) during dissociation removes the normal cell-matrix interactions and cell death can ensue (Marchionini et al., 2003; Reddig et al., 2005). Furthermore, the length of time that tissue is stored prior to transplantation is important to cell survival. Prolonged periods of storage can be detrimental to cell survival and efficacy, and should be avoided in future clinical trials (Freed et al., 2001; Freed et al., 2011). Moreover, the use of appropriate culture media and storage in a cool place are both factors that can help improve cell survival pre-transplantation (Watts et al., 1998). The passing of cells through cannula during transplantation is another point of cell loss which can mainly be attributed to its traumatic effect on cells and/or incomplete delivery. A study by Steiner et al. (2008) showed that the use of ultrathin cannula with a diameter of 0.26 mm significantly increased the number of surviving cells. Subsequently, Nikkhah et al. (2009) showed that the use of
glass capillary cannula, rather than steel cannula improved cell survival by four-fold. However, the safety of glass capillaries in human application is yet to be determined.

1.1.5.2.2 Cell death immediately post-transplantation

Fetal cell replacement therapy in Parkinson’s disease involves the removal of dopaminergic neurons from the VM of the developing embryo and subsequent transplantation into the denervated adult striatum. Thus, these cells are being removed from a trophic factor rich environment at the height of neurogenesis and placed into a trophic factor depleted adult striatum. The trophic activity of the brain and in particular, the striatum, is known to decrease with age (Ling et al., 2000). As a result, transplanted cells undergo trophic withdrawal, being deprived of the factors normally present throughout target innervation and development (Abeliovich et al., 2007). Moreover, numerous studies suggest that the critical time-point in which 80-90% of dopaminergic neurons die, is the first 4 days post-transplantation, and that it is not until after this point that dopaminergic neuron survival is stabilised (Barker et al., 1996; Emgard et al., 1999; Sortwell et al., 2001; Sortwell et al., 2000).

1.1.5.2.3 Cell death post-transplantation

The innate immune response is the body’s defence mechanism against foreign bodies. Identification of a foreign body results in the activation of microglia and the subsequent release of pro-inflammatory molecules and cytokines, including TNF-α, IL-1β and INF-γ. These molecules can then enhance the immune surveillance of the brain by dramatically increasing the BBB permeability (Fabry et al., 1995). As a result, a cycle of neuroinflammation ensues, causing cell death. Experimental studies have shown that the injection of exogenous cells into the brain evokes an elevated host immune response
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(Barker et al., 1996; Duan et al., 1997; Duan et al., 1993; Hudson et al., 1994; Shinoda et al., 1995). Additionally, in line with the prominent cell death seen immediately post-transplantation, microglial activation, lymphocyte infiltration and major histocompatibility complex (MHC) expression all increased over the first 4 days post-transplantation (Duan et al., 1995). Moreover, the immune response may intensify over-time as the immune cells in the brain are not stationary and inactive, but rather provide a continuous inflammatory response (Shinoda et al., 1995). Post-mortem analysis from the double-blind, placebo-controlled clinical trials, as well as an earlier study, detected prominent activated microglial staining around the graft site (Freed et al., 2001; Kordower et al., 1997; Olanow et al., 2003). This immune response against the transplanted cells may have hindered their survival and consequently had an effect on their clinical efficacy. This seems increasingly possible in the Olanow et al. (2003) trial where deterioration of clinical benefit began after the withdrawal of immunosuppression. Moreover, a recent study suggests that long-term immunosuppression (up to 2 years) is beneficial to the long term relief of motor symptoms (Kefalopoulou et al., 2014).
Figure 1.4. Points of dopaminergic cell death. Dopaminergic cell death occurs at numerous points throughout the transplantation process by factors such as 1) detachment from the extracellular matrix during tissue dissection, known as anoikis, 2) immediate growth factor deprivation upon transplantation into the adult striatum, and 3) the recruitment of host neuro-immune cells to the established exogenous graft. Embryonic stage (E).

1.1.6 STEM CELLS AS AN ALTERNATIVE CELL SOURCE

1.1.6.1 Rationale for stem cell therapy

Despite the encouraging results found experimentally and clinically, the widespread use of fetal VM tissue is hindered by the significant ethical and logistical concerns raised around the procurement and use of embryonic tissue. While fetal VM grafts have provided proof-of-principle for cell replacement therapy in Parkinson’s disease, new cell sources are
clearly necessary for this to become a widespread therapy. The therapeutic potential of stem cells has raised great interest in recent years. Stem cells are undifferentiated, non-specialised cells that are capable of self-renewal over long periods of time. Moreover, they can give rise to specialised and fully functional mature cell types.

The primary goal of any stem cell based therapy for Parkinson’s disease must be to generate a safe, reliable and stable cell source that has a steady karyotype, is free from microbiological contamination, does not undergo uncontrolled proliferation and does not form large numbers of non-nigral cells. Importantly, these cells must be dopaminergic-like cells that express appropriate markers such as TH, DAT and GIRK2, physiologically behave like nigral dopaminergic neurons and produce sufficient dopamine. Upon transplantation they must survive long term in the brain, ameliorate motor symptoms, sufficiently innervate the host striatum, be functionally similar, or better than, fetal VM grafts and not migrate extensively throughout the CNS (Barker, 2014).

The use of stem cell therapies has the potential to generate large cell numbers necessary for transplantation, as well as high standardisation of the transplantation procedure. Several types of stem cells have been investigated in this respect, with embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) showing the greatest potential thus far to deliver dopaminergic neurons to replace those lost in Parkinson’s disease (Fig 1.5).
Figure 1.5. Potential sources of dopaminergic neurons for cell-based therapies. ESCs can be differentiated into midbrain dopaminergic neuron progenitors that will mature into functional dopaminergic neurons after transplantation (A). Somatic cells such as fibroblasts can be reprogrammed, either via iPSCs that are subsequently differentiated into dopamine neuron progenitors or directly converted into neurons for transplantation (B,C). This allows for HLA-matched donors (B) or personalised treatments using the patient’s own cells (C). DA: dopaminergic. Taken from Parmar (2018)
1.1.6.2 Embryonic stem cells

Human ESCs (hESCs) are derived from the inner cell mass of the blastocyst following donation through in vitro fertilisation. They are pluripotent cells and can give rise to cells from all three germ layers, endoderm, mesoderm and ectoderm (Thomson et al., 1998). Dissimilar to normal somatic cells, hESCs can proliferate indefinitely in an undifferentiated state, a process that is then lost upon differentiation into fully differentiated somatic cells. Therefore, hESCs have the potential to provide an unlimited cell source for cell replacement therapies (Zeng et al., 2007). Mouse ESCs were first isolated in 1981 (Evans et al., 1981), and were followed by the isolation of hESCs in 1998 (Thomson et al., 1998). There are two major pathways for the neuronal differentiation of ESCs, the formation of embryoid bodies (Park et al., 2004) and co-culture with a layer of feeder cells that can induce differentiation (Kawasaki et al., 2000). The differentiation protocol for hESCs has undergone vigorous optimisation over the years to increase the dopaminergic yield, with the most successful protocols involving a combination of stromal feeder cells and the expression of key transcription factors, giving a TH+ population of 60-75% (Perrier et al., 2004; Roy et al., 2006; Schulz et al., 2004). Impressively, Cho et al. (2008) generated a significantly higher dopaminergic yield (86%) through the formation of spherical neural masses. Moreover, of these TH+ cells, the majority expressed markers of nigral origin. The transplantation of undifferentiated ESCs results in spontaneous differentiation into dopaminergic neurons in vivo and behavioral recovery (drug-induced rotations). However, this led to major concern as teratoma-like tumor generation was observed in some animals (Bjorklund et al., 2002). The potential of tumor formation with hESC transplantation (Nussbaum et al., 2007) is a challenge that must be abolished if these cells are to undergo clinical translation. The in vitro differentiation of ESCs prior to
transplantation has shown to reduce the incidence of tumor formation (Brederlau et al., 2006; Grealish et al., 2014), however, extensive differentiation and maturity could affect their survival post-transplant. Other efforts to reduce tumor formation include the blockage of cell proliferation pathways (Parish et al., 2005), the insertion of ‘suicide genes’ (Schuldiner et al., 2003) and advancements in cell sorting (Chung et al., 2006). Notably, the detailed attention that differentiation protocols have received in recent years has led to a number of experimental studies reporting the efficacy of hESC-derived dopaminergic transplants to be comparable to the preclinical efficacy and potency of fetal VM grafts, without tumor generation (Grealish et al., 2014; Kirkeby et al., 2012; Kriks et al., 2011; Steinbeck et al., 2015). However, it must be noted that their propensity for tumor generation and the ethical issues surrounding their procurement are major issues that must be considered when approaching clinical application.

1.1.6.3 Induced pluripotent stem cells

iPSCs are somatic cells, such as fibroblasts, which can be reprogrammed into pluripotent cells and then subsequently differentiated into dopaminergic neurons (Nishikawa et al., 2008). Their discovery in 2006 was hailed a major scientific breakthrough when Yamanaka and Takahashi reported that cells with a similar developmental potential to ESCs can be generated from mouse somatic cells using a cocktail of just 4 transcription factors – OCT3/4, SOX2, KLF4 and c-MYC (Takahashi et al., 2006), and within a year, two groups had reported on the generation of iPSCs from human fibroblasts (Takahashi et al., 2007; Yu et al., 2007a). These discoveries generated great excitement in the field of cell therapy as their use, unlike ESCs, negates any ethical issues regarding their procurement. Moreover, it opens up the avenue of patient-specific cell therapies, where the use of genetically compatible transplants would avoid host immune rejection. iPSCs have
already been shown to differentiate into dopamine neurons of midbrain character through reprogramming, and after intra-striatal transplantation, were capable of survival, integration with the host system and restoration of rotational behaviour in an 6-OHDA rat model of Parkinson's disease (Wernig et al., 2008). Additionally, Kikuchi et al. (2011) reported the survival of human iPSC-derived functional dopaminergic neurons for up to 6 months in the primate brain. A major concern related to the use of human iPSC-derived transplants is the use of viral vectors to insert genes into the genome at multiple sites during re-programming, a process that is potentially clinically incompatible. However, Soldner et al. (2009) demonstrated that by using Cre-recombinase excisable viruses, dopaminergic neurons could be differentiated from iPSCs without the use of viral reprogramming factors. Moreover, these factor-free human iPSCs remain in a pluripotent state and have a gene expression similar to ESCs. Similarly, Shi et al. (2008) showed that iPSCs from mouse-ESC-derived neural precursor cells could be generated with fewer genetic manipulations and the addition of other small molecules such as BIX-01294. As with ESCs, iPSCs also pose the risk of tumour formation after transplantation. The risk of tumorigenesis from grafted cells can be minimised by separating contaminating pluripotent cells and committed neural cells using fluorescence-activated cell sorting, thus leaving the more differentiated cells for transplantation (Wernig et al., 2008). While there are many challenges that need to be overcome before the clinical application of such iPSC therapies, evidence for the potential of dopaminergic neurons derived from iPSCs is growing. A recent study showed for the first time that dopaminergic neurons derived from human iPSCs survived and functioned as midbrain dopaminergic neurons for an extended period of time (2 years) in an MPTP primate model of Parkinson’s disease (Kikuchi et al., 2017).
Thus, while dopaminergic neurons from both ESCs and iPSCs show extraordinary potential as restorative cell therapies in Parkinson’s disease, numerous challenges such as ethical restrictions (ESCs), potential tumorigenesis (ESC, iPSC) and genetic manipulation (iPSCs) hinder their clinical use. Bearing the lessons learnt from fetal cell transplantations in mind, it is important not to rush clinical investigation until protocol optimisation is fully complete. Moreover, further investigation is required to determine whether the limitations associated with fetal VM transplantation, namely poor survival and GIDs, will also affect stem cell therapies.

1.2 NEUROTROPHIC THERAPY FOR PRIMARY DOPAMINERGIC NEURONS

1.2.1 RATIONALE FOR NEUROTROPHIC THERAPY

As discussed above, cell replacement therapy shows extraordinary potential as a restorative treatment for Parkinson’s disease. Numerous studies have shown that transplanted dopaminergic neurons can survive, integrate with the host system, produce dopamine and restore motor function in rodents, non-human primates and Parkinson’s disease patients. However, as highlighted above, their use is hindered by their extremely poor survival post-transplantation, which consequently raises concern regarding the large number of fetal donors required to reach therapeutic efficacy. A leading cause of this vast cell death seen immediately post-transplantation is the removal of primary dopaminergic neurons from the developing fetal brain which is in the height of neurogenesis and their subsequent transplantation into the adult trophic factor deprived brain.
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In an effort to tackle the cell death caused by trophic withdrawal, a number of studies have looked at the inclusion of neurotrophic support in cell replacement strategies. Neurotrophic factors are endogenous proteins that are involved in the development of the nervous system and are crucial for neuron survival and the establishment of host connections in the brain (Huang et al., 2001). Several studies have demonstrated the beneficial effects that neurotrophic therapy has on the survival and efficacy of midbrain dopaminergic neurons, thus highlighting the potential of trophic factors to improve cell replacement therapies. While many neurotrophic factors are available, focus will be placed on GDNF, a trophic factor that has shown to exert prominent neuroprotective and neurorestorative effects on dopaminergic neurons.

1.2.2 Discovery and classification of GDNF

In 1974, Schubert and colleagues reported that an unidentified factor in the conditioned media of a rat glial cell line had positive effects on dopaminergic neurons (Schubert et al., 1974). Later, in 1993, Lin and colleagues successfully cloned and purified this factor, now termed GDNF (Lin et al., 1993). In this paper, GDNF was described as a glycosylated, disulfide-bonded homodimer which relatively selectively enhanced the survival and morphological differentiation of dopaminergic neurons. GDNF belongs to the GDNF family of growth factors which also includes, artemin, neurturin and persephin (Kotzbauer et al., 1996). They are classified based on their structural composition of seven conserved cysteine residues with similar spacing that form dimers to function (Ibanez, 1998) and are considered distant members of the TGFβ superfamily due to their 20% homology. They function through a two component receptor complex including the GDNF family receptor α (GFRα), which is a glucosylphosphoinositol-linked surface receptor, and RET, a receptor
tyrosine kinase. There are four identified GFRα subtypes (GFRα 1-4) and members of the GDNF family bind to these receptors differentially, holding no specificity (Airaksinen et al., 1999).

The relative specificity at which GDNF positively affects dopaminergic neurons sparked major scientific interest into its potential use in Parkinson’s disease, as it is a disease predominantly characterised by the relatively selective and progressive loss of nigrostriatal dopaminergic neurons (Schapira et al., 2011). Since its discovery, a plethora of studies have demonstrated its neuroprotective effects on the survival of midbrain dopaminergic neurons (Beck et al., 1995; Espejo et al., 2000b; Lin et al., 1993; Perez-Bouza et al., 2017). Moreover, given its prominent neuroprotection, the addition of GDNF to dopamine cell replacement therapies has gained significant interest.

1.2.3 GDNF EXPRESSION THROUGHOUT DEVELOPMENT

Since its discovery, GDNF has been coined as a target-derived neurotrophic factor for the developing dopaminergic neurons of the substantia nigra (Lin et al., 1993). Supporting this, many studies have reported the expression of GDNF throughout neonatal development (Blum et al., 1995; Golden et al., 1999; Oo et al., 2005; Schaar et al., 1993; Stromberg et al., 1993). While GDNF mRNA is highly expressed in the embryonic brain between E7.5 - E10.5 (mouse) of embryogenesis, expression is not found in dopamine cell bodies of the ventral midbrain or dopaminergic target areas at this stage (Hellmich et al., 1996). However, it is known that nigrostriatal dopaminergic neurons are only generated between E10-12 (mouse) and innervate the rostral striatum from E16 to the early postnatal period (Hegarty et al., 2013; Van den Heuvel et al., 2008). Furthermore, GDNF expression was found in the striatal and ventral limbic dopaminergic target areas of the E20 – P7 rat,
but not in the adult rat brain (Stromberg et al., 1993). Moreover, post-mortem analysis reported that GDNF could not be detected in the adult brain, most probably due to the very low levels of GDNF in the adult human brain (Hunot et al., 1996). GDNF levels are much higher in the striatum than the substantia nigra (Oo et al., 2005), supporting the idea that GDNF is a target-derived trophic factor for dopaminergic neurons. Furthermore, upon intra-striatal injection, GDNF is transported to the dopaminergic cell bodies in the substantia nigra, further suggesting that GDNF acts endogenously as a target-derived trophic factor for dopamine neurons (Tomac et al., 1995). In line with this, numerous studies have reported the detection of Ret and GFRα1 in GDNF responsive regions, including midbrain dopamine neurons (Nosrat et al., 1997; Trupp et al., 1997).

The developmental programmed cell death of midbrain dopaminergic neurons occurs during postnatal development through a biphasic process where the first peak occurs at postnatal day 2 and the second at postnatal day 14 (Burke, 2003). Indeed, GDNF is a target-derived trophic factor which regulates this natural cell death event, and is capable of supporting the viability of postnatal midbrain dopaminergic neurons by inhibiting apoptotic cell death (Burke et al., 1998). To further examine the role of GDNF in nigrostriatal development, Granholm and colleagues transplanted GDNF -/- VM dopamine neurons into the denervated adult striatum, resulting in significant disturbance to their postnatal survival and outgrowth (Granholm et al., 2000). Likewise, administration of GDNF antibodies to the striatum during this natural cell death process enhances dopaminergic cell death (Oo et al., 2003), while GDNF over-expression during the same period increases the number of surviving dopaminergic neurons (Kholodilov et al., 2004). Taken together, there is a wealth of evidence highlighting the importance of GDNF during
dopaminergic development (af Bjerken et al., 2007; Kramer et al., 2007; Pascual et al., 2008).

Given that afferent activity is thought to regulate the expression of neurotrophic factors, dopamine is a likely candidate for the control of GDNF expression (Hughes et al., 1999). In support of this, D2 knockout mice exhibit reduced expression of striatal GDNF (Bozzi et al., 1999), while the addition of dopamine (McNaught et al., 2000) or D1 and D2 receptor agonists (Ohta et al., 2010) increases the synthesis and/or secretion of GDNF in primary astrocyte cultures. Thus, the depletion of dopamine during nigrostriatal degeneration would lead to a long-term decrease in striatal GDNF expression as dopamine afferent activity is lost. While the levels of GDNF increase initially over the first two or three weeks after nigrostriatal injury (neuroprotective effects), after that they begin to subside until a decreased expression is observed (Nakajima et al., 2001; Yurek et al., 2002). Ultimately, the transplantation of dopaminergic neurons into the Parkinsonian adult brain potentially provides them with an even lower trophic environment than that of the normal aged brain.

1.2.4 GDNF FOR PRIMARY DOPAMINERGIC NEURON TRANSPLANTATION

The potent neuroprotective effects of GDNF on dopaminergic neurons derived from the VM of embryonic tissue (Lin et al., 1993) has made it an attractive neuroprotective factor for the combined use with primary dopaminergic neuron transplantation. While GDNF has shown to be advantageous to the survival and efficacy of VM transplants, an optimised route of administration has not yet been identified. GDNF cannot penetrate the BBB and therefore must be delivered directly to the brain. This has led to a plethora of studies exploring various methods of intra-cranial delivery, including both once-off and repeated
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exposure. As follows, the application of GDNF neurotrophic therapy to cell replacement strategies in Parkinson’s disease has taken various forms; 1) the pre-treatment of cells/tissue with GDNF prior to transplantation, 2) the simultaneous delivery of GDNF alongside dopaminergic cell transplantation or 3) prior treatment of the host striatum with GDNF using viral vectors.

The rationale behind pre-treating dopaminergic neurons with GDNF prior to transplantation stems from the clinical limitations surrounding embryo procurement. The difficulty in simultaneously obtaining multiple fetal donors for a single transplant often results in the necessity to store tissue before transplantation. While long-term storage is linked with sizeable cell death prior to transplantation, the hibernation of tissue in GDNF enriched media has shown to reduce the level of cell death, while enhancing cell survival, innervation and functional recovery after transplantation (Apostolides et al., 1998; Granholm et al., 1997; Mehta et al., 1998). Hebb and colleagues reported that the positive effects of GDNF pre-treatment can last for up to 9 days prior to transplantation (Hebb et al., 2003), while, Andereggen and colleagues showed that GDNF pretreatment led to a tendency towards a higher fraction of GIRK2 (A9 subtype) positive TH⁺ cells (Andereggen et al., 2009). Moreover, Mendez and colleagues reported a 30% increase in the survival time of human fetal dopaminergic tissue following 6 days of incubation with GDNF (Mendez et al., 2000). Following subsequent transplantation into two Parkinson’s disease patients, PET analysis exhibited enhanced graft survival with a 107% increase in flurodopa uptake at 12 months post-transplant (Mendez et al., 2000).

In contrast, the delivery of GDNF alongside dopamine cell transplants aims to directly combat the vast majority of cell death that occurs within the first 4 days post-transplantation. Chaturvedi and colleagues showed that the co-transplantation of
recombinant GDNF (2 µg) with fetal VM cells resulted in increased TH+ cell numbers, enhanced fiber density, restoration of dopamine levels and functional recovery (Chaturvedi et al., 2003). In an effort to combat GDNF’s short half-life in vivo, a number of studies have looked at the long-term delivery of recombinant GDNF using successive injections or in situ placed osmotic pumps (Rosenblad et al., 1996; Sinclair et al., 1996; Wang et al., 1996; Yurek, 1998). While this approach significantly improves graft survival and efficacy, it requires multiple invasive procedures and/or the implantation of a delivery device. Another approach to the sustained delivery of recombinant GDNF is its encapsulation in biodegradable capsules (Garbayo et al., 2009). Using this method, GDNF-releasing biodegradable microspheres were capable of providing the transplanted cells with sustained GDNF delivery for up to 56 days (Clavreul et al., 2006). Moreover, when the microspheres were delivered at a distance from the transplantation site, an increase in graft survival and function was reported (Clavreul et al., 2006).

As an alternative to the delivery of recombinant GDNF, numerous studies have looked at the co-transplantation of GDNF-producing cells alongside VM-derived dopaminergic neurons (Deng et al., 2013; Espejo et al., 2000b). Several tissue sources have been explored for their ability to produce neurotrophic factors including GDNF. One such cell source is the carotid body (Villadiego et al., 2005). Carotid body type 1 cells are a prototypical source of GDNF and their co-transplantation with VM cells provides cells with an endogenous GDNF “biological pump” (Rodriguez-Pallares et al., 2012). Similarly, olfactory ensheathing cells have been shown to express GDNF, along with other neurotrophic factors (Woodhall et al., 2001) and their co-transplantation with VM cells resulted in significant increases in graft efficacy (Agrawal et al., 2004). Likewise, many groups have applied the use of GDNF genetically modified cells to co-transplantation
strategies where the genetically modified cells supply an indefinite supply of GDNF to the target area and transplanted cells (Deng et al., 2013; Duan et al., 2016; Espejo et al., 2000a; Perez-Bouza et al., 2017).

In a similar manner, the modification of the host system prior to transplantation can provide an indefinite supply of GDNF at the target site. The delivery of plasmid DNA encoding for GDNF to the striatum prior to transplantation resulted in a dramatic increase in cell survival with significant functional recovery (Yurek et al., 2009). Comparatively, viral vector delivery of GDNF is another avenue explored to overexpress GDNF at the target transplantation site and its inclusion with VM cell transplantation has shown to improve the survival and efficacy of transplanted dopaminergic neurons in both rodent and non-human primate studies (Kauhausen et al., 2013; Redmond et al., 2013; Torres et al., 2005).

Together, these studies highlight the extremely potent effects of GDNF on the survival and efficacy of transplanted dopaminergic cells. However, despite the plethora of experimental evidence, the optimal route of GDNF delivery alongside transplanted dopaminergic cells is yet to be determined. Notably, the delivery of GDNF, at least in cell replacement therapies, appears to be most beneficial when applied at the time of or shortly after grafting, rather than chronic administration to mature grafts (Georgievska et al., 2004; Georgievska et al., 2002; Winkler et al., 2006). Thus, the application of a localised and transient delivery of GDNF to dopaminergic cell replacement strategies, sooner than continuous long-term delivery, should provide greater efficacy overall.
1.3 POTENTIAL OF BIOMATERIALS TO IMPROVE CELL REPLACEMENT THERAPY

1.3.1 RATIONALE FOR THE USE OF BIOMATERIALS

While cell replacement therapy shows extraordinary potential in the treatment of Parkinson’s disease and other neurodegenerative disorders, its use as a routine clinical procedure faces significant hurdles. It is therefore imperative that novel and superior delivery strategies are adopted in order to enhance the survival and efficacy of cells after transplantation. Biomaterials, that is, materials that have been specifically engineered to interact with biological systems for therapeutic purposes, have the potential to substantially improve such cell based strategies (Orive et al., 2009).

As described previously, transplanted primary dopaminergic neurons undergo a large degree of cell death throughout their transplantation, with a survival rate of < 20%. Notably, this cell death does not occur as a result of one single insult, or at one specific time-point, but instead occurs at distinct stages throughout the whole transplantation process (Sortwell et al., 2000) (Fig. 1.4).

Firstly, pre-transplantation cell death (stage 1) occurs as a result of tissue handling, dissection, dissociation and storage. The generation of a single cell suspension for transplantation results in the detachment of cells from their anchor proteins of the extracellular matrix (Marchionini et al., 2003). The cell death that ensues, known as anoikis, was first termed by Frisch and Francis when they showed that the loss of integral cell-matrix interactions is a major trigger of apoptotic cell death (Frisch et al., 1994). Hence, the delivery of cells in a biomaterial matrix to which they can adhere may provide them with the necessary support needed both during and after transplantation.
Secondly, the transplantation of cells into the growth factor deprived adult striatum (stage 2) is the point immediately post-transplantation where the vast majority of cell death occurs (Abeliovich et al., 2007; Sortwell et al., 2001). As already highlighted, the incorporation of growth factors, namely GDNF, to dopaminergic cell transplantation strategies can dramatically improve cell survival and efficacy (Apostolides et al., 1998; Chaturvedi et al., 2003; Deng et al., 2013; Redmond et al., 2013; Rosenblad et al., 1996; Yurek et al., 2009). The encapsulation of GDNF in a biomaterial matrix holds the potential to further enhance its intra-cranial delivery by providing a supportive, direct and controllable delivery system. Moreover, the encapsulation of cells in a growth factor-loaded biomaterial matrix has the potential to enhance cell survival and efficacy by providing the transplanted cells with localised, site-specific and prolonged access to GDNF upon transplantation and the period of target innervation.

Finally, cells which survive the transplantation process are then subjected to the hostile host environment where the host immune cells can trigger neuroinflammation and further cell death within the cell graft (stage 3) (Barker et al., 1996; Duan et al., 1995; Hudson et al., 1994; Shinoda et al., 1995). Encapsulation of cells within a supportive biomaterial matrix may protect the transplanted cells from the hostile host environment by forming a physical barrier between the transplanted cells and the host neuro-immune cells.

Each of these stages provides an intervention point at which graft survival could be improved (Fig. 1.6). It must be duly noted, that since cell death in primary dopaminergic cell grafts occurs over a number of distinct stages throughout the whole transplantation process, for a biomaterial scaffold to be advantageous to the delivery, survival and efficacy of cell replacement efforts, it should be capable of 1) providing a supportive environment for cell adhesion, 2) providing a reservoir for localised and sustained growth factor
delivery and 3) creating a physical barrier between the transplanted cells and the host neuro-immune cells.

**Figure 1.6. Stages of biomaterial intervention.** For a biomaterial scaffold to enhance the transplantation of VM cells, it should be capable of providing a supportive environment for cell adhesion (1), providing a reservoir for localised and sustained growth factor delivery (2) and creating a physical barrier between the transplanted cells and the host neuro-immune cells (3).

### 1.3.2 Desirable properties of biomaterials for brain repair

Repairing the damaged brain can be a daunting task, yet recent advances in tissue engineering and cell-based therapies are bringing us closer to clinical translation. The diversity and adaptability of biomaterial scaffolds makes them an attractive strategy for
neural cell replacement therapy (Orive et al., 2009), however, any material used for intra-cranial delivery should exhibit a number of desirable characteristics. Such materials should
1) be capable of relatively non-invasive delivery 2) be biomimetic in order to encourage cell survival and host integration, 3) not themselves elicit an exaggerated host immune reaction that can instigate neuroinflammation around the transplantation site, 4) be structurally stable for prolonged periods in situ but ultimately biodegrade without leaving any undesirable foreign remnants, 5) be modifiable in relation to adhesion molecules, pore size, molecular charge, and functionalisation, 6) be non-toxic to any cellular components of brain tissue or the encapsulated cells and 7) be capable of controlled and sustained delivery of therapeutic factors.

1.3.3 Injectable Hydrogels as a Biomaterial Scaffold

The large range of available biomaterials coupled with their high adaptability leads to the generation of application specific materials, making biomaterials a very attractive avenue in the field of cell-based therapies (Kim et al., 2012). Biomaterials can be characterised under two main subtypes, natural materials or synthetic materials. Natural materials are derived from biological sources including, chitosan, alginate, methylcellulose, hyaluronan, fibrin and collagen. The advantages of their use stems from their natural roles in the biological system. Many contain endogenous binding sites that allow for natural cell adhesion (Heino et al., 2009), while their biological source minimises the activation of the host immune response (Mano et al., 2007). In comparison, synthetic materials are chemically manufactured and can therefore be more readily manipulated and standardised (Lutolf et al., 2005). Indeed, many biomaterials that use natural materials, such as alginate, fibrin or collagen, as their primary framework are often crosslinked with synthetic
polymers, such as polymer polyethylene glycol (PEG), giving rise to a new class of biosynthetic materials which possess the biological properties of the protein and the mechanical stability afforded by the chemical cross-linking (Delgado et al., 2015). It is of utmost importance when choosing a biomaterial, whether it be from a natural or synthetic origin, to take their individual characteristics into consideration, as properties such as adhesion potential, degradability, shape, pore size, hydrophilicity and delivery potential will render them suitable or unsuitable for specific applications.

In relation to the CNS, injectable hydrogels are the most widely investigated and promising biomaterial scaffolds for the delivery of therapeutic agents and/or cells to the brain in regenerative therapies (Burdick et al., 2016). Hydrogels are three-dimensional networks of hydrophilic polymers which can be chemically crosslinked to form insoluble polymer matrices (Hoffman, 2002). The ability of hydrogels to form in situ in response to temperature and pH changes makes them injectable, an extremely attractive property which allows for their relatively non-invasive intra-cranial delivery (Pakulska et al., 2012). Furthermore, hydrogels can be chemically crosslinked to alter their physical properties to specific applications. Indeed, the degree of chemical crosslinking used can directly affect the level of gelation (strength of in situ formation), porous structure and degradation (Drury et al., 2003). The alteration of a hydrogel’s porous structure allows for control over nutrient infusion to encapsulated cells and therapeutic factor diffusion to surrounding tissues (Lee et al., 2016), while simultaneously minimising host immune cell infiltration. Additionally, by using biomaterials that undergo natural degradation, they will eventually be eliminated from the body, while the degree of chemical crosslinking used can control the hydrogel’s degradation rate and therefore its persistence in situ (Davidenko et al., 2015). Depending on their biological source, hydrogels may naturally support cell
adhesion or be manipulated to support cell attachment through the addition of adhesion factors (Hersel et al., 2003). Moreover, growth factors can be added to further support cell survival and function after transplantation (Burdick et al., 2016).

The potential of injectable hydrogels to enhance the delivery of both trophic factors and cells to the Parkinson’s disease brain has received heightened interest in recent years. Hydrogels from a variety of sources, both natural and synthetic, have shown to successfully deliver trophic factors to the brain in a site-specific, controlled and sustained manner (Chierchia et al., 2017; Fon et al., 2014; Li et al., 2016). Further to the enhanced delivery of GDNF in injectable hydrogels (Fon et al., 2014), many approaches have investigated the use of hollow micro-particles to achieve sustained GDNF release from a single administration (Agbay et al., 2014; Garbayo et al., 2016; García-Caballero et al., 2017; Lampe et al., 2011). Moreover, the delivery of GDNF containing microspheres in an injectable fibrin hydrogel enhanced the length of GDNF release in situ to 2 weeks compared to 3 days with “free” GDNF (Wood et al., 2013). Similarly, hydrogels have also been successfully used to enhance cellular delivery (Aguado et al., 2012; Ballios et al., 2015; Das et al., 2016; Freudenberg et al., 2009).

Taken together, this literature highlights the potential of biomaterial hydrogel scaffolds to improve the outcome of reparative cell therapies for Parkinson’s disease. Moving towards a clinical therapy, the use of biomaterial scaffolds from natural polymers, namely collagen, offers significant translatability owing to it already being approved for use in a variety of clinical applications (Chajra et al., 2008; Chattopadhyay et al., 2014; Patino et al., 2002; Solish, 2010). Thus, the work in this thesis will focus on the pre-clinical evaluation of injectable collagen hydrogels in dopamine cell replacement strategies.
1.3.4 Injectable Collagen Hydrogels

Forming 20-30% of the body’s protein component, the collagen family is the body’s most abundant protein group, making collagen one of the most investigated natural biomaterials (Khan et al., 2013). There are many distinct types of collagen found throughout the body, however, 90% of collagen in the human body is type I (Henriksen et al., 2016). Type 1 collagen can be extracted with ease from animal tissues including tendons and skin. It is a fibrous protein composed of three polypeptide chains (α subunits) that are wound together using hydrogen bonds to form a triple helix structure (Bhattacharjee et al., 2005). The transition of collagen from a liquid to a solid state through the structural change of the triple helix to highly compacted coils as a result of physiological conditions such as temperature and pH, makes it an attractive biomaterial for CNS delivery (Sargeant et al., 2012). Furthermore, this in situ gelation makes collagen hydrogels an ideal delivery scaffold for both trophic and cellular regenerative therapies.

While collagen is capable of naturally forming a hydrogel in situ, its weak mechanical properties make it highly susceptible to rapid degradation in the brain. In an effort to better control the mechanical stability of collagen, the use of synthetic polymers to form biosynthetic hydrogels that possess the biological properties of the protein and the mechanical stability afforded by the chemical crosslinking has been investigated (Delgado et al., 2015; Sargeant et al., 2012). The synthetic polymer polyethylene glycol (PEG) is hydrophilic, non-toxic, non-immunogenic (Veronese et al., 2005) and importantly, already FDA approved for a number of clinical applications (Alconcel et al., 2011). Furthermore, the chemical crosslinking of collagen with PEG has been shown to improve the mechanical stability, degradation rate and protein diffusion from these biosynthetic hydrogels, while also maintaining the protein’s biological function (Doillon et al., 1994; Lee et al., 2000;
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Sargeant et al., 2012; Weber et al., 2009). Thus, the ability of crosslinked collagen hydrogels to mimic the extracellular matrix, while also having control over the strength of gelation, rate of degradation and diffusion of encapsulated factors makes them an extremely attractive biomaterial scaffold for cell replacement therapies.

Keeping with this, collagen biomaterials have already been approved for use in a variety of applications including drug delivery, wound repair, burn treatment, dentistry and bone reconstruction (Blume et al., 2011; Chajra et al., 2008; Chattopadhyay et al., 2014; El-Chaar, 2016; Helary et al., 2010; Khan et al., 2013; Parenteau-Bareil et al., 2010; Patino et al., 2002; Solish, 2010) and could therefore be relatively easily adopted to neural applications. While the intra-cranial use of collagen hydrogels has been looked at to a lesser extent, Hoban and colleagues have recently shown that a PEG crosslinked collagen hydrogel successfully reduces the host immune response to encapsulated GDNF-overexpressing MSCs upon delivery to the Parkinsonian rat brain (Hoban et al., 2013). Thus, given the clinically biocompatible, immunoprotective and supportive properties of collagen hydrogels, they hold immense potential to improve the survival and efficacy of dopaminergic cell replacement therapies in Parkinson’s disease.
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1.4 HYPOTHESIS

Therefore, given the growing evidence in support of the potential of biomaterial scaffolds to improve cell and growth factor therapies, we hypothesise that the delivery of dopaminergic neurons in a trophic factor enriched injectable collagen hydrogel will increase the survival, outgrowth and function of dopaminergic neurons by creating an environment which mimics our extracellular matrix, provides a prolonged and site-specific delivery of growth factor support, while also reducing the host immune response to the transplanted cells.

1.5 THESIS OBJECTIVES

To this end, the overarching aim of this thesis was to develop a GDNF-loaded injectable collagen hydrogel for the delivery of dopaminergic neurons to the Parkinsonian brain.

Specifically we aimed to:

1. Optimise an injectable collagen hydrogel crosslinked with 4s-StarPEG for the intracranial delivery of dopaminergic neurons.

2. Assess the effect of a GDNF-loaded collagen hydrogel on the survival and efficacy of E14 VM-derived dopaminergic neurons in hemi-Parkinsonian rats.

3. Assess the effect of a GDNF-loaded collagen hydrogel on the survival and efficacy of E12 VM-derived dopaminergic neurons in hemi-Parkinsonian rats.

4. Assess the effect of a GDNF-loaded collagen hydrogel on the survival and efficacy of human iPSC-derived dopaminergic neurons in hemi-Parkinsonian rats.
Chapter 2: Materials & Methods

2.1 ETHICAL STATEMENT

All procedures involving the use of animals were 1. Approved by the Animal Care and Research Ethics Committee (ACREC) at the National University of Ireland, Galway, 2. Completed under 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. Niamh Moriarty (AE19125/I087), and 3. 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 a GDNF-loaded injectable collagen hydrogel for the delivery of dopaminergic neurons to the Parkinsonian brain (Fig. 2.1). After first optimising an injectable collagen hydrogel for the intra-cranial delivery of cells and trophic factors (Chapter 3), we proceeded to investigate the effect of our optimised collagen hydrogel on the long-term survival and efficacy of primary dopaminergic neurons derived from the VM (Chapter 4 & 5) or dopaminergic neurons derived from human iPSCs (Chapter 6).

In Chapter 3, we sought to assess the optimal level of collagen hydrogel crosslinking for the intra-cranial delivery of encapsulated cells. Once this was determined, we proceeded to assess the effect of encapsulation on primary dopaminergic cell survival and outgrowth, as well as growth factor retention in the transplanted striatum. These studies allowed us to determine the cytocompatibility of our selected collagen hydrogel and its suitability for
growth factor delivery. Once established, we proceeded to carry out long-term functional studies using an optimised growth factor-loaded collagen hydrogel.

In Chapter 4, we sought to assess the effect of our optimised GDNF-loaded collagen hydrogel on the long term survival, outgrowth and efficacy of encapsulated E14 primary dopaminergic neurons. This study allowed us to determine whether our growth factor-loaded collagen hydrogel could enhance the survival and efficacy of dopaminergic neurons by providing cells with a growth factor-rich microenvironment that forms a physical barrier between the transplanted cells and the host immune response.

In Chapter 5, we sought to assess the effect of our optimised GDNF-loaded collagen hydrogel on the long term survival, outgrowth and efficacy of encapsulated E12 primary dopaminergic neurons. This study allowed us to determine whether our growth factor-loaded collagen hydrogel could provide additional support to dopaminergic neurons derived from younger donor age tissue (including their attached meningeal layer) and therefore further enhance graft survival.

In Chapter 6, we sought to assess the effect of our optimised GDNF-loaded collagen hydrogel on the long term survival, outgrowth and efficacy of encapsulated dopaminergic neurons derived from human iPSCs. This study allowed us to determine whether our growth factor-loaded collagen hydrogel could enhance the survival and efficacy of alternatively sourced dopaminergic neurons and therefore be applied to future cell replacement therapies in Parkinson’s disease which will utilise stem-cell derived dopaminergic neurons.
Detailed experimental designs of each study are provided in the relevant results chapters, while this chapter will provide details of the different methodologies used throughout this thesis.

**Figure 2.1. Primary thesis objective.** A schematic depicting the overall aim of this thesis: to develop a GDNF-loaded injectable collagen hydrogel for the delivery of dopaminergic neurons to the Parkinsonian brain.

### 2.3 ANIMAL HUSBANDRY

A total of 185 Sprague Dawley rats were used to complete this research. All animals were sourced from Charles River, UK. Unless otherwise stated, all animals were housed in groups of four per cage, in plastic bottom cages (50.50 x 13 x 24 cm) with a wire grid lid.
and sawdust (pups) or 3Rs lab basic bedding (weaned and adult rats) as standard bedding material. Each cage contained sizzle-nest and circular hollow plastic tunnels as environmental enrichment. Animals were kept on a 12:12 h light:dark cycle (lights on at 08:00), at 19-23°C, with relative humidity levels maintained between 40 and 70%. For the duration of the experiments, animals were allowed food and water ad libitum. All behavioural testing and ex vivo analyses were carried out blind to the treatment of the animals.

2.4 FABRICATION OF TYPE 1 BOVINE COLLAGEN HYDROGELS

During the preparation of collagen hydrogels, all components were maintained on ice to prevent premature gelation. For a final volume of 100 µl, 40 µl of 5 mg/ml type 1 collagen (Vornia Biomaterials), neutralised with 1 M NaOH until pH 7 reached, was added to 20 µl of 10x 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 cell transplantation media (un-seeded hydrogels), cell suspension (seeded hydrogels) or GDNF enriched cell suspension (GDNF-loaded hydrogels) was then added to the collagen/PBS/crosslinker solution and mixed thoroughly. For in vitro experiments, 50 µl samples were transferred to a previously sterilised (UV radiation) super hydrophobic surface (Teflon®) and placed at 37°C to gel. For in vivo experiments, the un-seeded and cell-seeded collagen hydrogels were maintained on ice prior to transplantation to prevent premature gelation.
2.5 CELL CULTURE & ASSAYS

2.5.1 CULTURE OF GFP-MSCS

GFP transgenic MSCs from the green transgenic rat SD-Tg (CAG-EGFP) CZ-004Osb were kindly donated by Prof. Anthony Windebank, Mayo Clinic, USA and characterised by Dr. Gemma Rooney as previously described (Rooney et al., 2008). Cells were plated at a cell density of 5.7 x 10^3 cells cm^-2 in complete rat MSC medium (44.5% Alpha minimum essential media (MEM); 44.5% F12; 10% fetal bovine serum (FBS); 1% penicillin/streptomycin) and cultured at 37°C in 5% CO2 at 90% humidity. After 2 days, any cells which were not adhered to the tissue culture plastic were washed away and the remaining adherent cells were fed with fresh complete medium. The medium was subsequently changed every 3-4 days. Once the colonies began to merge into a confluent monolayer, the cells were deemed ready for subculture (in vitro cytocompatibility experiments) or in vivo transplantation.

2.5.2 VENTRAL MESENCEPHALON DISSECTION, PREPARATION AND CULTURE

The VM was obtained from embryonic tissue as previously described (Dunnett et al., 1997). Briefly, time-mated female Sprague-Dawley rats (sourced from Charles River, UK) were quickly decapitated using a guillotine under isoflurane anesthesia (5% in 0.5 L O2). E12 or E14 embryos in the uterine horn were removed from the pregnant female by laparotomy, using a rat tooth grasping forceps and a large scissors. The uterine horn containing embryos was then submersed in Hank’s Balanced Salt Solution (HBSS) (containing sodium bicarbonate and without phenol red, Ca^{2+} or Mg^{2+}; Sigma H6645) in a 9 cm petri dish and kept on ice. Further dissection of the VM was performed using a dissecting microscope. Using a curved forceps, small scissors and a fine-point forceps,
each embryo was removed from the uterine horn and their embryonic sac, and subsequently washed three times in ice cold HBSS (to ensure the removal of all blood). Embryos were then placed in the lid of a 9 cm petri dish containing ice cold HBSS for dissection (Fig. 2.2). Using a curved forceps and a dissecting micro-scissors, the mesencephalon was dissected out by making an incision at the midbrain-hindbrain boundary, and at the forebrain-midbrain boundary. The dorsal mesencephalon was then cut lateral to the midline, opening the neural tube and exposing the ventral mesencephalon in its centre. An incision was made at the point between the mid-lateral to medial mesencephalon on both sides and the meningeal layer was removed (E14 only). Cranial and caudal cuts were made to the medial mesencephalon to ensure that no forebrain or hindbrain tissue was included. Dissected ventral mesencephalon tissue was stored on ice in a 15 ml tube containing hibernation medium (Hibernate-E; Gibco) until all embryos were dissected. Once all dissections were complete, tissue preparation was performed immediately.

Dissected VM tissue was centrifuged at 1100 rpm for 5 min at 4°C. The tissue pellet was incubated in 40% trypsin-Hank’s balanced salt solution (HBSS) for 4 min, at 37°C with 5% CO2. Fetal calf serum (FCS) was then added to the tissue and centrifuged at 1100 rpm for 5 min at 4°C. The cell pellet was then resuspended in 1 ml of plating media (Dulbecco’s modified Eagle’s medium/F12, 0.6% D-glucose, 1% L-glutamine, 1% FCS and 2% B27), first using a P1000 Gilson pipette, followed by a 25 gauge needle and syringe. Extra caution was taken to ensure that no air bubbles were introduced to the cell suspension. Once a single cell suspension was obtained, a 10 µl sample of the cell suspension was taken and mixed with 90 µl of trypan blue. Cells were then counted using a haemocytometer. Once counted, cell suspension was centrifuged at 1100 rpm for 5 min at
4°C and the cell pellet was resuspended in the appropriate volume of transplantation media (surgery) or plating media (cell culture). For in vitro studies cells were resuspended at 2000 cells/µl and for in vivo studies cells were resuspended at a range between 83,333 cells/µl and 166,666 cells/µl.

Figure 2.2. Ventral mesencephalon dissection. Firstly, the embryo is removed from uterine horn (A). An incision is then made at the midbrain-hindbrain boundary (B), and at the forebrain-midbrain boundary (C), exposing the neural tube (D). The dorsal mesencephalon was then cut lateral to the midline (E), opening the neural tube and exposing the ventral mesencephalon. An incision is then made at the points between the mid-lateral to medial mesencephalon on both sides and the meningeal layer was removed (E14 only) (F). Cranial and caudal cuts were made to the medial mesencephalon to ensure that no forebrain or hindbrain tissue was included (G-H). Dissected ventral mesencephalon (I) was then maintained in ice cold hibernation media until all dissections were complete.
2.5.3 **Human iPSC Derived Dopaminergic Neurons**

Human iPSC derived dopaminergic precursors were generated as previously described (Devine et al., 2011) and kindly donated at differentiation stage (D) 11 by Dr. Tilo Kunath, Centre for Regenerative Medicine, Edinburgh.

Upon thawing, D11 cells were placed in wash media (50% Neurobasal media, 50% DMEM/F12, 1:100 L-glutamine, 1:100 B27, 1:200 N2 and 1:1000 Y2) and centrifuged at 1100 rpm for 5 min at 4°C. The spent media was aspirated, while extra care was taken not to disrupt the cell pellet. Cells were subsequently resuspended in plating media (Neurobasal media, 1:200 B27, 1:100 L-glutamine, 1:1000 Y2, 1:1000 ascorbic acid, 1:1000 FGF8, 1:1000 BDNF, 1:1000 heparin and 1:2000 GDNF), plated in laminin-111 coated wells of a 24 well plate, and incubated in 37°C in 5% CO₂. Two days later, the media was changed using feeding media (Neurobasal media, 1:200 B27, 1:100, L-glutamine, 1:1000 FGF8 1:1000 BDNF, 1:1000 heparin and 1:2000 GDNF) to remove Y2, and every 2-3 days thereafter. On D16, cells were incubated with Accutase for 8 min, at 37°C in 5% CO₂ and re-suspended in plating media (Neurobasal media, 1:200 B27, 1:100 L-glutamine, 1:1000 Y2, 1:1000 ascorbic acid, 1:1000 dbcAMP, 1:1000 BDNF, 1:2000 GDNF and 1:10,000 DAPT). Two days later, the media was changed using feeding media (Neurobasal media, 1:200 B27, 1:100 L-glutamine, 1:1000 ascorbic acid, 1:1000 dbcAMP, 1:1000 BDNF, 1:2000 GDNF and 1:10,000 DAPT) to remove Y2, and then every 2-3 days thereafter, to a maximum of D35. When the required differentiation stage was reached (D16 and D35 for tolerisation and D16 cells for transplantation) cells were incubated with Accutase for 8 min, at 37°C in 5% CO₂, and re-suspended in plating media at the necessary concentrations.
2.5.4 CELL VIABILITY ASSAYS

In order to determine the effect of the collagen hydrogels on MSC viability, MSCs were seeded at a density of 20,000 cells per well of a 24 well-plate and left overnight to attach. MSCs were then either incubated with unseeded collagen hydrogels (2 x 50 µl gels per well) of various 4s-StarPEG concentrations (1-12 mg/ml) for 48 h or left untreated. As an indicative measure of cell viability, metabolic activity of the cells was assessed using the alamarBlue® assay as previously described (Newland et al., 2013). Briefly, 100 µl of a 10% solution of alamarBlue® (Invitrogen) in HBSS was added to each well and incubated for 3h. Absorbance was read at 570 nm and 600 nm using a Varioskan Flash plate reader (Thermo Scientific) with SkanIt® software. MSC viability was assessed by normalisation of all results to controls.

To determine the effect of collagen hydrogels on VM cell viability, E14 VM cells were seeded on poly-L-lysine (Sigma) coated 24 well plates, at a density of 100,000 cells per well in 500 µl of plating media at 37°C with 5% CO₂ for 48 h. As above, the E14 VM cells were either incubated with unseeded collagen hydrogels (2 x 50 µl gels per well) of various 4s-StarPEG concentrations (1-12 mg/ml) for 48 h or left untreated. Once again, metabolic activity was assessed using the alamarBlue® assay as described above.

2.5.5 VM CELL IMMUNOCYTOCHEMISTRY

Dopaminergic neuron survival and outgrowth was assessed 48 h after treatment with collagen hydrogels (as described above) using tyrosine hydroxylase (TH) and beta-III tubulin immunocytochemistry, respectively. VM cultures were fixed with 4% paraformaldehyde for 30 min, followed by three washes in Tris-buffered saline (TBS) with 0.2% triton-X-100 for permeabilisation. Cultures were then incubated in blocking serum.
(5% bovine serum albumin in TBS with 0.2% triton-X-100) for 1 h at room temperature, before being subsequently incubated with primary antibody (Mouse anti-TH, 1:1000, Millipore; Mouse anti-beta III tubulin, 1:333, Millipore) diluted with 1% bovine serum albumin in TBS with 0.2% triton-X-100 at room temperature overnight. Following 3 x 10 min washes with TBS, cultures were incubated in rabbit anti-mouse AF 488 conjugated secondary antibodies (1:1000, Biosciences) in 1% bovine serum albumin in TBS, at room temperature for 3 h in darkness. Cultures were then counterstained with DAPI (1 µg/ml in TBS, Sigma) for 5 min. Following 3 x 10 min washes in TBS, cultures were stored in 0.1% TBS azide at 4°C until imaging. Negative controls, where no primary antibody was added were also prepared.

2.5.6 Ex vivo image analysis

All ex vivo image analysis was performed using ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA). Analysis consisted of counting the number of TH⁺ cells and the area of beta-III tubulin fluorescence. TH⁺ cell numbers were quantified from five randomly selected sample sites per well, in three technical replicates per experimental condition, with three biological replicates. The level of beta-III tubulin fluorescence was quantified by measuring the threshold area of three randomly selected sample sites per well, in three technical replicates per experimental condition, with three biological replicates.

2.6 Neonatal desensitisation

Desensitisation of neonatal rats to xenogenic tissue (ie. human iPSC derived dopaminergic precursors) was carried out as described previously (Heuer et al., 2016; Kelly et al., 2009)
(Fig. 2.3). In short, pups were briefly separated from their mother on postnatal day (PND) 2 and 4 and given an i.p injection of 100,000 differentiated human iPSC derived dopaminergic cells (50% D16 : 50% D35) in 1 µl sterile media, followed by immediate return to their mother. To reduce the potential risk of rejection by the mother, all pups were handled minimally using disposable nitrile gloves. Cells were delivered to each pup using a 10 µl Hamilton syringe with 26 gauge needle.

**Figure 2.3. Neonatal desensitisation.** Schematic representation of neonatal desensitisation in PND 2 and 4 pups (100,000 human iPSC cells/1µl). Rats were subsequently allowed to mature before receiving intra-striatal human iPSC transplants. Image modified from Heuer et al., (2016).
2.7 SURGERY

2.7.1 STEREOTAXIC SURGERY

All surgeries were performed under isoflurane anaesthesia (5% in O2 for induction and 2% in O2 for maintenance) in a stereotaxic frame with the nose bar set at -4.5 (intra-MFB) or -2.3 (intra-striatal). Firstly, the site of surgery was shaved and the animal was secured to the stereotaxic frame using ear bars. Following disinfection with iodine, an incision was made through the skin on the head using a scalpel to expose the skull. 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 using polyethene tubing (0.28 mm inner diameter) and filled with saline. An electric drill was used to expose dura, from which the cannula was lowered to the correct dorso-ventral coordinates. The required cell suspension or neurotoxin was slowly and carefully delivered to the desired region through the monitoring of movement of a deliberately made air bubble in the polyethene tubing between the saline and required suspension. The plunger on the Hamilton was depressed at a steady rate of 1 µl/min using an automated pump (Harvard Apparatus). Following injection, the incision was sutured closed, a topical anaesthetic was applied and animals were monitored throughout recovery before being placed back in their home cage.

2.7.2 INTRA-MFB LESION SURGERIES

For lesion induction, 6-OHDA was weighed out and dissolved in 0.01% sterile ascorbate saline. Prior to infusion the 6-OHDA suspensions were kept on ice and in the dark. All 6-OHDA lesion surgeries were performed under isoflurane anaesthesia (5% in O2 for induction and 2% in O2 for maintenance) in a stereotaxic frame with the nose bar set at -4.5. The MFB was infused unilaterally with 6-OHDA (12 µg) at coordinates AP -4.0, ML -
1.3 (from bregma) and DV -7.0 below dura (Fig. 2.4). Infusions were completed at a total volume of 3 µl at a rate of 1 µl/min with a further 2 min allowed for diffusion.

2.7.3 INTRA-STRIATAL TRANSPLANTATION SURGERIES

For the transplantation of cells (MSCs and VM cells), trophic factors (GDNF) and collagen hydrogels (with or without cells and/or GDNF), all surgeries were performed under isoflurane anaesthesia (5% in O₂ for induction and 2% in O₂ for maintenance) in a stereotaxic frame with the nose bar set at -2.3. The striatum was infused unilaterally or bilaterally at coordinates AP = 0.0, ML ±3.7 (from bregma) and DV -5.0 below dura (Fig. 2.4). Infusions were completed at a total volume of either 3 µl (MSC transplants) or 6 µl (VM transplants and collagen hydrogels) at a rate of 1 µl/min with a further 2 min allowed for diffusion.
Figure 2.4. Stereotaxic medial forebrain bundle and striatal target sites. Schematic of the rodent nigro-striatal pathway highlighting the medial forebrain bundle and striatal injection sites. SVZ: Subventricular zone; Str: Striatum; mfb: Medial Forebrain bundle; SNC: Substantia nigra pars compacta. Image modified from Cova et al., (2011).

2.8 AMPHETAMINE-INDUCED ROTATIONAL BEHAVIOR

Dopaminergic asymmetry was assessed via amphetamine-induced rotational behavior as described previously (Ungerstedt et al., 1970) (Fig 2.5). In brief, rats were removed from their home cage and placed into plastic basins containing standard bedding and allowed to habituate to their new environment for 10 mins. Once the habituation period was finished, rats were injected intraperitoneal (i.p) with 2.5 mg/kg methamphetamine. Full body ipsiversive and contraversive rotations were manually counted for 60 mins in 10 x 1 min time bins. Data was expressed as net ipsilateral turns/min.
Figure 2.5. Amphetamine-induced rotational behavior. The number of ipsilateral and contralateral rotations per minute were counted for 60 minutes in 10 x 1 min time bins after i.p administration of methamphetamine (2.5 mg/kg).

2.9 IMMUNOHISTOCHEMISTRY

2.9.1 TISSUE PROCESSING

Animals were sacrificed by terminal anaesthesia (50 mg/kg pentobarbital i.p) and transcardially perfused with 100 ml heparinised saline (5000 units/Litre) followed by 150 ml of ice cold 4% paraformaldehyde (PFA). Brains were rapidly removed and placed in 4% PFA overnight before being cryoprotected in 25% sucrose plus 0.1% sodium azide solution. Serial coronal sections (30 μm) were cut using a freezing stage sledge microtome (Bright, Cambridgeshire, UK) and collected in a series of 12.
2.9.2 IMMUNOHISTOCHEMISTRY

Free floating immunohistochemistry (IHC) was performed using the streptavidin-biotin-peroxidase method as previously described (Hoban et al., 2013; Naughton et al., 2016). In brief, endogenous peroxidise activity was quenched using a solution of 3% hydrogen peroxidase and 10% methanol in distilled water. Non-specific binding was blocked using 3% normal serum (serum origin was dependent on 2° antibody host) in TBS with 0.2% Triton-X-100 at room temperature for 1 hour. Primary antibody (see Table 2.1) was diluted in TBS with 0.2% triton-X-100 and 1:100 normal serum, added to sections and incubated at room temperature overnight. Sections were then incubated in the corresponding secondary antibody (diluted in TBS) and 1:100 normal serum (see Table 2.2) for 3 hours at room temperature. A streptavidin-biotin-horseradish peroxidise solution (Vector, UK) was subsequently added to sections and allowed to incubate for 2 hours. The development of staining was carried out using a 0.5% solution of diaminobenzidine tetra hydrochloride (DAB) (Sigma, Ireland) in TNS containing 0.3 µl/ml of hydrogen peroxide. Sections were mounted onto gelatin-coated slides, dehydrated in a series of ascending alcohols, cleared in xylene and finally coverslipped using DPX mountant for DAB stained sections (Sigma) or ‘fluoromount’ fluorescent mounting medium for GFP-expressing sections (Sigma).
<table>
<thead>
<tr>
<th>Target</th>
<th>Primary Antibody</th>
<th>Source</th>
<th>Host</th>
<th>Dilution</th>
<th>Application</th>
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<td>IHC/ICC</td>
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<td>Rabbit</td>
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<td>IHC</td>
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<td>cd11b</td>
<td>Chemicon</td>
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<td>1:333</td>
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<tr>
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</table>

Table 2.1. A list of primary antibodies used in this thesis. IHC: Immunohistochemistry; ICC: Immunocytochemistry

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<tbody>
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<td>Horse</td>
<td>Mouse</td>
<td>1:200</td>
<td>IHC</td>
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<tr>
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<td>Mouse</td>
<td>1:1000</td>
<td>ICC</td>
</tr>
</tbody>
</table>

Table 2.2. A list of secondary antibodies used in this thesis. IHC: Immunohistochemistry; ICC: Immunocytochemistry
2.10 HISTOLOGICAL QUANTIFICATION

All histological image analysis was performed using ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA). In the instances of graft volume, re-innervation volume, collagen volume, GDNF volume and the volume of microgliosis/astrocytosis, measurements were quantified using an equation based on Cavalieri’s Principle (Section 2.10.1). For MSC graft volume, the transplant was identified directly by GFP expression from fluorescent photomicrographs, whereas VM graft, collagen, GDNF, cd11b and GFAP expression were identified in DAB stained sections. Additionally, microglial reactivity (cd11b) and astrocytic reactivity (GFAP) were also quantified using optical density measurements. Finally, the total number of transplanted dopaminergic cells was determined by counting individual TH⁺ cell bodies in the transplanted region and correcting using Abercrombie’s equation (Section 2.10.2).

2.10.1 CAVALIERI’S PRINCIPLE

Cavalieri’s Principle was used to perform all volumetric analysis in this thesis. It states that ‘‘the volume of an arbitrary shaped object can be estimated in an unbiased manner from the product of the distance between planes and the sum of the areas on systematic random parallel sections through the object’’ (Garcia et al., 2007). The following equation was used to determine graft volume, re-innervation volume, collagen volume, GDNF volume and the volume of microgliosis/astrocytosis throughout this thesis.

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

\[ T_T = NS \times D, \]

- \( V \) is volume
- \( T_T \) is the product of the distance between the planes
- \( A_i \) is Area and was determined using ImageJ software as described below.
- \( NS \) is the number of sections in the series (i.e. 6 series)
- \( D \) is the known distance between tissue sections (i.e. 30 µm)

2.10.2 Abercrombie’s Principle

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

- $T$ 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. 30µm)
- $D$ is average cell diameter.

### 2.10.3 Quantification of Area/Volume

ImageJ software was used to measure area and subsequently calculate volume throughout this thesis. In order to determine GFP-MSC graft volume, fluorescent photomicrographs of striatal sections containing the GFP-MSCs were taken using an Olympus IX81 fluorescent microscope (Olympus UK, London, United Kingdom). At the same time, an image of a graticule was captured at the same magnification and saved at the same pixel quality as the fluorescent photomicrographs. Using this graticule image, the ImageJ program was calibrated by associating the number of pixels with a known distance. Using the ImageJ freehand drawing tool, the area of GFP-MSC graft was measured in each striatal section of a 1 in 6 series (Fig. 2.6). Using these area measurements, graft volume was assessed according to Cavalieri’s Principle (Section 2.10.1). This method was also used to measure the area and volume of TH, cd11b, GFAP, GDNF and collagen staining. In these incidences all striatal sections containing DAB staining were measured in a 1 in 6 series (unless otherwise stated).
Figure 2.6. Screen grab of the ImageJ software used to determine area. The GFP-MSC graft was outlined using the freehand drawing tool (yellow outline in photomicrograph). Since the pixel number of the photomicrograph corresponds to a known distance (calibrated using a graticule image that was captured at the same magnification and pixel conditions), the graft area could be determined for each section.

2.10.4 QUANTIFICATION OF TYROSINE HYDROXYLASE-POSITIVE CELL BODIES

The point counter tool in ImageJ was used to count the number of surviving transplanted TH-positive cell bodies in the denervated striatum (Fig. 2.7). Photomicrographs of the transplanted region were captured using an Olympus microscope BX40 and Olympus C5060 digital camera. The complete unilateral denervation of the striatum using a 6-OHDA intra-MFB lesion allowed for the easy identification of transplanted TH+ cell bodies. For each animal, the number of TH+ cell bodies were counted in each striatal
section of a 1 in 6 series containing a dopaminergic cell transplant. The average cell diameter was determined using ImageJ and the total number of transplanted cells was then corrected for using Abercrombie’s Principle (Section 2.10.2).

![ImageJ software screenshot]

**Figure 2.7.** Screen grab of the ImageJ software used to determine the number of TH+ cell bodies in the transplanted striatum. Red counter dots on the image represent the location of a TH+ cell body.

### 2.10.5 Quantification of Striatal Density

Throughout this thesis, optical density measurements were taken to determine the response of the brain’s inflammatory cells, namely microglia and astrocytes, to the transplanted grafts. In all instances, photomicrographs were obtained using a Nikon SMZ800 microscope with a DXM1200C digital camera. All images were carefully taken under the
same conditions of magnification, exposure and pixel quality. Prior to analysis, all images were converted to 8-bit black and white using the ImageJ program. To measure optical density, a representative coronal image through the transplant was chosen for each animal. The site of transplantation was located based on the visible transplant (TH, GDNF and/or collagen immunostaining) and/or visible needle tract sites. An oval shape was centered over the site of transplantation and the density of staining was measured (Fig. 2.8). To determine the specific staining density, the optical density readings were corrected for non-specific background density. To do this, an oval shape was placed over an unstained region in the section and the optical density was measured.
2.11 STATISTICAL ANALYSIS

Throughout the results section, all data are expressed as mean ± standard error of the mean (SEM) and were analysed using a one-way analysis of variance (ANOVA), two-way ANOVA or a two-way repeated measures ANOVA as appropriate. One-way ANOVA was used to compare more than two groups on one factor, whereas a two-way ANOVA was
used to compare two or more groups on two factors simultaneously. Behavioral data were analyzed using a two-way repeated measures ANOVA with within subject factor of time and between subject factor of group. Bonferroni post-hoc analyses was carried out when required as indicated in the text. In all cases, analysis were deemed significant at $P<0.05$.

Throughout the results section, the main effects from the initial ANOVA are cited in the body of the results, while the results of the post-hoc analyses are shown on the corresponding figure and explained in the figure legend.
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3.1 INTRODUCTION

The relatively selective loss of dopaminergic neurons from the substantia nigra pars compacta makes Parkinson’s disease an ideal candidate for cell replacement therapies (Schapira et al., 2011). To date, the focus of cell therapies in Parkinson’s disease has been on the transplantation of dopamine neuron-rich fetal VM grafts which have shown to both survive and re-innervate the striatum post-transplantation, whilst also restoring motor function (Brundin et al., 1986; Freed et al., 1992; Kefalopoulou et al., 2014; Kordower et al., 1998; Lindvall et al., 1990; Olanow et al., 2003). However, despite long-term symptomatic relief in some patients, significant limitations, including poor survival post-transplantation, prevent this therapy being utilised as a potential restorative approach for Parkinson’s disease (Barker et al., 2013). Dopaminergic neurons account for 30-40% of the VM cell population, and strikingly, only 5 - 20% of these neurons survive the transplantation process (Brundin et al., 2000a). Thus, poor survival, necessitating the use of up to 6 human fetal donors per grafted hemisphere, and the associated ethical concerns and logistical complications, has highlighted an urgent need for improved methodologies to enhance dopamine neuron survival rates post-transplantation.

Biomaterials - that is, materials that have been specifically engineered to interact with living systems for therapeutic purposes - have the potential to substantially improve cell replacement therapies (Orive et al., 2009). Their diversity and adaptability renders them as highly tuneable scaffolds that can therefore be specifically modified to a therapeutic need (Kim et al., 2012). A vast array of different biomaterials are available, however, any
material intended for intra-cranial delivery should 1) be capable of relatively non-invasive delivery, 2) not themselves elicit an exaggerated host immune reaction that can instigate neuroinflammation around the transplantation site, 3) be biomimetic in order to encourage cell survival and host integration, 4) be structurally stable for prolonged periods in situ but ultimately biodegrade without leaving any undesirable foreign remnants, 5) be modifiable in relation to adhesion molecules, pore size, and functionalisation, 6) be non-toxic to brain tissue or the encapsulated cells and 7) be capable of controlled and sustained delivery of therapeutic factors (Orive et al., 2009).

Naturally-derived biomaterials, such as collagen hydrogels, hold the advantage of being characteristically similar to the body’s native tissue, making them highly biocompatible and biodegradable, while also naturally supporting cell adhesion (Khan et al., 2013). Collagen is also capable of forming in situ gelling (and therefore injectable) hydrogels (Sargeant et al., 2012), thus making it an attractive candidate for improving cell replacement therapies in neurodegenerative disorders such as Parkinson’s disease. Collagen-derived biomaterials have the benefit of already having clinical approval for a wide variety of applications, however, their intra-cranial use has been investigated to a lesser degree.

In light of this, the aim of this chapter was to determine the optimal collagen hydrogel composition for the intra-striatal delivery of VM-derived dopaminergic neurons and adjunctive trophic factor (GDNF) therapy. Special focus was placed on the survival of, and striatal re-innervation from, encapsulated dopaminergic neurons.
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3.2 METHODS

3.2.1 EXPERIMENTAL DESIGN

The studies presented in this chapter were designed to assess the optimal level of collagen hydrogel crosslinking for the delivery of E14 primary dopaminergic neurons to the hemi-Parkinsonian brain. Initially, in vitro/ex vivo cytocompatibility studies were carried out to assess the biocompatibility of our crosslinked collagen hydrogels. Following this, preliminary in vivo studies used GFP-MSCs to assess graft survival when encapsulated in collagen hydrogels of various crosslinker concentrations. GFP-MSCs were used in these initial in vivo studies as they are easily detected post-transplantation and are therefore ideal to assess whether the intensity of gelation is suitable for graft survival. Subsequently, an in vivo study using single cell suspensions of E14 VM cultures was carried out to assess the potential impact (if any) of hydrogel encapsulation on VM derived dopaminergic neuron outgrowth. Finally, we assessed the impact of hydrogel encapsulation on the striatal retention of transplanted human recombinant GDNF. Detailed experimental designs of the above studies are outlined below.

3.2.1.1 Preliminary in vitro/ex vivo cytocompatibility studies

Before undertaking in vivo studies, in vitro and ex vivo studies were completed in order to determine the cytocompatibility of the collagen hydrogels in two-dimensional (2D) cultures. This was assessed using the alamarBlue® cell viability assay (see section 2.5.4) and ICC (see section 2.5.5) on MSC and/or E14 primary VM cell cultures. Prior to analysis, MSCs were seeded at a density of 20,000 cells per well of a 24 well-plate and left overnight to attach. MSCs were then either incubated with unseeded collagen hydrogels (2
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x 50 µl gels per well) of various 4s-StarPEG concentrations (1-12 mg/ml) for 48 hours or left untreated. Similarly, E14 primary VM cell cultures were seeded at a density of 100,000 cells per well of a 24 well-plate and left for 48 hours to attach. The VM cells were then either incubated with unseeded collagen hydrogels (2 x 50 µl gels per well) of various 4s-StarPEG concentrations (1-12 mg/ml) for 48 hours or left untreated. A schematic of this experimental design is shown in Fig. 3.1.

Figure 3.1. Preliminary in vitro/ex vivo cytocompatibility studies. Schematic representation of the in vitro/ex vivo experimental design. MSCs (20,000 cells/well) were left for 24 hours to attach and were then incubated with unseeded collagen hydrogels (2 x 50 µl gels per well) of various 4s-StarPEG concentrations (1-12 mg/ml) for 48 hours or left untreated. E14 primary VM cell cultures (100,000 cells/well) were left for 48 hours to attach and were then either incubated with unseeded collagen hydrogels (2 x 50 µl gels per well) of various 4s-StarPEG concentrations (1-12 mg/ml) for 48 hours or left untreated.
3.2.1.2 *In vivo* assessment of the impact of hydrogel crosslinking on grafted cell viability

As the concentration of 4s-StarPEG used to crosslink collagen will affect the intensity of gelation, it was important to determine to what extent the hydrogel can be crosslinked without negatively impacting graft survival. The final level of 4s-StarPEG crosslinking was chosen based on successful graft survival. In order to determine the optimum level of hydrogel crosslinking for cell delivery, an *in vivo* pilot study using male Sprague-Dawley rats (n=24) was carried out. Rats were divided into groups to receive bilateral intra-striatal transplants of GFP-MSCs (30,000 cells in 3 µl) delivered in either transplantation media or encapsulated in a collagen hydrogel of various 4s-StarPEG concentrations (1-12 mg/ml). Animals were then sacrificed for *post mortem* analysis at days 1, 4 and 7 post transplantation (n=4 per group, per time point). A schematic of this experimental design is shown in Fig. 3.2.
Figure 3.2. Preliminary *in vivo* study to assess impact of crosslinking on MSC survival. Schematic representation of MSC encapsulation in a collagen hydrogel. MSCs (30,000/animal) were delivered bilaterally to the striatum in either transplantation media or encapsulated in collagen hydrogels of various 4s-StarPEG concentrations (1-12 mg/ml). Graft survival was assessed at days 1, 4 and 7 post-transplant.

3.2.1.3 *In vivo* assessment of the impact of the collagen hydrogel on grafted E14 VM survival and neurite outgrowth

Once the optimal level of hydrogel crosslinking was determined, an *in vivo* pilot study was carried out to assess whether the collagen hydrogel will impact the survival of, and neural outgrowth from, encapsulated VM cells. Male Sprague-Dawley rats (n=24) received unilateral intra-MFB 6-OHDA lesions (12 µg in 3 µl). Two weeks later, all rats were subjected to methamphetamine-induced rotations. Based on these results, rats were
performance matched into six groups (n=4 per group) to receive unilateral intra-striatal transplants of E14 VM cells of various densities (200,000, 300,000 or 400,000 cells/6 μl) in transplantation media or encapsulated in a collagen hydrogel (crosslinked with 4 mg/ml 4s-StarPEG). The animals were then sacrificed two-weeks post-transplantation for post mortem assessment. A schematic of this experimental design is shown in Fig. 3.3.

Figure 3.3. Preliminary in vivo study to assess the impact of the collagen hydrogel on grafted VM survival and neurite outgrowth. Schematic representation of E14 VM cell encapsulation in collagen hydrogel. E14 VM cells of various densities (200,000, 300,000 or 400,000 cells/6 μl) were encapsulated in collagen hydrogel (4 mg/ml) and delivered to the unilaterally 6-OHDA lesioned striatum. Graft survival and neural outgrowth were assessed at two weeks post-transplant.
3.2.1.4 *In vivo* assessment of GDNF retention within the collagen hydrogel

To assess the striatal retention of human recombinant GDNF when encapsulated within the collagen hydrogel, male Sprague-Dawley rats (n=12) received bilateral intra-striatal infusions of GDNF (1000 ng) as either a bolus or encapsulated in a crosslinked collagen hydrogel (4 mg/ml 4s-StarPEG). The animals were then sacrificed for *post mortem* assessment at days 1, 2 and 4 post transplantation (n=4 per group, per time point). A schematic of this experimental design is shown in **Fig. 3.4**.

**Figure 3.4. Preliminary *in vivo* study to assess GDNF retention within the collagen hydrogel.**

Schematic representation of GDNF delivery in a collagen hydrogel. Human recombinant GDNF (1000 ng) was encapsulated in a crosslinked collagen hydrogel (4 mg/ml) and delivered bilaterally to the intact striatum. The striatal retention of transplanted GDNF was then assessed at days 1, 2 and 4 post-transplantation.
3.3 RESULTS

3.3.1 IN VITRO/EX VIVO ASSESSMENT OF THE IMPACT OF DIFFERENTLY CROSSLINKED COLLAGEN HYDROGELS ON CELL VIABILITY

3.3.1.1 The effect of crosslinking on collagen hydrogel gelation time

Prior to conducting any studies, collagen hydrogels crosslinked with different 4s-StarPEG concentrations were formulated on the bench on a super hydrophobic surface (Teflon®) and placed at 37°C. It was shown that the higher the concentration of 4s-StarPEG used, the shorter the time taken for gelation (Fig. 3.5; Group, $F_{(4,10)} = 1101$, $P < 0.0001$).

![Figure 3.5. The effect of 4s-StarPEG crosslinking on collagen hydrogel gelation time. The crosslinking of hydrogels with rising levels of 4s-StarPEG significantly decreased the time required for gelation. Data are represented as mean ± SEM and were analysed by one-way ANOVA with post-hoc Bonferroni. ***P<0.001, **P<0.01 vs. preceding concentration.](image)

3.3.1.2 The effect of crosslinked collagen hydrogels on cellular metabolic activity

In order to determine if the collagen hydrogels of increasing crosslinker concentrations (1, 2, 4, 6 or 12 mg/ml 4s-StarPEG) had any detrimental effects on cellular metabolic activity,
both MSCs and E14 VM cells were incubated with pre-formed collagen hydrogels (2 x 50 µl/well) for 48 hours in a 2D culture. None of the collagen hydrogels were found to have a negative impact on MSC (Fig. 3.6a; Group, $F_{(5,12)} = 0.39$, $P>0.05$) or E14 VM cell metabolic activity (Fig. 3.6b; Group, $F_{(5,12)} = 0.76$, $P>0.05$).

Figure 3.6. The effect of crosslinked collagen hydrogels on cellular metabolic activity. The incubation of collagen hydrogels crosslinked with various 4s-StarPEG concentrations had no negative effect on the metabolic activity of MSCs (A) or VM cells (B). Data are represented as mean ± SEM and were analysed by one-way ANOVA with post-hoc Bonferroni.

3.3.1.3 The effect of crosslinked collagen hydrogels on the survival and neural outgrowth of primary dopaminergic neurons

Once it was confirmed that the differently crosslinked collagen hydrogels did not impact cell viability, we then wanted to confirm that the presence of collagen hydrogels in VM cultures did not affect the survival or neural outgrowth of TH$^+$ primary dopaminergic neurons. When the survival of dopaminergic neurons within these cultures was assessed, the hydrogels were found to have no negative effect on the number of surviving TH$^+$ cells (Fig. 3.7a; Group, $F_{(5,24)} = 1.27$, $P>0.05$) and importantly, the presence of the hydrogels did not hinder the neural outgrowth from these TH$^+$ dopaminergic neurons (Fig. 3.7b; Group, $F_{(5,18)} = 0.29$, $P>0.05$). This indicates that the increasing level of crosslinker in
these collagen hydrogels is cytocompatible with these VM-derived primary dopaminergic neurons, at least when they are in an ex vivo cell culture system.

**Figure 3.7. The effect of crosslinked collagen hydrogels on the survival and neural outgrowth of primary dopaminergic neurons.** The incubation of collagen hydrogels crosslinked with various 4s-StarPEG concentrations had no negative effect on the number of surviving TH⁺ primary dopaminergic neurons (A) or the neural outgrowth (B) from these cells. Photomicrographs (C, D) represent TH and βIII tubulin immunofluorescent staining, respectively, counterstained with DAPI. Scale bar represents 100 μm. Data are represented as mean ± SEM and were analysed by one-way ANOVA with post-hoc Bonferroni.
3.3.2 In vivo assessment of the impact of hydrogel crosslinking on grafted cell viability

3.3.2.1 The effect of crosslinked collagen hydrogels on MSC graft survival.

Having determined that the collagen hydrogels are cytocompatible in vitro, we then sought to determine the optimal level of 4s-StarPEG crosslinking for the encapsulation of cells in a collagen hydrogel. To investigate this, the survival of GFP-MSCs either delivered in control transplantation media or encapsulated in collagen hydrogels crosslinked with 1, 2, 4, 6 or 12 mg/ml 4s-StarPEG was assessed at days 1, 4 and 7 post-transplantation. Since the MSCs were extracted from the bone marrow of GFP transgenic rats, the cellular grafts could be easily visualised in the striatum using fluorescent microscopy. Upon analysis, it was found that not all hydrogel groups exhibited successful graft survival, particularly with hydrogels of high crosslinker concentrations (6 and 12 mg/ml) (Fig. 3.8). Intense gelation due to the higher level of 4s-StarPEG crosslinking resulted in cellular graft loss and striatal tissue damage at the transplantation site, with no significant difference between the groups (Fig. 3.9; Group, \( F_{(1,18)} = 0.18, P>0.05 \)). For the lower levels of crosslinker (1-4 mg/ml), MSC graft volume was similar between each group at each time-point (Fig. 3.10; Group, \( F_{(3,36)} = 0.29, P>0.05 \)) indicating that the encapsulation of cells inside these collagen hydrogels, of lower crosslinker concentrations, did not impact graft survival.
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![Graph showing the number of surviving MSC grafts.

Figure 3.8. Number of surviving MSC grafts. Lower levels of 4s-StarPEG collagen hydrogel crosslinking allowed for the successful survival of MSC grafts, while the higher levels of 4s-StarPEG collagen hydrogel crosslinking were detrimental to cell survival.

![Graph showing the damage volume over time.

Figure 3.9. The effect of high crosslinking on MSC graft survival. High levels of 4s-StarPEG crosslinking (6 and 12 mg/ml) were detrimental to graft survival. Intense gelation resulted in cellular graft loss and striatal tissue damage at the transplantation site. Photomicrographs represent striatal damage caused by 6 and 12 mg/ml crosslinking. Scale bar represents 100 µm. Data are represented as mean ± SEM and were analysed by two-way ANOVA with post-hoc Bonferroni.
Figure 3.10. The effect of low crosslinking on MSC graft survival. The encapsulation of GFP-MSCs inside collagen hydrogels of lower crosslinker concentrations (1-4 mg/ml) had no negative impact on graft survival. Photomicrographs represent successful graft survival (GFP fluorescence) with lower crosslinking concentrations. Scale bar represents 100 µm. Data are represented as mean ± SEM and were analysed by two-way ANOVA with post-hoc Bonferroni.
3.3.2.2 *In situ* gelation of collagen hydrogels

Once it was confirmed that collagen hydrogels crosslinked with 1-4 mg/ml of 4s-StarPEG successfully permitted the survival of encapsulated MSCs after intra-striatal delivery, we next assessed their ability to undergo *in situ* gelation. Bovine collagen immunostaining confirmed the presence of collagen hydrogels at the transplantation site. Furthermore, photomicrographs showed strong, defined and uniform staining with no significant variations between the groups (**Fig. 3.11**: Group, $F_{(2,23)} = 0.49$, $P>0.05$), suggesting that the hydrogels successfully underwent chemical crosslinking *in situ*. Importantly, the collagen hydrogels did not undergo rapid degradation and were still present 7 days post-transplant (**Fig. 3.11b**).

**Figure 3.11. In situ gelation of collagen hydrogels.** Collagen hydrogels crosslinked with 1-4 mg/ml of 4s-StarPEG successfully formed *in situ* and were still present 7 days post-infusion. Representative photomicrographs show striatal collagen staining at 7 days post-transplantation. Scale bar represents 100 µm. Data are represented as mean ± SEM and were analysed by two-way ANOVA with *post-hoc* Bonferroni.
3.3.2.3 Host immune response to collagen hydrogels.

It was important to determine if the collagen hydrogels crosslinked with 1-4 mg/ml of 4s-StarPEG were biocompatible and did not themselves evoke an exaggerated host immune response. Immunostaining confirmed that while cell transplantation did elicit an astrocytic and microgliotic response in the host brain, the encapsulation of cells in crosslinked collagen hydrogels did not evoke an exaggerated astrocytic (Fig. 3.12; Group, $F_{(3,31)} = 4.36, P<0.05$) or microglial (Fig. 3.13; Group, $F_{(3,30)} = 7.51, P<0.05$) reaction, suggesting that the 4s-StarPEG crosslinked collagen hydrogels are biocompatible with intra-cranial delivery.

**Figure 3.12. Astrocytic response to collagen hydrogels.** GFAP immunostaining confirmed that the encapsulation of cells in a crosslinked collagen hydrogel did not elicit an exaggerated astrocytic response. Representative photomicrographs show GFAP staining at the transplantation site 7 days post-transplantation. Scale bar represents 100 µm. Data are represented as mean ± SEM and were analysed by two-way ANOVA with post-hoc Bonferroni.
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Figure 3.13. Microglial response to collagen hydrogels. Cd11b immunostaining confirmed that the encapsulation of cells in a crosslinked collagen hydrogel did not elicit an exaggerated microglial response. Representative photomicrographs show cd11b staining at the transplantation site 7 days post-transplantation. Scale bar represents 100 μm. Data are represented as mean ± SEM and were analysed by two-way ANOVA with post-hoc Bonferroni.

3.3.3 In vivo assessment of the impact of the collagen hydrogel on grafted VM cells

Having demonstrated that the collagen hydrogels of lower 4s-StarPEG crosslinking (1-4 mg/ml) are highly cytocompatible (with MSCs) and are well tolerated in vivo, all further studies were carried out using a collagen hydrogel crosslinked with 4 mg/ml of 4s-StarPEG.

3.3.3.1 The effect of encapsulation on primary dopaminergic neuron survival

Once it was confirmed that our collagen hydrogel crosslinked with 4 mg/ml 4s-StarPEG was compatible with intra-striatal MSC delivery, we sought to determine the impact of the collagen hydrogel on the survival and neural outgrowth from encapsulated TH+ primary dopaminergic neurons derived from E14 VM cultures. E14 VM cell densities of 200,000; 300,000 or 400,000 cells were delivered either within control transplantation media or
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encapsulated in the collagen hydrogel. TH\(^+\) cell survival and neural outgrowth was then assessed at 14 days post-transplantation. The delivery of VM cells in the collagen hydrogel did not have any significant impact on the survival of TH\(^+\) dopaminergic neurons (Fig. 3.14a; Group, \(F_{(1,12)} = 2.58, P>0.05\)) or importantly on their neurite outgrowth (Fig. 3.14b; Group, \(F_{(1,12)} =1.08, P>0.05\)). Thus, although the hydrogel was not successful at improving the number of surviving TH\(^+\) dopaminergic neurons, it was not detrimental to graft survival and did not impede outgrowth of neurons from within the hydrogel, confirming that it is possible to deliver a high density of VM cells (400,000 cells in 6 \(\mu\)l) without any negative effects on graft survival.
**Figure 3.14. The effect of the collagen hydrogel on encapsulated primary dopaminergic neurons.** The encapsulation of VM cells of various cell densities (200,000; 300,000 or 400,000 cells) in a collagen hydrogel had no negative effect on the survival of primary dopaminergic neurons (A) or their ability to re-innervate the striatum (B). Photomicrographs show TH* graft survival at the transplantation site 14 days post-transplantation (C). Scale bar represents 1 mm. Data are represented as mean ± SEM and were analysed by two-way ANOVA with post-hoc Bonferroni.

### 3.3.3.2 Biodegradability of collagen hydrogels *in vivo*

Strong, uniform and defined collagen immunostaining at the transplantation site confirmed that our collagen hydrogels had successfully formed *in situ* and were still present 14 days
post-transplantation. Importantly, the encapsulation of rising cell densities had no impact on their gelation (Fig 3.15; Group, $F_{(2,9)} = 0.55, P>0.05$).

**Figure 3.15. Biodegradability of collagen hydrogels *in vivo*.** Collagen hydrogels successfully formed *in situ* and were still present 14 days post-transplantation. Photomicrographs represent striatal collagen staining at 14 days post-transplantation. Scale bar represents 1 mm. Data are represented as mean ± SEM and were analysed by one-way ANOVA with post-hoc Bonferroni.

### 3.3.3.3 Host immune response to encapsulated primary dopaminergic neurons

Knowing that our collagen hydrogels do not themselves elicit an exaggerated host immune response, we sought to establish whether encapsulation of VM cells in a collagen hydrogel
could create a physical barrier between the transplanted VM cells and host neuro-immune cells and thereby reduce the host immune response to the transplanted graft. As expected, the cells delivered in control transplantation media elicited a substantial host immune response. However, the delivery of cells in the collagen hydrogel significantly decreased the volume of astrocytosis around the graft site (Fig. 3.16; Group, \( F_{(1,18)} = 34.01, P<0.0001 \)). Similarly, the delivery of cells in the collagen hydrogel significantly decreased the volume of microgliosis around the graft site (Fig. 3.17; Group, \( F_{(1,17)} = 23.03, P<0.05 \)).

Figure 3.16. Astrocytic response to encapsulated primary dopaminergic neurons. The delivery of cells in the collagen hydrogel significantly decreased the volume of astrocytosis around the graft site. Representative photomicrographs show GFAP staining 14 days post-transplantation in response to the delivery of 400,000 VM cells. Scale bar represents 1 mm. Data are represented as mean ± SEM and were analysed by two-way ANOVA with post-hoc Bonferroni. \(^*P<0.05, \quad **P<0.01\) vs. relative control.
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3.3.4 *In vivo* assessment of GDNF retention within the collagen hydrogel

Having established that the collagen hydrogel is suitable for the intra-striatal delivery of VM cells, we then sought to determine its impact on GDNF retention in the surrounding striatum. Intra-striatal GDNF was delivered either as a bolus or in a collagen hydrogel and assessed at days 1, 2 and 4 post-transplantation. Although the volume of striatal GDNF...
declined over time (Fig 3.18; Time, $F_{(2,18)} = 28.62, P<0.0001$), delivery within the collagen hydrogel significantly improved its retention at the early time points (Fig. 3.18; Group, $F_{(1,18)} = 20.32, P<0.001$).

**Figure 3.18.** *In vivo* assessment of GDNF retention in the collagen hydrogel. GDNF immunostaining showed that despite the significant depletion of GDNF by day 4, the encapsulation of GDNF in a collagen hydrogel significantly increased the volume of striatal GDNF at days 1 and 2 post-transplantation. Representative photomicrographs show striatal GDNF at Day 1 post-transplantation. Scale bar represents 1 mm. Data are represented as mean ± SEM and were analysed by two-way ANOVA with post-hoc Bonferroni. *$P < 0.05$, **$P < 0.01$ vs. relevant control; *$P < 0.05$, ###$P < 0.001$ vs. relevant day 1; **$P < 0.01$ vs. relevant day 2.
3.4 DISCUSSION

Dopaminergic cell replacement therapy is an extremely promising therapeutic strategy that is currently under investigation for the treatment of Parkinson’s disease. However, its use as a routine clinical procedure is hindered by the extremely poor survival of dopaminergic neurons after transplantation (Brundin et al., 2000a). It is therefore imperative that novel and superior delivery strategies are adopted in order to enhance the survival and efficacy of dopaminergic cell transplantation (Winkler et al., 2005a). Biomaterials, particularly collagen-based biomaterials, show extraordinary potential to improve such cell replacement strategies (Moriarty et al., 2018b; Moriarty et al., 2018c; Orive et al., 2009). Thus, the aims of this chapter were to 1) determine the suitability of collagen hydrogels for the intra-cranial delivery of encapsulated cells, 2) determine the optimal level of 4s-StarPEG crosslinking for intra-striatal cell survival, 3) determine the effect of encapsulation on VM cell survival and outgrowth, and 4) determine the effect of encapsulation on GDNF persistence in the brain. Through a series of pilot in vivo studies, we identified the optimal level of crosslinking for the encapsulation and delivery of cells to be 4 mg/ml 4s-StarPEG. Furthermore, in these preliminary in vivo studies we showed that our optimised collagen hydrogel is well tolerated in the brain, significantly reduces the host response to our transplanted cells, and permits the survival and outgrowth of primary dopaminergic neurons, while also acutely retaining GDNF in the striatum at a significantly higher volume (Moriarty et al., 2017).

Before any biomaterial can be used in the brain it must undergo essential toxicity and cytocompatibility assessment. The alamarBlue® assay is an indicator of metabolic function and can therefore be used as a measure of cellular health in in vitro/ex vivo viability studies
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(Hamid et al., 2004; Rampersad, 2012). Our preliminary in vitro/ex vivo assessments showed that the incubation of pre-formed collagen hydrogels with MSC and mesencephalic cultures did not have any negative effect on the cells metabolic activity. Importantly, the increasing concentration of 4s-StarPEG in our collagen hydrogels had no detrimental effects, confirming that neither the collagen nor crosslinker component of our hydrogels were toxic to our cell cultures. Moreover, these results are in line with previous literature reporting the in vitro cytocompatibility of 4s-StarPEG crosslinked collagen hydrogels (Hoban et al., 2013). Neural outgrowth from TH⁺ dopaminergic neurons is essential for the establishment of a new terminal network (Grealish et al., 2010), so one of our most important in vitro findings was that the presence of our crosslinked collagen hydrogels did not hinder the ability of these cells to form neural outgrowths. Notably, although measuring the area of beta-III tubulin fluorescence is not a direct measurement of neurite length, when compared directly to our control group (which were simultaneously cultured without any hydrogel incubation), it gives a strong indication that our hydrogels of increasing crosslinker concentration have no negative impact on neural neurite outgrowth.

Collagen hydrogels possess many favourable properties that make them suitable for neuroregenerative approaches, including their characteristic similarity to the body’s native tissue, making them highly biocompatible and biodegradable, and their ability to naturally support cell adhesion (Khan et al., 2013). However, it is collagen’s ability to form in situ gelling (and therefore injectable) hydrogels that makes them an attractive strategy for the intra-cranial delivery of cellular and neurotrophic therapies (Sargeant et al., 2012). While collagen is capable of naturally forming a hydrogel in situ, its weak mechanical properties make it highly susceptible to rapid degradation in the brain. In an effort to better control the mechanical stability of collagen, the use of synthetic polymers allows for a greater
level of control over the hydrogel’s stability upon gelation (Delgado et al., 2015; Sargeant et al., 2012). PEG is a synthetic polymer used to crosslink numerous materials, including collagen. It holds the advantage of being non-toxic, non-immunogenic and importantly, already FDA approved (Alconcel et al., 2011; Veronese et al., 2005). In order to identify the optimal level of 4s-StarPEG crosslinking for cell encapsulation, we encapsulated GFP-MSCs in collagen hydrogels of rising 4s-StarPEG levels (1-12 mg/ml). Notably, GFP-MSCs were strategically chosen for these early optimisation studies. Their survival at early time-points post-transplantation is well cited in the literature (Hoban et al., 2015; Moloney et al., 2010b; Pollock et al., 2016), while their GFP expression allows for easy graft identification in the striatum (Moloney et al., 2010a). Moreover, in comparison to mesencephalic grafts, MSCs can be efficaciously transplanted into the non-lesioned striatum, eliminating the need for nigrostriatal lesioning prior to transplantation.

While the simple addition of 4s-StarPEG crosslinking to collagen hydrogels will improve the mechanical stability of collagen, resulting in the formation of strong, well defined and uniform hydrogels in situ (see Fig. 3.11 & 3.15), the rising levels of crosslinking also generates a significantly greater intensity of gelation. As shown in our preliminary in vivo studies, the higher levels of 4s-StarPEG crosslinking were detrimental to graft survival, which was evident by the absence of GFP-MSC fluorescence in the transplanted striatum, coupled with the presence of site-specific tissue damage (likely due to the dislodgement of the intensely gelled hydrogel upon tissue sectioning). In contrast, the lower levels of 4s-StarPEG crosslinking still generated well defined and uniform hydrogels in situ (see Fig. 3.11 & 3.15), however, they permitted successful graft survival, showing no significant changes to graft volume when compared to the control group. Based on these findings, we chose the 4s-StarPEG concentration of 4 mg/ml to progress into future studies because it
permits successful graft delivery, while the higher crosslinker concentration will increase the hydrogels resistance to enzymatic degradation in the brain.

Knowing that our optimised collagen hydrogel successfully forms in situ and permits the survival of encapsulated MSCs after transplantation, it was imperative to assess the survival and outgrowth of encapsulated mesencephalic cells. Dopaminergic neurons account for 30-40% of the VM cell population, necessitating the requirement to deliver high densities of cells (and thus, multiple fetal donors) to reach functional benefit (Brundin et al., 2000a). Thus, we sought to determine to what extent we can load our hydrogel with mesencephalic cells without affecting their survival post-transplantation. We showed that a high density of mesencephalic cells (range of 200,000 – 400,000) can be encapsulated and delivered to the striatum without there being any negative effects on the number of surviving dopaminergic neurons. Moreover, although the cells are resident within the formed hydrogel, their neurite outgrowths are capable of extending past the constraints of the hydrogel and re-innervating the striatum. Although the delivery of cells within collagen materials has been reported (Hoban et al., 2013; Sakai et al., 2003; Suuronen et al., 2006; Wakitani et al., 1989), this shows for the first time that primary dopaminergic neurons can be delivered in a 4s-StarPEG crosslinked collagen hydrogel without hindering their ability to re-innervate the striatum. This is imperative because in order to gain functional recovery the transplanted cells must establish a new terminal network with the host striatum – a process that is essential if dopamine neurotransmission is to be restored.

Furthermore, one of the major obstacles facing the transplantation of fetal-derived cells is the substantial host immune response that occurs at the site of transplantation (Barker et al., 1996; Barker et al., 2004). Here, we showed that the exaggerated host response to the transplanted cells was attenuated by the encapsulation of cells in the collagen hydrogel. To
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the best of our knowledge, this is the first report of a reduced immune response to mesencephalic grafts using a collagen hydrogel and is in line with the findings of Hoban and colleagues who reported that a collagen hydrogel can significantly attenuate the immune response to transplanted MSCs (Hoban et al., 2013).

However, it must be duly noted that although the collagen hydrogel is well tolerated in the brain, permits the survival and outgrowth of dopaminergic neurons and protects cells from the host immune response, it was unable to increase the number of surviving dopaminergic neurons. Most likely, this is because cell death in mesencephalic grafts is multifactorial and does not occur at one single time-point (Sortwell et al., 2000). While the elevated host response plays a key role in the extensive cell death seen post-transplantation, studies suggest that the critical time-point in which 80-90% of dopaminergic neurons die is immediately post-transplantation (Sortwell et al., 2001), with trophic factor withdrawal upon transplantation a major contributor. GDNF is a target-derived trophic factor that is highly expressed during prenatal and early postnatal development but is virtually absent in the adult brain (Hunot et al., 1996; Stromberg et al., 1993). It has been well reported that GDNF is advantageous to the survival and protection of dopaminergic neurons and most likely exerts survival promoting activity during the period of target innervation (Chaturvedi et al., 2003; Kauhausen et al., 2013; Lin et al., 1993; Rosenblad et al., 1996). Here we showed that the encapsulation of human recombinant GDNF in our collagen hydrogel considerably increased the level of GDNF immunostaining in the striatum immediately post-transplantation. Although striatal levels of encapsulated GDNF were similar to bolus delivery at day 4, the acute striatal retention immediately post-transplantation could provide cells with enhanced trophic factor support upon
transplantation – the time-point at which the large extent of dopaminergic cell death occurs (Sortwell et al., 2001).

Despite the significant clinical benefits of dopaminergic cell replacement therapies experienced by patients, the extremely poor survival of grafted dopaminergic neurons presents a major obstacle to their clinical translation. The resultant need for novel and enhanced therapeutic strategies has provoked research into the potential of biomaterial-based scaffolds to improve cell replacement therapies. In this chapter, we have shown that our optimised collagen hydrogel is well tolerated in the brain, can significantly reduce the host response to our transplanted cells, and permits the survival and outgrowth of primary dopaminergic neurons, while also acutely retaining GDNF in the striatum at a significantly higher volume (Moriarty et al., 2017). Bearing in mind that the majority of dopaminergic neurons die within the first 4 days post-transplantation, the enhanced delivery of GDNF to the striatum, combined with the attenuation of the host-immune response could in theory increase the survival of dopaminergic neurons by intervening with two essential apoptotic triggers. Thus, in an effort to enhance the functionality of cell replacement therapy in Parkinson’s disease and reduce the ethical concerns associated with the use of large volumes of fetal tissue, the following chapters in this thesis will assess the potential of a GDNF-loaded collagen hydrogel to improve the long-term survival and efficacy of grafted dopaminergic neurons.
Chapter 4: Delivery of E14 primary dopaminergic neurons in a GDNF-loaded collagen hydrogel.

4.1 INTRODUCTION

Cell replacement therapy for Parkinson’s disease has evolved from a relatively simple conceptual framework – if the cardinal motor symptoms of the disease are caused by the relatively selective loss of dopaminergic neurons in the substantia nigra and the subsequent loss of dopamine neurotransmission in the striatum, then the transplantation of healthy and viable dopaminergic neurons to the Parkinsonian brain should (in theory) restore the brain’s dopamine neurotransmission and therefore, provide permanent symptomatic relief to patients. Grafted dopaminergic neurons have been shown to survive, mature and function after transplantation into the adult striatum (Brundin et al., 1987; Dowd et al., 2004; Kefalopoulou et al., 2014; Lindvall et al., 1990). Moreover, numerous studies, both experimentally and clinically, have demonstrated that grafted dopaminergic neurons are capable of the synthesis, release and uptake of dopamine (Rose et al., 1985), exerting electrical firing patterns (Wuerthele et al., 1981), re-innervating the host striatum, and developing graft-host synaptic connections (Bolam et al., 1987). However, despite this, major obstacles hinder its clinical translation.

The extremely poor survival of primary dopaminergic neurons after transplantation is a significant hurdle to its widespread clinical use. With only 5 – 20% of cells surviving the transplantation process, this necessitates the need to transplant large cell densities, resulting in a requirement for large quantities of donor fetal tissue (as many as 6 fetal donors per transplanted hemisphere) and therefore, raising numerous logistical and ethical
Chapter 4: Encapsulation of E14 primary dopaminergic neurons

Concerns (Winkler et al., 2005a). As a result, while the efficacy of dopamine neuron-rich fetal mesencephalic grafts are still being investigated clinically through the TRANSEURO consortium (Barker et al., 2013), the field of cell replacement therapy in Parkinson’s disease is moving towards more readily available dopaminergic cell sources, such as those derived from embryonic stem cells and induced pluripotent stem cells (Stoker et al., 2017). Although these cells show extraordinary regenerative potential, their use is still in the experimental stages and has not yet reached a clinical setting. With this in mind, dopamine neuron-rich fetal mesencephalic grafts are an extremely well established cell type and are therefore optimal for testing the potential of biomaterial scaffolds to improve the survival and efficacy of such cell regenerative therapies.

The majority of cell death in fetal-derived mesencephalic grafts occurs through apoptosis at various points of the transplantation process (Sortwell et al., 2000) by factors such as detachment from the extracellular matrix during tissue dissection (Reddig et al., 2005), growth factor deprivation upon transplantation (Collier et al., 1999) and recruitment of host neuro-immune cells to the exogenous graft after transplantation (Barker et al., 2004). Each of these stages provides a target point of intervention at which graft survival could be improved. Injectable scaffolds, such as in situ forming hydrogels, may provide a delivery platform to improve grafted cell survival after transplantation. These hydrogels could potentially increase cell engraftment by providing a supportive environment for cell adhesion, creating a physical barrier between the transplanted cells and the host neuro-immune cells and by providing a reservoir for localised growth factor delivery (Orive et al., 2009).

A particular scaffold of interest, collagen, is a clinically accepted, highly abundant and natural extracellular matrix that is used for a variety of applications (Khan et al., 2013).
Chapter 4: Encapsulation of E14 primary dopaminergic neurons

The injectable nature of collagen hydrogels, coupled with their ability to support and immunoisolate cells, whilst simultaneously delivering trophic factors in a localised manner, creates a natural scaffold with the potential to improve the transplantation of dopaminergic neurons (Moriarty et al., 2018b; Moriarty et al., 2018c). Therefore, the aim of this chapter was to determine whether the encapsulation and delivery of mesencephalic cells in a trophic factor-loaded collagen hydrogel could enhance the long-term survival and efficacy of dopaminergic neurons.
4.2 METHODS

4.2.1 EXPERIMENTAL DESIGN

Having established that the 4 mg/ml crosslinked collagen hydrogel is well tolerated *in vivo*, supports cell survival and outgrowth, reduces the host immune response and retains GDNF at a higher volume in the striatum, the study presented in this chapter was designed to assess the long-term effect of a GDNF-loaded collagen hydrogel on the survival and efficacy of encapsulated primary dopaminergic neurons. To do this, 40 adult male Sprague-Dawley rats received a unilateral intra-MFB 6-OHDA lesion (3 μl). Two weeks later, rats underwent post-lesion methamphetamine-induced rotations (2.5 mg/kg). Based on these results, rats were performance matched into four groups to receive intra-striatal transplants of E14 VM cells alone (400,000 per 6 μl), E14 VM cells with GDNF (1000 ng), E14 VM cells encapsulated in a collagen hydrogel (crosslinked with 4 mg/ml 4s-StarPEG) or E14 VM cells encapsulated in a GDNF-loaded collagen hydrogel. Methamphetamine-induced rotations resumed three weeks post-transplantation and were carried out at three week intervals for a total of twelve weeks. The animals were then sacrificed for *post mortem* assessment. A schematic of this experimental design is shown in Fig. 4.1.
Figure 4.1 *In vivo* assessment of the impact of a GDNF-loaded collagen hydrogel on the survival and efficacy of primary dopaminergic neurons. Schematic representation of the delivery of E14 VM cells in a GDNF-loaded collagen hydrogel. E14 VM cells (400,000 cells/6 µl) were encapsulated in a GDNF (1000 ng)-loaded collagen hydrogel and delivered to the unilaterally 6-OHDA lesioned striatum. Graft survival, neural outgrowth and the host immune response were assessed at twelve weeks post-transplantation.
4.3 RESULTS

4.3.1 IMPACT OF THE GDNF-LOADED COLLAGEN HYDROGEL ON E14 PRIMARY DOPAMINERGIC NEURON SURVIVAL

In order to evaluate the survival of transplanted grafts, the number of surviving TH⁺ dopaminergic cells throughout the striatum were counted. TH immunostaining identified the successful transplantation of dopaminergic neurons in each group (Fig. 4.2 & Fig 4.3), however, not all groups expressed the same number of surviving neurons (Fig. 4.4a; Group, $F_{(3,33)}= 18.36, P<0.0001$). In line with expectations, the delivery of GDNF with VM cells showed a significant increase in the number of surviving dopaminergic neurons. However, interestingly, when cells were delivered in a GDNF-loaded collagen hydrogel, there was a significant (five-fold) increase in the number of surviving cells when compared to the delivery of VM cells alone (Fig. 4.3). Additionally, cell survival in a GDNF-loaded collagen hydrogel was significantly greater than that of the VM & GDNF group (1.7 fold).

4.3.2 IMPACT OF THE GDNF-LOADED COLLAGEN HYDROGEL ON E14 PRIMARY DOPAMINERGIC NEURON STRIATAL RE-INNERVATION

We then sought to assess the ability of these surviving cells to form neural outgrowths in situ and re-innervate the striatum. Using TH immunostaining, the volume of striatal tissue occupied by innervation from the transplanted dopaminergic cells was measured. All VM grafts did successfully re-innervate a portion of the lesioned striatum (Fig. 4.2), however, not all groups expressed the same magnitude of re-innervation (Fig. 4.4b; Group; $F_{(3,33)}=9.86, P<0.0001$). As expected, the volume of re-innervation was significantly increased with the delivery of GDNF with VM cells. Interestingly, in line with the five-
fold increase in cell survival (above), the magnitude of striatal re-innervation was also significantly greater (four-fold) from the delivery of cells alone when cells were encapsulated in a GDNF-loaded collagen hydrogel (Fig. 4.2). Furthermore, striatal re-innervation from cells in the GDNF-loaded collagen hydrogel was significantly greater than that of cells delivered with GDNF alone (two-fold), showing that the GDNF-loaded collagen hydrogel is not only capable of increasing the number of surviving dopaminergic cells, but also the volume of innervation from these cells.
Figure 4.2. Representative photomicrographs of E14 primary dopaminergic striatal re-innervation. TH immunostaining showed that all groups did successfully re-innervate a portion of the denervated striatum. However, when cells were delivered in a GDNF-loaded collagen hydrogel the magnitude of striatal re-innervation was significantly greater (three-fold vs. VM alone). Scale bar represents 1 mm.
Figure 4.3. Representative photomicrographs of E14 primary dopaminergic neuron survival. TH immunostaining identified the presence of surviving dopaminergic cell bodies in each group. However, when cells were delivered in a GDNF-loaded collagen hydrogel there was a significantly greater yield of surviving cells. Scale bar represents 1 mm and 200 µm (inset).
Chapter 4: Encapsulation of E14 primary dopaminergic neurons

Figure 4.4. *In vivo* assessment of the impact of the GDNF-loaded collagen hydrogel on primary dopaminergic neuron survival and striatal re-innervation. TH immunostaining was used to identify the number of surviving dopaminergic cells throughout the striatum and their ability to re-innervate the lesioned striatum. When cells were delivered in a GDNF-loaded collagen hydrogel there was a significantly greater level of cell survival (A; five-fold increase vs. VM alone) and striatal re-innervation (B; four-fold increase vs. VM alone). Data are represented as mean ± SEM and were analysed by one-way ANOVA with *post-hoc* Bonferroni. *P* < 0.05, ***P* < 0.001 vs. VM alone; *P* < 0.05, ###*P* < 0.001 vs. VM in hydrogel; *P* < 0.05 vs. VM & GDNF.
4.3.3 IMPACT OF THE GDNF-LOADED COLLAGEN HYDROGEL ON GRAFT FUNCTIONALITY

The ability of the transplanted graft to restore motor function to the unilaterally lesioned animals was assessed at three-weekly intervals for 12 weeks post-transplantation using methamphetamine-induced rotations. In line with previous results, we found that the delivery of VM cell grafts significantly reduced the number of ipsilateral rotations in each group (Fig. 4.5; Time, $F_{(4,144)} = 309.5, P<0.0001$). However, the encapsulation of VM cells in a GDNF-loaded collagen hydrogel provided a significantly greater level of functional recovery at both 9 and 12 weeks post-transplantation.
Figure 4.5. Impact of the GDNF-loaded collagen hydrogel on graft functionality. Transplantation of VM grafts significantly decreased the number of ipsilateral turns made in each group. However, the delivery of cells in a GDNF-loaded collagen hydrogel resulted in a significantly greater level of functional recovery at 9 and 12 weeks post-transplantation. PL; post-lesion, PT; post-transplant. Data are represented as mean ± SEM and were analysed by two-way repeated measures ANOVA with post-hoc Bonferroni. *$P < 0.05$ VM alone vs. VM & GDNF in hydrogel; ###$P < 0.001$, #$P < 0.05$ VM in hydrogel vs. VM & GDNF in hydrogel; +$P < 0.05$ VM & GDNF vs. VM & GDNF in hydrogel.
4.3.4 CORRELATION BETWEEN E14 VM GRAFT SURVIVAL AND FUNCTION

Having established that the encapsulation of VM cells in a GDNF-loaded collagen hydrogel was advantageous to their survival and outgrowth, it was important to determine whether the increased number of surviving dopaminergic neurons correlated with the observed improvement in graft function.

When we looked at the relationship between the number of surviving dopaminergic neurons and the volume of striatal re-innervation, there was a strong correlation (Fig. 4.6a; \( r = 0.81, P<0.0001 \)), indicating that the three-fold increase in striatal re-innervation was likely related to the improved cell survival caused by the encapsulation of cells in the GDNF-loaded collagen hydrogel.

The encapsulation of cells in a GDNF-loaded collagen hydrogel did not only result in an increased cell survival and re-innervation but also a greater level of behavioural recovery. This significant behavioural recovery correlated strongly with the number of surviving \( \text{TH}^+ \) cells (Fig. 4.6b; \( r = 0.67, P<0.0001 \)) and also to the volume of striatal re-innervation (Fig. 4.6c; \( r = 0.71, P<0.0001 \)). This indicates that the significant reduction of net ipsilateral turns seen is likely related to the enhanced delivery and efficacy of cells in the GDNF-loaded collagen hydrogel.
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Figure 4.6. Correlation between graft survival and functionality. A strong positive correlation was found between the number of surviving TH\(^+\) neurons and the volume of striatal re-innervation (A: \(r = 0.81\)). Additionally, a strong negative correlation was found between the number of net ipsilateral turns taken and the number of surviving TH\(^+\) cells (B: \(r = 0.67\)) and striatal re-innervation (C: \(r = 0.71\)).
4.3.5 Impact of the GDNF-Loaded Collagen Hydrogel on Host Immune Response

In line with the results from our preliminary study (see section 3.3.3.3), the transplantation of VM cells elicited a host immune response in the striatal tissue surrounding the graft site (Fig. 4.7 & 4.8). However, when the VM cells were delivered encapsulated in a collagen hydrogel or a GDNF-loaded collagen hydrogel there was a significant decrease in striatal microgliosis (Fig. 4.7a; Volume: Group, $F_{(3,30)} = 9.79, P<0.0001$; Optical density: Fig. 4.7b; Group, $F_{(3,32)} =5.84, P<0.01$) and astrocytosis (Fig. 4.8a; Volume: Group, $F_{(3,28)} =6.66, P<0.01$; Optical density: Fig 4.8b; $F_{(3,32)} = 9.64, P<0.01$), indicating that the loading of a collagen hydrogel with GDNF does not hinder its ability to act as a protective matrix to the grafted cells, reducing the host immune response.

4.3.6 Biodegradability of the Collagen Hydrogel in Vivo

The absence of collagen immunohistochemical staining 12 weeks post intra-striatal delivery showed that the collagen hydrogel is biodegradable at the implanted site (not shown).

4.3.7 Expression of Human-GDNF In Situ

The absence of human-GDNF immunohistochemical staining 12 weeks post intra-striatal delivery showed that the transplanted GDNF was cleared from the brain (not shown).
Figure 4.7. Impact of the GDNF-loaded collagen hydrogel on the host microglial response. Cd11b immunostaining for microglial activity at the transplantation site showed that the volume (A) and density (B) of microgliosis was significantly decreased with the encapsulation of cells in a collagen hydrogel or a GDNF-loaded collagen hydrogel (C). Scale bar represents 100 μm. Data are represented as mean ± SEM and were analysed by one-way ANOVA with post-hoc Bonferroni. *P < 0.05, **P < 0.01 vs. VM alone; #P < 0.05, ###P < 0.001 vs. VM & GDNF.
Figure 4.8. Impact of the GDNF-loaded collagen hydrogel on the host astrocytic response. GFAP immunostaining for astrocyte activity at the transplantation site showed that the volume (A) and density (B) of astrogliosis was significantly decreased with the encapsulation of cells in a collagen hydrogel or a GDNF-loaded collagen hydrogel (C). Scale bar represents 100 μm. Data are represented as mean ± SEM and were analysed by one-way ANOVA with post-hoc Bonferroni. *P < 0.05, **P < 0.01 vs. VM alone; #P < 0.05, ###P < 0.001 vs. VM & GDNF.
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4.4 DISCUSSION

Primary dopaminergic neurons derived from the VM of fetal tissue are an exogenous cell source with great potential as a cell replacement strategy in Parkinson’s disease. Numerous studies have shown their ability to re-innervate the striatum and restore motor function in rodents and non-human primates (Björklund et al., 1980b; Brundin et al., 1986; Dowd et al., 2004; Dunnett et al., 1981; Torres et al., 2007a). While, clinical trials have shown that motor function can be dramatically improved by the transplantation of VM tissue (Freed et al., 2001; Kefalopoulou et al., 2014; Lindvall et al., 1990; Olanow et al., 2003), the poor survival of cells post-transplantation means that multiple fetal donors are required to obtain a level of cell survival necessary for functional benefit. The sheer scarcity of fetal donors and associated ethical and logistical constraints hinders its translation to a routine therapeutic procedure (Winkler et al., 2005a). Therefore, in an effort to lessen the ethical and logistical concerns associated with the use of multiple fetal donors, this chapter aimed to assess the potential of a trophic factor-loaded collagen hydrogel to enhance the long-term survival and efficacy of primary dopaminergic neurons. Overall, the work described in this chapter shows that the encapsulation of cells derived from the E14 VM in a GDNF-loaded collagen hydrogel resulted in a 5-fold increase in primary dopaminergic neuron survival and a 3-fold increase in striatal re-innervation, which correlated with a significantly greater level of functional recovery (Moriarty et al., 2017). Overall, this highlights the potential of growth factor enriched biomaterial matrices to enhance cell replacement therapies in neurodegenerative diseases such as Parkinson’s disease.

It was previously assumed that cell death in VM grafts was predominantly necrotic, occurring as a result of cell insult during the tissue dissection, preparation and transplantation process. While some necrotic cell death does occur during the cell
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preparation, the large extent of cell death in VM grafts occurs post-transplantation through apoptosis and is predominantly driven by external factors in the cells environment rather than a physiologic insult (Mahalik et al., 1994; Schierle et al., 1999; Sortwell et al., 2001). Apoptosis can be triggered at numerous stages of the grafting process (Sortwell et al., 2000). Firstly, cell death can be triggered during the cell dissection/dissociation process by anoikis (detachment from extracellular matrix). Secondly, the vast extent of cell death is triggered immediately post-transplantation due to the lack of trophic factors in the adult striatum, and finally, cell death also occurs post-transplantation with the onset of the host-immune response to the established graft. Each of these stages provides a point of intervention at which graft survival could be improved.

Even with the clean and efficient dissection of VM tissue, mechanical destruction caused by the cells detachment from the extracellular matrix (known as anoikis) during dissociation removes the normal cell-matrix interactions and cell death can ensue (Marchionini et al., 2003; Reddig et al., 2005). While many biomaterial scaffolds require chemical manipulation to improve cell adherence (Carlisle et al., 2000; Krijgsman et al., 2002; Woerly et al., 1995), collagen mimics the extracellular matrix and contains the natural Arg-Gly-Asp (RGD) tripeptide sequence that facilitates cell adhesion and thus provides an advantageous environment for cell delivery (Hersel et al., 2003; Hoban et al., 2013).

Numerous studies have shown that the delivery of exogenous cells to the brain evokes an elevated host response (Barker et al., 1996; Hudson et al., 1994; Olanow et al., 2003; Shinoda et al., 1995). Moreover, in line with the vast extent of cell death seen within the first 4 days after transplantation, increases in microglial activation, lymphocyte infiltration and MHC expression have been recorded during this period (Duan et al., 1995). In Chapter
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3 we demonstrated that collagen hydrogels, cross linked with 4s-StarPEG, are immune-neutral upon transplantation, while also attenuating the host response to the transplanted graft through the formation of a physical barrier between the transplanted cells and the host neuro-immune cells (Moriarty et al., 2017). Similarly, in this study we have shown that our GDNF-loaded collagen hydrogel possesses the same immuno-protective properties. Interestingly, this attenuation of the host response outlives the degradation of the collagen hydrogel which occurred previous to this 12 week time-point. While unclear, it is possible that the slow degradation of collagen (afforded by the enhanced mechanical strength using 4s-StarPEG) provided cells with a protective environment throughout their maturation and establishment of a new terminal network. Mature cells that are integrated with the host system may be more resilient to the host response and/or be characteristically less immunogenic. However, it must be noted that immuno-histological analysis, namely volume and density, is only a gross measure of astrocytosis and microgliosis and while it gives a strong indication of the level of immune response generated, it does not provide detailed information on cell activation. Further analysis would be required to carry out a detailed assessment of the hydrogel’s attenuation of the host response.

Although significant increases in cell survival were not observed with cell encapsulation alone (discussed in Chapter 3), it is important to note that apoptosis post-transplantation is triggered by more than one factor and not solely the host immune response (Sortwell et al., 2000). Primary dopaminergic neurons are derived from the VM of the developing embryo, and are therefore being removed from a trophic rich environment at the height of neurogenesis and subsequently placed into a trophic depleted adult striatum. In line with previous reports, we have shown that the addition of GDNF to VM cell suspensions enhances the survival and re-innervation of dopaminergic neurons. Interestingly, cell
survival and re-innervation was further enhanced (5-fold and 3-fold vs. control, respectively) when cells were delivered in a GDNF-loaded collagen hydrogel. Despite not finding any GDNF staining 12 weeks post-transplantation due to its relatively short half-life (3-4 days), preliminary in vivo studies (reported in Chapter 3) showed that the encapsulation of GDNF in the collagen hydrogel significantly retained GDNF in the striatum immediately post-transplantation. Assuming that this was also the case when cells were encapsulated in the GDNF-loaded hydrogel, the enhanced, site-specific retention of GDNF in the striatum provided primary dopaminergic neurons with critical trophic support upon transplantation and throughout target innervation.

Graft functionality was assessed using methamphetamine-induced rotational behaviour as it is the ‘gold standard’ measure of striatal dopamine depletion in the MFB lesion model (Ungerstedt et al., 1970). The MFB lesion model of Parkinson’s disease is long established, and a single injection of the neurotoxin 6-OHDA is usually sufficient to degenerate >90% of dopaminergic neurons (Torres et al., 2011). A threshold of, on average, 6 turns per minute is indicative of a ‘successful lesion’ and is widely considered as the threshold required for the generation of a stable lesion. In this case, animals were rotating greater than 6 turns per minute, which is above the established threshold. This was further confirmed by extensive dopamine loss in the dorsal and ventral striatum (areas that did not receive cell transplantations), strongly suggesting that animals had extensive nigrostriatal lesions and as a result, any re-innervation seen is from the transplanted cells. As expected, the delivery of VM cells in each group resulted in significant behavioural recovery, suggesting that all grafts were successfully producing dopamine upon transplantation. However, at 12 weeks post-transplantation, animals who received VM cells encapsulated in a GDNF-loaded collagen hydrogel exhibited a much greater level of
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functional recovery. The strong correlation between this enhanced functional recovery and the 5-fold increase in dopaminergic cell survival and the 3-fold increase in striatal re-innervation, suggests that the enhanced delivery of cells in a GDNF-loaded collagen hydrogel results in increased striatal dopamine levels, and subsequently enhanced functional efficacy. Notably, while the efficacy of cell replacement strategies in Parkinson’s disease is dependent on the enhanced survival and axonal outgrowth of transplanted dopaminergic neurons, a corresponding enhanced restoration of striatal dopamine neurotransmission (and functional recovery) is crucial for such therapies to reach their full potential.

While tackling each of these triggers alone may not be sufficient to improve graft function, the summation of enhanced cell delivery, increased trophic factor support and the attenuation of the immune response results in a significant improvement in dopaminergic cell survival, efficacy and importantly, motor function (Moriarty et al., 2017). Moreover, this data is in line with two recent studies of biomaterial approaches to dopaminergic neuron survival after transplantation in the brain (Adil et al., 2017; Wang et al., 2016). In the study by Wang et al., (2016), a GDNF-functionalised composite poly(l-lactic acid)/xyloglucan hydrogel was shown to enhance survival of, and striatal re-innervation from, transplanted mouse VM grafts in Parkinsonian mice, while in Adil et al., (2017), a heparin/RGD functionalised hyaluronic acid hydrogel was shown to improve the survival of transplanted human embryonic stem cell-derived dopaminergic neurons. Taken together with the current study, this literature highlights the potential of biomaterial hydrogel scaffolds to improve the outcome of reparative cell therapies for Parkinson’s disease.

In recent years the influence of donor age on VM graft efficacy has received heightened interest. A study by Torres and colleagues demonstrated that the use of younger embryonic
donor tissue (E12 vs the conventional E14 used in this study) generates a significantly larger yield of surviving dopaminergic neurons after transplantation (Torres et al., 2007b). It was hypothesised that the enhanced survival seen with this younger donor age may be a result of trophic support from the attached meningeal layer, a component that is easily removed in E14 preparations. Thus, given that the margin for improvement in cell therapies is considerable, the following chapter aims to determine whether our GDNF-loaded collagen hydrogel can provide additional support to dopaminergic neurons derived from younger age donor tissue and therefore further increase graft survival and efficacy.
Chapter 5: Delivery of E12 primary dopaminergic neurons in a GDNF-loaded collagen hydrogel

5.1 INTRODUCTION

For several decades dopaminergic neurons derived from the VM of human embryonic donors have shown to survive, integrate and function when transplanted ectopically into the Parkinsonian brain. While open label clinical trials have provided ‘proof of principle’ evidence of the ability of these cells to alleviate motor symptoms in patients (Brundin et al., 2000b; Freed et al., 1992; Lindvall et al., 1990; Lindvall et al., 1994), poor survival and inadequate striatal re-innervation have halted their clinical progression (Freed et al., 2011; Winkler et al., 2005a). Thus, if fetal-derived dopaminergic neuron cell transplantation is to become an efficacious therapeutic procedure, novel and improved strategies to enhance cell survival after transplantation must be achieved. In an effort to improve the survival and integration of grafted dopaminergic neurons, numerous studies have investigated the use of VM cells derived from younger embryonic donors (Bye et al., 2012; Gates et al., 2006; Somaa et al., 2015; Torres et al., 2008; Torres et al., 2007b).

For the successful generation of dopaminergic neuron-rich grafts after transplantation, the VM must be collected during a specific time frame of neurogenesis to allow for the level of maturity required for commitment to a dopaminergic neuron phenotype, while also avoiding over-differentiation prior to transplantation (Torres et al., 2007b). In experimental studies (rat), the conventional donor age has become E14 (or equivalent age in mice), as it was perceived to coincide with peak dopamine neurogenesis in the developing brain (Dunnett, 1991; Gates et al., 2006; Hegarty et al., 2013). However, in recent years, the
influence of donor age on graft efficacy has received heightened interest. Numerous studies have shown that the transplantation of E12 (or equivalent age in mice) VM cell suspensions results in a higher fraction of surviving dopaminergic neurons and striatal re-innervation (Bye et al., 2012; Somaa et al., 2015; Torres et al., 2007b), and a higher proportion of A9 dopaminergic neurons (subtype involved in the restoration of motor function) (Grealish et al., 2010). It was hypothesised that such improvements were related to the increased number of surviving dopaminergic neurons, alongside the post-transplantation differentiation of dopaminergic precursors present in E12 VM cell preparations (Torres et al., 2007b). Furthermore, a recent study has shown that the inclusion of meningeal cells from young embryonic donors with VM preparations enhanced both the survival and axonal outgrowth of transplanted dopaminergic neurons (Somaa et al., 2015), suggesting that the enhanced survival with VM cell suspensions of younger donor age may be a result of trophic support from the attached meningeal layer and adjacent neural floor plate, components that are easily removed in older, E14, preparations. While the exact mechanism through which meningeal cells support dopaminergic neurons remains to be elucidated, these studies highlight the potential benefits of using VM tissue from younger embryonic donors in future studies, both experimentally and clinically.

We have already shown that the summation of enhanced cell delivery, increased trophic factor support and attenuation of the host immune response afforded by our GDNF-loaded \textit{in situ} gelling collagen hydrogel resulted in a 5-fold increase in E14 VM cell survival, a 3-fold increase in striatal re-innervation, and a significantly greater level of functional recovery (Moriarty et al., 2017). Thus, given that the margin for improvement in dopamine cell therapies is considerable, and to further determine the potential of biomaterials to
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improve such dopaminergic cell replacement therapies, the following chapter aims to determine whether our GDNF-loaded collagen hydrogel can provide additional support to dopaminergic neurons derived from younger age donor tissue (including their attached meningeal layer) and therefore further enhance graft survival and efficacy.
Chapter 5: Encapsulation of E12 primary dopaminergic neurons

5.2 METHODS

5.2.1 EXPERIMENTAL DESIGN

In light of the results presented in the previous chapter, which showed for the first time that a GDNF-loaded collagen hydrogel could dramatically enhance the survival, re-innervation and functionality of primary dopaminergic neurons through a reduction in the host immune response to the transplanted cells and a simultaneous increase in the acute retention of GDNF at the transplantation site, the study presented in this chapter was designed to assess the long-term effect of a GDNF-loaded collagen hydrogel on the survival and efficacy of younger donor age primary dopaminergic neurons, namely E12 VM cells. To do this, 21 adult male Sprague-Dawley rats received a unilateral intra-MFB 6-OHDA lesion (12 µg in 3 µl). Two weeks later, rats underwent post-lesion methamphetamine-induced rotations (2.5 mg/kg) and based on these results, were performance matched into four groups (n= 5-6 per group) to receive unilateral intra-striatal transplants of E12 VM cells alone (200,000 per 6 µl), E12 VM cells with GDNF (1000 ng), E12 VM cells encapsulated in a collagen hydrogel (crosslinked with 4 mg/ml 4s-StarPEG) or E12 VM cells encapsulated in a GDNF-loaded collagen hydrogel. Methamphetamine-induced rotations resumed three weeks post-transplantation and were carried out at three week intervals for a total of twelve weeks. The animals were then sacrificed for post mortem assessment. A schematic of this experimental design is shown in Fig. 5.1.
Figure 5.1. *In vivo* assessment of the impact of a GDNF-loaded collagen hydrogel on the survival and efficacy of E12 primary dopaminergic neurons. Schematic representation of the delivery of E12 VM cells in a GDNF-loaded collagen hydrogel. E12 VM cells (200,000 cells/6 µl) were encapsulated in a GDNF (1000 ng)-loaded collagen hydrogel and delivered to the unilaterally 6-OHDA lesioned striatum. Graft survival, neural outgrowth and the host immune response were assessed at twelve weeks post-transplant.
5.3 RESULTS

5.3.1 IMPACT OF THE GDNF-LOADED COLLAGEN HYDROGEL ON E12 PRIMARY DOPAMINERGIC NEURON SURVIVAL

In order to assess the survival of transplanted E12 VM cells, the number of surviving TH+ dopaminergic cells throughout the striatum were counted. TH immunostaining identified the successful transplantation of dopaminergic neurons in each group (Fig. 5.2 & Fig. 5.3), however, not all groups expressed the same number of surviving neurons (Fig. 5.4a; Group, $F_{(3,17)} = 21.78, P<0.0001$). In contrast to our previous findings, the delivery of GDNF with E12 VM cells did not increase the number of surviving dopaminergic neurons. However, interestingly when cells were delivered encapsulated in a collagen hydrogel, there was a positive trend towards an increase in the number of surviving cells when compared to the delivery of VM cells alone. Moreover, the encapsulation of cells in a GDNF-loaded collagen hydrogel resulted in a significant increase in cell survival (4.1-fold vs. VM alone). Additionally, cell survival in a GDNF-loaded collagen hydrogel was significantly greater than encapsulation in a collagen hydrogel without GDNF (2.3-fold vs. VM in hydrogel).

5.3.2 IMPACT OF THE GDNF-LOADED COLLAGEN HYDROGEL ON E12 PRIMARY DOPAMINERGIC NEURON STRIATAL RE-INNERVATION

We then sought to assess the ability of these surviving cells to form neural outgrowths in situ and re-innervate the striatum. Using TH immunostaining we showed that all E12 VM grafts did successfully re-innervate a portion of the lesioned striatum (Fig. 5.3), however, not all groups expressed the same magnitude of re-innervation (Fig. 5.4b; Group;
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$F_{(3,17)}=8.85, P<0.0001)$. Again, in contrast to our previous findings, the delivery of GDNF with E12 VM cells had no impact on the volume of striatal re-innervation. However, in a similar manner to the survival of TH$^+$ E12 dopaminergic neurons (above), the delivery of cells in a collagen hydrogel resulted in a tendency towards a greater magnitude of striatal re-innervation when compared to the delivery of cells alone. Moreover, the delivery of cells in a GDNF-loaded collagen hydrogel resulted in a significantly greater level of striatal re-innervation (5.4-fold vs. VM alone). This shows that in a similar manner to the delivery of E14 VM cells, the GDNF-loaded collagen hydrogel is capable of increasing both the survival and re-innervation capacity of younger donor age, E12, dopaminergic cells.
Figure 5.2. Representative photomicrographs of E12 primary dopaminergic striatal re-innervation. Using TH immunostaining we showed that all E12 VM grafts did successfully re-innervate a portion of the lesioned striatum. However, when cells were delivered in a GDNF-loaded collagen hydrogel there was a significantly greater magnitude of striatal re-innervation. Scale bars represent 1 mm.
Figure 5.3. Representative photomicrographs of E12 primary dopaminergic neuron survival. TH immunostaining was used to identify the number of surviving E12 dopaminergic cells throughout the graft. The delivery of E12 VM cells in a GDNF-loaded collagen hydrogel resulted in a significantly greater level of cell survival. Scale bars represent 200 μm.
Figure 5.4. *In vivo* assessment of the impact of the GDNF-loaded collagen hydrogel on E12 primary dopaminergic neuron survival and striatal re-innervation. TH immunostaining was used to identify the number of surviving E12 dopaminergic cells throughout the striatum and their ability to re-innervate the lesioned striatum. When cells were delivered in a GDNF-loaded collagen hydrogel there was a significantly greater level of cell survival (A) and striatal re-innervation (B). Data are represented as mean ± SEM and were analysed by one-way ANOVA with post-hoc Bonferroni. **P < 0.01, ***P < 0.001 vs. VM alone; **P < 0.01 vs. VM in hydrogel; **P < 0.01, ***P<0.001 vs. VM & GDNF.

5.3.3 Impact of the GDNF-Loaded Collagen Hydrogel on E12 Graft Functionality

Methamphetamine-induced rotational behavior was carried out at three-weekly intervals for a total of 12 weeks to assess the ability of the transplanted E12 VM grafts to restore motor function to unilaterally lesioned animals. In line with previous literature, the intra-striatal delivery of E12 VM cell grafts significantly reduced the number of ipsilateral rotations in each group (*Fig. 5.5*. Time, $F_{(4,68)} = 106.3, P<0.0001$). However, the delivery of E12 cells in collagen hydrogels resulted in a significantly greater level of functional recovery at 6, 9 and 12 weeks post-transplantation (*Fig. 5.5*. Group x Time, $F_{(12,68)} = 5.04,$
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Moreover, the delivery of E12 cells in a GDNF-loaded collagen hydrogel resulted in an even greater level of functional recovery at 12 weeks post-transplantation.

**Figure 5.5. Impact of the GDNF-loaded collagen hydrogel on graft functionality.** Transplantation of E12 VM grafts significantly decreased the number of ipsilateral turns made in each group. However, the delivery of E12 cells in collagen hydrogels resulted in a significantly greater level of functional recovery at 6, 9 and 12 weeks post-transplantation. Moreover, the delivery of E12 cells in a GDNF-loaded collagen hydrogel resulted in an even greater level of functional recovery at 12 weeks post-transplant. PL; post-lesion, PT; post-transplant. Data are represented as mean ± SEM and were analysed by two-way repeated measures ANOVA with post-hoc Bonferroni. *P<0.05, **P<0.01, ***P<0.001 vs. VM alone; #P<0.05, ##P<0.01, ###P<0.001 vs. VM & GDNF; +P<0.05 vs. VM in hydrogel.
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5.3.4 CORRELATION BETWEEN E12 VM GRAFT SURVIVAL AND FUNCTION

Having established that, similar to the delivery of E14 VM cells, the encapsulation of E12 cells in our GDNF-loaded collagen hydrogel significantly increased the survival and re-innervation of dopaminergic neurons, we next assessed whether the enhanced delivery of E12 dopaminergic neurons correlated with their significantly greater functional recovery.

When we looked at the relationship between the number of surviving TH⁺ dopaminergic neurons and the volume of striatal re-innervation, we found a strong positive correlation (Fig. 5.6a; r = 0.75, P<0.0001), indicating that the enhanced re-innervation seen is likely related to the enhanced delivery and survival of cells in a GDNF-loaded collagen hydrogel.

When we looked at the relationship between cell survival or re-innervation and behavioral recovery, we found that the significant behavioral recovery correlates strongly with the number of surviving TH⁺ dopaminergic neurons (Fig. 5.6b; r = 0.79, P<0.0001) and also the volume of striatal re-innervation (Fig. 5.6c; r = 0.87, P<0.0001). This indicates that the significant reduction in ipsilateral turns after transplantation is likely related to the enhanced survival and re-innervation capacity of E12 cells delivered in a GDNF-loaded collagen hydrogel.
Figure 5.6. Correlation between E12 graft survival and functionality. A strong positive correlation was found between the number of surviving TH⁺ dopaminergic neurons and the magnitude of striatal re-innervation (A: $r = 0.75$). Moreover, a strong negative correlation was found between the number of net ipsilateral turns taken and the number of TH⁺ dopaminergic cells (B: $r = 0.79$) and striatal re-innervation (C: $r = 0.87$).
Chapter 5: Encapsulation of E12 primary dopaminergic neurons

5.3.5 Biodegradability of the collagen hydrogel in vivo

As previously described (Chapter 4), the absence of collagen immunostaining 12 weeks post intra-striatal delivery indicates that the collagen hydrogel is biodegradable at the transplantation site (not shown).

5.3.6 Expression of human-GDNF in situ

As previously described, the absence of human-GDNF immunostaining 12 weeks post intra-striatal delivery indicates that the transplanted human-GDNF is cleared from the brain prior to this 12 week time-point (not shown).

5.3.7 Comparison between E12 and E14 cell survival

In order to evaluate the magnitude of survival seen with VM cells derived from younger donor age embryos, we compared the number of surviving E12 TH⁺ dopaminergic cells in the present study to the number of surviving E14 TH⁺ dopaminergic neurons from the previous study (Chapter 4, section 4.3.1). When comparing the delivery of 400,000 E14 VM cells and 200,000 E12 VM cells, we found that the E12 VM cell grafts generated a similar number of surviving TH⁺ dopaminergic neurons when compared to their E14 counterpart (Fig. 5.7a; Group, \( F_{(1,48)} = 8.16, P<0.01 \)). Interestingly, the survival of E12 cells delivered in a collagen hydrogel was significantly greater than the delivery of E14 cells in the same manner. Moreover, given that significantly less E12 cells were delivered than E14 cells (200,000 vs. 400,000 cells), when TH⁺ cell numbers were expressed as a percentage of the total number of VM cells transplanted, the survival rate of dopaminergic neurons from E12 grafts was significantly greater than E14 grafts (Fig. 5.6b; Group, \( F_{(1,48)} = 87.80, P<0.0001 \)).
In a similar manner, the volume of striatal re-innervation from E12 cell grafts, containing 200,000 VM cells, was similar to that of E14 cell grafts, containing 400,000 VM cells (Fig. 5.7c; Group, $F_{(1,48)} = 3.70, P<0.0001$). Interestingly, when E12 cells were delivered in a collagen hydrogel or a GDNF-loaded collagen hydrogel there was significantly greater level of striatal re-innervation than seen with the delivery of E14 cells in the same manner.

In line with previous literature, this indicates that the use of VM cells from E12 donor tissue generates significantly larger dopaminergic grafts, and moreover, the delivery of E12 VM cells in a collagen hydrogel and in particular, a GDNF-loaded collagen hydrogel results in an even greater level of survival and re-innervation.
Figure 5.7. Comparison between E12 and E14 cell survival. The number of surviving dopaminergic neurons and the volume of striatal re-innervation was similar between E12 and E14 VM grafts. However, given that significantly less E12 cells were delivered than E14 cells (200,000 vs. 400,000 cells), when cell numbers were expressed as a fraction of the total number of transplanted cells, the survival rate of dopaminergic neurons from E12 grafts was significantly greater than E14 grafts. Data are represented as mean ± SEM and were analysed by two-way ANOVA with post-hoc Bonferroni. *P<0.05, **P<0.01, ***P<0.001 vs. relative E14 group.
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5.4 DISCUSSION

Despite numerous ‘proof of principle’ studies demonstrating the efficacy of embryonic-derived dopaminergic neurons for brain repair in Parkinson’s disease, the extremely poor survival associated with their transplantation has prevented them from reaching their full potential (Brundin et al., 2000a). In an effort to improve the survival and integration of grafted dopaminergic neurons, several studies have demonstrated that the use of VM cells derived from younger embryonic donor tissue yields larger numbers of surviving dopaminergic neurons, as well as greater levels of striatal re-innervation (Somaa et al., 2015; Torres et al., 2008; Torres et al., 2007b). Thus, in light of the positive effects that our GDNF-loaded collagen hydrogel had on the survival and efficacy of cells derived from the conventional donor age, E14 (Moriarty et al., 2017), this chapter aimed to determine if our GDNF-loaded collagen hydrogel could provide additional support to dopaminergic neurons derived from younger age donor tissue (E12) and therefore further enhance graft survival and efficacy. Overall, the work described in this chapter showed that the encapsulation of E12 VM cells in a GDNF-loaded collagen hydrogel resulted in a 4.1-fold increase in primary dopaminergic cell survival, a 5.4-fold increase in striatal re-innervation and a significantly greater level of functional recovery (Moriarty et al., 2018a). Moreover, while not significant, when cells were encapsulated in a GDNF-free collagen hydrogel, there was a positive trend towards an increase in the number of surviving cells and the volume of striatal re-innervation. Overall these findings further highlight the potential of biomaterial scaffolds to enhance cell replacement therapies in neurodegenerative diseases such as Parkinson’s disease.

Embryonic donor age is an important factor that must be considered during VM cell transplantation. For the successful collection of transplantable primary dopaminergic
neurons, the VM must be collected during a specific time frame of neurogenesis to allow for the level of maturity required for commitment to a dopaminergic neuron phenotype, while also avoiding over-differentiation prior to transplantation. Early studies identified the upper limits of donor age to be E17-18 (or equivalent in mice) when using solid VM tissue pieces (Simonds et al., 1990) and E15-16 (or equivalent in mice) when using single VM cell suspensions (Brundin et al., 1985). From here, E14 (or equivalent in mice) became the conventional donor age as it was perceived to coincide with peak dopamine neurogenesis. However, in an effort to enhance VM cell transplantation techniques, studies have reported that the use of younger E12 donor tissue (or equivalent in mice) yields significantly larger grafts (Torres et al., 2008; Torres et al., 2007b). Indeed, findings in this chapter support these reports. When we compared the delivery of untreated E12 and untreated E14 VM cells we found that the delivery of E12 VM cells resulted in a 4.5-fold increase in the number of surviving dopaminergic neurons. Moreover, the delivery of E12 VM cells alone generated dopaminergic cell yields similar to the delivery of E14 VM cells with GDNF.

While the use of younger donor age VM tissue results in enhanced yields of surviving dopaminergic neurons, cells are still subject to unfavorable conditions upon transplantation, such as, 1) matrix detachment during tissue dissection and dissociation (anoikis) (Reddig et al., 2005), 2) growth factor (and/or possibly differentiation factor) deprivation upon transplantation into the adult brain (Collier et al., 1999) and 3) the host immune response (Barker et al., 1996). We have previously shown that a GDNF-loaded collagen hydrogel can significantly improve E14-derived dopaminergic cell survival and efficacy through the summation of enhanced cell delivery, increased trophic factor support and the attenuation of the host immune response (Moriarty et al., 2017). Thus, if a GDNF-loaded collagen hydrogel could provide younger donor age cells (E12) with similar
support, the yield of surviving dopaminergic neurons and thus the dependency on multiple fetal donors could be dramatically improved.

In contrast to our previous findings, the delivery of GDNF alone with E12 VM cells had no significant effect on the number of surviving neurons. GDNF is known to be a potent neurotrophic factor for dopaminergic neurons, however, its inability to increase E12 VM cell survival could in part be due to their earlier developmental stage. In a study by Torres and colleagues, the authors demonstrated that while over-expression of GDNF in the transplanted striatum significantly increased the survival of E14-derived dopaminergic neurons, it had little effect on E12-derived dopaminergic neurons. In contrast, the over-expression of sonic hedgehog (SHH), a known dopaminergic differentiation factor, significantly increased the number of E12-derived dopaminergic neurons but had no effect on E14-derived neurons (Torres et al., 2005). The majority of dopaminergic neurons derived from E14 VM tissue are post-mitotic at the time of transplantation (Sinclair et al., 1999), however, E12 VM tissue contains a large fraction of actively dividing cells which can differentiate into dopaminergic neurons post-transplantation (Bye et al., 2012; Gates et al., 2006; Jonsson et al., 2009). Taken together with the present data, this suggests that cells derived from the E12 VM are too young to respond to GDNF treatment.

Interestingly, the delivery of cells in a GDNF-free collagen hydrogel showed a positive trend towards a higher number of surviving dopaminergic neurons and a higher level of striatal re-innervation. These findings are in contrast to the delivery of E14 cells in a collagen hydrogel where no effect on cell survival was seen (Moriarty et al., 2017). We hypothesised that the collagen hydrogel had no effect on E14 cell survival because these cells, although protected from the host response, were denied the necessary neurotrophic support upon transplantation (Sortwell et al., 2001). However, unlike the E14 VM, the E12
Chapter 5: Encapsulation of E12 primary dopaminergic neurons

VM contains a tightly adhered meningeal layer (and adjacent neural floor plate) that is easily removed in older donor age (E14) preparations. These cells are known to secrete proteins involved in neurogenesis, cell migration, cell differentiation and axonal growth, including retinoic acid, bone morphogenic protein-7, fibroblast growth factor-2 and stromal derived factor-1 (Choe et al., 2012; Reiss et al., 2002; Siegenthaler et al., 2009; Zhang et al., 2003; Zhu et al., 2002), as well as proteins involved in extracellular matrix formation, including collagen and laminin (Montagnani et al., 2000). Numerous studies have highlighted the positive effects of meningeal cells (and their secreted factors) in the CNS (Decimo et al., 2012; Siegenthaler et al., 2011), and particularly on the development of dopaminergic neurons (Cohen et al., 1997; Hayashi et al., 2008; Hynes et al., 1995; Schwartz et al., 2012; Yu et al., 2007b). Furthermore, an important study by Somaa and colleagues recently showed that the inclusion of young (E10 mouse), but not older (E12 mouse), meningeal cells in VM preparations increased the differentiation, survival and outgrowth of dopaminergic neurons (Somaa et al., 2015). Thus, we hypothesised that the inclusion of meningeal cells with our E12 VM cell suspensions provided transplanted cells with essential differentiation cues that therefore enhanced the number of surviving dopaminergic neurons. Moreover, assuming that our collagen hydrogel could act as a microenvironment reservoir, acutely retaining factors secreted from meningeal cells in the same manner as it did GDNF (Moriarty et al., 2017), then this could provide enhanced and site specific developmental support to transplanted cells, resulting in the positive trends seen in both cell survival and outgrowth when cells were encapsulated in a collagen hydrogel.

Dissimilar to the effects of GDNF alone on E12 cell survival, the encapsulation of cells in a GDNF-loaded hydrogel resulted in a significant increase in the survival of dopaminergic
neurons and their capacity to re-innervate the striatum. We hypothesise that the encapsulated meningeal cells provided enhanced developmental support to the also encapsulated dopamine precursors, causing an increase in post-transplantation differentiation and therefore a higher yield of dopaminergic neurons, which could subsequently benefit from the enhanced and site-specific delivery of GDNF. In summary, while GDNF may not be beneficial to E12 dopamine precursors, it could be advantageous to cells which have undergone post-transplantation differentiation, a process that is likely increased by the encapsulation of meningeal cells and retention of secreted factors. However, further studies are required to determine whether the collagen hydrogel does indeed retain secreted meningeal factors at the transplantation site and if so, which factors.

Unsurprisingly, the enhanced cell survival and striatal re-innervation seen with the delivery of cells in a collagen hydrogel and a GDNF-loaded collagen hydrogel strongly correlate with a greater level of functional recovery. In part, this could be associated with a greater proportion of A9 cells in younger donor age grafts. Many studies have shown that the birth of A9 dopaminergic neurons (subtype involved in motor function) predates the birth of A10 dopaminergic neurons and therefore the use of younger VM tissue generates A9 rich dopamine grafts (Bye et al., 2012; Grealish et al., 2010). Moreover, the findings from Somaa and colleagues suggest that young meningeal cells play an important role in providing cues for the early development of A9 cells (Somaa et al., 2015). Thus, the encapsulation of E12 meningeal cells in our collagen hydrogel and GDNF-loaded collagen hydrogel alongside dopamine precursor cells could have a positive effect on A9 cell development and therefore generate a greater level of functional recovery. It was beyond the scope of the current study to assess the specific dopaminergic subtypes in our encapsulated grafts, however, it will be the focus of further studies in our laboratory.
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Taken together with the previous chapter, these studies highlight the potential of biomaterial hydrogel scaffolds to improve the outcome of reparative cell therapies for Parkinson’s disease (Moriarty et al., 2018a; Moriarty et al., 2018b; Moriarty et al., 2017; Moriarty et al., 2018c). However, given the ethical and logistical limitations of using fetal-derived tissue for brain repair, in future studies, it will be important to determine if collagen scaffolds can also improve the outcome of stem cell-derived dopaminergic neuron transplants in the Parkinsonian brain. Thus, the aim of the following Chapter is to determine the effect of our GDNF-loaded collagen hydrogel on the survival and efficacy of human iPSC-derived dopaminergic neurons.
Chapter 6: Delivery of human iPSC-derived dopaminergic precursors in a GDNF-loaded collagen hydrogel

6.1 INTRODUCTION

Over the last three decades primary dopaminergic neurons derived from the ventral mesencephalon of embryonic tissue have exhibited vast neurorestorative potential in cell replacement therapies. Early preclinical studies demonstrated that grafted VM cells not only survived in the host brain after transplantation but that they also led to significant functional recovery in 6-OHDA lesioned animals (Bjorklund et al., 1983; Brundin et al., 1987; Perlow et al., 1979a). Moreover, further studies showed that the behavioural recovery seen was a result of the dopaminergic component of the grafted cells (Dunnett et al., 1988b; Grealish et al., 2010), and their ability to receive and make synapses within the host brain (Clarke et al., 1988). These pivotal studies provided the necessary ‘proof-of-concept’ that warranted progression to clinical trials.

While initial open label phase 1 clinical trials mirrored the positive results found experimentally, with the vast majority of patients experiencing sustained long-term clinical benefit with a cessation of oral dopamine medication (Brundin et al., 2000b; Freed et al., 1992; Kordower et al., 1998; Wenning et al., 1997), conversely two subsequent double-blind clinical trials yielded varied results, with some patients exhibiting no significant clinical improvements and a high proportion suffering graft-induced dyskinesias (Freed et al., 2001; Olanow et al., 2003). Despite these sub-optimal results, it was apparent that fetal-derived VM cell replacement therapy was efficacious in some patients, namely those with less advanced Parkinson’s disease and adequate immunosuppression post-
transplantation, leading to the establishment of TRANSEURO, a consortium aimed at analysing all available experimental and clinical data to determine whether this therapy held sufficient merit to warrant further investigation. The findings of this consortium established how far the field was from knowing how to optimally deliver such tissue and as a result the full potential of fetal-derived VM cells had yet to be determined (Freed et al., 2011; Winkler et al., 2005b). This led to the commencement of refined and optimised phase 1 clinical trials in 2012.

While the results of the TRANSEURO trial will provide further insight into the potential of fetal-derived VM cells in neurorestorative therapies, having to use multiple fetal donors for a single transplant prevents it from ever becoming a widespread clinical therapy. However, decades of fetal-derived VM transplantation has provided invaluable proof-of-principle evidence that dopamine-producing cells can be efficacious in Parkinson’s disease and has subsequently led to a new stem cell era, with the aim of finding alternative and clinically feasible sources of dopaminergic neurons (Stoker et al., 2016).

Following a decade of enormous progress, iPSCs show great potential as a source of transplantable dopaminergic neurons (Sonntag et al., 2018). Their generation, firstly from mouse somatic cells (Takahashi et al., 2006), and then from human fibroblasts (Takahashi et al., 2007), was hailed a major scientific breakthrough and sparked great excitement in the field of cell therapy, as their use, unlike primary fetal tissue and ESCs, negates any ethical issues regarding their procurement. Moreover, iPSCs have already been shown to differentiate into dopamine neurons of midbrain character via reprogramming, and after intra-striatal transplantation were capable of survival, integration with the host system and behavioural recovery in Parkinsonian animal models (Kikuchi et al., 2017; Kikuchi et al., 2011; Soldner et al., 2009; Wang et al., 2015; Wernig et al., 2008).
Chapter 6: Encapsulation of iPSC-derived dopamine precursors

While iPSC-derived dopaminergic neurons show extraordinary potential experimentally, bearing in mind the lessons learnt from fetal-derived cell transplantation, it is important to not prematurely rush clinical investigation (Barker et al., 2016; Xiao et al., 2016). It is first important to determine whether some of the limitations associated with fetal VM transplantation, such as poor survival, will also affect stem cell therapies, and if so, can post-transplant survival and re-innervation be improved by strategies similar to those of primary dopaminergic neurons.

We have already shown that a GDNF-loaded collagen hydrogel can significantly enhance the survival, re-innervation and functionality of both E12 and E14 primary dopaminergic neurons through the provision of a localised growth factor enriched environment that is capable of shielding cells from the hostile host environment (Moriarty et al., 2018a; Moriarty et al., 2017). While these studies highlight the potential of growth factor enriched biomaterial matrices to enhance dopaminergic cell replacement therapies in Parkinson’s disease (Moriarty et al., 2018b; Moriarty et al., 2018c), it is important to determine if such collagen scaffolds can also improve the outcome of stem cell-derived dopaminergic grafts.

Therefore, the aim of this chapter was to assess whether the encapsulation of human iPSC-derived dopaminergic neurons in a GDNF-loaded collagen hydrogel could enhance their long-term survival and efficacy after transplantation to the Parkinsonian rat brain.
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6.2 METHODS

6.2.1 EXPERIMENTAL DESIGN

Having already established that our GDNF-loaded collagen hydrogel is advantageous to the long-term survival and function of primary dopaminergic neurons after transplantation, the studies presented in this chapter were designed to assess whether a GDNF-loaded collagen hydrogel could afford the same benefits to dopaminergic neurons derived from human iPSCs. The iPSC-derived dopaminergic precursors used in this chapter were generated and differentiated in the laboratory of Dr. Tilo Kunath, as previously described (Devine et al., 2011), and generously supplied to us at D11.

**Experiment 1**

In an effort to avoid graft rejection upon transplantation, neonatal rats were desensitised with a mixture of D16 and D35 human iPSC-derived dopaminergic neurons (100,000 cells per rat (50% D16 and 50% D35)) at PND 2 and 4. Upon maturation (minimum 6 weeks later) 28 Sprague-Dawley rats (20 males & 8 females) received a unilateral intra-MFB 6-OHDA lesion (12 µg in 3 µl). Two weeks later, rats underwent post-lesion methamphetamine-induced rotations (2.5 mg/kg) and based on these results were performance matched into four groups (n = 7 per group) to receive unilateral intra-striatal transplants of D16 human iPSCs alone (75,000 cells per rat), with GDNF (1000 ng), encapsulated in a collagen hydrogel (crosslinked with 4 mg/ml 4s-starPEG) or encapsulated in a GDNF-loaded collagen hydrogel. Behavioral testing was then carried out every four weeks for a total of 18 weeks. The animals were then sacrificed for *post mortem* assessment. A schematic of this experimental design is shown in Fig. 6.1.
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**Experiment 2**

This experiment followed the same experimental design as described for experiment 1 (above) with the following exceptions – 36 Sprague Dawley rats (24 males & 12 females) received unilateral intra-striatal lesions at 12 weeks of age and two weeks later were performance matched into groups (n = 9 per group) to receive unilateral intra-striatal transplants of D16 human iPSCs alone (200,000 cells per rat), with GDNF (1000 ng), encapsulated in a collagen hydrogel (crosslinked with 4 mg/ml 4s-starPEG) or encapsulated in a GDNF-loaded collagen hydrogel. Following behavioral assessment, animals were sacrificed at 18 weeks post-transplant for *post mortem* assessment. A schematic of this experimental design is shown in Fig. 6.1.

<table>
<thead>
<tr>
<th>Study</th>
<th>Animals / Group</th>
<th>Males / Group</th>
<th>Females / Group</th>
<th># Cells Transplanted</th>
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<tr>
<td>1.</td>
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<td>5</td>
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<tr>
<td>2.</td>
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<td>6</td>
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**Table 6.1.** Specific details of the two independent experiments of human iPSC transplantation that are presented in this chapter, including, group size, gender balance within groups, and the total number of cells transplanted per animal.
Figure 6.1. *In vivo* assessment of the impact of a GDNF-loaded collagen hydrogel on the survival and efficacy of human iPSC-derived dopaminergic neurons. Schematic representation of the intra-striatal delivery of D16 human iPSCs (75,000 or 200,000 cells/6 µl) encapsulated in a GDNF-loaded (1000 ng) collagen hydrogel (crosslinked with 4 mg/ml 4s-starPEG). Graft survival, neural outgrowth and the host immune response were assessed at 18 weeks post-transplant.
6.3 RESULTS

6.3.1 EXPERIMENT 1

6.3.1.1 Impact of the GDNF-loaded collagen hydrogel on human iPSC-derived dopaminergic neuron survival

In order to assess the survival of transplanted human iPSC-derived dopaminergic neurons, the number of surviving HuNu$^+$ and TH$^+$ cells throughout the striatum were assessed using immunohistochemistry. Qualitative analysis of HuNu and TH immunostaining determined the absence of viable human cells (Fig. 6.2) and dopaminergic neurons (Fig 6.3), respectively, throughout all groups. Thus, because there were no surviving grafts, we could not determine the impact of the GDNF-loaded collagen hydrogel on the survival of iPSC-derived dopaminergic neurons. However, personal communication with Dr. Andreas Heuer and Dr. Mariah Lelos, suggests that the morphology of staining is consistent with non-specific hemosiderin staining, indicative of cell death/graft rejection.
Figure 6.2. Representative images of HuNu staining. Qualitative analysis of HuNu immunostaining determined the absence of viable intra-striatal human iPSC grafts. Scale bar represents 100 µm.
Figure 6.3. Representative images of TH staining. Qualitative analysis of TH immunostaining determined the absence of viable dopaminergic neurons in the transplanted striatum. Scale bar represents 100 µm.
6.3.1.2 Impact of the GDNF-loaded collagen hydrogel on human iPSC-derived dopaminergic neuron functionality

Methamphetamine-induced rotational behavior was carried out at four-weekly intervals for a total of 18 weeks to assess the ability of the transplanted iPSC-derived dopaminergic grafts to restore motor function to unilaterally lesioned animals. The delivery of cells alone, with GDNF, encapsulated in a collagen hydrogel or encapsulated in a GDNF-loaded collagen hydrogel, did not result in behavioral recovery at any point post-transplantation (Fig 6.4; Group x Time, $F_{(10,125)} = 0.38$). In line with HuNu and TH qualitative analysis (above), this suggests that transplanted cells were not functional after transplantation.
Figure 6.4. Impact of the GDNF-loaded collagen hydrogel on graft functionality. The intra-striatal transplantation of D16 human-derived iPSCs had no effect on the number of ipsilateral turns made in each group. PL; post-lesion, PT; post-transplant.
6.3.1.3 Impact of the GDNF-loaded collagen hydrogel on the host immune response

In order to assess the host immune response to the transplanted human iPSCs, cd11b and GFAP immunostaining was carried out 18 weeks post-transplantation. Because there were no surviving grafts, we could not assess whether the GDNF-loaded hydrogel had any effect on the host immune response to the transplanted cells, however, qualitative analysis showed that while some microgliosis (Fig. 6.5) and astrocytosis (Fig. 6.6) was present in each group, there was not an elevated immune response to the transplanted cells at this time point.

![Image of immunostaining results](image_url)

**Figure 6.5. Representative images of the host microglial response.** Qualitative analysis of cd11b immunostaining determined that there was not an exaggerated microglial response at the transplantation site, in any group. Scale bar represents 100 µm.
Figure 6.6. Representative images of the host astrocytic response. Qualitative analysis of GFAP immunostaining determined that there was not an exaggerated astrocytic response at the transplantation site, in any group. Scale bar represents 100 µm.

6.3.2 EXPERIMENT 2

6.3.2.1 Impact of the GDNF-loaded collagen hydrogel on human iPSC-derived dopaminergic neuron survival

Following the poor graft survival seen in the previous experiment (section 6.3.1), we next assessed the number of surviving HuNu+ and TH+ cells throughout the striatum when a higher number of human iPSC-derived dopaminergic neurons were grafted (200,000 vs. 75,000). However, similar to previous findings, qualitative analysis of HuNu and TH immunostaining determined the absence of viable human cells (Fig. 6.7) and dopaminergic
neurons (Fig 6.8), respectively, in all groups. Therefore, because there were no surviving grafts, the impact of the GDNF-loaded collagen hydrogel on the survival of iPSC-derived dopaminergic neurons could not be determined. Again, the morphology of staining is consistent with non-specific hemosiderin staining, indicative of cell death/graft rejection.

**Figure 6.7. Representative images of HuNu staining.** Qualitative analysis of HuNu immunostaining determined that absence of viable human iPSC grafts 18 weeks post-transplantation. Scale bar represents 100 µm.
6.3.2.2 Impact of the GDNF-loaded collagen hydrogel on D16 human iPSC-derived dopaminergic neuron functionality

In a similar manner to experiment 1, methamphetamine-induced rotational behavior was carried out at 4 week intervals post-transplantation, for a total of 18 weeks. The intra-striatal transplantation of D16 iPSCs alone, with GDNF, encapsulated in a collagen hydrogel or encapsulated in a GDNF-loaded collagen hydrogel, failed to result in behavioral recovery at any time-point post-transplantation (Fig 6.9; Group x Time, $F_{(15,160)} = 0.28$), suggesting that the iPSC grafts were not functional after transplantation.
Figure 6.9. Impact of GDNF-loaded collagen hydrogel on graft functionality. The intra-striatal transplantation of D16 human iPSCs had no effect on the number of ipsilateral turns made in each group. PL; post-lesion, PT; post-transplant.
6.3.2.3 Impact of the GDNF-loaded collagen hydrogel on the host immune response

Cd11b and GFAP immunostaining was used to assess the host microglial and astrocytic response to the transplanted cells, respectively. Due to the absence of surviving grafts, we could not assess whether the GDNF-loaded hydrogel had any effect on the host immune response to the transplanted cells. However, qualitative analysis showed that while some microgliosis (Fig. 6.10) and astrocytosis (Fig. 6.11) was present in each group, there was not an elevated immune response to the transplanted cells at this time point.
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Figure 6.10. Representative images of the host microglial response. Qualitative analysis of cd11b immunostaining determined that there was not an exaggerated microglial response at the transplantation site, in any group. Scale bar represents 100 µm.

![Cells alone](image1)
![Cells in hydrogel](image2)
![Cells & GDNF](image3)
![Cells in GDNF hydrogel](image4)

Figure 6.11. Representative images of the host astrocytic response. Qualitative analysis of GFAP immunostaining determined that there was not an exaggerated astrocytic response at the transplantation site, in any group. Scale bar represents 100 µm.
6.4 DISCUSSION

Decades of extensive research into the transplantation of primary dopaminergic neurons have paved the way for cell replacement therapies in Parkinson’s disease (Stoker et al., 2016). These cells have provided invaluable proof-of-principle that dopaminergic neurons can survive, integrate, and function in the host brain after transplantation (Bolam et al., 1987; Brundin et al., 1987; Dowd et al., 2004; Kefalopoulou et al., 2014; Lindvall et al., 1990; Rose et al., 1985). However, variable outcomes between clinical trials, accompanied by extremely poor cell survival, inadequate striatal re-innervation, the occurrence of GIDs and host-to-graft disease transfer, have highlighted the need for improved and standardised transplantation procedures (Barker et al., 2015; Stoker et al., 2017). While we have shown that the delivery of primary dopaminergic neurons in a growth factor-loaded injectable collagen hydrogel can improve their survival, re-innervation and functionality (Moriarty et al., 2018a; Moriarty et al., 2017), the nature of their embryonic origin will prevent them from ever becoming a widespread therapy. As a result, investigation into more readily available and standardised sources of dopaminergic neurons began, and has thus led to a new stem cell era.

Stem cells, particularly iPSCs, show vast potential as a source of transplantable dopaminergic neurons. Experimentally they have shown to differentiate into, and function as, dopaminergic neurons, possessing the ability to survive, integrate with the host system, produce dopamine, and restore behavioral function in Parkinsonian animal models (Kikuchi et al., 2017; Kikuchi et al., 2011; Soldner et al., 2009; Wang et al., 2015; Wernig et al., 2008). However, it is yet to be determined whether some of the issues that hindered the efficacy of primary dopaminergic neurons, namely poor survival and inadequate re-innervation, will also affect stem cell-derived dopaminergic neuron therapies. Thus, given
the positive effects of our biomaterial scaffold on the delivery and efficacy of primary dopaminergic neurons (Moriarty et al., 2018a; Moriarty et al., 2017), and to further highlight the potential of biomaterials to improve cell replacement therapies in Parkinson’s disease (Moriarty et al., 2018b; Moriarty et al., 2018c), the aim of this chapter was to assess whether the encapsulation of human iPSC-derived dopaminergic cells in a GDNF-loaded collagen hydrogel could improve their survival and functionality after transplantation to the Parkinsonian brain.

Xenotransplantation of human-derived cells into experimental animal models is an important stage of the preclinical validation of therapeutic potential. However, without adequate immunosuppression, neural xenografts in the adult brain will be rejected within a period of just 3 weeks (Brundin et al., 1988). This is owing to the primary function of the immune system, which is to protect the host organism from all potential threats, whether that be bacterial, viral or the appearance of foreign entities. In order to fulfil this function, the adult immune system has the ability to identify between ‘self’ and ‘non-self’ antigens, and it is this distinguishment of ‘non-self’ antigens in transplanted xenografts that results in their identification and removal from the host system. While the brain has traditionally been considered as a relatively immunologically privileged site, owing to 1) its tightly sealed BBB, which shields it from the infiltration of circulating immune cells, and 2) the absence of antigen-presenting cells, that are vital in the initiation of an immune response, this immunological privilege is only present in the healthy CNS (Bauer et al., 2014; Carson et al., 2006; Galea et al., 2007). Indeed, the invasive process of transplantation disrupts the BBB, exposing the brain to circulating immune cells. This loss of immune-privilege was demonstrated when an established graft (implanted during the neonatal period) was rejected in adulthood after the loss of BBB integrity, and subsequent immune cell influx (Pollack et al., 1990).
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A number of strategies have been implemented to dampen the host response to xenografts, including the use of immunosuppressive drugs such as cyclosporine A. However, cyclosporine A requires daily i.p injections that in the long-term is extremely taxing on animals and eventually results in a decline of their health, thus limiting its administration to 18-20 weeks (Andoh et al., 1998; Bertani et al., 1987; Kelly et al., 2009; Murray et al., 1985). Since human neurons require months to fully mature, integrate with the host system and induce behavioral recovery, an animal model involving drug-induced immunosuppression is incompatible with such long-term functional studies.

Alternatively, in an effort to evade neural xenograft rejection, we implemented the concept of neonatal desensitisation (Roberton et al., 2015). Neonatal desensitisation is based on the phenomenon that the transplantation of cells to the neonatal brain, while the immune system is still underdeveloped, results in the cells being recognised as ‘self’ antigens and are therefore not rejected, even upon the maturation of the immune system (Englund et al., 2002; Kallur et al., 2011; Lund Raymond et al., 1987). The induction of neonatal tolerance in rodents dates back over several decades, with many studies reporting that the rejection of skin allografts can be avoided through the exposure of the host to donor cells during early neonatal development (Adkins et al., 2004; Billingham et al., 1956; Billingham et al., 1953; Modigliani et al., 1997). More recently, this concept has been applied to xenotransplantation studies. Zhang et al., (2013) demonstrated that neonatal desensitisation supports the long-term survival of hESC-MSCs that were seeded in a collagen bilayer scaffold and delivered to rat joint cartilage. Moreover, this concept was given neural application when Kelly and colleagues showed that neural transplants could survive long term in the adult rat brain following neonatal desensitisation with human cortical tissue prior to PND 5 (Kelly et al., 2009). Similarly, Heuer et al., (2016) showed that the desensitisation of rats prior to PND 5 with differentiated human ESCs resulted in the long-
term survival of human ESCs-derived neural progenitors. Interestingly, Heuer and colleagues made significant modifications to the protocol of Kelly et al., (2009) opting for repeated cell injections at both PND 2 and 4 for desensitisation, and also delivering a mixture of cells of different differentiation stages (Heuer et al., 2016). These modifications maximise the likelihood of animals receiving adequate cell doses for desensitisation and also exposes the rats to different epitopes that are expressed on cells at the various differentiation stages.

While numerous studies have now implemented the desensitisation approach, not all have seen successful (Jablonska et al., 2013; Janowski et al., 2012; Mattis et al., 2014; Roberton et al., 2013a). Unfortunately, the two studies presented in this chapter fall among the reports of graft rejection after desensitisation. The mechanisms which underlie desensitisation remain elusive, and as a result it is unclear as to why success is seen in some cases, but not all. There are many possible reasons why desensitisation may fail in animal models, with species/strain differences, donor cell type (including variations in epitope expression), and cell number, all being likely candidates (Roberton et al., 2015). The literature shows a variance in the efficacy of xenogenic desensitisation between rats and mice. While success has been found in studies utilising rats (Heuer et al., 2016; Kelly et al., 2009), studies using mice show very poor survival after desensitisation, levels similar to the transplantation of cells into naive mice (Janowski et al., 2012; Mattis et al., 2014; Roberton et al., 2013b). Moreover, the rejection of donor tissue in mouse hosts is reported to be more rapid than in rats (Roberton et al., 2013b), suggesting that the mouse has a more hostile environment and may therefore partly explain the variations between species. However, in the studies presented here, we utilised the same strain of inbred rat (Sprague Dawley) used by both Kelly et al., (2009) and Heuer et al., (2016). Furthermore, in an effort to maximise the chances of adequate exposure to cells during the
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desensitisation process, we implemented the modifications undertaken by Heuer et al., (2016) and delivered two cell injections, firstly at PND 2 and then at PND 4. Similarly, in an effort to cover the changes in cell marker expression over time, we also delivered a mixture of cell differentiation stages, with each injection consisting of 50% D16 neural progenitors and 50% D35 mature dopaminergic neurons. Notably, we also delivered cells at a dose of $1 \times 10^5$ cells/injection, as reported by Kelly et al., (2009) and Heuer et al., (2016). Thus, given that a similar protocol to that reported by Heuer et al., (2016) was followed and invaluable advice/training was provided by both Dr. Claire Kelly and Dr. Andreas Heuer prior to the commencement of desensitisation, it is probable that the failure to see survival after desensitisation in this case was associated with the variation in donor cell used, rather than solely the desensitisation protocol.

Both Kelly et al., (2009) and Heurer et al., (2016) have reported success using human primary fetal cells and human ESCs, respectively, whereas in these studies we used human iPSCs as a source of dopaminergic neurons. Given the uncertainty that surrounds the mechanisms of desensitisation, it has been suggested that differences in the type of donor cells used may affect efficacy. Indeed, there have been reports of unsuccessful desensitisation using fetal-derived human glial-restricted precursor (hGRP) cells, human umbilical cord blood-derived neural stem cells (HUCB-NSC), and human fetal neural precursors (hNPC) (Jablonska et al., 2013; Janowski et al., 2012; Mattis et al., 2014). Interestingly, while there are not many reports of desensitisation using iPSC-derived donor cells, Mattis and colleagues found rapid graft rejection of human iPSC-derived neural precursor cells (iPSC-NPCs) following neonatal transplantation, with no graft survival past 21 days, in contrast to the long-term presence of surviving grafts in immunodeficient mice (Mattis et al., 2014). However, it must be noted that as this study only used a mouse host,
it is unclear whether graft rejection is a consequence of strain difference (mouse vs. rat) or donor cell type.

The varied success between studies to date, suggests that the success of desensitisisation may be dependent on the host model and the type of cells used. While we may speculate, as the mechanisms which underlie this process remain unclear, it is difficult to underpin why we have seen rejection after desensitisisation when other groups have reported success. Notably, it is possible that our donor cells (iPSC-derived dopaminergic neurons) do not hold the same transplantation efficacy as other cell lines. While these cells show good differentiation in vitro (Devine et al., 2011), this was the first attempt at in vivo transplantation and thus further studies are required to determine their differentiation and survival post-transplantation. Additionally, it must also not be ruled out that the viability and general health of cells in culture prior to transplantation could have affected their transplantation.

Unfortunately, the absence of surviving cells post-transplantation made it impossible to assess the effect of our collagen hydrogel on the survival of iPSC-derived dopaminergic neurons. The inability to pinpoint why desensitisation was unsuccessful, means that extra steps should be taken to ensure success if this experiment is to be repeated. The inclusion of immunosuppressive drug groups, such as cyclosporine A administered rats, will allow us to determine the donor cells transplant efficacy, independent of desensitisisation. It would also provide immunosuppressive control groups when assessing the outcome of further desensitisisation efforts. In an attempt to further maximise the efficacy of desensitisisation, it may be justified to give multiple neonatal injections each day prior to PND 5, as in theory, the more exposure the animal gets to the donor cells during early development, the higher the chance of recognition as ‘self’ antigens by the immune system. The inclusion of more differentiation stages within the ‘cell cocktail’ that is delivered during the desensitisisation
process would also increase the host’s antigen exposure. As cells would be transplanted at D16 and expected to differentiate into mature dopaminergic neurons \textit{in vivo} (reported as \textasciitilde{}D35 \textit{in vitro}), it is possible that the host may be presented with more epitope cell markers that those expressed solely at D16 and D35, a process that could lead to graft rejection prior to dopaminergic neuron maturation.

With the addition of the above modifications, hopefully the chances of graft survival after neonatal desensitisation will be increased, and the potential of growth-factor loaded collagen hydrogels to improve the long-term survival and function of human iPSC-derived dopaminergic neurons in the Parkinsonian brain can be assessed.
Chapter 7: General Discussion

The work presented in this thesis sought to determine the potential of injectable growth factor-loaded collagen hydrogels to improve dopaminergic cell replacement therapies in Parkinson’s disease. Particularly, we focussed on the ability of these hydrogels to provide the encapsulated cells with a localised growth factor reservoir upon transplantation and their ability to reduce the host immune response to the transplanted cells.

The main findings from this body of work are: 1) injectable collagen hydrogels successfully form \textit{in situ}, are well tolerated in the brain, and permit the survival and outgrowth of encapsulated cells (Moriarty \textit{et al.}, 2017), 2) the encapsulation of cells in a collagen hydrogel significantly reduces the host immune response to the transplanted cells (Moriarty \textit{et al.}, 2017), 3) the loading of collagen hydrogels with GDNF prior to transplantation results in the acute retention of GDNF in the striatum immediately post-transplantation (Moriarty \textit{et al.}, 2017), and 4) the encapsulation of primary dopaminergic neurons (of different embryonic ages) in a GDNF-loaded collagen hydrogel results in a dramatic increase in cell survival, striatal re-innervation and behavioural recovery (Moriarty \textit{et al.}, 2018a; Moriarty \textit{et al.}, 2017). Moreover, the significant increases in cell survival and striatal re-innervation strongly correlate, both with each other, and the experienced behavioural recovery, suggesting that the enhanced delivery of cells in the GDNF-loaded collagen hydrogel results in a higher proportion of re-innervated striatum and consequently a greater level of behavioural recovery. These findings highlight the potential of biomaterial scaffolds to improve cell replacement therapies in Parkinson’s disease (Moriarty \textit{et al.}, 2018b; Moriarty \textit{et al.}, 2018c).

Although remarkable progress has been made thus far in the search of novel treatments for Parkinson’s disease, there remains a serious unmet clinical need for effective neuroprotective, neurorestorative or neurorepairative therapies for this condition. Although efficacious, the pharmacological therapies routinely used in clinical practice solely provide
symptomatic relief to patients and fail to address the underlying disease pathology, resulting in a waning of therapeutic efficacy overtime. Due to the increasing longevity of the worldwide population, the incidence of Parkinson’s disease is expected to rise from 4.1 million to 9.3 million by 2030 (Dorsey et al., 2007). As a result of this ever-increasing aging society, the economic burden of diagnosis, treatment and care of patients will also continue to rise. This inevitable phenomenon combined with the stark insufficiencies of current pharmacotherapies has prompted the search for novel therapeutic strategies that adequately address the symptoms of the disease, relieve the associated motor complications and target the underlying disease pathology. While the search for disease-modifying therapies that halt and/or reverse disease progression is a daunting task, in parallel, a search for novel strategies that not only symptomatically treat the disease but ultimately repair the diseased brain is underway and making significant progress.

Cell replacement therapy is a neuroreparative approach that has shown vast promise in the treatment of Parkinson’s disease. The relatively selective loss of nigrostriatal dopaminergic neurons in Parkinson’s disease makes it an ideal candidate for cell replacement therapy (Fearnley et al., 1991), a therapy that has emerged from a relatively simple conceptual framework - if the primary pathological feature of a disorder is the degeneration and subsequent loss of a specific neuronal subtype, then the replacement of such cells should, in theory, repair the brain and restore function to the patient. The focus of cell transplantation in Parkinson’s disease started with fetal dopaminergic neurons derived from the developing ventral mesencephalon. Pioneering pre-clinical studies provided proof-of-concept that these primary dopaminergic neurons could survive and function in the host brain following transplantation (Björklund et al., 1980a; Bjorklund et al., 1983; Brundin et al., 1987; Brundin et al., 1986; Dunnett et al., 1997; Dunnett et al., 1981; Dunnett et al., 1988a). This led to the quick progression into clinical trials just a decade later where significant benefits were reported by patients in a number of small open-label trials (Brundin et al., 2000b; Freed et al., 1992; Hauser et al., 1999; Lindvall et al., 1990;
Lindvall *et al.*, 1994; Spencer *et al.*, 1992; Wenning *et al.*, 1997). However, unfortunately these promising findings were challenged when two larger double-blind trials failed to reach their primary endpoint (Freed *et al.*, 2001; Olanow *et al.*, 2003), alongside the emergence of GIDs in many patients. Despite these disappointing results, the emergence of longitudinal follow-up studies confirmed that long-term transplantation (up to 18 years) was efficacious in a subset of patients (Hallett *et al.*, 2014; Kefalopoulou *et al.*, 2014; Ma *et al.*, 2010; Mendez *et al.*, 2008). Moreover, re-evaluation of previous clinical data highlighted major technical discrepancies between trials and identified a number of aspects, including patient selection, disease severity, pre-transplant dyskinesias, cell preparation and cell storage, that must be addressed before any improvements to this neuroreparative approach can be seen (Barker *et al.*, 2013). These factors are currently being addressed in the new TRANSEURO open-labelled clinical trials (www.transeuro.org.uk), however, there are many fundamental ethical and logistical issues surrounding the procurement and use of fetal-derived tissue, that are further complicated by the extremely poor survival of cells after transplantation (5 - 20%) (Brundin *et al.*, 2000a). Therefore, in order for the full potential of primary dopaminergic neurons, as well as dopaminergic neurons-derived from alternative sources, to be realised, efforts must be made to improve the number of surviving cells after transplantation.

Biomaterials, that is, materials that have been specifically engineered to interact with biological systems for therapeutic purposes, have the potential to substantially improve such neurorestorative strategies (Moriarty *et al.*, 2018b; Moriarty *et al.*, 2018c; Orive *et al.*, 2009; Wang *et al.*, 2012). Their diversity and adaptability makes it possible to choose and modify a scaffold based on its desired application (Table 7.1).
### Desirable characteristics of biomaterial scaffolds for intra-cranial delivery

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<tr>
<td>1.</td>
<td>Capable of relatively non-invasive delivery</td>
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<td>2.</td>
<td>Biomimetic in order to encourage cell survival and host integration</td>
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<td>3.</td>
<td>Not themselves elicit an exaggerated host immune reaction</td>
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<td>4.</td>
<td>Structurally stable for prolonged periods <em>in situ</em></td>
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<td>5.</td>
<td>Modifiable in relation to adhesion molecules, pore size, molecular charge, and functionalisation</td>
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<td>6.</td>
<td>Non-toxic to any cellular components of brain tissue or the encapsulated cells</td>
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<td>7.</td>
<td>Capable of controlled and sustained delivery of therapeuic factors.</td>
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**Table 7.1. Desirable characteristics of biomaterial scaffolds for intra-cranial delivery.** Based on Orive *et al.*, (2009)

In Chapter 3, we optimised a collagen hydrogel that exhibited a number of characteristics that make it a desirable scaffold for the intra-cranial neurorestorative therapy that is cell transplantation (Moriarty *et al.*, 2017). Any material that is selected for intra-cranial delivery must be capable of relatively non-invasive delivery. The ability of our collagen hydrogel, like many other hydrogel scaffolds (Drury *et al.*, 2003), to form *in situ* in response to temperature changes makes it injectable, allowing for relatively non-invasive delivery. Furthermore, we have shown that our collagen hydrogel is well tolerated in the brain and does not itself elicit an exaggerated host immune reaction. This is a vital characteristic, because as the transplantation of cells to the brain alone instigates a state of neuroinflammation, causing cell death (Barker *et al.*, 1996), any exaggeration of this response would be detrimental to graft survival. This low immunogenicity is owing to the primary component of the hydrogel, collagen. Collagen is a naturally occurring material and is one of the most abundant proteins in the human body, making it highly biomimetic and one of the most investigated natural biomaterials (Khan *et al.*, 2013). Keeping with
this, collagen biomaterials have already been approved for use in a variety of applications including drug delivery, wound repair, burn treatment, dentistry and bone reconstruction (Blume et al., 2011; Chajra et al., 2008; Chattopadhyay et al., 2014; El-Chaar, 2016; Helary et al., 2010; Patino et al., 2002; Solish, 2010), and could therefore, in theory, be relatively easily adopted to neural applications.

While collagen is capable of naturally forming a hydrogel in situ, its weak mechanical properties make it highly susceptible to rapid degradation in the brain. While eventual degradation in the brain is favorable, structural stability upon transplantation and throughout its therapeutic window is required in order to provide cells with a supportive environment. We showed that by using a synthetic polymer, we could improve the mechanical stability of the hydrogel, while also maintaining the protein’s biological function (Moriarty et al., 2017). Through a serious of in vivo pilot studies, we showed that the crosslinking of our collagen hydrogels with an optimised concentration of 4s-StarPEG resulted in the formation of strong, well-defined, uniform and stable hydrogels in situ. Moreover, these hydrogels did not undergo rapid degradation (within 2 weeks), but were ultimately degraded (prior to 12 weeks) without leaving any unwanted foreign remnants.

The application of polymer crosslinking to natural biomaterials means that a scaffold can be modifiable in relation to pore size (Delgado et al., 2015). This modification can be extremely advantageous in terms of immune cell infiltration and therapeutic factor release. We demonstrated that the encapsulation of primary dopaminergic neurons in a crosslinked collagen hydrogel significantly reduced the level of microgliosis and astrocytosis at the transplantation site (Moriarty et al., 2017). This strongly suggests that the hydrogel is capable of isolating cells from the hostile host environment and preventing the infiltration of immune cells and inflammatory mediators. These results are similar to previous findings, where Hoban et al., (2013) reported a reduction in immunogenicity to MSCs using a 4s-StarPEG crosslinked hydrogel, however, to the best of our knowledge, this is
the first report of reduced immunogenicity to mesencephalic grafts using a collagen scaffold.

Alongside the prevention of immune cell infiltration, we hypothesised that the mechanical stability afforded through the crosslinking of the collagen hydrogel could also prolong the presence of encapsulated growth factors. It is well reported that GDNF is beneficial to the survival of dopaminergic neurons (Lin et al., 1993) and has been advantageous to cell replacement strategies (Andereggen et al., 2009; Apostolides et al., 1998; Chaturvedi et al., 2003; Rosenblad et al., 1996; Torres et al., 2005). We showed that the encapsulation of GDNF in our crosslinked collagen hydrogel resulted in the acute striatal retention of GDNF at the implantation site immediately after transplantation (Moriarty et al., 2017). While GDNF was still cleared from the brain within 4 days, the higher levels of GDNF within the hydrogels environment could increase growth-factor exposure to encapsulated cells during a critical period of cell vulnerability (Sortwell et al., 2001).

These preliminary optimisation studies described in Chapter 3, gave us an invaluable insight into the workings and potential application of our collagen hydrogel. The ability to deliver cells intra-cranially without having any detrimental effects to cell survival or without hindering the natural outgrowth of neural processes, coupled with the hydrogel’s ability to dampen the host response, immunoisolating cells and also acutely retaining growth-factors at the transplantation site, provided encouragement as to the hydrogel’s potential to improve cell replacement strategies (Moriarty et al., 2017).

This led to the most important question addressed in this body of research, could the application of a GDNF-loaded collagen hydrogel improve the long-term survival and efficacy of dopaminergic neurons after transplantation. We have shown that the encapsulation of mesencephalic cells of two different embryonic donor ages, E14 (chapter 4) and E12 (chapter 5), resulted in a dramatic increase to the survival, re-innervation and function of dopaminergic neurons (Moriarty et al., 2018a; Moriarty et al., 2017). The findings throughout this body of work, particularly in the optimisation stages, suggests that
our collagen hydrogel is capable of improving the efficacy of dopaminergic transplantation by 1) providing a supportive environment throughout transplantation, 2) providing a localised trophic factor reservoir at the time of transplantation and 3) providing protection from the hostile host environment during the establishment of a new terminal network.

Cell death in mesencephalic grafts can be triggered at numerous stages of the grafting process (Sortwell et al., 2000). These cells are dissected from the developing mesencephalon at the height of neurogenesis and placed into the adult striatum which is known to be a trophic deficient environment. By doing this, cells, which have now undergone anoikis, are denied trophic support that would ordinarily be necessary for their natural development and maturation. It is within these first 4 days post-transplantation, that the vast majority of cell death occurs (Sortwell et al., 2001; Sortwell et al., 2000). Furthermore, any dopaminergic neurons that survive the initial transplantation are then subjected to the hostile host response and further cell death can ensue (Barker et al., 1996; Barker et al., 2004). As a result, survival after transplantation is extremely poor with <20% of cells surviving the transplantation process (Brundin et al., 2000a). Here, we have shown that by providing a supportive environment for cell adhesion, creating a physical barrier between the transplanted cells and the host neuro-immune cells and providing a reservoir for localised growth factor delivery, the survival and efficacy of dopaminergic neurons can be substantially improved. However, while the properties of the GDNF-loaded hydrogel remain the same, their applications to primary mesencephalic cells of different donor ages differs somewhat (Fig 7.1).

In Chapter 4, using the conventional donor age, E14, we showed that the encapsulation of cells in a GDNF-loaded collagen hydrogel resulted in a 5-fold increase in cell survival, a 3-fold increase in striatal re-innervation and a greater level of functional recovery (Moriarty et al., 2017). Notably, the encapsulation of E14 cells in a GDNF-free collagen hydrogel did not result in any increases in cell survival, despite the obvious reduction in immunogenicity at the graft site. We hypothesised that this was a result of the still present
trophic deficiency in the host brain, as cell death upon transplantation is not caused by one single factor. Instead, it was the combination of reduced immunogenicity and the all-important acute retention of GDNF at the transplantation site immediately post-transplantation afforded by the GDNF-loaded collagen hydrogel that led to these dramatic increases in cell survival (Fig 7.1). This hypothesis was further supported when we showed that the level of survival seen was significantly greater than that of the delivery of GDNF alone with cells.

Similarly, in chapter 5, we showed that the delivery of younger donor age cells, E12, also resulted in dramatic increases in cell survival (4-fold), re-innervation (5-fold) and functionality (Moriarty et al., 2018a). In line with previous literature (Torres et al., 2008; Torres et al., 2007b), the number of surviving cells was significantly greater when using younger donor tissue in place of the conventional E14 cells (Moriarty et al., 2018a; Moriarty et al., 2017). However, in contrast to our previous findings, the delivery of GDNF alone with E12 VM cells had no significant effect on the number of surviving neurons. GDNF is known to be a potent neurotrophic factor for dopaminergic neurons (Lin et al., 1993), however, its inability to increase E12 VM precursor survival could in part be due to their earlier developmental stage (Torres et al., 2005). Somewhat perplexing, was that although GDNF failed to increase E12 cell survival, dramatic increases were still seen with our GDNF-loaded hydrogel, suggesting that other factors may also be at play.

Interestingly, converse to that seen with E14 delivery, the encapsulation of E12 cells in a GDNF free collagen hydrogel showed a positive trend towards increases in the survival and re-innervation of dopaminergic neurons. This is most probably owing to the decision to include the tightly adhered meningeal layer in our cell preparations. Numerous studies have highlighted the positive effects of meningeal cells (and their secreted factors) on the development and survival of dopaminergic neurons (Cohen et al., 1997; Hayashi et al., 2008; Hynes et al., 1995; Schwartz et al., 2012; Somaa et al., 2015). Thus, we hypothesised that the inclusion of meningeal cells with our E12 VM cell suspensions
provided transplanted cells with essential differentiation cues that therefore enhanced the number of surviving dopaminergic neurons. Moreover, assuming that our collagen hydrogel could act as a microenvironment reservoir, acutely retaining factors secreted from meningeal cells in the same manner as it did GDNF, then this could provide enhanced and site specific developmental support to transplanted cells, resulting in an increase in post-transplantation differentiation and therefore a higher yield of dopaminergic neurons. Thus, while GDNF may not be beneficial to E12 dopaminergic precursors, it could be advantageous to these cells which have undergone post-transplantation differentiation, a process that is likely increased by the encapsulation of meningeal cells and retention of their secreted factors (Fig. 7.1).

These results highlight the efficiency of collagen hydrogels to act as a growth factor reservoir, whether it be to purposely encapsulated growth factors such as GDNF, or naturally secreted factors such as those from meningeal cells.
Figure 7.1. Schematic representation of the hypothesised mechanisms through which the GDNF-loaded hydrogel improves primary dopaminergic survival and efficacy. DA: dopaminergic; E: embryonic age; GDNF: glial-derived neurotrophic factor; VM: ventral mesencephalon.
Future Directions

The ethical and logistical concerns associated with the procurement of mesencephalic tissue from multiple fetal donors will prevent it from ever becoming a routine clinical procedure. However, decades of intense research have not been in vain, as it has provided invaluable proof-of-concept that dopaminergic neurons can survive and function in the Parkinsonian brain, and provide symptomatic relief to patients. As a result, in recent years, the field of cell transplantation has moved away from fetal-derived dopaminergic neurons and towards dopaminergic neurons-derived from stem cell sources such as iPSCs (Stoker et al., 2017).

It is undeniable that iPSCs are a promising source of transplantable neurons (Sonntag et al., 2018), having been shown to generate dopaminergic neurons that can survive and function in a manner similar to that of primary dopaminergic neurons (Kikuchi et al., 2017; Kikuchi et al., 2011; Soldner et al., 2009; Wang et al., 2015; Wernig et al., 2008). However, bearing the lessons learnt from fetal-derived strategies in mind, it is important not to rush clinical investigation before optimal differentiation protocols are established (Barker et al., 2016). Primarily, any stem cell based therapy for Parkinson’s disease must utilise a safe, reliable and stable cell source that has a steady karyotype, is free from microbiological contamination, does not undergo uncontrolled proliferation and does not form large numbers of non-nigral cells (Barker, 2014). Importantly, these cells must be dopaminergic-like cells that express appropriate markers such as TH, DAT and GIRK2, physiologically behave like nigral dopaminergic neurons and sufficiently produce dopamine. Upon transplantation they must survive long term in the brain, ameliorate motor symptoms, sufficiently innervate the host striatum, be functionally similar, or better than, fetal VM grafts and not migrate extensively throughout the CNS (Barker, 2014). Indeed, monumental progress is being made experimentally in relation to each of the above points, bringing us ever-closer to clinical investigation and the likelihood of a routine clinical therapy.
The TRANSEURO consortium is currently underway, addressing multiple aspects of trial design, including patient selection, disease severity and the occurrence of pre-transplant dyskinesias. The outcomes of these trials will shed light on the full potential of primary dopaminergic neurons, but more importantly, it will provide information on the subset of patients that are most likely to benefit from future cell transplantation strategies. Furthermore, the generation of a safe, reliable and stable cell source that does not include large numbers of non-nigral cells, has the potential to ameliorate the potential occurrence of GIDs, as pre-clinical, as well as retrospective clinical assessments, indicate that the presence of serotonergic neurons within the donor preparations (a consequence of poor/broad tissue dissection) were likely responsible for GIDs in patients.

What remains unclear, is whether stem cell-derived dopaminergic neurons will also experience extremely poor survival and sub-optimal re-innervation after transplantation. While a readily available cell source will remove any issues surrounding cell procurement, making it easier to deliver high numbers of nigral neurons, these cells will still be placed into the same hostile host environment as fetal-derived cells, raising questions as to the efficiency of host integration and functionality.

While there was a strong rationale behind the application of a GDNF-loaded collagen hydrogel to fetal-derived cell replacement strategies (Moriarty et al., 2018b; Moriarty et al., 2018c), the specific requirements of iPSC-derived dopaminergic neurons after transplantation remain elusive. Stem cell-derived neurons take weeks, or even months to fully mature into efficacious dopaminergic neurons and as a result it is questionable whether the acute trophic support (within the first few days post-transplantation), supplied by our GDNF-loaded hydrogel, will be sufficient to support and enhance such a long process. Modifications to our hydrogel scaffold that allow for prolonged and sustained trophic release could provide cells with differentiation support throughout the maturation process. Indeed, in an effort to prolong GDNF presence, Wang et al., (2016) incorporated short nanofibers with immobilised GDNF within their hydrogel scaffold, thus providing
cells with a long-term supply to GDNF. Moreover, Adil et al., (2017) reported increased survival of hESC-derived neurons through the addition of heparin, which is known to have neurotrophic factor binding properties. Additionally, many studies have demonstrated the use of microspheres to provide prolonged release of therapeutic factors to the transplantation site (Agbay et al., 2014; Garbayo et al., 2016; García-Caballero et al., 2017; Lampe et al., 2011), many of which can also be incorporated into cell encapsulating hydrogel scaffolds (Wood et al., 2013). Besides increasing the length of trophic support, the combined use other trophic factors such as BDNF (Baquet et al., 2005), or therapeutic factors that are normally included in their in vitro differentiation mediums (Devine et al., 2011), with GDNF, could provide cells with superior and more applicable trophic support throughout maturation.

The diversity and relative ease with which biomaterials can be modified, means that a core biomaterial, such as our GDNF-loaded hydrogel, has the potential to be adapted not only to their application, but also to specific cell types. While widespread graft rejection in Chapter 6 meant that we were unable to assess the potential of our GDNF-loaded collagen hydrogel (as a starting point) to improve iPSC cell-derived neuron survival, there is mounting evidence to suggest that biomaterials can improve stem cell-based therapies by providing a supportive, trophic-rich and immuno-neutral environment for transplanted cells (Adil et al., 2017; Moriarty et al., 2018a; Moriarty et al., 2017; Wang et al., 2016).

Concluding remarks

As cell therapies for Parkinson’s disease once again propel towards the clinic, it is of increasing importance to address strategies to ensure maximal survival, integration and functional efficacy of newly implanted tissue. In this regard, it is clear that evidence is mounting that supports the potential of biomaterial scaffolds to enhance neurorestorative strategies. The findings presented here by ourselves using a GDNF-loaded collagen hydrogel (Moriarty et al., 2018a; Moriarty et al., 2017), as well as recent studies by Wang et al., (2016), using a GDNF-functionalised composite poly(l-lactic acid)/xyloglucan
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hydrogel to deliver mouse VM grafts, and Adil et al., (2017), using a heparin/RGD functionalised hyaluronic acid hydrogel to deliver human embryonic stem cell-derived dopaminergic neurons, highlight the variety and adaptability of biomaterial scaffolds. Further work remains to be carried out to identify the ideal biomimetic scaffold and determine optimal strategies to functionalise these matrices – targeted at supporting cell transplantation for neural repair.
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Appendices

Appendix I: An overview of Parkinson’s disease

A.1 Parkinson’s disease

Parkinson’s disease is one of the most common neurological disorders and is the second most common neurodegenerative disease in the world with a prevalence ranging between 0.1 and 0.2% of the population (Tysnes et al., 2017). The incidence of the disease before the age of 50 years old is rare and dramatically increases in the aging population, with 3% of the population over 65 years of age affected and 4-5% of the population over 85 years of age affected (de Lau et al., 2006; von Campenhausen et al., 2005). The vast majority of Parkinson’s disease diagnoses are sporadic idiopathic, while only 10% of diagnosed patients are found to have hereditary factors or genetic links (Schulte et al., 2011).

Unfortunately no diagnostic test yet exists for the conclusive diagnosis of Parkinson’s disease, meaning that certainty of diagnosis cannot occur during the patient’s lifetime and post-mortem analysis of the patient’s brain tissue for Lewy body inclusions still remains the sole method of definitive disease confirmation (Dickson et al., 2009). Instead, diagnosis is based on the appearance of the clinical symptoms of the disease, which only materialise when ~40% of dopaminergic neurons have degenerated and dopamine levels have already been depleted by ~80%.

The appearance of two or more cardinal motor symptoms combined with the presence of some secondary motor symptoms remains the primary criteria for the clinical diagnosis of Parkinson’s disease (Jankovic, 2008). The cardinal motor symptoms include a resting tremor, rigidity, bradykinesia and postural instability. Additionally, patients may also present with one or more secondary motor symptoms including akinesia, freezing,
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shuffling of gait, speech impairment, and dystonia. Despite this, it is important to remember that Parkinson's disease is not solely a disorder of motor dysfunction and patients also experience many non-motor symptoms, including sensory features such as olfactory deficits, visual disturbances, somatosensory disturbances and pain; neuropsychiatric features such as anxiety, depression, dementia, apathy and fatigue; autonomic features such as bladder and gastrointestinal dysfunction, as well as many sleep disorders (Schapira et al., 2017). The non-motor symptoms of the disease have received heightened interest in recent years and it is understood that many of these symptoms may proceed the onset of the motor syndrome. Despite this, their occurrence is grossly overlooked in comparison to the cardinal motor features and often go unreported, frequently leading to delayed treatment.

A.1.1 Pathophysiology

Despite the growing recognition of non-motor symptoms, Parkinson’s disease is predominantly recognised as a movement disorder. Although the motor dysfunctions associated with the disease have been very well characterised, there is still an extremely poor understanding of the underlying disease pathogenesis. The appearance of these characteristic motor symptoms are understood to be a result of dopaminergic cell loss from the nigrostriatal pathway, and the accumulation of intra-neural Lewy body inclusions. Neuroinflammation, mitochondrial dysfunction and oxidative stress are all thought to play a role in nigrostriatal degeneration (Galvan et al., 2008; Schapira et al., 2011).

A.1.1.1 Nigrostriatal Degeneration

Dopaminergic neuron loss from the nigrostriatal pathway is one of the primary neuropathological features of Parkinson’s disease. Despite an early understanding that this slow and progressive neurodegeneration began in the substantia nigra pars compacta,
extending rostro-caudally to the cell terminals of the striatum (Damier et al., 1999a; Damier et al., 1999b), recent data suggests that striatal cell terminals are more vulnerable to neurodegeneration, and their degeneration precedes the loss of nigral cell bodies (Burke et al., 2013; Caminiti et al., 2017; Kordower et al., 2013). As a consequence of this nigrostriatal degeneration, an imbalance in striatal dopamine levels disrupts the basal ganglia motor circuitry which is vital for the modulation of normal motor control.

A.1.1.1.1 The basal ganglia

The basal ganglia consists of a group of subcortical nuclei that are primarily responsible for the modulation of motor control, as well as motor learning and emotions. They 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 reticulate (SNr); and the subthalamic nucleus (STN) (Lanciego et al., 2012). Gamma-aminobutyric acid (GABA)-ergic medium spinal neurons (MSN) are the most abundant neuronal cell type in the striatum, consisting of >90% of all striatal neurons and are the major target neurons of nigrostriatal dopaminergic projections (Deutch et al., 2007; Dubé et al., 1988). These MSNs express both D1 and D2 receptors and stimulation by dopamine will either activate the direct pathway or inhibit the indirect pathway, respectively (Fig A.1) (Gerfen et al., 1990).

The direct pathway is an excitatory pathway whereby the action of striatal dopamine on the D1 receptors of GABAergic MSNs results in an exaggerated inhibition of the GPi and the SNr. This inhibition therefore blocks the normal thalamic inhibition from the GPi and SNr, increasing the activity of thalamo-cortical projections and thereby facilitating movement. In comparison, the indirect pathway is an inhibitory pathway where the activation of D2 receptors on striatal GABAergic MSNs results in an abating of GPe inhibition. As a result,
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the normal inhibitory actions of the GPe on the STN are increased, resulting in an increased activation of the GPi, which in turn enhances thalamus inhibition, thereby inhibiting movement (Calabresi et al., 2014).

Normal motor control requires the efficient modulation of both direct and indirect pathways (Fig A.1a). In Parkinson’s disease the depletion of striatal dopamine levels results in a basal ganglia imbalance towards the indirect pathway (Day et al., 2006; DeLong et al., 2007), causing over activation of the GPi, excessive thalamus inhibition and subsequently an inhibition of motor cortex activation (Fig A.1b). This basal ganglia imbalance and resultant motor dysfunction is attributable to the progressive loss of dopaminergic neurons in the nigrostriatal pathway.

Figure A.1: The basal ganglia circuitry in the normal and Parkinsonian brain. In the normal brain (A), the direct and indirect basal ganglia circuits exert opposing functions to control movement. In the Parkinsonian brain (B), decreased striatal dopamine levels disrupt the natural balance of the basal ganglia causing a decreased activation of the direct pathway and excessive activation of the indirect pathway, ultimately reducing movement. Modified from Lewis et al., (2004)
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A.1.1.2 Dopaminergic cell loss

The degeneration of dopaminergic neurons is known to commence in the sub-clinical phase of the disease, several years prior to the clinical presentation of motor dysfunction. By the time a patient first portrays the initial signs of motor dysfunction (Jankovic, 2008), the level of degeneration is so extensive that dopaminergic cell loss has reached 40-50% and the subsequent loss of dopamine in the striatum results in striatal dopamine levels that are 80% less than basal levels (Damier et al., 1999b; Fearnley et al., 1991).

The absence of motor dysfunction for several years after the commencement of dopaminergic degeneration is a testament to the extraordinary compensatory capacity of the human body, particularly, the dopaminergic system (Blesa et al., 2017). This masking of striatal dopamine depletion is thought to occur by several compensatory mechanisms, which may not be considered mutually exclusive. Firstly, an upregulation of dopamine release, turnover and transmission is seen in surviving dopaminergic neurons. It was first shown by Bernheimer et al. (1965) that in Parkinson’s disease patients with slight motor dysfunction, tissue concentrations of dopamine metabolites were less affected than concentrations of dopamine. This up-regulation of dopamine transmission was considered to be a possible sub-clinical compensatory mechanism and has since been consistently found in many studies (Lee et al., 2000; Piffl et al., 2006; Zigmond et al., 1990). As well as increased dopamine transmission, a reduction in dopamine uptake by the dopamine transporter (DAT) is thought to be another striatal compensatory mechanism. Down regulation of DAT mRNA has been reported in Parkinson’s disease patients (Adams et al., 2005; Joyce et al., 1997; Uhl et al., 1994), while one asymptomatic LRRK2 carrier showed to have reduced DAT binding (Lee et al., 2000) and in a larger study, LRRK2 carriers showed to have lower DAT binding (Wile et al., 2017). Dopamine denervation is also linked with alterations in dopamine receptor levels, namely D2 receptors. Striatal
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dopamine depletion has shown to increase the density of D2 receptors on MSNs (Chefer et al., 2007; Decamp et al., 1999; Graham et al., 1990; Sun et al., 2013). Finally, the axons of nigro-striatal projections exhibit extremely dense striatal branching, resulting in dopamine release from hundreds of neurons at the same striatal site (Matsuda et al., 2009). Under normal physiological conditions synaptic dopamine levels are controlled by strict reuptake and catabolism but in Parkinson’s disease denervation of dopaminergic neurons results in less synaptic terminals and DAT levels (Wile et al., 2017), causing increased diffusion of dopamine to areas of the striatum with reduced innervation.

A.1.1.2 Lewy bodies and α-synuclein

Alongside the degeneration of dopaminergic neurons in the nigro-striatal pathway, the presence of Lewy bodies and/or Lewy neurites is another primary neuropathological feature of Parkinson’s disease. The accumulation of these round, intra-neuronal, eosinophilic inclusions in brain regions outside of the substantia nigra of Parkinson’s disease patients was first identified by Frederic H. Lewy (1913). Later, in 1919, Konstantin Nikolaevich identified similar aggregations in the substantia nigra of Parkinson’s disease patients and proceeded to call these protein inclusions ‘corps de Lewy’ after Frederic H. Lewy (Lees et al., 2008). Since their naming, Lewy bodies have been found in the brains of Parkinson’s disease patients (both sporadic and familial) as well as other neurodegenerative disorders (Goedert et al., 2012), suggesting that they play an important role in disease onset and progression (Mezey et al., 1998; Polymeropoulos et al., 1997).

Lewy bodies are found in the remaining dopaminergic neurons of the substantia nigra and are primarily composed of the insoluble protein α-synuclein (Spillantini et al., 1997), ubiquitin, ubiquitinated proteins (Kuzuhara et al., 1988), and neurofilaments (Schmidt et al., 1991). Lewy neurites are abnormal neurites that contain granular material and α-
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Synuclein filaments (Braak et al., 1999). They are more abundant than Lewy bodies and are mainly found in the striatum of Parkinson’s disease patients (Duda et al., 2002). The primary component, α-synuclein, is a 140 amino acid protein that is found extensively in presynaptic terminals (Burre, 2015). In healthy brains, α-synuclein is found in a soluble state and likely regulates synaptic release. Although its normal function is not fully understood, it is evident that normal α-synuclein expression is essential. In Parkinson’s disease, α-synuclein undergoes extensive conformational transition to form pathogenic insoluble forms of α-synuclein, namely dimers, trimers and oligomers. These pathogenic species aggregate into protofibrils and form the basis of Lewy bodies (Luk et al., 2014).

Pivotal studies by Braak and colleagues has given rise to a greater understanding of the development and spread of Lewy pathology to the central nervous system (CNS) (Rietdijk et al., 2017). It was postulated that an unknown pathogen in the gut could be responsible for the instigation of sporadic Parkinson’s disease and proposed a staging system based on the specific pattern of α-synuclein spread and its correlation with the appearance of clinical motor symptoms (Braak et al., 2003). In Braak’s hypothesis, α-synuclein is thought to spread from the neurons of the gut and nasal cavity via the vagal nerve to lower brainstem regions. The subsequent appearance of Lewy pathology in the dorsal motor nucleus and olfactory system causes autonomic and olfactory dysfunction, associated with the subclinical phase of the disease, while it is the progression of α-synuclein from lower brain regions to the neocortex that coincides with the appearance of the clinical syndrome.

Clinically, Parkinson’s disease patients present with gastrointestinal (Pfeiffer, 2011) and olfactory problems (Doty, 2012), while Lewy pathology has been found in neurons of the olfactory tract and enteric nervous system (ENS) (Beach et al., 2008; Braak et al., 2006), in line with Braak’s hypothesis. Further to this, it is suggested that the spread of α-synuclein occurs by cell-cell transmission, in a prion-line manner. This hypothesis gained
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heightened interest when α-synuclein pathology was found in neurons of transplanted fetal grafts (Li et al., 2008), alongside substantial in vitro and in vivo studies exhibiting this pathological spread (Freundt et al., 2012; Hansen et al., 2011; Holmqvist et al., 2014; Pan-Montojo et al., 2012). Together these studies highlight the important role of abnormal α-synuclein in the onset and progression of Parkinson’s disease.

A.1.1.3 Neuroinflammation

There is significant evidence to suggest that neuroinflammation plays an integral role in the progression of Parkinson’s disease. This first came to light when McGeer et al. (1988) demonstrated the presence of activated microglial cells in the substantia nigra of post-mortem Parkinsonian brains, which was soon followed by the identification of astrocyte upregulation in the substantia nigra (Damier et al., 1993). Since these early findings, a plethora of studies have demonstrated the key role of microglia and astrocytes in Parkinson’s disease pathology (Gerhard et al., 2006; Saijo et al., 2009; Tansey et al., 2007; Yamada et al., 1992), as well as significant elevations in pro-inflammatory mediators including tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interferon-gamma (INF-γ) (Mogi et al., 1994a; Mogi et al., 1994b).

Microglia are resident immune cells of the innate immune response, forming 5-20% of all glial cells. They are known as ‘surveillance cells’ and are the first line of defence against bacterial and/or viral pathogens. Activation by such threats via Toll-like receptor signalling results in an increased number of microglia, as well as a change in cell morphology from a ramified ‘resting’ state to an amoeboid ‘active’ state (Lull et al., 2010). In comparison, astrocytic responses are relatively slower than microglial activation (Pekny et al., 2014). While microglia initiate the inflammatory reaction, astrocytes are activated by the pro-inflammatory mediators released from activated microglia, and once activated, they
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proceed to further amplify the pro-inflammatory response. This microglial and astrocytic activation is necessary for normal CNS maintenance, but chronic over-activation, causing an over-production of pro-inflammatory mediators, can be catastrophic to CNS integrity and function (Hirsch et al., 2012).

Although abundant, microglial distribution throughout the brain is not uniform, and strikingly, the substantia nigra is a more densely populated region with 4 – 5 times the amount of microglia in comparison to other regions (Kim et al., 2000; Lawson et al., 1990; Yang et al., 2013). Neurons, specifically dopaminergic neurons, in these densely populated regions are extremely sensitive to microglial activation, so whether the initial insult be α-synuclein aggregation, environmental or toxic (Gao et al., 2002; Lofrumento et al., 2011; Zhang et al., 2017), the exacerbated degeneration of dopaminergic neurons causes an exaggerated microglial and subsequent astrocytic response that causes further cell death, which in turn causes further neuroinflammation, and thus a vicious cycle of neurodegeneration and neuroinflammation ensues (Gao et al., 2008) (Fig. A.2). Hence, although neuroinflammation is not thought to be the primary cause of dopaminergic cell death in Parkinson’s disease, it plays an important role in disease progression (Tansey et al., 2010; Wang et al., 2015).
Figure A.2. Cyclic neuroinflammation and cell death. Neurotoxic insults or inflammatory triggers can instigate a vicious cycle of chronic neuroinflammation and neuronal damage that can cause progressive dopaminergic degeneration over time. Taken from Block et al., (2007).

A.1.1.4 Mitochondrial dysfunction and oxidative stress

Mitochondrial dysfunction and the concomitant oxidative stress are further contributing factors that play an important role in the pathogenesis of Parkinson’s disease. Known as the powerhouse of the cell, mitochondria are intracellular organelles that are involved in the production of energy in the form of adenosine triphosphate (ATP). The ATP produced by mitochondria accounts for over 90% of the total energy requirements of eukaryotic cells (Brown, 1992; Erecinska et al., 1982). In particular, the physiological output of neuronal cells requires large quantities of energy, resulting in a large number of intracellular mitochondria. The increased quantity of mitochondria in neuronal cells makes them highly susceptible to mitochondrial dysfunction (Villace et al., 2017). Further to this, dopaminergic neurons are particularly sensitive to mitochondrial dysfunction. Dopamine is
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an unstable neurotransmitter which generates reactive oxygen species (ROS) upon oxidation and can therefore be highly neurotoxic (Hastings, 2009).

A significant breakthrough in the understanding of the mechanisms underlying Parkinson’s disease pathogenesis came in the 1980s when cases of induced Parkinsonism were linked with the illicit self-administration of the heroine analog 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1983). Within days of administration, Parkinsonian symptoms appeared and post-mortem analysis later confirmed the loss of dopaminergic neurons from the substantia nigra. The MPTP metabolite, 1-methyl-4-phenylpyridinium (MPP+), is highly selective for dopaminergic neurons through its dependence on the dopamine transporter (DAT) for uptake into neurons (Singer et al., 1988). Once inside the neuron, MPP+ inhibits complex 1 of the mitochondrial respiratory chain, resulting in oxidative stress (Nicklas et al., 1987).

Oxidative stress reflects an imbalance in the accumulation of ROS and the body’s ability to actively detoxify these reactive chemical species. The mitochondria’s electron transport chain is the main source of ROS in the brain. Under normal physiological conditions, as oxygen is metabolised, superoxide is produced by both complex I and II. Superoxide is then subsequently converted to hydrogen peroxide ($\text{H}_2\text{O}_2$) by manganese superoxide dismutase (MmSOD). Enzymes resident in the mitochondria further break down $\text{H}_2\text{O}_2$ into water and molecular oxygen. Under stressful conditions such as complex 1 inhibition, the level of ROS surpasses the antioxidant capacity of the cell, causing cell death (Hauser et al., 2013).

It has been shown that both complex 1 activity (Schapira et al., 1990) and antioxidant (glutathione) levels (Sian et al., 1994) are decreased in the substantia nigra of Parkinson’s disease patients. While it is widely accepted that there is complex 1 dysfunction in the mitochondria of Parkinson’s disease patients, the cause of this dysfunction is not well
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understood. Exposure to environmental agents such as complex 1 inhibiting pesticides, rotenone and paraquat, have been linked to Parkinson’s disease (Tanner et al., 2011), however, MPTP exposure is the only confirmed environmental cause of Parkinson’s disease.

A.1.2 Etiology

The etiology of Parkinson’s disease is now widely accepted to be multifactorial, having no single causative factor. Instead, a variety of risk factors, such as, aging, exposure to environmental toxins and genetic predisposition are all thought to play a role in disease manifestation.

A.1.2.1 Age

Parkinson’s disease is the second most common age related neurodegenerative disorder in the world, second only to Alzheimer’s disease (Tysnes et al., 2017). Aging remains the single largest risk factor for the development of Parkinson’s disease, with the average age of onset being 65 years of age. It is estimated that 3% of the population over 65 years of age are affected, with this number rising to 4-5% of the population over 85 years of age (de Lau et al., 2006; von Campenhausen et al., 2005). A European meta-analysis reported that 600 per 100,000 individuals living with Parkinson’s disease are within the ages of 65 and 69, a figure that rose significantly to 2,600 per 100,000 within the ages of 85 to 89 (de Rijk et al., 2000). Furthermore, with the increasing longevity of the worldwide population, the incidence of Parkinson’s disease is expected to rise from 4.1 million to 9.3 million by 2030 (Dorsey et al., 2007). As a result of the ever increasing aging society, the economic burden of diagnosis, treatment and care will also continue to rise.

While the mechanisms underlying the relationship between the ageing population and the prevalence of Parkinson’s disease are not very well understood, it has been suggested that
the dopaminergic population of neuronal cells are particularly vulnerable to the natural ageing process. A study containing elderly individuals (mean age 88 years of age) without clinically diagnosed Parkinson’s disease found that ~33% of individuals exhibited mild to severe cell loss in the substantia nigra, while 10% showed Lewy body pathology (Buchman et al., 2012). Moreover, an age related decline in mitochondrial activity, oxidative stress due to dopamine metabolism, and the accumulation of damaged proteins in neurons of the substantia nigra are all likely risk factors associated with advanced aging (Reeve et al., 2014). However, the incidence of Parkinson’s disease is not ubiquitous within the aging population and although ageing is the single largest risk factor associated with the development of Parkinson’s disease, it is not a single causative factor, meaning that disease initiation is most likely attributable to a combination of aging, exposure to environmental toxins and genetic predisposition.

A.1.2.2 Environment

The etiology of Parkinson’s disease is widely accepted to be multi-factorial, with the environment playing a pivotal role in disease susceptibility and onset. Epidemiology studies have identified a number of environmental factors associated with the development of Parkinson’s disease, including, exposure to bacterial and/or viral infections, exposure to industrial chemicals and the exposure to herbicides and/or pesticides. Interestingly, a number of environmental factors have also been identified as protective against the development and progression of Parkinson’s disease.

A number of viruses have been associated with the development of both acute and chronic Parkinsonism, including, influenza, Japanese encephalitis B, western equine encephalitis, coxsackie, herpes and those that lead to human immunodeficiency virus (HIV) (Jang et al., 2009). The suggestion of virus related Parkinsonism dates back to the end of World War 1
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when patients developed a Parkinsonian-like syndrome secondary to viral encephalitis during the 1918 influenza epidemic (Dourmashkin, 1997). Intriguingly, these patients responded extremely well to levodopa, the gold standard treatment for Parkinson’s disease (Sacks, 1983).

The most persuasive evidence identifying the environment as a causative factor in the development of Parkinson’s disease was in the 1980s when a group of intravenous drug users developed Parkinsonian symptoms within days of MPTP administration (outlined in section 1.1.1.4). Moreover, the deleterious effects of MPTP administration were later reversed by Levodopa treatment (Langston et al., 1983). MPTP exposure remains the only confirmed cause of Parkinsonism and was soon utilised as a compound to experimentally model Parkinson’s disease in vivo (Langston, 2017; Langston et al., 1984). Strikingly, MPTPs metabolite, MPP+, was found to have a remarkable structural resemblance to the worldwide used herbicide, Paraquat (Sandy et al., 1988), an observation that lead to the search for specific environmental causative factors.

Numerous epidemiological studies have examined pesticides as a risk factor for the development of Parkinson’s disease, highlighting a correlation between pesticide exposure and the incidence of neurodegeneration and disease development (Brown et al., 2006). Interestingly, the occupational use of pesticides and rural living, where the probability of pesticide exposure is significantly higher, have both been associated with an increased risk of Parkinson’s disease (Priyadarshi et al., 2001; Yitshak Sade et al., 2015). However, the common exposure of individuals to more than one pesticide hinders the identification of singular causative agents. A recent study was carried out to examine the potential of a number of pesticides (which cause mitochondrial dysfunction or oxidative stress) to cause Parkinson’s disease. From the selection of compounds examined, exposure to two pesticides, paraquat and rotenone, increased the risk of developing Parkinson’s disease.
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(Tanner et al., 2011; Tanner et al., 2009). Both rotenone (Betarbet et al., 2000) and paraquat (McCormack et al., 2002) have been successfully used to model Parkinson’s disease in experimental animals, where administration results in the relatively specific degeneration of the nigrostriatal pathway and appearance of Parkinsonian motor dysfunction. The use of such compounds to experimentally model the disease further reiterates their involvement in the etiology of Parkinson’s disease.

Conversely, some environmental compounds have been identified as beneficial to disease development and exhibit neuroprotective effects. Studies have found a strong negative correlation between the incidence of Parkinson’s disease and smoking. It is reported that cigarette smokers are 50% less likely to develop Parkinson’s disease than age/gender matched non-smokers (Fratiglioni et al., 2000). Although not well understood, the major component of cigarette smoke, nicotine, is thought to induce biological protection to nigral dopaminergic neurons through its modulation of dopamine transmission and regulation of striatal activity (Ma et al., 2017). Similarly, the moderate consumption of caffeine has shown to reduce the likelihood of Parkinson’s disease (Ascherio et al., 2001), raising question as to its promising potential as a therapeutic tool. While studies do suggest that caffeine may improve motor aspects of Parkinson’s disease (Altman et al., 2011; Postuma et al., 2012), long term trials are required for further investigation.

A.1.2.3 Genetic predisposition

Parkinson’s disease was originally considered to be a solely sporadic disease, however, epidemiology studies have identified familial forms of the disease that affect 5-10% of patients (Schulte et al., 2011). Familial Parkinson’s disease can be inherited in an autosomal dominant or autosomal recessive form, and to date, genome wide association studies have identified mutations of more than 20 distinct chromosomal loci that have been
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associated with Parkinson’s disease and a growing list of genes that increase the risk of Parkinson’s disease (Nalls et al., 2014; Simon-Sanchez et al., 2009). Of these, six genes are unequivocally linked to hereditable monogenic Parkinson’s disease. SNCA (PARK1) and LRRK2 (PARK8) are responsible for autosomal dominant forms of Parkinson’s disease, while Parkin (PARK2), PINK1 (PARK6), DJ-1 (PARK7) and ATP13A2 (PARK9) are associated with autosomal recessive forms of Parkinson’s disease. SNCA, the gene encoding α-synuclein which is the main component of the protein inclusions known as Lewy bodies (Spillantini et al., 1997), was the first solid evidence of a mutated gene that caused autosomal dominant Parkinson’s disease (Polymeropoulos et al., 1997). Since its discovery, mutations of many genes have been identified as the driving factors of familial Parkinson’s disease (Table A.1).
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<td>Unreported</td>
<td>Juvenile</td>
<td>(Edvardson et al., 2012; Koroglu et al., 2013)</td>
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**Table A.1.** Genes that are involved in the etiology of Parkinson’s disease. Modified from several sources (Bonifati, 2014; Funke et al., 2013; Puschmann, 2013; Spatola et al., 2014)
A.2 Current Pharmacological Treatment for PD

Although remarkable progress has been made thus far in understanding the etiology and pathogenesis of Parkinson’s disease, this knowledge has not yet translated to the discovery of a cure. Currently the treatment of Parkinson’s disease is solely symptomatic and does not target the underlying neurodegeneration. As a result, disease pathophysiology continues to progress, even throughout treatment, causing the patients symptomatic relief to be eventually lessened. Current therapies are complex and patient specific, with age of onset, disease progression and quality of life all being considering factors when choosing a treatment regimen. Although existing therapies focus on dopamine replacement strategies that will ameliorate the motor symptoms of the disease, ideally, new therapies are required that not only symptomatically treat the disease, but ultimately repair the diseased brain, improving disease prognosis and the patient’s quality of life.

A.2.1 Levodopa

The discovery of levodopa therapy in 1967 still remains one of the most significant advancements in the pharmacotherapy of Parkinson’s disease (Cotzias et al., 1969). 50 years after its discovery, levodopa therapy remains the gold standard treatment for Parkinson’s disease. Its efficacy is undeniable and arrived on the scene as a ‘miracle’ drug, reviving patients who had been immobilised for years by the crippling symptoms of this extremely progressive neurodegenerative disease.

The story of levodopa therapy began in 1939 when it was discovered that levodopa was a dopamine precursor that could be converted to dopamine in the brain by the enzyme dopa decarboxylase (Holtz, 1939). At this point it was assumed that dopamine’s sole role was in the biosynthetic pathway of adrenaline and noradrenaline. However, a remarkable breakthrough by Arvid Carlsson proved dopamine to be of great importance in the control
Appendix I: Overview of Parkinson’s disease

of movement (Carlsson et al., 1957). Moreover, while Carlsson’s concepts implicating dopamine in Parkinson’s disease were initially rejected, a 1960 post-mortem analysis of Parkinson’s disease patients showed a significant depletion of dopamine in the striatum (Ehringer et al., 1960). From here the first clinical trial using levodopa showed that its administration abolished the bradykinesia and rigidity symptoms of the disease (Birkmayer et al., 1961).

Despite its superlative effects in the early stages of disease, continued disease progression hinders the efficacy of levodopa and it becomes increasingly difficult to deliver a therapeutic dose that proves symptomatic relief without the emergence of drug-induced dyskinesias and motor fluctuations (Marsden, 1994). While they are not well understood, these non-avoidable dyskinesias appear in all patients within 10 years of treatment and are thought to arise as a result of a pulsatile stimulation of dopaminergic receptors. A younger age of onset, disease severity and high levodopa dose are all considered to increase their risk of occurrence (Fabbrini et al., 2007).

A.2.2 Dopamine Agonists

Dopamine agonists exhibit their anti-Parkinsonian effects by acting on post-synaptic dopamine receptors, directly mimicking the effects of dopamine. Dopamine agonists include those that are ergoline derived, such as bromocriptine, pergolide, lisuride, and cabergoline and those that are non-ergoline derived, such as ropinirole, pramipexole and apomorphine (Brooks, 2000). Initially, dopamine agonists were introduced as an adjunctive treatment to levodopa therapy in patients who suffered from motor fluctuations and dyskinesias as a result of chronic treatment (Poewe, 1998). Their addition to treatment regimens allows for a 20-30% reduction in levodopa dose, lessening the disabling complications associated with chronic treatment. Dopamine agonists are also used
Appendix I: Overview of Parkinson’s disease

successfully as a monotherapy, providing adequate symptomatic relief in early stage Parkinson’s disease (Bracco et al., 2004; Guttman, 1997). For younger patients with mild to moderate motor symptoms, dopamine agonists are suggested as the first line therapy, as they provide symptomatic relief equivalent to levodopa therapy and will minimise or delay the complications associated with chronic levodopa treatment.

A.2.3 MAO/COMT Inhibitors

The inactivation of dopamine in the brain involves the actions of three enzymes, monoamine oxidase (MAO), catechol-O-methyl transferase (COMT) and aldehyde dehydrogenase (ALDH) (Meiser et al., 2013). They act to breakdown dopamine into three inactive metabolites, 3,4-dihydroxyphenyl-acetaldehyde (DOPAC), 3-methoxytyramine (3-MT) and homovanillic acid (HVA). The use of enzyme inhibitors, particularly MAO and COMT inhibitors, as pharmacotherapies in Parkinson’s disease helps prevent the breakdown of dopamine in the brain and consequently prolongs the availability of dopamine in the striatum (Muller, 2015; Riederer et al., 2011). Selective irreversible MAO-B inhibitors, such as selegiline and rasagiline have shown to be efficacious in the treatment of akinesia and motor fluctuations experienced by Parkinson’s disease patients. They are often used as a monotherapy to delay levodopa therapy (Biglan et al., 2006; Myllyla et al., 1997) or as an adjunctive therapy to levodopa (Elizan et al., 1989; Rascol et al., 2005) in order to prolong and reduce the dose of levodopa required for symptomatic relief. Similarly, the COMT inhibitor, entacapone, has shown efficacy in the treatment of motor fluctuations caused by chronic levodopa treatment. While it is not efficacious as a monotherapy, its combination with levodopa and carbidopa is clinically approved for the treatment of late-stage levodopa-induced motor fluctuations (Brooks, 2008; Seeberger et al., 2009).
Appendix I: Overview of Parkinson’s disease

A.2.4 Non-dopaminergic Treatments

The use of anti-cholinergic drugs to address the symptoms of Parkinson’s disease precedes all current dopaminergic replacement strategies (Rezak, 2007). Since the discovery of their anti-Parkinsonian effects in 1867, they were the sole first line of treatment for nearly a century (Katzenschlager et al., 2003). Moreover, despite the discovery of newer dopaminergic therapies, anti-cholinergic drugs are still used for the treatment of Parkinson’s disease today (as a monotherapy or adjunct to levodopa therapy). However, given the depth of unwanted side effects (see section 1.2.6), their use is preferably reserved for tremors resistant to dopaminergic therapy. Anti-cholinergic drugs currently on the market include trihexyphenidyl, benztropine, and procyclidine.

The anti-viral agent, amantadine, has shown to be effective in the treatment of Parkinson’s disease. It is often used as a monotherapy or adjunctive therapy, but does not offer the same symptomatic relief as dopaminergic therapies (Crosby et al., 2003). It exerts its effects through the non-competitive inhibition of NMDA receptors and the subsequent blockage of NMDA mediated excitotoxity in the basal ganglia that is associated with dopaminergic neuron cell death. The chronic treatment of amantadine when given as an adjunct to levodopa therapy is thought to improve levodopa-induced dyskinesias (Blanchet et al., 1998; Metman et al., 1998).

A.2.5 Deep Brain Stimulation

Deep brain stimulation (DBS) is a neurosurgical approach in which an implanted device, known as a neurostimulator, sends electrical impulses to specific brain regions through implanted electrodes (Perlmutter et al., 2006). Stimulation of the STN or the GPi has shown to substantially reduce bradykinesia, rigidity, tremor, and gait difficulties in patients (Anderson et al., 2005). Similarly, stimulation of the ventral intermediate nucleus of the
thalamus can dramatically relieve tremor (Ohye et al., 1977). DBS is most often considered when a patient’s symptomatic relief is no longer under the control of dopaminergic therapy and further optimisation of their treatment regime is not an option. While DBS provides remarkable and immediate benefit to patients, this treatment is still solely symptomatic and does not address the underlying neurodegeneration and progression of the disease (Limousin et al., 2008). Moreover, the implantation of such electrodes is a highly invasive procedure and patient candidacy is therefore limited by surgical risk factors.

A.2.6 Limitations of Current Treatments

Despite the superlative relief that levodopa therapy provides to Parkinson’s disease patients, its long term use is hindered by the development of severe adverse reactions (Fabbrini et al., 2007). Chronic administration of therapeutic doses of levodopa are associated with disabling motor fluctuations and drug induced dyskinesias that are often worse than the disease symptoms themselves. These adverse reactions are evident in 70-90% of patients within 10 years of levodopa therapy (Lopez et al., 2010). As a result, despite it being the most efficacious drug available for the symptomatic treatment of Parkinson’s disease, its use is commonly reserved for advanced Parkinson’s disease, in a hope to delay or avoid such motor complications. Moreover, the conversion of levodopa to dopamine in the peripheral system causes a host of further adverse reactions such as nausea, vomiting and hypertension. While carbidopa (a peripherally restricted DOPA-decarboxylase inhibitor) administration alongside levodopa helps control these peripheral side effects, they are still experienced by many patients (Fahn, 2008).

In an effort to combat the debilitating motor complications associated with levodopa treatment, therapies such as dopamine agonists, enzyme inhibitors and non-dopaminergic
Appendix I: Overview of Parkinson’s disease

treatments were applied either as monotherapies to delay levodopa use or adjunctive therapies to lessen such motor complications. Unfortunately, despite their benefits, their use is also associated with unwanted adverse reactions. Contrary to the benefits of dopamine agonists, as a result of their interactions with non-dopaminergic neurons they are known to cause non-motor adverse reactions such as sudden sleep onset, constipation, nausea, dizziness, hallucinations, peripheral oedema and heart valve fibrosis (Antonini et al., 2009; de Smet et al., 1982). Additionally, their use can also cause impulse control disorder, which affects 1 in 7 patients (Weiss et al., 2012). These compulsive behaviours (gambling, shopping, sex etc.) can have devastating effects on the life of both the patient and their family.

Conversely, although MAO-B inhibitors are much better tolerated with few side effects, they are limited therapeutically as their efficacy is inferior to both levodopa and dopamine agonists (Lees, 1995). While entacapone, a COMT inhibitor, is often used in conjunction with levodopa and carbidopa to treat the ‘end of dose’ motor fluctuations of levodopa, it has more recently been linked with an increase in levodopa-induced dyskinesias, hindering its use (Stocchi et al., 2010).

In a similar manner, although anti-cholinergic drugs have been used to treat Parkinson’s disease for over a century, their use is associated with an unfavourable adverse event profile, including confusion, agitation, hallucinations and dementia (de Smet et al., 1982). As a result, their use is reserved for tremors resistant to dopaminergic therapy. Likewise, while the anti-viral agent, amantadine, has shown to improve levodopa-induced dyskinesias when given as an adjunct to levodopa therapy, its use is associated with mental status changes, livedo reticularis and lower extremity edema.
Appendix I: Overview of Parkinson’s disease

It is important to note that despite the remarkable advances made in the treatment of Parkinson’s disease, there is still a serious unmet therapeutic need. All current pharmacological therapies fail to target the underlying pathophysiology of the disease, resulting in a purely symptomatic treatment where disease progression swiftly continues. Therefore, while the search for disease-modifying and neuroprotective drugs continues, a parallel search for novel strategies that not only symptomatically treat the disease but ultimately repair the diseased brain is underway.
A.3 Reference list


Appendix I: Overview of Parkinson’s disease


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Appendix II: Buffers for Perfusion - Fixation

1. **0.2M Phosphate buffer solution**

   A: Sodium dihydrogen phosphate monohydrate (NaH2PO4.H2O MW=137.99)
   
   B: Disodium hydrogen phosphate dehydrate (Na2HPO4.2H2O MW=177.99)

   → For 500ml PBS
   A: 27.598g/L x 0.095L = 2.621 g
   B: 35.598g/L x 0.405L = 14.417 g
   Dissolve both in 500ml of dH2O

   → For 1000ml PBS
   A: 27.598g/L x 0.19L = 5.2436 g
   B: 35.598g/L x 0.81L = 28.834 g
   Dissolve both in 1000ml of dH2O

   → For 2000ml PBS
   A: 27.598g/L x 0.38L = 10.4872 g
   B: 35.598g/L x 1.62L = 57.668 g
   Dissolve both in 2000ml of dH2O

   **Note:** *To be used in a 1:1 dilution with PFA*

2. **Fixative: 4% Paraformaldehyde (4L) (in fume hood)**

   1. Heat 1.5L of dH2O to 60°C
   2. Add 160g of PFA powder (40 g/L)
   3. Add a few NaOH pellets in order to dissolve PFA
   4. Stir until clear
   5. Fill to 2L with dH2O
   6. Stir until clear
   7. Add 2L of 0.2M PBS to give a final volume of 4L
   8. PH to 7.4 and cool to 4°C
3. **Heparinised Saline**

1 ml heparin is added per 1 L saline

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

4. **25% w/v Sucrose Solution (1 L)**

1. Dissolve 5 PBS tablets in ~500 ml dH$_2$O (1 tablet per 200 ml water)
2. Add 250 g of sucrose
3. Stir until dissolved, apply heat if necessary
4. Make up to 1 L with dH$_2$O

5. **0.1% w/v TBS-Azide**

1. Weigh out 12 g Trizma, 9 g NaCl, and 1 g Sodium Azide
2. Add to 1 L dH$_2$O and dissolve
3. PH to 7.4
## Solutions for immunohistochemistry

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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 5mins
   - Methanol 5ml
   - 30% H₂O₂ 5ml
   - Distilled H₂O 40ml

3. Wash 3*5min with TBS

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

5. Draw off excess and incubate in primary antibody in 1% serum at RT overnight (e.g. 1:1000)
   - 1µl/ml of 1º in 10µl/ml serum in TXTBS
Appendix III: Immunohistochemistry Protocol

Day 2

6. Wash 3*10min with TBS

7. Incubate in biotinylated secondary in 1% serum for 3 hours (e.g. 1:200)
   - 5µl/ml of 2º in 10µl/ml serum in TBS

8. Make ABC complex. Wash 3*10mins with TBS
   - 5µl of solution A and 5µl of solution B per ml in 10µl/ml serum in TBS

9. Incubate in ABC complex for 2hours

10. Wash 3*10mins with TBS

11. Wash with TNS (freshly made up) overnight @ 4ºC

Day 3

12. Incubate in H$_2$O$_2$/DAB solution until the colour develops
   - TNS 40ml
   - DAB 20mg (frozen in 2ml aliquots)
   - 30% H$_2$O$_2$ 12µl

13. Wash 3*5min with TNS

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

15. Dehydrate in an ascending series of alcohols
   - 50% EtOH for 5 min
   - 70% EtOH for 5 min
   - 100% EtOH for 5 min
   - 100% EtOH for 5 min

16. Clear in Xylene in the fume hood and coverslip using DPX mountant
   - 1$^{st}$ Xylene for 5 min
   - 2$^{nd}$ Xylene for 5 min
Appendix III: Immunohistochemistry Protocol

Slide Subbing

Materials

- Gelatin (10 g/L)
- Chromic potassium Sulphate (500 mg/L)
- Distilled H₂O
- Slides

Method

1. Heat H₂O 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 IV: General Immunocytochemistry Protocol

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

Solutions

*Sodium Azide (10% Stock Solution)*

- 1 g Sodium azide in 10 ml TBS

*TBS containing 1% BSA*

- 1 g BSA in 100 ml TBS

Step by step process

Day 1

1. Wash wells 1*5 min in TBS.

2. Incubate cells in blocking solution for 1 hour at RT.

   **40ml Blocking Solution**

   0.4 g Bovine Serum Albumin
   2 ml Normal Rabbit Serum (NRS) (dependant on secondary host)
   120 µl Triton X 100
   40 µl Sodium Azide (10% Stock Solution (0.01% is final conc))
   37.84 ml TBS (can use stock 0.1g BSA in 10ml then omit 0.4g BSA above)
3. Remove Blocking solution and incubate wells in primary antibody at RT overnight.

**Primary – mouse anti-TH (1:000)**
6 µl TH antibody
6 µl Sodium Azide (from 10% Stock Solution)
5988 µl TBS containing 1% BSA (can use stock 0.1g BSA in 10ml)

**Primary – mouse anti-βIII tubulin (1:333)**
18 µl βIII tubulin antibody
6 µl Sodium Azide (from 10% Stock Solution)
5976 µl TBS containing 1% BSA (can use stock 0.1g BSA in 10ml)

*Day 2*

1. Remove primary antibody.

2. Wash wells 3 *10 min with TBS.

3. Incubate wells in fluorophore labelled secondary antibody for 3 hours at RT.

**Secondary – Rabbit anti-mouse A.F. 488**
75 µl secondary antibody
150 µl NRS
14775 µl TBS

4. Wash wells 3*5 min with TBS.

5. Incubate sections in 1 µg/ml DAPI in TBS for ~5 min.

6. Wash wells 3*10 min TBS.

7. Store wells in 0.1% TBS Azide in the fridge.

8. Image cells
Encapsulation of primary dopaminergic neurons in a GDNF-loaded collagen hydrogel increases their survival, re-innervation and function after intra-striatal transplantation

Niamh Moriarty1, Abhay Pandit2 & Eilís Dowd1

Poor graft survival limits the use of primary dopaminergic neurons for neural repair in Parkinson’s disease. Injectable hydrogels have the potential to significantly improve the outcome of such reparative approaches by providing a physical matrix for cell encapsulation which can be further enriched with pro-survival factors. Therefore, this study sought to determine the survival and efficacy of primary dopaminergic grafts after intra-striatal delivery in a glial-derived neurotrophic factor (GDNF)-loaded collagen hydrogel in a rat model of Parkinson’s disease. After intra-striatal transplantation into the lesioned striatum, the GDNF-enriched collagen hydrogel significantly improved the survival of dopaminergic neurons in the graft (5-fold), increased their capacity for striatal re-innervation (3-fold), and enhanced their functional efficacy. Additional studies suggested that this was due to the hydrogel’s ability to retain GDNF in the microenvironment of the graft, and to protect the transplanted cells from the host immune response. In conclusion, the encapsulation of dopaminergic neurons in a GDNF-loaded hydrogel dramatically increased their survival and function, providing further evidence of the potential of biomaterials for neural transplantation and brain repair in neurodegenerative diseases such as Parkinson’s disease.

The relatively selective loss of dopaminergic neurons from the substantia nigra pars compacta makes Parkinson’s disease an ideal candidate for cell replacement therapies12. To date, the focus of cell therapies in Parkinson’s disease has been on the transplantation of dopamine neuron-rich foetal ventral mesencephalon (VM) grafts which have shown to both survive and re-innervate the striatum post-transplantation, whilst also restoring motor function3-7. However, despite long-term symptomatic relief in some patients, significant limitations, including poor survival post-transplantation, prevent this therapy being utilised as a potential restorative approach for Parkinson’s disease8. VM grafts contain diverse cell populations, the least abundant of which is dopaminergic neurons, and less than 20% of these neurons survive transplantation9. Thus, poor survival, the sheer volume of human foetal tissue required (10 per grafted hemisphere), and the associated ethical concerns has highlighted an urgent need for improved methodologies to enhance dopamine neuron survival rates post-transplantation.

While the efficacy of dopamine neuron-rich foetal VM grafts is still being investigated clinically through the TRANSEURO consortium10, the field of cell replacement therapy in Parkinson’s disease is moving towards more readily available dopaminergic cell sources, such as those derived from embryonic stem cells and induced pluripotent stem cells11. While these cells show extraordinary regenerative potential, their use is still in the experimental stages and has not yet reached a clinical setting. With this in mind, dopamine neuron-rich foetal VM grafts are an

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extremely well established cell type and are therefore optimal for testing the potential of biomaterial scaffolds to improve the survival and efficacy of such cell regenerative therapies.

The majority of cell death in VM grafts occurs through apoptosis at various points of the transplantation process by factors such as detachment from the extracellular matrix during tissue dissection, growth factor deprivation upon transplantation, and recruitment of host neuro-immune cells to the exogenous graft. Each of these stages provides a target point of intervention at which graft survival could be improved. Injectable scaffolds, such as in situ forming hydrogels, may provide a delivery platform to improve grafted cell survival after transplantation. These hydrogels could potentially increase cell engraftment by providing a supportive environment for cell adhesion, creating a physical barrier between the transplanted cells and the host neuro-immune cells and by providing a reservoir for localised growth factor delivery. A particular scaffold of interest, collagen, is a clinically accepted, highly abundant and natural extracellular matrix that is used for a variety of applications. The injectable nature of collagen hydrogels, coupled with their ability to support and immunosegregate cells, whilst simultaneously delivering trophic factors in a localised manner, creates a natural scaffold with the potential to improve the transplantation of dopaminergic neurons. Despite this, the intra-cerebral use of collagen hydrogels has not been well established as a delivery platform in its own right.

Thus, this study aimed to assess the use of a glial-derived neurotrophic factor-loaded collagen hydrogel for the transplantation of primary dopaminergic neurons to the Parkinsonian brain. GDNF was selected as the growth factor in this study as it is well established as a neurotrophin for developing dopaminergic neurons. We hypothesised that the type 1 collagen hydrogel would provide a local GDNF reservoir and reduce the host immune response to the transplanted cells, thereby improving the overall survival, re-innervation and functionality of primary dopaminergic neurons after intra-striatal transplantation.

Methods

In vitro/ex vivo experimental design. Before undertaking in vivo studies, in vitro and ex vivo studies were completed in order to determine the cytocompatibility of the collagen hydrogels. This was assessed using alamarBlue cell viability assay and immunocytochemistry on bone marrow-derived mesenchymal stem cells (MSC) and/or primary embryonic day 14 (E14) VM cell cultures. Subsequently a series of in vivo studies to optimise the collagen hydrogel for VM cell transplantation were conducted.

In vivo experimental designs. Preliminary in vivo assessment of the impact of hydrogel cross-linking on grafted cell viability. As the concentration of poly(ethylene glycol) ether tetracuccinimidy glutarate (4s-StarPEG) used to crosslink collagen will affect the intensity of gelation, it was important to determine to what extent the hydrogel can be cross-linked without negatively impacting graft survival. The final level of cross-linking will be chosen based on successful graft survival. In order to determine the optimum level of hydrogel cross-linking for cell delivery, an in vivo pilot study using male Sprague-Dawley rats (n = 24) was carried out. Rats were divided into two groups to receive either a bilateral intra-striatal transplant of green fluorescent protein (GFP)-MSCs (30,000 cells/3 µl) delivered in transplantation media or encapsulated in a collagen hydrogel of various 4s-StarPEG concentrations (1, 2, or 4 mg/ml). The animals were then sacrificed for post-mortem analysis at days 1, 4 and 7 post transplantation (n = 4 per group, per time point). GFP-MSCs were used as they are easily detected post-transplantation and are ideal to assess whether the intensity of gelation is suitable for graft survival. A schematic of this experimental design is shown in the results section.

Preliminary in vivo assessment of the impact of the collagen hydrogel on encapsulated VM cells. Once the optimal level of hydrogel cross-linking was determined, an in vivo pilot study was carried out to assess if the collagen hydrogel will impact the striatal re-innervation from encapsulated VM cells. Male Sprague-Dawley rats (n = 24) received a unilateral intra-medial forebrain bundle (MFB) 6-hydroxydopamine (6-OHDA) lesion (3 µl). Two weeks later, all rats were subjected to methamphetamine-induced rotations. Based on these results, rats were then performance matched into six groups (n = 4 per group) to receive intra-striatal transplants of E14 VM cells of various densities (200,000, 300,000 or 400,000 cells per 6 µl) in transplantation media or encapsulated in a collagen hydrogel (cross-linked with 4 mg/ml 4s-StarPEG). The animals were then sacrificed two weeks post-transplantation for post-mortem assessment. A schematic of this experimental design is shown in the results section.

Preliminary in vivo assessment of GDNF retention within the collagen hydrogel. To assess the striatal retention of human recombinant GDNF when encapsulated within the collagen hydrogel, male Sprague-Dawley rats (n = 12) received bilateral intra-striatal infusions of GDNF (1000 ng) as either a bolus or encapsulated in a collagen hydrogel (6 µl per rat). The animals were then sacrificed for post-mortem assessment at days 1, 2 and 4 post-transplantation (n = 4 per group, per time point). A schematic of this experimental design is shown in the results section.

Main in vivo study to assess the long-term survival, reinnervation and functionality of grafted VM cells encapsulated in a GDNF-loaded collagen hydrogel. After the initial in vitro, ex vivo and in vivo analyses, the main in vivo study was conducted to assess the long term survival and efficacy of E14 VM cells encapsulated in a GDNF-loaded collagen hydrogel. Male Sprague-Dawley rats (n = 40) received a unilateral intra-MFB 6-OHDA lesion (3 µl). Two weeks later, rats underwent post-lesion methamphetamine-induced rotations. Based on these results, rats were performance matched into four groups to receive intra-striatal transplants of VM cells alone (400,000 per 6 µl), VM cells with GDNF (1000 ng), VM cells encapsulated in a collagen hydrogel (cross-linked with 4 mg/ml 4s-StarPEG) or VM cells encapsulated in a GDNF-loaded collagen hydrogel. Methamphetamine-induced rotations resumed three weeks post-transplantation and were carried out at three week intervals for a total of twelve weeks. The animals were then sacrificed for post-mortem assessment. A schematic of this experimental design is shown in Fig. 1.
Animals. All experiments involving the use of animals for procedures and cell preparations were carried out in accordance with relevant guidelines and regulations, were completed under licence by the Irish Department of Health and Children and the Irish Health Products Regulatory Authority, were performed in compliance with the European Union Directive 2010/63/EU and S.I No. 543 of 2012, and were approved by the Animal Care and Research Ethics Committee at the National University of Ireland, Galway. Male Sprague-Dawley rats (weighing 200–225 g on arrival) and time-mated female Sprague-Dawley rats were sourced from Charles River, UK. Animals were housed in groups of four per cage, on a 12:12 h light/dark cycle, at 19–23 °C, with relative humidity levels maintained between 40 and 70%. For the duration of the experiment, animals were allowed food and water ad libitum. All behavioural testing and ex vivo analyses were carried out by an experimenter who was blind to the treatment of the animals.

Cell culture. For E14 VM cultures, E14 embryos were obtained by laparotomy from time-mated female Sprague-Dawley rats following quick decapitation under isoflurane (5% in 0.5 L O2). The VM was micro-dissected from each embryo as previously described. Dissected VM tissue was centrifuged at 1100 rpm for 5 min at 4 °C. The tissue pellet was incubated in 40% trypsin-Hank's balanced salt solution (HBSS) for 4 min, at 37 °C with 5% CO2. Foetal calf serum (FCS) was then added to the tissue and centrifuged at 1100 rpm for 5 min at 4 °C. The cell pellet was then resuspended in 1 ml of plating media (Dulbecco's modified Eagle's medium/F12, 0.6% D-glucose, 1% L-glutamine, 1% FCS and 2% B27), first using a P1000 Gilson pipette, followed by a 25 gauge needle and syringe. Cell density was estimated using a haemocytometer. For in vitro experiments cells were resuspended at 2000 cells/µl and for in vivo studies cells were resuspended at 166,666 cells/µl.

Bone marrow-derived MSCs were extracted from the femora and tibiae of GFP transgenic Sprague-Dawley rats and characterized as MSCs as described previously. MSCs were then cultured in 1:1 Dulbecco's modified Eagle's medium:alpha-minimum essential media (DMEM:Alpha-MEM) mix containing 10% FCS and 1% penicillin/streptomycin at 37 °C with 5% CO2.

Fabrication of cross-linked type 1 bovine collagen hydrogels. During the preparation of collagen hydrogels, all components were maintained on ice to prevent premature gelation. For a final volume of 100 µl, 40 µl of 5 mg/ml type 1 collagen (Vornia Biomaterials), neutralised with 1 M NaOH until PH 7 reached, was added to 20 µl of 10x phosphate buffer saline (PBS) containing 4s-StarPEG. 40 µl of transplantation media (un-seeded hydrogels), cell suspension (seeded hydrogels) or human recombinant GDNF enriched cell suspension (GDNF-loaded hydrogels) was then added to the collagen/PBS/cross-linker solution and mixed thoroughly. For in vitro experiments, 50 µl samples were transferred to a previously sterilised (UV radiation) super hydrophobic
surface (Teflon®) and placed at 37 °C to gel. For in vivo experiments, the cell-seeded collagen hydrogel was maintained on ice prior to transplantation to prevent premature gelation.

**Cell viability assays.** In order to determine the effect of the collagen hydrogels on MSC viability, MSCs were seeded at a density of 20,000 cells per well of a 24 well-plate and left overnight to attach. MSCs were then either incubated with unseeded collagen hydrogels (2 × 50 µl gels per well) of various 4s-StarPEG concentrations (1, 2, or 4 mg/ml) for 48 h or left untreated. As an indicative measure of cell viability, metabolic activity of the cells was assessed using the alamarBlue® assay as previously described28. Briefly, 100 µl of a 10% solution of alamarBlue® (Invitrogen) in HBSS was added to each well and incubated for 3 h. Absorbance was read at 550 nm and 595 nm using a Varioskan Flash plate reader (Thermo Scientific) with SkanIt® software. MSC viability was assessed by normalisation of all results to controls.

To determine the effect of collagen hydrogels on VM cell viability, E14 VM cells were seeded on poly-D-lysine (Sigma) coated 24 well plates, at a density of 100,000 cells per well in 500 µl of plating media at 37 °C with 5% CO₂ for 48 h. As above, the E14 VM cells were either incubated with unseeded collagen hydrogels (2 × 50 µl gels per well) of various 4s-StarPEG concentrations (1, 2, or 4 mg/ml) for 48 h or left untreated. Once again, metabolic activity was assessed using the alamarBlue® assay as described above.

**E14 VM cell immunocytochemistry.** Dopaminergic survival and outgrowth was assessed 48 h after treatment with collagen hydrogels (as described above) using tyrosine hydroxylase (TH) and beta-III tubulin immunocytochemistry, respectively. VM cultures were fixed with 4% paraformaldehyde (PFA) for 30 min, followed by three washes in tris-buffered saline (TBS) with 0.2% triton-X-100 for permeabilization. Cultures were then incubated in blocking serum (5% bovine serum albumin in TBS with 0.2% triton-X-100) for 1 h at room temperature, before being subsequently incubated with primary antibody (Mouse anti-TH, 1:1000, Millipore; Mouse anti-beta III tubulin, 1:200, Millipore) diluted with 1% bovine serum albumin in TBS with 0.2% triton-X-100 at room temperature overnight. Following 3 × 10 min washes with TBS, cultures were incubated in rabbit anti-mouse AF 488 conjugated secondary antibodies (1:1000, Biosciences) in 1% bovine serum albumin in TBS, at room temperature for 3 h in darkness. Cultures were then counterstained with 4′,6-diamidino-2-phenylindole (DAP) (1 µg/ml in TBS, Sigma) for 5 min. Following 3 × 10 min washes in TBS, cultures were stored in 0.1% TBS azide at 4 °C until imaging. Negative controls, where no primary antibody was added were also prepared (data not shown).

**6-OHDA lesions, transplantation and rotational behaviour.** All surgeries were performed under iso-flurane anaesthesia (5% in O₂ for induction and 2% in O₂ for maintenance) in a stereotaxic frame with the nose bar set at −4.5 (intra-MFB) or −2.3 (intra-striatal). The striatum was infused unilaterally or bilaterally at coordinates Anterior-Posterior (AP) = 0.0, Medial-Lateral (ML) ±3.7 (from bregma) and Dorsal-Ventral (DV) = −5.0 below dura, while the MFB was infused unilaterally at coordinates AP = −4.0, ML = −1.3 (from bregma) and DV = −7.0 below dura. Infusions were completed at a total volume of either 3 µl (6-OHDA lesion and MSC transplants) or 6 µl (VM transplants and collagen hydrogels) at a rate of 1 µl/min with a further 2 min allowed for diffusion. Dopaminergic asymmetry in lesioned and transplanted rats was assessed via rotational behaviour using the dopaminergic stimulant methamphetamine (5 mg/ml i.p.) as previously described30,50.

**Immunohistochemistry (IHC).** Animals were sacrificed by terminal anaesthesia (50 mg/kg pentobarbital intraperitoneal (i.p.)) and transcardially perfused with 100 ml heparinised saline followed by 150 ml of 4% PFA. Brains were rapidly removed and placed in 4% PFA overnight before being cryoprotected in 25% sucrose solution. Serial coronal sections (30 µm) were cut using a freezing stage sledge microtome (Bright, Cambridgeshire, UK) and free floating IHC for TH, collagen, GDNF, microglial activation (cd11b) and astrocyte recruitment (glial fibrillary acidic protein (GFAP)) was performed as previously described28,31. In short, endogenous peroxidase activity was quenched using a solution of 3% hydrogen peroxide and 10% methanol in distilled water. Non-specific binding was blocked using 3% normal horse serum (TH, GDNF and cd11b) or normal goat serum (Collagen and GFAP) in TBS with 0.2% Triton-X-100. Primary antibody (Mouse anti-TH, 1:1000, Millipore; Rabbit anti-collagen, 1:1000, Abcam; Mouse anti-GDNF, 1:200, R & D systems; Mouse anti-cd11b, 1:400, Millipore; Rabbit anti-GFAP, 1:2000, Dako) was diluted in TBS with 0.2% Triton-X-100, added to sections and incubated at room temperature overnight. Sections were incubated in secondary antibody (Horse anti-mouse, 1:200, Vector; Goat anti-rabbit, 1:200, Jackson ImmunoResearch) for 3 h at room temperature. A streptavidin-biotin-horseradish peroxidise solution (Vector, UK) was subsequently added to sections and allowed to incubate for 2 h. The development of staining was carried out using a 0.5% solution of diaminobenzidine tetra hydrochloride (DAB, Sigma, Ireland) in TBS containing 0.3 µl/ml of hydrogen peroxide. Sections were mounted onto gelatin-coated slides, dehydrated in a series of ascending alcohols, cleared in xylene and finally coverslipped using DPX mountant for DAB stained sections (Sigma) or ‘fluoromount’ fluorescent mounting medium for GFP-expressing sections (Sigma).

**Image analysis.** In vitro image analysis consisted of counting the number of TH⁺ cells and the area of beta-III tubulin fluorescence. TH⁺ cells were quantified from five randomly selected sample sites per well, in three technical replicates per experimental condition, with three biological replicates. The level of beta-III tubulin fluorescence was quantified by measuring the threshold area of each image using ImageJ software.

Graft volume, re-innervation volume, collagen volume, GDNF volume and the volume of microgliosis/astrocytosis were assessed using ImageJ software, as described previously31. For MSC graft volume, the transplant was identified directly by GFP expression from fluorescent photomicrographs, whereas VM graft, collagen, GDNF, cd11b and GFAP expression were identified in DAB stained sections. For each of these experimental outcomes, volume was measured using cross-sectional areas measured on a one in six series of sections throughout the
rostral-caudal axis of the striatum. All sections containing either GFP expression or elevated DAB staining were used in the analysis. The total number of transplanted VM cells was determined by counting individual TH+ cell bodies in the transplanted region and correcting using Abercrombie’s equation. All sections containing TH+ cell bodies were used.

Statistical analysis. All data are expressed as mean ± standard error of the mean, and were analysed using 1-way, 2-way or 2-way repeated measures analysis of variance (ANOVA) as appropriate, with post hoc Bonferroni test when required. Throughout the results text, the main effects from the initial ANOVA are cited in the body of the results, while the results of the post-hoc analyses are shown on the corresponding figure and explained in the figure legend.

Data availability. All data generated or analysed during this study are included in this published article.

Results

In vitro/ex vivo assessment of the impact of differently cross-linked collagen hydrogels on cell viability. Prior to conducting in vivo experiments, collagen hydrogels of different 4s-StarPEG concentrations were formulated to determine their in vitro cytocompatibility. It was shown that the higher the concentration of 4s-StarPEG used, the shorter the time taken for gelation (Fig. 2A; Group, \( F_{(2,6)} = 404.30, P < 0.0001 \)).

In order to determine if the collagen hydrogels of rising cross-linker concentrations (1, 2 or 4 mg/ml 4s-StarPEG) had any detrimental effects on the viability of MSCs, they were incubated with pre-formed collagen hydrogels. None of the collagen hydrogels were found to have a negative impact on cell survival (Fig. 2B; Group, \( F_{(2,6)} = 0.14, P > 0.05 \)).

Once it was confirmed that the differently cross-linked collagen hydrogels did not impact cell survival, we then wanted to confirm that the presence of collagen hydrogels in VM cultures did not affect survival or neural outgrowth of the TH+ primary dopaminergic neurons. The presence of hydrogels did not have any negative impact on overall VM cell viability (Fig. 2C; Group, \( F_{(2,6)} = 0.5757, P > 0.05 \)), and when the survival of dopaminergic neurons within these cultures was assessed, the hydrogels also had no negative effect on the number of surviving TH+ cells (Fig. 2D; Group, \( F_{(3,6)} = 0.5143, P > 0.05 \)) and importantly, the presence of the hydrogels did not hinder the neural outgrowth from these TH+ dopaminergic neurons (Fig. 2E; Group, \( F_{(1,3)} = 0.0865, P > 0.05 \)). This indicates that increasing levels of cross-linker in hydrogels is cytocompatible with these cells, at least when they are in an ex vivo cell culture system.

In vivo assessment of the impact of hydrogel cross-linking on grafted cell viability. Having determined that the collagen hydrogels are cytocompatible in vitro, we then sought to determine the optimal level of 4s-StarPEG cross-linker for encapsulation of cells in a collagen hydrogel. To investigate this, the survival of GFP-MSCs either delivered in control transplantation media or encapsulated in collagen hydrogels cross-linked with 1, 2 or 4 mg/ml 4s-StarPEG was assessed at days 1, 4 and 7 post-transplantation. A schematic of this experimental design is shown in Fig. 3A. Since the MSCs were extracted from the bone marrow of GFP transgenic rats, the cellular grafts could be easily visualised in the striatum using fluorescent microscopy. MSC graft volume was measured at each time-point (Fig. 3B; Group, \( F_{(3,6)} = 1.183, P > 0.05 \)) indicating that the encapsulation of cells inside the collagen hydrogels, of any cross-linker concentration, did not impact graft survival. Furthermore, the collagen hydrogels successfully formed in situ, were still present 7 days post-infusion (Fig. 3D) and did not result in any striatal damage (not shown). This confirmed that the collagen hydrogels were suitable for intra-striatal cell delivery.

In vivo assessment of the impact of the collagen hydrogel on encapsulated VM cells. Having demonstrated that the collagen hydrogels are highly cytocompatible (with MSCs) and are well tolerated in vivo, further studies were carried out using a collagen hydrogel cross-linked with 4 mg/ml of 4s-StarPEG. We then sought to determine the impact of the collagen hydrogel on the striatal re-innervation from different densities of VM cells encapsulated within it (to ensure that the hydrogel did not impede striatal reinnervation). A schematic of this experimental design is shown in Fig. 4A.

The optimised collagen hydrogel successfully formed in situ after intra-striatal delivery and was still present 14 days post-transplantation (Fig. 4B). VM cells at cell densities of 200,000; 300,000 or 400,000 cells were delivered either within control transplantation media or the collagen hydrogel. TH+ cell survival and striatal re-innervation was then assessed at 14 days post-transplantation. The delivery of VM cells in the collagen hydrogel did not have any significant impact on either the survival of cells (Fig. 4C; Group, \( F_{(2,12)} = 1.23, P > 0.05 \)) or on their ability to re-innervate the striatum (Fig. 4D; Group, \( F_{(2,12)} = 1.83, P > 0.05 \)). Thus, although the hydrogel was not successful at improving the number of surviving TH+ cells, it was not detrimental to graft survival and did not impede striatal re-innervation from within the hydrogel, confirming it is possible to deliver the highest density of VM cells (400,000 cells) without any negative effects on graft survival. We then sought to establish whether encapsulation of cells in a collagen hydrogel would create a physical barrier between the transplanted VM cells and host neuro-immune cells and thereby reduce the host immune response to the transplanted graft. As expected, the cells delivered in control transplantation media elicited a substantial host immune response. The delivery of cells in the collagen hydrogel significantly decreased the volume of microgliosis around the graft site (Fig. 4F; Group, \( F_{(2,18)} = 23.03, P < 0.05 \)). Similarly, the delivery of cells in the collagen hydrogel significantly decreased the volume of astrocytosis around the graft site (Fig. 4G; Group, \( F_{(2,18)} = 34.01, P < 0.0001 \)).
In vivo assessment of GDNF retention within the collagen hydrogel. Having established that the collagen hydrogel is suitable for the intra-striatal delivery of VM cells, we then sought to determine the impact on GDNF retention in the surrounding striatum. Intra-striatal GDNF was delivered either as a bolus or in a collagen hydrogel and assessed at days 1, 2 and 4. A schematic of this experimental design is shown in Fig. 5A. Although the volume of striatal GDNF declined over time (Fig. 5B; Time, $F_{(2,12)} = 25.63, P < 0.0001$), delivery within the hydrogel significantly improved retention at the early time points (Fig. 5B,C; Group, $F_{(2,12)} = 3.95, P < 0.05$).

Pivotal in vivo study to assess the long-term survival, re-innervation and functionality of grafted VM cells encapsulated in a GDNF-loaded collagen hydrogel. Having established that the collagen hydrogel is well tolerated in vivo, supports cell survival and striatal re-innervation, reduces the host immune response and retains GDNF at a higher volume in the striatum, we then wanted to investigate the effect...
of a GDNF-loaded collagen hydrogel on dopaminergic cell survival, striatal re-innervation and functional efficacy. A schematic of this experimental design is found in Fig. 1.

Impact of the GDNF-loaded collagen hydrogel on host immune response. In line with the results from our preliminary study (Fig. 4F,G), the VM cells elicited a host immune response in the striatal tissue surrounding the graft site (Fig. 6). However, when the VM cells were delivered in a collagen hydrogel or a GDNF-loaded collagen hydrogel there was a significant decrease in striatal microgliosis (Fig. 6A; Volume: Group, $F_{(3,30)} = 9.792, P < 0.0001$; Optical density: Group, $F_{(3,30)} = 5.834, P < 0.01$) and astrocytosis (Fig. 6B; Volume: Group, $F_{(3,28)} = 6.659, P < 0.01$; Optical density: $F_{(3,32)} = 7.198, P < 0.01$). This shows that the loading of a collagen hydrogel with GDNF does not affect its ability to act as a protective matrix to the grafted cells, reducing the host immune response.

Impact of the GDNF-loaded collagen hydrogel on primary dopaminergic neuron survival and striatal re-innervation. In order to evaluate the survival of transplanted grafts, the number of surviving TH+ dopaminergic cells throughout the striatum were counted. TH IHC identified successful transplantation of dopaminergic neurons in each group (Fig. 7A,C; Group, $F_{(3,31)} = 15.91, P < 0.0001$). In line with expectations, the delivery of GDNF with VM cells showed a significant increase in the number of surviving dopaminergic neurons. However, interestingly when cells were delivered encapsulated in a GDNF-loaded collagen hydrogel, there was a significant (five-fold) increase in the number of surviving cells when compared to the delivery of VM cells alone. Additionally, cell survival in a GDNF-loaded collagen hydrogel was significantly greater than that of the VM & GDNF group (1.7 fold).

We then sought to assess the ability of these surviving cells to re-innervate the striatum. Using TH IHC, the volume of striatal tissue occupied by innervation from the transplanted dopaminergic cells was measured. All VM grafts did successfully re-innervate a portion of the lesioned striatum (Fig. 7B,C; Group, $F_{(3,36)} = 12.42$, 6F,G).

Figure 3. In vivo assessment of the impact of hydrogel cross-linking on grafted cell viability. 30,000 MSCs were delivered bilaterally to the striatum in either transplantation media or a collagen hydrogel of various 4s-StarPEG concentrations and graft volume was assessed at days 1, 4 and 7 post-transplantation (A). MSCs were extracted from the bone-marrow of GFP transgenic rats, meaning grafts could be easily identified using fluorescent microscopy. The encapsulation of MSCs in hydrogels of increasing levels of 4s-StarPEG had no negative effect on graft volume in each group, at each time-point (B,C). Collagen IHC confirmed the in situ polymerisation of hydrogels and their presence at 7 days post-transplantation (D). Scale bar represents 100 µm. Data are represented as mean ± SEM and were analysed by two-way ANOVA with post-hoc Bonferroni.
Figure 4. In vivo assessment of the impact of the collagen hydrogel on encapsulated VM cells. VM cells of various cell densities (200,000; 300,000 or 400,000 cells) were delivered unilaterally to the lesioned striatum in either transplantation media or a collagen hydrogel (cross-linked with 4 mg/ml 4s-StarPEG) and assessed for graft survival, efficacy and host-immune response 14 days post-transplantation (A). Collagen IHC confirmed the in situ formation of hydrogels and their presence at 14 days post-transplantation (B). The encapsulation of VM cells in a collagen hydrogel had no negative effect on the survival of primary dopaminergic neurons (C,E) or their ability to re-innervate the striatum (D,E). This indicates that the collagen hydrogel supports the in vivo delivery of a high density of VM cells. The host response to the transplanted graft was assessed using CD11b and GFAP IHC. The encapsulation of cells within the collagen hydrogel significantly decreased the volume of microgliosis (F) and astrocytosis (G). This indicated that the collagen hydrogel can act as a physical barrier between the transplanted cells and the host neuro-immune cells. This reduction in microgliosis and astrocytosis to the encapsulated VM cells (400,000 cells) can be seen in photomicrographs F and G, respectively. Scale bar represents 1 mm. Data is represented as mean ± SEM and were analysed by two-way ANOVA with post-hoc Bonferroni. *P < 0.05, **P < 0.01 vs. relative control.
As expected, the volume of re-innervation was significantly increased with the delivery of GDNF with VM cells. Interestingly, in line with the five-fold increase in cell survival (above), the magnitude of striatal re-innervation was significantly greater (three-fold) from the delivery of cells alone. Additionally, striatal re-innervation from cells in the GDNF-loaded collagen hydrogel was significantly greater than that of cells delivered with GDNF alone (1.5 fold), showing that the GDNF-loaded collagen hydrogel is not only capable of increasing the number of surviving dopaminergic cells, but also the volume of innervation from these cells.

**Impact of the GDNF-loaded collagen hydrogel on graft functionality.** The ability of the transplanted graft to restore function to unilaterally lesioned animals was assessed at three-weekly intervals for 12 weeks post-transplantation using methamphetamine-induced rotations. Because methamphetamine induces release of dopamine, in rats with unilateral nigrostriatal lesions, dopamine is released primarily from one side leading to rotational bias. In lesioned rats with VM cell grafts significantly reduced the number of ipsilateral rotations in each group (Fig. 8A; Group x Time, F(3,14) = 130.7, P < 0.0001). However, encapsulation of the VM cells in a GDNF-loaded collagen hydrogel provided a greater level of functional recovery at 9 and 12 weeks post-transplantation.
Figure 6. Impact of the GDNF-loaded collagen hydrogel on host immune response. The host immune response to the transplanted cells was assessed by measuring the volume and density of striatal astrogliosis and microgliosis present 12 weeks post-transplantation. IHC analysis showed that the level of both microgliosis (Ai, ii & iii) and astrogliosis (Bi, ii & iii) was significantly decreased by the encapsulation of VM cells in a collagen hydrogel. Importantly, this attenuation was not affected by the loading of the collagen hydrogel with GDNF. Scale bar represents 100 µm. Data are represented as mean ± SEM and were analysed by one-way ANOVA with post-hoc Bonferroni. *P < 0.05, **P < 0.01 vs. VM alone; ***P < 0.001 vs. VM & GDNF.

Figure 7. Impact of the GDNF-loaded collagen hydrogel on primary dopaminergic neuron survival and striatal re-innervation. Each animal received a unilateral transplantation of 400,000 VM cells to the lesioned striatum and both cell survival and striatal re-innervation were assessed at 12 weeks post-transplantation. TH+ IHC was used to identify the number of surviving dopaminergic cells throughout the striatum and their ability to re-innervate the lesioned striatum. As expected, the delivery of GDNF with cells significantly increased their survival. However, when cells were delivered in a GDNF-loaded collagen hydrogel there was a significantly greater level of cell survival (Fig. 7A,C; five-fold increase vs. VM alone). Similarly, the delivery of cells with GDNF significantly increased their striatal re-innervation. However, the delivery of cells in a GDNF-loaded collagen hydrogel resulted in a significant increase in the volume of striatal re-innervation (Fig. 7B,C; three-fold increase vs. VM alone). Scale bars represent 1 mm or 100 µm (insert). Data are represented as mean ± SEM and were analysed by one-way ANOVA with post-hoc Bonferroni. *P < 0.05, ***P < 0.001 vs. VM alone; **P < 0.05, ****P < 0.001 vs. VM & GDNF.
Correlation between VM graft survival and function. Having established that the encapsulation of VM cells in a GDNF-loaded collagen hydrogel was advantageous to their survival and outgrowth, it was important to determine whether the increased number of surviving dopaminergic neurons correlated with the observed improvement in graft function.

When we looked at the relationship between the number of surviving dopaminergic neurons and the volume of striatal re-innervation, there was a strong correlation (Fig. 8B; r = 0.81, P < 0.0001), indicating that the three-fold increase in striatal re-innervation was directly related to the improved cell survival caused by the encapsulation of cells in the GDNF-loaded collagen hydrogel.

The encapsulation of cells in a GDNF-loaded collagen hydrogel did not only result in an increased cell survival and re-innervation but also a greater level of behavioural recovery. This significant behavioural recovery correlates strongly with the number of surviving TH+ cells (Fig. 8C; r = 0.67, P < 0.0001) and also to the volume of striatal re-innervation (Fig. 8D; r = 0.67, P < 0.0001). This indicates that the significant reduction of net ipsilateral turns seen is directly related to the enhanced delivery and efficacy of cells in the GDNF-loaded collagen hydrogel.

Figure 8. Impact of the GDNF-loaded collagen hydrogel on graft functionality. Each rat received a unilateral intra-MFB 6-OHDA lesion two weeks prior to VM transplantation. Methamphetamine induced rotations (5 mg/kg) were carried out prior to transplantation and at 3 week intervals for 12 weeks post-transplantation. Transplantation of VM grafts significantly decreased the number of ipsilateral turns made in each group. However, the delivery of cells in a GDNF-loaded collagen hydrogel resulted in a significantly greater level of functional recovery at 9 and 12 weeks post-transplantation (A). Given the dramatic graft survival seen with the GDNF-loaded collagen hydrogel, the relationship between cell survival, striatal re-innervation and behavioural recovery was assessed. A strong positive correlation was found between the number of surviving TH+ neurons and the volume of striatal re-innervation (B: r = 0.81). Additionally, a strong negative correlation was found between the number of net ipsilateral turns taken and the number of surviving TH+ cells (C: r = 0.67) and striatal re-innervation (D: r = 0.71). This indicates that the greater level of behavioural recovery seen with the GDNF-loaded collagen hydrogel is a result of the increased striatal re-innervation caused by the significant increase in TH+ cell survival. PL: post-lesion. Data are represented as mean ± SEM and were analysed by two-way repeated measures ANOVA with post-hoc Bonferroni (A). *P < 0.05 VM alone vs. VM & GDNF in hydrogel; **P < 0.001, ***P < 0.001 VM in hydrogel vs. VM & GDNF in hydrogel; +P < 0.05 VM & GDNF vs. VM & GDNF in hydrogel.
Biodegradability of the collagen hydrogel in vivo. The absence of collagen immunohistochemical staining 12 weeks post intra-striatal delivery showed that the collagen hydrogel is biodegradable at the implanted site (not shown).

Expression of human-GDNF in situ. The absence of human-GDNF immunohistochemical staining 12 weeks post intra-striatal delivery showed that the transplanted GDNF was cleared from the brain prior to this 12 week time-point (not shown). This is in line with our preliminary studies (Fig. 5).

Discussion

This present study sought to determine the impact of encapsulation in a GDNF-loaded collagen hydrogel on the survival and functional efficacy of primary dopaminergic neurons after transplantation into the Parkinsonian brain. We found that the collagen hydrogel was well tolerated in the brain, acutely retained GDNF at the injection site, and reduced the host response to the grafted cells, while also facilitating primary dopaminergic neuron survival and striatal re-innervation. Moreover, the GDNF-loaded hydrogel resulted in a 5-fold increase in primary dopaminergic neuron survival and a 3-fold increase in striatal re-innervation, which correlated with a significantly greater level of functional recovery. Given that the clinical translation of foetal-derived dopaminergic neuron regenerative therapies in Parkinson’s disease is limited by the extremely poor survival of cells post-transplantation and the consequent requirement of multiple foetal donors per transplant, if a single infusion of an injectable, non-toxic and GDNF-rich hydrogel can dramatically increase cell survival and efficacy, it can potentially reduce the number of foetal donors needed per striatal transplant. Overall this highlights the potential of growth factor enriched biomaterial matrices to enhance cell replacement therapies in neurodegenerative diseases such as Parkinson’s disease.

Cell-based therapies have emerged from a relatively simple conceptual framework as a viable therapeutic option for Parkinson’s disease: since the disease is associated with degeneration of the nigrostriatal dopaminergic neurons, then replacement of these neurons through transplantation should alleviate the disease’s motor symptoms. Over the past 30 years, a number of clinical trials have provided ‘proof-of-principle’ for this supposition, and have shown that dopaminergic neurons taken from the developing ventral mesencephalon of human foetuses can survive, integrate and function after transplantation into the adult Parkinsonian brain. In spite of this, the number of patients that have benefited is small, in part due to our current dependence on VM tissue dissected from human foetuses donated after elective abortions and poor dopaminergic neuron survival after intra-cerebral transplantation. It is now well established that the majority of transplanted dopaminergic neurons die as a result of the transplantation process through matrix detachment, growth factor deprivation, and immune rejection, all of which could be addressed using biomaterial technology.

In the present study, we used an in situ forming, GDNF-enriched, type I collagen hydrogel as a biomaterial matrix for encapsulation and transplantation of dissociated VM tissue into the striatum of Parkinsonian rats, which resulted in a dramatic increase in dopaminergic neuron survival and striatal reinnervation, with a corresponding improvement in motor function. Several features of the injectable hydrogel may be responsible for the enhanced delivery of primary dopaminergic neurons. Numerous studies suggest that the critical time-point in which 80–90% of dopaminergic neurons die, is the first 4 days post-transplantation and that it is not until after this point that dopaminergic neuron survival is stabilised. Initially some apoptosis is caused by the detachment of cells from the extracellular matrix (anoikis) during tissue preparation. While many biomaterial scaffolds require chemical manipulation to improve cell adherence, collagen mimics the extracellular membrane and contains the natural Arg-Gly-Asp (RGD) tripeptide sequence that facilitates cell adhesion and thus provides an advantageous environment for cell delivery. Secondly, given that the elevated host immune response to the exogenous graft is one of the leading triggers of apoptosis post-transplantation, it is of utmost importance that any delivery platform itself does not evoke an elevated host response. Collagen hydrogels, cross-linked with 4s-StarPEG have shown to be immune-neutral upon transplantation and throughout their degradation. Moreover, the delivery of cells in a GDNF-loaded hydrogel significantly decreased the host response to the transplanted graft through the formation of a physical barrier between the transplanted cells and the host neuro-immune cells. Although significant increases in cell survival were not observed with cell encapsulation alone, it is important to note that apoptosis post-transplantation is triggered by more than one factor and not solely the host immune response. While the host immune response can trigger apoptotic cell death in the established graft, the transplantation of cells into a trophic factor deprived adult striatum is associated with the vast cell death seen immediately post-transplantation. Despite not finding any GDNF staining 12 weeks post-transplantation due to its relatively short half-life (3–4 days), preliminary in vivo studies showed that the encapsulation of GDNF in the collagen hydrogel significantly retained GDNF in the striatum immediately post-transplantation. Assuming that this was also the case when cells were encapsulated in the GDNF-loaded hydrogel, the enhanced, site-specific retention of GDNF in the striatum provided primary dopaminergic neurons with critical trophic support upon transplantation and throughout target innervation. While tackling each of these triggers alone may not be sufficient to improve graft function, the summation of enhanced cell delivery, increased trophic factor support and the attenuation of the immune response results in a significant improvement in dopaminergic cell survival, efficacy and importantly, motor function.

In summary, the present study shows, for the first time, that a growth factor-infused collagen hydrogel dramatically enhances the survival, re-innervation and functionality of primary dopaminergic neurons by reducing the host immune response to the transplanted graft and simultaneously increasing the acute retention of GDNF in the striatum. This study is therefore ‘proof-of-principle’ of the significant potential of biomaterials as a means of improving regenerative cell therapies in Parkinson’s disease, as well as other neurodegenerative diseases and therefore, warrants further investigation. Moreover, this data is in line with two recent studies of biomaterial approaches to dopaminergic neuron survival after transplantation in the brain. In the study by Wang et al.,
2016, a GDNF-functionalised composite poly(l-lactic acid)/xyloglucan hydrogel was shown to enhance survival of, and striatal reinnervation from, transplanted mouse VM grafts in Parkinsonian mice, while in Adil et al., 2017, a heparin/RGD functionalised hyaluronic acid hydrogel was shown to improve the survival of transplanted human embryonic stem cell-derived dopaminergic neurons. Taken together with the current study, this literature highlights the potential of biomaterial hydrogel scaffolds to improve the outcome of reparative cell therapies for Parkinson's disease. Moving towards a clinical therapy, the use of bovine collagen is already approved for a variety of applications including drug delivery, wound healing, burn repair, dentistry and bone reconstruction and could therefore, be relatively easily adopted for neural applications. However, given the ethical and logistical limitations of using fetal-derived tissue for brain repair, in future studies, it will be important to determine if collagen scaffolds can also improve the outcome of stem cell-derived dopaminergic neuron transplants in the Parkinsonian brain.

References


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Author Contributions
We confirm that this manuscript has been read and approved by all named authors and that all named authors have fulfilled the required criteria for authorship. N.M. contributed to conception, execution, analysis and interpretation of data, E.D. contributed to conception, design, analysis and interpretation of data, and A.P. contributed to analysis and interpretation of data.

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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

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Abstract
Biomaterials have been shown to significantly improve the outcome of cellular reparative approaches for Parkinson’s disease in experimental studies because of their ability to provide transplanted cells with a supportive microenvironment and shielding from the host immune system. However, given that the margin for improvement in such reparative therapies is considerable, further studies are required to fully investigate and harness the potential of biomaterials in this context. Given that several recent studies have demonstrated improved brain repair in Parkinsonian models when using dopaminergic grafts derived from younger foetal donors, we hypothesized that encapsulating these cells in a supportive biomaterial would further improve their reparative efficacy. Thus, this study aimed to determine the impact of a GDNF-loaded collagen hydrogel on the survival, reinnervation, and functional efficacy of dopaminergic neurons derived from young donors. To do so, hemi-Parkinsonian (6-hydroxydopamine-lesioned) rats received intrastriatal transplants of embryonic day 12 cells extracted from the rat ventral mesencephalon either alone, in a collagen hydrogel, with GDNF, or in a GDNF-loaded collagen hydrogel. Methamphetamine-induced rotational behaviour was assessed at three weekly intervals for a total of 12 weeks, after which rats were sacrificed for postmortem assessment of graft survival. We found that, following intrastriatal transplantation to the lesioned striatum, the GDNF-loaded collagen hydrogel significantly increased the survival (4-fold), reinnervation (5.4-fold), and functional efficacy of the embryonic day 12 dopaminergic neurons. In conclusion, this study further demonstrates the significant potential of biomaterial hydrogel scaffolds for cellular brain repair approaches in neurodegenerative diseases such as Parkinson’s disease.

KEYWORDS
biomaterials, cell replacement therapy, neurotrophic support, Parkinson’s disease

Abbreviations: 4s-StarPEG, poly(ethylene glycol) ether tetrascuvinimidyl glutarate; E12, Embryonic day 12; E14, Embryonic day 14; GDNF, Glial-derived neurotrophic factor; MFB, Medial forebrain bundle; TH, Tyrosine hydroxylase; VM, Ventral mesencephalon.

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1 | INTRODUCTION

For several decades, dopaminergic neurons derived from the ventral mesencephalon (VM) of human foetal donors have been shown to survive, integrate, and function when transplanted ectopically into the Parkinsonian brain (Boronat-Garcia, Guerra-Crespo, & Drucker-Colin, 2017). While open label clinical trials have provided “proof-of-principle” evidence of the ability of these cells to alleviate motor symptoms in patients (Brundin et al., 2000; Freed et al., 1992; Lindvall et al., 1990, 1994), poor survival and inadequate striatal reinnervation have halted their clinical progression (Freed, Zhou, & Breeze, 2011; Winkler, Kirik, & Bjorklund, 2005). In recent years, experimental studies have begun to demonstrate the remarkable potential of biomaterial scaffolds to improve the survival and functional efficacy of dopaminergic grafts (Adil et al., 2017; Moriarty, Pandit, & Dowd, 2017; Wang et al., 2016). In our own work, we recently demonstrated that injectable collagen hydrogels enriched with the dopaminergic neurotrophin, glial-derived neurotrophic factor (GDNF), dramatically improved the survival, striatal reinnervation, and motor restorative capacity of foetal-derived dopaminergic grafts in Parkinsonian rats (Moriarty et al., 2017). This was due to the hydrogel’s ability to provide the delicate nascent neurons with (a) a structural matrix during the transplantation process, (b) a supportive GDNF-rich microenvironment after transplantation into the neurotrophin-depleted adult brain, and (c) protection/shielding from the aversive host immune system. Despite this however, the margin for improvement in such reparative therapies remains considerable, and as such, further studies are required to investigate the potential of biomaterials in this context.

Several recent studies have also demonstrated improved brain repair in Parkinsonian models when using dopaminergic grafts derived from younger foetal donors (Bye, Thompson, & Parish, 2012; Gates, Torres, White, Fricker-Gates, & Dunnett, 2006; Somaa, Bye, Thompson, & Parish, 2015; Torres, Dowd, & Dunnett, 2008; Torres, Monville, Gates, Bagga, & Dunnett, 2007). For the successful generation of dopaminergic neuron-rich grafts after transplantation, the VM must be collected during a specific time frame of neurogenesis to allow for the level of maturity required for commitment to a dopaminergic neuron phenotype, while also avoiding overdifferentiation prior to transplantation (Brundin et al., 1985; Torres et al., 2007). In experimental studies, the conventional donor age has become embryonic age (E) 14, as it was perceived to coincide with peak dopamine neurogenesis in the developing brain (Dunnett, 1991; Gates et al., 2006; Hegarty, Sullivan, & O’Keefe, 2013). However, in recent years the influence of donor age on graft efficacy has received heightened interest. Numerous studies have shown that the transplantation of E12 VM cell suspensions results in a higher fraction of surviving dopaminergic neurons and striatal reinnervation (Bye et al., 2012; Somaa et al., 2015; Torres et al., 2007), a higher proportion of A9 dopaminergic neurons (subtype involved in the restoration of motor function) (Grealish et al., 2010), and functional recovery (Torres et al., 2008). It was hypothesized that such improvements were related to the increased number of surviving dopaminergic neurons, alongside the posttransplantation differentiation of dopaminergic neuroblasts present in E12 VM cell preparations (Torres et al., 2007). Furthermore, a recent study has shown that the inclusion of meningeal cells from young foetal donors with VM preparations enhanced both the survival and axonal outgrowth of transplanted dopaminergic neurons (Somaa et al., 2015). This suggests that the enhanced survival with VM cell suspensions of younger donor age may also result from trophic support from the attached meningeal layer and adjacent neural floor plate, components that are typically removed in older preparations. Taken together, these studies highlight the potential benefits of using VM tissue from younger foetal donors in future studies, both experimentally and clinically.

Thus, given the potential of biomaterials to improve the outcome of cellular reparative therapies for Parkinson’s disease, and the benefits of using younger donor age cells, the aim of this study was to determine whether a GDNF-loaded collagen hydrogel could provide additional support to dopaminergic neurons derived from younger donor tissue and therefore further enhance graft survival and efficacy.

2 | METHODS

2.1 | Experimental design

This study was conducted to assess the long-term effect of a GDNF-loaded collagen hydrogel on the survival and efficacy of younger donor age primary dopaminergic neurons, namely those derived from E12 VM cells. To do this, 21 adult male Sprague-Dawley rats received a unilateral intramedial forebrain (MFB) 6-hydroxydopamine lesion. Two weeks later, rats underwent postlesion methamphetamine-induced rotations and, based on these results, were performance matched into four groups (n = 5–6 per group) to receive unilateral intrastratial transplants of E12 VM cells alone (200,000 per 6 μl), E12 VM cells encapsulated in a collagen hydrogel (cross-linked with 4 mg/ml 4s-StarPEG), E12 VM cells with GDNF (1,000 ng), or E12 VM cells encapsulated in a GDNF-loaded collagen hydrogel. Methamphetamine-induced rotations resumed 3 weeks posttransplantation and were carried out at 3-week intervals for a total of 12 weeks. The animals were then sacrificed for postmortem assessment. A schematic representation of this experimental design is shown in Figure 1.
2.2 | Animals

All procedures involving the use of animals were approved by the Animal Care and Research Ethics Committee at the National University of Ireland, Galway, were completed under licence by the Irish Department of Health and Children and the Irish Health Products Regulatory Authority, and were carried out in compliance with the European Union Directive 2010/63/EU and S.I No. 543 of 2012. 21 Male Sprague-Dawley rats (weighing 200–225 g on arrival) and three time-mated female Sprague-Dawley rats were sourced from Charles River, UK. Animals were housed in groups of four per cage, on a 12:12 hr light/dark cycle, at 19–23°C, with relative humidity levels maintained between 40% and 70%. For the duration of the experiment, animals were allowed food and water ad libitum. All behavioural testing and ex vivo analyses were carried out by an experimenter who was blind to the treatment of the animals.

2.3 | Preparation of E12 VM cell suspension

Time-mated pregnant rats were anaesthetized using isoflurane (5% in 0.5 L O₂) and quickly decapitated. The uterine horn containing embryonic day 12 (E12) embryos was removed via laparotomy and the VM was microdissected from each embryo as previously described for E14 preparations (Moriarty et al., 2017) with the exception that the meningeal layers were not removed. Dissected VM tissue was centrifuged at 200 g for 5 min at 4°C. The tissue pellet was incubated in 40% trypsin-Hank’s balanced salt solution (HBSS) for 4 min, at 37°C with 5% CO₂. Foetal calf serum (FCS) was then added to the tissue and centrifuged at 1,100 rpm for 5 min at 4°C. The cell pellet was then resuspended in 1 ml of plating media (Dulbecco’s modified Eagle’s medium/F12, 0.6% D-glucose, 1% L-glutamine, 1% FCS, and 2% B27), first using a P1000 Gilson pipette, followed by a 25 gauge needle and syringe. Extra caution was taken to ensure that no air bubbles were introduced to the cell suspension.
2.4 Fabrication of cross-linked type 1 collagen hydrogels

During the preparation and transplantation of collagen hydrogels, all components were maintained on ice to prevent premature gelation. For a final volume of 100 μl, 40 μl of 5 mg/ml type 1 bovine collagen (Vornia Biomaterials), neutralized with 1 M NaOH until PH 7 reached, was added to 20 μl of 10× phosphate buffer saline (PBS) containing 0.4 mg of poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4s-StarPEG). An aliquot of 40 μl of cell suspension (seeded hydrogels) or human recombinant GDNF-enriched cell suspension (GDNF-loaded hydrogels) was then added to the collagen/PBS/cross-linker solution and mixed thoroughly.

2.5 6-OHDA lesions, transplantation, and rotational behaviour

All surgeries were performed under isoflurane anaesthesia (5% in O2 for induction and 2% in O2 for maintenance) in a stereotaxic frame with the nose bar set at −4.5 (intra-MFB) or −2.3 (intrastriatal). To induce Parkinsonism, rats received a unilateral intramedial forebrain bundle (MFB) lesion using 6-hydroxydopamine (12 μg in 3 μl 0.1% ascorbate saline) at stereotaxic coordinates AP −4.0, ML −1.3 (from bregma) and DV −7.0 below dura. For transplant surgery, rats received unilateral intrastriatal transplants of E12 VM cells alone (200,000 cells), with GDNF (1,000 ng), encapsulated in a collagen hydrogel (2 mg/ml cross-linked with 4 mg/ml 4s-StarPEG) or encapsulated in a GDNF-loaded collagen hydrogel at stereotaxic coordinates AP 0.0, ML −3.7 (from bregma) and DV −5.0 below dura in a total volume of 6 μl. Animals were administered Buprenorphine (0.05 mg/kg in 0.9% saline) 30 min prior to surgery and then every 8–12 hr for 24–48 hr postoperative. Animals were regularly monitored until full health was recovered. Methamphetamine (2.5 mg/kg in 0.9% saline i.p.) was used to induce rotational behaviour 2 weeks after lesion surgery and at three weekly intervals after transplant surgery for a total of 12 weeks.

2.6 Immunohistochemistry

Animals were sacrificed by terminal anaesthesia (50 mg/kg pentobarbital i.p.) and transcardially perfused with 100 ml heparinized saline followed by 150 ml of paraformaldehyde (4% in PBS). Brains were rapidly removed and placed in 4% paraformaldehyde overnight before being cryoprotected in 25% sucrose solution. Serial coronal sections (30 μm) were cut using a freezing stage sledge microtome and free floating immunohistochemistry for tyrosine hydroxylase (TH), collagen, and GDNF was performed as previously described (Hoban et al., 2013; Moriarty et al., 2017). In short, endogenous peroxidise activity was quenched using a solution of 3% hydrogen peroxidase and 10% methanol in distilled water. Nonspecific binding was blocked using 3% normal horse serum (TH and GDNF) or normal goat serum (Collagen) in tris-buffered saline (TBS) with 0.2% Triton-X-100. Primary antibody (Mouse anti-TH, 1:1,000, Millipore; Rabbit anti-collagen, 1:1,000, Abcam; Mouse anti-GDNF, 1:200, R & D systems) was then added to TBS with 0.2% triton-X-100, added to sections, and incubated at room temperature overnight. Secondary antibody (Horse anti-mouse, 1:200, Vector; Goat anti-rabbit, 1:200, Jackson ImmunoResearch) was then added to TBS, added to sections, and incubated overnight. A streptavidin-biotin-horseradish peroxidise solution (Vector, UK) was subsequently added to sections and allowed to incubate for 2 hr. The development of staining was carried out using a 0.5% solution of diaminobenzidine tetra hydrochloride (DAB, Sigma, Ireland) in TNS containing 0.3 μl/ml of hydrogen peroxide. Sections were mounted onto gelatin-coated slides, dehydrated in a series of ascending alcohols, cleared in xylene, and finally coverslipped using DPX mountant (Sigma).

2.7 Image analysis

Graft volume, reinnervation volume, collagen volume, and GDNF volume were assessed using ImageJ software as previously described (Moriarty et al., 2017). For each of these experimental outcomes, volume was measured using cross-sectional areas measured on a one in six series of sections throughout the rostrocaudal axis of the striatum. All sections containing elevated DAB staining were used in the analysis. The total number of transplanted VM cells was determined by counting individual TH+ cell bodies in the transplanted region and correcting using Abercrombie’s equation. All sections containing TH+ cell bodies were used. As expected, there was no collagen or GDNF immunostaining remaining at the protracted time-point for sacrifice used in this study indicating their clearance from the brain. Therefore, these data are not shown in the results section.

2.8 Statistical analysis

All data are expressed as mean ± standard error of the mean, and were analysed using 1- or 2-way (with repeated measures) analysis of variance (ANOVA) as appropriate, with post hoc Bonferroni test when required. Throughout the results text, the main effects from the initial ANOVA are cited in the body of the results, while the results of the post hoc analyses are shown on the corresponding figure and explained in the figure legend.
3 | RESULTS

3.1 Impact of the GDNF-loaded collagen hydrogel on E12 primary dopaminergic neuron survival, reinnervation, and functional efficacy

In order to assess the survival of transplanted E12 VM cells, the number of surviving TH⁺ dopaminergic cells throughout the striatum was counted. TH immunostaining identified the successful transplantation and outgrowth of dopaminergic neurons in each group (Figure 2a and b; Group, \( F_{(3,17)} = 21.78, p < 0.0001 \)). Although there was a strong trend towards an improvement in survival when the cells were implanted in the collagen hydrogel, neither implantation of the cells in the collagen hydrogel alone nor with GDNF alone significantly improved their survival. In contrast, when the cells were implanted in the GDNF-loaded collagen hydrogel, there was a dramatic and significant improvement in cell survival (4-fold vs. VM alone).

We then sought to assess the ability of these surviving cells to form neural outgrowths in situ and reinnervate the striatum. Again, TH immunostaining demonstrated that all E12 VM grafts did successfully reinnervate a portion of the lesioned striatum (Figure 2a and c; Group; \( F_{(3,17)} = 8.81, p < 0.0001 \)). Again, although there was a strong trend for an improvement in striatal reinnervation from cells implanted in the collagen hydrogel, ultimately neither implantation of the cells in the collagen hydrogel alone nor with GDNF alone significantly improved their reinnervation. In contrast, delivery of cells in a GDNF-loaded collagen hydrogel resulted in a significantly greater magnitude of striatal reinnervation (5.4-fold vs. VM alone).

Methamphetamine-induced rotational behaviour was carried out at three weekly intervals for a total of 12 weeks to assess the ability of the transplanted E12 VM grafts to restore motor function to unilaterally lesioned animals. As expected, the intrastriatal delivery of E12 VM cell grafts significantly reduced the
number of ipsilateral rotations in each group (Figure 3; Group x Time, $F_{(3,17)} = 106.30$, $p < 0.0001$). Here, consequent to the trend for improved survival and striatal reinnervation, delivery of E12 cells in the collagen hydrogel resulted in a significantly greater level of functional recovery at 6, 9, and 12 weeks post-transplantation. However, delivery in the GDNF-loaded collagen hydrogel resulted in an even greater level of functional recovery at 12 weeks posttransplantation.

### 3.2 Correlation between E12 VM graft survival and function

Having established that encapsulation of E12 cells in the GDNF-loaded collagen hydrogel significantly increased the survival and reinnervation of dopaminergic neurons, we next assessed whether the enhanced delivery of E12 dopaminergic neurons correlated with their significantly greater functional recovery. When we looked at the relationship between the number of surviving TH$^+$ dopaminergic neurons and the volume of striatal reinnervation, we found a strong positive correlation (Figure 4a; $r = 0.75$, $p < 0.0001$), indicating that the enhanced reinnervation seen was likely related to the enhanced delivery and survival of cells in a GDNF-loaded collagen hydrogel. When we looked at the relationship between cell survival or reinnervation and behavioural recovery, we found that the significant behavioural recovery correlated strongly with the number of surviving TH$^+$ dopaminergic neurons (Figure 4b; $r = 0.79$, $p < 0.0001$) and also with the volume of striatal reinnervation.

**Figure 3** Impact of the glial-derived neurotrophic factor (GDNF)-loaded collagen hydrogel on E12 graft functionality. Transplantation of E12 VM grafts significantly decreased the number of ipsilateral turns made in each group. However, the delivery of E12 cells in collagen hydrogels resulted in a significantly greater level of functional recovery at 6, 9, and 12 weeks posttransplantation. Moreover, the delivery of E12 cells in a GDNF-loaded collagen hydrogel resulted in an even greater level of functional recovery at 12 weeks posttransplant. PL: postlesion; PT: posttransplant. Data are represented as mean ± SEM and were analysed by two-way repeated measures ANOVA with post hoc Bonferroni. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. VM alone; $p < 0.05$, $p < 0.01$, $p < 0.001$ vs. VM & GDNF; $p < 0.05$ vs. VM in hydrogel.

**Figure 4** Correlation between E12 graft survival and functionality. A strong positive correlation was found between the number of surviving TH$^+$ dopaminergic neurons and the magnitude of striatal reinnervation ($a: r = 0.75$). Moreover, a strong negative correlation was found between the number of net ipsilateral turns taken and the number of TH$^+$ dopaminergic cells ($b: r = 0.79$) and striatal reinnervation ($c: r = 0.87$). This indicates that the greater level of behavioural recovery seen with the GDNF-loaded collagen hydrogel is likely a result of the increased striatal reinnervation caused by the significant increase in TH$^+$ cell survival. GDNF: glial-derived neurotrophic factor.
of striatal reinnervation (Figure 4c; $r = 0.87$, $p < 0.0001$). This indicates that the significant reduction in ipsilateral turns after transplantation was likely related to the enhanced survival and reinnervation capacity of E12 cells delivered in a GDNF-loaded collagen hydrogel.

4 | DISCUSSION

Recent experimental studies have demonstrated the significant potential of biomaterials to improve the outcome of cellular brain repair for Parkinson’s disease (Adil et al., 2017; Moriarty et al., 2017; Wang et al., 2016) through their ability to provide cells with a structural matrix during transplantation, a supportive microenvironment after transplantation, and protection from the host immune response. To further improve such reparative therapies, we wanted to harness the inherently enhanced reparative capacity of younger donor age tissue (Somaa et al., 2015; Torres et al., 2007, 2008) by transplanting these cells in a GDNF-loaded collagen hydrogel. Overall, the work described in this study showed that the encapsulation of E12 VM cells in a GDNF-loaded collagen hydrogel resulted in a 4-fold increase in primary dopaminergic cell survival, a 5.4-fold increase in striatal reinnervation and a greater level of functional recovery. Moreover, while not significant, when cells were encapsulated in a GDNF-free collagen hydrogel, there was a positive trend towards an increase in the number of surviving cells and the volume of striatal reinnervation, which translated to a significant improvement in functional recovery. Overall, these findings further highlight the potential of biomaterial scaffolds to enhance cell replacement therapies in neurodegenerative diseases such as Parkinson’s disease.

Foetal donor age is an important factor that must be considered during VM cell transplantation. For the successful collection of transplantable primary dopaminergic neurons, the VM must be collected during a specific time frame of neurogenesis to allow for the level of maturity required for commitment to a dopaminergic neuron phenotype, while also avoiding overdifferentiation prior to transplantation. Early studies using rat VM identified the upper limits of donor age to be E17–18 when using solid VM tissue pieces (Simonds & Freed, 1990) and E15–16 when using single VM cell suspensions (Brundin et al., 1985). From here, E14 became the conventional donor age as it was perceived to coincide with peak dopamine neurogenesis. However, in an effort to enhance VM cell transplantation techniques, recent studies have reported that the use of younger donor tissue yields significantly larger grafts (Torres et al., 2007, 2008). While, the use of younger donor age VM tissue results in enhanced yields of surviving dopaminergic neurons, these cells are still subject to unfavourable conditions upon transplantation, such as, (a) matrix detachment during tissue dissection and dissociation (anoikis) (Reddig & Juliano, 2005), (b) growth factor (and/or possibly differentiation factor) deprivation upon transplantation into the adult brain (Collier & Sortwell, 1999), and (c) the host immune response (Barker, Dunnett, Faissner, & Fawcett, 1996). We have previously shown that a GDNF-loaded collagen hydrogel can significantly improve E14-derived dopaminergic cell survival and efficacy through the summation of enhanced cell delivery, increased trophic factor support, and the attenuation of the host immune response (Moriarty et al., 2017). Thus, if a GDNF-loaded collagen hydrogel could provide younger donor age cells (E12) with similar support, the yield of surviving dopaminergic neurons and thus the dependency on multiple foetal donors could be dramatically improved.

In this study, in contrast to our previous findings (Moriarty et al., 2017), the delivery of GDNF alone with VM cells had no significant effect on the number of surviving neurons. GDNF is known to be a potent neurotrophic factor for dopaminergic neurons (Lin, Doherty, Lile, Bektesh, & Collins, 1993); however, its inability to increase E12 VM cell survival could in part be due to their earlier developmental stage. In a study by Torres and colleagues, the authors demonstrated that while overexpression of GDNF in the transplanted striatum significantly increased the survival of E14-derived dopaminergic neurons, it had little effect on E12-derived dopaminergic neurons. In contrast, the overexpression of sonic hedgehog, a known dopaminergic differentiation factor (Wang et al., 1995), significantly increased the number of E12-derived dopaminergic neurons but had no effect on E14-derived neurons (Torres, Monville, Lowenstein, Castro, & Dunnett, 2005). The majority of dopaminergic neurons derived from E14 VM tissue are postmitotic at the time of transplantation (Sinclair, Fawcett, & Dunnett, 1999); however, E12 VM tissue contains a large fraction of actively dividing cells which can differentiate into dopaminergic neurons post-transplantation (Bye et al., 2012; Gates et al., 2006; Jonsson, Ono, Bjorklund, & Thompson, 2009). Thus, together with the present data, this suggests that cells derived from the E12 VM are too young to respond to GDNF treatment.

Interestingly, the delivery of cells in a GDNF-free collagen hydrogel showed a positive trend towards a higher number of surviving dopaminergic neurons and a higher level of striatal reinnervation which translated to improved functional recovery. These findings are in contrast to the delivery of E14 cells in a collagen hydrogel where no effect on cell survival was seen (Moriarty et al., 2017). In that study, we hypothesised that the collagen hydrogel had no effect on E14 cell survival because these cells, although protected from the host response, were denied the necessary neurotrophic support upon transplantation (Sortwell, Camargo, Pitzer, Gyawali, & Collier, 2001). However, unlike the E14 VM, the E12 VM contains a tightly adhered meningeal layer that is easily removed in older donor age preparations. These cells...
are known to secrete proteins involved in neurogenesis, cell migration, cell differentiation, and axonal growth, including retinoic acid, bone morphogenic protein-7, fibroblast growth factor-2, and stromal derived factor-1 (Choe, Siegenthaler, & Pleasure, 2012; Reiss, Mentlein, Sievers, & Hartmann, 2002; Siegenthaler et al., 2009; Zhang, Smith, Yamamoto, Ma, & McCaffery, 2003; Zhu et al., 2002), as well as proteins involved in ECM formation, including collagen and laminin (Montagnani, Castaldo, Di Meglio, Sciorio, & Giordano-Lanza, 2000). Numerous studies have highlighted the positive effects of meningeal cells (and their secreted factors) in the central nervous system (CNS) (Decimo, Fumagalli, Berton, Krampera, & Bifari, 2012; Siegenthaler & Pleasure, 2011), and particularly on the development of dopaminergic neurons (Cohen & Sladek, 1997; Hayashi et al., 2008; Hynes, Poulsen, Tessier-Lavigne, & Rosenthal, 1995; Schwartz et al., 2012; Yu, Gu, Huang, & Wen, 2007). Furthermore, an important study by Somaa and colleagues recently showed that the inclusion of young (E10 mouse), but not older (E12 mouse), meningeal cells in VM preparations increased the differentiation, survival, and outgrowth of dopaminergic neurons (Somaa et al., 2015). Thus, given that the collagen hydrogel acts as a reservoir capable of acutely retaining secreted factors in the transplant microenvironment (Moriarty et al., 2017), it is probable that it provided the nascent dopaminergic neurons with enhanced meningeal-derived developmental support, resulting in the positive outcome observed when cells were encapsulated in a collagen hydrogel alone.

The most striking and important finding of the present study was that encapsulation of E12 VM cells in a GDNF-loaded hydrogel resulted in a significant increase in the survival of dopaminergic neurons, their capacity to reinnervate the striatum, as well as their ability to restore motor function in hemi-Parkinsonian rats. Given that neither GDNF nor the collagen hydrogel significantly improved dopaminergic cell survival nor striatal reinnervation in their own right, it is intriguing that such pronounced effects were observed when the cells were implanted in the GDNF-loaded hydrogel. We suggest that this resulted from sequential reservoir-related benefits afforded by the collagen hydrogel. Specifically, given the capacity of the hydrogel to retain trophic factors within the graft microenvironment (Moriarty et al., 2017), it is probable that any meningeal-derived trophic or developmental factors were retained at the graft site, thus allowing for enhanced survival and posttransplantation differentiation of the dopaminergic neuroblasts (Torres et al., 2007). This would produce a higher yield of more developmentally mature, and GDNF-responsive, dopaminergic neurons, which could then benefit from exposure to the GDNF retained in the GDNF-loaded collagen hydrogel. Thus, while GDNF may not be beneficial to E12 dopamine precursors, it could be advantageous to cells which have undergone posttransplantation differentiation, a process that is likely increased by the encapsulation of meningeal cells and retention of their secreted factors.

In summary, this study demonstrates that the encapsulation of VM cells derived from younger foetal donors in a GDNF-loaded collagen hydrogel results in a dramatic increase in dopaminergic neuron survival, reinnervation, and function. When taken together with previous literature (Adil et al., 2017; Moriarty et al., 2017; Wang et al., 2016), these studies provide “proof-of-principle” of the potential of hydrogel scaffolds to improve the outcome of reparative cell therapies. However, given the ethical and logistical limitations of using foetal-derived tissue, the field of cell replacement therapy in Parkinson’s disease is moving towards the use of alternatively sourced dopaminergic neurons, such as those derived from embryonic stem cells and induced pluripotent stem cells. It will therefore be imperative to determine whether in situ gelling collagen hydrogels can also improve the outcome of stem cell-derived dopaminergic neuron transplants in the Parkinsonian brain.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

DATA ACCESSIBILITY

All data generated or analysed during this study are included in this published article.

AUTHOR CONTRIBUTIONS

We confirm that this manuscript has been read and approved by all named authors and that all named authors have fulfilled the required criteria for authorship. N.M contributed to the conception, execution, analysis, and interpretation; S.C. and V.M contributed to the execution; A.P contributed to the analysis and interpretation; E.D contributed to conception, design, analysis, and interpretation.

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REFERENCES


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 intrastrial transplantation. *Scientific Reports, 7*(1), 16033. https://doi.org/10.1038/s41598-017-15970-w


Reiss, K., Mentlein, R., Sievers, J., & Hartmann, D. (2002). Stromal cell-derived factor 1 is secreted by meningeal cells and acts as chemotactic factor on neuronal stem cells of the cerebellar


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INTRODUCTION

Cell replacement therapy for neurodegenerative diseases has emerged from a relatively simple conceptual framework—if the primary pathological feature of a disorder is the degeneration and subsequent loss of a specific neuronal subtype, then the replacement of these cells should repair the brain and restore function to the patient. The relatively selective loss of dopaminergic neurons from the substantia nigra makes Parkinson’s disease an ideal candidate for cell replacement therapy (Fearnley & Lees, 1991). To date, cellular brain repair for Parkinson’s disease has been developed using tissue dissected from the embryonic ventral mesencephalon (VM) which incorporates the developing dopaminergic neurons from the substantia nigra and ventral
tegmental area. As this is the current “gold standard” source of dopaminergic neurons for brain repair in Parkinson’s disease, this will be the focus of this review. However, as it will be essential in the future to move towards a more standardised cell source (such as neurons derived from pluripotent stem cells), the reader is also directed to the article by Malin Parmar et al. in this Special Issue for a review of developments in stem cell-derived dopaminergic neurons for brain repair in Parkinson’s disease.

2 | CELLULAR BRAIN REPAIR FOR PARKINSON’S DISEASE – PRECLINICAL STUDIES

The transplantation of primary dopaminergic neurons began in the 1970s when Björklund et al. transplanted small pieces of tissue (containing dopaminergic, serotonergic and noradrenergic neurons) dissected from the foetal rat brain into the cerebral cortex and hippocampus of unlesioned adult rats. These cells showed good survival after transplantation with substantial axonal outgrowth that formed extensive fibre patterns within the graft itself and with adjacent brain tissue (Björklund, Stenevi, & Svendgaard, 1976; Stenevi, Björklund, & Svendgaard, 1976). Then, in unilateral rodent models of Parkinson’s disease, Perlow et al. (1979) transplanted solid tissue pieces into the lateral ventricle in contact with the caudate-putamen, while Björklund, Schmidt, and Stenevi (1980) transplanted tissue pieces to the dorsal cortical cavity overlying the caudate-putamen. Both studies noted improvement in amphetamine-induced rotational behaviour, and postmortem analysis revealed survival and reinnervation of the lesioned striatum. A setback to these original studies was the use of pieces of dissected brain tissue, necessitating the need to create a highly vascularised cavity within the brain, while also preventing the targeting of deep brain structures (Dunnett, Björklund, Stenevi, & Iversen, 1981). This led to the generation and use of dissociated cell suspensions, which allowed for cell transplantation at multiple sites of the striatum, resulting in more widespread innervation and better functional recovery in rotational behaviour as well as sensorimotor tests (Björklund, Stenevi, Schmidt, Dunnett, & Gage, 1983a; Björklund et al., 1980; Schmidt, Björklund, Stenevi, Dunnett, & Gage, 1983). Interestingly, it was shown that functional recovery caused by the transplantation of foetal tissue was specific to cells derived from the dopamine-rich VM. This confirmed that the effects seen were dependent on dopamine replacement in the striatum and not on nonspecific stimulation by the foetal tissue (Dunnett, Hernandez, Summerfield, Jones, & Arbuthnott, 1988). From here, the field of cell replacement therapy in Parkinson’s disease rapidly expanded.

It is now well established that the efficacy of cell replacement strategies in Parkinson’s disease is dependent on (a) the survival and maturation of dopaminergic neurons in the host brain; (b) appropriate axonal outgrowth from the transplanted cells; (c) integration with the host system; and (d) restoration of dopamine transmission. Numerous studies have shown that dopaminergic neurons from the developing VM can mature and function in the host adult striatum following transplantation (Annett et al., 1997; Brundin & Björklund, 1987; Dowd & Dunnett, 2004; Hahn, Timmer, & Nikkhah, 2009; Kauhausen, Thompson, & Parish, 2013; Parish et al., 2008; Torres, Dowd, & Dunnett, 2008). Additionally, electrophysiological and neurochemical studies have shown that grafted dopaminergic neurons are capable of the synthesis, release and uptake of dopamine (Rose, Gerhardt, Stromberg, Olson, & Hoffer, 1985; Schmidt, Ingvar, Lindvall, Stenevi, & Björklund, 1982; Zetterström et al., 1986), exerting electrical firing patterns (Wuerthele et al., 1981), reinnervating the host striatum and developing graft-to-host synaptic connections (Bolam, Freund, Björklund, Dunnett, & Smith, 1987; Mahalik, Finger, Stromberg, & Olson, 1985). Moreover, dopamine release from transplanted neurons in the striatum can restore basal dopamine levels towards normal (Piccini et al., 1999). Overall, while transplanted dopaminergic neurons are capable of survival and extensive afferent and efferent connectivity with the host brain, it must be noted that there are a number of factors which can affect the efficacy of VM transplantation including graft survival, placement, neuronal subtype and donor age.

2.1 | Impact of graft placement

Preliminary in vivo studies have given significant insight into how the specific placement of foetal VM grafts in the brain can affect the efficacy of transplantation. In the normal physiological scenario, dopaminergic cell bodies reside in the substantia nigra, from which they extend long axonal projections along the trajectory of the medial forebrain bundle (MFB) to the striatum where dopamine transmission is required. To date, ectopic placement of grafts in the striatum has been favoured over homotopic, intranigral delivery. This is because intranigral transplantation is associated with poorer cell survival and insufficient striatal innervation, most likely caused by the absence of guidance cues along the already developed nigrostriatal pathway (Bentlage, Nikkhah, Cunningham, & Björklund, 1999) and the corresponding presence of inhibitory factors (Kauhausen, Thompson, & Parish, 2015; Victorin, Brundin, Gustavii, Lindvall, & Björklund, 1990). Early studies showed that while dopaminergic neurons survive intranigral grafting, they failed to extend axons along the nigrostriatal pathway or produce any behavioural recovery (Björklund, Stenevi, Schmidt, Dunnett, & Gage, 1983b). These failings were understood to be due to the restrictive host environment and not a reflection of the cells capacity to grow. Proof-of-principle arose from the microtransplantation
of VM cells to the substantia nigra of 6-hydroxydopamine lesioned neonates, where nigrostriatal reconstruction was observed upon adulthood (Nikkhah, Cunningham, Cenci, McKay, & Bjorklund, 1995). Moreover, the transplantation of “bridge” grafts of Schwann cells that stretch from the transplantation site to the striatum alongside intranigral VM grafts showed that grafted dopamine neurons had the intrinsic potential to extend axons to the denervated striatum (Brecknell et al., 1996; Wilby et al., 1999). Xenografting studies have also shown that human VM cells placed into the substantia nigra of hemiparkinsonian adult rats could innervate regions of the host striatum, alluding to the concept that this was a result of the outgrowing axons’ failure to recognise species-specific inhibitory factors (Isacson et al., 1995). Additionally, recent studies using VM tissue from GFP transgenic mice have shown a notable pattern of axon growth towards the striatum, along with the normalisation of rotational behaviour (Gaillard et al., 2009; Kauhausen et al., 2013; Thompson, Grealish, Kirik, & Bjorklund, 2009).

2.2 | Impact of neuronal subtype

Dopaminergic neuron subtype is another factor that holds influence over the efficacy of VM transplants. As they are dissected from primary tissue, VM grafts are heterogeneous with respect to cell type, with the dopaminergic neurons being just one component. Dopaminergic neurons can be further divided into three major cell groups, A8, A9 and A10, based on the classification of cerebral monoamine neurons by Dahlstrom and Fuxe (1964). A10 neurons are phenotypically small round cells that send projections to the cortical and limbic structures including the amygdala, nucleus accumbens, hippocampus and prefrontal cortex to form the mesocorticolimbic structure. A9 neurons are phenotypically larger angular cells that send projections predominantly to the dorsolateral striatum to form the nigrostriatal pathway. While, A8 neurons innervate limbic and striatal structures and provide local innervation to both A9 and A10 neurons (Bjorklund & Dunnett, 2007). The A9 neurons are the most vulnerable to degeneration in Parkinson’s disease, while the A10 neurons are relatively resistant to disease pathology and are one of the last to degenerate (Damier, Hirsch, Agid, & Graybiel, 1999). Moreover, the A9 component of VM grafts has been found to be the most important for functional recovery due to their exceptional ability to target the dorsolateral striatum which is involved in movement (Grealish et al., 2010), highlighting the importance of a subtype ratio that is favourable to A9 neurons. Quantification of DA neuronal subtypes expressing A9 and A10 marker proteins (G protein-gated inwardly rectifying potassium channel [Girk2] and calcium-binding protein [calbindin], respectively) shows that intrastriatal VM grafts are comprised of 60%–70% A9 neurons and 30%–40% A10 neurons (Bye, Thompson, & Parish, 2012). Interestingly, it was found that A9 neurons precede the birth of A10 neurons and as a result, the use of younger embryonic donor tissue generated grafts which were composed of ~75% A9 neurons (Bye et al., 2012). Furthermore, recent studies have demonstrated that A9 neurons from younger embryonic donor tissue are more responsive to environmental cues at the transplantation site when adopting a dopaminergic phenotype during differentiation postgrafting (Fjodorova, Torres, & Dunnett, 2017; Kauhausen et al., 2013).

2.3 | Impact of donor age

As highlighted above, embryonic donor age is of particular importance in VM transplantation. The VM must be collected during a specific time frame of neurogenesis to allow for the level of maturity required for commitment to a dopaminergic neuron phenotype, while also avoiding significant axonal outgrowth prior to tissue dissociation (as dissecting at this stage will sever the axons and kill the neurons). Early experiments established the upper limits of donor age for VM cell suspensions to be embryonic day (E) 15-16 (rat; Brundin et al., 1985), and in more recent years, the conventional donor age has become E14 (rat) as it was perceived to coincide with peak dopamine neurogenesis (Hegarty, Sullivan, & O’Keeffe, 2013). More recently, the influence of donor age on graft efficacy has received heightened interest. Torres et al. investigated the yield of dopaminergic neurons from intrastriatal grafts of VM dissected at E11, E12, E13, E14 and E15, and found that the E12 preparations yielded the highest dopaminergic neurons at fivefold that of the E14 preparation (Torres, Monville, Gates, Bagga, & Dunnett, 2007). The authors hypothesised that the enhanced survival with this younger donor age may be a result of trophic support from the attached meningeal layer, a component that is ordinarily removed in E14 preparations. Moreover, a recent study showed that the addition of meningeal cells from young donors with VM preparations enhanced both the survival and axonal outgrowth of dopaminergic neurons (Somaa, Bye, Thompson, & Parish, 2015).

3 | CELLULAR BRAIN REPAIR FOR PARKINSON’S DISEASE – CLINICAL STUDIES

The positive results found in the early preclinical studies led to a swift movement to clinical trials, with the first open-label clinical trial taking place less than 10 years after dopaminergic neurons from VM grafts were first shown to be suitable for transplantation. The first clinical trials were carried out in the 1980s, first by Madrazo et al. (1988) in Mexico and then by Lindvall et al. (1989) in Sweden. In both studies, two patients received an intrastriatal transplantation of
human VM tissue from 12- to 14- and 8- to 10-week-old embryos, respectively. While Madrazo et al. reported dramatic motor improvements, particularly in the disappearance of rigidity and dyskinesia, Lindvall et al. noted minimal clinical improvement. This leads to refinements in surgical technique such as a reduction in the diameter of implantation device and an increase in the number of implantation sites. As a result, in a subsequent study, Lindvall et al. (1990) reported a significant reduction in the rigidity and bradykinesia of two patients, with a marked decrease in the patients “on-off” phenomenon. Moreover, long-term follow-up showed that the grafted VM cells were capable of surviving and exerting functional benefit 3 years after transplantation (Lindvall et al., 1994). This led to several subsequent open-label trials which reported significant improvements in UPDRS scores, quality of life and levodopa requirements (Brundin et al., 2000; Freed et al., 1992; Hauser et al., 1999; Peschanski et al., 1994; Spencer et al., 1992; Wenning et al., 1997). Importantly, these positive results were associated with an increase in fluorodopa uptake, as measured by $^{[18F]}$-DOPA positron emission tomography (PET) which is widely used as a measure of graft viability (Piccini et al., 2005). Likewise, the binding of $[^{11C}]$-raclopride, a D$_2$ receptor antagonist, showed that the transplanted grafts restored D$_2$ receptor occupancy to normal levels. Encouragingly, the grafts’ capacity to release dopamine can be maintained for at least a decade, despite their exposure to the ongoing disease progression (Piccini et al., 1999). Furthermore, short-term (18 and 19 months; Kordower et al., 1995, 1998) and long-term (14 years; Mendez et al., 2008) postmortem analysis has shown that grafted dopaminergic neurons can survive the procurement and transplantation process, are capable of reinnervating the denervated striatum and can form graft-to-host synapses. Moreover, Kefalopoulou et al. (2014) recently reported on the long-term (18 years after transplantation) symptomatic relief in two patients along with their discontinuation of any antiparkinsonian medication.

The success of the open-label trials led to the initiation of two double-blind, placebo-controlled trials in the early 2000s. The first, by Freed et al. (2001), included 40 patients between the age of 34 and 75 years with a mean disease duration of 14 years. They were randomly assigned to receive bilaterally either a foetal cell transplantation (tissue from two embryos/side, each between 7 and 8 weeks of age) or sham surgery. No immunosuppression was given pre- or postoperatively, and follow-up continued for 12 months post-transplantation. The primary outcome was a subjective self-report global rating of clinical improvement at 12 months post-transplantation. Although $^{[18F]}$-DOPA PET and postmortem analysis confirmed the survival and growth of transplanted grafts, the study failed to reach its primary endpoint, with no significant difference found in total UPDRS scores between the treatment and placebo groups. However, significant improvement in “off” state UPDRS and motor scores was found in transplant patients below 60 years of age. Of concern, five of 33 patients who received a transplant (including those from the sham group who elected to have the surgery after the study) developed dyskinesias within the first year. These dyskinesias persisted after the reduction or cessation of dopaminergic medication and are now known as graft-induced dyskinesias (GIDs). Subsequent preclinical assessments, as well as retrospective clinical assessments of patient transplants, indicated that the presence of serotonergic neurons within the donor preparations (a consequence of poor/broad tissue dissection and isolation of hindbrain nuclei), as well as uneven striatal reinnervation by the grafts, was likely responsible for the observed GID (Carlsson, Carta, Winkler, Bjorklund, & Kirik, 2007; Carlsson et al., 2006; Hagell et al., 2002). The second placebo-controlled trial included 34 patients between the ages of 30 and 75 years of age (Olanow et al., 2003). Patients were randomly assigned to receive bilaterally either a foetal cell transplantation (from one or four embryos/side, each between 6 and 9 weeks) or sham surgery. All patients received immunosuppression with cyclosporine 2 weeks preoperatively and up to 6 months postoperatively. The primary outcome measure was a significant difference in the “off” state UPDRS score from baseline to the final 24-month visit. Again, the primary endpoint was not achieved, although there was a trend towards improved motor scores in patients who received a four embryo transplant. Stratification based on disease severity also showed significant improvement in motor scores in less severe patients who received four embryo transplants. Patients also showed significant motor improvement at 6 and 9 months post-transplant with deterioration afterwards, which may be a result of the cessation of immunosuppression. Postmortem analysis did show good survival of dopaminergic neurons and reinnervation of the striatum, while PET analysis revealed increases in fluorodopa uptake. However, worryingly, within a year, GIDs were found in several grafted patients, similar to the previous findings of Freed et al. (2001). Further to this, numerous postmortem reports (>10 years post-transplant) have shown that a number of dopaminergic neurons grafted into the putamen of patients with Parkinson’s disease display Lewy body pathology that is indistinguishable from those seen in the host brain (Chu & Kordower, 2010; Kordower, Chu, Hauser, Freeman, & Olanow, 2008; Kordower, Chu, Hauser, Olanow, & Freeman, 2008; Li et al., 2008, 2010), highlighting that disease progression continues in the face of transplantation, a process that could have detrimental effects to the long-term efficacy of grafted cells.

The results of these trials raised concerns over the efficacy and safety of foetal VM transplants in Parkinson’s disease, and while the reasons behind the negative outcomes and GIDs remain unknown, patient selection, tissue preparation, tissue placement, immunosuppression and follow-up time are
all thought to be contributing factors. The best results from these placebo-controlled trials were found in patients with lower disease severity (rated by UPDRS scores) and good levodopa response pretransplantation, suggesting that disease severity is a factor that could affect the efficacy of transplantation. Additionally, large variations in tissue preparations were seen between the studies. Freed et al. (2001) delivered a lower volume of tissue that had been stored for 4 weeks prior to transplant, while Olanow et al. (2003) delivered larger quantities of tissue that was only stored for 2 days prior to transplant. The long storage of tissue prior to transplant may have been deleterious to cell survival, which could be further affected by the lower quantity of tissue delivered. Further to this, donor age (range of 6–9 weeks) and tissue composition (strands vs. pieces) are other factors that could have affected graft efficacy. Freed et al. (2001) also implemented a new trajectory to the striatum which may have affected cell distribution. The breach of the blood–brain barrier (BBB) during surgery and the subsequent addition of a cell transplant are both factors that can instigate a host immune response; therefore, the decision to implement (Olanow et al., 2003) or not (Freed et al., 2001) an immunosuppressive regimen may have affected graft survival. The decision to stop immune suppression after 6 months in the Olanow et al. (2003) study is notable because up until this point, grafted patients had improved at a rate similar to that seen in the open-label studies. The deterioration after cessation of immunosuppression could be explained by a delayed immune response that compromised long-term survival. Concern also rose over the decision to base the primary outcome on results found at just 1 year. Open-label studies had shown that graft-induced recovery can take months to years to develop. A follow-up of the Olanow et al. (2003) study at 2 and 4 years post-transplant showed significant improvements in UPDRS motor scores and fluorodopa uptake. Moreover, the increase in fluorodopa uptake over the course of the study correlates with the clinical outcome (Ma et al., 2010).

4 | CELLULAR BRAIN REPAIR FOR PARKINSON’S DISEASE – POTENTIAL OF BIOMATERIALS

One of the major issues that have limited the efficacy of foetal VM grafts since the initial pioneering studies began ~40 years ago is the poor survival and engraftment of the dopaminergic neurons after transplantation into the brain. The clinical and preclinical experiences gained so far have all reported poor survival of dopaminergic neurons after transplantation, estimated at less than 20%, see reviews—Castilho, Hansson, and Brundin (2000); Olanow, Kordower, and Freeman (1996). This extremely poor survival results in the need for multiple foetal donors per transplant, thus further exacerbating the ethical concerns over the use of tissue from elective abortions.

It was previously assumed that cell death in VM grafts was predominantly necrotic occurring as a result of cell insult during the tissue dissection and transplantation process. However, while some necrotic cell death does occur, the large extent of cell death in VM grafts occurs post-transplantation through apoptosis and is predominantly driven by external factors in the cells’ environment rather than a physiologic insult (Castilho et al., 2000; Mahalik, Hahn, Clayton, & Owens, 1994; Schierle et al., 1999; Sortwell, Pitzer, & Collier, 2000; Zawada et al., 2001). Apoptotic cell death is triggered at various points of the transplantation process by factors such as (a) detachment from the extracellular matrix during tissue dissection (Reddig & Juliano, 2005); (b) shearing of the cells upon delivery, via fine cannulas, into the host parenchyma (Barker, Fricker, Abrous, Fawcett, & Dunnett, 1995); (c) immediate growth factor deprivation upon transplantation into the adult striatum (Collier & Sortwell, 1999); and (d) the recruitment of host neuroimmune cells to the graft (Duan, Widner, & Brundin, 1995). Each of these stages provides an intervention point at which graft survival could be improved. Consequently, a number of studies explored the benefit of antiapoptotic agents such as caspase inhibitors/lazaroids and JNK inhibitors (Karlsson, Emgard, & Brundin, 2002; Rawal, Parish, Castelo-Branco, & Arenas, 2007; Schierle et al., 1999), as well as prosurvival proteins inclusive of glial-derived neurotrophic factor (GDNF) and neurturin, which have shown to be advantageous to dopaminergic neuron survival, at the stages of cell preparation, implantation and/or integration—see review Deierborg, Soulet, Roybon, Hall, and Brundin (2008). More recently, however, data have begun to emerge which show that injectable biomaterial scaffolds, such as in situ forming hydrogels, have the potential to improve the engraftment and survival of these grafts in the brain through provision of a supportive microenvironment for cell adhesion, growth and protection from the host immune response. Thus, since cell death in primary dopaminergic cell grafts occurs over a number of distinct stages throughout the whole transplantation process, for a biomaterial scaffold to be advantageous to the delivery, survival and efficacy of cell replacement efforts, it should be capable of (a) providing a supportive environment for cell adhesion; (b) providing a reservoir for localised and sustained growth factor delivery; and (c) creating a physical barrier between the transplanted cells and the host neuroimmune cells (Figure 1).

4.1 | Cell-matrix adhesion

Prior to transplantation, embryonic tissue must be collected, dissected and dissociated into a cell suspension. Even with the clean and efficient dissection of VM tissue, mechanical destruction caused by cellular detachment from the
extracellular matrix during dissociation removes the normal cell-matrix interactions and cell death can ensue (Marchionini et al., 2003; Reddig & Juliano, 2005). This process, known as anoikis, was first termed by Frisch and Francis when they showed that the loss of integral cell-matrix interactions is a major trigger of apoptotic cell death (Frisch & Francis, 1994). Hence, the delivery of cells in a biomaterial matrix to which they can adhere may provide them with the necessary support needed both during and after transplantation. Indeed, studies have shown that the attachment of neural cells to biomaterial scaffolds (particularly compressive biosynthetic materials) prior to transplantation can provide cells with an adherent surface throughout the transplantation process, therefore reducing the detrimental effects of anoikis on cell survival (Béduer et al., 2015; Jgamadze et al., 2012; Moriarty, Cabre, Alamilla, Pandit, & Dowd, 2018; Moriarty & Dowd, 2018; Moriarty, Pandit, & Dowd, 2017; Newland et al., 2015).

4.2 | Growth factor provision

Foetal cell replacement therapy in Parkinson’s disease involves the removal of dopaminergic neurons from the VM of the developing embryo and subsequent transplantation into the adult striatum. As the trophic activity of the brain and in particular, the striatum, is known to decrease with age (Ling et al., 2000), these cells are removed from a trophic-rich environment at the height of neurogenesis and placed into the depleted striatum. As a result, transplanted cells undergo trophic withdrawal, being deprived of the factors normally present throughout target innervation and development (Abeliovich & Hammond, 2007). Indeed, numerous studies suggest that the critical time-point in which 80%–90% of dopaminergic neurons die is the first 4 days post-transplantation, and that it is not until after this point that dopaminergic neuron survival is stabilised (Barker, Dunnett, Faissner, & Fawcett, 1996; Emgard, Karlsson, Hansson, & Brundin, 1999; Rawal et al., 2007; Sortwell, Camargo, Pitzer, Gyawali, & Collier, 2001; Sortwell et al., 2000). The incorporation of growth factors, such as brain-derived growth factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), growth/differentiation factor 5 (GDF5) and GDNF, has been investigated for their potential to improve dopaminergic cell transplantation strategies (Clayton & Sullivan, 2007; Feng et al., 1999; Hyman et al., 1994; Jaumotte & Zigmond, 2014; Lin, Doherty, Lile, Bektesh, & Collins, 1993). While they have all shown to improve dopamine neuron survival in VM cultures, GDNF has been established as an extremely potent neurotrophic factor, significantly improving cell survival and efficacy (Apostolides, Sanford, Hong, & Mendez, 1998; Chaturvedi et al., 2003; Deng et al., 2013; Redmond et al., 2013; Rosenblad, Martinez-Serrano, & Bjorklund, 1996; Yurek, Flectcher, Kowalczyk, Padegimas, & Cooper, 2009).
However, the encapsulation of cells in a growth factor-loaded biomaterial matrix has the potential to further enhance cell survival and efficacy by providing the transplanted cells with localised, site-specific and prolonged access to growth factors upon transplantation and the period of target innervation.

Hydrogels from a variety of sources, both natural and synthetic, have shown to successfully deliver trophic factors to the brain in a site-specific, controlled and sustained manner (Chierchia et al., 2017; Fon et al., 2014; Li et al., 2016). Further to the enhanced delivery of GDNF in injectable hydrogels (Fon et al., 2014), many approaches have investigated the use of hollow microparticles to achieve sustained GDNF release from a single administration (Agbay, Mohtaram, & Willerth, 2014; Garbayo et al., 2016; García-Caballero et al., 2017; Lampe, Kern, Mahoney, & Bjugstad, 2011). Moreover, the delivery of GDNF containing microspheres in an injectable fibrin hydrogel enhanced the length of GDNF release in situ to 2 weeks compared to 3 days with “free” GDNF (Wood et al., 2013). Similarly, hydrogels have also been successfully used to enhance cellular delivery (Aguado, Mulyasasmita, Su, Lampe, & Heilshorn, 2012; Ballios et al., 2015; Das et al., 2016; Freudenberg et al., 2009). Three recent studies have highlighted the potential of injectable hydrogels to improve dopaminergic cell replacement strategies. First, in the study by Wang et al. (2016), a GDNF-functionalised composite poly(l-lactic acid)/xyloglucan hydrogel, where GDNF was blended into and/or covalently attached to the scaffold, was shown to enhance survival of, and striatal reinnervation from, transplanted mouse VM grafts in parkinsonian mice. Second, Adil et al. (2017) showed that a heparin/RGD-functionalised hyaluronic acid hydrogel could improve the survival of transplanted human embryonic stem cell-derived dopaminergic neurons. Finally, Moriarty et al. (2017, 2018) demonstrated that encapsulating VM grafts in a GDNF-loaded collagen hydrogel resulted in a dramatic increase in the survival of dopaminergic neurons and that this correlated with enhanced striatal reinnervation and restoration of motor function in hemiparkinsonian rats.

### 4.3 Immune shielding

Experimental studies have shown that the injection of exogenous cells into the brain evokes an elevated host immune response (Barker et al., 1996; Duan, Cameron, Brundin, & Widner, 1997; Duan, Widner, Bjorklund, & Brundin, 1993; Hudson, Hoffman, Stromberg, Hoffer, & Moorhead, 1994; Shinoda et al., 1995). Additionally, in line with the prominent cell death seen immediately post-transplantation, microglial activation, lymphocyte infiltration and major histocompatibility complex (MHC) expression all increased over the first 4 days post-transplantation (Duan et al., 1995). Moreover, the immune response may intensify overtime as the immune cells in the brain are not stationary and inactive, but rather provide a continuous inflammatory response (Shinoda et al., 1995). Postmortem analysis from the double-blind, placebo-controlled clinical trials, as well as an earlier study, detected prominent activated microglial staining around the graft site (Freed et al., 2001; Kordower et al., 1997; Olanow et al., 2003). This immune response against the transplanted cells may have hindered their survival and consequently had an effect on their clinical efficacy. This seems increasingly possible in the Olanow et al. (2003) trial where deterioration of clinical benefit began after the withdrawal of immunosuppression. Thus, encapsulation of cells within a supportive biomaterial matrix may protect the transplanted cells from the hostile host environment by forming a physical barrier between the transplanted cells and the host neuroimmune cells. Indeed, Hoban et al. (2013) and Moriarty et al. (2017) have both demonstrated a dramatic reduction in the recruitment and proliferation of microglia and astrocytes around intrastriatal cell grafts when encapsulated in a collagen hydrogel.

### 5 Cellular Brain Repair for Parkinson’s Disease – Which Biomaterial to Use?

Repairing the damaged brain can be a daunting task, yet recent advances in tissue engineering and cell-based therapies are bringing us closer to clinical translation. The diversity and adaptability of biomaterial scaffolds make them an attractive strategy for neural cell replacement therapy (Orive, Anitua, Pedraz, & Emerich, 2009); however, any material used for intracranial delivery should exhibit a number of desirable characteristics. Such materials should (a) be capable of relatively noninvasive delivery; (b) be biomimetic in order to encourage cell survival and host integration; (c) not themselves elicit an exaggerated host immune reaction that can instigate neuroinflammation around the transplantation site; (d) be structurally stable for prolonged periods in situ and where appropriate biodegrade without leaving any undesirable foreign remnants; (e) be modifiable in relation to adhesion molecules, pore size, molecular charge, surface topography and functionalisation; (f) be nontoxic to any cellular components of brain tissue or the encapsulated cells; and (g) be capable of controlled and sustained delivery of therapeutic factors (based on Orive et al. (2009) and Wang, Forsythe, Parish, and Nisbet (2012)).

#### 5.1 Injectable hydrogels as a biomaterial scaffold

The large range of available biomaterials coupled with their high adaptability leads to the generation of application-specific materials, making biomaterials a very attractive avenue in the field of cell-based therapies (Kim, Cooke,
Biomaterials can be characterised under two main subtypes, natural materials or synthetic materials. Natural materials are derived from biological sources including, chitosan, alginate, methylcellulose, hyaluronan, fibrin and collagen. The advantages of their use stem from their natural roles in the biological system. Many contain endogenous binding sites that allow for natural cell adhesion (Heino & Käpylä, 2009), while their biological source minimises the activation of the host immune response (Mano et al., 2007). In comparison, synthetic materials are chemically manufactured and can therefore be more readily manipulated and standardised (Lutolf & Hubbell, 2005). Indeed, many biomaterials that use natural materials, such as alginate, fibrin or collagen, as their primary framework are often cross-linked with synthetic polymers, such as polymer polyethylene glycol (PEG), giving rise to a new class of biosynthetic materials which possess the biological properties of the protein and the mechanical stability afforded by the chemical cross-linking (Delgado, Bayon, Pandit, & Zeugolis, 2015). It is of utmost importance when choosing a biomaterial, whether it be from a natural, synthetic or biosynthetic origin, to take their individual characteristics into consideration, as properties such as adhesion potential, degradability, shape, pore size, hydrophilicity and delivery potential will render them suitable or unsuitable for specific applications.

In relation to the CNS, injectable hydrogels are the most widely investigated and promising biomaterial scaffolds for the delivery of therapeutic agents and/or cells to the brain in regenerative therapies (Burdick, Mauck, & Gerecht, 2016). Hydrogels are three-dimensional networks of hydrophilic polymers which can be chemically cross-linked to form insoluble polymer matrices (Hoffman, 2002). The ability of hydrogels to form in situ response to temperature and pH changes makes them injectable, an extremely attractive property which allows for their relatively noninvasive intracranial delivery (Pakulska, Ballios, & Shoichet, 2012). Furthermore, hydrogels can be chemically cross-linked to alter their physical properties to specific applications. Indeed, the degree of chemical cross-linking used can directly affect the level of gelation (strength of in situ formation), porous structure and degradation (Drury & Mooney, 2003). The alteration of a hydrogel’s porous structure allows for control over nutrient infusion to encapsulated cells and therapeutic factor diffusion to surrounding tissues (Lee, Tong, & Yang, 2016), while simultaneously minimising host immune cell infiltration. Additionally, using biomaterials that undergo natural degradation, they will eventually be eliminated from the body, while the degree of chemical cross-linking used can control the hydrogels’ degradation rate and therefore its persistence in situ (Davidenko et al., 2015). Depending on their biological source, hydrogels may naturally support cell adhesion or be manipulated to support cell attachment through the addition of adhesion factors (Hersel, Dahmen, & Kessler, 2003). Moreover, growth factors can be added to further support cell survival and function after transplantation (Burdick et al., 2016) with studies now demonstrating the capacity to temporally control the release of multiple growth factors simultaneously or sequentially, dependent on the requirement of the host and/or implanted cells (Bruggeman, Rodriguez, Parish, Williams, & Nisbet, 2016).

### 5.2 Injectable collagen hydrogels

Forming 20%–30% of the body’s protein component, the collagen family is the body’s most abundant protein group, making collagen one of the most investigated natural biomaterials (Khan & Khan, 2013). There are many distinct types of collagen found throughout the body; however, 90% of collagen in the human body is type I (Henriksen & Karsdal, 2016). Type I collagen can be extracted with ease from animal tissues including tendons and skin. It is a fibrous protein composed of three polypeptide chains (α subunits) that are wound together using hydrogen bonds to form a triple-helix structure (Bhattacharjee & Bansal, 2005). The transition of collagen from a liquid to a solid state through the structural change in the triple helix to highly compacted coils as a result of physiological conditions such as temperature and pH makes it an attractive biomaterial for CNS delivery (Sargeant, Desai, Banerjee, Agawu, & Stopak, 2012). Furthermore, this in situ gelation makes collagen hydrogels an ideal delivery scaffold for both trophic and cellular regenerative therapies.

While collagen is capable of naturally forming a hydrogel in situ, its weak mechanical properties make it highly susceptible to rapid degradation in the brain. In an effort to better control the mechanical stability of collagen, the use of synthetic polymers to form biosynthetic hydrogels that possess the biological properties of the protein and the mechanical stability afforded by the chemical cross-linking has been investigated (Delgado et al., 2015; Sargeant et al., 2012). The synthetic polymer PEG is hydrophilic, nontoxic, nonimmunogenic (Veronese & Pasut, 2005) and importantly, already FDA approved for a number of clinical applications (Alconcel, Baas, & Maynard, 2011). Furthermore, the chemical cross-linking of collagen with PEG has shown to improve the mechanical stability, degradation rate and protein diffusion from these biosynthetic hydrogels, while also maintaining the proteins biological function (Doillon, Cote, Pietrucha, Laroche, & Gaudreault, 1994; Lee et al., 2000; Sargeant et al., 2012; Weber, Lopez, & Anseth, 2009). Thus, the ability of cross-linked collagen hydrogels to mimic the extracellular matrix, while also having control over the strength of gelation, rate of degradation and diffusion of encapsulated factors, makes them an extremely attractive biomaterial scaffold for cell replacement therapies.

Keeping with this, collagen biomaterials have already been approved for use in a variety of applications including...
drug delivery, wound repair, burn treatment, dentistry and bone reconstruction (Blume et al., 2011; Chajra et al., 2008; Chattopadhyay & Raines, 2014; El-Chaar, 2016; Helary et al., 2010; Khan & Khan, 2013; Parenteau-Bareil, Gauvin, & Berthod, 2010; Patino, Neiders, Andreana, Noble, & Cohen, 2002; Solish, 2010) and could therefore be relatively easily adopted to neural applications. While the intracranial use of collagen hydrogels has been looked at to a lesser extent, Hoban et al. (2013) and Moriarty et al. (2017, 2018) have recently demonstrated the efficacy of PEG cross-linked collagen hydrogels for brain repair in Parkinson’s disease. Thus, given the clinically biocompatible, immunoprotective and supportive properties of collagen hydrogels, they hold immense potential to improve the survival and efficacy of dopaminergic cell replacement therapies in Parkinson’s disease. Taken together, this literature highlights the potential of biomaterial hydrogel scaffolds to improve the outcome of reparative cell therapies for Parkinson’s disease. Moving towards a clinical therapy, the use of biomaterial scaffolds from natural polymers, namely collagen, offers significant translatability owing to it already being approved for use in a variety of clinical applications (Chajra et al., 2008; Chattopadhyay & Raines, 2014; Patino et al., 2002; Solish, 2010).

6 | CONCLUSION

As cell therapies for Parkinson’s disease and other neurodegenerative disorders propel towards the clinic, it is of increasing importance to address strategies to ensure maximal survival, integration and functional efficacy of the newly implanted tissue. In this regard, it is clear that evidence is mounting that supports the potential of biomaterial scaffolds to enhance brain repair for Parkinson’s disease. Further work remains to be carried out to identify the ideal biomimetic scaffold and determine optimal strategies to functionalise these matrices—targeted at supporting cell transplant for neural repair.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

We confirm that this manuscript has been read and approved by all named authors and that all named authors have fulfilled the required criteria for authorship. N.M., C.P. and E.D contributed equally to the drafted manuscript.

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REFERENCES


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. https://doi.org/10.1038/s41598-017-15970-w


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Brain repair for Parkinson’s disease: is the answer in the matrix?

Two hundred years after James Parkinson first described the cardinal motor symptoms of the disorder that would later bear his name, there is still an irrefutable need for a therapy that targets the underlying pathophysiology of the disease and not solely its symptoms. Parkinson’s disease (PD) is classically characterised by Lewy body formation and a relatively selective degeneration of nigrostriatal dopaminergic neurons (Schapira and Jenner, 2011). The loss of dopaminergic neurons from the substantia nigra pars compacta causes a consequential depletion of the neurotransmitter dopamine from the striatum, and it is this loss that causes the motor symptoms experienced by patients. To date, all treatments for this condition are symptomatic in that they simply endeavour to correct the neurochemical and/or electrical anomalies caused by striatal dopaminergic deafferentation in an attempt to improve motor function (LeWitt and Fahn, 2016). While such symptomatic approaches show extraordinary efficacy in the early years after initiating treatment, the underlying disease pathology continues to progress, and eventually their efficacy subsides. In view of this, there remains an urgent need for an alternative treatment approach that is capable of protecting or repairing the brain in order to provide a more sustained benefit to patients.

Brain repair for PD: Brain repair for PD has developed from a relatively simple conceptual framework - if a primary pathological hallmark of the disease is the degeneration and death of dopaminergic neurons, then it should be possible to replace these neurons with healthy, viable cells. Over the last 30 years, cell replacement therapy for PD has focused on the transplantation of primary dopaminergic neurons sourced from the ventral mesencephalon of fetal donor tissue. Experimental studies in rodents to non-human primates have illustrated the ability of these cells to survive, integrate with the host system, release dopamine and restore motor function; results that have since translated to clinical trials in PD patients (Barker et al., 2015). However, despite the potential of brain repair for PD, the use of human fetal tissue, obtained from elective abortions, raises many ethical and logistical concerns, which are exacerbated by the extremely poor survival of these cells in the brain post-transplantation (Sortwell et al., 2000). With a survival rate of only 5–10% of implanted cells, there is a requirement for as many as 12 fetal donors per patient which is clearly an impediment to the more widespread roll-out of this approach to patients (Barker et al., 2013). Several factors, occurring at various points of the transplantation process, are thought to contribute to the poor survival of the implanted fetal cells. These include 1) detachment from the extracellular matrix during tissue dissection, 2) growth factor deprivation upon transplantation into the adult striatum, and 3) the host brain’s neuroinflammatory response to the implanted cells (Moriarty et al., 2017).

The potential of biomaterials for brain repair for PD: Biomaterials - that is, materials that have been specifically engineered to interact with living systems for therapeutic purposes - have the potential to substantially improve brain repair approaches for PD. Structural biomaterials can be used as scaffolds to provide a supportive matrix for transplanted cells, and can be functionalised for delivery of therapeutic molecules that can enhance survival, axonal outgrowth and connectivity of transplanted cells. A vast array of different biomaterials are available, and while their characteristics may render them more suitable for some applications than others, in general, they are highly tuneable scaffolds and can therefore be specifically modulated to a therapeutic need (Orive et al., 2009). Naturally-derived biomaterials, such as collagen hydrogels, hold the advantage of being characteristically similar to the body’s native tissue, making them highly biocompatible and biodegradable, while also naturally supporting cell adhesion without the need for further chemical alterations which may disrupt the immunogenicity of the scaffold. Collagen is also capable of forming in situ gelling (and therefore injectable) hydrogels, thus making it an attractive candidate for improving cell replacement therapies in nervous disorders such as PD. Natural or engineered biomaterials have the benefit of already having clinical approval for a wide variety of applications (Bhat and Kumar, 2013). In theory, collagen hydrogels have the potential to increase the engraftment of cells by intervening at various points throughout the transplantation process where cell death occurs, such as, 1) providing a supportive matrix environment for cell adhesion, 2) providing a reservoir for localised growth factor delivery, 3) creating a physical barrier between the transplanted cells and the host neuro-immune cells (Figure 1).

Biomaterials improve brain repair in PD models: We have recently embarked on a series of studies to determine if the conceptual benefits of biomaterial hydrogels can be realised in experimental studies (Ho- ban et al., 2013; Newland et al., 2013; Samal et al., 2013; Krittavy et al., 2017). In the first instance, we found a dramatic reduction in the host’s immune response to transplanted cells (mesenchymal stem cells or primary dopaminergic neurons) when these are injected into the brain in an in situ gelling collagen hydrogel (Hoban et al., 2013; Morriarty et al., 2017). This was manifest through a significant reduction in the recruitment and proliferation of both microglia and astrocytes at the transplantation site. Given that intracerebral transplantation of these cells usually stimulates a substantial host immune response, the collagen hydrogel was clearly capable of shielding the grafted cells by forming a physical barrier between the cells and the host brain’s immune cells. However, despite the significant reduction in glialosis at the transplant site, this was not sufficient to improve the survival of either mesenchymal stem cell or primary dopaminergic transplants. We hypothesised that this was due to the lack of trophic support provided by the collagen hydrogel during transplantation, as this is the critical period where the vast majority of cell death is known to occur. Therefore, we then sought to determine if the collagen hydrogel was capable of providing a growth factor reservoir in the brain by functionalising the gels with the dopaminergic neurotrophin, glial-derived neurotrophic factor (GDNF). Injection of GDNF within the hydrogel resulted in a significantly enhanced acute retention of the trophic factor in the brain when compared with a bolus injection of GDNF (Moriarty et al., 2017). We then hypothesised that the GDNF-functionalised hydrogel could provide implanted cells with the localised and sustained growth factor required immediately post-transplantation which is lacking during the conventional delivery of ventral mesencephalic tissue alone. Strikingly, when we transplanted primary dopaminergic neurons in the GDNF-functionalised in situ gelling collagen hydrogel, we found that cell survival was significantly and substantially (5-fold) enhanced, and that this was associated with a greater extent of striatal reinnervation from the grafted cells which translated to a greater level of functional recovery (Figure 2, Morriarty et al., 2017). Taken together, these data indicate that collagen hydrogel scaffolds can be functionalised for delivery of GDNF by providing cells with a supportive environment throughout transplantation that is rich in trophic support and capable of guarding the cells from the hostile host environment. Consistent with these findings, other research groups have also recently reported the benefits of biomaterial application to cell replacement therapies in PD models. Wang et al. (2016) demonstrated the enhanced survival and re-innervation of transplanted fetal ventral mesencephalon grafts through their encapsulation in a GDNF containing composite scaffold consisting of a xylolucan hydrogel and electroporated short nanofibers. An interesting addition to this scaffold was the tethering of GDNF to short nanofibers, alongside the presence of soluble GDNF throughout the hydrogel, thus providing long-term GDNF delivery at the graft site and sustained release from the hydrogel. Moreover, since the tethering of GDNF to the short nanofibers alone did not result in improved cell survival or re-innervation, this highlights the importance of GDNF release from the graft core to the surrounding striatum, where it can guide and support neurite outgrowth. Adil et al. (2017) have also recently demonstrated that a hyaluronic acid hydrogel can enhance the survival of, and neurite outgrowth from, embryonic mesencephalic dopaminergic neurons. This hydrogel was additionally functionalised with extracellular membrane derived ligands, RGD and heparin, in an effort to assist cell attachment and trophic factor binding, respectively. Moreover, this study demonstrated that the hyaluronic acid hydrogel to improve the efficacy of dopaminergic neuronal differentiation, with a higher fraction of dopaminergic cells obtained in vitro, and an increase in the number of surviving cells during enzymatic cell harvest, a step that is thought to be a major contributing factor to pre-transplantation cell death in stem cell therapies. Encouragingly, this further demonstrates the potential of biomaterial applications to future stem cell-based cell replacement therapies.

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The future of biomaterials for brain repair for PD: It is clear that evidence is mounting that supports the potential of biomaterial scaffolds to enhance brain repair for PD. As cell therapies for PD and other neurodegenerative disorders propel towards the clinic, simultaneously, the area of biomaterial science is also making monumental progress; and the question remains: “is the answer in the matrix?” While further work must be carried out to determine the optimal material for dopaminergic cell replacement therapies, it is indisputable that great potential lies within biomaterial scaffolds and their application to neuroregenerative therapies.

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Comments to authors: The perspective article highlights the potential implications of biomaterials for enhancing neuronal repair. In particular, authors discuss the potential use of GDNF-loaded collagen hydrogel scaffolds for the transplantation of primary dopaminergic neurons to the improve the outcome of reparative cell therapies for PD. The article is very interesting, and has its merit and represents a valuable contribution to the literature.

References