Defining the potential of Gene Therapy with Bone Morphogenetic Proteins as a novel therapeutic approach in Parkinson’s disease

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Defining the potential of Gene Therapy with Bone Morphogenetic Proteins as a novel therapeutic approach in Parkinson’s disease.

Thesis presented by
Susan R. Goulding BSc, MSc.
Department of Biological Sciences
Under the supervision of
Dr Louise Collins, Prof Gerard O’Keeffe,
Dr Caitriona Guinane, Dr Brigid Lucey
For the degree of
Doctor of Philosophy (PhD)
January 2021
Declaration

This is to certify that all work presented in this thesis is original and entirely my own. The work was carried out under the supervision of Dr Louise Collins, Prof Gerard O’Keeffe, Dr Caitriona Guinane and Dr Brigid Lucey between October 2017 and September 2020. This dissertation has not been submitted in whole or in part for any other degree, diploma or qualification at Cork Institute of Technology or elsewhere.

Author contributions

All procedures described in this thesis were carried out accurately, and without bias, to the highest standards. The procedures were performed solely by Susan, with the exception of:

- Chapter 3.0, section 3.4.6 – Dr Noelia Morales Prieto carried out densitometric analysis.
- Chapter 4.0 – Dr Ruth Concannon performed stereotactic surgery and behavioural testing, Dr Sean Wyatt assisted with RT-qPCR, Dr Francisca Villalobos-Manriquez supported with HPLC.
- Chapter 7.0 - Dr Noelia Morales Prieto assisted with stereotactic surgery, Dr Francisca Villalobos-Manriquez supported with HPLC.

Susan Goulding

January 2021
Dissemination of this research:

Abstracts

Gene co-expression analysis of the human substantia nigra identifies BMP2 as a novel neurotrophic factor for axonal neuroprotection in Parkinson’s disease.

Susan R. Goulding, Gerard W. O’Keeffe, Louise M. Collins
Anatomical Society Summer Meeting, Oxford 2018

Gene co-expression analysis of the human substantia nigra identifies BMP2 as a novel neurotrophic factor for axonal neuroprotection in Parkinson’s disease.

Susan R. Goulding, Gerard W. O’Keeffe, Louise M. Collins
Young Neuroscience Ireland, Dublin 2018

Therapeutic efficacy of GDF5 viral vectors in the α-synuclein rat model of Parkinson’s disease.

BNA Festival of Neuroscience, Dublin 2019

Targeting the BMP signalling pathway as a novel therapeutic approach in Parkinson's disease.

Susan R. Goulding, Aideen M. Sullivan, Gerard W. O’Keeffe, Louise M. Collins
NECTAR, Cardiff 2019
Targeting the BMP signalling pathway as a novel therapeutic approach in Parkinson's disease.

**Susan R. Goulding**, Aideen M. Sullivan, Gerard W. O’Keeffe, Louise M. Collins

New Horizons UCC, Cork 2019

GDF5 exerts neuroprotection in an α-synuclein rat model of Parkinson’s disease.


Young Neuroscience Ireland, Virtual Conference 2020

GDF5 exerts neuroprotection in an α-synuclein rat model of Parkinson’s disease.


NECTAR, Virtual Conference 2020

**Publications**

Gene co-expression analysis of the human substantia nigra identifies BMP2 as a neurotrophic factor that can promote neurite growth in cells overexpressing wild-type or A53T α-synuclein.

**Susan R. Goulding**, Aideen M. Sullivan, Gerard W. O’Keeffe, Louise M. Collins

*Parkinsonism and Related Disorders* (2019), Vol. 64, Pages 194-201.
The potential of bone morphogenetic protein 2 as a neurotrophic factor for Parkinson’s disease.

**Susan R. Goulding**, Aideen M. Sullivan, Gerard W. O’Keeffe, Louise M. Collins

**Neural Regeneration Research** (2020), Vol. 15, Pages 1432-1436

GDF5 exerts neuroprotection in an α-synuclein rat model of Parkinson’s disease.


**Brain** (2020), [https://doi.org/10.1093/brain/awaa367](https://doi.org/10.1093/brain/awaa367)

STRAP and NME1 mediate the neurite growth-promoting effects of the neurotrophic factor GDF5.


**Submitted**

Quinacrine and Niclosamide promote neurite growth in midbrain dopaminergic neurons through the canonical BMP-Smad pathway and protect against neurotoxin and α-synuclein-induced neurodegeneration.

**Susan R. Goulding**, Aideen M. Sullivan, Martin Levésque, Louise M. Collins, Gerard W. O’Keeffe,
Growth differentiation factor 5: a neurotrophic factor with neuroprotective potential in Parkinson’s disease.

Susan R. Goulding, Jayanth Anantha, Louise M. Collins, Aideen M. Sullivan, Gerard W. O’Keeffe

NME1 protects against neurotoxin, α-synuclein and LRRK2-induced neurite degeneration in cell models of Parkinson’s disease.

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A huge thank you to everyone in the Anatomy and Neuroscience department and the Biological Services Unit in UCC. In particular, míle buíochas to Tara Foley for your invaluable help with the animal study, from the surgeries, to the culls and everything in between. We even managed a few laughs despite the stress!

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No project is complete without its collaborators and I would like to express my huge gratitude to Professor Aideen Sullivan for all your wisdom and insight, leaving each project better than when you found it. Thank you to Dr Sean Wyatt in Cardiff, Dr Francisca Villalobos-Manriquez in the APC and to Dr Martin Lévesque in Quebec for hosting me in your lab for two memorable weeks of science and touristing!

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To Ryan – my best friend, my partner in crime, my soulmate, my rock. You are the most hard-working person I know and luckily for me, it rubs off. You are my favourite study buddy and I could not have gotten through this PhD without you. What a team we make! There are no words that can sum up how thankful I am for the laughs, the memories, the holidays, the takeaways, the breakdowns, the late nights, the hangovers and everything in between! Long may the adventure continue. I love you.

To my siblings, Lisa, Padraig and Daniel - thank you all so much for always supporting me in everything I do and for feigning interest in my work in the family WhatsApp, much appreciated! Ye are the best.
Finally, to my parents, Margaret and Patrick. Where would I be without you. Your unconditional love and support gives me the confidence and strength to chase my dreams and not look back. Thank you for your constant enthusiasm about my work and for telling everyone and anyone who will listen to ye about it. Thank you from the bottom of my heart for all you have done and continue to do for me. More importantly, thanks for not listening to me in my first year of college when I wanted to switch from science to law – good call! I love you both to bits and I dedicate this thesis to you.
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AAV-BMP2 causes the loss of dopaminergic striatal terminals and exacerbates α-synuclein-induced motor deficits in the α-synuclein rat model of Parkinson’s disease.
Abstract

Parkinson’s disease is a neurodegenerative disorder, characterised by the progressive degeneration of midbrain dopaminergic neurons, and the intracellular aggregation of the α-synuclein protein in neurons throughout the nervous system. These changes result in the characteristic motor impairments in Parkinson’s disease. Current treatments are solely symptomatic and therefore there is an unmet clinical need to develop new disease-modifying therapies that can alter disease progression.

For over 25 years, one proposed experimental therapy has focused on the delivery of proteins called neurotrophic factors to the brain to prevent dopaminergic neuron degeneration. However, to date, clinical trials using the most well-known neurotrophic factors have not met their primary endpoints, likely as a result of the down regulation of their signalling receptor called Ret. Therefore, the aim of this thesis was to investigate the efficacy of two Ret-independent neurotrophic factors called GDF5 and BMP2 using in vitro and in vivo models of PD.

The main findings of this thesis were that GDF5 and BMP2 protected dopaminergic neurons and their axons from neurotoxin- and α-synuclein-induced degeneration, which is the protein that accumulates in the brains of people with Parkinson’s disease. Moreover, intranigral delivery of an AAV vector carrying the human GDF5 transgene protected dopaminergic neurons and their terminals from α-synuclein-induced degeneration in vivo. This thesis has also identified two clinically approved drugs that were capable of modulating the signalling pathway used by GDF5 and BMP2 to exert their effects on dopaminergic neurons. These compounds, called Quinacrine and Niclosamide, promoted dopaminergic neuron survival, and protected these neurons from neurotoxin- and α-synuclein-induced axonal degeneration.
The wider implications of these findings are that they support the conclusion that neurotrophic factor therapy remains a viable therapeutic approach for patients with PD, and they have identified new therapeutics for the future development of neurotrophic factor therapy in Parkinson’s disease.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>2-Mercaptoethanol</td>
<td>β-ME</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>AADC</td>
<td>Aromatic amino acid decarboxylase</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno associated virus</td>
</tr>
<tr>
<td>ALK</td>
<td>Activin receptor-like kinase</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>Anteroposterior</td>
</tr>
<tr>
<td>AV</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived Neurotrophic Factor</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>BMPR</td>
<td>Bone Morphogenetic Protein Receptor</td>
</tr>
<tr>
<td>BMPR2DN</td>
<td>BMPR2 dominant negative</td>
</tr>
<tr>
<td>BP</td>
<td>Binding Potential</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromodeoxyuridine</td>
</tr>
<tr>
<td>BRE</td>
<td>BMP Response Element</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CBA</td>
<td>Chicken β-actin</td>
</tr>
<tr>
<td>CDNF</td>
<td>Cerebral Dopamine Neurotrophic Factor</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyltransferase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>Co-Smad</td>
<td>Common-mediator Smad</td>
</tr>
<tr>
<td>CTPB</td>
<td>N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-Diaminobenzidine</td>
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<tr>
<td>DAPI</td>
<td>4'-6-Diamidino-2-phenylindole</td>
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<tr>
<td>DAT</td>
<td>Dopamine Transporter</td>
</tr>
<tr>
<td>DAT⁺</td>
<td>DAT-positive</td>
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<tr>
<td>DBS</td>
<td>Deep Brain Stimulation</td>
</tr>
<tr>
<td>DD</td>
<td>Destabilising domain</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Deionised water</td>
</tr>
<tr>
<td>DIV</td>
<td>Days <em>in vitro</em></td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DOPAC</td>
<td>3,4-Dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal Root Ganglia</td>
</tr>
<tr>
<td>DV</td>
<td>Dorsoventral</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
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<td>Ethylene diamine tetra acetic acid</td>
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<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EOPD</td>
<td>Early onset Parkinson’s disease</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
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FDA  Food and Drug Administration
FDR  False Discovery Rate
FGF  Fibroblast Growth Factor
FMC  Foetal mesencephalic cells
GABA  γ-aminobutyric acid
GAPDH  Glyceraldehyde 3-Phosphate Dehydrogenase
GDF  Growth and Differentiation factor
GDNF  Glial cell-line derived neurotrophic factor
GFAP  Glial fibrillary acidic protein
GFAP+  GFAP-positive
GFL  GDNF family of ligands
GFP  Green Fluorescent Protein
GFR  GDNF family receptor
GPI  Glycosylphosphatidylinositol
GS  Glycine/Serine
h  hour / human
H₂O₂  Hydrogen Peroxide
HAT  Histone Acetyl Transferase
HBSS  Hank’s Balanced Salt Solution
HCl  Hydrochloric acid
HDAC  Histone Deacetylase
HDI  HDAC Inhibitor
HES  Hairy enhancer of split
HPLC  High Performance Liquid Chromatography
HPRA  Health Products Regulatory Authority
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>HVA</td>
<td>Homovanillic acid</td>
</tr>
<tr>
<td>iPSCs</td>
<td>Induced Pluripotent Stem Cells</td>
</tr>
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<td>Inhibitory Smads</td>
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<tr>
<td>ITRs</td>
<td>Inverted terminals repeats</td>
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<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
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<td>Lentivirus</td>
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<tr>
<td>MANF</td>
<td>Mesencephalic Astrocyte-derived Neurotrophic Factor</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
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<td>MFB</td>
<td>Median Forebrain Bundle</td>
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<td>Min</td>
<td>Minutes</td>
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<td>Multiplicity of Infection</td>
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<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>N</td>
<td>Number of replicates</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NEN</td>
<td>Niclosamide Ethanolamine</td>
</tr>
<tr>
<td>NME1</td>
<td>Nucleoside diphosphate kinase</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural Stem Cell</td>
</tr>
<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>NTF</td>
<td>Neurotrophic factor</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>PBS containing 0.1% Tween® 20</td>
</tr>
<tr>
<td>PBS-Tx</td>
<td>PBS containing 0.02% Triton-X100</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>Platelet-derived growth factor BB</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission topography</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>Phospho</td>
<td>Phosphorylated</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-induced kinase 1</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
</tr>
<tr>
<td>PPF</td>
<td>Preformed fibrils</td>
</tr>
<tr>
<td>pSmad</td>
<td>Phosphorylated-Smad</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RCF</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>RET</td>
<td>Rearranged during transfection</td>
</tr>
<tr>
<td>rh</td>
<td>Recombinant human</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio Immunoprecipitation Assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RPM</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>RRF</td>
<td>Retrorubral field</td>
</tr>
<tr>
<td>R-Smads</td>
<td>Receptor-regulated Smads</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SBE</td>
<td>Smad Binding Element</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague Dawley</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Smad</td>
<td>Signalling mothers against decapentaplegic</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia nigra</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>SNpc</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>STR</td>
<td>Striatum</td>
</tr>
<tr>
<td>STRAP</td>
<td>Serine threonine receptor-associated protein kinase</td>
</tr>
<tr>
<td>SYN1</td>
<td>Synapsin-1</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-Buffered Saline</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TH⁺</td>
<td>TH-positive</td>
</tr>
<tr>
<td>TXTBS</td>
<td>TBS containing 0.02% Triton-X100</td>
</tr>
<tr>
<td>UPDRS</td>
<td>Unified Parkinson's Disease Rating Scale</td>
</tr>
<tr>
<td>VMAT2</td>
<td>Vesicle monoamine transporter</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
</tr>
<tr>
<td>WPRE</td>
<td>Woodchuck hepatitis virus post-transcriptional regulatory element</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>Zeb</td>
<td>Zinc finger E-box-binding homeobox</td>
</tr>
</tbody>
</table>
Chapter 1.0

General Introduction
1.1 Foreword

Neurological disorders are the leading cause of disability worldwide. Parkinson’s disease (PD) is continuously growing in prevalence, disability, and deaths and is second only to Alzheimer’s disease in terms of global incidence (Dorsey et al., 2018). There is no cure for PD and available therapies are solely symptomatic (Poewe et al., 2017). Consequently, there is an unmet clinical need to develop disease-modifying therapies that can slow down, stop or even reverse the disease progression.

1.2 Epidemiology of PD

PD is a chronic idiopathic neurodegenerative disorder, first described over 200 years ago by James Parkinson in his work ‘An Essay on the Shaking Palsy’ (Parkinson, 1817, Parkinson, 2002). PD currently affects more than 6 million people worldwide and almost 2% of the global population over the age of 65 (Dorsey et al., 2018), increasing to over 3% above the age of 80 (Pringsheim et al., 2014). The incidence of PD in 2016 was more than twice that in 1990 and is set to double by 2030, due in part to changing demographics leading to longer disease duration, and exposure to possible environmental risk factors (Dorsey et al., 2013). The estimated economic burden of PD in the US in 2017 was $51.9 billion (Yang, 2019.), while in Europe the annual cost was estimated at €13.9 billion in 2013 (Dorsey et al., 2013).

The aetiology of PD is still largely unknown, however certain genetic and environmental influences have been linked to the onset of the disease. The leading risk factor is age, with the average age of onset occurring above 60 years (Beitz, 2014, Schapira and Jenner, 2011). However, PD can also manifest itself in persons younger than 50 years, in what is known as Early Onset PD (EOPD) (Schrag and Schott, 2006). Furthermore, PD can affect persons under the age of 20, recognised as Juvenile PD, however this is not common (Klein and Westenberger, 2012). Gender is also implicated
in PD with males being twice as likely to develop the disease than females (Poewe et al., 2017). Other notable risk factors include ethnicity, exposure to environmental pesticides, obesity, β-blockers, previous head injuries, agricultural employment, well-water drinking and rural living (Beitz, 2014, Dick et al., 2007, Van Den Eeden et al., 2003). In contrast, smoking and caffeine have been associated with a decreased risk of developing PD (Li et al., 2015, Hernán et al., 2002).

While over 90% of PD cases are sporadic in origin, approximately 5-10% of cases can be attributed to hereditary genetic mutations (Klein and Westenberger, 2012, Toulouse and Sullivan, 2008). To date, six genes have been explicitly linked to inherited monogenic forms of PD. These include mutations in SNCA and LRRK2, both of which cause autosomal-dominant forms of PD and whose symptoms typically align with those of sporadic PD, while mutations in PRKN, PINK1, DJ-1 and ATP13A2 are attributed to autosomal-recessive patterns of inheritance and are linked with EOPD and slow disease progression (Klein and Westenberger, 2012, Farrer, 2006, Toulouse and Sullivan, 2008). In addition, changes in several other genes are considered to be risk factors for developing PD including UCHL1, GYGYF2, HTRA2, PLA2G6, FBX07, PARK16, GAK, MAPT, GBA, NAT2, INOS2A, GAK, HLA-DRA and APOE (Klein and Westenberger, 2012, Billingsley et al., 2018, Reed et al., 2019).

1.3 Pathophysiology of PD

PD is a progressive degenerative neurological disorder, the signature pathologies of which include the extensive loss of dopaminergic neurons and their axons from the substantia nigra pars compacta (SNpc) and the abnormal aggregation of the α-synuclein protein inside neuronal cell bodies and their presynaptic nerve terminals, known as Lewy bodies or Lewy neurites (Gibb and Lees, 1988, Kordower et al., 2013, Dickson, 2018, Spillantini et al., 1997).
1.3.1 Midbrain dopaminergic neurons

Dopaminergic neurons within the midbrain are categorised into three subpopulations – A8 in the retrorubral field (RRF), A9 in the substantia nigra and A10 in the ventral tegmental area (VTA) (German and Manaye, 1993) (Fig. 1.1). A9 midbrain dopaminergic neurons in the SN par compacta (SNpc) project to the dorsal striatum (or caudate putamen) via the nigrostriatal pathway and play a key role in the regulation of voluntary movement and motor control as part of the basal ganglia circuitry (Luo and Huang, 2016, Hegarty et al., 2013c). In contrast, A8 and A10 midbrain dopaminergic neurons project to the nucleus accumbens (ventral striatum), limbic systems, and the prefrontal cortex via the mesolimbic and mesocortical pathways. These mesocorticolimbic projections regulate a range of behaviours, including emotion, motivation, reward, and addiction (Tzschentke and Schmidt, 2000). Consequently, abnormalities within these A8 and A10 midbrain dopaminergic neurons have been implicated in psychiatric disorders such as schizophrenia and depression (Meyer-Lindenberg et al., 2002). However, the degeneration of the A9 group of midbrain dopaminergic neurons is the neuropathological hallmark of PD (Iarkov et al., 2020). These neurons project to γ-aminobutyric acid (GABA) neurons in the striatum to regulate motor control (Sumit and Puneet, 2019). Therefore, it is this selective loss of dopaminergic neurons, leading to a loss of striatal dopaminergic innervation, that forms the aetiological basis of the motor impairments associated with the disease including; resting tremor, muscular rigidity, bradykinesia, akinesia and postural and gait instability (Kordower et al., 2013, Goetz, 2011, Moustafa et al., 2016). In addition, several non-motor symptoms exist in PD, which commonly present themselves prior to the onset of the classical motor symptoms. These include olfactory dysfunction, gastrointestinal issues, sleep disorders, cognitive impairment, psychiatric symptoms, as well as pain and fatigue (Kalia and Lang, 2015, Pfeiffer, 2016).
Fig. 1.1 Schema depicting the dopaminergic pathways in the neurotypical and Parkinson’s disease brain. A9 dopaminergic neurons in the SNpc project to the dorsolateral striatum via the nigrostriatal pathway. A10 dopaminergic neurons in the VTA project to the prefrontal cortex and ventral striatum. A8 dopaminergic neurons in the RRF project to the ventral striatum. In PD there is a progressive loss of A9 dopaminergic neurons from the SNpc leading to the degeneration of the nigrostriatal pathway. Created with BioRender.com.
1.3.2 Axonal degeneration in PD

The loss of dopaminergic neurons from the SNpc is central to the clinical manifestation of PD, however where the degeneration initially occurs within the nigrostriatal pathway is unclear (O'Keeffe and Sullivan, 2018, Cheng et al., 2010). As mentioned, the axons of dopaminergic neurons extend to the dorsolateral striatum forming the nigrostriatal pathway and there now exists an array of pre-clinical, in vivo imaging and post-mortem evidence suggesting that early axonal degeneration is a key feature of PD that precedes dopaminergic neuronal loss (Caminiti et al., 2017, Burke and O'Malley, 2013, Tagliaferro and Burke, 2016, Kordower et al., 2013, Dijkstra et al., 2015). In addition, it is commonly estimated that at the time of PD onset, over 50% of dopaminergic neurons have been lost from the SN (Ross et al., 2004, Dauer and Przedborski, 2003, Lang and Lozano, 1998). However, 3 independent studies using linear regression to analyse dopaminergic neurons in the SN versus duration of PD in post-mortem brains have estimated the loss of dopaminergic neurons at the time of clinical onset to be closer to ~30% (Greffard et al., 2006, Ma et al., 1997, Fearnley and Lees, 1991), while the loss of striatal dopamine is estimated to be ~70% (Riederer and Wuketich, 1976, Cheng et al., 2010, Burke and O'Malley, 2013).

Several imaging studies have used positron emission tomography (PET) to elucidate the relationship between striatal terminals and motor symptoms (Tagliaferro and Burke, 2016). In particular, tracers for vesicular monoamine transporter (VMAT2) or dopamine transporter (DAT) have been used, as they are predominantly expressed in dopaminergic axons but are also present in cell bodies (Hersch et al., 1997, Nandhagopal et al., 2008, Fu et al., 2019). By measuring the uptake of $^{18}$F-DTBZ using PET, one study examined the level of VMAT2 activity in both the putamen and SN of patients diagnosed with PD five years previous and compared their findings to neurotypical age-matched controls.
(Hsiao et al., 2014). This study found a severe reduction of 63-76% VMAT2 in the posterior putamen while in the SN the loss was much lower at 13-29% (Hsiao et al., 2014). In support of this finding, a subsequent study examining DAT loss by measuring the uptake of $^{18}$F-FE-PE21 using PET, found a 3-fold reduction of DAT activity in the putamen and a 1.5-fold reduction in the SN of PD patients with a mean disease duration of 3 years (Fazio et al., 2015). Furthermore, a study examining $^{11}$C]FeCIT PET imaging to measure DAT activity in 36 PD patients with a mean disease duration of 22 months found a severe loss of DAT in the putamen while the SN was shown to be less affected (Caminiti et al., 2017). A substantial reduction in dopamine network connectivity was also recorded between the dorsal putamen and SN (Caminiti et al., 2017). These studies support the hypothesis that there is an extensive loss of striatal terminals and degeneration of the nigrostriatal pathway in the early stages of PD which may be central to disease progression.

In addition to the evidence from the imaging studies, post-mortem evaluation of nigrostriatal integrity has also been performed (Kordower et al., 2013, Chu et al., 2012). In this study, the brains of 28 PD patients with disease durations ranging from 1 – 27 years were analysed for tyrosine hydroxylase (TH) and DAT expression in the SN and putamen. Their findings showed that striatal fibres were only moderately affected 1 year post diagnosis but almost completely absent by years 4-5. In addition, quantification of striatal innervation at 1-3 years post diagnosis revealed a 35-75% loss in TH and DAT immunoreactivity, consistent with that seen in the Hsiao et al study above. In the SN however, there was a loss of 50-90% TH positive (TH+) neurons from the earliest time points with only subtle loss found thereafter (Kordower et al., 2013). Moreover, the same lab previously reported that the number of TH+ neurons in the SN of PD patients at stage 1 on the Hoehn and Yahr scale were not significantly different to neurotypical controls.
However, a severe loss of striatal terminals was observed (Chu et al., 2012), further implicating the loss of dopaminergic axons as a key early event in the pathogenesis of PD. Kordower et al noted that a residual population of TH+ neurons remained in the SN even decades after initial diagnosis despite the complete loss of striatal fibres (Kordower et al., 2013), suggesting that therapeutic strategies targeting these surviving dopaminergic neurons with a goal of axonal regeneration may offer potential as a disease-modifying therapy.

1.3.3 α-synuclein

α-synuclein is a protein encoded by the SNCA gene which is predominantly expressed in presynaptic terminals in the central nervous system (CNS) (Burré, 2015). The role of endogenous α-synuclein remains elusive, but increasing evidence suggests that α-synuclein is involved in synaptic plasticity and neurotransmitter release (Venda et al., 2010). α-synuclein was first linked to PD in 1997 when a point mutation in the SNCA gene was located on chromosome 4, in which alanine was replaced by threonine at position 53 (later termed A53T), was discovered in families living with an autosomal dominant inheritance of PD (Polymeropoulos et al., 1997). Since that time several other point mutations associated with autosomal dominant PD have been discovered including A30P (Kruger et al., 1998), E46K (Zarranz et al., 2004), H50Q (Appel-Cresswell et al., 2013), G12D (Lesage et al., 2013), A53E (Pasanen et al., 2014) and A53V (Yoshino et al., 2017). In addition, duplications (Chartier-Harlin et al., 2004) or triplications (Singleton et al., 2003) of the SNCA gene are also associated with autosomal dominant forms of PD, while linkage and genome-wide association studies have also identified polymeric variants in the SNCA gene in patients with idiopathic PD (International Parkinson Disease Genomics et al., 2011, Billingsley et al., 2018). α-synuclein was further linked to PD when it was discovered that aggregates of misfolded α-synuclein
were the major component of abnormal protein inclusions found throughout the CNS and peripheral nervous system (PNS) (Spillantini et al., 1997). These atypical aggregates are found in neuronal cells and their presynaptic terminals known respectively as Lewy bodies and Lewy neurites, which constitute major pathological hallmarks of PD (Spillantini et al., 1997, Meade et al., 2019). However, the pathological function of these α-synuclein inclusions remains unclear (Shults, 2006).

α-synuclein inclusions are predominately found in the neuritic processes prior to neuronal soma in the early stages of PD (Stefanis, 2012, Orimo et al., 2008, Braak et al., 1999, Burke and O'Malley, 2013). Thus, α-synuclein has been strongly linked to the axonal degeneration of dopaminergic neurons in PD (O'Keeffe and Sullivan, 2018, Burke and O'Malley, 2013). This is supported by several experimental studies, one of which utilised a transgenic mouse model expressing truncated human α-synuclein (α-Syn120) and showed that by 12 months of age, α-synuclein was heavily distributed in neuritic processes which also displayed beaded and dystrophic morphologies (Tofaris et al., 2006). In addition, there was a significant reduction in striatal dopamine levels while there was no loss in dopaminergic neurons in the SN (Tofaris et al., 2006). In this animal model, the pathology is also associated with an age-dependent relocation of synaptic proteins and a decline in dopamine release (Garcia-Reitbock et al., 2010). Likewise, in line with the pathology of human PD, transgenic mice expressing mutant A30P or A53T α-synuclein have also shown axonal degeneration to be an early and predominant feature of the disease in these animal models (Giasson et al., 2002, Lee et al., 2002, Gomez-Isla et al., 2003). Additional pre-clinical evidence using adeno-associated viral (AAV) delivery of wild-type (WT) or A30P α-synuclein into the optic nerve of rats demonstrated accelerated axonal degeneration in those axons overexpressing α-synuclein (Koch et al., 2015). While intranigral AAV-mediated delivery of A53T α-synuclein demonstrated
dystrophic axons with degenerative bulbs as early as 4 weeks post-surgery (Chung et al., 2009). In addition, significant changes in the distribution of proteins related to synaptic transmission and axonal transport were also seen in the SN and striatum (Chung et al., 2009), further implicating the role of α-synuclein in axonal degeneration in PD.

Additional in vitro studies investigating the effects of α-synuclein on primary dopaminergic neurons and induced pluripotent stem cells (iPSCs) are also shedding light on the role of α-synuclein in axonal degeneration (Koch et al., 2015, Oliveira et al., 2015). The overexpression of WT, A30P or A53T α-synuclein in primary cultures of dopaminergic neurons from embryonic day (E)14 Wistar rats was shown to cause detrimental effects on neurite morphology, autophagy, vesicle transport and axonal degeneration after only 5 days in vitro (DIV) (Koch et al., 2015). iPSCs can be generated and reprogrammed from somatic cells and differentiated into neurons (Takahashi et al., 2007). Consequently, this has led to advent of somatic cells from patients with idiopathic or familial PD being differentiated into neurons to help better understand and characterise the disease pathology, including the role of α-synuclein in axonal degeneration (Soldner et al., 2011, Devine et al., 2011, Singh Dolt et al., 2017). Such studies have uncovered impaired neuronal differentiation, lower neuronal connectivity and spine formation in iPSC derived neurons from patients with SNCA gene triplication compared to neurotypical controls (Oliveira et al., 2015). Similarly, other studies on iPSCs with SNCA triplication have shown anomalies in neurite length as well as axonal degeneration in the form of axonal blebbing and fragmentation (Lin et al., 2016b).

Moreover, iPSCs derived from patients with EOPD with an A53T SNCA mutation demonstrated Lewy-like neurites, α-synuclein aggregation and displayed varicosities positive for α-synuclein expression, neurite swelling and fragmentation as well as a compromised ability to form synapses (Kouroupi et al., 2017). These findings are
consistent with previous studies showing knockdown of α-synuclein results in increased neurite length and neurite number in iPSCs derived from PD patients with SNCA triplication (Oliveira et al., 2015). Moreover, transgenic mice expressing human α-synuclein exhibit synaptic dysfunction in striatal dopaminergic terminals and a reduction in dopamine release over time (Garcia-Reitbock et al., 2010, Hunn et al., 2015). Collectively, these studies demonstrate a fundamental link between α-synuclein pathology and synaptic and axonal degeneration.

1.4 Management of PD

Despite decades of intense investigation, there is no cure or disease-modifying therapy available for the treatment of PD. Currently, existing pharmacological treatments are solely symptomatic and centre around replenishing dopamine levels or delaying its metabolism (Raza et al., 2019, Schapira et al., 2014). Therefore, these interventions do not halt the progression of the disease nor do they reverse or restore the neuronal loss seen in the SNpc or the loss of dopaminergic axon terminals from the striatum. Since its discovery in the 1960’s, systemic delivery of Levodopa, the precursor to dopamine, remains the most potent and effective symptomatic treatment strategy for PD (Olanow, 2008, LeWitt and Fahn, 2016). Unlike dopamine, Levodopa can cross the blood brain barrier (BBB) where it is converted to dopamine and acts to alleviate the motor symptoms associated with PD (Fig. 1.2). However, prolonged treatment with Levodopa causes unwanted adverse effects such as motor response oscillations and chronic dyskinesia, which can be worse than the disease itself (Olanow, 2008). These side effects are believed to be result of discontinuous drug delivery due to the rapid half-life of Levodopa and the variability associated with its gastrointestinal absorption and transport across the BBB (Abbott, 2010). Therefore, in clinical practice Levodopa is usually withheld from the patient until all other viable options have been exhausted. However, novel sustained-
release or continuous delivery formulations of Levodopa have been developed to address these issues (Abbott, 2010).

Additional or alternative pharmacological treatment options available include aromatic amino acid decarboxylase (AADC) inhibitors such as Benzerazide or Carbidopa as well as Catechol-\(O\)-methyltransferase (COMT) inhibitors including Entacapone which act to inhibit the metabolism of Levodopa in the PNS in order to maximise the level of Levodopa reaching the CNS (Jankovic and Aguilar, 2008, Connolly and Lang, 2014). In addition, oxidation by monoamine oxidase (MAO) type B (MAOB) in glial cells plays a major role in the breakdown of synaptically released dopamine. Therefore, inhibitors of MAOB, such as Selegiline and Rasagiline, act to prolong synaptic dopamine concentrations and increase pre-synaptic reuptake of dopamine (Poewe et al., 2017). Furthermore, dopamine agonists, such as Pramipexole and Apomorphine, mimic endogenous dopamine and directly target and activate pre- and post-synaptic dopamine receptors. Dopamine agonists also have a long half-life and are often used to allow for the delayed administration of Levodopa (Jankovic and Poewe, 2012). In addition, several non-dopaminergic pharmacological therapies are also available for the treatment of motor symptoms in PD (Poewe et al., 2017).

Other treatment options accessible to PD patients include physiotherapy, speech therapy, diet therapy, exercise (Yang et al., 2015, Jankovic and Poewe, 2012). Surgical interventions are also available to alleviate motor symptoms including; Deep Brain Stimulation (DBS), which involves the targeted delivery of electrical impulses to stimulate areas of the brain involved in motor control, namely the subthalamic nucleus (Groiss et al., 2009), Thalamotomy, which involves the surgical ablation of a portion of the ventrolateral nucleus of the thalamus (Speakman, 1963) and Pallidotomy, whereby a small area in the Globus Pallidus is surgically destroyed (Lozano and Lang, 1998).
**Fig. 1.2** Dopaminergic drug targets in Parkinson disease. Presynaptic targets include Levodopa substitution in combination with peripherally active inhibitors of AADC or COMT. MAOB inhibitors act to increase the synaptic availability of dopamine while dopamine agonists act post-synaptically. Dashed arrow from blood to brain designates BBB transport of Levodopa. Dashed arrow through the DAT denotes reuptake of dopamine from the synaptic cleft. $3\text{-O-M-DOPA} = 3\text{-O-methyl-DOPA}; \text{D1R} = \text{dopamine D1 receptor}; \text{DOPAC} = 3,4\text{-dideoxy-phenylacetic acid. Adapted figure and legend (Poewe et al., 2017).}$
1.5 Emerging therapies under investigation

Given the distinct degeneration of the A9 population of dopaminergic neurons in PD, several lines of research are being investigated to combat this specific loss. Some of the most promising potential strategies include a) Cell replacement therapy which involves the transplantation of new dopaminergic neurons, generated from foetal tissue or stem cells, to the brain to replace those that have been lost (Wijeyekoon and Barker, 2009, Ganz et al., 2011, Fan et al., 2020) and b) Neurotrophic factor therapy to protect the remaining dopaminergic neurons in the SN and to regenerate the nigrostriatal dopaminergic pathway (Sullivan and O’Keeffe, 2016) (Fig. 1.3). The focus of this thesis will be on Neurotrophic factor therapy.

Fig. 1.3 The potential of Neurotrophic factor therapy in PD. A Showing the progressive degeneration of the nigrostriatal pathway in PD due to the loss of dopaminergic neurons innervating the striatum. B The administration of neurotrophic factors has the potential to protect dopaminergic neurons as well as maintain and regenerate the innervation of the nigrostriatal pathway. Adapted figure and legend (Hegarty et al., 2014b).
1.6 Neurotrophic factors – a history

Neurotrophic factors (NTFs) are naturally occurring, small secreted proteins or cytokines that regulate neuronal differentiation and maturation during development and adulthood (Sullivan and O’Keeffe, 2016, Holtzman and Mobley, 1994). Within the SN, NTFs for dopaminergic neurons are intrinsically involved in the generation of the dopaminergic transmitter phenotype, the proliferation of dopaminergic cells and their subsequent migration to the striatum (Kriegstein et al., 1995, Hefti, 1994). During this migration, NTFs are required to support axon elongation for successful innervation of their target. Neurotrophic support is therefore crucially involved in all aspects of the development, growth, proliferation, survival, migration and maturation of dopaminergic neurons (Unsicker et al., 1996). Given the loss of dopaminergic neurons in PD, the disease lends itself to NTF therapy (Hefti, 1994, Sullivan and Toulouse, 2011). Thus, the goal of NTF therapy is the application of growth factors that can protect the remaining dopaminergic neurons from degeneration and promote re-innervation of the striatum through the regeneration of the nigrostriatal pathway (Hegarty et al., 2014b, Sullivan and O’Keeffe, 2016).

However, there are several problems associated with the administration of NTFs. Systemic delivery of NTFs is not feasible due to poor bioavailability and their large molecular weight, which prevents their transportation across the BBB (Pardridge, 2007). In addition, exogenous delivery of NTFs to the brain is hampered by rapid metabolism by endogenous enzymes within the brain (Sullivan and Toulouse, 2011). Gene therapy using viral vectors to deliver neurotrophic factors to targeted areas of the brain offers the potential to evade these issues. Viral vectors have many attractive attributes which make them suitable for gene transfer including targeted delivery to specific areas of the brain reducing unwanted off-target effects, the ability to transduce neuronal cells causing long-
term transgene expression and the relatively small titre and volume needed which avoids the circulation of immunogenic material (Bartus, 2015, Kirik et al., 2017). While several approaches exist for NTF delivery, the typical strategy is the use of engineered non-replicating viral vectors including adenoviruses (AV), lentiviruses (LV) and AAV (Kelly et al., 2015, Lin et al., 2017). AAVs are predominately used to target neurons in the CNS in neurological disorders such as PD due to their natural tropism for neurons, their low rates of pathogenicity and toxicity and their ability to provide long-term targeted transgene expression (Burger et al., 2005, Lundstrom, 2018, Mijanović et al., 2020). AAVs belong to the parvovirus family and are single stranded viruses with a genome size of 4.7kb (Naso et al., 2017, Mandel et al., 2008). AAVs are replicative defective as all viral sequences are removed except for the inverted terminal repeats (ITRs), which are required for genome packaging into the AAV capsid (Mandel et al., 2006, Weitzman et al., 2003). Consequently, AAVs incite very little host immune responses. A limitation of AAV compared to AV or LV is its limited packaging capacity of 4.7kb (Kelly et al., 2015, Grieger and Samulski, 2005). However, even with this small genome size, AAVs are capable of encoding most NTFs (Shevtsova et al., 2005). AAV2 represents the prototypical AAV serotype of choice and has been used extensively in preclinical and clinical studies of PD (White, 2012, Shevtsova et al., 2005, Gasmi et al., 2007, Ren et al., 2013, Kordower et al., 2006, Marks et al., 2010, Warren Olanow et al., 2015, Bäck et al., 2013, Richardson et al., 2011, Gombash et al., 2013, Bartus et al., 2011, Su et al., 2009, Bartus et al., 2013, Herzog et al., 2007, Decressac et al., 2011, Quintino et al., 2019). A brief history of the most prevalent NTFs under review for the treatment of PD will now be discussed.
1.7 The GDNF Family

The glial cell-line derived neurotrophic factor (GDNF) family of ligands (GFLs) is a distant and distinct member of the transforming growth factor beta (TGF-β) protein superfamily comprised of four NTFs including GDNF, Neurturin, Artemin and Persephin (Airaksinen and Saarma, 2002). Unlike most members of the TGF-β family which signal via the Smad pathway, GFLs activate signalling through binding to different glycosylphosphatidylinositol (GPI)-anchored GDNF family receptor alpha (GFRα) complexes which then signal through a transmembrane receptor RET (REarranged during Transfection) tyrosine kinase (Mulligan, 2019). The GFL-GFRα complexes interact with RET’s extracellular domain which promotes dimerization and activation of its intracellular kinase domain, causing the activation of multiple downstream pathways including mitogen-activated protein kinase (MAPK) and PI3 (Creedon et al., 1997). GFLs are known to play a number of roles in cell survival and differentiation as well as neurite growth and migration in several neuronal populations. Specifically, GDNF and Neurturin have shown potent neurotrophic ability for dopaminergic neurons affected in PD (Kordower and Bjorklund, 2013)

1.7.1 Glial Cell-Line Derived Neurotrophic Factor

GDNF is a glycosylated, disulfide-bonded homodimer and was discovered and characterised in 1993 after demonstrating promising results in promoting the survival of cultured midbrain dopaminergic neurons (Lin et al., 1993). These findings were consistently mirrored in subsequent in vitro studies including cellular models of PD (Clarkson et al., 1995, Eggert et al., 1999, Widmer et al., 2000, Clarkson et al., 1997). In addition, GDNF has been shown to promote neurite growth in primary midbrain dopaminergic neurons (Lin et al., 1993, Hegarty et al., 2014c). Consequently, GDNF was tested in adult rodent and primate models where it was found to reliably improve the
survival of embryonic ventral mesencephalic (VM) dopaminergic neuronal grafts to the striatum (Apostolides et al., 1998, Espejo et al., 2000, Granholm et al., 1997, Yurek, 1998, Sullivan et al., 1998, Tomac et al., 1995, Kholodilov et al., 2004, Eslamboli et al., 2005, Dowd et al., 2005). Given such promising results in vivo, GDNF was subsequently tested in clinical trials (Nutt et al., 2003, Gill et al., 2003, Lang et al., 2006, Patel et al., 2005, John T. Slevin et al., 2005, Whone et al., 2019a, Whone et al., 2019b). However, despite initial triumphs in open-label trials (Gill et al., 2003, John T. Slevin et al., 2005), GDNF has thus far failed to reach its primary endpoints in randomised placebo-controlled studies. In addition, AAV-mediated or LV-mediated delivery of GDNF was found to be unable to protect dopaminergic neurons from α-synuclein-induced degeneration in the α-synuclein pre-clinical model of the disease (Decressac et al., 2011). A phase I open-label safety trial delivering AAV2-GDNF to patients with advanced PD is currently ongoing and will be completed in 2022 (ClinicalTrials.gov identifier: NCT01621581).

1.7.2 Neurturin

Neurturin is a naturally occurring analogue of GDNF and has been shown to demonstrate comparable neurotrophic action on midbrain dopaminergic neurons in vitro (Horger et al., 1998, Akerud et al., 1999). In addition, Neurturin is also highly potent in vivo against animal models of PD (Tseng et al., 1998, Hoane et al., 1999, Oiwa et al., 2002, Herzog et al., 2007, Kordower et al., 2006). This evidence led to the first clinical trials using AAV-mediated delivery of Neurturin to targeted areas of the brain (Bartus et al., 2013, Marks et al., 2010, Marks et al., 2008, Bartus et al., 2011). However, despite demonstrating safety and success in open label trials, the therapy failed to reach its primary endpoint in double-blind, randomised, controlled trials (Warren Olanow et al., 2015).
The failure of the GDNF and Neurturin clinical trials to date is speculated to be attributable to factors surrounding patient age and selection, catheter design and positioning as well as drug dosage and diffusion (Kordower et al., 1999, Salvatore et al., 2006, Decressac et al., 2011). Additionally, from a molecular point of view, accumulation of α-synuclein in PD (Spillantini et al., 1997, Kontopoulos et al., 2006, Stefanis, 2012), has been shown to cause downregulation of Nurr1 and its downstream effector protein, RET, the co-receptor for GDNF and Neurturin signalling (Decressac et al., 2012a, Drinkut et al., 2016). This may offer an explanation as to why both NTFs have shown limited efficacy in clinical trials, as RET has also been shown to be downregulated in PD (Decressac et al., 2012a), however evidence to the contrary has also been reported (Su et al., 2017). Therefore, it has been proposed that RET-independent NTFs should be considered in the study of NTF therapy for PD and pre-clinical evaluation of NTFs should include the α-synuclein model of PD (Warren Olanow et al., 2015).

1.8 Brain Derived Neurotrophic Factor

Brain Derived Neurotrophic Factor (BDNF) belongs to the Neurotrophin family and is produced in the endoplasmic reticulum of neural cells throughout the CNS (Palasz et al., 2020). Here, BDNF and other Neurotrophins support neuronal differentiation and maturation as well as serving as potent inhibitors of apoptosis-mediated cell death through the activation of BDNF-TrkB and IP3K/Akt kinase signalling cascades (Yoo et al., 2017). BDNF has been found to be downregulated in the blood serum and brain of PD patients, which is correlated with a greater loss of dopaminergic neurons from the SN (Huang et al., 2018, Scalzo et al., 2010, Ventriglia et al., 2013, Wang et al., 2016). BDNF has been trialed in animal models of PD through direct injection (Hung and Lee, 1996, Tsukahara et al., 1995), viral vector delivery, including transgenes to enhance
endogenous BDNF expression in the brain (Klein et al., 1999, Nam et al., 2015, Sun et al., 2005, Tronci et al., 2017, Kim et al., 2012) or non-viral vehicles (Hernandez-Chan et al., 2015, Razgado-Hernandez et al., 2015). While several studies have found BDNF to be protective of dopaminergic neurons when administered prior to neurotoxic insult (Nam et al., 2015, Kim et al., 2012, Tsukahara et al., 1995, Klein et al., 1999), others have reported no therapeutic protection of dopaminergic neurons when delivered after the insult has occurred (Hernandez-Chan et al., 2015, Kim et al., 2012, Yoshimoto et al., 1995). Yet despite the lack of effect on dopaminergic cell numbers, others have noted improved striatal dopaminergic levels (Hernandez-Chan et al., 2015) as well as better behavioural output (Kim et al., 2012, Klein et al., 1999, Hernandez-Chan et al., 2015, Razgado-Hernandez et al., 2015).

To date, BDNF has not been trialed in humans for PD but has been trialed for patients with amyotrophic lateral sclerosis (ALS), however the study failed to reach statistical significance, despite positive findings in phase I/II trials (Bradley, 1999, Ochs et al., 2000). Notably, BDNF expression and trafficking has been shown to be negatively affected by α-synuclein through the impairment of the BDNF-TrkB signalling pathway, leading to dopaminergic neuronal cell death (Fang et al., 2017, Kang et al., 2017). This raises important questions concerning the use of cellular models and animal models to mimic synucleinopathies. Investigating potential NTFs using an α-synuclein model of PD in pre-clinical studies is needed to overcome this issue and to highlight discrepancies early in the discovery phase.
1.9 Cerebral Dopamine Neurotrophic Factor and Mesencephalic astrocyte-derived neurotrophic factor

Cerebral Dopamine Neurotrophic Factor (CDNF), formerly known as Conserved Dopamine Neurotrophic factor, and Mesencephalic Astrocyte-derived Neurotrophic Factor (MANF) form a novel evolutionary conserved family with selective neurotrophic potential for dopaminergic neurons (Lindholm and Saarma, 2010). MANF is the founding member of the family and was originally identified from the culture medium of rat Type-1 astrocyte ventral mesencephalic cell line 1 when it was found to selectively promote dopaminergic neuronal survival in embryonic neuronal cultures (Petrova et al., 2003). Interestingly, MANF expression in drosophila flies is known to be crucial for the maintenance of dopaminergic neurites and dopamine levels (Palgi et al., 2009). CDNF was then later discovered as a specific paralogue to MANF (Lindholm et al., 2007). Both CDNF and MANF have been shown to reduce motor deficits and protect dopaminergic neurons in the SN and their axonal terminals in the striatum in animal models of PD (Lindholm et al., 2007, Voutilainen et al., 2009, Airavaara et al., 2012).

In addition, several studies have used viral vectors to deliver CDNF or MANF in pre-clinical models of PD and have achieved encouraging results (Bäck et al., 2013, Ren et al., 2013, Cordero-Llana et al., 2015, Hao et al., 2017). Striatal delivery AAV2-CDNF has demonstrated neuroprotection of dopaminergic neurons and restoration of behavioural deficits when delivered to the striatum in animal models of PD (Bäck et al., 2013, Ren et al., 2013). Similarly, AAV9-MANF has been shown to halt neurodegeneration and regenerate lost nerve terminals in the striatum of lesioned animals (Hao et al., 2017). An additional study investigated the combined effect of LV-mediated delivery of both CDNF and MANF to the nigra and found significant synergistic effects that resulted in a robust reduction in behavioural deficits and protection of dopaminergic
neurons and striatal fibres in lesioned animals (Cordero-Llana et al., 2015). The first clinical trial using CDNF was started in 2017 (Huttunen and Saarma, 2019). In this study, patients with moderately advanced PD were given intermittent monthly bilateral intraputamenal infusions of CDNF in randomized placebo-controlled phase I–II clinical studies (ClinicalTrials.gov identifier: NCT03295786) (Huttunen and Saarma, 2019). Preliminary results indicate that the therapy is safe for human use and comprehensive results are expected to be published shortly.

1.10 Platelet-derived Growth Factor-BB

Platelet-derived growth factor BB (PDGF-BB) is a homodimer of PDGF isoform B which has been demonstrated to have significant restorative effects on the nigrostriatal pathway in animal models of PD (Mohapel et al., 2005, Zachrisson et al., 2011). These results led to randomized, placebo-controlled phase I–IIa clinical studies in which continuous intracerebroventricular infusion of PDGF-BB was given to patients with moderate PD over a 14-day period (Paul et al., 2015). The initial results found this treatment to be safe and well received. However, no differences were found in the Unified Parkinson’s Disease Rating Scale (UPDRS) scores between treatment groups in a follow up after 3 months. Although patients who received high doses of the treatment exhibited significant increases in DAT ligand binding in PET scans compared with patients who received the placebo (Paul et al., 2015). However, after significant delays in initiating subsequent clinical trials and problems relating to the medical device and commercialisation of the therapy, the continued development of PDGF-BB therapy by Newron Pharmaceuticals SpA was discontinued in 2015 (Huttunen and Saarma, 2019).
1.11 The Bone Morphogenetic Protein family

The bone morphogenetic protein (BMP) family is the largest subgroup of the TGF-β superfamily of proteins and is comprised of at least 20 growth factors, including the Growth and Differentiation factor (GDF) family (Kawabata et al., 1998, Chen et al., 2004). BMPs were first identified by their presence in extracts of demineralized bone (Wozney, 1992) and have since been shown to induce a sequential developmental cascade of cartilage and bone formation (Nishimura et al., 2012, Miyazono et al., 2010, Wu et al., 2016), as well as playing roles in various other developmental processes (Chen et al., 2004). Much experimental evidence has demonstrated that BMPs are involved in many biological processes across various cell types, including monocytes, epithelial cells, mesenchymal cells, and importantly, neuronal cells (Miyazono and Shimanuki, 2008, Wang et al., 2014). Within these cells, BMPs are known to regulate growth, differentiation, chemotaxis and apoptosis, while also playing vital roles in the morphogenesis of almost all organs and tissues throughout embryogenesis (Hogan, 1996, Wang et al., 2014). Additionally, BMPs are crucial regulators of axonal growth in many distinct neuronal populations (Gratacos et al., 2002, Parikh et al., 2011, Hegarty et al., 2013a).

1.11.1 BMP Signalling

BMPs signal through a canonical pathway that involves transcription factors known as Smads. In this pathway, BMPs bind to a heterotetrameric complex of cell surface transmembrane type I and type II serine/threonine kinase receptors, causing the subsequent activation of Smad signalling (Weiss and Attisano, 2013, Nohe et al., 2004) (Fig. 1.4). Depending on the cellular context, BMP signalling can also occur via non-canonical pathways involving the activation of many intracellular pathways, including GTPases, MAPK and PI3K pathways (Zhang, 2009). To date, there are seven identified
type I receptors, the activin receptor-like kinases (ALK) 1–7, and five type II receptors (Hegarty et al., 2013a, Sieber et al., 2009, Miyazono and Shimanuki, 2008). There are eight distinct Smad proteins, which constitute three separate functional groupings. These include the receptor-regulated Smads (R-Smads) -1, -2, -3, -5, and -8/9, the common-
mediator Smad (Co-Smad) 4 and the inhibitory Smads (I-Smads) 6 and 7 (Wrana and Attisano, 2000, Heldin et al., 1997). Following ligand binding to the heterotetrameric receptor complex, which is composed of a type I and type II receptor dimer, the
constitutively active type II receptors intracellularly transphosphorylates the type I receptors at serine and threonine residues via a highly conserved glycine/serine (GS) domain (Miyazono et al., 2010). The phosphorylation of type I receptors leads to the recruitment and subsequent phosphorylation of the R-Smads, which can then form a heterotrimeric complex with Co-Smad 4. The I-Smads function as major negative
regulators of BMP-Smad signalling and compete with the R-Smads for both receptor binding and Co-Smad 4 binding. The I-Smads also target the receptors for degradation via the proteasome (Shi and Massague, 2003, Miyazono et al., 2005). The signalling pathway culminates with the R-Smads and Co-Smad 4 complex translocating to the nucleus, where it binds to DNA and regulates the transcription of target genes (Wrana and Attisano, 2000).

However, although there are many ligands of the TGF-β superfamily, there are very few receptors available to accommodate ligand binding. Thus, the type I and type II receptors are required to bind to more than one ligand type (Shi and Massague, 2003). There are two known type I BMP receptors (BMPRs), BMPR1A (also known as ALK3) and BMPR1B (also known as ALK6); however, BMPs are also capable of binding to ALK1 and 2. Furthermore, BMPs can bind to three type II receptors: BMPR2, activin
type 2A receptor (ACVR2A) and ACVR2B (Hegarty et al., 2013a, Miyazono et al.,
Recent studies have identified dynamin-dependent endocytosis to be a crucial regulator of the BMPRs at the surface of the cell membrane and to play a central role in the activation, temporal kinetics and magnitude of BMP signal transduction (Hegarty et al., 2017b).

The type I and II BMPRs can form the heterotetrameric complex required for Smad activation in various ways; consequently, BMP ligands have varying affinities for these combinations. For example, BMP2 binds to BMPR1A and BMPR1B with a higher affinity than it does to BMPR2. Similarly, GDF5 binds to BMPR2 and BMPR1B with greater affinity than to BMPR1A. In fact, the affinity of GDF5 for BMPR1B is at least 15-fold higher than it is for BMPR1A, signifying the fundamental importance of BMPR1B for GDF5 signalling (Nickel et al., 2005). Such varying affinities for multiple receptor complexes is relevant when studying the effects of potential NTFs in different cell lines. In addition, various studies on the structural and functional features of the BMPRs have highlighted a cytoplasmic loop segment, known as the L45 loop, located adjacent to the GS domain on type I receptors, which is involved in R-Smad binding. A corresponding loop, the L3 loop, is also present on the R-Smads and is involved in determining the specific Smad substrates for the type I receptor kinases. Smads 1, 5 and 8 are the pivotal effectors that are primarily linked to members of the BMP/GDF subfamily, while Smads 2 and 3 are typically associated with TGF-βs, Activins and Nodals (Mueller and Nickel, 2012).
Fig. 1.4 Schematic of BMP-Smad signalling from cell membrane to the nucleus.

BMP ligands bind to a heterotetrameric complex of type I and type II serine/threonine kinase cell surface receptors causing the constitutively active type II receptor to intracellularly phosphorylate the type I receptors. R-Smads and I-Smads compete for binding at the receptor complex for phosphorylation and subsequent complexation with Co-Smad 4. The complex formed with Co-Smad 4 translocates to the nucleus where it binds to the DNA and alters transcription (Goulding et al., 2020).
1.11.2 BMP expression in the nervous system

Several studies have characterized the temporal expression profiles of the various BMPs and their receptors in the developing nervous system. *BMPR1* and *BMPR2* are stably expressed in the brain throughout adulthood, in several regions including the cortex, striatum, hippocampus and SN (Chen et al., 2003, Miyagi et al., 2011). The expression of *BMPR1B* and *BMPR2* receptors in the rat VM is present from E11 onwards, after which it is continuous right through to adulthood to at least post-natal day (P) 90 (Hegarty et al., 2014a). Similarly, *BMPR1B* and *BMPR2* transcripts are expressed in the mouse VM from E12 onwards (Hegarty et al., 2017c). Notably, this BMPR temporal expression profile parallels the growth of nigrostriatal dopaminergic projections as TH, a key marker of dopaminergic neurons, is expressed at E13 and peaks at E15 (O'Keeffe et al., 2004b). In addition, both BMPR1b and BMPR2 are found to be colocalised with TH in VM dopaminergic neurons (Hegarty et al., 2014a). These findings suggest that BMP-Smad signalling plays an active role in regulating nigrostriatal projections towards their target, the dorsal striatum. Indeed, BMP-Smad signalling has been shown to be involved in neural crest formation, modelling of the dorsal spinal cord, and has been heavily implicated in neuronal and glial development (Hegarty et al., 2013a). The sustained expression of the BMPRs through to adulthood would also suggest their role in the maintenance of dopaminergic neurons. In strong support of this, a study using BMPR2 dominant negative (BMPR2DN) adult male mice highlighted a significant 20% reduction in the number of VM dopaminergic neurons and a radical loss of almost 90% in striatal innervation. These mice also displayed lower locomotor ability when compared WT controls (Chou et al., 2008a). Therefore, it would appear that a neurotrophic role for endogenous BMP-Smad signalling exists in dopaminergic neuronal development and maintenance.
It is now also known that the initial blocking of BMP-Smad signalling in embryonic and adult iPSCs can lead to high rates of dopaminergic neuronal generation (Chambers et al., 2009, Cai et al., 2013). Yet, the role of BMP-Smad signalling in the later stages of midbrain dopaminergic development, until very recently, remained unclear. Jovanovic et al (Jovanovic et al., 2018) have now described experimental evidence to support BMP-Smad signalling as a critical regulator of mammalian dopaminergic neurogenesis in vivo, through modulating the proliferation and differentiation of progenitor cells. Here, they found that Smad 1 inactivation in the neural stem cells (NSCs) of mice in vivo caused an obstruction to progenitor cells differentiating into dopaminergic neurons, through the prevention of cell cycle exit of SN neurons (Jovanovic et al., 2018).

In addition to the BMPRs, transcripts for the ligands BMP2-8 are expressed from E12 to E19 in several pivotal regions in the CNS (Jordan et al., 1997) while transcripts for BMP2, -4, -5, -6 and -7 are shown to continually express throughout the cortex, hippocampus, striatum and nigrostriatal system in the adult rat brain (Jordan et al., 1997, Soderstrom and Ebendal, 1999, Chen et al., 2003). Likewise, GDF5 (also known as BMP14) mRNA and protein expression begins at E12 in the VM and striatum, where it reaches a peak at E14 and continues through to adulthood (Gavin et al., 2014, Krieglstein et al., 1995, O’Keeffe et al., 2004b). Furthermore, mRNA transcripts for BMPs -2, -3, -4, -6, -7 and GDF-5 have shown to be heavily abundant in the E14 rat dorsal root ganglia (DRG) and are implicated in the modulation of neurotrophins on sensory neurons (Farkas et al., 1999). Interestingly, like the BMPRs, transcripts for BMP2, BMP6 and GDF5 are found to be expressed in the developing midbrain floor region during dopaminergic neurogenesis, a time when neurotrophic support is crucial, prior to target innervation (Jordan et al., 1997, Hegarty et al., 2014c). Given that the temporal expression profiles
for several BMPs evolve in the VM and coincide with the generation of dopaminergic neurons, this suggests that BMPs are neurotrophic factors for this specific neuronal class. In support of this, several BMPs have been shown to possess potent neurotrophic potential for midbrain dopaminergic neurons (Table 1.1). Within the BMP family, the most promising and extensively studied members include GDF5 and BMP2. Therefore, the focus of this thesis will be on these two ligands. Evidence for the neurotrophic action of both GDF5 and BMP2 in vitro and in vivo will be discussed below.

Table 1.1 The effect of BMP ligands on dopaminergic neurons in vitro and in vivo

<table>
<thead>
<tr>
<th>BMP Ligand</th>
<th>Study Type</th>
<th>Effect on dopaminergic neurons</th>
<th>Reference</th>
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<tbody>
<tr>
<td>BMP2</td>
<td>In vitro</td>
<td>Promotes survival and differentiation of midbrain dopaminergic neurons</td>
<td>(Jordan et al., 1997, Stull et al., 2001, Reiriz et al., 1999, Hegarty et al., 2014a)</td>
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<tr>
<td></td>
<td>In vitro</td>
<td>Increases differentiation of NSCs into dopaminergic neurons</td>
<td>(Yan et al., 2016)</td>
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<td></td>
<td>In vitro</td>
<td>Promotes differentiation of SH-SY5Y cells</td>
<td>(Hegarty et al., 2013b)</td>
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<td></td>
<td>In vivo</td>
<td>Increases survival of dopaminergic neurons in 6-OHDA lesioned rat model</td>
<td>(Espejo et al., 1999)</td>
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<td></td>
<td>In vivo</td>
<td>Decreases rotational behaviour in 6-OHDA lesioned rat model</td>
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<td>BMP4</td>
<td>In vitro</td>
<td>Promotes survival of dopaminergic neurons</td>
<td>(Jordan et al., 1997)</td>
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<td></td>
<td>In vitro</td>
<td>Induces dopaminergic differentiation</td>
<td>(Stull et al., 2001)</td>
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<td>BMP5</td>
<td><strong>In vitro</strong></td>
<td>Promotes survival of dopaminergic neurons</td>
<td>(Jordan et al., 1997, Brederlau et al., 2002)</td>
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<td></td>
<td></td>
<td>Increases the number of TH+ neurons in iPSCs and NSCs</td>
<td>(Jovanovic et al., 2018)</td>
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<tr>
<td></td>
<td><strong>In vivo</strong></td>
<td>Promotes dopaminergic neurogenesis</td>
<td>(Jovanovic et al., 2018)</td>
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<td>BMP6</td>
<td><strong>In vitro</strong></td>
<td>Promotes survival of dopaminergic neurons</td>
<td>(Jordan et al., 1997, Brederlau et al., 2002)</td>
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<td></td>
<td></td>
<td>Induces dopaminergic differentiation</td>
<td>(Stull et al., 2001)</td>
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<td>BMP7</td>
<td><strong>In vitro</strong></td>
<td>Promotes survival of dopaminergic neurons</td>
<td>(Jordan et al., 1997, Brederlau et al., 2002)</td>
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<td></td>
<td></td>
<td>Antagonises effects of Methamphetamine neurotoxicity</td>
<td>(Chou et al., 2008b)</td>
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<tr>
<td></td>
<td></td>
<td>Increases the number of TH+ neurons in iPSCs and iNSCs</td>
<td>(Jovanovic et al., 2018)</td>
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<td></td>
<td><strong>In vivo</strong></td>
<td>Decreases rotational behaviour in 6-OHDA lesioned rat models</td>
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<td></td>
<td></td>
<td>Restores KCl evoked dopamine release to striatum</td>
<td>(Harvey et al., 2004)</td>
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<td>Partially protects dopaminergic neurons after 6-OHDA administration</td>
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<td>Antagonises effects of Methamphetamine</td>
<td>(Chou et al., 2008b)</td>
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<td>Promotes dopaminergic neurogenesis</td>
<td>(Jovanovic et al., 2018)</td>
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<td>BMP12</td>
<td><strong>In vitro</strong></td>
<td>Promotes survival of dopaminergic neurons</td>
<td>(Jordan et al., 1997)</td>
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<tr>
<td>GDF5</td>
<td><strong>In vitro</strong></td>
<td>Increases survival of dopaminergic neurons</td>
<td>(Krieglstein et al., 1995, Jaumotte and Zigmond,</td>
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<tr>
<td>Protects dopaminergic neurons against MPP⁺ neurotoxic insult</td>
<td>Krieglstein et al., 1995</td>
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<tr>
<td>Promotes differentiation of dopaminergic neurons</td>
<td>Hegarty et al., 2014a, O'Keeffe et al., 2004a</td>
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<tr>
<td>Promotes survival and differentiation of SH-SY5Y cells</td>
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<td>Protects SH-SY5Y cells against 6-OHDA neurotoxicity</td>
<td>Toulouse et al., 2012</td>
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<td>Protects dopaminergic neurons against iron toxicity</td>
<td>Lingor et al., 1999</td>
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<td>Protects dopaminergic neurons against 6-OHDA neurotoxicity</td>
<td>O'Sullivan et al., 2010</td>
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<td>Decreases rotational behaviour in 6-OHDA lesioned rat models</td>
<td>Sullivan et al., 1998, Hurley et al., 2004, O'Sullivan et al., 2010, Costello et al., 2012</td>
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<tr>
<td>Increased survival of dopaminergic neurons in 6-OHDA lesioned rat models</td>
<td>Sullivan et al., 1998, Hurley et al., 2004, Costello et al., 2012</td>
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<td>Promotes survival of dopaminergic neurons after 6-OHDA lesion</td>
<td>Hurley et al., 2004</td>
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<td>Increases mean somal area and total neurite length in TH⁺ neurons</td>
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<td>Increases total neurite length of dopaminergic neurons</td>
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1.1.3 Evidence for GDF5 and BMP2 as neurotrophic factors for dopaminergic neurons

1.1.3.1 Evidence for the neurotrophic effects of GDF5 in vitro

GDF5 and its signalling receptors share a co-expression pattern with TH in VM dopaminergic neurons, suggesting that GDF5 may offer trophic support to these neurons to promote their survival and growth. Many studies have investigated this premise, the first of which examined the effect of 20ng/ml of GDF5 in its active dimeric form on E14 VM cultures for up to 8 DIV and found there was a 1.6-fold increase in the survival of TH⁺ neurons compared to untreated controls (Krieglstein et al., 1995). Consistent with this, additional studies using 1ng/ml or 10ng/ml GDF5 induced a 3.1- and 2.6-fold increase in TH⁺ neurons, respectively (O’Keeffe et al., 2004a), while also significantly increasing axon length and branching. Furthermore, both doses of GDF5 led to...
noteworthy increases in the number of astrocytes present in the culture, suggesting a possible association between astrocytes and GDF5 induced signalling, as also documented in previous studies (Krieglstein et al., 1995, Wood et al., 2005). However, the positive effects of GDF5 on TH+ neuron can also occur independently of astrocytes indicating that GDF5 acts directly on dopaminergic neurons themselves, as has been shown in glial depleted E14 VM cultures (Wood et al., 2005). In this study, the authors aimed to compare the neurotrophic effects of GDF5 with that of GDNF using E14 VM cultures. Here, 1, 10 or 100ng/ml doses of GDNF or GDF5 were added to normal cultures and to glial depleted cultures every 48 hours for a period of 8 DIV. GDF5 increased the survival of TH+ neurons at all doses up to 6 DIV, while 100ng/ml was also effective at 8 DIV. The same results were achieved for GDNF (Wood et al., 2005). In glial depleted cultures, 1ng/ml GDF5 was tested as this dose was shown to significantly increase both TH+ neurons and GFAP-positive (GFAP+) astrocytes. Here, the authors found no significant difference in the number of TH+ neurons compared with normal cultures at any time point. GDNF was not tested in these cultures as no significant increases in GFAP+ astrocytes were found for any dose of GDNF. These results illustrate that while treatment of GDF5 may contribute to astroglial proliferation, indicating a role for GDF5 in the regulation of neural progenitors, this is independent of its trophic effects on dopaminergic neurons. Interestingly, the authors also investigated a co-treatment of E14 VM cultures with 1ng/ml GDNF and 1ng/ml GDF5 and found a substantial increase of 11.6% in TH+ cell number compared to cultures treated with GDF5 (5.1%) or GDNF (4.9%) alone (Wood et al., 2005). The reason for this additive effect was postulated to be due to the differential expression patterns of the receptors required by these NTFs to elicit signalling, indicating that a combination therapy of NTFs may warrant investigation.
Given that GDF5 is capable of promoting TH+ survival in mesencephalic cultures, the question remained of whether GDF5 could also protect these TH+ neurons from neurotoxic insult. To answer, one study employed E14 VM cultures treated with 20ng/ml GDF5 one day prior to the administration of 0.5mM of the selective dopaminergic toxin 1-methyl-4-phenylpyridinium (MPP+) for 4 DIV (Krieglstein et al., 1995). MPP+ was found to reduce TH+ neuronal survival by 67% while co-treatment with GDF5 saw a reduction of just 30%, indicating a partial protective effect of dopaminergic neurons (Krieglstein et al., 1995). An additional study investigated the potential of GDF5 to protect against free radical damage given the evidence of their role in neurodegenerative disorders (Lingor et al., 1999). Here, E14 VM cultures were given 10ng/ml GDF5 for one week prior to addition of 30uM FeCl$_2$ or 10uM sodium-nitroprusside (SNP) on day 7 for 24 h. Treatment with GDF5 induced a significant 1.7-fold increase in TH+ cell survival compared to controls. However, no protection was achieved against SNP (Lingor et al., 1999). The suggested reason for this was due to a difference in cell death pathway between FeCl$_2$ and SNP, where GDF5 may not be effective. However, this study was the first of its kind to highlight the potential of GDF5 in rescuing dopaminergic neurons from free radical injury and death. This is important given the evidence of elevated iron deposits in the brains of PD patients (Hirsch et al., 1991, Sofic et al., 1991).

In contrast to these positive findings, other research groups have reported unfavourable outcomes. One particular study treated E14 VM cultures with 30uM of the selective dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA) for 6 DIV, prior to the addition of GDF5 on day 7 (Brederlau et al., 2002). After assessing thymidine incorporation, immunohistochemistry and immunofluorescence, GDF5 was found to have had no protective or survival promoting effects on these cells. However, the authors note that caution is needed when making conclusions about 6-OHDA work in vitro, as
glial cells, which offer potent protective effects, can also be affected. While, in vivo, this compound is known to act more selectively on dopaminergic neurons (Brederlau et al., 2002). To coincide with this work, a recent study employed cultures of the SN isolated from P0 pups and investigated the effects of five different NTFs against exposure to 10-500µM MPP⁺ (Jaumotte et al., 2016). Here, cultures were exposed to MPP⁺ and treated either 1 h prior to MPP⁺ exposure, during MPP⁺ exposure or 48 h after MPP⁺ exposure with 100ng/ml GDNF, BDNF, TGF-β, fibroblast growth factor 2 (FGF-2) or GDF5. However, no survival enhancing effects were seen for any of the NTFs when tested alone. However, given in combination, a significant increase in survival of TH⁺ cells in the SN cultures was seen at 32.6 ± 5.13% after exposure to 10µM MPP⁺ for 30 minutes, and 29.1 ± 8.56% after exposure to 500µM MPP⁺ for 30 minutes (Jaumotte et al., 2016). This apparent synergy could be as a result of elevated NTF receptor ‘hits’ on dopaminergic cells, altered signalling cascades and the fact that each NTF not only acts on dopaminergic neurons, but also non-dopaminergic cells whose paracrine signalling may serve as a critical influence to dopaminergic neuron survival (Jaumotte et al., 2016). The fact that no NTF alone was found to promote survival in this study may be due to oversaturation of the relevant receptors above certain doses of each NTF (Jaumotte et al., 2016), rendering high concentrations of these factors null and void. Still, these results again reiterate the potential of a combination therapy using a cocktail of NTFs for the treatment of PD.

1.11.3.2 Evidence for the neurotrophic effects of GDF5 in vivo

To complement the neurotrophic effects of GDF5 seen in vitro, the actions of GDF5 in vivo are also quite compelling. The first group to investigate GDF5 in vivo examined both the monomeric (inactive) and dimeric (active) form of the protein in the 6-OHDA rat
model of PD (Sullivan et al., 1997). Here, 50ug of the monomer or dimer was injected into the left SN and left lateral ventricle prior to a 6-OHDA lesion into the left median forebrain bundle (MFB). One-week post-surgery, animals underwent behavioural testing in the form of amphetamine induced rotations. Animals which had received the 6-OHDA lesion as well as animals receiving the lesion combined with the monomeric form of GDF5 displayed a rotation rate indicative of a 95% depletion of the nigrostriatal pathway. In contrast, control animals and animals who had been administered the dimeric form of GDF5 with 6-OHDA did not rotate at all, inferring an intact nigrostriatal system (Sullivan et al., 1997). A subset of each experimental group also underwent PET scanning using a dopaminergic transporter tracer to measure binding potential (BP). Again, animals which had received the 6-OHDA lesion and animals receiving the lesion combined with the monomeric form of GDF5 had a BP suggestive of a complete loss of dopaminergic uptake within the striatum. While control animals and animals administered the dimeric form of GDF5 with 6-OHDA exhibited an intact striatum (Sullivan et al., 1997). Post-mortem High Performance Liquid Chromatography (HPLC) analysis examining dopamine and its metabolites, 3,4-Dihydroxyphenylacetic acid (DOPAC) and Homovanillic acid (HVA), found similar levels between the control group and GDF5 dimer with 6-OHDA group while the 6-OHDA group and 6-OHDA with GDF5 monomer group had a marked decrease across all 3 metabolites. Furthermore, TH immunostaining confirmed that the 6-OHDA and GDF5 monomer with 6-OHDA had extensive losses of TH⁺ neurons within the SN and VTA while TH⁺ neurons in the GDF5 dimer with 6-OHDA group were largely spared (Sullivan et al., 1997). These results demonstrate that GDF5 promotes survival against dopaminergic toxins *in vivo* and can protect not only the nigral cell bodies of dopaminergic neurons but their striatal nerve terminals too. These effects are also corroborated in a functional capacity, as demonstrated by the behaviour testing and
dopaminergic reuptake. Thus, establishing GDF5 early on as a strong candidate for NTF therapy for treatment of PD.

In a follow up study, the group investigated the effectiveness of GDF5-treated foetal mesencephalic grafts in the 6-OHDA lesioned rat model versus GDNF-treated or untreated transplants (Sullivan et al., 1998). Here, rats were lesioned in the MFB 4 weeks prior to grafting. Rats received untreated mesencephalic transplants or transplants which had been suspended in solutions containing 500ug of GDNF or GDF5 (monomer or dimer) in buffer, prior to transplantation into the striatum. Amphetamine-induced rotation rates were assessed over an 11-week period. The mean rate for lesion-only animals was 12.6 ± 2.8, graft-only animals was 2 ± 0.4, GDF5 monomer-treated animals was 1.6 ± 0.3 while animals receiving GDF5 dimer-treated or GDNF-treated grafts displayed no rotations (Sullivan et al., 1998). Furthermore, PET scans were undertaken to assess the BP in the striatum. Intact control rats had a BP of 0.71 ± 0.07, while all 6-OHDA lesioned animals prior to transplant had a mean BP of 0.3 ± 0.04. Post-graft, animals receiving the graft only had a mean BP of 0.4 ± 0.04, GDF5 monomer-treated grafts had a mean BP of 0.36 ± 0.04, GDF5 dimer-treated grafts had a mean BP of 0.65 ± 0.04 while GDNF-treated grafts had a mean BP of 0.67 ± 0.06 (Sullivan et al., 1998). In terms of TH⁺ cell survival, a deposit of TH⁺ cells was visible in all groups who had received a VM transplant. However, the grafts pre-treated with GDF5 dimer or GDNF displayed a significant increase in number and density of TH⁺ neurons compared with the graft alone or the GDF5 monomer treated graft (Sullivan et al., 1998). This work was the first of its kind to show the neurotrophic and neuroprotective effect of GDF5 on treated foetal mesencephalic transplants. Notably, this study also highlighted that GDF5 treatment is equally as beneficial as GDNF which is important in the pursuit of new functional therapies given the failures of GDNF in clinical trials thus far.
While GDF5 has been shown to be effective in protecting TH+ neurons from intrastriatal 6-OHDA lesioning when administered concurrently, the question of whether GDF5 is restorative post-lesion, is a major area of interest. In an effort to answer this, one study administered 25µg/5µl GDF5 into the left striatum or left SN, one or two weeks post 6-OHDA lesion into the left striatum (Hurley et al., 2004). Amphetamine-induced rotation testing showed all 6-OHDA lesioned animals rotated at least 5 turns/minute after lesioning, indicative of a partial striatal lesion. Animals receiving GDF5 to the striatum at one-week post-lesion, but not at two weeks, had significantly reduced rotations. Animals receiving GDF5 into the SN at one and two weeks post-lesion also had significantly decreased rotation rates to 6-OHDA alone, three weeks post-surgery (Hurley et al., 2004). Post-mortem analysis of the left SN showed a 60% loss of TH+ neurons in the 6-OHDA group in comparison to its contralateral side, while animals injected with GDF5 one-week post lesion into the SN or striatum had a 30-35% loss of TH+ neurons from the SN. However, animals injected with GDF5 two weeks post lesion into the SN or striatum conferred no protection to nigral dopaminergic neurons. In the striatum of 6-OHDA animals, dopaminergic fibre density was reduced to 50% of the contralateral side, and animals injected with GDF5 one or two weeks post lesion into the SN or striatum were not significantly different to 6-OHDA animals (Hurley et al., 2004). The partial lesion model used in this study is more reflective of the pathological degeneration seen in PD, compared to complete lesion via the MFB. However, the fact that GDF5, delivered via the SN or striatum, was ineffective in sparing striatal fibres is not entirely surprising considering when 6-OHDA is injected into the striatum, the degeneration of axons begins immediately and is complete within 24 h (Rosenblad et al., 1999). Therefore, administration of GDF5 one or two weeks post-lesion, is most likely too late for axonal protection using this model of PD, a result also found with intranigral delivery of GDNF.
(Kirik et al., 2001). The fact remains however that a single dose of GDF5 injected into the SN or striatum, can significantly protect nigral cell soma from injury or death induced by 6-OHDA lesioning weeks prior - a major breakthrough.

Given that NTFs are rapidly metabolised in vivo, one study has looked at the overexpression of GDF5 in E13 VM tissue using cationic lipid-mediated gene delivery (O'Sullivan et al., 2010). Here, cells were transfected with plasmids for enhanced green fluorescent protein (EGFP) as the control, GDF5 or pcDNA (mock transfection). Twenty four h post transfection, cells were treated with 50uM 6-OHDA for 30 minutes, then processed for immunocytochemistry. Analysis of the cultures revealed that the cells transfected with the GDF5 plasmid had significantly lower levels of TH+ cell death. The number of TH+ cells was also significantly higher than the control or mock transfected cultures (O'Sullivan et al., 2010). Given the efficacy of this system, the authors tested these GDF5 transfected E13 VM cultures in the 6-OHDA model of PD. Animals received a 6-OHDA lesion to the striatum two weeks prior to receiving the GDF5 transfected cells, mock-transfected cells or freshly dissected tissue. Amphetamine induced rotations were used to assess motor function (O'Sullivan et al., 2010). Post-lesion, all animals rotated ipsilaterally with 8 turns per minute. Two weeks post-graft, those receiving the GDF5 transfected transplant displayed significantly lower rotation rates than that of 6-OHDA alone, control animals or mock transfected animals. Moreover, post-mortem analysis showed the presence of TH+ cells in all rats who had received a graft. While somal area and total neurite length were significantly higher in animals receiving GDF5 transfected cell transplants compared to mock transfected or freshly dissected transplant groups (O'Sullivan et al., 2010). This study was the first to demonstrate the feasibility of transfections prior to transplantation of foetal tissue, which could easily be applied to
other cell types such as neural progenitors or stem cells, given the quantity of the foetal tissue required for viable grafts.

In an additional study, a Chinese hamster ovary (CHO) cell line was stably transfected with the human GDF5 gene in order to examine the effects of GDF5 production after transplantation into the rat striatum (Costello et al., 2012). Here, E14 VM cultures were supplemented with concentrated medium from GDF5-CHO cells or Mock-CHO cells. In E14 VM cultures with GDF5-CHO medium, GDF5 precursors and mature GDF5 were secreted for at least 15 days. There was also a 2-fold increase in the number of TH+ cells. For E14 VM cells with Mock-CHO medium, there was no GDF5 secretion and no increase to TH+ cell number (Costello et al., 2012). To test these cells in vivo, rats received a 6-OHDA lesion to the MFB. GDF5-CHO or Mock-CHO cells were then delivered in three ways: injected into the striatum at the same time as 6-OHDA lesion, injected into the striatum one week after 6-OHDA lesion, or injected into the SN one week after 6-OHDA lesion. Rats receiving GDF5-CHO transplants expressed GDF5 for at least one week. Amphetamine-induced rotation testing revealed 6-OHDA animals and 6-OHDA+Mock-CHO (same time) animals rotated at a rate of 8 turns/minute. 6-OHDA+GDF5-CHO (same time) animals had significantly lower rotations than both 6-OHDA alone and 6-OHDA+Mock-CHO animals (Costello et al., 2012). Animals receiving GDF5-CHO into the striatum or SN 7 days post-lesion also displayed significantly decreased rotations compared with 6-OHDA or 6-OHDA+Mock-CHO. Four weeks post-surgery, animals who had received GDF5-CHO transplants at the same time as the 6-OHDA lesion or one week post-lesion into the striatum or SN, had significantly higher numbers of TH+ neurons in the SN compared to 6-OHDA alone or 6-OHDA+Mock-CHO (Costello et al., 2012). This data once again highlights the
protective and restorative effects of GDF5 and shows that sustained, targeted and long-term delivery of GDF5 can be achieved.

1.1.3.3 Evidence for the neurotrophic effects of BMP2 in vitro

The first evidence for a neurotrophic effect of BMP2 came in 1997 when recombinant human (rh)BMP2 was demonstrated to exert neurotrophic effects on cultured E13-15 rat VM midbrain dopaminergic neurons (Jordan et al., 1997). Specifically, treatment with 10ng/ml rhBMP2 increased the survival of TH+ midbrain dopaminergic neurons, by 1.5-fold compared to that in controls, after 8 DIV. The same study demonstrated that BMP2 treatment of midbrain dopaminergic cultures resulted in increased numbers of 5-bromodeoxyuridine (BrdU)-positive and GFAP+ cells, markers of cell proliferation and astrocytes, respectively. These early findings indicated not only does BMP2 promote the survival of midbrain dopaminergic neurons, but that it also appears to play a selective role in the proliferation and differentiation of neural progenitors. A further study also demonstrated positive effects of BMP2 treatment on midbrain dopaminergic neurons in E14 VM cultures. Specifically, cultures treated with BMP2 displayed a concentration-dependent increase in the number of midbrain dopaminergic neurons, reaching a maximum increase of 237% after treatment with 10ng/ml, compared to vehicle-treated controls after 7 DIV (Reiriz et al., 1999). In addition, BMP2 treatment increased the number of primary neurites and neurite branching in TH+ cells in a concentration-dependent manner, reaching a maximal effect with 1ng/ml rhBMP2 (Reiriz et al., 1999). Similarly, other studies have shown a significant increase in the neurite length of TH+ neurons in E14 rat VM primary cultures after treatment with 200ng/ml rhBMP2 (Hegarty et al., 2014a).
One study investigated whether BMP2-induced effects were mediated by astroglial cells and reported that addition of 10ng/ml BMP2 had no effect on the number or morphology of astroglial cells in E14 VM cultures (Reiriz et al., 1999). They further showed that BMP2-induced increases in TH⁺ cell numbers were not altered in the presence of 30µM α-amino adipic acid, a gliotoxin that eliminates GFAP-immunoreactive astrocytes (Reiriz et al., 1999). Interestingly however, this study did report that treatment with 100ng/ml BMP2 led to significant increases in both the number and morphology of astrocytes (Reiriz et al., 1999). These findings suggest that, at low doses, BMP2 treatment promotes both the number and morphological differentiation of midbrain dopaminergic neurons, while at higher doses, it also influences the number and phenotype of astroglial cells (Reiriz et al., 1999). Several studies have reported that the neurotrophic effects of BMP2 and BMP signalling often occur in tandem with, and are amplified by, other NTFs. For example, BMP2-induced signalling in E19 rat striatal neurons was found to be facilitated by co-administration of either BDNF or Neurotrophin-3 (NT-3) (Gratacos et al., 2001). However, there is limited data available on the interactions of BMPs with other NTFs in the context of dopaminergic neurons, and this will be an important point to consider in future research.

In addition to the neurotrophic effects of BMP2 reported in studies on primary VM cultures, similar effects have been demonstrated in SH-SY5Y cells, a cell line which, despite some limitations, is a widely-used in vitro model of human midbrain dopaminergic neurons (Xie et al., 2010, Hegarty et al., 2016b, Xicoy et al., 2017, Goulding et al., 2019). Specifically, SH-SY5Y cells treated with 200ng/ml rhBMP2 for 4 DIV displayed an increase in total neurite length (Hegarty et al., 2013b). This supported additional studies which showed that BMP2 treatment induced neuronal differentiation of midbrain dopaminergic neurons (Hegarty et al., 2014a, Reiriz et al., 1999). Treatment
with BMP2 was also found to activate the canonical Smad 1/5/8 signalling pathway in SH-SY5Y cells to induce neurite growth (Hegarty et al., 2013b). A causal link between neurite growth and BMP signalling was demonstrated when it was shown that pretreatment with dorsomorphin, a selective small molecule inhibitor of BMPR1 receptors (Yu et al., 2008, Hegarty et al., 2013b), completely prevented BMP2-induced differentiation of SHSY5Y cells (Hegarty et al., 2013b). In addition, dorsomorphin treatment completely blocked BMP2-induced Smad activation. Similar experiments on E14 VM cultures showed that dorsomorphin blocked both morphological differentiation of midbrain dopaminergic neurons and activation of the Smad pathway, which had been induced by BMP2 treatment (Hegarty et al., 2013b, Hegarty et al., 2014a). Collectively, these studies show that the phenotypic effects exerted by BMP2 are directly dependent on the activation of the Smad 1/5/8 signalling pathway in midbrain dopaminergic neurons. (Hegarty et al., 2013b, Hegarty et al., 2014a).

1.11.3.4 Evidence for the neurotrophic effects of BMP2 in vivo

To support the positive neuroprotective effects of BMP2 seen in the in vitro studies, there are some promising data regarding its in vivo effects. One study reported that pretreatment with 50ng/ml rhBMP2 could maintain cell viability in cultures of E14 VM for up to 6 h, compared to untreated control cultures which demonstrated a 25% decrease in cell viability over the same period (Espejo et al., 1999). A subsequent in vivo study showed that treatment of E14 rat VM with 50ng/ml rhBMP2 prior to grafting into the striatum of 6-OHDA-lesioned rats resulted in a significant increase in the survival of the grafted dopaminergic neurons (Espejo et al., 1999). This neuroprotective effect of BMP2 pretreatment in vivo was substantial at one week after grafting, and remained, although at a lower level, after four weeks (Espejo et al., 1999). After one week, the rats that had
received BMP2-treated VM grafts showed no difference in motor impairment, as measured by apomorphine-induced rotations, than those that had received untreated VM grafts; however after four weeks, rats with BMP2-treated VM grafts displayed significant reductions in rotations, compared to those with untreated grafts. Furthermore, the morphology of the grafted dopaminergic neurons was notably more differentiated in the rhBMP2 pre-treated group than in the untreated graft group at four weeks post-grafting (Espejo et al., 1999). In light of these studies showing the in vivo potential of BMP2 treatment, it will be important to test both recombinant BMP2 and viral vectors carrying the BMP2 transgene in neurotoxin and α-synuclein in vivo models of PD.

As well as the neurotrophic effects of BMP2 described above, another study has highlighted the potential of BMP2 to induce differentiation of neural stem cells (NSCs) in vitro (Yan et al., 2016). This is an important finding, given the low survival rate, and considering the ethical and logistical difficulties involved, of human foetal VM tissue grafts for treatment of PD (Olanow et al., 2003, Ishii and Eto, 2014). Specifically, the Yan study employed five doses of BMP2 (that is 0.1, 1, 10, 20 and 100 ng/ml) over a 14-day period, to treat NSCs harvested from E14 VM cells. This study found that 20 and 100 ng/ml doses of BMP2 induced the highest increases in the number of dopaminergic neurons, while also increasing their neurite length (Yan et al., 2016). This effect of BMP2 on NSC differentiation was induced via miR-145-mediated upregulation of Nurr1, a key player in the maintenance of dopaminergic neurons (Yan et al., 2016). NSCs offer a viable therapeutic approach to PD therapy as the cells are self-renewing and can be differentiated into all neural lineage cells, therefore showing promising potential to be used for replacement of those midbrain dopaminergic neurons that are lost from the PD brain.
Collectively, GDF5 and BMP2 have been shown to be potent neurotrophic factors for dopaminergic neurons and have demonstrated neuroprotective capabilities against several commonly used dopaminergic neurotoxins including MPP+ and 6-OHDA. The substantial evidence reviewed here in support of these morphogens would suggest that their neurotrophic properties warrant further investigation as novel therapeutic agents against PD. In addition, the fact that both GDF5 and BMP2 mediate their neurotrophic effects exclusively via the BMP-Smad signalling is also important as the authors of the recent Neurturin clinical trial have suggested that “better results might be achieved with other trophic factors that are not RET dependent” (Warren Olanow et al., 2015). However, there has been little evidence to date on the neurotrophic effects of GDF5 and BMP2 on promoting dopaminergic axon growth and protecting these axons against neurotoxin induced axonal degeneration. In addition, there is no evidence to date on the potential neuroprotective action of GDF5 and BMP2 against α-synuclein-induced degeneration in vitro or translational models of PD. Moreover, whether α-synuclein disrupts the BMP-Smad signalling pathway is currently unknown. These issues are important to address in order to justify the further development of GDF5 and BMP2, or any other BMP ligand, as therapeutic interventions for PD.

1.12 Small molecule activators of the BMP signalling pathway
Currently, the only practical method of delivering NTFs to the brain is via direct injection. While this approach is safe for patients and clinically feasible (Bartus et al., 2014, Marks et al., 2008, Bartus et al., 2013), it is dependent on the remaining dopaminergic neurons in the SN expressing the full complement of proteins needed to respond to the specific NTF being administered. The consequences of this requirement were highlighted by the failure of GDNF to protect against α-synuclein in vivo due to the down regulation of the
RET receptor required for pathway activation (Decressac et al., 2012a, Decressac et al., 2011). One way to circumvent this challenge is by identifying small molecule drugs or biologics that are can cross the BBB and that are capable of selectively activating the signalling pathway of the desired NTF or their downstream effectors, which would bypass the need for the NTF itself and allow for the advent of non-invasive therapies (O’Keeffe et al., 2017).

In this regard, small molecule activators of the BMP-Smad signalling pathway have previously been identified. CTPB (\((N-(4\text{-chloro-3-trifluoromethyl-phenyl})\text{-2-ethoxy-6-pentadecyl-benzamide})\) is a small molecule activator of p300/CBPs, which are histone acetyltransferases (HATs) known to interact with Smad effectors to modulate BMP-Smad mediated gene transcription (Pouponnot et al., 1998, Pearson et al., 1999, Balasubramanyam et al., 2003). In SH-SY5Y cells, CTPB has been shown to promote neurite growth to a similar extent to that of GDF5 and BMP2 and promotes survival against 6-OHDA-induced cell death (Hegarty et al., 2016a). In addition to HATs, histone deacetylase (HDACs) inhibitors (HDIs) are potent transcriptional regulators whose neurotrophic effects on dopaminergic neurons have been widely characterised (Gardian et al., 2004, Kidd and Schneider, 2011, St Laurent et al., 2013, Kontopoulos et al., 2006, Outeiro et al., 2007). Class-IIa HDIs have recently been shown to promote neurite length in SH-SY5Y cells and E14 cultures of dopaminergic neurons and protects these cells from neurotoxin-induced degeneration (Collins et al., 2015). Moreover, HDIs have been demonstrated as activators of the BMP-Smad pathway and regulators of neurite growth via BMP signalling (Collins et al., 2015, Mazzocchi et al., 2019). Pre-clinical testing of HDACs in animal models of PD is currently underway and will further elucidate the potential use of HDACs to target dopaminergic neurons as a therapeutic approach in PD.
In addition to downstream transcripational regulators of the BMP-Smad pathway, modulators of BMP expression have also been discovered. A recent study has identified Quinacrine and Niclosamide, both of whom are Food and Drug Administration (FDA) approved medicinal drugs, as potent modulators of BMP2 expression in cultures of human tendon tissue and in a BMP2 reporter cell line (Ghebes et al., 2017). Quinacrine is an acridine derivative that has previously been used as an antimalarial compound but has since been superseded by Chloroquine in recent years (Ehsanian et al., 2011). Quinacrine has also been used as an anthelmintic and as a therapy for giardiasis. In addition, Quinacrine has been used to treat connective tissue diseases, including lupus erythematosus and rheumatoid arthritis and was once used as a form of contraception/sterilisation (Macfarlane and Manzel, 1998, Gurova, 2009, Chumanevich et al., 2016, Gardner and Hill, 2001, Wozniacka et al., 2002, Lippes, 2015). Quinacrine has been shown to activate p53 and superoxide dismutase and is an inhibitor of NF-κB and phospholipase A2 (Gurova et al., 2005, Gasparian et al., 2011, Al Moutaery and Tariq, 1997, Kara et al., 2014, Jani et al., 2010). Consequently, these anti-inflammatory and anti-cancer properties have led to Quinacrine being used in the treatment of several cancers (Jani et al., 2010, Dermawan et al., 2014, Fasanmade et al., 2001, Preet et al., 2012). Importantly, Quinacrine has been shown to protect dopaminergic neurons against neurotoxic insult in animal models of PD (Tariq et al., 2001).

Niclosamide is a salicylamide derivative and an anthelmintic drug, most widely used to treat tapeworm infections (Kadri et al., 2018). The mechanism of action of Niclosamide is poorly understood however several studies have discovered that Niclosamide is a potent uncoupler of oxidative phosphorylation and can modulate Wnt/β-catenin, mTORC1, STAT3, NF-κB and Notch signalling pathways (Chen et al., 2018, Frayha et al., 1997, Satoh et al., 2016, Li et al., 2014). Consequently, there is growing
interest in the therapeutic potential of Niclosamide for a range of diseases and disorders including Type 2 Diabetes, Mileus, Bacterial Infections, Viral Infections, Cancer, Neuropathic pain, Endometriosis and Rheumatoid Arthritis (Chen et al., 2018, Kadri et al., 2018, Arend et al., 2016, Tao et al., 2014, Lin et al., 2016a, Lu et al., 2011, Sack et al., 2011, Rajamuthiah et al., 2015, Wu et al., 2004, Prather et al., 2016). Niclosamide has also been investigated for potential use in the treatment of PD, as Niclosamide activates PTEN-induced kinase 1 (PINK1) signalling in cultured cortical neurons (Barini et al., 2018). This is important given that mutations in PINK1 are linked with autosomal recessive EOPD (Valente et al., 2004).

Given that Quinacrine and Niclosamide have been found to be potent modulators of BMP2 expression in primary tendon cells, together with the evidence for BMP2-mediated neurotrophic action in dopaminergic neurons, this suggests that Quinacrine and Niclosamide may also demonstrate neurotrophic action in dopaminergic neurons through the upregulation of BMP2 expression and/or the activation of the BMP-Smad signalling pathway. Therefore, these compounds warrant further investigation for their potential use as a treatment for PD.
1.13 Conclusion and Objectives

Neurotrophic factor therapy holds significant promise as a disease-modifying therapy in PD, however clinical trials to date using GFLs has yielded disappointing outcomes. In light of this, it has been suggested that better results may be realised using RET-independent NTFs. GDF5 and BMP2 are members of the BMP family which activate the RET-independent, canonical Smad signalling pathway. GDF5 and BMP2 have shown potent potential as NTFs in dopaminergic neurons against *in vitro* and pre-clinical models of PD but have not yet been investigated against α-synuclein-induced degeneration either *in vitro* or *in vivo*. In addition, experimental therapies to date, both protective and restorative, have notoriously targeted dopaminergic cell loss with no focus on the degeneration of axons. Therefore, the initial aim of this thesis is to identify the specific neurotrophic effects of GDF5 and BMP2 on dopaminergic neurons and their axons *in vitro* against cellular models of PD, including α-synuclein. Following these experiments, the second objective will be to evaluate the neuroprotective effect of viral mediated delivery of GDF5 and BMP2 against α-synuclein-induced degeneration *in vivo* in the α-synuclein rat model of PD. The final objective of this thesis will be to investigate the therapeutic potential of the FDA approved small molecule drugs, Quinacrine and Niclosamide, in dopaminergic neurons as a novel therapeutic approach for the treatment of PD.
Chapter 2.0

Materials & Methods
2.1 Cell culture

All experiments were performed in a sterile laminar flow Class II Microflow Biological Safety cabinet, wearing appropriate garbing and gloves and using aseptic techniques. All materials used were of tissue culture grade or had been sterilised via autoclaving at 121°C or filtered through a 0.20μm filter.

2.1.1 Harvesting and dissociation of the SH-SY5Y cell line

SH-SY5Y cells were stored in vials of freezing medium consisting of 70% Dulbecco’s modified Eagle’s medium (DMEM) F-12 Ham (Sigma), 20% foetal bovine serum (FBS) (Sigma) and 10% dimethyl sulfoxide (DMSO) (Sigma), under liquid nitrogen. For use in cell culture experiments, cells were thawed at room temperature (RT) and immediately centrifuged at 500 rotations per minute (rpm) for 5 minutes (min). The supernatant was then discarded, and cells were resuspended in 20 ml of fresh SH-SY5Y cell culture medium. The culture medium used was DMEM F-12 Ham, supplemented with 10% FBS, 100nM L-glutamine (Sigma) and 100U/ml penicillin and 10μg/ml streptomycin (Sigma). The cells were grown in T75 flasks and incubated in a humidified atmosphere containing 5% CO₂ at 37°C. At 80-90% confluency the cells were passaged. Cells were passaged three times prior to use in experiments. To passage the cells, the spent medium was removed; the cells were briefly washed with 3 ml of Hank’s Balanced Salt Solution (HBSS) to remove residual media before enzymatic cleavage of the cells in 3 ml of 0.2% Trypsin for 5 min at 37°C. 7 ml of serum-containing fresh media was added to the flask to neutralise the trypsin and was mixed to ensure homogeneity using a pipette. The cell solution was transferred into a 15 ml sterile tube and was centrifuged at 500 rpm for 5 min to pellet the cells. The supernatant was then removed, 1 ml of fresh media was added to the sterile tube and the cells were resuspended using a flame polished Pasteur pipette. A sample of the cell solution was then diluted 1:10 in fresh culture medium. 10 μl of this
solution was then used to count the cells using a haemocytometer and the following formula:

$$\frac{Cells\ counted \times 10 \ (dilution\ factor) \times 10^4 \ (haemocytometer\ constant)}{5 \ (number\ of\ grids\ counted)} = cells/ml$$

The cells were cultured such that each T75 flask contained 4 million cells in 20 ml fresh medium. For experiments in 24 well plates, 500 µl of the required cell solution was added per well. In 12 well plates, 1 ml was added per well and in 6 well plates 2 ml was added per well. This process was repeated as required. Where indicated, SH-SY5Y cultures were treated with 0-200ng/ml rhBMP2 (Gibco), 0-200ng/ml rhGDF5 (Peprotech), 0-10nM Niclosamide (Sigma), 0-10nM Quinacrine (Sigma), 0-15µM 6-OHDA (Sigma) or 0-1.5mM MPP⁺ (Sigma) or pre-treated with 1µg/ml dorsomorphin (Sigma) 30 min prior to the addition of rhBMP2, rhGDF5, Niclosamide or Quinacrine. Virkon solution was added to SH-SY5Y cells, spent media and other cell culture waste prior to discarding into an autoclave waste bin.

### 2.1.2 Poly-D-lysine coating of culture plates

24-well plates were coated using 130 µl of a 0.1mg/ml solution of Poly-D-lysine (Sigma) and incubated in the cell culture hood for 15 minutes. The solution was then removed, and the surface of each well was washed 3 times with sterile water. The coated plates were allowed to dry for 1-2 hours (h) before experimental use or were stored at -20°C until required. Prior to use, coated plates were incubated at 37°C for 30 min and placed in the cell culture hood to evaporate any condensation before introducing cells and media.

### 2.1.3 Harvesting of the E14 rat VM

Time-mated Sprague-Dawley (SD) rats (Biological Services Unit, UCC) were terminally anaesthetised using isoflurane (Abbott Laboratories Ltd.) in a fit-for-purpose bell jar
inside a Class I laminar flow cabinet. Once anaesthetised, the rats were decapitated using a guillotine. The E14 embryos were removed from the pregnant rat via laparotomy and placed in a 90mm petri dish containing HBSS and kept on ice. This procedure was carried out under license with full ethical approval. The embryos were then removed from their yolk sac and their crown-to-rump length was measured to verify the correct age with a 1.0-1.2cm crown-to-rump length indicative of E14. The mesencephalon was dissected out by incision at the midbrain-hindbrain boundary, and at the forebrain-midbrain boundary. The dorsal mesencephalon was then cut along the midline, and the mesencephalon was then levelled out to display the VM in the centre. A cut at a point between the lateral to medial VM was then made on both sides to isolate the VM. The meninges were removed, leaving the VM intact. Dissected VMs were stored in a sterile 15 ml Eppendorf tube in HBSS on ice until all the embryos were dissected. Primary cultures of the E14 rat VM were then established immediately upon completion of the VM dissection.

2.1.4 Primary cultures of the E14 rat VM

E14 VM tissue was immediately centrifuged post dissection at 1100 rpm for 5 min. The HBSS was removed and the pelleted tissue pieces were enzymatically dissociated in 1.5ml Trypsin-HBSS and incubated for 5 min at 37°C with 5% CO$_2$. 1ml of the Trypsin-HBSS was removed and replaced by 1ml FBS to neutralise the trypsin. The tissue pieces were then centrifuged at 1100 rpm for 5 min. The supernatant was discarded, and the pellet of tissue was then mechanically dissociated in 1ml of DMEM F-12 containing 100nM L-Glutamine, 6mg/ml D-Glucose, 100 U/ml Penicillin, 10μg/ml Streptomycin, with 2% B-27 supplement, and 1% FBS, using a p1000 pipette, followed by a 25 gauge needle and 1 ml syringe. Cell density was calculated as per section 2.1.1. To allow the cells to adhere to the wells, 50 μl of the required cell suspension was added to each well
of a poly-D lysine coated plate and incubated for 1 h at 37°C and 5% CO₂. The wells were then ‘flooded’ with 450μl of fresh medium and incubated at 37°C and 5% CO₂ for the length of the experiment. Fresh medium was applied to the cells every 2-3 days.

### 2.2 Fixation, blocking and immunocytochemical staining of cultured cells

Spent media was aspirated from each well and SH-SY5Y cells were fixed in 4% Paraformaldehyde (PFA; Sigma) for 15 min at RT. E14 VM cultures were fixed in ice cold methanol, or 4% PFA in the case of transfections or viral infections, for 15 min at RT. The cells were then washed using 0.02% Triton X-100 (Sigma) in 10mM phosphate buffered saline (PBS-Tx), 3 times for 5 min per wash. The cells were blocked using 5% bovine serum albumin (BSA) in 10mM PBS for 1 h at RT. Depending on the experiment, the cells were then incubated overnight at 4°C in the appropriate primary antibodies including; TH (1:2000; Abcam or Merck Millipore), α-synuclein (1:2000; Merck Millipore), Phospho-Smad (1:200; Cell Signalling Technologies), Total-Smad (1:200; Abcam), BMPR1A (1:200; R&D Systems or Thermo Fisher), BMPR1B (1:200; R&D Systems or Thermo Fisher), BMPR2 (1:200; R&D Systems or Thermo Fisher), GDF5 (1:200; Abcam) or BMP2 (1:200; Abcam) diluted in 1% BSA. The cells were then washed 3 times with PBS-Tx for 5 min each. The appropriate fluorescent secondary antibodies: Goat anti-Mouse IgG H&L Alexa Fluor® 488 or 594 (1:500; Invitrogen) or Goat anti-Rabbit IgG H&L Alexa Fluor® 488 or 594 (1:500; Invitrogen) were diluted 1:500 in 1% BSA in 10mM PBS and applied to the cells for 2 h in the dark at RT. Following three 5 min washes in PBS-Tx, 4′-6-Diamidino-2-phenylindole (DAPI) was diluted (1:3000) in 10mM PBS and applied to the cells to stain the nuclei for 5 min at RT in the dark. The cells were then washed with PBS, for three 5 min washes. PBS was added to each well to prevent dehydration and the plates were stored at 4°C until visualised using the Olympus IX71 inverted microscope.
2.3 MTT Cell Viability Assay

SH-SY5Y cells were plated at a density of 5×10⁴ cells per well and treated for 3 DIV prior to performing the assay. Media was slowly aspirated and 300μL of 0.5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) in HBSS was added to each well and the cells were incubated for 3 h at 37°C in 5% CO₂. The MTT-HBSS solution was then carefully aspirated and 100μL of DMSO was added to each well to permeabilise any formazan produced by the cells. 75μL of the DMSO/formazan solution in each well was pipetted into a 96 well plate and the absorbance of each sample was measured at 620nm using an A600 plate reader (Thermo Fisher).

2.4 Transfection of cultured cells

SH-SY5Y cells and E14 VM cultures were transfected with 0.5–1 μg of plasmid deoxyribonucleic acid (DNA) or 250ng of the Smad Binding Element (SBE)-GFP reporter assay (Qiagen) using the TransIT-X2® Dynamic Delivery System (Mirus Bio) according to the manufacturer’s instructions. SH-SY5Y cells were seeded at a density of 2.5×10⁴ cells per well and allowed to reach ≥80% confluency prior to transfection. E14 VM cultures were seeded at a density of 1.5×10⁵ cells per well. The TransIT-X2:DNA complex was prepared according to the manufacturer’s specifications using serum-free medium, TransIT-X2 reagent and 0.5–1μg/μl stock of a control EGFP plasmid (Control-GFP) or EGFP-α-synuclein-WT (αSynWT-GFP) or EGFP-α-synuclein-A53T (αSynA53T-GFP) plasmids. These were a kind gift from David Rubinsztein (Addgene plasmids #40822 and #40823). The transfection efficiency for SH-SY5Y cells and E14 VM cells was 31% and 3%, respectively.
2.5 Phospho-Smad Enzyme Linked Immunosorbent Assay (ELISA)

Activation of the Smad pathway by rhBMP2 was shown using the SMAD1 (Total/Phospho) Human InstantOne™ ELISA Kit (Invitrogen). In brief, SH-SY5Y cells were seeded at a density of 5\times10^4 cells per well and treated with 50ng/ml rhBMP2 or rhGDF5, 1µg/ml dorsomorphin or pre-treated with 1µg/ml dorsomorphin 30 min prior to treatment with rhBMP2 or rhGDF5 for 2 h. Cells were then lysed and prepared for the ELISA as per the manufacturer’s instructions.

2.6 Transduction of E14 VM cultures

E14 VM cultures were plated at a density of 1.0\times10^5 per well of a 24 well plate and transduced with AAV2/5-GDF5, AAV2/5-BMP2, AAV2/6-GFP or AAV2/6-α-synuclein to achieve a multiplicity of infection (MOI) of 5.0\times10^4 to 2.0\times10^5, as indicated in the text.

2.7 Analysis of neuronal complexity and nuclear intensity

5 randomly-selected cell bodies or neurites were measured in no less than 15 randomly-selected fields per N, yielding at least 225 cell bodies or neurites analysed per experiment. To verify the number of TH^+ cells and total cells, TH and DAPI counts were completed in 15 randomly-selected fields and the numbers of TH^+ cells were expressed as percentages of the total DAPI-stained cells. All experiments were repeated at least 3 times and all analyses were performed using Image J software.

2.8 Western Blotting

2.8.1 Protein extraction from SH-SH5Y cells and E14 VM Cells

Spent medium was aspirated from 24-well plates containing SH-SY5Y cells or E14 VM cultures. The cells were then washed with 1 ml PBS to remove residual media. 200 µl of RIPA lysis buffer (50 mM Tris pH 7-8, 150 mM sodium chloride (NaCl), 0.1% SDS,
0.5% sodium deoxycholate, 1 mM Sodium Fluoride, 1mM Sodium Orthovanadate) was added to each well and the plate. The adhered cells were scraped from each well using a cell scraper and aliquoted into eppendorf tubes, which were incubated on ice for 1 h. The eppendorf tubes were then centrifuged at 16000 rpm for 10 min to pellet any cell debris and the supernatant was stored at -80°C.

### 2.8.2 BCA assay

This was performed using the Pierce™ Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher) as per the manufacturer’s instructions. Briefly, to prepare the BCA working reagent, 10 ml of Reagent A and 200 µl of Reagent B were added to a centrifuge tube and mixed thoroughly using a pipette. 2 mg/ml BSA was then diluted in series using 500 µl dH₂O to prepare a set of standards. The final concentrations of the standards were as follows: 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 and 0.03125 mg/ml. 20 µl of each standard was added in duplicate to a 96 well plate. 20 µl of each protein sample of interest were also added in duplicate. 200 µl of the BCA reagent was added to each well, the plate was then covered and incubated at 37°C for 30 mins. The absorbance of each well was measured at 562nm using a plate reader. The protein concentration of each sample was determined using Microsoft Excel.

### 2.8.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Using the Bio-Rad Discontinuous System approach, SDS-PAGE was performed using a 10% resolving gel and 5% stacking gel in 1X Running buffer (80% deionised (d)H₂O, 20% 5X Running buffer (7.2% Glycine, 1.5% Tris Base, 0.5% SDS in 1L dH₂O)) on the Power-Pac Basic (Bio-Rad, USA). The protein samples were prepared in 2X Sample
Loading Buffer (40mM Tris-Hydrochloric Acid (HCl) pH 6.8, 2% SDS, 15% Glycerol, 6% 2-Mercaptoethanol (β-ME), 0.025% Bromophenol blue in dH2O) and loaded onto the gel and electrophoresed at 80V for 30 min, followed by 120V for 1 h 30 min. The proteins were transferred from the gel using Transfer buffer (60% dH2O, 20% Methanol, and 20% 5X Transfer Buffer (7.2% Glycine, 1.5% Tris Base)) onto a methanol pre-soaked polyvinylidene difluoride (PVDF) membrane at 100V for 1 h. Prior to transfer, the PVDF membrane was soaked in methanol for 15-30 seconds to activate the membrane, followed by immersion in dH2O for 5 min and finally soaking in transfer buffer for 15 min. Post transfer, the membrane was stained using the Ponceau S removable stain in order to visualise the protein bands. The membrane was then washed with Phosphate Buffered Saline containing 0.1% Tween® 20 (PBS-T) 3 times for 10 min per wash. The membrane was blocked in PBS-T containing 3% BSA for 1 h at RT or overnight at 4°C. The membrane was then incubated in primary antibody for: GAPDH (1:1000; SCBT) and α-synuclein (1:5000; Merck Millipore), diluted in 1% BSA, for 1 h at RT or overnight with shaking at 4°C. The primary antibody was removed, and the membrane was washed 3 times with PBS-T for 10 min per wash. The membrane was then incubated with the appropriate secondary antibody for 1 h, with shaking, at RT. The membrane was washed 3 times with PBS-T for 10 min per wash and protein bands were visualised using enhanced chemiluminescence.

### 2.8.4 Enhanced Chemiluminescence (ECL)

The Pierce™ ECL Western Blotting Substrate kit was utilised as per the manufacturer’s instructions (Thermo Fisher). Briefly, each detection reagent was added at a 1:1 ratio to an eppendorf and mixed thoroughly using a pipette. The membrane was then coated with the mixed solution for 1 min. The excess solution was then removed, and the membrane
was covered with a clear plastic sheet for protection. The membrane was then exposed to an AGFA CP1000 film which was developed using an automatic developer.

2.9 Bacterial transformations and plasmid purification

2.9.1 Bacterial transformations

100ng of plasmid DNA (EGFP, EGFP-α-synuclein-WT or EGFP-α-synuclein-A53T) was added to 50µl of competent DH5α cells (Sigma) on ice 30 min. The bacteria and plasmid mixture was then heat shocked at 42°C for 45 seconds and replaced on ice for 2 min. 250µl of Luria-Bertani (LB) Broth (Sigma) without antibiotic was added to each bacteria and the plasmid mixture was incubated at 37°C shaking at 250 oscillations per min for 45 min. The bacteria and plasmid mixture was then spread on an LB Agar (Sigma) 96 mm petri dish containing 100µg/ml ampicillin (Sigma) or 50µg/ml kanamycin (Sigma), depending on the plasmid, and incubated upside-down at 37°C overnight.

2.9.2 Isolation and culture of transformed bacteria

A single colony of transformed bacteria was isolated from the LB Agar petri dish using a pipette tip to inoculate a starter culture containing 5ml of LB broth containing the appropriate antibiotic. This culture was incubated at 37°C, with vigorous shaking (~250 oscillations per min) for 8 h. 1ml of starter culture was then aseptically transferred into a conical flask of 200ml LB Broth containing the appropriate antibiotic as indicated in section 2.9.1 and incubated at 37°C shaking (~250 oscillations per min) overnight.

2.9.3 Plasmid DNA purification

Plasmid purification was carried out using the Plasmid Midi Kit (Qiagen) as per the manufacturer’s instructions. Briefly, the bacterial cells were harvested by centrifugation at 6,000 relative centrifugal force (rcf) for 15 min at 4°C. The bacterial pellet was re-
suspended and lysed using the buffers provided, for the specified time. Lysis buffer was added for 5 min and the resulting mixture was incubated at RT before the addition of the neutralisation buffer to precipitate out the genomic DNA, proteins and cell debris. This mixture was incubated on ice for 15 min before centrifugation at 20,000 rcf for 30 min at 4°C. The supernatant was then removed and centrifuged at 20,000 rcf for 30 min at 4°C. The clear supernatant was then passed through the Qiagen anion-exchange tip where the plasmid DNA selectively bound to the resin and impurities in the flow-through were discarded. The resin was washed twice with 10 ml of QC buffer before the plasmid DNA was eluted in 5 ml of QF elution buffer. 3.5 ml of isopropanol (Sigma) was added to precipitate the DNA, and the precipitate was centrifuged at 15,000 rcf for 30 min at 4°C. The supernatant was removed, and the pellet was washed with 2 ml of 70% ethanol. The pellet was re-dissolved in 200 μl of nuclease-free water, and the DNA concentration in ng/μl was determined using a Nanodrop.

2.10 Bioinformatics

Human SN gene expression data from healthy controls (GSE:60863) (Ramasamy et al., 2014) and age-matched control and PD patients (GSE:49036) (Dijkstra et al., 2015) were analysed separately using the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl). Pearson correlation analysis with a false discovery rate (FDR) multiple testing correction was used to identify all genes that were co-expressed with five dopaminergic neuronal markers: ALDH1A1, NR4A2, KCNJ6, TH and LMX1B in the SN, and four markers enriched in Purkinje cells in the cerebellum: PDE1A1, CBLN1, PDE9A, CALB1 (GSE:60863).
2.11 GDF5 and BMP2 In Vivo Studies

2.11.1 Ethical approval

Procedures concerning live animals were performed under licence in accordance with Irish and European directive 2010/63/EU following full ethical approval by University College Cork Animal Experimentation Ethics Committee and a personal and project license issued from the Health Products Regulatory Authority (HPRA) (AE19130/P057).

2.11.2 Animal Husbandry and Study Design

Adult female SD rats were purchased from Envigo, UK, and maintained on a 12h:12h light:dark cycle with access to food and water ad libitum. In the case of the GDF5 study, animals were assigned to one of the following four experimental groups: AAV-Control (n=20), AAV-α-synuclein (n=20), AAV-GDF5 (n=20) or AAV-α-synuclein+AAV-GDF5 (n=20). In the case of the BMP2 study, animals were assigned to one of the following four experimental groups: AAV-Control (n=20), AAV-α-synuclein (n=20), AAV-BMP2 (n=20) or AAV-α-synuclein+AAV-BMP2 (n=20). Rats were housed in groups of four in standard housing cages containing environmental enrichment. All experiments were conducted fully in accordance with the European Directive 2010/63/EU and under an authorisation granted by the HPRA.

2.11.3 Virus Preparation

An α-Synuclein plasmid was generously donated from Dr Eilis Dowd (National University of Ireland, Galway) and Professor Deniz Kirik (Lund University, Sweden). AAV2/6-α-synuclein, AAV2/5-GDF5, AAV2/5-BMP2, AAV2/5-Null and AAV2/6-Null viral vectors were produced by Vector Biosystems Inc, Philadelphia, USA. Briefly, AAV2 ITRs coding for human WT α-synuclein or human GDF5 or BMP2 were packaged.
using AAV6 or AAV5 capsid proteins, to produce AAV2/6 and AAV2/5 viral vectors and the corresponding empty control vectors. Transgene expression was driven by synapsin-1 promoter (SYN1) and enhanced using woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). The final titre for AAV2/5-Null was $1.0 \times 10^{13}$ gc/ml, for AAV2/6-Null it was $5.3 \times 10^{12}$ gc/ml, for AAV2/6-α-synuclein it was $5.2 \times 10^{13}$ gc/ml, for AAV2/5-GDF5 it was $1.3 \times 10^{13}$ gc/ml and for AAV2/5-BMP2 it was $5.0 \times 10^{12}$ gc/ml.

### 2.11.4 Stereotaxic Surgery

All surgeries were performed using aseptic procedures, including sterile gloves, masks, sterile instruments and aseptic techniques. Aseptic surgery was performed using procedures that limit microbial contamination to lessen the possibility of any significant infection or suppuration. This included preparation of the animal, preparation of the person performing the procedure, sterilisation of instruments, supplies, and implanted materials, and the use of operative techniques to reduce the likelihood of infection. Good surgical technique included asepsis, gentle tissue handling, minimal dissection of tissue, appropriate use of instruments, effective haemostasis, and correct use of suture materials and patterns. Aseptic technique was followed through for all procedures. Preoperative analgesia was administered by the non-steroidal anti-inflammatory analgesic drug carprofen (Rimadyl®, 5mg/kg) in the animals’ drinking water. Stereotaxic surgery was conducted under general anaesthesia induced by the inhalation agent isoflurane. Once anaesthetised, the head was shaved, the animals were positioned in a stereotaxic frame and the scalp was sterilised using 70% ethanol and betadine. Local anaesthetic (Lidocaine) was applied to the incision site. Using a sterile scalpel, a small incision (approximately 1.5-2 cm) was made in the midline of the scalp. The skin was gently
retracted to expose the skull surface. Once bregma was located and with the aid of a stereotaxic guide and the use of a small electric drill, a small hole of approximately 1 mm in diameter was made through the skull over the brain region of interest.

All vector combinations were given unilaterally into the SN at coordinates anteroposterior (AP) -5.3, mediolateral (ML) ± 2.0, and dorsolateral (DV) -7.2 relative to bregma, at an infusion rate of 1µl/min with an additional 2 min for diffusion. At the end of surgery, the skull was gently swabbed with sterile physiological saline and the skin sutured closed. The sutured wound was then cleaned with sterile saline. Animals then received the Carprofen subcutaneously and 5% glucose solution via intraperitoneal injection. Following surgery, the animals were transferred to clean cages which were kept on heating pads until the animals were moving normally about the cage. Animals were treated with oral carprofen once daily for a further two days following surgery. The animals were closely monitored for any signs of pain, infection or distress for 7 days post-surgery and once weekly thereafter for 6 weeks using a HPRA-approved scoring rubric.

For the GDF5 study:

AAV-Control animals were administered 2 µL AAV2/5-Null (1.0×10^{10} vg/µL) + AAV2/6-Null (5.3×10^{9} vg/µL). AAV-α-synuclein animals were administered 2 µL AAV2/6-α-synuclein (5.2×10^{10} vg/µL) + AAV2/5-Null (1.0×10^{10} vg/µL). AAV-GDF5 animals received 2 µL AAV2/5-GDF5 (1.3×10^{10} vg/µL) + AAV2/6-Null (5.3×10^{9} vg/µL). AAV-α-synuclein+AAV-GDF5 animals received 2 µL AAV2/6-α-synuclein (5.2×10^{10} vg/µL) + AAV2/5-GDF5 (1.3×10^{10} vg/µL).
For the BMP2 study:

AAV-Control animals were administered 3 µL AAV2/5-Null (1.0\times10^{10} \text{ vg/µL}) + AAV2/6-Null (5.3\times10^9 \text{ vg/µL}). AAV-α-synuclein animals were administered 3 µL AAV2/6-α-synuclein (5.2\times10^{10} \text{ vg/µL}) + AAV2/5-Null (1.0\times10^{10} \text{ vg/µL}). AAV-BMP2 animals received 3 µL AAV2/5-BMP2 (5.0\times10^9 \text{ vg/µL}) + AAV2/6-Null (5.3\times10^9 \text{ vg/µL}). AAV-α-synuclein+AAV-BMP2 animals received 3 µL AAV2/6-α-synuclein (5.2\times10^{10} \text{ vg/µL}) + AAV2/5-BMP2 (5.0\times10^9 \text{ vg/µL}).

2.12 Behavioural Testing

Motor function was assessed using the Stepping Test of forelimb akinesia (Olsson et al., 1995), the Whisker Test of sensorimotor integration (Schallert et al., 2000), the Corridor Test of lateralised response (Dowd et al., 2005) or the Cylinder Test of independent forelimb use (Schallert et al., 2000). Each of which will be described below.

2.12.1 Stepping Test

The stepping test of forelimb akinesia was performed as previously described (Olsson et al., 1995). Animals were held with both hindlimbs and one forelimb restrained with the remaining free forelimb resting on the countertop. The animal was then moved sideways across a countertop at a steady pace (90cm in 15 seconds) and the numbers of adjusting steps taken with the unrestrained forelimb over both directions was counted.

2.12.2 Whisker Test

The Whisker Test measures sensorimotor integration and has been previously characterised (Schallert et al., 2000). This test is based on the sensorimotor reflex underlying vibrissae-elicited movement whereby the animal’s whiskers are brushed
against a surface (e.g. corner of a table) and this elicits a reflex which causes the rat’s ipsilateral limb to move, which results in the animal placing its paw on the table surface. During testing, the animal’s whiskers on one side of the body were gently brushed off the corner of the table, triggering the animal to place their paw on the tabletop (“vibrissae-elicited forelimb placings”). This was performed 10 times for each side of the body and the number of placings were recorded.

2.12.3 Cylinder Test

The Cylinder test was used to assess forelimb use, as previously described (Schallert et al., 2000). The test measures a rat’s forepaw use while exploring a novel cylindrical environment. Since this test takes advantage of an animal’s drive to explore a novel environment by standing on the hindlimbs and leaning towards the enclosing walls of a cylinder, no habituation to the test itself was required. To perform this test, animals were placed individually into a glass cylinder (4L beaker: 17cm diameter and 27cm height). The number of wall contacts performed independently with the left and the right forepaw were counted per rat. The test was deemed complete once a total of 20 wall contacts had been made or until an allotted time of 5 min had elapsed. The number of wall contacts per forelimb and the time taken were recorded. The number of times the rat touched the side walls with its contralateral paw was then expressed as a percentage of the total touches made.

2.12.4 Corridor Test

The Corridor Test measures contralateral sensorimotor neglect and was devised and characterised as previously described (Dowd et al., 2005). As is a normal part of this procedure, rats were food restricted to 85-90% of their free feeding body weight by being
deprived of food for 12 h prior to testing. The corridor apparatus consisted of two long, narrow chambers (length 50 cm, height 24.5 cm, width 7 cm). The test involved a habituation and testing period. During the habituation period (5 min), animals were allowed to freely explore the first empty corridor with CocoPops® scattered on the floor. During the testing period, 10 pairs of pots were placed adjacently and at equal intervals along the floor edge of the second corridor and 2-3 CocoPops® were placed in each of these. Animals were placed in the test corridor and allowed to freely retrieve CocoPops® from pots on either side. The trial finished after either 5 min s had elapsed, or the animal had retrieved 20 food rewards.

2.13 Tissue Processing

Animals were sacrificed 20 or 24 weeks post-surgery by either decapitation under isoflurane anaesthesia (5% in O₂) for quantitative real-time polymerase chain reaction (RT-qPCR) and HPLC analyses, or transcardially perfused with 10mM PBS followed by 4% PFA under terminal pentobarbital anaesthesia (50 mg/kg) for immunohistochemical analyses. For RT-qPCR, the SN were microdissected from each brain and stored at -80°C until needed. For HPLC analyses, the striata were microdissected from each brain and stored in the appropriate buffer at -20°C until required. For immunohistochemical analyses, the brains were post-fixed in 4% PFA for 24 h and cryoprotected in 30% sucrose solution containing 0.1% sodium azide. Coronal sections (30 μm thickness) were cut on a freezing stage sledge microtome and were then processed for immunohistochemistry.

2.14 HPLC

HPLC analysis was performed on striatal samples, as previously described (Clarke et al, 2013). Dissected striatal brain tissue was sonicated in 1 ml of chilled mobile phase, spiked with 2ng/20ul of N-Methyl 5-HT (Sigma, UK) as internal standard. The mobile phase
contained 0.1M citric acid, 0.1M sodium dihydrogen phosphate, 0.01mM ethylene diamine tetra acetic acid (EDTA) (Alkem/Reagecon, Cork, Ireland), 5.6mM octane-1-sulphonic acid (Sigma) and 9% (v/v) methanol (Alkem/Reagecon), and was adjusted to pH 2.8 using 4N sodium hydroxide (Alkem/Reagecon). Homogenates were then centrifuged at 14,000 rpm for 20 min at 4°C and 20μl of the supernatant injected onto the HPLC system which was coupled to an electrochemical detector. A reverse-phase column (Kinetex 2.6u C18 100 x 4.6mm, Phenomenex, UK) maintained at 30°C was employed in the separation (Flow rate 0.9ml/min), the glassy carbon working electrode combined with an Ag/AgCL reference electrode (Shimadzu) was operated at +0.8V and the chromatograms generated were analysed using Class-VP 5 software (Shimadzu). Dopamine was identified by its characteristic retention time as determined by standard injections which were run at regular intervals during the sample analysis.

**Analyte:** Internal standard peak height ratios were measured and compared with standard injections to calculate the results.

### 2.15 RT-qPCR

Total ribonucleic acid (RNA) was extracted from samples using the Qiagen RNeasy Universal Plus kit according to the manufacturer’s instructions. Levels of *Th*, *Dat*, *NeuN*, *Ret*, *Bmp2* *Bmpr2*, *Bmpr1b* and *Smad1* messenger (m)RNAs were quantified by real-time PCR relative to a geometric mean of mRNAs for the reference genes *glyceraldehyde phosphate dehydrogenase* (*Gapdh*), *ubiquitin C* (*Ubqc*) and *tata binding protein* (*Tbp*). 5 μl of SN total RNA was reverse-transcribed for 1 h at 45°C using the AffinityScript kit (Agilent, Berkshire, UK) in a 25 μl reaction according to the manufacturer’s instructions. 2 μl of cDNA was amplified in a 20 μl reaction volume using Brilliant III ultrafast qPCR master mix reagents (Agilent Technologies). PCR products were detected using dual-
labelled (FAM/BHQ1) hybridization probes specific to each of the cDNAs (MWG/Eurofins, Ebersberg, Germany). The PCR primers used were as follows:

<table>
<thead>
<tr>
<th></th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>Th</td>
<td>5'-AGA GAT TGC CTT CCA GTA-3'</td>
<td>5’-CCT TCA GCG TGA CAT ATA C-3’</td>
</tr>
<tr>
<td>Dat</td>
<td>5'-CAC CAC CTC CAT TAA CTC-3’</td>
<td>5’-CAG GAT AGA TGATGA AGA TGA G-3’</td>
</tr>
<tr>
<td>NeuN</td>
<td>5'-CAC TCT GTC CGT TTG-3'</td>
<td>5’-CCG ATG GTA TGA TGG TAG-3’</td>
</tr>
<tr>
<td>Ret</td>
<td>5'-TGT TCT CTT CCT CCA TTT CA-3’</td>
<td>5’-AGT TCT CCA CGC AAA CTT-3’</td>
</tr>
<tr>
<td>Bmp2</td>
<td>5’-GGA GAT TCT TTA ATT TAA G</td>
<td>5’-ACT GCT ATT GTT TCC TAA-3’</td>
</tr>
<tr>
<td>Bmpr2</td>
<td>5’-CAA CTT CAC TGA GAA CTT C-3’</td>
<td>5’-GCT AAT ACA GAA ACC GAT G-3’</td>
</tr>
<tr>
<td>Bmpr1b</td>
<td>5’-AAA GGT AGC TGT GAA AGT</td>
<td>5’-ATG ATA GTC TGT GAT GAG G</td>
</tr>
<tr>
<td>Smad1</td>
<td>5’-TGG TCA TCA ACG GGA AAC-3’</td>
<td>5’-CCA CGA CAT ACT CAG CAC-3’</td>
</tr>
<tr>
<td>Gapdh</td>
<td>5’-TGG TCA TCA ACG GGA AAC-3’</td>
<td>5’-GAT GCA ATG AAA CTT GTT A-3’</td>
</tr>
<tr>
<td>Ubqc</td>
<td>5’-TGG TCA TCA ACG GGA AAC-3’</td>
<td>5’-GAT GCA ATG AAA CTT GTT A-3’</td>
</tr>
<tr>
<td>Tbp</td>
<td>5’-AGA GAT TGC CTT CCA GTA-3’</td>
<td>5’-CCT TCA GCG TGA CAT ATA C-3’</td>
</tr>
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Dual-labelled probes used were:

<table>
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<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Th</td>
<td>5'-FAM-AAG CAC GGT CAA CCA ATTCC-BHQ1-3’;</td>
<td></td>
</tr>
<tr>
<td>Dat</td>
<td>5'-FAM-CTT CTC CTC TGG CCT CGT CGT-BHQ1-3’;</td>
<td></td>
</tr>
<tr>
<td>NeuN</td>
<td>5'-FAM-ATC AGC AGC CGC ATA GAC TC-BHQ1-3’;</td>
<td></td>
</tr>
<tr>
<td>Ret</td>
<td>5'-FAM-CGT GTC TGT GCC TGT C-BHQ1-3’;</td>
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</tr>
<tr>
<td>Bmp2</td>
<td>5’-FAM-CCC ACG GAG GAG TTT ATC ACC ACC-BHQ1-3’;</td>
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<tr>
<td>Bmpr2</td>
<td>5’-FAM-CAC CTC CTG ATA CAA CAC CAC TC-BHQ1-3’;</td>
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<tr>
<td>Bmpr1b</td>
<td>5’-FAM-TCA CCA CGG AGG AGG CCA-BHQ1-3’;</td>
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<tr>
<td>Smad1</td>
<td>5’-FAM-CGG CAG GAG GAG TTT ATC ACC ACC-BHQ1-3’;</td>
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<td>Gapdh</td>
<td>5’-FAM-CTT CTC TGT GCT TGT TGG TAA G-3’;</td>
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<td>Ubqc</td>
<td>5’-FAM-CTT CTC TGT GCT TGT TGG-TGG-BHQ1-3’;</td>
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<tr>
<td>Tbp</td>
<td>5’-FAM-TCC TGT CAC ACC AGC CTC TG -BHQ1-3’;</td>
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Forward and reverse primers were used at a concentration of 150 nM and dual-labelled probes were used at a concentration of 200 nM. PCR was performed using the Mx3000P platform (Agilent) using the following conditions: 95°C for 3 min followed by 40 cycles
of 95°C for 12 s and 60°C for 35 s. Standard curves were generated for each cDNA for every real-time PCR run, by using serial threefold dilutions of reverse-transcribed adult rat brain total RNA (Zyagen, San Diego, USA). Relative mRNA levels were quantified in SN dissected from 5 separate animals for each experimental condition. Primer and probe sequences were designed using Beacon Designer software (Premier Biosoft, Palo Alto, USA).

2.16 Immunohistochemistry

Sections were washed for 10 min using tris-buffered saline (TBS) solution. To remove endogenous peroxidase activity, sections were quenched using 3% Hydrogen Peroxide (H₂O₂)/10% Methanol in distilled water for 5 min followed by 3 x 5 min washes with TBS. Non-specific antibody binding was blocked for 1 h using 3% Goat, Horse or Rabbit serum as required diluted in TBS containing 0.02% Triton-X100 (TXTBS). Sections were incubated overnight at RT with primary antibody diluted in TXTBS containing 1% serum. Primary antibodies were TH (1:1000, Merck Millipore), DAT (1:200, SCBT), α-synuclein (1:1000, Millipore), and pSer129-α-synuclein (1:1000, Abcam). Following 3 x 5-min TBS washes, sections were incubated in secondary antibody diluted in TXTBS containing 1% serum for 2 h. For chromogen staining, biotinylated goat-anti-rabbit IgG (1:200, Jackson Immunoresearch Lab), horse-anti-mouse IgG (1:200, Vector Labs) or rabbit-anti-goat IgG (1:200, Vector Labs) secondary antibodies were used. Following 3 x 5-min TBS washes, sections were incubated in a Streptavidin–biotin–horseradish peroxidase solution (Vector Labs) for 2 h. Sections were then washed 3 x 5 min with TBS before developing with 3,3′-Diaminobenzidine (DAB) (Vector Labs). The sections were dehydrated using increasing concentrations of Ethanol, cleared in Xylene and coverslipped using DPX mounting media (BDH Chemicals). Images of the sections were taken.
using the Olympus BX53 Upright Microscope and the Olympus FV1000 Confocal Laser Scanning Biological Microscope.

### 2.17 Immunofluorescence
Sections were washed for 10 min using TBS solution. Non-specific antibody binding was blocked for 1 h using 3% Goat, Horse or Rabbit serum as required diluted in TXTBS. Sections were incubated overnight at RT with primary antibody diluted in TXTBS containing 1% serum. Primary antibodies used include TH (1:1000, Merck Millipore or Abcam), GDF5 (1:100, Abcam), BMP2 (1:200, Abcam), α-synuclein (1:1000, Millipore) and Phospho-Smad (1:100, Cell Signalling Technology). Following 3 x 5-min TBS washes, sections were incubated in secondary antibody diluted in TXTBS containing 1% serum for 2 h. For immunofluorescence, Alexa Fluor 488- or 594-conjugated secondary antibodies (1:200; Invitrogen) were used. Sections were washed for 3 x 5-min using TBS and cover-slipped using fluorescent mounting media (Dako Diagnostics). Images of the sections were taken using the Olympus BX53 Upright Microscope and the Olympus FV1000 Confocal Laser Scanning Biological Microscope.

### 2.18 Statistical Analysis
Statistical analysis was performed using GraphPad Prism 8 (©2020 GraphPad Software, CA USA). An Unpaired Student’s t-test or one-way analysis of variance (ANOVA) or two-way ANOVA with post hoc Dunnett’s, Tukey’s, Bonferroni, or Holm-Sidak’s test or Uncorrected Fisher’s LSD test, were used as indicated to determine significant differences between groups. Results were deemed to be significant when $p < 0.05$. All data are presented as mean ± SEM.
GDF5 protects midbrain dopaminergic neurons against MPP\(^+\), 6-OHDA and α-synuclein induced axon degeneration.

3.1 Aims of Study

- Identify the most effective concentration of rhGDF5 for promotion of neurite length
- Establish the neurotrophic effect of rhGDF5 against cellular models of PD in SH-SY5Y cells
- Determine the neurotrophic effect of rhGDF5 against cellular models of PD in primary VM cultures
- Evaluate the potential neurotrophic effects of AAV2/5-GDF5 in primary dopaminergic neurons
3.2 Abstract

Introduction: PD is a chronic neurological disorder causing the progressive loss of dopaminergic neurons from the SN and consequently a loss of striatal innervation. Current therapies are solely symptomatic and do not stop or slow the dopaminergic degeneration. One strategy to combat this loss is the targeted delivery of neurotrophic factors in an effort to protect remaining dopamine neurons and regenerate the nigrostriatal pathway. GDF5 is a member of the TGF-β protein superfamily and has previously been shown to harbour neurotrophic potential for dopaminergic neurons.

Methods: In this study, SH-SY5Y cells and primary VM cultures were used to evaluate the effect of rhGDF5 on neurite length in cultures treated with either 6-OHDA or MPP+, or overexpressing WT α-synuclein.

Results: rhGDF5 demonstrated a significant increase in neurite length in both SH-SY5Y cells and E14 VM cells. In addition, while MPP+, 6-OHDA and the overexpression of WT α-synuclein had a detrimental effect on neurite length, co-treatment with rhGDF5 significantly protected SH-SY5Y and primary cell terminals against the neurotoxic and α-synuclein-induced axon degeneration. Moreover, GDF5 signalling mediators were found to be unaffected by α-synuclein overexpression. Finally, AAV2/5-GDF5 was shown to proficiently infect TH+ neurons and markedly increase the neurite length of these cells.

Conclusion: These findings are particularly noteworthy given the increasing evidence of axon degeneration preceding dopaminergic neuronal cell loss in PD, potentially mediated by α-synuclein. Taken together with previous findings, these results justify the further investigation of GDF5 in an α-synuclein translational model of PD.
3.3 Introduction

PD is a progressive neurodegenerative disorder, characterised by the progressive
degeneration of nigrostriatal dopaminergic neurons from the SN and their axonal
terminals in the striatum (Kalia and Lang, 2015). Dopaminergic neurons in the SN project
to the dorsal striatum via the nigrostriatal pathway and play a key role in the regulation
of voluntary movement and motor control as part of the basal ganglia circuitry (Luo and
Huang, 2016). It is this selective loss of dopaminergic neurons, and hence a loss of striatal
dopaminergic innervation, that gives rise to the motor impairments associated with the
disease including; bradykinesia, resting tremor, rigidity and postural instability
(Kordower et al., 2013). However, despite decades of intensive research, no disease
modifying therapy exists and clinical therapies currently available are purely
symptomatic. Therefore, there is an urgent clinical need for disease-modifying therapies
that offer long term neuroprotection and/or neuroregeneration of the remaining
dopaminergic neurons in the SN (Hegarty et al., 2014b). One promising approach is the
targeted delivery of NTFs to protect the remaining dopaminergic neurons and their axons
and to regenerate the nigrostriatal pathway (Sullivan and O’Keeffe, 2016).

The TGF-β protein superfamily holds several members known to be potent NTFs
for dopaminergic neurons (Hegarty et al., 2014c). Indeed, the most well-known NTFs to
date, GDNF and Neurturin, are distant members of the TGF-β superfamily (Saarma,
2000). However, despite promising results in pre-clinical models of PD, to date both
NTFs have failed to reach their primary endpoints in clinical trials (Decressac et al., 2011,
Gill et al., 2003, Granholm et al., 1997, Lang et al., 2006, Nutt et al., 2003, Patel et al.,
2005, Herzog et al., 2007, Kordower et al., 2006, Marks et al., 2010, Marks et al., 2008,
Warren Olanow et al., 2015, Whone et al., 2019b, Whone et al., 2019a). Recently, the
lack of clinical efficacy of GDNF and Neurturin has been postulated to be caused by the
downregulation of their common co-receptor, Ret, by α-synuclein (Decressac et al., 2012a). It has therefore been suggested that NTFs that are Ret independent, may achieve better clinical results (Warren Olanow et al., 2015). The BMP family is the largest subgroup of the TGF-β protein superfamily (Chen et al., 2004). Members of the BMP family play key roles in the development and function of a variety of tissues, and were initially discovered as inducers of bone and cartilage formation (Nishimura et al., 2012) and for their role in skeletal development (Miyazono et al., 2010, Wu et al., 2016). Since then, BMPs have emerged as key regulators of neuronal function in a host of neuronal populations (Gratacos et al., 2002, Parikh et al., 2011, Hegarty et al., 2013a). Crucially, members of the BMP family signal in a Ret independent manner, through the activation of the canonical Smad signalling pathway (Weiss and Attisano, 2013, Hegarty et al., 2013a).

GDF5 is among the most extensively studied NTF in the BMP family and has been shown to promote dopaminergic survival and protect dopaminergic neurons from neurotoxin-induced degeneration both in vitro and in vivo (Krieglstein et al., 1995, O’Keeffe et al., 2004a, Wood et al., 2005, Lingor et al., 1999, O’Sullivan et al., 2010, Costello et al., 2012, Sullivan et al., 1998, Sullivan et al., 1997). However, to date the protective effect of GDF5 against neurotoxin-induced axonal degeneration remains unclear. In addition, the effect of GDF5 on neuronal cells in an α-synuclein model of PD are unknown. This is important given that α-synuclein is the principle component of Lewy bodies and Lewy neurites (Spillantini et al., 1997) and α-synuclein overexpression has been shown to induce axonal degeneration in cultured dopaminergic neurons (Koch et al., 2015, Kouroupi et al., 2017). Therefore, in this study, SH-SY5Y cells and E14 VM cultures were used to assess the ability of GDF5 to protect dopaminergic neurons and their axons from neurotoxin and α-synuclein based degeneration.
3.4 Results

3.4.1 rhGDF5 promotes neurite growth in SH-SY5Y cells in a dose-dependent manner and activates Smad signalling.

In order to determine the most effective concentration of rhGDF5 for this study, SH-SY5Y cells, a commonly used model of midbrain dopaminergic neurons (Xicoy et al., 2017), were treated with 0-200ng/ml concentrations of rhGDF5 for 24h (Fig. 3.1A) or 48h (Fig. 3.1B, D). Total neurite length was then used as a readout of neurotrophic action. Statistical analysis showed that rhGDF5 promoted neurite growth at 10ng/ml or 50ng/ml concentration after 24h. After 48hr, 50ng/ml or 200ng/ml significantly promoted neurite growth relative to the untreated control. Given that 50ng/ml induced the greatest increase in neurite length at both time points, this concentration was chosen for further experiments. To investigate whether 50ng/ml rhGDF5 was capable of activating the Smad signalling pathway, a phospho-Smad ELISA kit was used whereby an increase in phospho-Smad expression was indicative of the activation of the Smad pathway (see Fig. 1.4 in chapter 1.0). Treatment of SH-SY5Y cells with 50ng/ml rhGDF5 for 2h led to a significant increase in phospho-Smad levels (Fig. 3.1C).

3.4.2 rhGDF5 partially protects SH-SY5Y cells against MPP+ and 6-OHDA-induced axon degeneration.

Given that rhGDF5 can promote neurite growth in SH-SY5Y cells, the next question was whether rhGDF5 could also promote neurite growth in cellular models of PD. SH-SY5Y cells were treated with the dopaminergic neurotoxins MPP+ and 6-OHDA at doses known to elicit neurite degeneration and were treated with and without 50ng/ml rhGDF5 daily for 72h after which neurite length was measured. rhGDF5 significantly increased neurite
growth compared to untreated controls while 1mM MPP$^+$ and 15µM 6-OHDA had a significant detrimental effect (Fig 3.2A, B). However, co-treatment with rhGDF5 for 72h led to a significant increase in neurite length compared to each neurotoxin alone. Yet, when compared to the untreated control, the neurite length of the co-treated groups was still significantly different. These results demonstrate that rhGDF5 can partially protect against MPP$^+$- and 6-OHDA-induced axon degeneration in SH-SY5Y cells.

3.4.3 rhGDF5 protects against WT α-synuclein overexpression in SH-SY5Y cells.

While 6-OHDA and MPP$^+$ are very useful models of distinct aspects of the cellular pathology of PD, they do not model the α-synuclein accumulation which is central to the disease progression. Therefore, to investigate whether rhGDF5 could protect SH-SY5Y cells against α-synuclein-induced neurite degeneration, SH-SY5Y cells were transfected with a control-GFP or a WT α-synuclein-GFP (αSynWT-GFP) overexpression plasmid for 24h (Fig 3.3). SH-SY5Y cells were then immunocytochemically stained for α-synuclein to show overexpression relative to control-GFP cultures (Fig 3.3A). After 24h, control-GFP and αSynWT-GFP expressing cells were treated with or without 50ng/ml rhGDF5 daily for a further 72h. Quantification of GFP-positive neurites demonstrated that rhGDF5 increased neurite growth while α-synuclein overexpression significantly decreased neurite growth relative to GFP alone group (Fig. 3.3B, C). However, co-treatment of αSynWT-GFP with rhGDF5 prevented the degenerative effects of α-synuclein. These results show for the first time a neuroprotective effect of rhGDF5 against α-synuclein-induced axon degeneration in SH-SY5Y cells.
3.4.4 rhGDF5 protects E14 dopaminergic neurons against MPP⁺- and 6-OHDA-induced degeneration.

Although SH-SY5Y cells are widely used as *in vitro* model of human dopaminergic neurons (Xicoy et al., 2017, Xie et al., 2010) they do not recapitulate all the features of midbrain dopaminergic neurons. For this reason, the effects of rhGDF5 were next examined on primary cultures harvested from the E14 rat VM to determine whether rhGDF5 could protect against the effects of 6-OHDA and MPP⁺ in primary dopaminergic neurons. E14 VM cultures were treated with 5µM 6-OHDA or 5µM MPP⁺ and cultured with or without 50ng/ml for 72 h. Here, MPP⁺ and 6-OHDA significantly reduced neurite length relative to the untreated control group (Fig. 3.4A). Treatment with rhGDF5 led to a significant increase in dopaminergic neurite length (Fig. 3.4A, B). Moreover, co-treatment with rhGDF5 protected against the neuritotoxic effects of both 6-OHDA and MPP⁺ (Fig. 3.4A, B). In addition, somal area was analysed and found that MPP⁺ and 6-OHDA had a significantly reduced somal area compared with untreated controls while rhGDF5 treatment increased soma size (Fig. 3.4C). In addition, when co-treatment with rhGDF5 returned somal area to control levels. Furthermore, the number of TH⁺ neurons in the culture was used as a readout of cell survival (Fig. 3.4D). Here, MPP⁺ and 6-OHDA treated cells had a significantly reduced number of TH⁺ neurons compared to control cells, while co-treatment with rhGDF5 prevented this. These data show that rhGDF5 can protect primary dopaminergic neurons against 6-OHDA and MPP⁺.

3.4.5 rhGDF5 protects E14 VM cultures against WT α-synuclein overexpression.

To investigate if rhGDF5 treatment could also protect against α-synuclein-induced neuritotoxicity in primary cultures, E14 VM cells were transfected with a Control-GFP
or a WT-α-synuclein-GFP overexpression plasmid. Representative photomicrographs revealed strong expression of α-synuclein in cells transfected with the WT-α-synuclein-GFP compared to cells transfected with the control-GFP plasmid (Fig. 3.5A). Firstly, to assess the effect of α-synuclein on cell bodies, the somal area of transfected cells was analysed from both groups. Here, no difference was found in αSynWT-GFP cells compared to Control-GFP cells after 72 h (Fig. 3.5B). Next, neurite length was analysed and showed that cells overexpressing αSynWT-GFP exhibited a significant decrease in neurite length compared to Control-GFP (Fig. 3.5C, D); this decrease was prevented by co-treatment with rhGDF5.

3.4.6 α-synuclein overexpression does not affect canonical Smad signalling in E14 VM cultures.

In view of the evidence relating to the downregulation of the RET receptor required for GDNF and Neurturin mediated signalling by α-synuclein (Decressac et al., 2012a), a critical factor for NTFs is that the effector molecules required for their signalling are not affected by α-synuclein. GDF5 signals through the canonical Smad signalling pathway through preferential binding to the BMPR1B and BMPR2 receptors (Fig. 3.6A). Therefore, to determine the effect, if any, of α-synuclein on the expression of BMPRs and Smad1/5 effector molecules, SH-SY5Y cells were transfected with Control-GFP and αSynWT-GFP overexpression plasmids for 72h. The cells were then immunocytochemically stained for BMPR2, BMPR1B and Smad1/5 and GFP positive cells were analysed for their expression (Fig. 3.6B). Here, while the expression of BMPR2 was significantly downregulated in αSynWT-GFP cells compared to Control-GFP cells, the expression of BMPR1B and Smad1/5 remained unchanged (Fig. 3.6B).
Furthermore, to determine if α-synuclein overexpression in primary cultures would affect rhGDF5 induced Smad signalling, E14 VM cells were again transfected with Control-GFP and αSynWT-GFP overexpression plasmids for 24h. The cultures were then treated with or without 50ng/ml rhGDF5 for 2h and immunocytochemically stained and analysed for phospho-Smad activation. Here, αSynWT-GFP cells maintained a similar expression of phospho-Smad as Control-GFP, while cultures co-treated with 50ng/ml rhGDF5 significantly increased phospho-Smad expression in both Control-GFP and αSynWT-GFP cells (Fig. 3.6C). These data shows that GDF5-Smad signalling is not impacted by the overexpression of α-synuclein in primary VM cultures.

3.4.7 AAV2/5-GDF5 co-expresses with TH⁺ neurons and promotes neurite growth.

Taking into consideration the problems associated with the delivery of recombinant proteins to the brain, viral mediated delivery of genes coding for neurotrophic proteins has become a promising therapeutic approach. Therefore, to assess the ability of AAV-GDF5 to integrate into TH⁺ neurons in primary VM cultures, E14 VM cells were infected with AAV-GDF5 using a multiplicity of infection (MOI) rate of 5×10⁴ for 10 DIV. Immunocytochemistry revealed strong expression of the AAV-GDF5 vector throughout the culture and importantly, inside TH⁺ neurons (Fig. 3.7A). It was also found that AAV-GDF5 significantly increased neurite length compared to AAV-GFP (Fig. 3.7B, C). In addition, AAV-GDF5 had no adverse effect on TH⁺ cell survival compared to AAV-GFP cells (Fig. 3.7D). These results indicate that the AAV-GDF5 viral vector can successfully target and integrate into TH⁺ neurons and elicit neurotrophic effects on neural projections without any adverse effects on cell survival.
3.5 Figures and Figure Legends

**Fig. 3.1 rhGDF5 promotes neurite growth in SH-SY5Y cells in a dose-dependent manner and activates Smad signalling.** Graphs showing the neurite length of SH-SY5Y cells treated with 0-200ng/ml rhGDF5 for A 24h and B 48h. C Graph showing phospho-Smad expression in SH-SY5Y cells after treatment with 50ng/ml rhGDF5 for 2h. D Representative photomicrographs of SH-SY5Y cells treated with 0-200ng/ml rhGDF5 for 48h. Scale bar = 50µm. All data are presented as mean ± SEM from at least three experiments. (* p < 0.05, ** p < 0.01, *** p < 0.001 vs. control or as indicated. One-way ANOVA with Tukey’s post hoc test (A, B,) or Student’s t-test (C)).
Fig. 3.2 rhGDF5 partially protects SH-SY5Y cells against MPP⁺- and 6-OHDA-induced axon degeneration. A Graph and B Representative photomicrographs of SH-SY5Y cells treated with 1mM MPP⁺ or 15µm 6-OHDA with or without 50ng/ml rhGDF5 for 72h. Scale bar = 50µm. All data are presented as mean ± SEM from at least three experiments. (** p < 0.01, *** p < 0.001 vs. control. ### p < 0.001 vs MPP⁺. $$$ p < 0.001 vs 6-OHDA. One-way ANOVA with Tukey’s post hoc test).
Fig. 3.3 rhGDF5 protects against WT-α-synuclein overexpression in SH-SY5Y cells.

A Representative photomicrographs of SH-SY5Y cells transfected with constructs expressing GFP (Control-GFP) or WT α-synuclein-GFP (αSynWT-GFP) immunocytochemically stained for α-synuclein after 24h. Scale bar = 25µm. B Graph and C Representative photomicrographs of SH-SY5Y cells expressing Control-GFP or αSynWT-GFP and treated with or without 50ng/ml rhGDF5 for 72h. Scale bar = 50µm. All data are presented as mean ± SEM from at least three experiments. (* p < 0.05 vs. control. # p < 0.05 vs αSynWT-GFP. One-way ANOVA with Tukey’s post hoc test).
Fig. 3.4 rhGDF5 protects E14 VM dopaminergic neurons against MPP⁺- and 6-OHDA-induced degeneration. A Graph of neurite length and B Representative photomicrographs C Graph of Somal Area and D Graph of TH⁺ neurons expressed as a percentage of the control in E14 VM dopaminergic neurons treated with 5µm MPP⁺ or 5µm 6-OHDA with or without 50ng/ml rhGDF5 for 72h. Scale bar = 50µm. All data are presented as mean ± SEM from at least three experiments. (*) p < 0.05, (**) p < 0.01, (***) p < 0.001 vs. control. (#) p < 0.05, (###) p < 0.001 vs MPP⁺. ($$) p < 0.05, ($$$) p < 0.001 vs 6-OHDA. One-way ANOVA with Tukey’s post hoc test).
Fig. 3.5 rhGDF5 protects E14 VM cultures against WT-α-synuclein overexpression.

A Representative photomicrographs of E14 VM cells transfected with constructs expressing Control-GFP or WT αSynWT-GFP immunocytochemically stained for α-synuclein after 24h. Scale bar = 25µm. B Graph of Somal Area C Graph of Neurite Length and D Representative photomicrographs of E14 VM cultures expressing Control-GFP or αSynWT-GFP and treated with or without 50ng/ml rhGDF5 for 72h. Scale bar = 50µm. All data are presented as mean ± SEM from at least three experiments. (** p < 0.01, *** p < 0.001 vs. control. ### p < 0.001 vs αSynWT-GFP. Unpaired Student’s t-test (B) One-way ANOVA with Tukey’s post hoc test (C)).
Fig. 3.6 α-synuclein overexpression does not affect canonical Smad signalling in E14 VM cultures. A Schema showing GDF5 mediated activation of Smad signalling. B Graph of BMPR2, BMPR1B and Smad1/5 effector molecules in SH-SY5Y cells overexpressing Control-GFP or αSynWT-GFP. C Graph of phospho-Smad expression in E14 VM cultures overexpressing Control-GFP or αSynWT-GFP. All data are presented as mean ± SEM from at least three experiments. (* p < 0.05, *** p < 0.001 vs. control. ### p < 0.001 vs αSynWT-GFP. Student’s t-test). n.s. = no significance.
Fig. 3.7 **AAV2/5-GDF5 co-expresses with TH⁺ neurons and promotes neurite growth.**

**A** Representative photomicrographs of AAV-GDF5 expression in TH⁺ neurons. Scale bar = 25µm. **B** Graph of neurite length. **C** Representative photomicrographs. **D** Graph of total TH⁺ neurons expressed as a percentage of total cells after transduction with either AAV-GFP or AAV-GDF5 for 10 DIV. Scale bar = 50µm. All data are presented as mean ± SEM from at least three experiments. (* p < 0.05 vs. control. Student’s t-test).
3.6 Discussion

In this study, SH-SY5Y cells were initially used to assess the neurotrophic effect of rhGDF5. rhGDF5 increased neurite length in a concentration dependent manner and activated the canonical Smad signalling pathway, consistent with previous findings in SH-SY5Y cells (Hegarty et al., 2013b) and dopaminergic neurons (O'Keeffe et al., 2004a, Hegarty et al., 2014a, Costello et al., 2012). In addition, rhGDF5 was found to be partially neuroprotective against MPP⁺ and 6-OHDA-induced reductions in neurite length in SH-SY5Y cells. Considering the emerging evidence of α-synuclein-mediated axon degradation in dopaminergic neurons (Koch et al., 2015, Kouroupi et al., 2017), rhGDF5 was evaluated for neurotrophic potential against the overexpression of WT α-synuclein in SH-SY5Y cells which found rhGDF5 prevented the α-synuclein induced axon degeneration.

While SH-SY5Y cells are an excellent resource for use in initial testing of neurotrophic potential, since they are catecholaminergic they do not replicate all properties of dopaminergic neurons. Therefore, primary cultures of the E14 rat VM were used to investigate the neuroprotective potential of rhGDF5 in vitro. rhGDF5 led to a significant increase in neurite length relative to the untreated control, which is consistent with previous studies (Hegarty et al., 2014a, Costello et al., 2012). In addition, rhGDF5 protected dopaminergic neurons from MPP⁺ and 6-OHDA-induced somal and axon degeneration and increased the dopaminergic neuron survival. The number of TH⁺ neurons in cultures treated with rhGDF5 alone however were not significantly different from the control, this is in line with previous findings showing rhGDF5 predominately works to differentiate neuronal cells rather than promoting increased survival (Hegarty et al., 2013b). However, in contrast, several studies have found GDF5 to increase the survival of TH⁺ neurons. Krieglstein et al were among the first to report a 1.6-fold
increase in TH⁺ cell survival after treatment with 20ng/ml GDF5 for 8 DIV (Krieglstein et al., 1995). While additional studies found that 1-10ng/ml GDF5 could increase survival up to 3.1-fold after 6 DIV (O'Keeffe et al., 2004a). Furthermore, varying doses (1, 10 or 100ng/ml) of GDF5 and GDNF were compared for their effect on dopaminergic neuronal survival and it was found that GDF5 was as effective at promoting TH⁺ cell viability as GDNF at all doses tested up to 6 DIV (Wood et al., 2005). A potential reason for the lack of effect in this study may be due to the duration of the experiment which was 3 DIV in comparison to that in earlier studies. In addition, this study found a significant decrease in TH⁺ cell viability of 26% for MPP⁺ and 22% in 6-OHDA treated cultures compared to the controls after 72h. However, co-treatment with rhGDF5 significantly attenuated this loss. This is consistent with previous findings that have shown GDF5 to be protective against selective dopaminergic toxins including MPP⁺ (Krieglstein et al., 1995) and free radical induced damage induced by FeCl₂ (Lingor et al., 1999) in E14 VM cultures.

In contrast to these positive findings, GDF5 was not protective against SNP induced free radical toxicity in E14 VM cultures (Lingor et al., 1999). This was suggested to be in part due to the different cell death pathways induced by the SNP and FeCl₂. In addition, another study found no survival promoting effect of GDF5 in 6-OHDA treated E14 VM cultures (Brederlau et al., 2002). However, in that study cultures were not exposed to GDF5 until after 6DIV making the cultures E20 in nature, a time when the expression of the GDF5 is in fact downregulated in vivo and thus neuronal cells may be unresponsive to the effects of exogenous GDF5 (O'Keeffe et al., 2004b). Additionally, a recent study assessed the effect of GDF5 against MPP⁺ treated SN cultures harvested from postnatal day P0 pups (Jaumotte et al., 2016). However, the authors noted no survival enhancing effects of GDF5 at any time point tested. Interestingly however, when
a combination of NTFs was used including GDNF, BDNF, TGF-β, FGF-2 and GDF5, the number of TH+ neurons in the SN cultures rose by 32.6 ± 5.13% after exposure to 10µM MPP+ for 30 minutes, and 29.1 ± 8.56% after exposure to 500µM MPP+ for 30 minutes (Jaumotte et al., 2016). This synergistic effect may be due to each NTF not only acting on dopaminergic neurons, but also non-dopaminergic cells which may influence dopaminergic neuron survival through paracrine signalling (Jaumotte et al., 2016). The fact that GDF5 was not found to be neuroprotective in this study may again result from the age of the culture as GDF5 is known to be downregulated at P0 (O'Keeffe et al., 2004b).

While MPP+ and 6-OHDA are valuable neurotoxins for modelling selective dopaminergic degeneration, they do not accurately represent the pathophysiology of PD. The accumulation of α-synuclein protein aggregates that spread throughout the CNS known as Lewy bodies and Lewy neurites (Spillantini et al., 1997) are a major pathological hallmark of PD. In addition, missense point mutations in the SNCA gene including A30P, A53T or duplications or triplications of SNCA are associated with familial forms of PD (Kruger et al., 1998, Polymeropoulos et al., 1997, Singleton et al., 2003, Chartier-Harlin et al., 2004, International Parkinson Disease Genomics et al., 2011). Therefore, to investigate the effects of α-synuclein in E14 VM cultures, cells were transfected with either a control-GFP or αSynWT-GFP expression plasmid. The overexpression of α-synuclein had no effect on somal area, consistent with previous findings (Koch et al., 2015) but significantly reduced neurite length, which was attenuated by co-treatment with rhGDF5. This finding agrees with several studies showing neuritic pathologies as important events in synucleinopathies including PD (Kalia and Kalia, 2015, Prots et al., 2013, Furlong et al., 2020). Indeed, it is now becoming widely accepted that axonal degeneration occurs early in PD (Kordower et al.,
2013, O’Keeffe and Sullivan, 2018, Hsiao et al., 2014, Caminiti et al., 2017) and may be a consequence of α-synuclein overload (Koch et al., 2015, Oliveira et al., 2015, Prots et al., 2013, Lin et al., 2016b, O’Keeffe and Sullivan, 2018).

Given the evidence supporting the α-synuclein mediated downregulation of RET, the co-receptor required for GDNF and Neurturin signalling (Decressac et al., 2012a), the effect of α-synuclein, on the regulators of BMP-Smad signalling was investigated. GDF5 is known to preferentially bind to the BMPRs, BMPR1B and BMPR2, to activate the Smad signalling pathway (Nishitoh et al., 1996, Hegarty et al., 2013b). In this study, SH-SY5Y cells overexpressing α-synuclein significantly reduced the expression of BMPR2 while the expression of BMPR1B and Smad1/5/8 remained unchanged compared to control-GFP cells. As GDF5 is known to elicit its phenotypic neurotrophic effects primarily through high affinity binding to BMPR1B (Hegarty et al., 2013b), this result was promising. rhGDF5 was also shown to increase phospho-Smad expression in dopaminergic neurons overexpressing α-synuclein, indicating that the BMP-Smad pathway machinery are not affected by WT α-synuclein overexpression in SH-SY5Y cells and primary VM cultures.

While NTFs are a promising therapeutic intervention, a significant problem with delivering recombinant proteins, including GDF5, is their need for direct administration to the brain due to rapid metabolism in the gut and moreover, the inability to cross the blood brain barrier due their large molecular weight (O’Keeffe et al., 2017). Although administration of recombinant NTFs to the brain is safe and clinically feasible, clinical trials using recombinant proteins thus far failed to meet their primary endpoints (Gill et al., 2003, Lang et al., 2006, John T. Slevin et al., 2005, Nutt et al., 2003). This is due to low levels of the protein of interest reaching its target area due to rapid biometabolism by endogenous enzymes. However, the advent of gene therapy using viral vectors
bypasses this issue by incorporating the gene for the protein of interest for targeted expression within the brain (Axelsen and Woldbye, 2018). This has led to pre-clinical studies using recombinant LV or AAV vectors to deliver NTFs in animals models of PD (Choi-Lundberg et al., 1997, Kordower et al., 2000, Kordower et al., 2006, Ramaswamy et al., 2007, Herzog et al., 2007, Su et al., 2009, Decressac et al., 2011, Bäck et al., 2013, Cordero-Llana et al., 2015, Ren et al., 2013). Translating this research into clinical trials however favours AAV2 vectors due to higher safety profiles within the clinic (Kelly et al., 2015, Bartus et al., 2013, Bartus et al., 2011, Marks et al., 2010, Warren Olanow et al., 2015). E14 VM cultures transduced with AAV2-GDF5 vector for 10 DIV demonstrated strong expression of GDF5 and co-expression with TH+ neurons in culture. Moreover, AAV2-GDF5 significantly increased the neurite length of TH+ neurons and dopaminergic cell viability was not impacted relative to AAV2-GFP cultures, indicating its safe use.

Collectively, this study demonstrates for the first time that rhGDF5 protects against 6-OHDA, MPP+ and α-synuclein induced axonal degeneration in SH-SH5Y cells and primary E14 VM cultures. In addition, this study provides further evidence of rhGDF5 promoting TH+ neuronal survival against 6-OHDA and MPP+ treated primary cultures. This study also shows that GDF5-mediated canonical BMP-Smad signalling is not affected by the overexpression of WT α-synuclein in SH-SY5Y cells and primary VM cultures. Finally, this study validates the efficacy of AAV2/5-GDF5 in targeting TH+ neurons and highlights the potential of AAV2/5-GDF5 to induce neurotrophic effects on TH+ cells. Taken together, this study provides robust evidence for the potent neurotrophic efforts of GDF5 and justifies the further investigation of AAV2/5-GDF5 in a translational model of PD, such as the α-synuclein rat model of the disease.
Chapter 4.0

GDF5 exerts neuroprotection in an α-synuclein rat model of Parkinson’s disease.

4.1 Aims of Study

- Investigate the long-term effects of AAV-mediated delivery of GDF5 in vivo
- Identify the effect of AAV-mediated delivery of α-synuclein to the SN
- Determine if there is a neuroprotective effect of AAV-GDF5 against AAV-αSyn
- Evaluate the effect of α-synuclein on the expression of key mediators in the Smad signalling pathway
- Determine the effect of α-synuclein on motor behaviour

Work featured in this chapter has been published in the following paper:

4.2 Abstract

**Introduction:** Neurotrophic factors hold significant promise as neuroprotective therapies for PD. While viral vector-mediated delivery of neurotrophic factors to the PD brain is safe and clinically feasible, clinical trials of two well-known neurotrophic factors, GDNF and Neurturin, have failed to meet their primary end-points. This study sought to determine whether viral delivery to the adult rat brain of a novel neurotrophic factor, GDF5, could protect dopaminergic neurons from α-synuclein–induced degeneration, which is the primary neuropathological hallmark of PD.

**Methods:** 80 adult SD female rats were used over two experiments. In experiment 1; animals received a unilateral injection of an AAV-Cont+AAV-Cont or AAV-GDF5+AAV-Cont vector combination into the SN. In experiment 2; animals received an AAV-αSyn+AAV-Cont or AAV-αSyn+AAV-GDF5 vector combination into the SN. Behavioural tests of sensorimotor function were carried out at 8, 12, 16 and 20 weeks, prior to post-mortem analysis of α-synuclein pathology and nigrostriatal integrity at 20 weeks.

**Results:** Intranigral delivery of AAV-α-synuclein led to significant reductions in the expression of transcripts for Th, Dat and Ret expression, but did not affect the expression of Bmpr2, Bmpr1b and Smad 1. In addition, AAV-α-synuclein caused a significant loss of TH⁺ and DAT⁺ neurons from the SN and a loss of striatal dopamine which were prevented by AAV-mediated delivery of GDF5.

**Conclusion:** The observed neuroprotective effects of GDF5 in this preclinical model support the theory that Ret-independent neurotrophic factors may have therapeutic benefit in PD and are worthy of further exploration.
4.3 Introduction

The concept of NTF therapy holds significant promise as a disease-modifying therapy for PD (Paul and Sullivan, 2019). Whone et al. have recently reported the results of a randomized, double-blind, placebo-controlled trial of intermittent intraputamenal convection-enhanced delivery of GDNF in PD patients (Whone et al., 2019a). Despite an extensive body of excellent work and significant improvements to dosing and delivery, this trial failed to reach its primary endpoint. This outcome is largely consistent with an earlier randomized, placebo-controlled trial of GDNF in PD (Lang et al., 2006). Preclinical studies have shown that delivery of AAV-GDNF to either the striatum or SN failed to protect dopaminergic neurons or their terminals in a rat model of PD generated through viral-mediated overexpression of human WT α-synuclein (AAV-αSyn) (Decressac et al., 2011) or A30P mutant α-synuclein (LV-αSyn) (Lo Bianco et al., 2004). This has been suggested to be due to αSyn-induced downregulation of Nurrl and Ret in the midbrain, measured at 2 weeks post-surgery (Decressac et al., 2012a). Similar αSyn-induced downregulation of Ret was reported in the SN of PD patients (Decressac et al., 2012a). This may explain the lack of clinical efficacy of GDNF, as the Ret receptor has been reported to be critical for the dopaminergic neurotrophic effects of GDNF in vivo (Drinkut et al., 2016). However, others found that transgenic overexpression of human α-synuclein in mice, or intranigral administration of AAV-αSyn in rats, did not downregulate Nurrl or TH expression after 8 weeks (Su et al., 2017). Therefore, the effect of α-synuclein on Ret expression remains an open and important question.

More generally, the lack of clinical efficacy of GDNF has raised the larger question of whether NTF delivery as a therapeutic approach remains viable, or if this approach requires further testing and evaluation in clinical settings (Whone et al., 2019b). Another
consideration is that if Ret receptors are downregulated or not functioning optimally in the PD brain, then Ret-independent neurotrophic factors may have therapeutic benefit, assuming that α-synuclein does not also affect the expression of their key signalling mediators. Thus, a critical requirement to justify the development of Ret-independent NTF therapy, is that these factors must display therapeutic benefit in the α-synuclein model of PD, which more closely mimics the disease pathology than other rodent models.

In chapter 3.0, GDF5 was shown to be neuroprotective against *in vitro* models of PD including the overexpression of α-synuclein. Importantly, the neurotrophic effects of GDF5 are known to be exclusively dependent on the canonical Smad signalling pathway, which is a Ret-independent mechanism (Hegarty et al., 2014a, Hegarty et al., 2013b). Therefore, this study investigated whether viral mediated intranigral delivery of GDF5 could protect dopaminergic neurons and their axons against the overexpression of α-synuclein *in vivo*. 
4.4 Results

4.4.1 Experimental Design

The approach to this study was two-fold. Firstly, in experiment 1, the safety and efficacy of AAV-mediated delivery of GDF5 was evaluated. In this study, animals received a unilateral injection of either an AAV2/5-Cont+AAV2/6-Cont (AAV-Cont) or an AAV2/5-GDF5+AAV2/6-Cont (AAV-GDF5) vector combination into the SN (Fig. 4.1A). Subsequently, experiment 2 evaluated the neurodegeneration induced by the overexpression of human WT α-synuclein and the potential neuroprotective effect of GDF5. Here, animals were unilaterally injected with an AAV2/6-αSyn+AAV2/5-Cont (AAV-αSyn) or an AAV2/6-αSyn+AAV2/5-GDF5 (AAV-αSyn/GDF5) vector combination into the SN (Fig. 4.1B). In both experiments, behavioural testing was carried out at four weekly intervals beginning 8 weeks after stereotactic surgery (Fig. 4.1C). Animals were sacrificed at 20 weeks and analysed for α-synuclein overexpression and nigrostriatal integrity.

4.4.2 No adverse effects of long-term AAV-GDF5 on nigrostriatal integrity for up to 20 weeks post-surgery.

In experiment 1, the safety and efficacy of AAV-mediated transfer of GDF5 was investigated relative to animals who had received an AAV-Cont vector unilaterally injected into the SN. Twenty weeks post-surgery, animals sacrificed via transcardial perfusion were immunohistochemically probed for TH to assess nigral and striatal dopaminergic integrity (Fig. 4.2A, E). Quantification of TH in the SN displayed no significant difference in the number of TH⁺ cell bodies between AAV-Cont and AAV-GDF5 groups (Fig. 4.2B). To confirm this result, SN sections were also stained for DAT and a statistical analysis showed no difference in the number of DAT-positive (DAT⁺)
cell bodies in the SN between AAV-Cont and AAV-GDF5 animals (Fig. 4.2C). To confirm the expression of the human GDF5 transgene, SN sections from AAV-GDF5 animals were stained for human GDF5 which revealed strong expression of GDF5 in TH+ cells (Fig. 4.2D). Furthermore, quantification of TH staining as a percentage of the contralateral intact striatum revealed there was no difference in striatal TH levels in AAV-Cont compared to AAV-GDF5 animals (Fig. 4.2E, F). In agreement with this, HPLC analysis of dopamine in the striatum showed consistent dopamine levels in both AAV-Cont and AAV-GDF5 animals (Fig. 4.2G). The results of this study indicate that long-term AAV-mediated expression of GDF5 is well tolerated in TH+ neurons and produces no adverse effects in the SN or striatum of adult rats, at least up to 20 weeks.

4.4.3 AAV-GDF5 maintains nigrostriatal integrity and striatal dopamine levels in the rat AAV-αSyn model of PD.

In experiment 2, adult rats received a stereotaxic unilateral intranigral injection of α-synuclein vector (AAV2/6-αSyn) and concomitantly either a control vector (AAV2/5-Cont) or a vector carrying the human GDF5 transgene (AAV2/5-GDF5) (Fig. 4.3A). Rats were sacrificed 20 weeks later for analysis of transgene expression and nigrostriatal integrity. There were no differences in α-synuclein expression levels between AAV-αSyn and AAV-αSyn/GDF5 treated animals in the striatum (Fig. 4.3B) or SN (Fig. 4.3C). It was then investigated whether nigral GDF5 overexpression could prevent the loss of striatal dopaminergic terminals, which is known to occur in this model (Decressac et al. 2011). To evaluate the neurodegeneration induced by overexpression of human WT α-synuclein and the potential neuroprotective effect of AAV-GDF5, the number of TH+ and DAT+ neurons in the ipsilateral and contralateral SN were measured. At 20 weeks post injection of AAV-αSyn, there was ~36% loss of TH+ (Fig. 4.3D, E) and ~30% loss of
DAT\(^+\) (Fig. 4.3F) neurons, compared to the intact side. AAV-mediated delivery of GDF5 to the SN prevented this loss of dopamine neurons (Fig. 4.3E, F). In addition, quantitative analysis of TH\(^+\) striatal fibre innervation revealed that nigral overexpression of α-synuclein induced ~45% reduction (Fig. 4.3G, H). In contrast, nigral overexpression of GDF5 by AAV-GDF5 had a protective effect on striatal dopaminergic terminal density (Fig. 4.3G, H). Moreover, analysis of striatal dopamine levels using HPLC after 20 weeks revealed that nigral overexpression of α-synuclein induced ~45% reduction in striatal dopamine levels (Fig. 4.3I). While AAV-mediated nigral overexpression of GDF5 had a protective effect on striatal dopamine levels (Fig. 4.3I). Collectively, these data show that intranigral injection of AAV-GDF5 protected dopaminergic neurons and their terminals, maintaining striatal dopamine levels in the AAV-αSyn rat model of PD.

4.4.4 α-synuclein downregulates Ret but not key mediators of BMP signalling in vivo.

Given that α-synuclein has been shown to downregulate the Ret receptor required for GDNF and Neurturin signalling, this study also investigated the effect of α-synuclein overexpression on the effectors of the BMP-Smad signalling pathway in the rat SN. AAV-αSyn resulted in the strong expression of α-synuclein throughout the midbrain and high α-synuclein immunoreactivity in the axonal terminals distributed throughout the striatum on the ipsilateral, but not contralateral side (Fig. 4.4A). In addition, pSer129-α-synuclein immunostaining for the pathological form of α-synuclein revealed strong expression in dopaminergic neuronal soma and neurites in the ipsilateral SN, characteristic of Lewy bodies and Lewy neurites (Fig. 4.4B). Furthermore, intranigral delivery of AAV-αSyn led to significant reductions in the expression of transcripts for Th (Fig. 4.4C) and Dat (Fig. 4.4D), but not NeuN (Fig. 4.4E). AAV-αSyn also induced a
significant reduction in Ret expression (Fig. 4.4F), but no change in the expression of Bmpr2, Bmpr1b and Smad 1, all of which are required for the dopaminergic neurotrophic effects of GDF5 (Fig. 4.4G). These results therefore add significant weight to the proposed use of Ret-independent neurotrophic factors as therapeutic interventions for PD.

4.4.5 Phospho-Smad expression in dopaminergic neurons is substantially reduced by AAV-αSyn transduction of the SN, but not when combined with AAV-GDF5.

Given that the overexpression of WT α-synuclein did not affect transcripts for key mediators of canonical BMP-Smad signalling in the SN, this study also looked at the level of phospho-Smad proteins in TH+ neurons in the ipsilateral SN of animals injected with either AAV-αSyn+AAV-Cont or AAV-αSyn+AAV-GDF5 (Fig. 4.5). In this experiment, SN sections were immunofluorescently stained for TH and α-synuclein and TH+ neurons were then quantified for the level of phospho-Smad intensity. Statistical analysis showed that the overexpression of α-synuclein caused a significant reduction in phospho-Smad expression after 20 weeks, relative to the contralateral intact SN (Fig. 4.5A, B). However, animals injected with α-synuclein in combination with GDF5, did not experience this marked decline and instead phospho-Smad intensity was maintained at the level of the contralateral intact SN (Fig. 4.5C, D). These results indicate that the overexpression of α-synuclein impacts phospho-Smad protein expression in vivo, which can be rescued by GDF5.
4.4.6 AAV-αSyn transduction of the SN leads to subtle sensorimotor impairments, that are attenuated by AAV-GDF5.

To determine whether the overexpression of α-synuclein had any effects on motor behaviour and whether this could be prevented by GDF5, animals underwent the whisker, stepping and corridor tests of sensorimotor function beginning 8 weeks after stereotaxic surgery and repeated at four weekly intervals for 20 weeks. When the effect of the AAV-GDF5 viral vector on motor function were examined, no significant result for AAV-GDF5 was found on performance in the whisker test ($F_{(1, 18)} = 0.1951; p=0.6639$) (Fig. 4.6A), stepping test ($F_{(1, 18)} = 1.221; p=0.2838$) (Fig. 4.6B) nor in the corridor test ($F_{(1, 18)} = 0.4855; p=0.4948$) (Fig. 4.6C). These data show that AAV-GDF5 did not have any adverse effects on behaviour for up to 20 weeks suggesting that the approach is safe and well tolerated. In contrast, a two-way repeated measures ANOVA of performance in the whisker test revealed that there was a significant effect of AAV-αSyn ($F_{(1, 18)} = 8.587; p = 0.0089$) and time ($F_{(3, 54)} = 4.227; p =0.0093$) but no significant interaction between both ($F_{(3, 54)} = 3.13; p = 0.1238$). Post-hoc analysis showed that the AAV-αSyn animals performed significantly worse than controls at 16 ($p < 0.05$) and 20 weeks ($p < 0.05$) (Fig. 4.6D). In contrast however, there was no significant effects of AAV-αSyn in the stepping test ($F_{(1, 18)} = 0.0002; p =0.9644$) (Fig. 4.6E) nor in the corridor test ($F_{(1, 18)} = 0.04416; p=0.8359$) (Fig. 4.6F). These data show that AAV-αSyn leads to subtle impairments only in the whisker test that only emerge 16 weeks post-surgery.

Given the effects of AAV-αSyn were only seen in the whisker test, the four groups were then directly compared at the 20 week time point (Fig. 4.6G). A one-way ANOVA at 20 weeks revealed a significant effect in the whisker test with post-hoc testing showing that the only group in which there was a significant reduction in performance
was in the AAV-αSyn+AAV-Cont group (Fig. 4.6G). Collectively these data show that the while AAV-αSyn leads only to subtle impairments in motor function relative to the controls, the subtle impairments that are seen in the AAV-αSyn+AAV-Cont group, are not seen in the AAV-αSyn+AAV-GDF5 group. These data show that although AAV-αSyn exerts only subtle effects on aspects of motor behaviour, these effects are attenuated by AAV-GDF5.

Finally, given that weight loss has been reported as an adverse effect of GDNF therapy (Nutt et al., 2003, Manfredsson et al., 2009), the average weight of the animals in each group at 20 weeks was analysed by one-way ANOVA and found no differences compared with AAV-Cont+AAV-Cont animals (Fig. 4.6H). These results indicate that the overexpression of GDF5, or indeed α-synuclein, causes no adverse or long-term effects on weight.
4.5 Figures and Figure Legends

Fig. 4.1 Experimental Design. A In Experiment 1, animals received a unilateral injection of an AAV-Cont+AAV-Cont or AAV-GDF5+AAV-Cont vector combination into the SN. N=20 per group. B In Experiment 2, animals received a unilateral injection of an AAV-αSyn+AAV-Cont or AAV-αSyn+AAV-GDF5 vector combination into the SN. N=20 per group. C Schema showing the study timeline for each experimental group from 0-20 weeks, including behavioural testing and post-mortem assessment measure. IHC = Immunohistochemistry.
Fig. 4.2 No adverse effects of long-term AAV-GDF5 on nigrostriatal integrity for up to 20 weeks post-surgery. A Representative photomicrographs of AAV-Control (Black) and AAV-GDF5 (Blue) TH-stained sections through SN on the ipsilateral side. Scale bar = 200µm. B, C Graph showing the numbers of B TH+ and C DAT+ neurons in the SN expressed as a percentage of the intact side. D Representative photomicrographs showing expression of the human (h)GDF5 transgene (green) in TH+ neurons (red; white arrows) in the ipsilaterally (injected) SN at 20 weeks’ post-injection of the AAV-GDF5 vector. Scale bar = 50µm. E Representative photomicrographs of TH-stained sections through the striatum on the ipsilateral side and the contralateral intact side. Scale bar = 1mm. F Graph showing the striatal TH immunoreactivity expressed as percentage of the intact side. G Graph of HPLC data showing striatal dopamine (DA) levels expressed as a
percentage of the intact side. All data are presented as mean ± SEM and analysed by a Student’s t-test.

Fig. 4.3 AAV-GDF5 maintains nigrostriatal integrity and striatal dopamine levels in the rat AAV-αSyn model of PD. A Representative striatal images of α-synuclein staining in animals injected with AAV-αSyn with either an AAV-Cont (purple) or AAV-GDF5 (blue) vector into the SN and B, C quantification of α-synuclein levels in B the Striatum and C the SN in both experimental groups. D Representative photomicrographs of TH-stained sections through SN on the ipsilateral side. Scale bar = 200µm. E, F Graph showing the numbers of E TH⁺ and F DAT⁺ neurons in the SN expressed as percentage of the intact side. G Representative photomicrographs of TH stained sections through the striatum on the ipsilateral (injected) side and the contralateral intact side. Scale bar = 1mm. H Graph showing striatal TH immunoreactivity expressed as
percentage of the intact side. **Graph of HPLC data showing striatal dopamine (DA) levels expressed as percentage of the intact side. All data are presented as mean ± SEM and analysed by a Student’s t-test; *p < 0.05, **p < 0.01 vs control.

**Figure 4.4 α-synuclein downregulates Ret but not key mediators of BMP signalling in vivo.** Representative images showing A WT α-synuclein (αSyn) immunostaining in rat striatum and midbrain, and B pSer129-αSyn staining in SN, at 20 weeks after unilateral stereotaxic injection of AAV-αSyn vector into the SN. Blue arrows indicate dopaminergic neuronal cell bodies and red arrows indicate processes. Scale bar = 1mm. Real-time PCR data showing expression of transcripts for C Th (**t = 4.211, d.f. = 8, P = 0.0029), D Dat (**t = 5.28, d.f. = 8, P = 0.0007) E NeuN, F Ret (*t = 2.45, d.f. = 8,
P = 0.0399) and G Bmpr2, Bmpr1b and Smad1, key mediators of BMP signalling in animals injected with an AAV-Cont+AAV-Cont (Black) or an AAV-αSyn+AAV-Cont (purple) vector combination (n = 5 per group). All data are presented as mean ± SEM and analysed by Student’s t-test; *p < 0.05, **p < 0.01, ***p<0.001 vs control.

Fig. 4.5 Phospho-Smad expression in dopaminergic neurons is substantially reduced by AAV-αSyn transduction of the SN, but not when combined with AAV-GDF5. Quantification of phospho-Smad expression as a percentage change from the contralateral (intact) side of animals injected with A AAV-αSyn+AAV-Cont or C AAV-αSyn+AAV-GDF5 at 20 weeks. Representative photomicrographs of TH+ (red) neurons in the SN expressing phospho-Smad (green) in the ipsilateral (injected) and contralateral (intact) side of animals injected with B AAV-αSyn+AAV-Cont or D AAV-αSyn+AAV-GDF5 at 20 weeks. (n=7 per group; ***p<0.001 vs intact control). All data are presented as mean ± SEM and analysed by Student’s t-test. Scale = 10µm.
Fig. 4.6 Long-term viral-mediated overexpression of WT α-synuclein in the rat SN leads to subtle sensorimotor impairments that are attenuated by AAV-GDF5. A-F Graphs showing the results of the A, D Whisker, B, E Stepping and C, F Corridor tests at four weekly intervals from 8 weeks up to 20 weeks after stereotactic injection with A-C an AAV-Cont+AAV-Cont (Black) or AAV-GDF5+AAV-Cont (Blue) or D-F an AAV-αSyn+AAV-Cont (Purple) or AAV-αSyn+AAV-GDF5 (Purple/Blue) vector into the SN. G Graph showing the combined experimental results of the whisker test at 20 weeks post stereotactic injection into the SN with AAV-Cont+AAV-Cont, AAV-GDF5+AAV-Cont, AAV-αSyn+AAV-Cont or AAV-αSyn+AAV-GDF5, as indicated. H Graph showing the
final weight of animals in all experimental groups. All data are mean ± SEM (* p < 0.05 vs control; one-way ANOVA with *post-hoc* (D) Bonferroni’s or (G) Tukey’s test (n=10 per group)).

**4.6 Discussion**

Given the recent failure of AAV-GDNF to protect dopaminergic neurons from α-synuclein overexpression *in vivo*, this study evaluated the effect of another potent dopaminergic neurotrophic factor, GDF5. This study initially investigated the safety and efficacy of viral-mediated delivery of GDF5 to the SN of adult rats. To confirm its expression within the ipsilateral SN, immunofluorescent staining revealed strong levels of the GDF5 transgene within TH⁺ neurons. Nigral integrity was assessed after 20 weeks and revealed no significant difference in the number of TH⁺ and DAT⁺ neurons between AAV-Cont and AAV-GDF5 animals. In addition, analysis of striatal integrity showed no difference in TH immunoreactivity between the two groups, substantiated by HPLC analysis of the striata which showed no difference in dopamine levels between AAV-Cont and AAV-GDF5 animals. Moreover, GDF5 overexpression had no adverse effect on motor behaviour relative to AAV-Cont animals. Notably, the weight of the animals was not affected after long-term exposure to GDF5, which is an important finding given that anorexia and weight loss were adverse effects associated with intracerebral GDNF delivery in monkeys (Nutt et al., 2003). These results support the conclusion that AAV-mediated delivery of GDF5 to the SN is a safe and well-tolerable approach.

This study then assessed the effect of viral mediated α-synuclein overexpression in the SN and whether AAV-GDF5 could protect dopaminergic neurons from α-synuclein-induced degeneration. Here, intranigral AAV-αSyn delivery led to widespread expression of α-synuclein throughout the midbrain and striatum on the ipsilateral side only.
Moreover, immunostaining for pSer129-αSyn for the pathological form of α-synuclein revealed strong expression in dopaminergic neuronal soma and neurites in the ipsilateral SN, a key signature of Lewy bodies and Lewy neurites (Anderson et al., 2006). This overexpression of α-synuclein led to a significant reduction in the number of TH⁺ and DAT⁺ neurons in the SN and TH⁺ fibre density in the striatum, verified by HPLC dopamine analysis. However, animals given AAV-αSyn in combination with AAV-GDF5 saw a significant increase in both TH⁺ and DAT⁺ neurons in the SN and also TH⁺ fibre density in the striatum. However, the level of α-synuclein overexpression was unchanged by GDF5. These results agree with previous studies showing GDF5 exerts neuroprotection to dopaminergic neurons against commonly used neurotoxin-based preclinical models of the disease (Costello et al., 2012, O'Sullivan et al., 2010, Sullivan et al., 1997, Sullivan et al., 1998, Hurley et al., 2004). Of note, one study in particular compared the efficacy of GDF5 dimer-treated, GDNF dimer-treated and untreated foetal mesencephalic grafts in the 6-OHDA lesioned rat model of PD. In this study, rats received the graft four weeks post-lesion to the MFB. Behavioural testing of amphetamine-induced rotation rates found the mean rate for lesion-only animals was 12.6 ± 2.8, graft-only animals was 2 ± 0.4, while animals receiving GDF5-treated or GDNF-treated grafts displayed no rotations (Sullivan et al., 1998). In determining TH⁺ cell survival, a deposit of TH⁺ cells was visible in all groups that had received a VM transplant. However, the grafts pre-treated with GDF5 dimer or GDNF displayed a significant increase in number and density of TH⁺ neurons compared with the graft alone (Sullivan et al., 1998). This study was the first to show the neurotrophic and neuroprotective effect of GDF5 on treated foetal mesencephalic transplants. Notably, this study also highlighted that GDF5 treatment was equally as beneficial as GDNF. Collectively, the in vivo evidence in support of GDF5 to date demonstrates that GDF5 is neuroprotective against 6-OHDA...
and α-synuclein models of PD, both of which are mechanistically different models of PD (Raza et al., 2019, Dijkstra et al., 2015). This implies that GDF5 may play a role in alleviating oxidative stress and mitochondrial dysfunction as well as a role in regulating axonal function.

Furthermore, given that the failure of GDNF and Neurturin has been attributed to the downregulation of their common co-receptor, Ret (Decressac et al., 2012a), this study also examined the effect of α-synuclein overexpression on the transcripts for mediators of the Smad signalling pathway. In this experiment, the expression of GDF5’s receptors and downstream transcription factor Smad1 was not affected by α-synuclein overexpression, at least at the 20-week time point examined. The observed neuroprotective effects of GDF5 in this preclinical model thus support the theory that Ret-independent neurotrophic factors may have therapeutic benefit in PD and are worthy of further exploration (Warren Olanow et al., 2015). However, in agreement with Decressac et al (2012), this experiment found that viral-mediated overexpression of α-synuclein led to a significant reduction in Ret expression in the adult rat SN at 20 weeks post-surgery. However, it is important to note that the reduction of 25% in this study was observed at 20 weeks, in contrast to the much greater (~65%) reduction reported by Decressac et al after 2 weeks. Since this study found that ~75% of Ret expression remained in the α-synuclein-overexpressing brain, these data suggest that the signalling capacity of GDNF may be retained, at least partially, in these animals. This is also consistent with the significant putamenal increase in $^{18}$F-DOPA uptake in GDNF-treated patients at Week 40 reported in the Whone et al study (Whone et al., 2019a). The key determinant of clinical efficacy of GDNF may therefore be the level of α-synuclein burden in the host brain. High levels of α-synuclein may lead to an early and strong downregulation of Ret, similar to that reported in the Decressac et al. study (2012),
whereas modest α-synuclein levels may lead in the short term to minimal reductions in Ret, as observed by Su and co-workers after 8 weeks (2017), but more substantial impairment of Ret expression in the longer term, as seen in this study.

Finally, to determine whether the overexpression of α-synuclein had any effects on motor behaviour and whether this could be prevented by GDF5, animals underwent the whisker, stepping and corridor tests of sensorimotor function (Schallert et al., 2000, Dowd et al., 2005) beginning 8 weeks after stereotaxic surgery and repeated every four weeks for 20 weeks. Here, animals injected with AAV-αSyn had no functional deficits in the corridor or stepping tests at any time point compared to AAV-Cont animals. However, there was subtle sensorimotor dysfunction seen in AAV-αSyn animals in the whisker test at 16 and 20 weeks compared to AAV-Cont animals, but this effect was not seen in animals injected with AAV-αSyn+AAV-GDF5. These results are in contrast to those found by Decressac et al (2011) where AAV-αSyn transduced animals displayed increased amphetamine-induced rotations at only 8 weeks post injection. Similarly, in another study, intranigral AAV-αSyn overexpression was found to significantly affect performance in the cylinder and stepping test 8 weeks post stereotactic surgery (Gombash et al., 2013). The discrepancies in the behavioural findings of this study compared to Decressac et al and Gombash et al were initially thought to be due to differences in the virus preparation. In this study, viruses were prepared using a SYN1 promoter and a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) to enhance expression. While, in the contrasting studies, a cytomegalovirus (CMV) enhancer hybrid synthetic chicken β-actin (CBA) promoter was used. However, in an additional study by Decressac et al (2012) AAV-αSyn with synapsin-1 and WPRE enhancer were used and again reported a significant effect on performance in the stepping test 3 weeks post-surgery and moreover, after 8 weeks there was significant motor dysfunction recorded in
the stepping, cylinder and rotation test (Decressac et al., 2012b). In addition, the authors reported a loss of over 50% of dopamine neurons from the SN after 8 weeks. However, in contrast, a study carried out using a similar virus found a non-significant 10% loss in dopaminergic neurons after AAV-αSyn injection to the SN at the same 8 week time point (Su et al., 2017). As is now known, motor impairments arise when there is a 50% loss of dopaminergic neurons and an 80% loss of striatal fibres (Cheng et al., 2010). In this study, α-synuclein overexpression resulted in a ~36% loss of TH+ neurons from the SN and ~45% loss in striatal integrity. Therefore, the inconsistency perhaps lies with the efficacy of the vector combination given i.e. AAV2/6 combined with AAV2/5 packaging units and perhaps a stronger behavioural phenotype would be realised using a higher titre of the virus.

Collectively, this study shows that long-term delivery of AAV-GDF5 to the SN is safe and provides targeted delivery of GDF5 to TH+ neurons. In addition, AAV-GDF5 did not affect motor function or induce weight loss in adult rats. AAV-αSyn delivery to the SN caused a significant reduction in TH+ number and striatal fibre density, that was not seen in animals co-transduced with AAV-GDF5. In addition, α-synuclein induced subtle sensorimotor function at the later time points which was attenuated by AAV-GDF5. Finally, the overexpression of α-synuclein did not affect the BMP receptors or effector molecules required for GDF5 signalling. In conclusion, GDF5 has now been shown to exert neuroprotective effects against MPP+, 6-OHDA and α-synuclein in vitro models of PD as well as the α-synuclein in vivo model of the disease. Moving forward, it will important to evaluate whether there are neurorestorative effects of GDF5 in the α-synuclein animal model after degeneration of the nigrostriatal system has already developed.
Chapter 5.0

Gene co-expression analysis of the human substantia nigra identifies BMP2 as a neurotrophic factor that can promote neurite growth in cells overexpressing wild-type or A53T α-synuclein.

5.1 Aims of Study

- Identify BMP family members that co-express with markers of dopaminergic neurons in the SN
- Test the lead BMP ligand against 6-OHDA, MPP⁺ and the overexpression of WT and mutant A53T α-synuclein in SH-SY5Y cells
- Assess the neurotrophic ability of the lead BMP ligand against these cellular models of PD in primary VM cultures

Work featured in this chapter has been published in the following paper:

5.2 Abstract

**Introduction:** α-synuclein-induced degeneration of dopaminergic neurons has been proposed to be central to the early progression of PD. This highlights the need to identify factors that are neuroprotective or neuroregenerative against α-synuclein-induced degeneration. GDF5 is a member of the BMP family and is neuroprotective against α-synuclein *in vivo*. Thus, this study hypothesized that other members of the BMP family have the potential to protect these cells against α-synuclein.

**Methods:** To identify the most relevant BMP ligands, unbiased gene co-expression analysis was used to identify all BMP family members having a significant positive correlation with five markers of dopaminergic neurons in the human SN. The ability of lead BMPs to promote neurite growth was tested in SH-SY5Y cells and in primary VM cultures of dopaminergic neurons, treated with either 6-OHDA or MPP⁺, or overexpressing WT or A53T α-synuclein.

**Results:** Only the expression of BMP2 was found to be significantly correlated with multiple dopaminergic markers in the SN. BMP2 treatment promoted neurite growth in SH-SY5Y cells and in dopaminergic neurons. Moreover, BMP2 treatment promoted neurite growth in both SH-SY5Y cells and VM neurons, treated with the neurotoxins 6-OHDA or MPP⁺. Furthermore, BMP2 promoted neurite growth in cells overexpressing WT or A53T-α-synuclein. Finally, this study found that GDF5 upregulated the expression of BMP2 in dopaminergic neurons both *in vitro* and *in vivo*.

**Conclusion:** These findings are important given that clinical trials of two neurotrophic factors, GDNF and neurturin, have failed to meet their primary endpoints. These findings are therefore a key first step in rationalising the further study of BMP2 as a potential NTF in α-synuclein-based translational models of PD.
5.3 Introduction

The degeneration of midbrain dopaminergic neurons leading to the loss of striatal innervation is central to the progression of PD (O’Keeffe and Sullivan, 2018). α-synuclein has been linked to PD since a number of mutations, such as the A30P (Kruger et al., 1998) or A53T mutation in SNCA (Polymeropoulos et al., 1997), as well as triplications (Singleton et al., 2003) or duplications (Chartier-Harlin et al., 2004) of SNCA, are associated with autosomal dominant PD, while SNCA is also a susceptibility gene for sporadic PD (International Parkinson Disease Genomics et al., 2011). In addition, α-synuclein is the main component of Lewy bodies and Lewy neurites, which are pathological hallmarks of PD (Spillantini et al., 1997). Overexpression of α-synuclein in individual cultured midbrain dopaminergic neurons has been shown to reduce neurite growth (Koch et al., 2015). This is also supported by the reduced connectivity and spine formation (Oliveira et al., 2015), and decreased neurite length and axonal degeneration (Lin et al., 2016b), found in iPSC-derived neurons with SNCA triplication. Moreover, iPSC-derived dopaminergic neurons carrying the A53T mutation also develop neurite degeneration (Kouroupi et al., 2017). These studies show that α-synuclein can lead to axonal and somal degeneration and highlight the need to identify factors that can promote dopaminergic neurite growth in neurons carrying an α-synuclein load.

BMPs are a group of 15 structurally-related proteins that are members of the TGF-β superfamily of ligands (Hegarty et al., 2014c). The BMPRs (BMPR2 with BMPR1A or BMPR1B) are expressed in embryonic and adult rat dopaminergic neurons (Hegarty et al., 2014a). Adult male BMPR2 dominant-negative mice display an almost complete loss (~90%) of striatal innervation, with reduced locomotor activity (Chou et al., 2008a). Previous studies have shown that BMP treatment increases dopaminergic neuron number in midbrain cultures in vitro (Jordan et al., 1997, Reiriz et al., 1999). Additionally, BMP
treatment, or overexpression of a constitutively-active BMPR1B, promotes neurite growth in SH-SY5Y cells (Hegarty et al., 2013b), and in primary cultures of midbrain dopaminergic neurons through the canonical Smad signalling pathway (Hegarty et al., 2014a, Hegarty et al., 2013a, Hegarty et al., 2013b). Moreover, knockdown of a negative regulator of BMP-Smad signalling during development promotes dopaminergic neurite growth and leads to dopaminergic hyperinnervation of the striatum (Hegarty et al., 2017c).

In chapter 3.0, a member of the BMP family known as GDF5 was shown to confer protection to a model of dopaminergic neurons and primary VM cultures against MPP+ and 6-OHDA induced axon, soma and cell degeneration as well as WT α-synuclein induced axon degeneration. Moreover, in chapter 4.0 delivery of AAV-GDF5 to the adult rat SN was shown to exert neuroprotective effects in the AAV-αSyn animal model of PD. Additionally, mediators of the BMP-Smad signalling were found to be unaffected by the overexpression of AAV-αSyn in vivo. Given these important findings, this study sought to identify the expression profiles of individual BMPs in midbrain dopaminergic neurons, and to test the neurotrophic ability of lead BMP ligands against cells overexpressing α-synuclein.
5.4 Results

5.4.1 Co-expression analysis of the human SN identifies an association between BMPs/BMPRs and markers of dopaminergic neurons.

An unbiased co-expression analysis was conducted to identify all BMP genes that were co-expressed with five markers of midbrain dopaminergic neurons in the human SN: \textit{ALDH1A1}, \textit{NR4A2/Nurr1}, \textit{KCNJ6/Girk2}, \textit{TH} and \textit{LMX1B} (Fig. 5.1A, B) (Ramasamy et al., 2014). These analyses revealed significant positive correlations only for a specific ligand and receptors of the BMP subfamily, namely \textit{BMP2}, \textit{BMPR2}, \textit{BMPRIA} and \textit{BMPRI1B}, from the lists of co-expressed genes (Fig. 5.1B-F). While all of the BMPRs were positively correlated with four markers of Purkinje neurons in the human cerebellum (\textit{PDE1A}, \textit{CBLN1}, \textit{PDE9A}, \textit{CALB1}), \textit{BMP2} was not (Fig. 5.1G). \textit{BMP2} was also expressed at significantly higher levels in the SN than in the putamen or cerebellum (Fig. 5.1H). Gene expression data from age- and gender-matched samples in GSE:49036 (Dijkstra et al., 2015) was also examined for \textit{BMP2} and \textit{BMPR} expression in the SN of patients at distinct stages of PD. These analyses showed a significant downregulation of \textit{BMP2} in the SN of PD patients at Braak stage 5/6 compared to controls (Fig. 5.1I), while no significant differences were observed for \textit{BMPRI1B} (data not shown) or \textit{BMPR2} (Fig. 5.1J). These data suggest that BMP2-BMPR signalling may play a functional role in dopaminergic neurons in the human SN.

5.4.2 rhBMP2 promotes neurite growth in SH-SY5Y cells in a dose-dependent manner and activates Smad signalling.

To establish the effects of rhBMP2 on individual neuronal cells, SH-SY5Y cells were initially used. SH-SY5Y cells were treated with 0-200ng/ml concentrations of rhBMP2
for 24 h (Fig. 5.2A) or 48 h (Fig. 5.2B, C). The total neurite length was used as a readout of neurotrophic action and was measured at each time point. Statistical analysis revealed that the effect of rhBMP2 on neurite growth is dose-dependent, with 50ng/ml rhBMP2 promoting the greatest increase in neurite growth at both 24 and 48h. To determine if 50ng/ml rhBMP2 could activate the Smad signalling pathway, a phospho-Smad ELISA kit was used as before in chapter 3.0. Here, SH-SY5Y cells were treated with 50ng/ml rhBMP2 for 2 h and processed for the ELISA. Statistical analysis demonstrated a significant increase in phospho-Smad after treatment with 50ng/ml rhBMP2 for 2 h (Fig. 5.2D, E). These results are in agreement with previous findings that rhBMP2 activates Smad-signalling and elicits neurotrophic effects on SH-SY5Y cells (Hegarty et al., 2013b) and provides further knowledge of the most potent concentration of rhBMP2 for neurite growth promotion.

5.4.3 MPP⁺ and 6-OHDA elicit concentration-dependent neurotoxic effects on SH-SY5Y cells.

In order to test the ability of rhBMP2 against cellular models of PD, the next step was to determine a concentration of the neurotoxins MPP⁺ and 6-OHDA that would elicit a significant decrease in neurite length and cell viability in SH-SY5Y cells. Here, SH-SY5Y cells were treated daily with increasing concentrations of MPP⁺ (0-1mM) or 6-OHDA (0-15µM) for 72 h. The effect of both neurotoxins was analysed using neurite length analysis and an MTT assay was used to measure cell viability, whereby a decrease in absorbance is indicative of a reduction in cell viability. Increasing concentrations of both MPP⁺ and 6-OHDA led to significant decreases in neurite length at all concentrations tested for MPP⁺ (Fig. 5.3A, B) and at 10 and 15µM concentrations of 6-OHDA (Fig. 5.3D, F). In addition, 1 and 1.5mM concentrations of MPP⁺ produced a
substantial decrease in cell viability (Fig. 5.3C) while 10 and 15µM concentrations of 6-OHDA also proved detrimental to cell viability (Fig. 5.3E). Given that 1mM MPP⁺ and 15µM 6-OHDA induced statistically significant decreases in both neurite length and cell viability, these concentrations were used in further experiments.

5.4.4 rhBMP2 promotes neurite growth and survival, in 6-OHDA- and MPP⁺-treated SH-SY5Y cells.

To determine if rhBMP2 treatment could promote neurite growth in cells treated with 6-OHDA or MPP⁺, SH-SH5Y cells were treated with 50 ng/ml rhBMP2 and cultured with or without 1mM MPP⁺ or 15µm 6-OHDA for 72 h. Here, rhBMP2 promoted neurite growth while treatment with MPP⁺ or 6-OHDA led to a significant decrease in neurite length compared to the untreated control (Fig. 5.4A, B). However, when cells treated with 6-OHDA or MPP⁺ were co-treated with rhBMP2, there was no significant difference in neurite length compared to the untreated control indicating a protective effect of rhBMP2 (Fig. 5.4A, B). The viability of the cells was also analysed via MTT assay and showed that MPP⁺ and 6-OHDA significantly decreased cell survival compared to the control (Fig. 5.4C). However, when co-treated with rhBMP2, the groups were not significantly different from the untreated control (Fig. 5.4C), despite the fact that a decrease was still present. These data show that rhBMP2 can protect both against the degenerative effects of MPP⁺ and 6-OHDA in SH-SY5Y cells.

5.4.5 rhBMP2 promotes neurite growth in SH-SY5Y cells overexpressing WT-α-synuclein or mutant A53T-α-synuclein.

Given that the majority of PD cases involve the cellular accumulation of α-synuclein, this study next sought to investigate whether rhBMP2 could promote neurite growth in
cells overexpressing WT- or A53T-α-synuclein. To do this SH-SH5Y cells were transfected with a control-GFP plasmid, or plasmids expressing GFP-tagged WT-α-synuclein or A53T-α-synuclein. Quantification of α-synuclein overexpression was confirmed by western blotting and immunocytochemistry which revealed strong expression of α-synuclein in cells transfected with either WT- or A53T-α-synuclein-GFP (Fig. 5.5A, B). Furthermore, quantification of GFP-positive neurites showed that both WT- and A53T-α-synuclein overexpression induced significant decreases in neurite length compared to the untreated GFP control, while rhBMP2 promoted neurite length (Fig. 5.5C, D). In addition, rhBMP2 co-treatment was found to protect neurite growth against the effects of both WT- and A53T-α-synuclein-overexpression in these cells (Fig. 5.5C, D). These data highlight that rhBMP2 exerts neurotrophic effects in neuronal cells overexpressing α-synuclein.

5.4.6 rhBMP2 protects cultured midbrain dopaminergic neurons against 6-OHDA and MPP+.

Although SH-SY5Y cells are catecholaminergic, and are widely used in cellular models of PD, they do not recapitulate all the features of dopaminergic neurons. For this reason, the effects of rhBMP2 in primary cultures established from the E14 rat VM were examined. Firstly, a dose response experiment was performed to confirm the best concentration of MPP+ and 6-OHDA that would induce axon degeneration in primary dopaminergic neurons in these cultures after 72 h in vitro. Here, it was determined that all doses tested after had a detrimental effect on neurite growth after 72h. Therefore, in keeping with chapter 3.0, 5μM MPP+ or 6-OHDA was used in subsequent experiments (Fig. 5.6A, B). Here, 50ng/ml rhBMP2 treatment induced significant increases in dopaminergic neurite growth, while MPP+ and 6-OHDA caused a significant reduction
compared to the untreated control (Fig. 5.6C, D). However, co-treatment with rhBMP2 completely prevented the 6-OHDA or MPP+-induced degeneration (Fig. 5.6C, D). The effect of MPP+ and 6-OHDA on somal area was then examined and a substantial reduction in somal size was found, which was not seen in cultures co-treated with rhBMP2 (Fig. 5.6E). After assessing cell viability, both neurotoxins were found to cause a significant decrease in the number of TH+ neurons, which was partially rescued by rhBMP2 (Fig 5.6F, H), while the total cell number was unaffected (Fig. 5.6G), indicating a partial neuroprotective effect of rhBMP2. Collectively these data show that rhBMP2 promotes neurite growth and partially protect primary midbrain dopaminergic neurons against 6-OHDA- and MPP+-induced degeneration.

5.4.7 rhBMP2 promotes neurite growth in WT-α-synuclein- and A53T-α-synuclein- overexpressing cultured midbrain dopaminergic neurons.

To investigate if rhBMP2 could also promote neurite growth in neurons overexpressing WT- or mutant A53T-α-synuclein, E14 rat VM cultures were transfected with both WT-α-synuclein-GFP and A53T-α-synuclein-GFP overexpression plasmids, or with a control-GFP plasmid. Immunocytochemistry (Fig. 5.7A) and subsequent quantification of α-synuclein staining (given the transfection efficiency was not sufficient for western blotting) showed that E14 VM cultures transfected with either the WT-α-synuclein-GFP or A53T-α-synuclein-GFP plasmid showed strong expression of α-synuclein compared to cells transfected with the control plasmid (Fig. 5.7B). While the overexpression of WT-α-synuclein-GFP or A53T-α-synuclein had no significant effect on somal area compared to the control-GFP group (Fig. 5.7C), both WT-α-synuclein-GFP and A53T-α-synuclein-GFP resulted in a significant decrease in neurite length compared to controls (Fig. 5.7D, E). In contrast, WT-α-synuclein-GFP or A53T-α-synuclein-GFP expressing
cells that were also treated with rhBMP2 were not significantly different from the controls. These data show that WT- or A53T-α-synuclein reduced neurite growth without affecting somal area, and that rhBMP2 can promote neurite growth in cultured midbrain dopaminergic neurons overexpressing WT- or A53T-α-synuclein.

5.4.8 GDF5 upregulates BMP2 in SH-SY5Y cells and in the adult rat SN.

Chapter 3.0 highlighted the neuroprotective effects of GDF5 against cellular models of PD and chapter 4.0 revealed GDF5 exerts neuroprotection in vivo against the AAV-αSyn rat model of PD. However, given that BMP2 was the only member of the BMP family found to be co-expressed with five key markers of dopaminergic neurons in the SN (Fig. 5.1) and given its neurotrophic effects against cellular models of PD (Fig. 5.3-5.7), the hypothesis arose that the neurotrophic action of GDF5 may be partially mediated via the increased expression of BMP2. To test this hypothesis, SH-SY5Y cells were cultured with or without 50ng/ml rhGDF5 for 24 h. RNA was then prepared for RT-qPCR analysis of BMP2 expression. RT-qPCR analysis showed that transcripts for BMP2 were significantly upregulated after rhGDF5 treatment compared to the untreated control (Fig. 5.8A). To substantiate this finding, SN tissue from animals unilaterally injected with either an AAV-Control or AAV-GDF5 vector for 20 weeks were immunofluorescently stained for BMP2 and TH (Fig. 5.8B). The expression of BMP2 within TH+ neurons was then quantified and expressed as a percentage of the contralateral intact side (Fig. 5.8C). This analysis revealed a significant increase in BMP2 expression in AAV-GDF5 animals compared to AAV-Control animals. These findings therefore add significant weight to the potential contribution of BMP2 to the neurotrophic actions of GDF5 in vivo.
5.5 Figures and Figure Legends

Fig. 5.1 BMP2-BMPRs are co-expressed with dopaminergic neuron markers in the human SN. A, B Pearson correlation analysis showing the r values and false discovery rate (FDR)-corrected p-values (in parentheses) from the SN (n=99). C-F Graphs showing the correlation between BMPR2, BMPR1B, BMPR1A, BMP2 and ALDH1A1 expression.
in the SN. All data are 2log expression values. G Results of the Pearson correlation analysis showing the \( r \) values and FDR-corrected p-values (in parentheses) of the correlation of \( BMPR2 \), \( BMPR1B \), \( BMPR1A \) and \( BMP2 \) with four Purkinje neuron markers. H Box plot showing \( BMP2 \) expression in the human SN (n=99), putamen (\( n = 121 \)) and cerebellum (\( n = 121 \)) (*** \( p < 0.001 \) and ### \( p < 0.001 \) vs. SN). I, J 2log expression values of I \( BMP2 \) and J \( BMPR2 \) in the SN of PD at distinct Braak stages of PD, and in age-matched controls (*\( p < 0.05 \) vs. Control). H-J One-way ANOVA with post-hoc Tukey’s test. Raw data was obtained from B-H GSE:60863 and I, J GSE:49036 and analysed using the R2: Genomics analysis and visualisation platform (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi).
Fig. 5.2 rhBMP2 promotes neurite growth in SH-SY5Y cells in a dose-dependent manner and activates Smad signalling. Graphs showing the neurite length of SH-SY5Y cells treated with 0-200ng/ml rhBMP2 for A 24 h and B 48 h. C Representative photomicrographs of SH-SY5Y cells treated with 0-200ng/ml rhBMP2 for 48 h. D Quantification of phospho-Smad expression and E Representative photomicrographs of SH-SY5Y cells treated with 50ng/ml rhBMP2 for 2 h and immunocytochemically stained for phospho-Smad 1/5. Scale bar = 50µm. All data are presented as mean ± SEM from at least three experiments. (* p < 0.05, ** p < 0.01, *** p < 0.001 vs. control or as indicated. One-way ANOVA with Tukey’s post-hoc test (A, B) or Unpaired Student’s t-test (C)).
Fig. 5.3 MPP⁺ and 6-OHDA elicit concentration-dependent neurotoxic effects on SH-SY5Y cells. A Representative photomicrographs B Neurite length and C Cell viability of SH-SY5Y cells following 72h daily treatment with increasing concentrations of MPP⁺. D Neurite length E Cell viability and F Representative photomicrographs of SH-SY5Y cells following 72h daily treatment with increasing concentrations of 6-
OHDA. Scale bar = 50μm. All data are presented as mean ± SEM from at least three experiments. (**p < 0.01, ***p < 0.001 vs. control or as indicated. One-way ANOVA with Tukey’s post-hoc test).
Fig. 5.4 rhBMP2 promotes neurite growth in MPP⁺-treated, 6-OHDA-treated SH-SY5Y cells. Graph of A Neurite length, B Representative photomicrographs and C Cell viability and of SH-SY5Y cells treated with 1mM MPP⁺ or 15μM 6-OHDA cultured with or without 50ng/ml rhBMP2 for 72 h. Scale bar = 100μm. All data are presented as mean ± SEM from at least three experiments. (⁎ p < 0.05, ⁎⁎ p < 0.01, ⁎⁎⁎ p < 0.001 vs. control or as indicated. One-way ANOVA with Tukey’s post-hoc test).
Fig. 5.5 rhBMP2 promotes neurite growth in WT and A53T α-synuclein-overexpressing SH-SY5Y cells. A Representative images of SH-SY5Y cells transfected with constructs expressing GFP (Control-GFP) or A53T-α-synuclein-GFP (αSynA53T-GFP) immunocytochemically stained for α-synuclein protein at 24h, scale bar = 10μm; quantification of this expression is shown in B. C Total neurite length and D Representative images of transfected SH-SY5Y cells expressing either control-GFP or αSynA53T-GFP and cultured with or without 50ng/ml rhBMP2 for 72h. Scale bar = 50μm. All data are presented as mean ± SEM from at least three experiments. (* p < 0.05, **p < 0.01, ***p < 0.001 vs. control or as indicated. One-way ANOVA with Tukey’s (A, B, C, G) or Dunnett’s (F) post hoc test).
Fig. 5.6 rhBMP2 promotes neurite growth and partially protects against dopaminergic neuron loss in MPP⁺-treated and 6-OHDA-treated primary cultures of rat VM. Total neurite length of dopaminergic neurons in E14 VM cultures following 72h treatment with A 6-OHDA or B MPP⁺, at the concentrations indicated. C Total neurite length and D Representative photomicrographs of dopaminergic neurons in E14 VM cultures treated with 50ng/ml rhBMP2 and cultured with or without 5μM MPP⁺ or 5μM 6-OHDA for 72 h. Scale bar = 50μm. E Somal area, F TH⁺ neurons as a percentage of total cells and G number of cells per field of view, in primary cultures of the E14 rat VM treated with 50ng/ml rhBMP2 and cultured with or without 5μM MPP⁺ or 5μM 6-OHDA for 72 h. H Representative photomicrographs of TH/DAPI-stained E14 VM.
cultures, treated as indicated. Scale bar = 50µm. All data are presented as mean ± SEM from at least three experiments. (* p < 0.05, **p < 0.01, ***p < 0.001 vs. control or as indicated. One-way ANOVA with Tukey’s *post-hoc* test).

**Fig. 5.7** **rhBMP2** promotes neurite growth in WT α-synuclein- and A53T α-synuclein-overexpressing cells in primary cultures of E14 rat VM. A Representative images of primary cultures of E14 rat VM transfected with constructs expressing GFP (Control-GFP), α-synucleinWT-GFP (α-synWT-GFP) or α-synuclein A53T-GFP (α-synA53T-GFP), then immunocytochemically stained for α-synuclein at 72h. Scale bar = 10µm. Quantification of B α-synuclein expression and C cell somal area in cultures
transfected with Control-GFP, α-synWT-GFP and α-synA53T-GFP. D Representative images (Scale bar = 50μm) and E quantification of total neurite length of E14 VM cultures transfected with Control-GFP, α-synWT-GFP and α-synA53T-GFP and cultured with or without 50ng/ml rhBMP2 for 72h. All data are presented as mean ± SEM from at least three experiments. (*** p < 0.001 vs. control or as indicated. One-way ANOVA with Tukey’s post-hoc test).
Fig. 5.8 GDF5 upregulates the expression of Bmp2 transcripts in SH-SY5Y cells and BMP2 protein expression in TH+ neurons of the adult rat SN. A Real time PCR data showing expression of transcripts for Bmp2 in SH-SY5Y cells after 24h with and without treatment of 50ng/ml rhGDF5. B Representative images of BMP2 expression in TH+ neurons in the adult rat SN of animals who received an AAV-Control or AAV-GDF5 vector and immunocytochemically stained for the expression of BMP2 (green) and TH (red). Scale bar = 25µm. C Quantification of BMP2 expression in the SN of AAV-Control or AAV-GDF5 animals, expressed as a percentage of the contralateral side. Data are mean ± SEM from n=8 rats (*p < 0.05, **p<0.01; Student’s t-test).
5.6 Discussion

The BMP family exerts a broad range of biological functions depending on the tissue type and cellular context (Nishimura et al., 2012, Wu et al., 2016, Hegarty et al., 2014a, Jordan et al., 1997, Farkas et al., 1999, Yan et al., 2016). In recent years BMPs have emerged as potent neurotrophic factors for midbrain dopaminergic neurons and can promote dopaminergic neuronal survival, axonal growth and protect against neurotoxic insult (Krieglstein et al., 1995, Jordan et al., 1997, Hegarty et al., 2014a, O'Keeffe et al., 2017). The axon growth promoting effects of BMPs are particularly interesting given that axonal degeneration is increasingly recognised as being central to early nigrostriatal degeneration in PD (Burke and O'Malley, 2013, O'Keeffe and Sullivan, 2018, Dijkstra et al., 2015), suggesting that BMPs may be useful in protecting the nigrostriatal pathway in PD. In chapters 3.0 and 4.0, this thesis presented evidence of the potent neurotrophic effects on midbrain dopaminergic neurons of a member of the BMP family, GDF5, in cellular models of PD and in the α-synuclein rat model of PD. This promising discovery is particularly important given the failure of GDNF to promote dopaminergic survival using the same animal model (Decressac et al., 2011). In light of this novel finding, a number of key questions arose; 1) which BMPs are functionally associated with midbrain dopaminergic neurons in the adult SN, and 2) can these BMPs protect dopaminergic neurons against neurotoxin-, and in particular, α-synuclein-induced degeneration.

In the present study, only the expression of the BMP ligand, BMP2, and its receptors, BMPR1A, BMPR1B and BMPR2, were shown to be positively correlated with five key markers of dopaminergic function in the human SN, suggesting a functional relationship between this BMP member and midbrain dopaminergic neurons. The study also highlights that BMP2 is highly expressed in the SN compared to the putamen or indeed the cerebellum, an area known to be particularly responsive to BMPs (Alder et al.,
In agreement with this, previous studies have shown the expression of transcripts for *BMP2, BMPR1B* and *BMPR2* receptors in the rat midbrain from E12, a period where neurotrophic support is critical, prior to target innervation (Hegarty et al., 2014a). Furthermore, BMP2 expression is identified to be significantly downregulated in the SN in PD patients at Braak stage 5/6, while there are no significant changes in the expression of its receptors. The findings of these studies suggested that endogenous BMP signalling, perhaps mediated by BMP2, may promote midbrain dopaminergic neuronal survival and/or maintain dopaminergic axonal integrity in the adult brain.

Using SH-SY5Y cells as an initial cellular model, the functional role of BMP2 was examined and found that similar to rhGDF5, rhBMP2 exerts a concentration-dependent effect on neurite growth, with 50ng/ml rhBMP2 exerting the greatest increase at both 24 and 48h time points. In support of this, rhBMP2 has previously been shown to enhance axonal outgrowth in both SH-SY5Y cells and in dopaminergic neurons in primary E14 VM cultures (Reiriz et al., 1999, Jordan et al., 1997, Hegarty et al., 2014a, Hegarty et al., 2013b, Nakamura et al., 2003). 50ng/ml rhBMP2 was also shown to initiate activation of the canonical BMP-Smad signalling pathway in SH-SY5Y cells after 2hr, analysed through the increased expression of phospho-Smad. This is in agreement with previous studies showing that rhBMP2 is dependent on the canonical Smad signalling pathway to elicit neurotrophic action (Boergermann et al., 2010, Yu et al., 2008).

Given that rhBMP2 promotes neurite length in SH-SY5Y cells, the effect of rhBMP2 on cellular models of PD was examined next. A dose response of the well-established dopaminergic neurotoxins MPP⁺ and 6-OHDA was firstly carried out to determine a concentration of either neurotoxin that would cause neurite as well as cell degeneration. Here, 1mM MPP⁺ and 15µM 6-OHDA were found to cause significant reductions in neurite length while also decreasing cell viability after 72h. However,
when either neurotoxin was co-treated with rhBMP2 for 72h, the degeneration of neurite length and cell viability was no longer realised and instead were maintained at the same level as an untreated control. To then establish the potential of rhBMP2 against α-synuclein, SH-SY5Y cells were transfected with either WT α-synuclein (αSynWT-GFP) or mutant A53T-α-synuclein (αSynA53T-GFP). The overexpression of α-synuclein was quantified by western blot and immunocytochemistry compared to a control-GFP plasmid. In addition, the overexpression of WT- and A53T-α-synuclein-GFP in SH-SY5Y cells caused a significant decrease in neurite length after 72h, consistent with chapter 3.0 and previous findings (Koch et al., 2015, Oliveira et al., 2015), which was not seen when the groups were co-treated with rhBMP2.

However, while SH-SY5Y cells are widely-used as an in vitro model of dopaminergic neurons, they are ultimately catecholaminergic cells and thus do not replicate all of the features of dopaminergic neurons. To address this, primary cultures of E14 rat VM were used. A dose-response for MPP⁺ and 6-OHDA was initially carried out and confirmed that 5µM MPP⁺ and 6-OHDA induced axonal degeneration in TH⁺ neurons, as in chapter 3.0. rhBMP2 treatment was found to promote neurite length of TH⁺ neurons, consistent with previous findings showing rhBMP2 can promote survival and growth in rodent neurons (Jordan et al., 1997, Reiriz et al., 1999, Hegarty et al., 2014a). In addition, rhBMP2 protected dopaminergic neurons against MPP⁺- and 6-OHDA-induced axonal degeneration. Furthermore, MPP⁺ caused a reduction to cell soma size, and both MPP⁺ and 6-OHDA reduced numbers of TH⁺ neurons, both of which were partially protected by rhBMP2. In support of these findings, rhBMP2-treatment has also been shown to increase the survival of transplanted E14 VM dopaminergic neurons and to decrease motor deficits, in 6-OHDA-lesioned rat models of PD (Espejo et al., 1999). Collectively, these data show that rhBMP2 can protect dopaminergic neurons against
neurotoxin-induced injury and thus may have significant potential as an NTF for use in PD therapy.

While MPP+ and 6-OHDA are very useful neurotoxins for modelling PD, they do not mimic the α-synuclein accumulation that is central to disease progression (Stefanis, 2012). Therefore, E14 VM cultures were transfected with WT- and A53T-α-synuclein and found a decrease in neurite length, in agreement with a previous study using E14 rat dopaminergic neurons (Koch et al., 2015). Here, rhBMP2 was found to promote neurite growth against this α-synuclein-induced axonal degeneration. In addition, while both WT- and A53T- α-synuclein had significant effects on neurite length, neither WT- or A53T-α-synuclein had an effect on somal size, which is in agreement with chapter 3.0 and a previous study (Koch et al., 2015). This is significant as it is increasingly recognised that the degenerative aetiology of PD may occur at both somal and axon levels (Burke and O’Malley, 2013). Indeed, there have been several studies describing axonal degeneration using iPSC-derived dopaminergic neurons isolated from patients with PD. Specifically, iPSCs derived from PD patients with SNCA triplications displayed decreased neuronal differentiation, activity and neurite outgrowth compared to control iPSCs (Oliveira et al., 2015), as well as atypical neurite length and axonal fragmentation (Lin et al., 2016b). Additionally, it has also been shown that PD patient iPSC-derived dopaminergic neurons display phenotypic characteristics of PD, including Lewy-like neurites comprised of pathological α-synuclein, protein aggregation, atypical neurite outgrowth, and fragmented axons containing swollen varicosities of α-synuclein or Tau, and have a reduced capability to form synapses (Kouroupi et al., 2017). These data agree with previous in vivo studies showing that transgenic mice expressing human α-synuclein develop synaptic dysfunction in striatal dopaminergic terminals and display an age-dependent reduction in dopamine release (Garcia-Reitbock et al., 2010, Hunn et al.,
Therefore, given this mounting evidence in support of the role of α-synuclein in axonal degeneration in PD, it is important to identify neurotrophic factors that can protect against α-synuclein-induced axonal degeneration.

Chapter 3.0 and chapter 4.0 have given evidence to show that a member of the BMP family, GDF5, has potent neurotrophic capabilities against *in vitro* models of PD as well as *in vivo* against the α-synuclein rat model of PD, in addition to previous findings (Krieglstein et al., 1995, Sullivan et al., 1997, O'Keeffe et al., 2004a, Wood et al., 2005, O'Sullivan et al., 2010, Costello et al., 2012). However, in this study only the expression of BMP2 was found to be correlated with five key markers of dopaminergic neurons in the SN. Interestingly, both GDF5 and BMP2 activate BMP-Smad signalling through preferential binding to BMPR1B and BMPR2 receptors (Hegarty et al., 2014a), both of which are also co-expressed with the five markers of dopaminergic neurons. Therefore, this study sought to establish whether a link existed between GDF5, BMP2 and Smad signalling. RT-PCR confirmed that rhGDF5 upregulated the expression of *BMP2* transcripts in SH-SY5Y cells. In addition, animals who had received intranigral injection of AAV-GDF5 had a significantly increased presence of BMP2 in TH⁺ neurons compared to AAV-Control animals, further highlighting an important association exists between GDF5 and BMP2. This finding is particularly interesting given the fact that AAV-GDF5 was neuroprotective against AAV-αSyn, suggesting that AAV-BMP2 may also offer significant protection to dopaminergic neurons.

In summary, this study identified that BMP2 is co-expressed with midbrain dopaminergic neuronal markers in the human SN and is downregulated in PD, and that treatment with rhBMP2 can promote dopaminergic neurite growth and protect against neurotoxin- and α-synuclein-induced degeneration in a model of dopaminergic neurons and in primary cultures of dopaminergic neurons. In addition, GDF5, which was
previously shown to be neuroprotective against the AAV-αSyn rat model of PD, was shown to upregulate the expression of BMP2 in dopaminergic neurons and in the SN of AAV-GDF5 treated animals. These findings are important given that clinical trials of two dopaminergic NTFs, GDNF and neurturin, in PD patients have failed to meet their primary endpoints (Hegarty et al., 2017a, Whone et al., 2019a, Whone et al., 2019b, Warren Olanow et al., 2015). In light of this, the findings in our study are an important first step in rationalising the further study of BMP2 as a potential neuroprotective therapy using α-synuclein-based translational models of PD.
Chapter 6.0

Quinacrine and Niclosamide promote neurite growth in midbrain dopaminergic neurons through the canonical BMP-Smad pathway and protect against neurotoxin and α-synuclein-induced neurodegeneration.

6.1 Aims of Study

- Investigate the effect of Quinacrine and Niclosamide on SH-SY5Y cells and primary VM cultures
- Identify the effect of Quinacrine and Niclosamide on the canonical Smad signalling pathway
- Determine the effect of Quinacrine and Niclosamide against cellular models of PD in primary VM cultures of dopaminergic neurons
- Evaluate the potential effect of Quinacrine and Niclosamide in primary dopaminergic neurons transduced with AAV2/6-α-synuclein
6.2 Abstract

**Introduction:** PD is a neurological disorder affecting over 10 million people worldwide. Currently, the available treatment options are solely symptomatic and do not slow or stop disease progression. This highlights the need to identify factors that are neuroprotective or neuroregenerative against the progressive dopaminergic degeneration. This thesis identified a member of the BMP family, known as BMP2, whose expression is significantly correlated with that of several key markers of dopaminergic neurons in the human SN and whose transcripts are downregulated in PD. In addition, chapter 5.0 showed that treatment with rhBMP2 is neuroprotective against dopaminergic degeneration in *in vitro* models of Parkinson’s disease.

**Methods:** Using SH-SY5Y cells and primary E14 VM cultures, this study investigated the neurotrophic profile of two FDA-approved drugs, Quinacrine and Niclosamide, that have previously been shown to be modulators of BMP2 expression.

**Results:** Here, Quinacrine and Niclosamide were found to promote neurite length in both SH-SY5Y cells and in primary cultures of dopaminergic neurons. In addition, the neurotrophic action of Quinacrine and Niclosamide was shown to be mediated through the activation of the canonical Smad signalling pathway. Finally, these two drugs were demonstrated to be neuroprotective against *in vitro* models of Parkinson’s disease including AAV-mediated delivery of α-synuclein.

**Conclusion:** Collectively this study identifies two drugs, that are safe for use in patients, that can mimic the effects of endogenous BMP2 and activate BMP-Smad signalling. This study therefore demonstrates the feasibility of using small molecule compounds to target BMP-Smad signalling to protect dopaminergic neurons in patients with Parkinson’s disease.
6.3 Introduction

PD is a common bradykinetic disorder characterised by the progressive degeneration of midbrain dopaminergic neurons, and the accumulation of intracellular aggregates of α-synuclein which are known as Lewy bodies and Lewy neurites (Lees et al., 2009). In recent years there has been considerable interest in developing NTF therapy for PD. This involves the delivery of NTF genes, or the recombinant proteins to the brain to protect midbrain dopaminergic neurons from degeneration (Kelly et al., 2015). Despite initial success in animal’s models and in open label trials (Choi-Lundberg et al., 1997, Kordower et al., 2000, Kordower et al., 2006, Ramaswamy et al., 2007, Su et al., 2009, Gill et al., 2003, John T. Slevin et al., 2005), randomised controlled trials of GDNF and a related factor Neurturin failed to meet their primary end points (Lang et al., 2006, Patel et al., 2005, Olanow et al., 2003, Warren Olanow et al., 2015, Whone et al., 2019a, Whone et al., 2019b). As such there has been increasing interest in identifying other NTFs that are capable of protecting dopaminergic neurons from degeneration. However, the challenge surrounding NTFs is that they require direct administration to the brain as they are rapidly metabolised in vivo and are unable to cross the blood brain barrier in adequate doses (O'Keeffe et al., 2017). One strategy to overcome this problem may be to identify small molecule drugs or biologics that are capable of crossing the blood brain barrier and selectively activating the downstream targets of specific neurotrophic factors.

BMPs are a group of neurotrophic factors that like GDNF, are also members of the TGF-β superfamily (for review see O'Keeffe et al., 2017). Additionally, gene co-expression analysis of the human SN in chapter 5.0 identified that BMPRs and the BMP2 ligand display a co-expression pattern with multiple markers of dopaminergic neurons in the human SN, suggesting a functional role for BMPs in this neuronal population. Moreover, rhBMP2 promoted neurite growth in SH-SY5Y cells and dopaminergic
neurons after treatment with the neurotoxins 6-OHDA and MPP⁺, or in those overexpressing α-synuclein. In addition, AAV-mediated delivery of the BMP ligand GDF5 was neuroprotective against α-synuclein overexpression in vivo and was shown to upregulate the expression of BMP2 in the SN in Chapter 4.0. These data suggest that clinically-approved drugs that modulate BMP2 expression or the BMP2 signalling pathway, may be a novel class of compounds for protecting dopaminergic neurons.

In a recent study, Ghebes et al carried out a screen of 1280 commercially available FDA-approved compounds in primary human tendon–derived cells to identify those capable of modulating the BMP2 signalling pathway, which is essential for tendon and ligament repair (Ghebes et al., 2017). This analysis identified two drugs called Niclosamide and Quinacrine that modulated the BMP2 pathway using a BMP2 reporter cell line (Ghebes et al., 2017). Niclosamide is an anthelmintic agent that has been used clinically to treat tape worm infections (Chen et al., 2018), while Quinacrine has a long history of clinical use in the treatment of malaria (Ehsanian et al., 2011). Given that Niclosamide and Quinacrine were found to modulate BMP2 signalling in primary tendon cells (Ghebes et al., 2017), and since rhBMP2 protects dopaminergic neurons in primary cultures of the rat VM from neurotoxin- and α-synuclein-induced degeneration, this study hypothesised that Niclosamide and Quinacrine may have neurotrophic and neuroprotective effects on dopaminergic neurons in in vitro models of PD.
6.4 Results

6.4.1 SH-SY5Y cells as a tool to study drugs affecting BMP-Smad signalling.

This study initially used SH-SY5Y cells as they are a widely used model of dopaminergic neurons and are a useful drug screening tool of relevance to PD (Xie et al., 2010, Xicoy et al., 2017). These cells express high levels of BMPR1B (Fig. 6.1A), BMPR2 (Fig. 6.1B) and Smad1/5/8 (Fig. 6.1C). These cells respond to a BMP stimulus evidenced by the fact that treatment with rhBMP2 led to a significant increase in phospho-Smad1/5 activation (Fig. 6.1D, E) and an increase in neurite growth which was used as a readout of neurotrophic action at an individual cell level (Fig. 6.1F, G). Moreover, a functional link between an increase in pSmad1/5 levels and neurite growth was demonstrated by the fact that pre-treatment with dorsomorphin, a small molecule inhibitor of type-1 BMPRs (Yu et al., 2008), prevented the effects of rhBMP2 on pSmad1/5 and on neurite growth (Fig. 6.1F, G). Collectively these data show that SH-SY5Y cells are a suitable tool to study the potential effects of Quinacrine and Niclosamide on the BMP-Smad pathway and on neurite growth.

6.4.2 Quinacrine and Niclosamide promote neurite growth in SH-SY5Y cells.

Given that rhBMP2 is known to promote axon length in a number of neuronal cell types, including dopaminergic neurons (Jordan et al., 1997, Reiriz et al., 1999, Hegarty et al., 2013b), and that Quinacrine and Niclosamide have been shown to upregulate BMP2 expression in tendon tissue (Ghebes et al., 2017), the ability of both compounds to promote neurite growth as a readout of neurotrophic action in SH-SY5Y cells was next
tested. To do this, a dose-response experiment was performed in which SH-SY5Y cells were treated daily with 0-10nM concentrations of either Quinacrine or Niclosamide or with 50ng/ml rhBMP2 as a positive control for 72 h. Comparable results were found for both compounds whereby a concentration of 1 or 10nM Niclosamide (Fig. 6.2A, C) or Quinacrine (Fig. 6.2D, F) significantly increased neurite length relative to untreated controls and to a similar extent to that of rhBMP2. Cell viability was also examined, and it was found that there were no significant differences in cell viability in those groups treated with up to 10nM Niclosamide (Fig. 6.2B, C) or Quinacrine (Fig. 6.2E, F) relative to the untreated controls. Collectively these data show that Quinacrine and Niclosamide promote neurite growth and do not induce any adverse effects on cell viability in SH-SY5Y cells, which rationalised their further study in primary dopaminergic neurons.

6.4.3 Neurotrophic effects of Quinacrine and Niclosamide on primary midbrain dopaminergic neurons.

Although SH-SY5Y cells are a useful screening tool, they do not recapitulate all features of dopaminergic neurons. Therefore, a dose-response experiment was performed in which increasing concentrations (0-10nM) of Quinacrine and Niclosamide were applied to primary cultures of the E14 rat VM for 72h. In this experiment, concentrations of 0.1-10nM Quinacrine (Fig. 6.3A, C) or Niclosamide (Fig. 6.3D, F) induced a significant increase in dopaminergic neurite growth relative to untreated controls, and at a comparable level to neurite growth induced by rhBMP2. Given that a 10nM concentration of Niclosamide or Quinacrine exerted the maximal increase in neurite length, TH* cell survival was also examined using this concentration and found that both compounds did not negatively affect the cell viability of dopaminergic neurons (Fig. 6.3B, C, E, F). Collectively, these data show that Quinacrine and Niclosamide are well tolerated by
dopaminergic neurons and are capable of eliciting phenotypic changes that are similar to those induced by the NTF rhBMP2. These data suggests that the effects of Quinacrine and Niclosamide may be mediated through the canonical BMP-Smad signalling pathway.

6.4.4 Quinacrine and Niclosamide activate BMP-Smad-dependent transcription in SH-SY5Y cells.

Given the finding that Quinacrine and Niclosamide activated the BMP2 pathway in tendon cells (Ghebes et al., 2017), this suggested that Quinacrine or Niclosamide may activate the canonical BMP-Smad signalling to promote neurite growth in dopaminergic neurons. To investigate this possibility, a reporter assay in which a BMP responsive element (BRE) drives the expression of GFP was used in which an increase in intracellular GFP expression indicates increased BMP-Smad-dependent transcription (Fig. 6.4A), as described in previous studies (Hegarty et al., 2017c, Hegarty et al., 2014a). To do this SH-SY5Y cells were transiently transfected with 250ng of the BRE-GFP reporter plasmid. At 24h post-transfection, 10nM of Niclosamide or Quinacrine, or 50ng/ml rhBMP2 (as a positive control) was added to the culture medium. Here, the addition of 10nM Quinacrine or Niclosamide significantly increased the expression of GFP, compared to untreated controls and was comparable to the increase induced by rhBMP2 after 24 h (Fig. 6.4B, C). These data show that Quinacrine and Niclosamide stimulate a Smad-dependent transcriptional response in SH-SY5Y cells.
6.4.5 Quinacrine and Niclosamide increase phospho-Smad levels which are required for their neurite growth promoting effects in cultured dopaminergic neurons.

Given that Quinacrine and Niclosamide activate BMP-Smad dependent transcription in SH-SY5Y cells (Fig. 6.4), the functional relevance of this was examined in primary dopaminergic neurons. The same reporter assay could not be performed as the transfection efficiency of primary cultures is very low, which is compounded by them being mixed cultures, therefore a modified approach was used. As phospho-Smad levels are indicative of BMP-Smad pathway activation, dopaminergic neurons were immunocytochemically stained for TH and pSmad-1/5/8 which allowed us to analyse phospho-Smad levels in dopaminergic neurons. In this experiment, treatment of E14 VM cultures with 10nM Quinacrine or 10nM Niclosamide for 24h led to significant increases in pSmad-1/5/8 levels in TH⁺ neurons, which was completely prevented by pre-treatment with the BMPR inhibitor, dorsomorphin (Fig. 6.5A, D). Similarly, Quinacrine- and Niclosamide-induced increases in neurite growth were prevented by pre-treatment with dorsomorphin (Fig. 6.5B, C). Collectively these data show that both Quinacrine and Niclosamide mediate their phenotypic effects on dopaminergic neurons via the BMP-Smad signalling pathway. As BMPs have been shown to have neuroprotective effects on dopaminergic neurons (Jordan et al., 1997, Reiriz et al., 1999, Krieglstein et al., 1995, Hegarty et al., 2013b), this suggested that Quinacrine and Niclosamide may protect against dopaminergic degeneration.
6.4.6 Quinacrine and Niclosamide protect dopaminergic neurons from MPP\(^+\) and 6-OHDA-induced degeneration

In chapter 5.0, rhBMP2 was shown to protect dopaminergic neurons from neurodegeneration induced by the dopaminergic neurotoxins, MPP\(^+\) and 6-OHDA. Therefore, we next tested whether Quinacrine and Niclosamide could also protect dopaminergic neurons against 6-OHDA- and MPP\(^+\)-induced degeneration. To do this, primary cultures of E14 rat VM were treated with 10nM of Quinacrine or Niclosamide and cultured with or without 5µM 6-OHDA or MPP\(^+\), for 72 h. For Niclosamide, a two-way repeated measures ANOVA revealed a significant effect of both neurotoxins (F(1, 3) = 169.9, p = 0.0010) on neurite length, but no effect of Niclosamide (F(1.234, 3.701) = 4.449, p = 0.1072) and no interaction between Niclosamide and the neurotoxins (F(1.326, 3.978) = 0.5978, p = 0.5291). Furthermore, post-hoc testing using an uncorrected Fisher’s LSD test revealed a significant reduction in neurite length of MPP\(^+\) (p = 0.0095) and 6-OHDA (p = 0.0018) versus the control (Fig. 6.6A, B). While no significant difference was found for MPP\(^+\) (p = 0.5321) and 6-OHDA (p = 0.1662) cultures co-treated with Niclosamide. For Quinacrine, a two-way repeated measures ANOVA revealed a significant interaction between Quinacrine x Neurotoxins (F(1.44, 4.32) = 10.52, p = 0.0247) and both neurotoxins (F(1, 3) = 161.6, p = 0.0011) on neurite length, but no effect of Quinacrine alone (F(1.049, 3.146) = 2.081, p = 0.2429). Furthermore, post-hoc testing using an uncorrected Fisher’s LSD test revealed a significant reduction in neurite length of MPP\(^+\) (p = 0.0008) and 6-OHDA (p = 0.0273) versus the control (Fig. 6.6C, E). While no significant difference was found for MPP\(^+\) (p = 0.7516) and 6-OHDA (p = 0.7713) cultures co-treated with Quinacrine.

We next sought to determine whether Quinacrine and Niclosamide can protect TH\(^+\) neuron number against the neurotoxins MPP\(^+\) or 6-OHDA. Here, a two-way repeated
measures ANOVA revealed a significant effect of the neurotoxins (F(1, 2.001) = 23.03, p = 0.0408) and the drug compounds (F(1.183, 2.366) = 21.16, p = 0.0316) on TH+ neuron number, however there was no significant interaction between the drug compounds and the neurotoxins on TH+ cell number (F(1.293, 2.585) = 1.22, p = 0.3863). In addition, post-hoc testing using an uncorrected Fisher’s LSD test revealed a significant reduction in neurite length of MPP+ (p = 0.0097) and 6-OHDA (p = 0.0263) versus the control (Fig. 6.6D). While no significant difference was found for MPP+ (p = 0.1210) and 6-OHDA (p = 0.964) cultures co-treated with Niclosamide and no significant difference was found for MPP+ (p = 0.1167) and 6-OHDA (p = 0.3655) cultures co-treated with Quinacrine. Collectively, these data show that Quinacrine and Niclosamide can protect dopaminergic neurons against neurotoxin-induced degeneration.

6.4.7 AAV-mediated delivery of α-synuclein to primary dopaminergic neurons induces axon degeneration in an MOI-dependent manner.

The next goal was to develop an in vitro model of α-synucleinopathy in primary dopaminergic neurons in order to test the hypothesis that Quinacrine and Niclosamide could protect against α-synuclein-induced neurodegeneration. In order to determine the effect of AAV-mediated delivery of α-synuclein in vitro, primary cultures of the E14 rat VM were transduced with increasing MOIs of an AAV vector carrying the human α-synuclein transgene (AAV-αSyn) for 5 DIV (Fig. 6.7). Cultures were then immunocytochemically stained for α-synuclein and TH which demonstrated that α-synuclein was expressed in TH+ cells at each MOI tested (Fig. 6.7A). To assess the effect of AAV-αSyn on neurite length, E14 VM cultures were transduced with either an AAV-GFP vector or AAV-αSyn at increasing MOIs for 5 DIV. Quantification of TH+ cells expressing α-synuclein revealed that the effect of AAV-αSyn-induced axon degeneration
was MOI-dependent. (Fig. 6.7B, C). In this experiment, transduction with AAV-αSyn led to a significant decrease in neurite length only at an MOI of 2.0×10⁵ when compared to AAV-GFP transduced TH⁺ cells. This experiment therefore showed that AAV-mediated delivery of α-synuclein successfully transduces TH⁺ neurons and induced axon-degeneration in these dopaminergic neurons.

6.4.8 Quinacrine and Niclosamide protect dopaminergic neurons from AAV-α-synuclein-induced axon degeneration.

In chapter 5.0 it was shown that rhBMP2 was protective against axon-degeneration induced by the overexpression of both WT and mutant A53T α-synuclein plasmids in E14 VM cultures. To assess whether Quinacrine and Niclosamide could also protect against α-synuclein-induced degeneration, E14 VM cultures were transduced with AAV-GFP or AAV2-αSyn at an MOI of 2.0×10⁵. To assess whether treatment with Quinacrine or Niclosamide could protect dopaminergic cells from α-synuclein-induced degeneration, Quinacrine or Niclosamide were administered using two different treatment paradigms in this model. In the first experiment, primary cultures of the E14 rat VM were transduced with either AAV-GFP or AAV-αSyn vectors and treated daily thereafter for 10 DIV in a concurrent treatment paradigm. A two-way ANOVA revealed a significant α-synuclein x drug interaction on the numbers of TH⁺ dopaminergic neurons in these cultures (F(2, 10) = 5.388, p = 0.0258). Post-hoc testing showed that α-synuclein resulted in a significant reduction in dopaminergic neuron number (p = 0.0015), that was not seen in cultures treated with Quinacrine (p = 0.4409) or Niclosamide (p = 0.4409) (Fig. 6.8A). Dopaminergic neurite length was also examined, and showed a significant effect of α-synuclein (F(1, 4) = 9.656, p = 0.0360) and drug treatment (F(2, 8) = 21.37, p = 0.0006), with no significant interaction (F(2, 8) = 1.861, p = 0.2170). Post-hoc testing showed that α-
synuclein resulted in a significant reduction in dopaminergic neurite length (p = 0.0315),
that was not seen in cultures treated with Quinacrine (p = 0.4922) or Niclosamide (p =
0.4922) (Fig. 6.8B). These data show that concurrent treatment with Quinacrine or
Niclosamide prevents α-synuclein-induced reductions in dopaminergic neuron number
and neurite length.

Experiment 2 involved a delayed drug treatment paradigm. Primary cultures of the
E14 rat VM were transduced with the AAV-GFP or AAV-αSyn vectors and left for 5
days. The cultures were then treated with Quinacrine or Niclosamide on day 5 and daily
thereafter for an additional 5DIV (10 days total). A two-way ANOVA showed a
significant α-synuclein x drug interaction on the numbers of TH⁺ dopaminergic neurons
in these cultures (F(2, 10) = 6.420, p = 0.0161). Post-hoc testing revealed that α-synuclein
resulted in a significant reduction in dopaminergic neuron number (p = 0.0016), that was
not seen in cultures treated with Quinacrine (p = 0.5259) or Niclosamide (p = 0.7871)
(Fig. 6.8C). An examination of dopaminergic neurite length revealed a significant effect
of α-synuclein (F(1, 4) = 13.83, p = 0.0205) and drug treatment (F(2, 8) = 43.65, p < 0.0001)
with no significant interaction (F(2, 8) = 4.103, p = 0.0594) (Fig. 4D, E). Post-hoc testing
showed that α-synuclein resulted in a significant reduction in dopaminergic neurite length
(p = 0.0040), that was not seen in cultures treated with Quinacrine (p = 0.1278) or
Niclosamide (p = 0.4256). These data show that Quinacrine or Niclosamide, when given
to neurons with an established α-synuclein load, can prevent α-synuclein-induced
reductions in dopaminergic neuron number and neurite length.
6.5 Figures and Figure Legends

Fig. 6.1 SH-SY5Y cells as a tool to study drugs affecting BMP-Smad signalling.

Representative photomicrographs showing immunocytochemical staining for A BMPR1B, B BMPR2 and C Smad1/5/8 expression in SH-SY5Y cells. D Representative photomicrographs of immunocytochemical staining of phospho-Smad and E Intensity of phospho-Smad as measured using ELISA in SH-SY5Y cells after treatment with 50ng/ml rhBMP2 with and without 1µg/ml dorsomorphin for 2 h. F Total neurite length and G
Representative photomicrographs of SH-SY5Y cells after treatment with 1µg/ml dorsomorphin with and without 50ng/ml rhBMP2 for 24 h. Scale bar = 25µm. All data are presented as mean ± SEM from at least three experiments. (* p < 0.05, ** p < 0.01, ***p < 0.001 vs. control; +++ p < 0.001 vs rhBMP2. One-way ANOVA with Tukey’s post-hoc test).

Fig. 6.2 Niclosamide and Quinacrine promote neurite growth in SH-SY5Y cells. A Total neurite length B Total cell number and C Representative photomicrographs of SH-SY5Y cells following treatment with Niclosamide at the concentrations indicated for 72h. D Total neurite length E Total cell number and F Representative photomicrographs of
SH-SY5Y cells following treatment with Quinacrine at the concentrations indicated for 72h. Scale bar = 50 µm. All data are presented as mean ± SEM from at least three experiments. (*p < 0.05, ** p < 0.01, *** p < 0.001 vs. control. One-way ANOVA with Tukey’s post-hoc test).

Fig. 6.3 Neurotrophic effects of Quinacrine and Niclosamide on primary midbrain dopaminergic neurons. A Total neurite length B TH+ neurons expressed as a percentage of the control and C Representative photomicrographs of E14 VM TH+ neurons following treatment of Niclosamide at the concentrations indicated for 72h. D Total neurite length,
E TH+ neurons expressed as a percentage of the control and F Representative photomicrographs of E14 VM TH+ neurons following treatment of Quinacrine at the concentrations indicated for 72h. Scale bar = 50 µM. All data are presented as mean ± SEM from at least three experiments. (*p < 0.05, **p < 0.01, ***p < 0.001 vs. control. One-way ANOVA with Tukey’s post-hoc test).

**Fig. 6.4 Niclosamide and Quinacrine activate BMP-Smad dependent transcription in SH-SY5Y cells.** A Schema showing BRE-GFP reporter system. B BRE-driven GFP expression expressed as a percentage of untreated control cells and C Representative photomicrographs of SH-SY5Y cells following 24h treatment of 10nM Niclosamide or Quinacrine or 50ng/ml rhBMP2. Scale bar = 10 µM. All data are presented as mean ± SEM from three experiments. (*p < 0.05, ***p < 0.001 vs. control or as indicated. One-way ANOVA with Tukey’s post-hoc test).
Fig. 6.5 Quinacrine and Niclosamide increase phospho-Smad levels which are required for their neurite growth promoting effects in cultured dopaminergic neurons. A Phospho-Smad activation and B Total neurite length of TH+ neurons in E14 VM cultures following 24h treatment of 10nM Niclosamide or Quinacrine with or without 1µg/ml dorsomorphin. C, D Representative photomicrographs of E14 VM cells, immunocytochemically stained for C TH and D pSmad1/5/8, following 24h treatment of 10nM Niclosamide or Quinacrine with or without 1µg/ml dorsomorphin. Scale bar = 50
µM. All data are presented as mean ± SEM from at least three experiments. (*** p < 0.001 vs. control; # p < 0.05, ### p < 0.001 vs Quinacrine; $$ p < 0.01 vs Niclosamide. One-way ANOVA with Tukey’s post-hoc test).

Fig. 6.6 Quinacrine and Niclosamide protect dopaminergic neurons from MPP⁺ and 6-OHDA-induced degeneration. A Representative photomicrographs and B Total neurite length of E14 VM cultures after 72 h treatment with 10nM Niclosamide with or without 5µM MPP⁺ or 6-OHDA. C Total neurite length and E Representative photomicrographs of E14 VM cultures after 72 h treatment with 10nM Quinacrine with or without 5µM MPP⁺ or 6-OHDA. D Numbers of TH⁺ neurons as a percentage of the control in E14 VM cultures after 72 h treatment with 10nM Quinacrine or Niclosamide with or without 5µM MPP⁺ or 6-OHDA. Scale bar = 50 µM. All data are presented as
mean ± SEM from at least three experiments. (* p < 0.05, ** p < 0.01, *** p < 0.001 vs. control; Two-way repeated measures ANOVA with Uncorrected Fisher’s LSD post-hoc test).

Fig. 6.7. AAV-mediated delivery of α-synuclein to primary dopaminergic neurons induces axon degeneration in an MOI-dependent manner. A Representative
photomicrographs of E14 VM cultures after transduction with AAV2/6-GFP or AAV2/6-α-synuclein and immunocytochemically stained for TH and α-synuclein, counterstained with DAPI. B Graph of and C Representative photomicrographs of TH+ neurons transduced with AAV2/6-GFP or AAV2/6-α-synuclein at the MOI’s indicated for 5 DIV. Scale bar = 50µm. All data are presented as mean ± SEM. (** p < 0.01 vs. AAV-GFP. Student’s t-test).

![Graphs and images of TH+ neurons](image)

**Fig. 6.8 Quinacrine and Niclosamide protect midbrain dopaminergic neurons against α-synuclein-induced axon degeneration.** A DA neuron number and B DA
neurite length of E14 TH+ neurons transduced with AAV-GFP or AAV-α-synuclein and cultured with or without 10nM Quinacrine or Niclosamide daily for 10 DIV. C DA neuron number and D DA neurite length and E Representative photomicrographs of E14 TH+ neurons infected with AAV-GFP or AAV-α-synuclein and cultured with or without 10nM Quinacrine or Niclosamide after 5 DIV daily up to 10 DIV. Scale bar = 50µm. All data are presented as mean ± SEM from at least three experiments. (* p < 0.05, ** p < 0.01 vs. control; Two-way ANOVA with Holm-Sidak’s post hoc test).

6.6 Discussion

NTF therapy has attracted significant interest as a potential disease-modifying approach for the treatment of PD. However, the application of neurotrophic factors in a clinical setting currently requires direct administration to the brain via invasive surgery and has had limited success to date (Nutt et al., 2003, Patel et al., 2005, Whone et al., 2019a, Whone et al., 2019b). BMP2 has been shown to have potent neurotrophic effects on dopaminergic neurons (Jordan et al., 1997, Kriegstein et al., 1995, Hegarty et al., 2013b). Similar to other members of the BMP family, BMP2 exerts its effects via the canonical Smad signalling pathway (Hegarty et al., 2014a, Weiss and Attisano, 2013). Therefore, the identification of small molecule compounds, capable of crossing the blood brain barrier, that could modulate the expression of BMP2 and/or BMP-Smad signalling in dopaminergic neurons has potential therapeutic relevance for PD. The clinical application of such agents through non-surgical routes would be hugely advantageous from a safety perspective and allow wider application to patients. The FDA approved small molecule drugs, Quinacrine and Niclosamide, have been shown to upregulate BMP2 expression in a dose-dependent manner in a BMP2 reporter cell line and in human tendon-derived tissue (Ghebes et al., 2017). Given the substantial evidence in chapter 5.0 in support of
BMP2 as an NTF for dopaminergic neurons, this study investigated the effect of Quinacrine and Niclosamide on dopaminergic neurons using SH-SY5Y cells and cultures of the E14 rat VM.

Firstly, SH-SY5Y cells were verified as an appropriate tool to screen drug compounds for neurotrophic ability and for activation of the canonical Smad signalling pathway. SH-SY5Y cells were shown to express high levels of BMP2’s receptors, BMPR1B and BMPR2, as well as Smad-1, -5 and -8, the effector molecules involved in initiating Smad signalling (Wrana and Attisano, 2000). In addition, SH-SY5Y cells treated with rhBMP2 resulted in the activation of the Smad pathway, as shown by increased phospho-Smad activation, as well as increased neurite length, and both of these effects were supressed by co-treatment with dorsomorphin. This finding is supported by several studies showing that rhBMP2 preferentially binds to BMPRI receptors (Weber et al., 2007, Mueller and Nickel, 2012, Wang et al., 2014) to activate canonical Smad signalling in C2C12 cells (Heinecke et al., 2009), SH-SY5Y cells (Hegarty et al., 2013b, Hegarty et al., 2017b) and primary VM dopaminergic neurons (Hegarty et al., 2014a). In this study, both Quinacrine and Niclosamide were also found to promote neurite growth in SH-SY5Y cells and in cultured dopaminergic neurons, to the same extent as rhBMP2. Neither of these two compounds affected cell viability, rationalising their safe application to dopaminergic neurons. In contrast to this finding, exposure to 5µM Quinacrine induced the cell death of human tendon cells after 7 DIV (Ghebes et al., 2017), however the concentration of Quinacrine used on dopaminergic neurons in this study was significantly lower at 10nM. In addition, Quinacrine and Niclosamide were also found to activate the Smad signalling pathway, as evidenced by upregulated Smad dependent transcription in SH-SY5Y cells, and increased phospho-Smad levels in cultured dopaminergic neurons. This effect on Smad signalling was not seen in human tendon cells (Ghebes et al., 2017),
however in that study the cells were exposed to Quinacrine an Niclosamide for 2h in contrast to the 24h timepoint used in this study. Moreover, the effects of both Quinacrine and Niclosamide on neurite length and on phospho-Smad levels were blocked by dorsomorphin, showing that they were mediated through activation of the canonical Smad signalling pathway. This is consistent with findings showing BMP neurotrophic effects were prevented by the use dorsomorphin which demonstrated their dependence on the canonical Smad signalling pathway to elicit neurotrophic action. (Hegarty et al., 2014a, Hegarty et al., 2013b)

Chapter 5.0 showed that rhBMP2 promotes TH⁺ cell survival and neurite length against the selective dopaminergic neurotoxins MPP⁺ and 6-OHDA in cultures of the E14 VM. In this study Quinacrine and Niclosamide also demonstrated significant neuroprotection against MPP⁺- and 6-OHDA-induced neurite degeneration and cell death. In support of this finding, Quinacrine has previously been shown to protect striatal dopamine levels in vivo against 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) striatal lesioning in Wistar rats and C57BL mice, respectively (Tariq et al., 2001). In the MPTP study, adult male mice were administered four different doses of Quinacrine via intraperitoneal (i.p.) injection 30 min prior to the administration of 30mg/kg MPTP daily for 5 days. Mice were sacrificed on day 5 and striata were collected for HPLC analyses. For the 6-OHDA study, Wister rats were administered four different doses of Quinacrine via i.p. injection 30 min prior to a unilaterally 6-OHDA lesion to the right striatum and were treated daily thereafter for 4 days. On day 5 animals were sacrificed and striata were collected for HPLC analyses. In both studies, they found MPTP and 6-OHDA significantly reduced striatal dopamine and glutathione (GSH), which was attenuated by co-treatment with Quinacrine in a dose-dependent fashion. In addition, Niclosamide fully prevented the degeneration induced by MPP⁺ and 6-OHDA
on neurite length, and was protective against the effect of MPP⁺, but not 6-OHDA, on TH⁺ cell survival. These findings demonstrate that Quinacrine and Niclosamide can protect midbrain dopaminergic neurons against neurotoxin-induced dopaminergic degeneration, which is the clinical hallmark of PD.

Chapter 5.0 also showed that rhBMP2 is protective against axon-degeneration induced by the overexpression of both WT and mutant A53T α-synuclein plasmids in E14 VM cultures. Therefore, in this experiment E14 VM cultures were transduced with an AAV2/6-α-synuclein vector at an MOI that induced axon degeneration in dopaminergic neurons. The approach to this study was two-fold; could Quinacrine and Niclosamide protect nerve terminals from α-synuclein, and if so, could these compounds restore dopaminergic nerve terminals after degeneration had already occurred. Treatment with Quinacrine or Niclosamide at the time of AAV2/6-α-synuclein infection significantly protected TH⁺ neurons against α-synuclein-induced axon degeneration. Moreover, treatment with Quinacrine or Niclosamide 5 days after the AAV2/6-α-synuclein infection, when degeneration had already occurred, significantly rescued TH⁺ neurons against α-synuclein-induced degeneration. These findings are particularly noteworthy as axon degeneration is thought to be a central early pathological event that precedes symptom onset and initiates nigrostriatal neuron loss in PD (Kordower et al., 2013, O'Keefe and Sullivan, 2018). Thus, the advent of new therapeutics that can substantially restore dopaminergic nerve terminals within the nigrostriatal pathway is of huge significance.

In addition to its effects on BMP signalling in dopaminergic neurons, it is worth noting that Quinacrine has also been shown to be a potent non-selective inhibitor of phospholipase A₂ (PLA₂) (Tariq et al., 2001, Talk et al., 1997). PLA₂ is an important enzyme in the inflammatory response and within the brain, PLA₂ regulates the conversion
of arachidonic acid into proinflammatory mediators and its reabsorption into the membrane (Farooqui et al., 2006). When \( \text{PLA}_2 \) activity becomes disrupted, abnormally high amounts of proinflammatory mediators are produced, resulting in oxidative stress and neuroinflammation analogous to neurological diseases such as PD (Farooqui et al., 2006). \( \text{PLA}_2 \) is also known to play a role in regulating the synaptic release of dopamine within the adrenal medulla (Kudo et al., 1996). A study investigating the effect of \( \text{PLA}_2 \) on animal behavioural demonstrated that rats who received a unilateral intranigral lesion of \( \text{PLA}_2 \) displayed significantly increased apomorphine-induced rotational behaviour, suggesting that \( \text{PLA}_2 \) can cause severe inhibition of dopamine release within the nigrostriatal pathway (Brunner and Gattaz, 1995). In support of this, another study observed that mice deficient in group IV cytosolic \( \text{PLA}_2 \) are resistant to the degenerative effects of the dopaminergic neurotoxin MPTP (Klivenyi et al., 1998). These studies support the hypothesis that inhibitors of \( \text{PLA}_2 \), like Quinacrine, could be a promising lead for the treatment of neurological disorders. Interestingly, it has been shown that TGF-\( \beta \) signalling in mesangial cells, leading to the activation of Smad proteins, attenuates \( \text{PLA}_2 \) signalling (Xin et al., 2004). Consequently, further work is needed to clarify whether a relationship exists between BMP-Smad signalling and \( \text{PLA}_2 \) activity in dopaminergic neurons, which may further elucidate the mechanism of action of Quinacrine.

Similarly, Niclosamide is also known to modulate several cell signalling cascades including Wnt/\( \beta \)-catenin, mTOR, JAK/STAT3 and NF-\( \kappa \)B and can cause mitochondrial depolarisation through the uncoupling of oxidative phosphorylation (Chen et al., 2018, Kadri et al., 2018, Alasadi et al., 2018). This is interesting as PINK1, a mitochondrial serine/threonine-protein kinase, regulates mitochondrial homeostasis and is activated by mitochondrial depolarisation (Kazlauskaite and Muqit, 2015). Furthermore, point mutations in the PINK1 gene are responsible for autosomal recessive forms of early onset
PD (Valente et al., 2004). Niclosamide treatment has also been shown to indirectly activate endogenous PINK1 signalling in cultured cortical neurons (Barini et al., 2018). These findings suggest a possible therapeutic role for Niclosamide to slow the progression of PD neuropathology through the activation of PINK1, as well as BMP signalling.

In summary, this study has shown that the FDA-approved drugs Quinacrine and Niclosamide elicit positive phenotypic changes in both SH-SY5Y cells and primary cultures of dopaminergic neurons. Specifically, these drugs confer neurotrophic effects on SH-SY5Y cell and cultured dopaminergic neurons by stimulating the canonical BMP-Smad signalling pathway. Furthermore, Quinacrine and Niclosamide have been shown to be protective against MPP⁺- and 6-OHDA-induced dopaminergic degeneration and can protect and restore dopaminergic axon degeneration caused by AAV-α-synuclein. Further work is needed to examine the effects of Quinacrine and Niclosamide in in vivo models of PD such as the AAV-α-synuclein rat model and to further explore the mechanism of action of these compounds. However, collectively this study adds considerable evidence to the potential use of these small molecule drugs to target and protect dopaminergic neurons and justifies their further investigation as a potential therapeutic agents for neuroprotection in PD.
Chapter 7.0

AAV-BMP2 causes the loss of dopaminergic striatal terminals and exacerbates α-synuclein-induced motor deficits in the α-synuclein rat model of Parkinson’s disease.

7.1 Aims of Study

- Evaluate AAV-BMP2 in primary dopaminergic neurons
- Investigate the long-term effects of AAV-mediated delivery of BMP2 in vivo
- Determine if there is a neuroprotective effect of AAV-BMP2 against α-synuclein overexpression
- Determine the effect of α-synuclein and AAV-BMP2 on motor behaviour
7.2 Abstract

Introduction: The use of viral vectors to achieve long-term, targeted delivery of NTFs to the brain of PD patients is an area of growing interest. However, the first clinical trials using gene delivery of Neurturin to the putamen and SN, has had disappointing outcomes to date. BMP2 is a member of the BMP family and represents a novel NTF for dopaminergic neurons. This study therefore evaluated the neuroprotective effect of intranigral gene delivery of AAV-BMP2 in the α-synuclein pre-clinical model of PD.

Methods: 80 adult female SD rats were used over two experiments. In experiment 1; animals received a unilateral injection of an AAV-Cont+AAV-Cont or AAV-BMP2+AAV-Cont vector combination into the SN. In experiment 2; animals received an AAV-aSyn+AAV-Cont or an AAV-aSyn+AAV-BMP2 vector combination into the SN. Behavioural tests of sensorimotor function were carried out at 16, 20 and 24 weeks, prior to post-mortem analysis of α-synuclein pathology and nigrostriatal integrity at 24 weeks.

Results: Intranigral delivery of AAV-BMP2 induced a substantial loss of striatal fibres and striatal dopamine levels at 24 weeks post-surgery. AAV-BMP2 animals also presented with extensive forelimb akinesia as measured by the stepping test of motor function. In addition, when given in combination with AAV-aSyn, AAV-BMP2 exacerbated the functional deficits induced by α-synuclein overexpression.

Conclusions: Intranigral AAV-BMP2 is not protective against α-synuclein and is toxic to striatal terminals at the viral titre used in this study. More work is now needed to evaluate the safety and effective viral load of AAV-BMP2 in vitro and in vivo in order to rationalise the further development of BMP2 for the treatment of PD.
7.3 Introduction

The current available therapies for the treatment of PD are solely symptomatic and therefore do not slow or stop the degeneration of dopaminergic neurons or the accumulation of α-synuclein (Raza et al., 2019). Therefore, there is an urgent need for disease modifying therapies that can replace the dopaminergic neurons that have been lost or regenerate those that remain. In reference to the latter, one promising therapeutic intervention is the application of NTFs. However, the administration of NTFs to the brain has thus far proven difficult given the rapid metabolism of neurotrophic factors in vivo, coupled with insufficient spread to the target area (Lang et al., 2006).

The advent of gene therapy using viral vectors to deliver a gene of interest into the brain has the potential to overcome these hurdles and achieve long-term, targeted delivery of neurotrophic factors (Lundstrom, 2018). There are several candidate NTFs that have achieved promising results in pre-clinical models using AAV or LV vectors including GDNF (Chen et al., 2008, Decressac et al., 2011, Lu-Nguyen et al., 2014), Neurturin (Gasmi et al., 2007, Herzog et al., 2007, Kordower et al., 2006, Bartus et al., 2011), CDNF (Cordero-Llana et al., 2015, Bäck et al., 2013, Ren et al., 2013), MANF (Cordero-Llana et al., 2015, Hao et al., 2017), and VEGF (Tian et al., 2007). However, the vast majority of these studies have evaluated the effect of the viral delivery of NTFs against MPTP and 6-OHDA lesioned animal models. While these animal models are indeed useful, they do not accurately mimic the α-synuclein pathophysiology that is central to PD progression (Stefanis, 2012, Ko and Bezard, 2017). Related to this is the recent failure of AAV-GDNF to protect dopaminergic neurons against a novel α-synuclein model of PD (Decressac et al., 2011), despite extensive pre-clinical evidence of GDNF neuroprotection against the classical toxin models of the disease. In addition, clinical trials using AAV-mediated delivery of Neurturin to the brain has thus far failed to reach its primary end-points.
(Marks et al., 2010, Warren Olanow et al., 2015). This failure emphasises the need to evaluate potential NTFs against an α-synuclein model to justify the further development of these therapies.

In chapter 4.0, the potential of intranigral AAV-mediated delivery of GDF5 in the α-synuclein animal model of PD was investigated. In that study, AAV-GDF5 exerted potent neuroprotection to dopaminergic neurons and their striatal terminals against α-synuclein and attenuated the motor deficits induced by the α-synuclein overexpression. GDF5 is a member of the BMP family, which is known to harbour several other NTFs for dopaminergic neurons, including BMP2 (Jordan et al., 1997, Reiriz et al., 1999, Hegarty et al., 2014a, Hegarty et al., 2014c). As seen in chapter 5.0, BMP2 was the only ligand in the BMP family that was found to be co-expressed with five key markers of dopaminergic neurons within the SN and moreover, the expression of BMP2 was shown to be downregulated in late stage PD, while the expression of its target receptors remained unchanged. rhBMP2 was demonstrated to promote TH+ cell viability against MPP+ and 6-OHDA and was neuroprotective against MPP+ and 6-OHDA induced somal and axonal degeneration. Moreover, rhBMP2 was shown to promote neurite length in dopaminergic neurons overexpressing WT and mutant A53T α-synuclein. In addition, GDF5 was shown to upregulate the expression of BMP2 both in vitro and in vivo, suggesting that BMP2 may contribute to the neurotrophic actions of GDF5. In light of the growing evidence in support of BMP2, this study aimed to evaluate the neurotrophic potential of intranigral delivery of AAV-BMP2 in the α-synuclein animal model of PD.
7.4 Results

7.4.1 AAV2/5-BMP2 transduces TH⁺ neurons and promotes neurite growth.

Owing to the substantial neurotrophic effects achieved with rhBMP2 in chapter 5.0 in vitro, to test the potential ability of AAV-BMP2 to integrate into TH⁺ neurons in vivo, E14 VM cultures were transduced with an AAV-BMP2 vector using an MOI of 5×10⁴ for 10 DIV. The cultures were then immunocytochemically stained for BMP2 and TH which showed strong expression of the AAV-BMP2 vector throughout the VM culture and notably, inside TH⁺ neurons (Fig. 7.1A). In order to assess the effect of AAV-BMP2 on TH⁺ neurons, E14 VM cultures were transduced with an AAV-GFP control vector or AAV-BMP2 vector for 10 DIV. Neurite length and TH⁺ cell survival were then analysed as a readout of neurotrophic potential. Here AAV-BMP2 was found to have significantly increased neurite length compared to AAV-GFP (Fig. 7.1B, C). In addition, there was no change in TH⁺ neuron viability in cultures transduced with AAV-BMP2 compared to AAV-GFP cells (Fig. 7.1D). These preliminary results indicated that the AAV-BMP2 viral vector can successfully transduce TH⁺ neurons and can mediate neurotrophic effects on neurites without adversely affecting cell survival.

7.4.2 Experimental Design

Having shown the transduction of TH⁺ neurons by the AAV-BMP2 vector in vitro, an in vivo experiment was then carried out. The approach to this animal study was modelled on chapter 4.0. However, given the lack of a substantial behavioural phenotype after α-synuclein overexpression, the viral titre of each vector was increased 1.5-fold. In experiment 1, the safety and efficacy of AAV-mediated delivery of BMP2 to the SN was evaluated. Animals received a unilateral injection of either an AAV2/5-Cont+AAV2/6-
Cont (AAV-Cont) or an AAV2/5-BMP2+AAV2/6-Cont (AAV-BMP2) vector combination into the SN (Fig. 7.2A). Concurrently, experiment 2 assessed whether BMP2 could offer neuroprotection against the neurodegeneration induced by the overexpression of human WT α-synuclein. Here, animals received a unilateral injection of an AAV2/6-αSyn+AAV2/5-Cont (AAV-αSyn) or an AAV2/6-αSyn+AAV2/5-BMP2 (AAV-αSyn/BMP2) vector combination into the SN (Fig. 7.2B). In both cases, behavioural testing was carried out at four-weekly intervals beginning 16 weeks after stereotactic surgery (Fig. 7.2C). This 16 week time-point was based on evidence from the AAV-GDF5 study in which a behavioural phenotype became apparent only at 16 weeks post-surgery. Animals were sacrificed at 24 weeks and analysed for transgene overexpression and nigrostriatal integrity.

### 7.4.3 Intranigral delivery of AAV-BMP2 leads to substantial loss of TH⁺ striatal fibres at 24 weeks post-surgery.

In experiment 1, the safety and efficiency of intranigral AAV-mediated delivery of BMP2 was evaluated relative to animals who had received an AAV-Cont vector. After 24 weeks, animals were sacrificed and assessed for nigrostriatal integrity by immunohistochemical staining for TH (Fig. 7.3B, F). To confirm the expression of the BMP2 transgene, SN sections from AAV-BMP2 animals were also immunohistochemically stained for BMP2 and TH which showed strong expression of BMP2 within TH⁺ neurons in the ipsilateral SN (Fig. 7.3A). Quantification of the number of TH immunoreactive neurons in the SN showed no significant difference between AAV-BMP2 and AAV-Cont animals (Fig. 7.3B, C). However, analysis of TH staining as a percentage of the contralateral intact striatum revealed a significant loss of ~40% TH⁺ fibres in the ipsilateral striatum of AAV-BMP2 animals compared to a reduction of ~18% in AAV-Cont animals after 24 weeks
In addition, HPLC analysis of dopamine levels in the striatum demonstrated a significant loss of ~33% of striatal dopamine in AAV-BMP2 animals relative to those injected with an AAV-Cont vector (Fig. 7.3E). The initial results of this study therefore indicate that AAV-BMP2, at least at the titre used in this study, leads to chronic degeneration of nigrostriatal TH⁺ fibres.

7.4.4 Intranigral AAV-αSyn delivery induces strong overexpression of α-synuclein in the SN and Striatum.

Concurrently, in experiment 2, animals received a unilateral intranigral injection of an AAV-αSyn vector and concomitantly either an AAV-Cont or an AAV-BMP2 vector (Fig. 7.4). To confirm overexpression of the α-synuclein transgene, SN and striatal sections from AAV-αSyn and AAV-αSyn/BMP2 animals were immunohistochemically stained for α-synuclein (Fig. 7.4A). Quantification of α-synuclein staining revealed wide-spread expression of α-synuclein throughout the dorsolateral striatum (Fig. 7.4A, B) and the SN (Fig. 7.4D) at 24 weeks post-surgery. In addition, the level of α-synuclein expression did not differ between AAV-αSyn and AAV-αSyn/BMP2 animals. To confirm the targeted delivery of α-synuclein to TH⁺ neurons, SN sections from AAV-αSyn animals were also immunohistochemically stained for α-synuclein and TH which showed α-synuclein overexpression within TH⁺ neurons (Fig. 7.4C). These results demonstrate that intranigral delivery of AAV-αSyn penetrates dopaminergic neurons and AAV-BMP2 does not attenuate the expression of α-synuclein.
7.4.5 AAV-BMP2 does not protect dopaminergic neurons from the rat AAV-αSyn model of PD.

At 24 weeks post-surgery, animals in experiment 2 were sacrificed and assessed for nigrostriatal integrity. To determine the neurodegeneration induced by the overexpression of α-synuclein and whether AAV-BMP2 was neuroprotective against this degeneration, the number of TH⁺ neurons in the ipsilateral SN were measured against the contralateral side. At 24 weeks post injection of AAV-αSyn, there was ~23% loss of TH⁺ neurons compared to the intact side (Fig. 7.5A, C). While animals injected with AAV-αSyn/BMP2 experienced ~20% loss of TH⁺ neurons compared to the intact side (Fig. 7.5A, C). In addition, quantitative analysis of TH⁺ striatal fibre density showed the overexpression of α-synuclein induced a ~35% loss in TH immunoreactivity in the ipsilateral striatum compared to the intact side (Fig. 7.5B, D) while co-delivery of AAV-αSyn with AAV-BMP2 led to ~39% loss in TH⁺ striatal density in the ipsilateral striatum compared to the intact side (Fig. 7.5B, D). Moreover, analysis of striatal dopamine levels using HPLC analysis revealed that intranigral delivery of AAV-αSyn in combination with AAV-BMP2 induced a further ~22% reduction in striatal dopamine compared to AAV-αSyn alone (Fig. 7.5E), however this result was not statistically significant (p<0.08).

Collectively, these data indicate that AAV-BMP2, at the titre tested in this study, is not neuroprotective against α-synuclein overexpression.
7.4.6 Long-term viral-mediated overexpression of WT α-synuclein in the rat SN leads to sensorimotor impairments that are exacerbated by AAV-BMP2.

To determine the effect of α-synuclein overexpression on motor behaviour and whether this could be prevented by AAV-BMP2, animals underwent a battery of behavioural tests including the whisker, stepping and cylinder tests of sensorimotor function beginning at 16 weeks post-surgery and repeated again at weeks 20 and 24. Firstly, using a two-way repeated measures ANOVA the effect of AAV-BMP2 and time on motor function were examined relative to AAV-Cont animals (Fig. 7.6A-C). In the whisker test, neither AAV-BMP2 (F(1, 22) = 2.752; p=0.1114) nor time (F(2, 44) = 2.482; p=0.7813) had an effect on performance relative to control animals (Fig. 7.6A). In addition, neither AAV-BMP2 (F(1, 22) = 0.4017; p=0.5327) nor time (F(2, 44) = 2.098; p=0.1349) had an effect on the cylinder test relative to control animals (Fig. 7.6C). However, there was a significant effect of AAV-BMP2 (F(1, 22) = 31.44; p<0.0001) but not time (F(2, 44) = 0.3075; p=0.7369) on performance in the stepping test (Fig. 7.6B). Post-hoc analysis using a Bonferroni correction showed that AAV-BMP2 animals performed significantly worse than controls in the stepping test at all time points examined; 16 weeks (p=0.0008), 20 weeks (p=0.0005) and 24 weeks (p=0.0121) (Fig. 7.6B).

The effect of α-synuclein overexpression and time on motor performance relative to control animals were also analysed using a two-way repeated measures ANOVA (Fig. 7.6D-F). In the whisker test, time (F(2, 44) = 3.621; p=0.0350) but not AAV-αSyn (F(1, 22) = 0.3256; p=0.5740) had an effect on performance relative to control animals (Fig. 7.6D). While in the cylinder test neither AAV-αSyn (F(1, 22) = 2.279; p=0.1454) nor time (F(2, 44) = 0.5089; p=0.6047) had an effect on performance relative to control animals (Fig. 7.6F).
However, there was a significant effect of AAV-αSyn ($F(1, 22) = 9.659; p=0.0051$) but not time ($F(2, 44) = 1.158; p=0.3234$) on performance in the stepping test (Fig. 7.6E). Post-hoc analysis using a Bonferroni correction revealed that AAV-αSyn animals performed significantly worse than control animals at the 16 week timepoint ($p=0.0379$), but not at the 20 week ($p=0.4867$) or 24 week ($p=0.1002$) timepoint (Fig. 7.6E).

Given the effects of AAV-αSyn and AAV-BMP2 were predominantly seen in the stepping test, the four groups were then directly compared at the 24 week time point (Fig. 7.6G). A one-way ANOVA at 24 weeks revealed there was a significant effect in the cylinder test with a post-hoc Tukey test showing a significant reduction in the number of placings of AAV-αSyn, AAV-BMP2 as well as AAV-αSyn/BMP2 animals relative to AAV-Cont animals (Fig. 7.6G). Moreover, compared to AAV-Cont animals, the effect of AAV-BMP2 ($p=0.0046$) and AAV-αSyn/BMP2 ($p<0.0001$) were worse than that of AAV-αSyn alone ($p=0.0336$) however AAV-BMP2 and AAV-αSyn/BMP2 were not significantly different from AAV-αSyn. Collectively these data show that intranigral AAV-BMP2 caused significant impairments to forelimb behaviour relative to AAV-Cont animals. In addition, while intranigral AAV-αSyn led to significant impairments in some aspects of forelimb function relative to AAV-Cont animals, this was in these cases exacerbated by co-delivery of AAV-BMP2.

In addition, given that weight loss has previously been shown to be an adverse effect of GDNF (Nutt et al., 2003, Manfredsson et al., 2009), the average weight of the animals in each group at 24 weeks was analysed by one-way ANOVA and showed there were no differences in weight relative to AAV-Cont animals (Fig. 7.6H). This result indicates that the overexpression of BMP2 or α-synuclein caused no adverse effect on weight.
7.5 Figures and Figure Legends

Fig. 7.1 AAV-BMP2 co-expresses with TH⁺ neurons and promotes neurite growth.

A Representative photomicrographs of BMP2 expression in TH⁺ neurons. Scale bar = 25µm. B Graph of neurite length C Representative photomicrographs D Graph of total TH⁺ neurons expressed as a percentage of total cells after transduction with either AAV-GFP or AAV-BMP2 for 10 DIV. Scale bar = 50µm. All data are presented as mean ± SEM from at least three experiments. (***p < 0.001 vs. control. Unpaired Student’s t-test).
A In Experiment 1, animals received a unilateral injection of an AAV-Cont+AAV-Cont or AAV-BMP2+AAV-Cont vector combination into the SN. N=20 per group. B In Experiment 2, animals received a unilateral injection of an AAV-αSyn+AAV-Cont or AAV- αSyn+AAV-BMP2 vector combination into the SN. N=20 per group. C Schema showing the study timeline for each experimental group from 0-24 weeks, including behavioural testing and post-mortem assessment measure. IHC = Immunohistochemistry.
Fig. 7.3 Intranigral delivery of AAV-BMP2 leads to substantial loss of TH+ striatal fibres at 24 weeks post-surgery. A Representative photomicrographs showing expression of the BMP2 transgene (green) in TH+ neurons (red) in the ipsilaterally (injected) SN at 24 weeks’ post-injection of the AAV-BMP2 vector. Scale bar = 50µm.
B Representative photomicrographs of AAV-Control (Black) and AAV-BMP2 (Pink) TH-stained sections through SN on the ipsilateral side. Scale bar = 200µm. C, D, Graph showing TH immunoreactivity in C the SN and B the striatum expressed as a percentage of the intact side. E Graph of HPLC data showing striatal dopamine (DA) levels expressed as a percentage of the intact side. F Representative photomicrographs of TH-stained sections through the striatum on the ipsilateral side and the contralateral intact side. Scale bar = 1mm. All data are presented as mean ± SEM and analysed by a Student’s t-test; *p< 0.05, **p < 0.001 vs control.
7.4. Intranigral AAV-αSyn delivery induces strong overexpression of α-synuclein in the SN and Striatum. A Representative photomicrographs and B Quantification of α-synuclein expression in the striatum of AAV-αSyn and AAV-αSyn/BMP2 animals 24 weeks post-surgery. Scale bar = 1mm. C Representative images of the SN of AAV-αSyn animals immunofluorescently stained for α-synuclein and TH. Scale bar = 50µm. D Quantification of α-synuclein expression in the SN. All data are presented as mean ± SEM and analysed by a Student’s t-test.
Fig. 7.5 AAV-BMP2 does not protect dopaminergic neurons from the rat AAV-αSyn model of PD. A, B Representative photomicrographs of TH staining in A the ipsilateral SN and B the striatum in animals injected with AAV-αSyn with either an AAV-Cont (purple) or AAV-BMP2 (pink) vector into the SN. Scale bar in A = 200µm; in B = 1mm. C, D Quantification of TH immunoreactivity expressed as a percentage of the contralateral intact side in C the SN and D the striatum for both experimental groups. E Graph of HPLC data showing striatal dopamine (DA) levels expressed as percentage of the intact side. All data are presented as mean ± SEM and analysed by a Student’s t-test.
Fig. 7.6 Long-term viral-mediated overexpression of WT α-synuclein in the rat SN leads to subtle sensorimotor impairments that are not protected by AAV-BMP2. A-F Graphs showing the results of the A, D whisker, B, E stepping and C, F cylinder tests at four weekly intervals from 16 weeks up to 24 weeks after stereotactic injection with A-C an AAV-Cont+AAV-Cont (Black) or AAV-BMP2+AAV-Cont (Pink) or D-F an AAV-αSyn+AAV-Cont (Purple) or AAV-αSyn+AAV-BMP2 (Purple/Pink) vector into the SN. G Graph showing the combined experimental results of the stepping test at 24 weeks post stereotactic injection into the SN with AAV-Cont+AAV-Cont, AAV-BMP2+AAV-Cont, AAV-αSyn+AAV-Cont or AAV-αSyn+AAV-BMP2, as indicated.
Graph showing the final weight of animals in all experimental groups (N=20). All data are mean ± SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control; A-F two-way ANOVA with post-hoc Bonferroni’s test; G one-way ANOVA with post-hoc Tukey’s test (n=12 per group)).

7.6 Discussion

The application of dopaminergic NTFs as a therapeutic intervention for PD is a key area of interest (Sullivan and O’Keeffe, 2016). However, neurotrophic factors themselves are not ideal candidates as they are rapidly metabolised in the brain by endogenous enzymes. Gene therapy using viral vectors to transfer the gene of interest into specific regions of the brain serves to circumvent this issue to achieve long-term, targeted delivery of neurotrophic factors (Axelsen and Woldbye, 2018, Lundstrom, 2018). Given the successful outcome of AAV-GDF5 exerting neuroprotection against the α-synuclein rat model of PD in chapter 4.0, this study aimed to evaluate the potential of intranigral delivery of AAV-BMP2 against the same animal model of the disease.

E14 VM cultures were initially transduced with AAV-BMP2 for 10 DIV and immunocytochemical staining revealed strong expression of BMP2 inside TH⁺ neurons. In addition, AAV-BMP2 promoted neurite length of TH⁺ neurons and did not affect the number of TH⁺ neurons in culture compared to the AAV-GFP control, indicating the safety and efficacy of AAV-BMP2 in E14 VM cultures. The approach to the animal study was then consistent with the AAV-GDF5 study in chapter 4.0. The safety and proficiency of intranigral delivery of AAV-BMP2 was assessed. Immunofluorescent staining for BMP2 and TH immunoreactivity showed strong expression of the transgene within dopaminergic neurons in the ipsilateral SN. Nigral integrity was assessed after 24 weeks and showed there was no significant change in the number of TH⁺ neurons in the
ipsilateral SN of AAV-BMP2 relative to AAV-Cont animals. However, analysis of striatal integrity showed a substantial loss of TH\(^+\) fibres in AAV-BMP2 animals relative to AAV-Cont animals, which was substantiated by HPLC analysis showing a significant reduction in dopamine levels within the striatum. However, it is also worth noting that AAV-Cont animals in this study had ~20% fewer TH\(^+\) neurons in the SN and ~14% fewer TH\(^+\) striatal fibres compared to AAV-Cont animals in the GDF5 study, likely to be a consequence of the increased viral titre used in this study. Moreover, while no difference was identified in the whisker and cylinder motor tests in animals injected with AAV-BMP2 compared to AAV-Cont animals, analysis of the stepping test revealed substantial, long-lasting impairments of the contralateral paw of AAV-BMP2 animals, at all of the time points tested. The results of this study therefore indicate that while intranigral delivery of AAV-BMP2 did not negatively affect dopaminergic neurons in the SN, it had a detrimental effect on dopaminergic terminals in the striatum and induced forelimb akinesia in affected animals.

In a concurrent study, the consequences of viral mediated α-synuclein overexpression in the SN and whether co-delivery with AAV-BMP2 could protect dopaminergic neurons from α-synuclein induced neurodegeneration was assessed. Intranigral AAV-αSyn caused widespread expression of α-synuclein in the dorsolateral striatum and midbrain and was co-expressed in TH\(^+\) neurons within the ipsilateral SN. The level of α-synuclein overexpression was consistent in AAV-αSyn and AAV-αSyn/BMP2 animals, demonstrating that AAV-BMP2 does not quell the expression of α-synuclein. Additionally, after assessing nigrostriatal integrity, the number of TH\(^+\) neurons in the ipsilateral SN as well as TH\(^+\) fibre density in the ipsilateral striatum were found to be the same for both AAV-αSyn and AAV-αSyn/BMP2 animals, however co-delivery with AAV-BMP2 did appear to worsen the loss of striatal fibres.
Interestingly, the degenerative effect of AAV-BMP2 in this study appears to be localised to striatal terminals and not dopaminergic neurons themselves, as evidenced by the consistent number of TH+ neurons in the ipsilateral SN of AAV-BMP2 and AAV-Cont animals. In addition, the number of TH immunoreactive neurons in the ipsilateral SN of AAV-αSyn animals was no different to that of AAV-αSyn/BMP2 animals suggesting that the loss of TH+ neurons from the SN was a result of α-synuclein overexpression alone. However, that BMP2 induces the degeneration of striatal terminals is inconsistent with a previous study that showed BMP2-treated foetal mesencephalic cells (FMCs) transplanted into the striatum of 6-OHDA lesioned rats increased the number and morphology of dopaminergic neurons and reduced apomorphine-induced rotations in adult rats (Espejo et al., 1999). These results are also unprecedented given the substantial in vitro evidence in support of the neurotrophic effects of BMP2 (Jordan et al., 1997, Reiriz et al., 1999, Hegarty et al., 2014a, Hegarty et al., 2013b, Stull et al., 2001, Yan et al., 2016), coupled with the fact that AAV-GDF5, which was neuroprotective in the α-synuclein model of PD, upregulates the expression of BMP2 in the adult rat SN. Taking everything into account, it appears likely that the problem may lie with the AAV-BMP2 virus itself, in that the viral titre used in this study may have been too potent and better results may be achieved using a refined viral load.

The apparent toxicity of AAV-BMP2 on striatal terminals could also be a result of increased astroglial activation resulting in a heightened immune response. Astrocytes play a crucial role in the maintenance of brain and CNS homeostasis as well as in neurogenesis and synaptogenesis and have been heavily implicated in neurodegenerative disorders (Bélanger and Magistretti, 2009, Phatnani and Maniatis, 2015). Indeed, high concentrations of BMP2 has previously been shown to significantly increase both the number and phenotype of astroglial cells in primary VM cultures (Reiriz et al., 1999) and
promotes the differentiation of oligodendroglial-astroglial progenitor cells into astrocytes (Mabie et al., 1997). Astrocytes are immunocompetent cells and act as important regulators of inflammation within the brain. When astrocytes become activated, known as astrogliosis, this stimulates an immune response in which immune modulators including cytokines and growth factors are released with potential neurotoxic effects (Bélanger and Magistretti, 2009, Farina et al., 2007). These include the release of potentially harmful reactive oxygen species (ROS) and the formation of glial scars, thought to disrupt axon regeneration and neurite outgrowth (Sofroniew, 2005). Thus, the disappointing outcome of intranigral AAV-BMP2 in this study may in part be due to an increased astroglial response induced by the high viral titre of BMP2.

In addition, the level of α-synuclein-induced degeneration in this study was shown to be less than that achieved in the GDF5 study in chapter 4.0. In the GDF5 study, α-synuclein overexpression caused a significant loss of ~36% of TH⁺ neurons from the SN and ~45% loss in striatal fibre density. Yet in this study using a higher viral titre, α-synuclein overexpression induced a loss of ~23% of TH⁺ neurons from the SN and ~36% loss in striatal fibre density. These results are surprising, and the reason is so far unclear but may be caused by increased transduction competition between AAV-αSyn and AAV-Cont vectors in TH⁺ neurons in the SN. However, efficient co-delivery of multiple AAV vectors has been achieved previously (Muramatsu et al., 2002, Shen et al., 2000, During et al., 1998). Although in these studies, the genes shared a common goal to convert TH into dopamine and thus recognising competing vectors in this case would prove difficult. In addition, in these successful studies, the vectors have been delivered to the striatum rather than the SN. Moreover, the overexpression of α-synuclein in this study did not affect the sensorimotor function of animals in the whisker test, as seen in the GDF5 study. However, AAV-αSyn did have a significant reduction in the number of placings with the
contralateral paw in the stepping test, which was found to be aggravated by co-delivery with AAV-BMP2. Given that α-synuclein overexpression did not affect performance in the corridor test of lateralised response in the GDF5 study, this test was replaced by the cylinder test of independent forelimb use in this study (Olsson et al., 1995). The cylinder test is recommended as the best measure of lateralised sensorimotor behaviour in lesioned rats (Björklund and Dunnett, 2019) however no change in the number of placings of AAV-αSyn, or indeed AAV-BMP2 animals, was detected in this study. In addition, similar to the GDF5 study, the behavioural results of animals injected with α-synuclein in this study are in direct contrast with the behavioural phenotype achieved by Decressac et al in a previous study at only 3 weeks post-surgery (Decressac et al., 2012b).

Collectively, the results of this study indicate that while AAV-BMP2 did not affect dopaminergic neurons in the SN, the viral titre used proved to be toxic to striatal fibres and striatal dopamine levels resulting in impaired motor function, perhaps mediated by increased astroglial activation. AAV-BMP2 was not neuroprotective against α-synuclein and instead exacerbated the α-synuclein-induced neurodegeneration and motor deficits. Further work is now needed to investigate this potential immune response and to assess the extent of neuroinflammation within the striatum. Moving forward with BMP2 gene therapy, it will be important to decipher a safe and efficacious viral load of AAV-BMP2 through additional safety studies conducted both in vitro and in vivo and analysed over various timepoints. As for the future use of the AAV-αSyn viral vector in animal models, it is clear that more work is needed to ensure an efficient transduction of TH+ cells in the SN that lead to a concrete, consistent behavioural phenotype in order to justify the further use of this animal model.
Chapter 8.0

Discussion
8.1 Summary, Final Discussion and Future Work

The initial objective of this thesis was to investigate the efficacy of GDF5 and BMP2 in \textit{in vitro} models of PD using SH-SY5Y cells and dopaminergic neurons in primary cultures of E14 VM cultures. The second objective of this thesis was to establish the potential of gene therapy using viral mediated delivery of GDF5 or BMP2 to protect dopaminergic neurons in the SN and their axons projecting to the striatum, in the \(\alpha\)-synuclein rat model of PD. The final aim of this thesis was to identify clinically approved drugs, capable of crossing the blood brain barrier, that could potentially modulate BMP signalling in dopaminergic neurons for use as a novel therapeutic approach in PD.

In chapter 3.0 and chapter 5.0, this thesis demonstrated that GDF5 and BMP2, respectively, exerted neurotrophic effects in a number of \textit{in vitro} models of PD, including in cells overexpressing WT and mutant \(\alpha\)-synuclein expression plasmids. However, at the time these studies were performed, the AAV-mediated \textit{in vitro} model of \(\alpha\)-synucleinopathy developed in chapter 6.0, was not yet established. This model is particularly useful given that the restorative effect of potential factors can be determined after \(\alpha\)-synuclein induced axonopathy has already occurred, as seen by the neuroprotective effects of delayed treatment of Quinacrine and Niclosamide. Therefore, going forward it will be important to examine the effects of both GDF5 and BMP2, and indeed other BMP ligands and potential molecules, on dopaminergic neurons using this \textit{in vitro} model. In addition, while it is now abundantly clear that GDF5 and BMP2 promote the neurite growth of dopaminergic neurites given the findings in this thesis and those contained in previous studies (Reiriz et al., 1999, Hegarty et al., 2014a, Hegarty et al., 2013b, Jordan et al., 1997, Goulding et al., 2019, O’Keeffe et al., 2004a), a better understanding of the molecular mechanisms mediating
these effects are needed. Some recent work has shed some new light on this, however. The Zinc finger E-box-binding homeobox (Zeb)2 (also known as Smad-interacting protein-1 (Sip1)) transcription factor has recently been identified as a negative regulator of Smad signalling in midbrain dopaminergic neurons (Hegarty et al., 2017c). Zeb2 was identified from a PCR screen probing for novel regulators of midbrain dopaminergic axon growth and was shown to be temporally regulated in the developing midbrain in conjunction with several components of BMP signalling during initial axon growth and striatal innervation. Moreover, Zeb2 overexpression in E14 VM neurons significantly reduced phospho-Smad levels, while knockdown of Zeb2 correlated with a significant increase in phospho-Smad expression as well as neurite growth promotion which was shown to be dependent on the activation of BMP signalling. These results were mirrored in vivo whereby Zeb2 knockout mice demonstrated dopaminergic hyperinnervation of the striatum (Hegarty et al., 2017c).

More recently, my work contributed to a newly published study which identified serine threonine receptor-associated protein kinase (STRAP) and nucleoside diphosphate kinase (NME)1 proteins as regulators of GDF5-induced neurite growth (Anantha et al., 2020). In this study, STRAP and NME1 were identified through an untargeted proteomic screen of SH-SY5Y cells after treatment with GDF5. Gene co-expression analysis of the neurotypical human SN also identified a co-expression pattern of STRAP and NME1 with multiple markers of dopaminergic neurons (Anantha et al., 2020). Moreover, analysis of the SN of PD patients demonstrated a significant loss of NME1 compared with controls. Furthermore, in vitro studies revealed that gene knockdown of STRAP and NME1 in SH-SY5Y cells prevented GDF5 induced increases in neurite growth, while treatment with recombinant NME1 was capable of increasing neurite growth in SH-SY5Y cells and
TH⁺ neurons in E14 VM cultures. In addition, AAV-mediated delivery of GDF5 to the SN of adult rats significantly increased the expression of NME1 in TH⁺ neurons (Anantha et al., 2020). When considered in the context of this thesis, this suggests that the beneficial effects of AAV-GDF5 in the rat α-synuclein model \textit{in vivo} (Goulding et al. 2020 \textit{in press}) may be due at least in part to upregulation of NME1 \textit{in vivo} (Anantha et al. 2020), however this requires experimental validation.

Beyond dopaminergic neurons, GDF5 has also been shown to enhance the dendritic complexity in cultured rat hippocampal pyramidal neurons (Osório et al., 2013). In this study, GDF5 was shown to regulate dendritic growth and morphology through the upregulation of the hairy enhancer of split (HES)5 transcription factor, as gene knockdown of \textit{HES5} completely prevented GDF5-mediated dendritic growth. While GDF5 null mice exhibited significantly shorter and less complex dendritic arbours, suggesting a crucial role for GDF5 in modulating dendritic growth \textit{in vivo} (Osório et al., 2013). Moreover, intracerebral infusion of GDF5 has been shown to protect hippocampal neurons against kainic acid-induced degeneration (Zhao et al., 2017). Collectively, these findings suggest that downstream effectors of GDF5 and the BMP-Smad signalling pathway represent targets that are worthy of investigation as potential disease-modifying therapeutic targets for PD. Future work on translating these findings including identifying potential small molecule inhibitors of Zeb2 or the investigation of the therapeutic potential of NME1 \textit{in vivo}, possibly via viral mediated delivery to the SN in animal models of PD, will be important.

In addition to downstream effectors, the identification of small molecule drugs or biologics that are capable of initiating Smad pathway activation in dopaminergic neurons holds significant potential and chapter 6.0 provides proof of principle of this concept. Quinacrine and Niclosamide are FDA approved, clinically available drugs
that were shown to modulate BMP2 expression in human tendon cells (Ghebes et al., 2017). In chapter 6.0, both Quinacrine and Niclosamide demonstrated potent neurite growth promoting effects in midbrain dopaminergic neurons against several in vitro models of PD including AAV-mediated delivery of α-synuclein, through the activation of the canonical Smad signalling pathway. In support of the use of small molecule drugs as therapies for PD, additional small molecules compounds which target and inhibit α-synuclein oligomer formation have also been shown to attenuate axonal degeneration in iPSCs expressing A53T-α-synuclein (Kouropi et al., 2017). While other recent studies have identified small molecule compounds that can inhibit α-synuclein misfolding and aggregation (Tóth et al., 2019, Perni et al., 2017, Pujols et al., 2018), restore α-synuclein-impaired phagocytosis, block the cellular transmission of α-synuclein (Tóth et al., 2019) and prevent the degeneration of dopaminergic neurons (Pujols et al., 2018). Such findings highlight the potential of a combination therapy using small molecule compounds, with each drug targeting a specific facet of the α-synuclein pathology in PD.

The use of small molecule drugs such as Quinacrine and Niclosamide is also quite significant from a patient safety perspective given that Quinacrine and Niclosamide are readily available for systemic administration, completely bypassing the need for invasive surgeries. However, to translate these findings into clinical trials, pre-clinical evaluation of efficacy, toxicity, pharmacokinetic as well as pharmacodynamic studies are required. To this effect, there have been some studies examining the bioavailability of both Quinacrine and Niclosamide in animal models. As mentioned in chapter 6.0, 6-OHDA-lesioned rats or MPTP-lesioned mice treated daily with an intraperitoneal injection of 10, 30 or 60mg/kg Quinacrine daily for 5 days partially protected striatal dopamine levels and no adverse effects were noted by
the authors (Tariq et al., 2001). However, systemic delivery of Quinacrine to the brain is challenging due to its rapid efflux by the P-glycoprotein transporter which is found in the endothelial cells of the CNS (Dohgu et al., 2004). To combat this issue, one study reported that 10mg/kg Quinacrine given in combination with 100mg/kg Cyclosporine A, a P-glycoprotein inhibitor, by oral gavage resulted in a 6-fold increase in quinacrine accumulation within the brain compared to Quinacrine alone (Ahn et al., 2012). However, this study noted that Quinacrine was effectively cleared from the CNS some hours after administration and suggests that higher or more frequent dosing regimens may be required to elicit beneficial changes within the brain (Ahn et al., 2012).

In addition, Niclosamide has a poor solubility profile which hampers its systemic bioavailability when administered orally (Whitesell, 1998). While there have been no studies investigating the pharmacokinetics of Niclosamide in the brain, some studies have evaluated methods of delivery to increase the bioavailability of Niclosamide. In one such study, a 100mg/kg of a nano-suspension of Niclosamide administered via oral gavage has been evaluated in mice for the treatment of ovarian cancer (Lin et al., 2016a). Here the treatment regimen consisted of 5 times weekly for 4 weeks and post-mortem analysis indicated no adverse effects of Niclosamide on weight, renal and hepatic function and histological analysis of vital tissue, including the brain, showed no impairments, suggesting the safety of nano-formulated Niclosamide in animals. In addition, the salt form of Niclosamide known as Niclosamide Ethanolamine (NEN), which has increased water solubility, is known to have an excellent safety profile as long-term oral treatment with high doses of NEN in rats and dogs did not show any adverse effects even after one year (Hecht and Gloxhuber, 1962, Andrews et al., 1982). Oral NEN has also been shown to be safe.
and effective in an animal model of type-2 diabetes (Tao et al., 2014). In addition, Niclosamide has also been evaluated as an anticancer agent for glioblastoma, the most common and aggressive malignant brain tumour in adults (Davis, 2016, Wieland et al., 2013). Here, severe combined immunodeficient (SCID) mice were given an intraperitoneal injection of 30mg/kg Niclosamide 3 times daily for 5 days and no adverse effects were noted by the authors (Wieland et al., 2013). While these results are indeed encouraging, it is clear that much work is needed to develop and evaluate the safety profile and targeted efficacy of both drugs within the brain parenchyma before evaluating their potential use in pre-clinical models of PD.

In chapter 7.0, the therapeutic efficacy of AAV-mediated delivery of BMP2 to the SN was investigated and revealed that AAV-BMP2 had a detrimental effect on striatal dopamine levels and TH+ striatal fibres, which led to consistent contralateral forelimb akinesia in the stepping test. Moreover, AAV-BMP2 exacerbated the pathological and behavioural effects of α-synuclein overexpression. The outcome of this study was particularly disappointing given the positive findings for BMP2 in vitro reported in chapter 5.0 and indeed elsewhere (Goulding et al., 2019, Goulding et al., 2020, Hegarty et al., 2014a, Hegarty et al., 2013b, Espejo et al., 1999, Jordan et al., 1997, Yan et al., 2016). In addition to the evidence that AAV-GDF5 upregulated the expression of BMP2 in vivo, as seen in chapter 4.0. The reason for this outcome is unclear but it seems likely to be due to the viral titre used in the study causing a protein overload and an increased immune response. This has been seen previously with adenoviruses whereby viral loads exceeding 1.0×10⁸ vg/ml were associated with chronic inflammation and vector mediated cytotoxicity (Thomas et al., 2001, Puntel et al., 2010). In addition, viral transduction with GFP has been linked with cellular toxicity, oxidative stress and immunogenicity, compounding its use as a molecular tag.
in vivo (Ansari et al., 2016, Goto et al., 2003, Striepeke et al., 1999, Koprich et al., 2010). Moreover, while AAV vectors are associated with an excellent safety profile in a clinical setting (Kelly et al., 2015), high doses of AAV vectors delivered to the CNS via direct injection has led to toxicity in animal models (Haery et al., 2019, Colella et al., 2017). In one particular study, the use of Cre-dependent ‘helper’ AAVs for monosynaptic tracing was evaluated through stereotactic delivery to the somatosensory cortex (Lavin et al., 2020). Here, the authors found that high titres of the AAV vectors were associated with severe toxicity due to the high expression of the ‘helper’ genes, which was not seen in diluted preparations that were optimised at specific concentrations (Lavin et al., 2020). In addition, toxicity associated with AAV-mediated transgene delivery to the retina has been shown to be entirely dependent on the viral titre used, and is completely prevented at low doses (Khabou et al., 2018). More relevant to PD, high doses of GDNF have been associated with chronic adverse effects experienced by participants in a previous clinical trial (Nutt et al., 2003, d’Anglemont de Tassigny et al., 2015) These findings, together with the results of AAV-BMP2 in chapter 7.0, indicate that the dosing threshold of AAV-BMP2 requires careful consideration and optimisation prior to further evaluation in pre-clinical models, which is currently ongoing. In addition, evaluation of the innate and adaptive immune response to the viral load, and to the vector itself, requires further characterisation (Mingozzi and High, 2013, Colella et al., 2017).

In chapter 4.0, it was demonstrated that AAV-GDF5 protects dopaminergic neurons and their axons as well as striatal dopamine levels in the α-synuclein animal model of PD. In addition, AAV-GDF5 attenuated the motor deficits induced by α-synuclein overexpression. However, only a subtle behavioural phenotype was seen in this model after 16 weeks compared to other studies using a similar viral construct and
viral titre (Decressac et al., 2012b, Decressac et al., 2011). Additional limitations of this model include the slow rate at which neurodegeneration develops and the level of α-synuclein expression required to bring about this degeneration, which far exceeds that seen in the human disease by almost 5-fold (Decressac et al., 2012c, Duffy et al., 2018). Therefore, in developing this finding it will be important to determine the effectiveness of AAV-GDF5 in an α-synuclein model with more robust motor impairments. One such model includes the use of preformed α-synuclein fibrils (PFFs) injected into the brain (Gómez-Benito et al., 2020). The use of PFFs is based on the evidence that when α-synuclein protofibrils are injected into the brain, they act as ‘seeds’ which trigger the aggregation, hyperphosphorylation and ubiquitination of endogenous α-synuclein (Luk et al., 2012, Patterson et al., 2019). These aggregates have been shown to contain phosphorylated α-synuclein and are spread throughout dopaminergic cell somas and neuronal processes (Luk et al., 2012, Paumier et al., 2015, Chu et al., 2019). In addition, the morphology of these α-synuclein aggregates is akin to that of human Lewy bodies and several studies have given additional evidence of colocalization with markers of Lewy bodies (Wakabayashi et al., 2013, Paumier et al., 2015, Chu et al., 2019). However, similarly to the AAV-α-synuclein model, a limitation of this model is the slow development of neurological changes within the brain and the onset of motor deficits (Luk et al., 2012, Paumier et al., 2015, Peelaerts et al., 2015). To combat this, one study has evaluated a combined approach in which AAV-α-synuclein and PFFs, at doses mimicking that found in human PD pathology, were compared with the injection of AAV-α-synuclein or PFFs alone (Thakur et al., 2017). In the combined study, AAV6-α-synuclein was unilaterally injected into the SN and VTA first, followed four weeks later by unilateral injection of PFFs into the same sites. The results of this study showed that the combined model
induced a profound loss of dopaminergic neurons, triggered the formation of Lewy-like aggregates as well as fibrillar inclusions in dopaminergic cell soma and processes, and induced a long-lasting inflammatory response, just 3 weeks post PFF injection (Thakur et al., 2017). These neurological changes were significantly worse than those achieved by AAV-α-synuclein or PFF alone. Moreover, the authors noted significant impairment in the contralateral forelimb of animals who received the combined model only 3 weeks post-injection compared to AAV-α-synuclein or PFF only and this deficit was even more pronounced after 12 weeks (Thakur et al., 2017). These results indicate that the concurrent use of AAV-α-synuclein and PFFs can reproduce the cardinal features of human PD, which other α-synuclein models have failed to replicate.

An additional model being evaluated to provide a more faithful representation of PD involves the viral mediated overexpression of mutant A53T-α-synuclein, which has been shown to induce nigrostriatal degeneration, Lewy-like pathology as well as motor deficits in mice (Ip et al., 2017). In this study, AAV1/2-A53T-α-synuclein was unilaterally injected into the SN. Behavioural output was then measured via the cylinder test at 5 and 9 weeks post-surgery which revealed a significant asymmetry of forepaw use, with animals favouring the ipsilateral side as early as 5 weeks (Ip et al., 2017). At 10 weeks post-surgery, there was a significant loss of TH+ striatal fibres and a substantial reduction in striatal dopamine levels as well as the dopaminergic metabolite DOPAC. The number of TH immunoreactive neurons in the SN was also dramatically reduced compared to control animals (Ip et al., 2017). This novel model of α-synuclein pathology therefore also fulfils several criteria required for robust pre-clinical testing of disease-modifying therapies, such as AAV-GDF5.
Additionally, given that AAV-mediated viral delivery of GDF5 was neuroprotective when given at the same time as the α-synuclein insult, going forward it will also be important to evaluate the neurorestorative properties of GDF5 \textit{in vivo} after α-synuclein-induced degeneration has already developed. This is critical because at the time of onset of motor symptoms in PD, it is estimated that over 30% of dopaminergic neurons have been lost from the SN but ~70% of striatal fibres have also degenerated (Cheng et al., 2010). Relevant to this, a recent study has demonstrated that the level of neuroprotection granted by LV-mediated delivery of GDNF to the 6-OHDA-lesioned striatum was subject to the degree of nigrostriatal degeneration (Quintino et al., 2019). While GDNF provided neuronal and behavioural protection to animals when expressed at the time of lesion, expression of GDNF in animals lesioned with 6-OHDA 6 weeks prior, offered no neurorestorative effects in terms of dopaminergic neuronal rescue or a reversal of motor deficits (Quintino et al., 2019). These findings align with the lacklustre results of previous GDNF clinical trials, the nature of which involves participants already experiencing profound nigrostriatal deterioration (Kordower et al., 1999, Lang et al., 2006, Nutt et al., 2003, Whone et al., 2019a, Whone et al., 2019b). Related to this, studies with GDF5 in the 6-OHDA animal model have previously shown a partial protective effect on dopaminergic neurons in the SN when given one week after 6-OHDA lesion, however no protective effect was seen when given two weeks after the lesion (Hurley et al., 2004).

Therefore, careful consideration also needs to be given to the type of patients that may ultimately benefit from GDF5 therapy. It is widely hypothesised that the failure of GDNF and Neurturin in clinical trials to date may be correlated with the fact that the majority of patients enrolled in these trials were at advanced stages of PD.
This idea is supported by segregated clinical data from individual participants from the recent AAV-Neurturin trials. Analysis of this data demonstrated that patients who had received the intervention less than 5 years after diagnosis benefitted the most, while those who had received AAV-Neurturin more than 10 years post-diagnosis experienced no clinical benefit (Warren Olanow et al., 2015, Bartus, 2015, Marks et al., 2010). This lack of clinical efficacy may be attributed to the chronic axonal degeneration which occurs early in PD (Cheng et al., 2010). NTF signalling requires communication between neuronal cells and their axonal terminals innervating their target structures (Ito and Enomoto, 2016). Therefore, at advanced stages of PD, it is possible that an inadequate number of axons are available for the transport of NTFs. In support of this, post-mortem studies conducted on some of the participants from the GDNF and Neurturin trials have shown there to be a substantial loss of dopaminergic terminals in the putamen (Bartus, 2015, Love et al., 2005). Equally, the limited number of axons remaining may themselves be undergoing the degenerative process which would significantly impact on their capability to effectively transport proteins such as NTFs, which is supported by evidence of impaired axonal transport in axons prior to their degeneration (Roy et al., 2005, De Vos et al., 2008, Chung et al., 2009). In light of this evidence, patients at earlier stages of PD, when the nigrostriatal system is not yet fully compromised, may be more likely to benefit from potential GDF5 gene therapies in the future.

An interesting approach to evaluate the neurorestorative approach of GDF5 while the nigrostriatal system is undergoing degeneration may involve the study paradigm used in the Quintino et al study described earlier. In this study GDNF was under the control of a destabilising domain (DD) which allowed for the regulation of
gene expression at a post-translational level (Tai et al., 2012, Iwamoto et al., 2010). GDNF was expressed as a fusion protein coupled with a coding sequence for a DD, which marks the fusion protein for degradation. Thus, expression of the GDNF-DD fusion protein in the brain resulted in full proteasomal degradation. However, systemic delivery of trimethoprim (TMP) blocks this proteasomal recognition, allowing for the regulated and stable expression of GDNF (Quintino et al., 2019). This approach could potentially be used to regulate the expression of, and further evaluate, AAV-mediated delivery of BMP2 in future studies. In addition, the study paradigm provides a novel approach to evaluate the neuroprotective and neurorestorative properties of NTFs in animal models of PD that could be replicated with α-synuclein rather than 6-OHDA. An additional benefit of using this animal model is that only one stereotactic surgery is required, as the fusion protein can be delivered at the same time as the insult and will only become active in the brain upon TMP administration. This would then allow for the regulation and assessment of GDF5 gene therapy when the nigrostriatal system has significantly deteriorated and thus give important insights into the therapeutic window of GDF5 neuroprotection and potential neurorestoration. Moreover, this approach could potentially be used to deliver NTFs, with controlled expression, in a clinical setting.

Finally, in chapters 3.0 and 4.0 it was demonstrated that the overexpression of α-synuclein did not affect the expression of the BMPRs and Smad mediators required by both GDF5 and BMP2 to activate the canonical signalling pathway, both in vitro and in vivo. In addition, animals overexpressing α-synuclein and GDF5 had significantly increased levels of phospho-Smad expression compared to animals overexpressing α-synuclein alone, suggesting target engagement and the integrity of the BMP-BMPR-Smad signalling pathway. However, whether α-synuclein affects
BMP-BMPR-Smad mediated transcription remains elusive. One way to determine this would be to overexpress α-synuclein in SH-SY5Ys and E14 VM cultures and in parallel, transflect these cultures with a Smad luciferase assay, such as the BRE-GFP reporter assay used in chapter 6.0. This would allow for the evaluation of the effect of α-synuclein overexpression on Smad dependent transcription after treatment with GDF5, BMP2 as well as Quinacrine and Niclosamide which also mediate their neurotrophic action exclusively through this pathway.

However, while transcripts for the BMPRs and Smad effector molecules were found to be unaffected by α-synuclein in chapter 4.0, transcripts for RET, the coreceptor required for GDNF and Neurturin induced signalling, was shown to be significantly downregulated after 20 weeks (Goulding et al., 2020 in press). While this finding agrees with that previously shown (Decressac et al., 2012a), the reduction in RET found in this study after 20 weeks was significantly less than that seen after 2 weeks in the previous study. In addition, it has been reported that α-synuclein leads to the downregulation of Nurr1 (Decressac et al., 2012a, Jia et al., 2020), a critical factor for the survival of dopaminergic neurons (Saucedo-Cardenas et al., 1998, Jiang et al., 2005, Jankovic et al., 2005). However, a separate study found no evidence for a reduction in Nurr1 in PD patients or after α-synuclein overexpression in mice (Su et al., 2017). Collectively, given the evidence it would appear that the level of α-synuclein in the host brain may play a key role in regulating Nurr1 and RET expression and potentially explains why Neurturin was found to be most beneficial in patients more recently diagnosed with PD (Marks et al., 2010, Warren Olanow et al., 2015, Bartus, 2015) when the α-synuclein load in the brain is not saturating. One way to perhaps test this theory would be to design an in vivo study in which different groups of animals received increasing titres of α-synuclein (Fig. 8.1). This would allow for
the evaluation at specific time points of whether α-synuclein load affects *Nurr1* and *RET*, as well as mediators of the BMP-Smad signalling pathway and beyond. This information could lead to major breakthroughs in developing targeted NTF therapies for patients at all stages of PD.

![Fig. 8.1 Schematic of potential in vivo study to evaluate the effect of α-synuclein overexpression on signalling mechanisms in dopaminergic neurons.](image)

Cohorts of rats receive an intranigral injection of α-synuclein at increasing titres A, B or C. Animals from each cohort are sacrificed at timepoints beginning 4 weeks post-injection up to 52 weeks. Nigrostriatal integrity is evaluated as well as transcripts for effector molecules required for specific pathway activation including *RET, BMPRs, Smad1/5* etc. Created with Biorender.com.
8.2 Conclusion

The concept of NTF therapy and whether it remains a viable approach for the treatment of PD has recently been called into question (Barker et al., 2020). This thesis has demonstrated novel neurotrophic action of BMP2 and GDF5 including the protection of dopaminergic cell bodies and their axons against several in vitro models of PD. This thesis has also shown profound neuroprotection in vivo with GDF5 against the degenerative effects of α-synuclein on the nigrostriatal pathway in the α-synuclein pre-clinical model of the disease. Moreover, the overexpression of α-synuclein was demonstrated to not affect the expression of the mediators required for the activation of the canonical Smad signalling pathway both in vitro and in vivo. Therefore, the collective findings in this thesis would suggest that NTF therapy using BMP ligands is a viable approach and the evidence for GDF5 in vivo provides justification for the further development of GDF5 gene therapy as a disease-modifying intervention for PD. Finally, this thesis has demonstrated that the small molecule drugs, Quinacrine and Niclosamide, have potent neurotrophic action against cellular models of PD as well as neurorestorative properties in dopaminergic neurons against α-synuclein-induced axonal degeneration in vitro. The effects of these compounds was shown to be dependent on the activation of the canonical Smad signalling pathway. Therefore, the findings in this thesis highlight the potential of using these small molecule drugs to target the BMP-Smad signalling pathway in dopaminergic neurons as a novel therapeutic approach for neuroprotection in PD.
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Appendices