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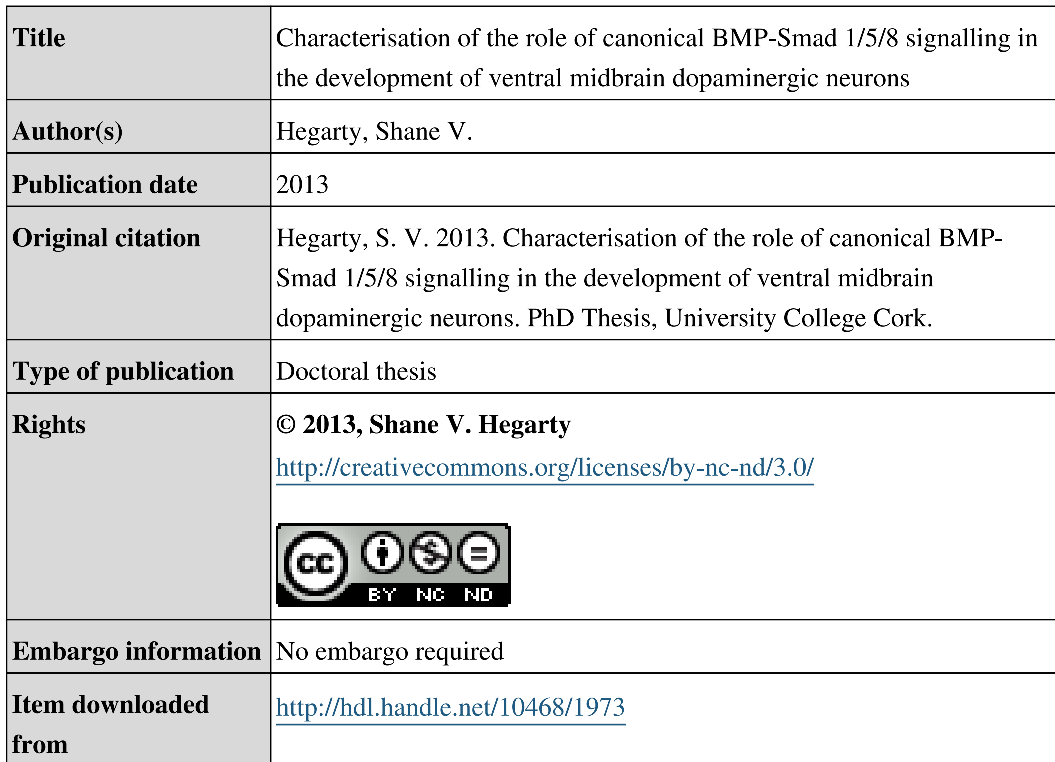

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Characterisation of the role of canonical BMP-Smad 1/5/8 signalling in the development of ventral midbrain dopaminergic neurons

Thesis presented by

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Department of Anatomy and Neuroscience

under the supervision of

Dr. Aideen Sullivan, Dr. Gerard O'Keeffe

for the degree of

Doctor of Philosophy (PhD)

December, 2013

Preface

All work presented in this thesis is original and entirely my own. The work was carried out under the supervision of Dr. Gerard O'Keeffe and Dr. Aideen Sullivan between October 2010 and December 2013 in the Department of Anatomy, University College Cork, Ireland. This dissertation has not been submitted in whole or in part for any other degree, diploma or qualification at any other University.

________________________________.

Shane Hegarty

December 2013

Prologue

This thesis examines the hypothesis 'Canonical Smad 1/5/8 signalling mediates the effects of BMP2 and GDF5 on the development of ventral midbrain dopaminergic (DA) neurons'. These neurons degenerate in Parkinson's disease (PD), for which the two most promising therapeutic options are 1) the application of neurotrophic factors to support the remaining DA neurons, and 2) the transplantation of midbrain DA neurons to replace those that are lost. The advancement of these therapies requires a greater understanding of the molecules and mechanisms that regulate DA neuron development. Bone morphogenetic protein (BMP) 2 and growth/differentiation factor (GDF) 5 are factors which may contribute to the induction, differentiation and survival of midbrain DA neurons. However, the molecular and cellular mechanisms mediating their effects on these neurons are unknown. It is essential to understand these mechanisms if BMP2 and GDF5 are to be used in a clinical context for the treatment of PD.

Herein, the introduction (Chapter 1) will first discuss PD and the potential restorative therapies, before extensively describing the molecular mechanisms regulating the development of ventral midbrain DA neurons. The BMP family, to which BMP2 and GDF5 belong, will then be reviewed, along with the roles that canonical BMP-Smad 1/5/8 plays during nervous system development. Finally, the introduction will focus on the roles this family of proteins play during midbrain DA neuronal development.

Chapter 2 investigates whether canonical Smad signalling mediates the neurotrophic effects of BMP2 and GDF5 in a model of human DA neurons, SH-SH5Y cells. Chapter 3 then assesses the potential role of BMP2 and GDF5 as regulators of the axonal growth of rat midbrain DA neurons, and determines whether the effects of these factors on DA neurite growth is mediated by the Smad signalling pathway. Chapter 4 investigates the mechanisms by which canonical BMP-Smad signalling may promote DA neurite growth. Finally, chapter 5 assesses the *in vitro* neurogenic potential of neural stem cells isolated from the ventral midbrain during DA neurogenesis, before examining the ability of BMP2 and GDF5 to induce a DA phenotype in these midbrain neural precursors.

Publications arising from this work

Abstracts

Neuronal differentiation from proliferating rat ventral mesencephalic neural stem cells requires radial glial-like stem cells.

Shane V. Hegarty, Gerard W. O'Keeffe, and Aideen M. Sullivan.

Neuroscience Ireland Conference 2011

Parkinson's disease: Can we move in the right direction?

Shane V. Hegarty, Gerard W. O'Keeffe, and Aideen M. Sullivan.

UCC Showcase 2012

GDF5 and BMP2 have differential effects on rat ventral mesencephalic neural stem cells, depending on their developmental stage.

Shane V. Hegarty, Aideen M. Sullivan, and Gerard W. O'Keeffe.

Neuroscience Ireland Conference 2012

BMP2 and GDF5 induce neurite extension in ventral midbrain dopaminergic neurons through a Smad dependent pathway.

Shane V. Hegarty, Aideen M. Sullivan, and Gerard W. O'Keeffe.

Young Life Scientists Conference 2013

BMP2 and GDF5 induce neuronal differentiation through a Smad dependant pathway in a model of human midbrain dopaminergic neurons.

Shane V. Hegarty, Aideen M. Sullivan, and Gerard W. O'Keeffe.

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Papers

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Midbrain dopaminergic neurons: A review of the molecular circuitry that regulates their development.

Shane V. Hegarty, Aideen M. Sullivan, Gerard W. O'Keeffe.

Developmental Biology (2013), Vol. 379, Pages 123-138.

BMP2 and GDF5 induce neuronal differentiation through a Smad dependant pathway in a model of human midbrain dopaminergic neurons.

Shane V. Hegarty, Aideen M. Sullivan, Gerard W. O'Keeffe.

Molecular and Cellular Neuroscience (2013), Vol. 56, Pages 263-271.

Ventral midbrain neural stem cells have delayed neurogenic potential *in vitro*.

Shane V. Hegarty, Katie Spitere, Aideen M. Sullivan, Gerard W. O'Keeffe.

Neuroscience Letters (2014), Vol. 559, Pages 193-198.

BMP and TGFβ superfamily members in midbrain dopaminergic neuron development.

Shane V. Hegarty, Aideen M. Sullivan, Gerard W. O'Keeffe.

Molecular Neurobiology (2014), Epub Date: 7 Feb 2014, DOI: 10.1007/s12035-014-8639-3.

Canonical BMP-Smad signalling promotes neurite growth in rat midbrain dopaminergic neurons

Shane V. Hegarty, Louise M. Collins, Aisling M. Gavin, Sarah L. Roche, Sean L. Wyatt, Aideen M. Sullivan, Gerard W. O'Keeffe.

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Submitted

Differential requirement for dynamin-dependent endocytosis by bone morphogenetic proteins in the promotion of neurite growth in a model of human midbrain dopaminergic neurons.

Shane V. Hegarty, Aideen M. Sullivan, Gerard W. O'Keeffe.

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1. Abstract

DA neurons of the VM play vital roles in the regulation of voluntary movement (A9), emotion and reward (A8 and A10), and are divided into the A8, A9 and A10 subgroups. A9 DA neurons project to the dorsal striatum via the nigrostriatal pathway to form part of the basal ganglia circuitry, and their progressive degeneration results in the motor dysfunction of PD. Therefore, the identification of the instructive factors that regulate the development of midbrain DA neurons, and the subsequent elucidation of the molecular bases of their effects, is vital. Such an understanding would facilitate both the generation of transplantable DA neurons from stem cells and the identification of developmentally-relevant neurotrophic factors, the two most promising therapeutic approaches for PD. Many DA neurotrophic factors are members of the transforming growth factor β (TGFβ) superfamily, the largest subfamily of which is the BMP family. BMPs signal via a canonical Smad 1/5/8 signalling pathway, and play diverse roles during nervous system development, including neural crest formation, spinal cord patterning, neural fate specification and neurite growth. Two related members of the BMP family, BMP2 and GDF5, have been shown to have neurotrophic effects on midbrain DA neurons both *in vitro* and *in vivo*, and may function to regulate VM DA neuronal development. However, the molecular (signalling pathway(s)) and cellular (direct neuronal or indirect via glial cells) mechanisms of their effects remain to be elucidated.

This thesis first investigated the neurotrophic effects of BMP2 and GDF5 using the SH-SH5Y human neuronal cell line, a model of human midbrain DA neurons, with the aim of determining the mechanisms of their effects. GDF5 and BMP2 induced neuronal differentiation and activated Smad signalling in these cells, which was prevented by BMP type I receptor inhibition. Furthermore, a constitutively active BMPRIb mimicked the neurite growth-promting effects of BMP2 and GDF5, which was dependent on Smad transcriptional activity. These findings demonstrated that BMP2 and GDF5 have direct neurotrophic effects that are dependent on BMP type I receptor activation of the canonical Smad 1/5/8 signalling.

Following these findings in a cell line model, the mechanisms regulating the neurotrophic effects of BMP2 and GDF5 on midbrain DA neurons were then examined. By characterising the temporal expression profiles of endogenous BMP

receptors (BMPR) in the developing and adult rat VM and striatum, BMP2 and GDF5 were identified as potential regulators of nigrostriatal pathway development. These BMPs activated Smad signalling and promoted neurite outgrowth in cultured embryonic DA neurons, while GDF5 was also shown to activate Smad signalling in the VM *in vivo*. Furthermore, through the use of noggin and dorsomorphin, which inhibit BMPR-activation, and BMPR/Smad plasmids, to alter canonical Smad signalling, this thesis demonstrated that GDF5- and BMP2-induced neurite outgrowth from midbrain DA neurons is dependent on BMP type I receptor activation of the Smad 1/5/8 signalling pathway. To further advance the present characterization of the DA neurotrophic effects of GDF5 and BMP2, this thesis aimed to define the molecular mechanisms regulating the phenotypic effects of canonical BMP-Smad signalling on midbrain DA neurons. BMP2 and GDF5 do not require glial cell-line derived neurotrophic factor (GDNF)-signalling to promote neurite growth, while BMP2-, but not GDF5-induced, Smad signalling and neurite growth is regulated by dynamin-dependent endocytosis. Furthermore, Smad-interacting protein-1 (Sip1) was identified as a novel regulator of neurite growth, which most likely acts to repress canonical BMP-Smad signalling-induced neurite growth of VM DA neurons.

Neural stem cells (NSCs) have been the focus of an intensive effort to direct their differentiation *in vitro* towards a VM DA phenotype for cell replacement therapy in PD. However, it is thought that NSCs derived from older embryos have limited neurogenic capacity and are restricted towards an astroglial fate. In the final part of this thesis, the neurogenic capacity of older VM NSCs was assessed. When the older NSCs were differentiated for three weeks, there were significant increases in the numbers of newly-born neurons at 14 and 21 days, as assessed by BrdU incorporation, which demonstrated that older NSCs retain significantly more neurogenic potential than was previously thought. The ability of GDF5 and BMP2 to induce these VM NSCs towards DA neuronal differentiation was then investigated. Both GDF5 and BMP2 induce the differentiation of VM NSCs in a similar fashion, but differentially induced a DA phenotype in VM NSC-derived neurons.

Taken together, these experiments identify GDF5 and BMP2 as novel regulators of midbrain DA neuronal induction, differentiation and survival, and demonstrate that their effects on DA neurons are mediated by canonical BMPR-Smad signalling.

2. Abbreviations

6-OHDA - 6-Hydroxydopamine

AADC - l-Aromatic Amino Acid Decarboxylase

ALK - Activin Receptor-like Kinases

A/P - Anterior-Posterior

BDNF - Brain-derived Neurotrophic Factor

BMP - Bone Morphogenetic Protein(s)

BMPR - BMP receptor(s)

BrdU - 5-bromo-2'-deoxyuridine

BSA - Bovine Serum Albumin

c/a – Constitutively Active

CNS - Central Nervous System

Co-Smads - Common-mediator Smads

CRT - Cell Replacement Therapy

DA – Dopaminergic/Dopamine

DAPI - 4'-6-Diamidino-2-phenylindole

DAT - Dopamine Transporter

DCC - Deleted In Colorectal Cancer

DD - Days Of Differentiation

DG - Dentate Gyrus

dI - Dorsal Interneuron

DIV - Days In Vitro

DMEM-F12 - Dulbecco's Modified Eagle Medium Nutrient Mixture F-12

DRG - Dorsal Root Ganglion

D/V - Dorso-Ventral

E - Embryonic Day

EGF - Epidermal Growth Factor

En - Engrailed

ENS - Enteric NS

ES - Embryonic Stem

FCS - Fetal Calf Serum

FGF - Fibroblast Growth Factor

Fzd - Frizzled

GDF - Growth/Differentiation Factor

GDNF - Glial Cell Line-Derived Neurotrophic Factor

GFAP - Glial Fibrillary Acidic Protein

GFP - Green Fluorescent Protein

HBSS - Hank's Balanced Salt Solution

I-Smads - Inhibitory Smads

MAPK - Mitogen-Activated Protein Kinase

MBP - Myelin Basic Protein

MFB - Medial Forebrain Bundle

MH - Mad Homology

MPP+ - 1-Methyl-4-Phenylpyridinium Ion

MPTP - 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine-HCl

MTT - Thiazolyl Blue Tetrazolium Bromide

N - Number Of Experimental Repetitions

NCC - Neural Crest Cell(s)

Ngn - Neurogenin

NP - Neural Progenitor(s)/ Precursor(s)

NRBE - NGFI-B Response Element

NS - Nervous System

NSC - Neuroepithelial/ Neural Stem Cell(s)

OPC - Oligodendrocyte Precursor Cell(s)

P - Post-Natal Day

PBS (-T) - Phosphate Buffered Saline (-Triton X)

PFA - Paraformaldehyde

PD - Parkinson's Disease

R-Smads - Receptor-regulated Smads

RNAi - RNA Interference

RRF - Retrorubal Field

RTemp - Room Temperature

RT-PCR - Reverse Transcriptase-Polymerase Chain Reaction (PCR)

RT-QPCR - Quantitative Real-time PCR

SANE - Smad1 Antagonistic Effector

SC - Spinal Cord

SCG – Superior Cervical Ganglion

SDS - Sodium Dodecyl Sulphate

Shh - Sonic Hedgehog

Sip1 - Smad-interacting Protein-1

si(sh)RNA - Small Interfering (Short Hairpin) RNA

SNpc - Substantia Nigra *Pars Compacta*

SSXS - Ser-Ser-Val/Met-Ser

SVZ - Subventricular Zone

T2020 - Transit 2020 Transfection Reagent

TBS (-T) - Tris-Buffered Saline (-Tween)

TGFβ - Transforming Growth Factor beta(s)

TH - Tyrosine Hydroxylase

VM - Ventral Midbrain/ Mesencephalon

VMAT2 - Vesicle Monoamine Transporter 2

VTA - Ventral Tegmental Area

VZ - Ventricular Zone

3. General Introduction

3.0 Foreword

The development of VM DA neurons is an area of major scientific relevance as their degeneration results in the motor dysfunction of PD. Understanding this developmental process is crucial for the establishment of promising restorative therapies for PD. To achieve this, the characterisation of the molecules which regulate VM DA neurogenesis is crucial.

3.1 Parkinson's Disease

PD is a slowly progressive neurodegenerative disorder affecting 0.5 to 1% of the population aged 65-69 years of age, and 1 to 3% of the population over 80 years of age making it the second most common neurodegenerative disorder (de Lau and Breteler, 2006). The four cardinal features of PD are tremor at rest, rigidity, bradykinesia and postural instability. Non-motor symptoms, including cognitive and autonomic disturbances, are also common features of the disease (Jankovic, 2008). Nigrostriatal DA neurons in the substantia nigra *pars compacta* (SNpc) of the midbrain are progressively degenerated in PD, resulting in loss of the neurotransmitter DA in the corpus striatum. This progressive degeneration causes the motor syndrome of PD (Gasser, 2009).

PD is pathologically characterised by the presence of abnormal, intracellular aggregates of the presynaptic protein α-synuclein, called Lewy bodies (Samii et al., 2004, Wakabayashi et al., 2007). The etiology of PD is largely unknown, however some genetic and environmental factors have been shown to contribute (Toulouse and Sullivan, 2008). Approximately 5-10% of PD cases are caused by inheritable genetic mutations, while the remaining 95% of cases are of idiopathic origin (Samii et al., 2004, Toulouse and Sullivan, 2008). When compared to idiopathic PD, most of the inherited forms of PD present with atypical clinical features such as earlier onset or lower prevalence of tremors (Vila and Przedborski, 2004). No unifying mechanism has emerged to date that explains the etiology of idiopathic PD, and aging remains the main risk factor (Toulouse and Sullivan, 2008).

Despite decades of extensive research, PD remains an incurable progressive disease with no efficient long-term treatment (Toulouse and Sullivan, 2008, Lees et al., 2009). Current treatments substantially improve quality of life and functional

capacity, however the beneficial effects of these treatments wear off over time, may lead to severe side effects, and do not halt the progression of the disease. Levodopa, in combination with a peripheral decarboxylase inhibitor and a cathecolamine-O-methyl tranferase inhibitor, is the most effective symptomatic therapy for the treatment of PD (Olanow, 2008). However, chronic treatment with levodopa is associated with the development of motor complications in the majority of cases (Ahlskog and Muenter, 2001). Invasive surgical therapies, such as thalamotomy, pallidotomy and deep brain stimulation, are also treatment options for PD (see table 3.1) (Olanow, 2002).

Table 3.1: Surgical procedures currently being performed or investigated in Parkinson's disease (taken from Olanow, 2002)

Ablative procedures

Thalamotomy

Pallidotomy

Subthalamotomy

Deep Brain Stimulation

Ventral intermediate nucleus of thalamus

Globus pallidus pars interna

Subthalamic nucleus

Restorative procedures

Transplantation

Trophic factors

Stem cells

Neuroprotective therapies for PD offer a means to preserve the remaining neurons, and if administered with other symptomatic treatments would improve the long-term outcome of PD patients (Mandel et al., 2003, Chen and Le, 2006, Toulouse and Sullivan, 2008). Despite research on various compounds, such as antioxidants (Fariss and Zhang, 2003, Young et al., 2007, Toulouse and Sullivan, 2008) and monoamine oxidase B inhibitors selegiline/rasagiline (Ebadi et al., 2002, Linazasoro, 2008), none have fulfilled the criteria of a true neuroprotectant, that is, a compound that could preserve the remaining neurons and stop the progression of the disease (Schapira and Olanow, 2004).

3.1.1 Restorative Therapies in PD

At present, the two most promising therapies for PD treatment involve (1) the application of neurotrophic factors to support the remaining DA neurons and protect them against the ongoing disease process, and (2) the transplantation of midbrain DA neurons to replace those degenerated (Sullivan and O'Keeffe, 2005). Neurotrophic factors are proteins that are important for the survival and function of the nervous system (Peterson and Nutt, 2008, Toulouse and Sullivan, 2008, von Bohlen und Halbach and Unsicker, 2009, Sullivan and Toulouse, 2011), and have the ability to protect neurons from insults, rescue damaged cells from neurodegeneration, or to allow newly-generated neurons to form in a tissue that has been injured. A number of molecules are under investigation for their neurotrophic effects on VM DA neurons including members of the epidermal growth factor (EGF) family, brain-derived neurotrophic factor (BDNF), members of the TGFβ superfamily, and some neurotrophins (Collier and Sortwell, 1999). GDNF and neurturin are related members of the TGFβ superfamily that have both undergone clinical trials based on their neurotrophic effects on midbrain DA neurons. Despite the initial success of GDNF in two open trials (Gill et al., 2003, Slevin et al., 2005), a randomised, double-blind, placebo-controlled trial did not find a significant clinical effect of GDNF (Lang et al., 2006), and GDNF-binding antibodies developed in half of the subjects (Tatarewicz et al., 2007). Similar to GDNF, an open-labeled clinical trial delivering AAV2-neurturin into the putamen of PD subjects reported a significant improvement of parkinsonism in the subjects (Marks et al., 2008), however a double-blind, randomised, controlled trial showed no significant improvement in the primary endpoint, with some patients developing serious adverse effects (Marks et al., 2010). Despite these disappointing results in clinical trials, there remains an optimism that neurotrophic factors will prove to be useful in PD therapy (Aron and Klein, 2011). Optimisation of delivery methods and surgical protocols, as well as careful patient selection, will be critical to the advancement of this promising therapeutic approach (Sullivan and Toulouse, 2011).

For the replacement of the lost DA neurons in PD by transplantation to achieve optimal functional recovery with minimal side-effects, the transplanted DA neurons must establish new synapses, integrate into the local circuitry, and release DA in an appropriate and responsive manner (Toulouse and Sullivan, 2008). A series

of experiments conducted around 1980 showed the feasibility of transplanting fetal tissue to replace lost DA neurons in the SNpc of animal models of PD (Bjorklund and Stenevi, 1979, Bjorklund et al., 1980a, Bjorklund et al., 1980b, Bjorklund et al., 1981), and led to the development of clinical trials in which human fetal mesencephalic tissue was transplanted into the striatum of PD patients. Initial assays produced unsatisfactory results (Lindvall et al., 1989), however transplantation of embryonic DA neurons to the striatum of PD patients has been shown to provide long-lasting relief of symptoms (Bjorklund et al., 2003), providing proof-of-principle for CRT in PD. Conversely, the results of two double-blind placebo-controlled trials failed to show significant improvement in transplanted patients, and raised concern about the appearance of disabling dyskinesias in some patients (Freed et al., 2001, Olanow et al., 2003), and about the use of sham-surgery (Dekkers and Boer, 2001). Furthermore, fetal mesencephalic neurons grafted into the stiatum of PD patients can develop Lewy body pathology (Kordower et al., 2008). Despite this, experts in this field have re-evaluated these results and are now in the process of undertaking a new clinical trial, which has been optimised from the successes and failures of the clinical trials to date (http://www.transeuro.org.uk/pages/disease.html 10/11/13).

The development of alternative cell sources for neural transplantation is crucial for this approach to be used widely as a PD treatment (Sullivan and O'Keeffe, 2005). Presently, 6-8 embryos are required for each transplant due to poor survival of transplanted fetal DA neurons, and due to the low percentage of A9 DA neurons present in the transplants (Hagell and Brundin, 2001). Such ethical and logistical issues highlight the need for an alternative cell source, such as neural and non-neural stem cells, and embryonic stem cells, however further research is required before these alternatives can be used clinically. Due to the poor survival of DA neurons after transplantation, various agents including neurotrophic factors are being examined for their ability to improve neuronal survival after transplantation (Brundin et al., 2000, Redmond, 2002, Liu and Huang, 2007, Deierborg et al., 2008). If restorative therapies are to become a realistic therapeutic option for PD, the development of VM DA neurons must first be characterised. Such an understanding would facilitate the induction of a midbrain DA phenotype in stem cells, and the identification of developmentally-relevant neurotrophic factors.

3.2 Midbrain DA neurons: a review of the molecular circuitry that regulates their development.

In the adult CNS, almost 75% of all DA neurons reside in the VM, with 400,000-600,000 found in the human VM and 20,000-30,000 in the mouse VM (Blum, 1998, German et al., 1983, Pakkenberg et al., 1991). During embryonic development, these DA neurons are generated in the floor plate region of the mesencephalon (Ono et al., 2007), and give rise to three distinct clusters of VM DA neurons which ultimately develop into anatomically and functionally distinct entities termed the A8, A9 and A10 groups. The A9 cluster gives rise to the SNpc, whose neurons project to the dorsal striatum via the nigrostriatal pathway. These neurons and their striatal projections are required for the control of voluntary movement, and the loss of these neurons is the pathological hallmark of PD, which is a neurodegenerative disorder characterised by impaired motor function (Toulouse and Sullivan, 2008, Lees et al., 2009). The other groups of DA neurons, the A10 and A8 clusters, develop into the ventral tegmental area (VTA) and the retrorubal field (RRF), respectively, whose neurons innervate the ventral striatum and the prefrontal cortex via the mesocorticolimbic system, and are involved in the regulation of emotion and reward (Tzschentke and Schmidt, 2000). Altered/defective neurotransmission of the mesocorticolimbic DA system has been associated with the development of schizophrenia, drug addiction and depression (Meyer-Lindenberg et al., 2002, Robinson and Berridge, 1993).

Interestingly, the A9 group of SNpc DA neurons, which undergo progressive degeneration in PD, are particularly vulnerable to cell death in comparison to the other VM DA neuronal populations (McNaught et al., 2004, Betarbet et al., 2000, Alavian et al., 2008, Farrer, 2006). The anatomical, functional and apparent sensitivity differences between these three populations of VM DA neurons likely results from subtle developmental differences during their ontogeny. However, little is known regarding the molecular mechanisms that regulate the phenotypic and functional diversities between these VM DA neuronal populations. Given the involvement of A9 DA neurons in PD, an intensive research effort over the last five decades has focused on identifying the molecules and mechanisms that regulate their development. This information is vital to advance efforts to generate SNpc DA neurons from stem cells for application in cell replacement therapy for PD. Through

the mutation of specific genes, and the subsequent analysis of VM DA neurogenesis and development, a number of molecular pathways have been shown to play key roles in the development of VM DA neurons. Herein we discuss the 'normal' developmental program that regulates VM DA neurogenesis, including the cellular and molecular determinants involved in their regional specification, induction, differentiation and maturation.

3.2.1 Early patterning of the ventral mesencephalon

The first key steps in VM DA generation are the early patterning events which lead to the formation of the VM region. During gastrulation, the dorsal ectoderm is restricted towards a neural fate in response to signals arising from the Speamann organizer (Hemmati-Brivanlou and Melton, 1997, Harland, 2000, Liu and Niswander, 2005). The resulting neural plate is then subdivided into restricted domains and subsequently closes to form the neural tube, which is specified by graded signals along the anterior-posterior (A/P) and dorso-ventral (D/V) axes (Simon et al., 1995, Puelles, 2001, Ulloa and Briscoe, 2007). The development of the VM region relies on appropriate A/P and D/V patterns of gene expression which are regulated by signals arising from two key structures in the early embryo: the floor plate of the midbrain and the isthmus organizer. Organisation of the VM region is initiated upon formation of these signalling centres.

The floor plate is present along the length of the neural tube and secretes the sonic hedgehog (Shh) signalling protein from around embryonic day (E) 8.5 onwards in the mouse (Ho and Scott, 2002, Hynes et al., 1995a, Echelard et al., 1993). Interestingly, the spatiotemporal expression pattern of *Shh* in the VM has been shown to contribute to the diverse populations of VM DA neurons, with the 'early medial pool' giving rise primarily to VTA, and very few SNpc, DA neurons and the 'later intermediate pool' giving rise to DA neurons of all three subgroups, but largely contributing to the SNpc (Joksimovic et al., 2009a). In the floor plate, the bHLH (basic helix-loop-helix) transcription factor Hes1 (also expressed by the isthmus organizer) has been shown to suppress proneural gene expression and induce cell cycle exit (Baek et al., 2006, Ono et al., 2010). Null mutation of *Hes1* results in a transient increase in the number of VM DA neurons between E11.5 and E12.5, followed by a significant reduction in their number from E13.5, compared to the wild-type (Kameda et al., 2011). Interestingly, another bHLH transcription factor

expressed in the floor plate, Nato3, has been shown to repress *Hes1* expression, and mutation of *Nato3* has been shown to result in a reduction in the number of VM DA neurons generated due to unchecked Hes-1-mediated suppression of proneural genes and the induction of cell cycle arrest (Ono et al., 2010).

The isthmus organizer is a unique signalling centre that separates the midbrain from the hindbrain and is necessary for the development of both of these brain regions (Rhinn and Brand, 2001, Liu and Joyner, 2001). The correct positioning of the isthmus organizer at the midbrain-hindbrain boundary is dependent on the mutual repression of two opposing homeodomain transcription factors: *Otx2* and *Gbx2* (Martinez-Barbera et al., 2001). *Otx2* is expressed in the forebrain and midbrain of the developing anterior neural tube (Matsuo et al., 1995, Simeone et al., 1992, Acampora et al., 1997), while *Gbx2* is expressed more posteriorly in the anterior hindbrain (Wassarman et al., 1997). *Gbx2* expression at the posterior border limits *Otx2* expression which creates the sharp boundary between the midbrain and the hindbrain (Millet et al., 1999).

Fibroblast growth factor (FGF) 8 is a diffusible factor secreted by the isthmus organizer (Rhinn and Brand, 2001), from around E8 until at least E12.5 in the mouse midbrain-hindbrain boundary (Crossley and Martin, 1995). Surprisingly, although *Otx2* and *Gbx2* are critical for the correct positing of the isthmus organiser, they are not required for the expression of *FGF8*, or for the induction of other isthmus organizer-genes, however they are essential for the correct positioning of the expression domains of these genes (Liu and Joyner, 2001, Brodski et al., 2003). This is highlighted by studies showing that if the position of the isthmus organiser is moved caudally as a result of ectopic *Otx2* expression in hindbrain, there is an increase in the number of VM DA neurons (Brodski et al., 2003). Similarly if its position is moved rostrally by depleting *Otx2* in the midbrain, there is a decrease in the number of VM DA neurons (Brodski et al., 2003), demonstrating the critical importance of isthmus organiser positioning for normal VM DA generation.

As *Otx2*- and *Gbx2*-dependent sharpening of the borders of the isthmus is occuring, a second group of transcription factors begin to be expressed in the isthmus organiser. These include the paired box gene *Pax2* (Urbanek et al., 1997), the lim-homeodomain factor *Lmx1b* (Adams et al., 2000, Smidt et al., 2000), the secreted glycoprotein *Wnt1* (Davis and Joyner, 1988, Adams et al., 2000, Wilkinson et al., 1987, Crossley and Martin, 1995), and *Engrailed-1* (*En1*) (Davis and Joyner, 1988).

Of these, *Pax2* is required for the induction of *FGF8* expression by the isthmus, whereas *Wnt1* and *En1* function cooperatively with *Otx2* and *Gbx2* to further refine the position of the expression domain of *FGF8* at the isthmus (Ye et al., 2001).

Shortly after the induction and positioning of *FGF8* expression, *Engrailed-2* (*En2*) and *Pax5* start to be expressed in the midbrain-hindbrain boundary. These genes play critical roles in the regional specification of the VM, and homozygous mutant mice null for *Otx2* (Acampora et al., 1995, Ang et al., 1996), *Wnt1* (Prakash et al., 2006, McMahon and Bradley, 1990), *Pax2* and *Pax5* (double mutant) (Schwarz et al., 1997), *En1* and *En2* (double mutant) (Liu and Joyner, 2001, Simon et al., 2001), or *Lmx1b* (Smidt et al., 2000) all display major VM defects, including partial or total loss of VM DA neurons (see table 3.2.1).

Table 3.2.1: Genetic mutations affecting the development of VM DA neurons

Mutation(s)	Effect(s) on DA development	Reference(s)
Otx2 -/-	loss of VM DA neurons (midbrain absent)	(Acampora et al., 1995, Ang et al., 1996)
Wnt1 -/-	severe reduction in VM DA neurons generated	(McMahon and Bradley, 1990, Prakash et al., 2006)
Pax2 -/- : **Pax5** -/-	loss of VM DA neurons (midbrain absent)	(Schwarz et al., 1997)
En1 -/- : **En2** -/-	VM DA neurons lost by E14 via apoptosis	(Liu and Joyner, 2001, Simon et al., 2001, Alberi et al., 2004)
Lmx1b -/-	substantial reduction in VM DA neurons generated, and disappearance of $Pitx3^-$ VM DA neurons by birth	(Smidt et al., 2000, Deng et al., 2011)
Hes1 -/-	transient increase (~E12) in VM DA neurons, followed by their significant reduction (from E13.5). dorsal migration and hindbrain invasion	(Kameda et al., 2011)
Nato3 -/-	reduction in VM DA neurons generated	(Ono et al., 2010)
FGFR -/-	deficit of VM DA neurons	(Lahti et al., 2012, Saarimaki-Vire et al., 2007)
FGF2 -/-	peri-/post-natal increase in VM DA neurons	(Ratzka et al., 2012)
Lmx1a -/-	substantial reduction in VM DA neurons generated	(Ono et al., 2007, Deng et al., 2011)
$En1^{Cre/+}$;	reduction in VM DA neurons due to failure of	(Omodei et al., 2008, Puelles et

Otx2$^{flox/flox}$	VM DA NP induction	al., 2004)
Ngn2 -/-	reduction in mature VM DA neurons generated	(Kele et al., 2006)
Oc1 -/- : **Oc2** -/-	reduction in VM DA neurons generated	(Chakrabarty et al., 2012)
Gli1 -/- : **Gli2** -/-	deficient VM DA neurogenesis (more severe than Gli2 -/-)	(Park et al., 2000)
Gli2 -/-	deficient VM DA neurogenesis	(Park et al., 2000)
Lmx1a -/- ; **Shh**$^{Cre/+}$; **Lmx1b** -/-	substantial reduction in VM DA neurons generated (more severe than Lmx1a -/-)	(Yan et al., 2011)
Lmx1a -/- : **Lmx1b** +/-	substantial reduction in the generation of VM DA neurons (more severe than Lmx1a -/-)	(Deng et al., 2011)
Lrp6 -/-	delayed differentiation of VM DA neurons	(Pinson et al., 2000, Castelo-Branco et al., 2010)
Fzd3 -/- : **Fzd6** -/-	deficient VM DA neurogenesis (severe midbrain defects)	(Stuebner et al., 2010)
Fzd3 -/-	transient reduction in VM DA neuron generated	(Stuebner et al., 2010)
Wnt2 -/-	reduction in VM DA neurons generated	(Sousa et al., 2010)
β-catenin (Th-IRES-Cre; β-Ctn$^{Ex3/+}$ **mutant)**	reduction in VM DA neurogenesis	(Tang et al., 2009)
Dkk1 -/-	severe loss of VM DA neurons	(Ribeiro et al., 2011)
L1 -/-	positional abnormalities of VM DA neurons	(Demyanenko et al., 2001)
Reelin -/-	failure of VM DA neuron lateral migration	(Nishikawa et al., 2003)
DCC -/-	aberrant VM DA neuron migration, dorsal shifting of ventral striatal DA projections, aberrant crossing of MFB fibers at caudal diencephalic midline, and reduction of prefrontal cortex DA innervation	(Xu et al., 2010)
Ebf1 -/-	impaired tangential migration of VM DA neurons	(Yin et al., 2009)
Nurr1 -/-	lack TH, AADC, VMAT2 and DAT expression in VM DA neurons, and their subsequently loss	(Castillo et al., 1998, Filippi et al., 2007, Saucedo-Cardenas et al., 1998, Smits et al., 2003, Wallen et al., 1999)

Nurr1 -/- **(at late stage of DA dev.)**	VM DA neuron degeneration (SNpc more vulnerable)	(Kadkhodaei et al., 2009)
FoxA2 -/-	increase in numbers of Nurr1^{+} TH^{-} neurons in VM	(Ferri et al., 2007)
FoxA2 +/-	aged mice develop PD-like symptoms and pathologies	(Kittappa et al., 2007)
Pitx3$^{ak/ak}$	deficit of SNc DA neurons from E12.5 (VTA largely unaffected)	(Hwang et al., 2003, Nunes et al., 2003, Smidt et al., 2004, van den Munckhof et al., 2003)
En1 +/- : **En2** +/+	progressive degeneration of VM DA neurons (8-24 weeks)	(Sonnier et al., 2007)
En1 +/- : **En2** -/-	progressive degeneration of VM DA neurons (more pronounced in SNpc)	(Sonnier et al., 2007, Sgado et al., 2006)
Nkx2.1 -/-	aberrant crossing of MFB fibers at caudal diencephalic midline	(Kawano et al., 2003)
DCC +/-	increased branching of VM DA fibers in prefrontal cortex	(Manitt et al., 2011)
Pbx1a -/-	partial misrouting of VM DA fibers	(Sgado et al., 2012)
Pax6 -/-	dorsal deflection of MFB fibers in the diencephalon	(Vitalis et al., 2000)
Slit1 -/- : **Slit2** -/-	aberrant crossing of MFB fibers at caudal diencephalic midline	(Bagri et al., 2002, Dugan et al., 2011)
Robo1 -/- : **Robo2** -/-	aberrant crossing of MFB fibers at caudal diencephalic midline, and abnormal dorsal trajectories of VM DA fibers	(Dugan et al., 2011, Lopez-Bendito et al., 2007)

3.2.2 Identity of VM DA neural precursors

Once the appropriate patterning of the VM region has occurred, a developmental program involving a sequential pattern of gene expression establishes the identity of VM DA neural precursors (NPs) that ultimately generate VM DA neurons (Fig. 3.2.1). The identity of these VM DA NPs has been the focus of intensive research in recent years, largely due to their potential to be used as a cell source to generate DA neurons for cell replacement therapy in PD (Kim, 2011, Morizane et al., 2008, Toulouse and Sullivan, 2008).

The origin of VM DA NPs has been debated for many years, with regions such as the diencephalon (Marin et al., 2005), isthmus (Marchand and Poirier, 1983) and VM basal plate (Hynes et al., 1995b, Hynes et al., 1995a) emerging as potential candidates. Despite this research, the precise identity of VM DA NPs remained elusive until recently, when a study showed that floor plate cells in the murine VM become neurogenic and subsequently give rise to DA neurons (Ono et al., 2007). This discovery was surprising as the floor plate was thought to consist of specialised non-neurogenic glial type cells that were largely involved in ventralizing the neural tube, mainly by secreting Shh (Jessell, 2000, Placzek and Briscoe, 2005, Fuccillo et al., 2006). This role in ventralisation seems to remain as the main function of floor plate cells caudal to the midbrain, as the hindbrain floor plate has been shown to be non-neurogenic (Joksimovic et al., 2009b, Ono et al., 2007). However, the VM floor plate is different to its caudal counterparts and attains neurogenic potential. Ono et al. (2007) demonstrated that *Otx2*, which is critical for the positioning of the isthmus organiser, is also essential for the neurogenic potential of VM floor plate cells. This finding expands the importance of this gene in determining the overall structure of the VM region.

However, although the region from which VM DA NPs arise had been determined, the specific floor plate cell type which is a DA NP cell remained to be identified. Fate-mapping studies using a marker for radial glia-specific marker GLAST, demonstrated that radial glial-like cells in the floor plate of the mouse VM were DA NPs (Bonilla et al., 2008). Shortly thereafter, a similar study using human VM tissue demonstrated that DA NPs in the VM floor plate showed radial glial characteristics, that is they expressed the radial glial markers, vimentin and BLBP, and displayed a radial morphology (Hebsgaard et al., 2009). VM DA neurons therefore arise from floor plate radial glial-like NPs. The radial-glial origin of DA neurons should not be considered peculiar due to its departure from the classical view of radial glia as a supportive glial cell type. Indeed, the separate identities of radial neuroectodermal stem cells and radial glial cells is regularly challenged in the literature, with some authors suggesting that they are in fact the same cell type (Kriegstein and Alvarez-Buylla, 2009).

3.2.3 Induction of a DA phenotype in VM NPs

While the floor plate and isthmus organiser are critical determinants of VM patterning, they are also crucial for the induction of a VM DA phenotype. Their role in the induction of a DA phenotype is dependent upon the interaction of floor plate-secreted Shh and isthmus-secreted FGF8 (Ye et al., 1998, Hynes et al., 1997).

FGF8

The mechanism by which FGF8 regulates VM DA development is still under investigation, however a recent study suggested that FGF8 is required to induce the correct patterning of VM DA NPs, as the loss of FGFRs (FGF receptors) resulted in altered patterning of the VM and failure of VM DA neuron maturation, with the DA domain adopting diencephalic characteristics (Lahti et al., 2012). In support of this, a similar study that mutated the *FGFRs* reported a reduction in the generation of VM DA NPs, and a disturbance in the maturation of VM DA neurons (Saarimaki-Vire et al., 2007). Another FGF, FGF2, has been shown to function in the regulation of SNc DA NPs proliferation, and also in the developmental cell death of mature SNc DA neurons (Ratzka et al., 2012).

Sonic hedgehog (Shh) signalling and *Lmx1* expression

The first sign of a DA phenotype in VM NPs is the initiation of expression of two key determinants of VM DA cell fate, the lim-homeodomain factor *Lmx1a* and the homeodomain transcription factor *Msx1*, at ~E9 in the mouse (Alavian et al., 2008). Shh induces the expression of *Lmx1a*, which subsequently induces the expression of its downstream effector Msx1 (Andersson et al., 2006). The overexpression of *Lmx1a* in the anterior VM results in the ectopic generation of DA neurons, while reduced expression results in a loss of VM DA neurons (Andersson et al., 2006). Additionally, null mutation of *Lmx1a* or the spontaneous mutation of *Lmx1a* in *dreher* mice results in substantial reductions in the numbers of VM DA neurons generated (Ono et al., 2007, Deng et al., 2011). *Lmx1a* expression is maintained in post-mitotic VM DA neurons until postnatal day (P) 180 in mice (Zou et al., 2009b), however *Msx1* expression is confined to VM DA NPs (Failli et al., 2002, Andersson et al., 2006). This is surprising considering that *Lmx1a* is upstream of *Msx1*, suggesting that the post-mitotic repression of *Msx1* expression somehow overrides the inductive effect of Lmx1a. *Msx1* contributes to DA neurogenesis by inducing the expression of the proneural gene, *neurogenin* (*Ngn*) *2*, and thus neuronal differentiation. *Ngn2* expression in VM NPs also appears to be under the control of

Otx2 expression, as conditional *Otx2* mutant mice display a loss of *Ngn2* expression in DA NPs (Vernay et al., 2005). This finding is not surprising, considering that *Otx2* induces the expression of *Lmx1a* in VM floor plate cells, suggesting that the loss of *Ngn2* expression in the *Otx2* knockout mouse is due to a failure of *Lmx1a* induction and subsequently *Msx1* expression, rather than a direct effect on *Ngn2* expression, but this remains to be determined (Ono et al., 2007). This suggestion is supported by recent findings showing that in the absence of *Otx2*, VM NPs fail to activate the expression of *Lmx1a*, *Msx1* and *Ngn2*, and therefore largely fail to differentiate into VM DA neurons (Omodei et al., 2008).

Support for the role of Ngn2 in DA induction comes from studies showing that loss of Ngn2 in mice results in a severe reduction in the expression of post-mitotic VM DA markers *Nurr1* and *tyrosine hydroxylase* (*TH*) (discussed later), demonstrating its importance in the generation of mature VM DA neurons (Kele et al., 2006). However, the role of Ngn2 is likely to be generally proneural, rather than specific for VM DA neuronal differentiation. In support of this, overexpression of *Ngn2* induces neurogenesis but not a DA phenotype in cultured VM NPs (Kim et al., 2007). These data suggest the existence of a developmental program consisting of an inductive effect of floorplate-secreted Shh on *Lmx1a* expression which subsequently induces the expression of *Msx1*, which in turn induces the expression of *Ngn2* which is required for neuronal differentiation of VM DA NPs. How and where *Otx2* fits into this cascade is unclear, but it is known that *Otx2* is required for the expression of *Lmx1a*. It has yet to be determined whether this is a parallel pathway cooperating with Shh or whether *Otx2* is a master regulator of Shh-induced *Lmx1a* expression. In support of a role of *Otx2* as a master regulator, studies involving the conditional knockout of *Otx2* in the midbrain have suggested that *Otx2* controls the fate of VM progenitors through the repression of *Nkx2.2* and maintenance of *Nkx6.1* expression (Puelles et al., 2004). FoxA2, involved in a feedback loop with Shh (discussed later), induces *Lmx1a* expression and also inhibits *Nkx2.2* (Lin et al., 2009). It is possible that *Otx2* functions via a similar pathway to FoxA2, or indeed that FoxA2 may function downstream of *Otx2* and Shh in the regulation of *Lmx1a* and *Nkx2.2* expression during VM DA neurogenesis.

Interestingly, a recent genome-wide gene expression profiling study has expanded the regulatory role of Lmx1a in this process by identifying novel transcription factors involved in the generation of the VM DA neuronal field. The Oc

transcription factors, *Oc1*, *2* and *3*, display similar expression profiles to that of *Lmx1a* in the developing VM, and their loss resulted in diminished generation of VM DA neurons (Chakrabarty et al., 2012). Whether these Oc transcription factors are regulated by Lmx1a, which is plausible given their overlapping expression patterns, or whether they act in parallel to regulate neuronal differentiation in the VM, will be an important question for future research.

Similar to Lmx1a, the related protein Lmx1b has also been shown to promote VM DA neurogenesis (Lin et al., 2009, Deng et al., 2011, Yan et al., 2011). *Lmx1a* and *Lmx1b* are co-expressed in VM DA NPs, and have been shown to mediate the initial steps of NP DA specification (Andersson et al., 2006, Smidt et al., 2000). Furthermore, *Lmx1a* and *Lmx1b* are co-expressed in the P0 VM, suggesting that they may function in the maturation of VM DA neurons also (Zou et al., 2009b). Similar to *Lmx1a*, *Lmx1b* can induce the ectopic production of VM DA neurons when ectopically expressed (Nakatani et al., 2010), and its loss results in a substantial reduction in the number of VM DA neurons (Smidt et al., 2000, Deng et al., 2011). A recent study using conditional knockout of *Lmx1a* and *Lmx1b* in mice demonstrated that Lmx1a and Lmx1b function cooperatively to regulate the proliferation of VM DA NPs and Ngn2 expression (Yan et al., 2011). This suggestion is supported by studies on *Lmx1a* null mice carrying one mutant *Lmx1b* allele (as double null mutations are embryonically lethal) which found that *Lmx1a* and *Lmx1b* function cooperatively in the generation of VM DA neurons. This study also showed that *Lmx1b* is involved in the generation of ocular motor neurons and red nucleus neurons in the VM (Deng et al., 2011) and it has been suggested that *Lmx1b* partially compensates for *Lmx1a* function in *dreher* mice, as only 46% of VM DA neurons are lost in these mutants (Ono et al., 2007). It will be important to understand whether Lmx1b exerts its effects in precisely the same way as Lmx1a, for example by modulating *Msx1* expression or that of *Oc1*, *2* and *3* (Chakrabarty et al., 2012).

Sonic hedgehog (Shh) signalling and FoxA expression

Floor plate-derived Shh has been shown to play a key role in induction of a DA phenotype by modulating the expression of the transcriptional regulator *FoxA2*, a well-known floor plate marker. The modulation of *FoxA2* expression is mediated by the downstream effector of Shh signalling, *Gli1* (Hynes et al., 1997). *Gli1* expression is upregulated by a related molecule Gli2 (activator) in response to Shh signalling,

which is required for generation of VM DA neurons, while *Gli3* (repressor) is suppressed by Shh to allow the de-repression of *FGF8* expression (Blaess et al., 2006). *Gli2* homozygous null-mutants demonstrate the importance of Gli2 in inducing ventral phenotypes, as these mice display clear deficits in VM DA neurogenesis (Park et al., 2000). Loss of both *Gli2* and *Gli1* resulted in a more severe phenotype (Park et al., 2000). *FoxA2*, along with *FoxA1*, is expressed in the VM and in differentiated DA neurons during development. Both have been shown to regulate the expression of *Ngn2* and to maintain the expression of *Lmx1a* and *Lmx1b*, which promotes VM DA neurogenesis (Lin et al., 2009, Ferri et al., 2007, Bayly et al., 2012). As aforementioned, Gli1 has been shown to induce the expression of *FoxA2* (Hynes et al., 1997), with *FoxA2* being reported as a downstream target of Shh signalling (Chung et al., 2009). However, *FoxA2* expression precedes that of *Shh* in the ventral neural tube and is proposed to regulate *Shh* expression (Echelard et al., 1993). These findings likely reflect a regulatory feedback loop between *Shh* and *FoxA2* expression, with Gli1 functioning downstream of Shh in this loop. In addition to Gli1, Nato3, a bHLH transcription factor that contributes to VM DA neurogenesis through the repression of *Hes1* (Ono et al., 2010), has been shown to integrate with the Shh-FoxA2 regulatory feedback loop in the SN4741 dopaminergic cell line (Nissim-Eliraz et al., 2012). It has recently been suggested that Shh is necessary and sufficient for lateral floor plate generation, and necessary but not sufficient for medial floor plate generation, while FoxA2 is necessary and sufficient to specify the entire floor plate, acting through both Shh-dependent and independent mechanisms (Bayly et al., 2012). This induction of *FoxA2* expression by Shh has also been proposed to function cooperatively with Lmx1a and Lmx1b in the generation of DA neurons from VM floor plate NPs (Nakatani et al., 2010). This is not surprising, considering that Shh and FoxA2 positively regulate *Lmx1a* and *Lmx1b* expression. *FoxA2* mutant mice have a defective floor plate, as well as notochord, and die at E9.5 (Ang and Rossant, 1994, Sasaki and Hogan, 1994), which precludes examination of their role in DA induction. Given the recent identification of VM radial-glial progenitors, it will be interesting to use targeted strategies to conditionally remove *FoxA2* in the midbrain while preserving its expression in the floor plate (possibly through the use of *GLAST*-cre mice), and thus examine its inductive effect on *Shh* expression and its specific role in DA neurogenesis.

Wnt signalling

The Wnt family of secreted glycoproteins have become increasingly recognised as key regulators of DA neuron induction. *Wnt1* is expressed in the isthmus organizer, in an area rostral to *FGF8* at E9.5 in mice (Wilkinson et al., 1987), and is also expressed in the developing midbrain (Wilkinson et al., 1987, Davis and Joyner, 1988), along with other members of the Wnt family (Parr et al., 1993, Andersson et al., 2008, Rawal et al., 2006). *In vitro*, Wnt1 has been shown to regulate the proliferation of VM DA NPs and to increase the number of DA neurons generated from these cells. Wnt3a has been shown to enhance VM DA NP proliferation but to inhibit their terminal DA differentiation, whereas Wnt5a regulates the acquisition of a DA phenotype to increase DA neuronal numbers (Castelo-Branco et al., 2003). Wnt5a in particular has recently been demonstrated to play a role in the acquisition of a DA phenotype in VM DA NPs *in vivo* (Andersson et al., 2008). The effect of Wnt5a on DA differentiation has been suggested to be regulated by the Rac1 guanosine exchange factor, Tiam1 (Cajanek et al., 2012), and Wnt5a has been proposed to be an important mediator of the DA inductive activity of VM glia (Castelo-Branco et al., 2006). Another Wnt, Wnt2, has been implicated as a novel regulator of VM DA NP proliferation as *Wnt2* null mice displayed reductions in DA neurogenesis (Sousa et al., 2010).

Given that *Wnt1* is expressed in the isthmus and developing midbrain, it is perhaps not surprising that null mice displayed a loss of most of the midbrain and the DA neurons therein (McMahon and Bradley, 1990). Subsequently it was shown that although *Wnt1* null mice develop VM DA NPs, these NPs fail to proliferate and differentiate appropriately, and the few DA neurons that are generated are lost shortly thereafter (Prakash et al., 2006). This is in agreement with data describing Wnt1 as a key regulator of VM DA NPs proliferation and subsequent differentiation (Castelo-Branco et al., 2003). There is now a large body of evidence describing the key role of Wnt signalling in DA generation. Loss of the Wnt receptor *Lrp6* replicates some of the *Wnt1* developmental abnormalities (Pinson et al., 2000, Castelo-Branco et al., 2010). Similarly, loss of the Wnt receptors, *frizzled (Fzd) 3* and *Fzd6*, severely impairs midbrain morphogenesis (Stuebner et al., 2010). Interestingly, the null mutation of *Fzd3* results in a transient reduction in the numbers of VM DA neurons generated, similar to that seen in the *Lrp6* null mutant (Stuebner et al., 2010, Castelo-Branco et al., 2010). Furthermore, the specific

inactivation of β-catenin, which mediates canonical Wnt signalling, mimics the midbrain-hindbrain deficits observed in *Wnt1* null mice (Brault et al., 2001, Chilov et al., 2010), suggesting that Wnt1 acts via β-catenin during midbrain-hindbrain development.

As a result of these studies, the molecular basis of Wnt-induced DA differentiation have been the focus of intensive research. Wnt1 has been shown to be essential for the maintenance of *En1* and *En2* expression (Danielian and McMahon, 1996, McMahon et al., 1992, McGrew et al., 1999), with *En1*/*En2* double knockout mice displaying a similar defective VM phenotype as the *Wnt1* null mutants (Simon et al., 2001), suggesting that the effects of loss of *Wnt1* may be due to a loss of *En* expression. This was subsequently confirmed when it was shown that *En1* was sufficient to rescue early midbrain deficits in *Wnt1* mutant mice (Danielian and McMahon, 1996). *En1* and *En2* are expressed in the ventral mesencephalon at the same time as *Wnt1* (~E8.0 in mice), however the overlapping expression domains of these three genes become restricted by E12 (Davis and Joyner, 1988). The expression of *Wnt1* in the *En1* expression domain (*En1*-*Wnt1* knock-in) causes a ventro-rostral and ventro-caudal expansion of *Wnt1* expression, which is usually restricted to the caudal VM (Danielian and McMahon, 1996), and results in an expansion of the most ventro-rostral DA cell group (Panhuysen et al., 2004). This cell group corresponds to the SNpc, thus these studies demonstrate that this cell group is the most robustly influenced by Wnt1 signalling. *En1* expression is detectable in VM DA neurons from the time point at which they initiate their differentiation and persists into adulthood, while *En2* is only expressed in a subset of DA neurons (Simon et al., 2001, Zhong et al., 2010).

As mentioned earlier, *Wnt1* is expressed in the isthmus organizer, in an area rostral to where *FGF8* is expressed (Wilkinson et al., 1987). Interestingly, FGF8 signalling has also been shown to regulate *En1* expression in the developing VM (Lahti et al., 2012). It is tempting to speculate that this may be achieved through the induction of Wnt-1. In support of this suggestion, a functional link between FGF-8 and Wnt signalling has recently been described, where it was shown that Wnt-β-catenin signalling positively regulated *FGF8* expression in the midbrain-rhombomere1 region (Chilov et al., 2010). It is possible that this may be an autoregulatory loop, similar to that of Shh and FoxA2, with FGF-8 inducing the expression of *Wnt1* and subsequently *En1*/*En2* expression in the midbrain.

Interestingly, new data have now shown that Lmx1a and Lmx1b function cooperatively to control the proliferation of VM DA NPs through the regulation of *Wnt1* expression (Yan et al., 2011).

A link between Shh and Wnt signalling has recently emerged with the proposal that canonical Wnt-β-catenin signalling is required to antagonize *Shh*, and that the subsequent reduced *Shh* levels allow the induction of VM DA NPs and the promotion of DA neurogenesis (Joksimovic et al., 2009b). The finding that Shh inhibits DA neurogenesis (Joksimovic et al., 2009b) is surprising, considering its well-established role in the induction of VM DA neurogenesis (Ye et al., 1998, Blaess et al., 2006, Hynes et al., 1995a, Andersson et al., 2006). However, Joksimovic et al. (2009) suggest that Shh is initially required for the early establishment of the VM DA NP pool, but that later it inhibits VM DA NP proliferation and neurogenesis. The current model suggests that once the Shh-induced VM DA NP pool has been established, Wnt-β-catenin signalling suppresses *Shh* levels in the VM to facilitate DA neurogenesis. Additionally Wnt signalling has been shown to induce *Otx2* and *Lmx1a* expression (Joksimovic et al., 2009b, Prakash et al., 2006). Furthermore, a Wnt1-Lmx1a autoregulatory loop has been identified which is proposed to regulate *Otx2* expression via β-catenin during VM DA neurogenesis (Chung et al., 2009). Interestingly, Otx2 has recently been suggested to regulate the proliferation of VM DA NPs via Wnt1 regulation (Omodei et al., 2008), suggesting a possible Otx2-Wnt1 regulatory feedback loop. In contrast to Joksimovic et al. (2009), Chung et al. (2009) suggested a mechanism by which the Wnt1-Lmx1a autoregulatory loop and a Shh-FoxA2 autoregulatory loop control VM DA neurogenesis synergistically. Despite this finding, a more recent paper described an antagonistic relationship between Wnt-β-catenin signalling and Shh signalling that is important in the progression of DA NPs into VM DA neurons (Tang et al., 2010), thus supporting the Joksimovic et al. (2009) theory. Furthermore, the stabilization of β-catenin in VM NPs, by the inhibition of GSK3β, leads to an increase in DA differentiation (Castelo-Branco et al., 2004, Tang et al., 2009) and targeted deletion of *β-catenin* in VM NPs (Th-IRES-Cre;β-Ctn$^{Ex3/+}$ mutant) results in reduced VM DA neurogenesis (Tang et al., 2009). Surprisingly, mice with mutations in the Wnt/β-catenin inhibitor *Dkk1* actually have a reduction in VM DA neurons (Ribeiro et al., 2011). This is surprising, given that the stabilization of β-catenin in VM NPs, through the inhibition of GSK3β, leads to an increase in DA differentiation (Castelo-

Branco et al., 2004, Tang et al., 2009). Collectively these data largely support the theory that Wnt signalling is required for DA induction, but it is also clear that this is likely to involve a complex interplay with Shh and FGF8 signalling, and potentially other extrinsic signalling factors which have been suggested to induce VM DA neurogenesis, including TGFβs (Farkas et al., 2003, Roussa et al., 2006, Roussa et al., 2009).

3.2.4 Development of post-mitotic VM DA neurons

Once NPs of the VM floor plate are specified towards a DA phenotype, these DA NPs gradually become post-mitotic from E10-E14 in mice (E12-E16 in rats) (Lumsden and Krumlauf, 1996, Lauder and Bloom, 1974), with the greatest proportion of VM DA NPs undergoing their final division at E12 in the rat (Gates et al., 2006). The induction of *TH* expression, the rate-limiting enzyme for DA synthesis, is the first sign of the acquisition of the DA neuronal phenotype, and occurs shortly after the final mitosis of VM DA NPs while they are actively migrating to their final positions (Specht et al., 1981a, Specht et al., 1981b, Puelles and Verney, 1998). This process of migration of VM DA neurons from the floor plate ventricular zone to the presumptive VTA and SNpc involves two steps: firstly, DA neurons migrate ventrally along tenascin-expressing radial glial processes which project to the pial surface, and secondly, once they have reached the basal part of the VM, they migrate laterally along tangentially orientated fibres to form the VTA and SNc (Shults et al., 1990, Kawano et al., 1995). These tangentially-orientated fibres express the neural cell adhesion molecule L1, while VM DA neurons express the chondroitin sulphate proteoglycan 6B4. A heterophilic interaction between L1 and 6B4 has been proposed to facilitate this process of lateral migration of VM DA neurons (Ohyama et al., 1998). There have been a variety of studies that show that this process of migration is crucial for the normal positioning of VM DA neurons. Specifically, VM DA neurons are abnormally located in *L1* knockout mice (Demyanenko et al., 2001). A role for Wnt signalling in this process has been implicated by a study showing that the targeted deletion of *β-catenin* in the VM disrupts the integrity of these radial glia, resulting in perturbed migration of VM DA neurons (Tang et al., 2009). It is unclear whether these migratory defects are secondary to a disrupted radial glial scaffold or whether Wnt signalling can also directly affect this process of migration. A number of other molecules involved in

neuronal migration in the developing CNS also appear to be involved in the migration of VM DA neurons. These include the well-known migrational regulator *Reelin*, as VM DA neurons fail to migrate laterally to the SNpc, in *reeler* (*Reelin* null) mice (Nishikawa et al., 2003), and the netrin receptor, *DCC*, which is expressed by migrating VM DA neurons in mice, and whose loss results in aberrant migration of these neurons (Xu et al., 2010). In terms of the molecular regulatory networks that control this migration, there have been a number of studies describing roles for Ebf1 (early B-cell factor 1) (Yin et al., 2009) and Hes1 (Kameda et al., 2011) in this process. A key goal for future research will be to understand the molecular networks that control this process of VM DA migration and how newly-identified molecules such as Ebf1 and Hes1 "fit" within this network. While much work has focused on identifying the molecular signals that are required for neuronal migration, it will also be important to understand what positional cues inhibit these processes so that VM DA neurons "know" when to stop.

Several transcription factors have been identified which are essential for the differentiation and subsequent long-term survival of VM DA neurons. These include Lmx1b, Nurr1, Pitx3, En1 and En2. Each of these factors are not individually capable of inducing a complete DA phenotype, suggesting that they function as part of a network (Fig. 3.2.1).

Lmx1b

The lim-homeodomain factor *Lmx1b* is broadly expressed in the presumptive midbrain before neural tube closure, and its expression becomes restricted to VM DA NPs at E10.5 in mice, where it is co-expressed with *Lmx1a* and *Msx1* (Smidt et al., 2000, Andersson et al., 2006). Surprisingly, *Lmx1b* expression disappears in the VM at around E11.5, but reappears at E16 in post-mitotic VM DA neurons. *Lmx1b* is subsequently co-expressed with *Pitx3* and *TH* into adulthood in the VM (Dai et al., 2008). Although loss of *Lmx1b* leads to a loss of VM DA neurons (Smidt et al., 2000), *Lmx1b* mutant mice express *Nurr1* and *TH* normally during early development, but fail to express *Pitx3*. These TH-positive VM neurons, which lack *Pitx3* expression, are lost by birth, suggesting a role for Lmx1b in the regulation of *Pitx3* expression and VM DA neuronal survival. Similarly in *Wnt1* null mice, the few TH-positive VM neurons generated lack *Pitx3* expression, and are subsequently lost before E12.5 (Prakash et al., 2006). A similar regulatory loop may exist between Wnt1 and Lmx1b, as Lmx1b induces and/or maintains the expression of *Wnt1*, an

important extrinsic factor in VM DA neurogenesis (see above), around the midbrain-hindbrain boundary (Adams et al., 2000, Matsunaga et al., 2002). Wnt1 has also been proposed to act downstream of Lmx1b in the potential regulation of *Pitx3* expression (Prakash et al., 2006). This maintenance of *Wnt1* expression by Lmx1b may be important in the generation of post-mitotic DA neurons, as Wnt1 is required for the terminal differentiation of VM DA neurons at later stages of embryogenesis (Prakash et al., 2006).

Nurr1

Nurr1 is a member of the nuclear receptor superfamily of steroid-thyroid hormone-activated transcription factors (Law et al., 1992), which atypically lacks both a ligand cavity and a canonical coactivator-binding site (Wang et al., 2003). *Nurr1* is expressed in the VM from E10.5 in the mouse, as VM DA NPs begin to become post-mitotic, one day before the appearance of *TH* (Zetterstrom et al., 1996), and *Nurr1* expression is maintained into adulthood (Backman et al., 1999). *Nurr1* expression levels show a sharp peak between E13 and E15 in the rat, at a stage of development when most VM DA neurons are undergoing terminal differentiation (Volpicelli et al., 2004). VM DA neurons in *Nurr1*-deficient animals, do not express *TH*, l-aromatic amino acid decarboxylase (*AADC*), the vesicle monoamine transporter 2 (*VMAT2*) or the dopamine transporter (*DAT*), all markers of a DA neuron that has acquired its DA neurotransmitter identity (Castillo et al., 1998, Smits et al., 2003, Filippi et al., 2007). Nurr1 has been shown to play a direct role in regulating the expression of these genes, and a number of well-established signalling pathways in VM DA development cooperate with Nurr1 to mediate this induction. Specifically, Nurr1 has been shown to induce *TH* expression by binding to a NRBE (NGFI-B response element) sequence in the *TH* promoter (Sakurada et al., 1999, Kim et al., 2003), and is known to induce *DAT* expression via an NRBE-independent mechanism (Sacchetti et al., 2001). These effects of Nurr1 on the expression of these genes are enhanced by Wnt-activated β-catenin, which has been shown to promote Nurr1-induced *TH* promoter activation, by interacting with Nurr1 at NRBEs, causing the dissociation of transcriptional co-repressors and recruitment of transcriptional co-activators (Kitagawa et al., 2007). Similarly, nuclear FGFR1 has been shown to cooperate with Nurr1 to promote activation of the *TH* promoter (Baron et al., 2012a). Collectively these data show that Nurr1 functions as a master regulator in the induction of the neurotransmitter phenotypic identity of VM DA neurons, and

controls the expression of the molecules that regulate the synthesis, vesicle packaging, axonal transport and reuptake of DA.

Aside from this role in DA identity, Nurr1 has also been shown to be crucial for long-term VM DA neuron survival. In *Nurr1*-deficient animals, VM DA neurons adopt a correct ventral position and express the DA markers *Lmx1b*, *Pitx3* and *En1* (Saucedo-Cardenas et al., 1998, Wallen et al., 1999), demonstrating that *Nurr1* is not required for all aspects of VM DA specification and differentiation. However, these *Pitx3*-expressing VM DA neurons are lost in *Nurr1*-deficient animals during later development (Saucedo-Cardenas et al., 1998), suggesting a role for *Nurr1* in the survival and maintenance of VM DA neurons. In support of these findings, *Nurr1* is expressed throughout the life of VM DA neurons and its heterozygous mutation increases the vulnerability of VM DA neurons to the parkinsonian toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-HCl (MPTP) (Le et al., 1999). Furthermore, the conditional ablation of *Nurr1* at a late stage of VM DA neuron development or in the adult brain results in loss of striatal DA, loss of VM DA markers and DA neurodegeneration, with SNpc DA neurons more vulnerable than those of the VTA (Kadkhodaei et al., 2009).

While Nurr1 has been shown to directly regulate the expression of *TH* and *DAT*, Nurr1 may promote VM DA neuron survival indirectly, by inducing the expression of genes essential for their survival. Nurr1 has been found to regulate the expression of the GDNF receptor, *cRet*, whose expression is lost in *Nurr1*-deficient animals (Castillo et al., 1998). GDNF is a well-known survival-promoting factor for VM DA neurons (Toulouse and Sullivan, 2008, Yasuhara et al., 2007, Lin et al., 1993). *Nurr1* has also been shown to regulate the expression of *VIP* (vasoactive intestinal peptide), which has been proposed to function in the survival of VM DA neurons (Luo et al., 2007). It remains to be determined whether the VM DA neuronal death in *Nurr1* null mutants is as a direct consequence of the absence of a Nurr1-mediated survival-promoting effect, and/or is induced by a lack of neurotransmission by these cells. Aside from its role in DA survival, Nurr1 has been suggested to play a role in target innervation by VM DA neurons (Wallen et al., 1999), however, this finding has been challenged (Witta et al., 2000). It will be important to assess the role of Nurr1 in striatal innervation *in vivo* using an approach where DA neurons can survive long term in the absence of Nurr1. Such a strategy has been employed successfully in the PNS, where Bax-deficient mice were used to analyse specifically

the effects on a gene of interest on target innervation independent of this genes role in neuronal survival (Barker et al., 2001, Glebova and Ginty, 2004, Middleton and Davies, 2001).

The molecular mechanisms by which *Nurr1* expression is induced and regulated in the VM are largely unknown, but Nurr1 has been shown to function independently of FGF8 and Shh signalling (Sakurada et al., 1999). However, recent data suggests that FoxA1 and FoxA2 may be critical to *Nurr1* induction. Through the analysis of single and double mutants, *FoxA1* and *FoxA2* have been reported to regulate the expression of *Nurr1* in a dose-dependent manner, with a dramatic decrease in *Nurr1* expression observed in double mutants (Ferri et al., 2007). In addition to this, a gain-of-function study has demonstrated that *FoxA2* mediates *Nurr1* expression (Lee et al., 2010). Lee at al. (2010) further demonstrated that FoxA2 cooperates with Nurr1 during VM DA neurogenesis, with both factors binding to the *TH* promoter. In support of this, the loss of *FoxA2* resulted in an increase in the numbers of Nurr1-positive, TH-negative, cells in the VM (Ferri et al., 2007). These more recent data suggest that Nurr1 function may not be independent of Shh signalling, as *FoxA2* is a downstream target of Shh, however FoxA2 can function independently of Shh. Similar to Nurr1, FoxA2 appears to be involved in the survival and maintenance of VM DA neurons, as aged mice with a heterozygous mutation in *FoxA2* develop PD-like symptoms and pathologies (Kittappa et al., 2007), but this may be due to altered FoxA2-induction of Nurr1 expression.

Pitx3

Pitx3 is a bicoid-related, homeodomain-containing transcription factor that is exclusively expressed in the mouse VM from E11.5, at a time when VM DA neurons are beginning to appear (Smidt et al., 1997). VM DA neurons only begin to express *Pitx3* when they arrive at their final ventral position, suggesting that Pitx3 is not involved in the early development or migration of VM DA neurons (Smidt et al., 2004). GDNF has been suggested to induce the expression of *Pitx3* in the VM (Peng et al., 2011, Lei et al., 2011) which is interesting as Nurr1 is known to regulate the expression of the GDNF receptor *cRet*, and is expressed before *Pitx3* in the VM. Nurr1 may therefore play an indirect, non-essential (*Pitx3* expression is retained in *Nurr1* null mutants) role in the induction of *Pitx3* expression. Pitx3 and Nurr1 have been shown to function cooperatively in the regulation of target genes involved in VM DA neurogenesis (discussed later) (Chakrabarty et al., 2012, Jacobs et al.,

2009a, Jacobs et al., 2009b, Hwang et al., 2009). Pitx3 is co-expressed in the TH-positive neurons of the VM (Smidt et al., 1997, Zhao et al., 2004, van den Munckhof et al., 2003). In *aphakia* mice, which have approx. 5% *Pitx3* mRNA expression due to deletions in the *Pitx3* gene, there is unaltered VM DA development until E12.5, at which time a deficit is observable in the lateral population of VM DA neurons which constitute the presumptive SNpc (Smidt et al., 2004, Nunes et al., 2003, Hwang et al., 2003, van den Munckhof et al., 2003). VTA DA neurons are largely unaffected in these mice. The specific absence of SNpc DA neurons in *Pitx3* null (*aphakia*) mice results in a loss of nigrostriatal projections to the dorsal striatum (Smidt et al., 2004, Nunes et al., 2003, Hwang et al., 2003, van den Munckhof et al., 2003), and suggests distinct developmental programs for SNpc and VTA DA neurons. Interestingly, it has been reported that lateral VM DA neurons express *Pitx3* prior to *TH*, while the medial VM DA neurons express *Pitx3* coincidently with *TH* (Maxwell et al., 2005). In addition to this, Pitx3 has been suggested to regulate *TH* expression (Lebel et al., 2001, Cazorla et al., 2000, Maxwell et al., 2005). Pitx3 may therefore be critical for the induction of *TH* expression in SNpc DA neurons, but not those of the VTA. In support of this, the absence of *Pitx3* results in a failure of SNpc DA neurons to express *TH*, while VTA neurons do so. Interestingly, *Pitx3* expression has been reported to be six times higher in VTA DA neurons than in those of the SNc (Korotkova et al., 2005). Perhaps this lower expression level of Pitx3 functions in the induction of *TH* expression in SNpc DA neurons, while it may also contribute to their inherent sensitivity. In support of the proposed role for Pitx3 in *TH* expression, Pitx3 has been shown to contribute to the neurotransmitter phenotype of VM DA neurons by inducing the expression of *DAT* and *VMAT2* (Hwang et al., 2009). However, the loss of TH-positive neurons in the ventro-lateral VM is not due to the loss of TH mRNA expression, but to neuronal loss (Hwang et al., 2003, Nunes et al., 2003, Smidt et al., 2004). Perhaps the selective neurodegeneration of SNc DA neurons in *aphakia* mice is not as a result of a failure of DA neurogenesis in the absence of *Pitx3*, but may reflect the characteristic sensitivity of this VM population in comparison to those of the VTA. Indeed, the VTA DA neurons in *aphakia* mice display a normal DA phenotype (Smidt et al., 2004). A recent study has demonstrated that Pitx3 induces the expression of *BDNF* in SNpc DA neurons, which may be important in the survival of these neurons (Peng et al., 2011). Peng et al. (2011) showed that loss of *BDNF* expression correlates with the SNpc neuronal

loss in *Pitx3* null mice, and that BDNF treatment induces the survival of *Pitx3* (-/-) VM DA neurons and protects them against the dopaminergic neurotoxin 6-hydroxydopamine. Pitx3 may therefore be critical in the maintenance and survival of SNpc DA neurons, acting via BDNF. Similarly, *BDNF* has been identified as a target gene of Nurr1 (Volpicelli et al., 2007).

Despite the lack of a direct role for Nurr1 in *Pitx3* expression, a recent set of studies has shown that Nurr1 regulates target gene expression cooperatively with Pitx3 during VM DA neurogenesis, with Pitx3 potentiating Nurr1 activity by releasing it from SMRT-mediated repression (Jacobs et al., 2009a, Jacobs et al., 2009b). Similarly, the same group demonstrated that Nurr1 and Pitx3 cooperatively regulate the expression of two cholinergic receptors, *Chrna3* and *Chrnb6*, which may play non-essential roles in VM DA neurogenesis (Chakrabarty et al., 2012). Furthermore, Pitx3 has been shown to induce the expression of the Nurr1-target genes, *VMAT2* and *DAT*, potentially in coordination with Nurr1 (Hwang et al., 2009). Pitx3 also induces the expression of aldehyde dehydrogenase 2 (*ADH2*), an enzyme which is highly expressed in SNpc DA neurons (Chung et al., 2005). In support of this combinatorial function, Nurr1 and Pitx3 have been shown to cooperatively promote terminal maturation of VM DA neurons in stem cell cultures (Martinat et al., 2006). Collectively, these data suggest Nurr1 and Pitx3 may cooperate to promote VM DA survival and acquisition of a mature DA neurotransmitter phenotype by cooperatively regulating the expression of DA neurotrophic factors, *BDNF* and *GDNF*, and of genes involved in DA neurotransmission respectively.

<u>En1 and En2</u>

En1 and *En2* are important in the formation of the isthmus organizer and in the generation of VM DA neurons (Simon et al., 2001, Liu and Joyner, 2001). Following their initial expression in the midbrain-hindbrain boundary (Davis and Joyner, 1988), VM DA neurons begin to express *En1* and *En2* between E11.5 and E14 in mice, and this expression is maintained into and throughout adulthood (Alberi et al., 2004). Interestingly, in *En1* and *En2* double mutants, VM DA neurons develop normally initially, but are lost by E14 due to caspase-dependent apoptosis, just after the expression of *En* begins in the wild type (Simon et al., 2001, Alberi et al., 2004). Alberi et al. (2004) demonstrated that *En1* and *En2* are required cell-autonomously in post-mitotic VM DA neurons to prevent apoptosis. However, further studies are

required to ascertain that VM DA neuronal loss in the *En* double mutants is not as a result of the large midbrain/hindbrain deletion in these mice. Despite this possibility, these data strongly suggest a role for *En1* and *En2* in the maintenance and survival of VM DA neurons. Indeed, intermediate genotypes between wild type and double *En* mutants show varying degrees of VM DA neuronal deficiencies (Simon et al., 2001, Sonnier et al., 2007, Sgado et al., 2006), as has been well-described in recent reviews (Alavian et al., 2008, Alves dos Santos and Smidt, 2011). The most notable phenotype was observed in *En1* (+/-)/*En2* (+/+) mutant mice, which display a progressive degeneration (between 8-24 weeks) of VM DA neurons that can be antagonized by recombinant En2 protein infusion (Sonnier et al., 2007). The progressive degeneration of VM DA neurons in *En1* heterozygotes (*En2* null background in Sgado et al. (2006) study) is more pronounced in the SNpc and results in reduced striatal DA and motor deficits, as is characteristic of PD pathology (Sgado et al., 2006, Sonnier et al., 2007). These findings further support the theory that *En1*/*En2* function as important survival-promoting factors for VM DA neurons.

Diversity in genetic regulation of DA neuron development

As mentioned earlier, the molecular mechanisms controlling phenotypic and functional diversity between the various VM DA neuronal subpopulations remain poorly understood. However, recent work has implicated *Otx2* as a factor that may contribute to these distinct developmental pathways. *FoxA2*, *En1*, *Lmx1b*, *Nurr1* and *Pitx3* are ubiquitously expressed in post-mitotic VM DA neurons throughout life, however *Otx2* expression is restricted to VTA DA neurons in the adult brain (Di Salvio et al., 2010b). *Otx2* was shown to regulate subtype identity in the VTA by antagonising the expression of *Girk2* and *DAT*, and was also shown to antagonise the neurotoxic effect of the MPTP in these VTA neurons (Di Salvio et al., 2010a). Interestingly, ectopic *Otx2* expression also provides SNpc neurons with neuroprotection to MPTP (Di Salvio et al., 2010a). This potential role of *Otx2* in VM DA neuronal subtype identity has been comprehensively described in a recent review (Simeone et al., 2011).

It is also necessary to mention that a proportion of DA neurons arise anterior to the VM in the diencephalon (Gonzalez et al., 1999, Lahti et al., 2012, Marin et al., 2005, Puelles and Verney, 1998, Smits et al., 2006, Verney, 1999, Verney et al., 2001, Vitalis et al., 2000), and develop earlier than those from the VM (Lahti et al., 2012, Marin et al., 2005). The diencephalic DA domain differs to that of the

midbrain. The DA NPs in the diencephalon are intermingled with non-DA Pou4f1+FoxP1+ cells, they lack *Pitx3* and *DAT* expression, and lose *En1/2* expression by E9.5 (Lahti et al., 2012), unlike those in the midbrain (Alberi et al., 2004). FGF8 regulates the diverse identities of the DA neurons from the VM and caudal diencephalon. This anterior-posterior patterning by FGF8 suppresses diencephalic identity and maintains midbrain identity (Lahti et al., 2012, Scholpp et al., 2003). A study using zebrafish showed that Nodal signalling was required for the specification of ventral diencephalic and pretectal catecholaminergic neurons (Holzschuh et al., 2003). Holzschuh et al. (2003) also demonstrated that FGF8 signalling was not required for the specification of these neurons but was important for their proliferation or survival, and that Shh signalling is required for pretectal DA development. Diencephalic DA neurons therefore seem to be subject to a different program of neurogenesis than those of the VM. Genetic fate-mapping studies are needed to verify whether, or to what extent, these diencephalic DA neurons contribute to the DA subpopulations of the VM.

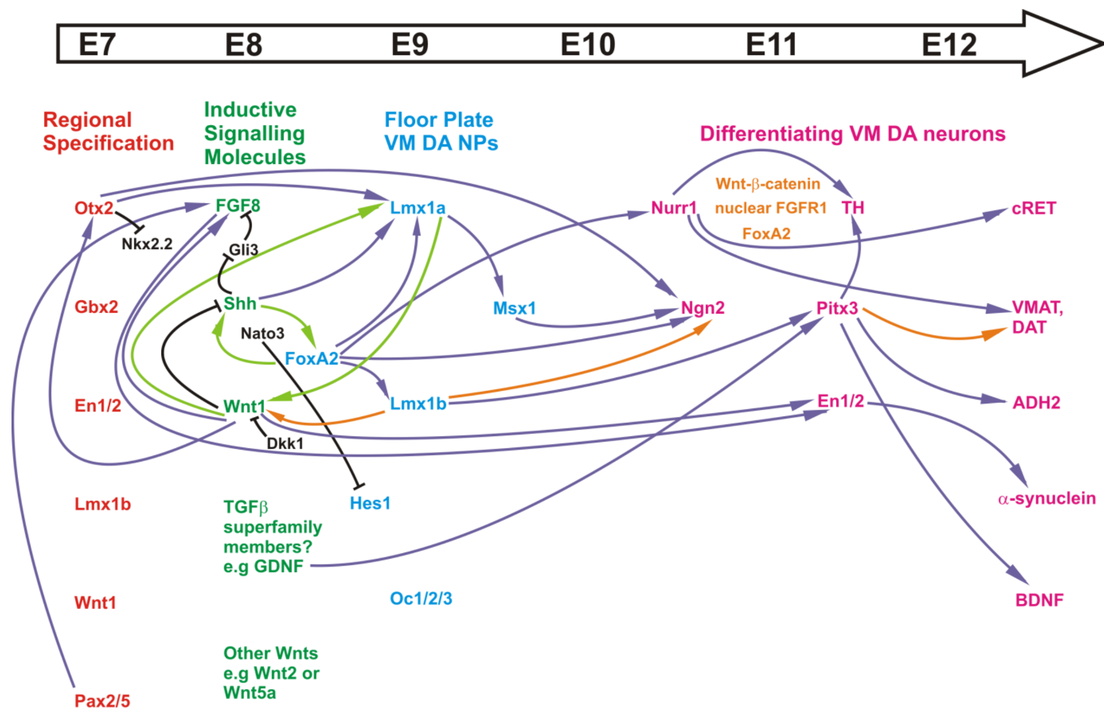


Figure 3.2.1: Molecular interactions in the genesis of VM DA neurons.

The sequence of appearance (see time-course arrow) of each of the factors involved in VM DA neuronal development, and their effects on each other (Note: the molecules in black text are not shown at the time point at which they appear). The arrows denote the effect on expression: purple = positive regulation, green = autoregulatory loop, orange = cooperative regulation, and black = negative regulation. The factors are colour-coded as per their role (listed above molecules). Otx2, Gbx2, En1/2, Lmx1b, Wnt1 and Pax2/5 play vital roles in the establishment of the midbrain/hindbrain region, including the isthmus organizer and VM floor plate, and the majority also play direct roles in VM DA neurogenesis. The diffusible signalling factors FGF8, Shh and Wnt1 induce VM DA neurogenesis in radial glial-like floor plate cells, through the induction of FoxA2, Lmx1a/1b, and Msx1 expression. Two autoregulatory loops, Shh-FoxA2 and Wnt1-Lmx1a, contribute to this process, with Hes1 being involved in the Shh-FoxA2 autoregulatory loop, and Lmx1b functioning cooperatively with Lmx1a. The expression of Nurr1 and Pitx3 promotes the differentiation of VM DA NPs into post-mitotic neurons. Nurr1 induces the expression of proteins that are key to the neurotransmitter phenotype of VM DA neurons. A number of factors facilitate the induction of TH by Nurr1 (listed in orange), and Pitx3 has been shown to cooperatively regulate a number of important genes involved in VM DA neurogenesis with Nurr1.

3.2.5 Establishment of DA projections from the VM

Following their generation, post-mitotic VM DA neurons undergo functional maturation, which involves axonal pathfinding and synaptogenesis. Axons from VM DA neurons, which arise at E11 in mice (E13 in rat), initially project dorsally but then deflect ventro-rostrally towards the forebrain, in response to extrinsic directional cues in the dorsal midbrain and repulsive cues in the caudal brain stem (Gates et al., 2004, Nakamura et al., 2000). The reorientated VM DA neuronal axons then extend towards the telencephalon, through the diencephalon, via the medial forebrain bundle (MFB) which has been reported to have a chemo-attractive effect on these axons (Gates et al., 2004). A recent paper has suggested that Nurr1 regulates the axonal extension of VM DA neurons through the regulation of the expression of the axon genesis gene *Topoisomerase IIβ* (*TopIIβ*) (Heng et al., 2012). Furthermore, a study using retrograde labelling suggested that Nurr1 plays a role in target innervation by VM DA neurons (Wallen et al., 1999). However, as mentioned before, this finding has been challenged (Witta et al., 2000). Gates et al. (2004) also demonstrated that the thalamus prevents entry of VM DA axons through the action of contact-dependent inhibitors, which likely function to maintain the orientation of these axons in the MFB. Furthermore, *Nkx2.1* mutant mice display aberrant midline crossing of MFB fibers at the caudal diencephalon, suggesting that chemo-repulsive factors involved in maintaining the ipsilateral trajectory of the MFB at the medial part of the caudal diencephalon are lost in this mutant (Kawano et al., 2003).

The VM DA neuronal axons run via the MFB into the telencephalon, where they terminate in the striatum and cerebral cortex (Specht et al., 1981a, Specht et al., 1981b, Zhao et al., 2004). In the case of the nigrostriatal pathway, chemoattraction from the striatum and chemorepulsion from the cortex have been suggested to facilitate appropriate striatal innervation by nigral DA neurons (Gates et al., 2004). The molecular signals that guide the axons of the various populations of VM DA neurons remain to be characterised, however a relatively recent review has comprehensively described the current understanding of the development of VM DA circuitry (Van den Heuvel and Pasterkamp, 2008). Molecules which are known to be involved in the establishment of the VM DA circuit are illustrated in Fig. 3.2.2.

Despite the current paucity of studies determining the molecular basis of the formation of VM DA projections, several molecules have been implicated to play a

role in this process. *EphrinB2* and its receptor *EphB1* have been shown to be expressed in a complementary pattern to facilitate nigro-striatal innervation, with *EphB1* expressed by VM DA neurons (with highest expression in the SNpc) and *ephrinB2* expressed in the striatum (Yue et al., 1999). Cell-surface tethered ephrins, and their Eph receptor tyrosine kinases, are known to play important roles in axonal guidance (Egea and Klein, 2007). Furthermore, Yue at al. (1999) showed that co-culture with ephrinB2-expressing NIH-3T3 cells reduced neurite outgrowth and induced death of SNpc, but not VTA, DA neurons. These results suggest that the interaction between ephrinB2 and EphB1 in the striatum ensures that SNc DA neurons are confined to the dorsal striatum. Conversely, *EphB1* expression has been shown to disappear in the SNpc from E18, and its null mutation resulted in no observable defects in the nigrostriatal pathway (Richards et al., 2007). These results challenge the role for EphB1 in the formation of the nigrostriatal pathway, however other Eph receptors may allow SNpc DA axons to detect ephrinB2 in the striatum. In support of a role for ephrinB2 in the correct target innervation of nigral DA neurons, the application of ephrinB2 to VM cultures resulted in an upregulation of *Nurr1* (Calo et al., 2005). This action by ephrinB2 could function to support and maintain correctly-innervated DA neurons. However, this effect was suggested to be mediated by the EphB1 receptor (Calo et al., 2005). Other ephrins and Ephs have also been implicated in DA pathway formation. For example, studies on genetically-altered EphAs and ephrinAs have shown that these molecules are important in the formation of VM DA projections (Sieber et al., 2004, Halladay et al., 2004, Van den Heuvel and Pasterkamp, 2008), with *ephrinA5* expression being reduced in the forebrain of *Nkx2.1* mutants (described above) (Marin et al., 2002). *EphrinA5* has been shown to be expressed in the developing telencephalon and striatum, in the vicinity of VM DA axons, and to have a repulsive effect on these axons, likely through the action of EphA5 (Deschamps et al., 2009). Conversely, another study has shown that ephrinA5-EphA5 signalling promotes DA axonal growth *in vitro* (Cooper et al., 2009). Perhaps ephrinA5 initially functions in the establishment of VM DA projections, but later functions to restrict these axons to their targets. Semaphorin signalling has also been proposed to function in VM DA axonal pathfinding (Hernandez-Montiel et al., 2008, Torre et al., 2010, Tamariz et al., 2010, Kolk et al., 2009), with a number of semaphorins and their receptors being expressed in VM DA neurons (Torre et al., 2010). Furthermore, *Sema3A* expression is reduced in *Nkx2.1*

mutants (Kawano et al., 2003), and the expression of its co-receptor *Neuropilin1* has been shown to be regulated by Nurr1 in the developing midbrain (Hermanson et al., 2006).

The netrin receptor *DCC* has been demonstrated to play an important role in the formation of VM DA axonal projections. *DCC* is expressed in the VM and in cultured VM DA neurons, as well as in VM DA targets, such as the striatum and prefrontal cortex (Livesey and Hunt, 1997, Xu et al., 2010, Lin et al., 2005), while *netrin1* is expressed in a complimentary fashion in VM, striatal and cortical neurons (Hamasaki et al., 2001, Livesey and Hunt, 1997, Manitt et al., 2011). Studies of heterozygous and homozygous *DCC* mutants have provided insights into how netrin-DCC signalling may regulate the formation of VM DA neuronal projections (Xu et al., 2010, Flores et al., 2005). DA innervation of the dorsal striatum is not affected in heterozygous and homozygous *DCC* mutants, while the ventral striatal DA projections are aberrantly shifted to a more dorsal location in null mutants. The innervation of the prefrontal cortex by VM DA neurons is significantly reduced in null mutants, suggesting that *DCC* is an important mediator of VM DA axonal guidance. Xu et al. (2010) also propose that DCC signalling is an important negative regulator of DA axon arborisation, demonstrating that DA innervation is maintained/increased despite significant VM DA neuronal loss in *DCC* deficient animals. In support of this, analysis of heterozygous *DCC* mutants has shown that DCC can selectively influence the branching of VM DA fibers in the prefrontal cortex at puberty, with a significant increase in the number of TH-positive varicosities present post-puberty in these heterozygotes (Manitt et al., 2011). This proposed role of DCC contradicts previous reports which had suggested that DCC mediates netrin1-promotion of axonal outgrowth in VM DA neuronal cultures (Lin et al., 2005). However, these contrasting results may reflect differences between the responses of VM DA neurons *in vitro* and *in vivo*. Furthermore, DCC receptors are known to mediate both attraction and repulsion aspects of the axon growth-promoting effects of netrins (Round and Stein, 2007). The atypical homeoprotein Pbx1a has been shown to regulate the expression of *DCC* in VM DA neurons, and its deficiency results in partial misrouting of VM DA fibers (Sgado et al., 2012). Interestingly, *DCC* null mice also display aberrant midline crossing of MFB DA fibers at the caudal diencephalon, which is similar to that observed in *Nkx2.1* mutant mice (Xu et al., 2010), likely reflecting a loss of chemorepellant(s) at the ventral

midline. In *Pax6* null mice, which display a ventro-dorsal expansion of netrin1 expression, VM DA axons within the MFB are deflected dorsally in the diencephalon, seemingly avoiding areas of ectopic *netrin1* expression (Vitalis et al., 2000). Netrin-DCC signalling may therefore act at the ventral midline of the diencephalon to repel VM DA projections, ensuring that they maintain their ipsilateral course in the MFB. Similarly, mice deficient in both *Slit1* and *Slit2* display abnormal ventral midline crossing of MFB fibers in the diencephalon, suggesting they may also act as ventral midline chemorepellants (Bagri et al., 2002, Dugan et al., 2011). In support of this theory, Slit2 repels VM DA neuronal axons and inhibits their growth *in vitro* (Lin et al., 2005, Dugan et al., 2011). *Nkx2.1* mutant mice display altered *Slit1* and *Slit2* expression, and a more severe phenotype than that of *Slit1*/*Slit2* double mutants (Marin et al., 2002). This suggests that *Nkx2.1* may regulate the expression of a number of important chemorepellants at the diencephalic ventral midline, such as *Slit1*/*Slit2*, *Sema3A*, *ephrinA5* and perhaps *netrin1*. However, *netrin1* expression is maintained in the subpallium of the *Nkx2.1* mutants (Marin et al., 2002), and has been reported to attract diencephalic (A11) DA neurons towards the ventral midline in the absence of repulsive Slit signals (Kastenhuber et al., 2009). Mice deficient in the Slit receptors, *Robo1* and *Robo2*, also display guidance errors in the MFB tract similar to those in the *Slit* double mutant (Lopez-Bendito et al., 2007, Dugan et al., 2011). However, Dugan et al. (2011) also demonstrated abnormal dorsal trajectories of VM DA fibers in *Robo1*/*2* knockout mice, which does not occur in the *Slit1*/*2* mutant, suggesting that these Robos also function Slit-independently. The expression patterns of Slits also propose a role for these molecules in VM DA axonal guidance, with *Slit1* expressed in the dorsal midbrain (Nakamura et al., 2000) and *Slit3* expressed in the caudal midbrain (Gates et al., 2004), suggesting that these Slits contribute to the ventro-rostral trajectory of VM DA fibers.

Upon innervation of their targets, the axons of VM DA neurons compete to establish functional synapses and survive. There are two peak postnatal periods of naturally-occurring cell death for VM DA neurons. Cell death begins close to birth, reaching an initial peak at P2, before a second peak of apoptosis occurs at P14, with this process largely subsiding around P20 in rodents (Jackson-Lewis et al., 2000, Oo and Burke, 1997, Burke, 2003). This programmed cell death pathway relies on the limited availability of target-derived neurotrophic factors (Burke, 2003), with striatal

and prefrontal cortex tissue being shown to promote VM DA neuronal survival when co-cultured *in vitro* (Hoffmann et al., 1983). The most well-established target-derived neurotrophic factor for VM DA neurons is GDNF (Lin et al., 1993, Beck et al., 1995, Gash et al., 1996, Tomac et al., 1995, Costantini and Isacson, 2000, Lei et al., 2011, Wang et al., 2010, Redmond et al., 2009, Akerud et al., 1999, Burke, 2003). Another member of the GDNF protein family, neurturin, also acts as a neurotrophic factor for VM DA neurons (Akerud et al., 1999, Horger et al., 1998, Oiwa et al., 2002, Tseng et al., 1998, Zihlmann et al., 2005). Other neurotrophic factors identified for VM DA neurons include TGFβs (Farkas et al., 2003), BDNF (Alonso-Vanegas et al., 1999), BMP2 and GDF5 (O'Keeffe et al., 2004a, Wood et al., 2005, Reiriz et al., 1999, Jordan et al., 1997, Sullivan et al., 1997, Sullivan et al., 1999, Hurley et al., 2004, O'Sullivan et al., 2010, Sullivan et al., 1998b, Espejo et al., 1999, Hegarty et al., 2014a). Interestingly, FGF2 has recently been shown to act as a target-derived regulator of VM DA innervation (Baron et al., 2012b).

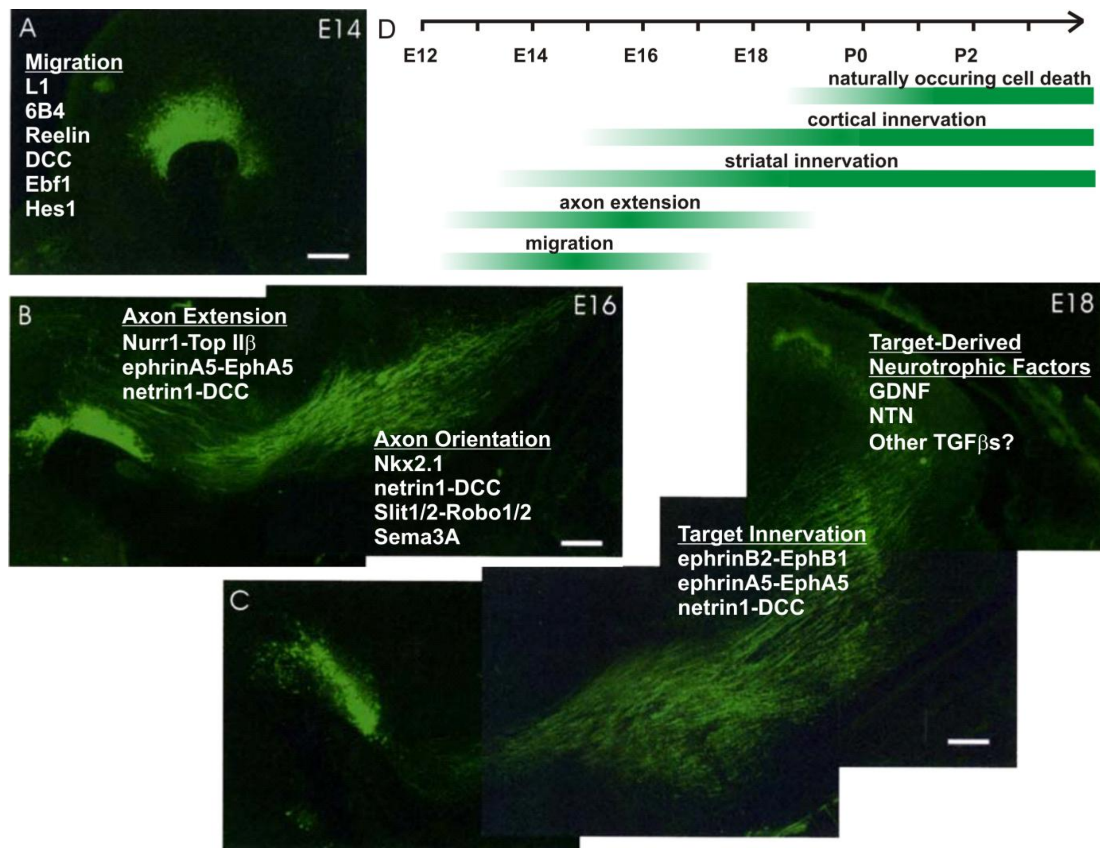


Figure 3.2.2: Molecules involved in the formation of the nigrostriatal DA circuitry.

Representative photomicrographs showing cryosections through the developing rat nigrostriatal pathway at (A) E14, (B) E16 and (C) E18, immunostained for TH. Molecules involved in the migration (A), axon extension (B), axon orientation (B), target innerevation (C) and survival (C) of VM DA neurons are labelled on the images where appropriate. Scale bar = 100µm. (D) Graphical representation of the time-course of DA circuitry formation. VM DA neurons begin to migrate and extend axons at E13 in the rat. These DA fibers begin to reach the striatum by E14, and the cortex at E16. The innervation of these targets continues into the first week after birth. Naturally occurring cell death begins close to birth, reaching a peak at P2 and P14, before subsiding around P20.

3.3 The BMP-Smad 1/5/8 Signalling Pathway

BMPs, such as BMP2 and GDF5, play roles in the development of midbrain DA neurons, but the mechanisms by which they achieve their DA neurotrophic effects are unclear.

3.3.1 Bone Morphogenetic Proteins and Their Receptors

Smad transcription factors are the pivotal intracellular effectors of the TGFβ superfamily members, which are dimeric, structurally-conserved proteins, that have pleiotropic functions in vitro and in vivo (Massague and Wotton, 2000). BMPs constitute the largest subgroup of the TGFβ superfamily, and consist of at least 20 phylogenetically-conserved growth factors, including GDF5 (Kawabata et al., 1998). BMPs are synthesised as large precursors of 400-500 amino acids, consisting of an N-terminal peptide which directs secretion, a prodomain which is involved in folding, and the C-terminal mature peptide which is cleaved from the prodomain upon secretion and dimerisation. The mature peptide has seven highly-conserved cysteine residues, six of which form three intramolecular disulfide bonds known as the cysteine-knot motif. The seventh forms a disulfide bond with another BMP monomer, resulting in dimerisation (Xiao et al., 2007, Peterson and Nutt, 2008, Sieber et al., 2009). A number of cleaved, mature BMP peptides have been shown to remain non-covalently associated with their prodomain. This interaction may function to target BMPs to the extracellular matrix, where the prodomains can bind to fibrillins, thus preventing the diffusion of BMPs away from the cell of origin (Sengle et al., 2008, Bragdon et al., 2011).

For signal transduction, BMP family members bind to two distinct types of serine/threonine kinase cell-surface receptors (Fig. 3.3.1), type I and type II receptors (BMPR), both of which are required for signal transduction (Yamashita et al., 1996, Shi and Massague, 2003). These BMPRs are composed of a short extracellular domain, a single transmembrane domain, and an intracellular serine/threonine kinase domain. There are five known distinct BMP type I receptors (BMPRI): activin receptor-like kinases (ALK) 1, ALK2, ALK3 (also known as BMPRIa), ALK4, ALK6 (also known as BMPRIb), and there are three type II receptors: BMP type II receptor (BMPRII), activin type IIa receptor, and activin type IIb receptor (Nohe et al., 2004, Bragdon et al., 2011). BMPRII has a long C-terminal tail at the end of its

serine/threonine kinase domain (Rosenzweig et al., 1995), which plays an important role in the functional regulation of several cytoskeletal proteins (Miyazono et al., 2005). C-terminal truncated slice variants that disrupt the function of BMPRII have been identified, and have been suggested to confer predisposition to primary pulmonary hypertension (Lane et al., 2000). Upon ligand binding, two molecules of the constitutively-active type II receptor kinase form a hetero-tetrameric complex with two molecules of the type I receptor kinase (Kirsch et al., 2000), and they then transphosphorylate the intracellular Gly-Ser domain of the type I receptors (Fig. 3.3.1). This phosphorylation activates the type I receptor which subsequently transmits intracellular signals by recruiting and phosphorylating Smad proteins (Fig. 3.3.1) (Miyazono et al., 2001, Miyazawa et al., 2002). The type I receptor thus determines the specificity of intracellular signalling. Unlike the TGFβs, in most cases it is the type I receptor that is the high affinity receptor for BMPs (Groppe et al., 2008, Heinecke et al., 2009). Co-receptors for the BMP receptors are also present at the cell surface, an example of which is the glycosylphosphatidylinositol-linked molecule DRAGON that interacts with BMPs and stabilizes the active BMPR complex to enhance BMP signalling (Samad et al., 2005, Wordinger and Clark, 2007).

3.3.2 The Smad Transcription Factors

The major signalling molecules acting downstream from the serine/threonine kinase receptors are the Smad proteins (Moustakas et al., 2001). These are classified into three subclasses based on function, receptor-regulated Smads (R-Smads), common-mediator Smads (Co-Smads), and inhibitory Smads (I-Smads) (Heldin et al., 1997). R-Smads are specifically phosphorylated by activated type I receptor kinases, and for the BMP family are Smad1, Smad5 and Smad8, referred to as BR-Smads. Co-Smads are recruited by activated, phosphorlyated R-Smads into heteromeric complexes which translocate into the nucleus. To date, only one Co-Smad, known as Smad 4, has been isolated from mammals (Zhang et al., 1997). The I-Smads, Smad6 and Smad7, negatively regulate R-Smad/Co-Smad signalling (Itoh et al., 2000, Miyazawa et al., 2002, Miyazono et al., 2005).

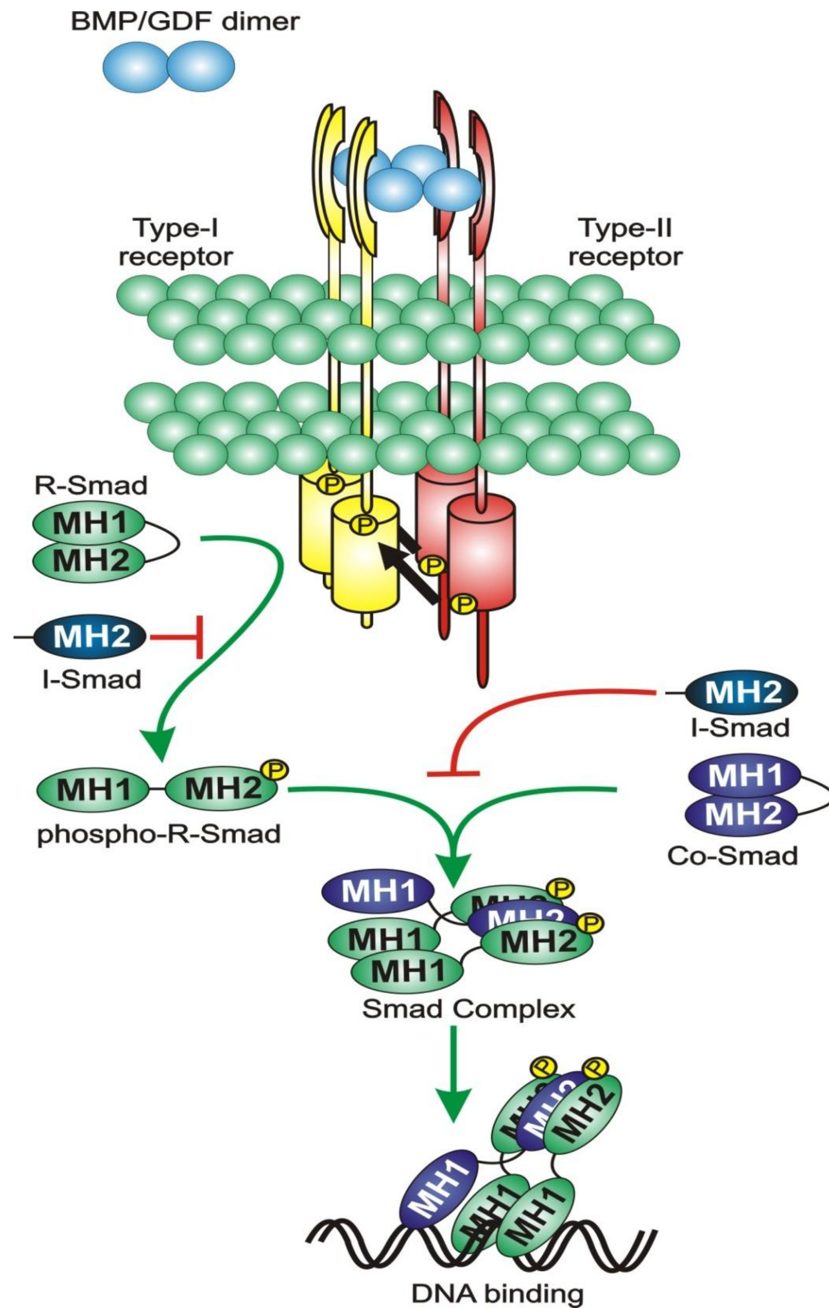


Figure 3.3.1: The BMP-Smad 1/5/8 signalling pathway.

Upon ligand-induced heteromeric complex formation and activation of type I and type II receptors, R-Smads are subsequently phosphoryled and their MH domains dissociate. The activated R-Smads form heteromeric complexes with the Co-Smads through their MH2 domains, and the R-Smad-Co-Smad hetero-trimer then translocates to the nucleus to regulate target gene expression through their MH1 domains. The I-Smads physically interact with activated type I receptors to block R-Smad activation, and with phosphorylated R-Smads to prevent R-Smad-Co-Smad complex formation.

Smads are composed of highly-conserved N-terminal Mad homology (MH) 1 domains and C-terminal MH2 domains, which are linked by a divergent proline-rich linker region of variable length that contains multiple phosphorylation sites each of which allow specific cross-talk with specific signalling pathways (Heldin et al., 1997, Massague and Wotton, 2000, Shi and Massague, 2003). I-Smads lack the MH1 domain (Souchelnytskyi et al., 1998), which is the DNA-binding domain of R-Smads and Co-Smads. The MH2 domains of R-Smads are responsible for their direct interaction with type I receptor kinases, and are also responsible for Smad complex formation. The L3 loop of the MH2 domain determines the specificity of receptor interaction (Lo et al., 1998). Additionally, Smad1 has the α-helix 1 in its MH2 domain to confer receptor specificity (Chen and Massague, 1999). R-Smads have Ser-Ser-Val/Met-Ser (SSXS) motifs in their most C-terminal parts which are phosphorylated by activated type I receptors. In the absence of receptor activation, MH1 and MH2 domains are physically associated with one another (Miyazawa et al., 2002, Miyazono et al., 2005). Upon receptor activation and subsequent R-Smad phosphorylation and MH domain dissociation, R-Smads form hetero-oligomers with Co-Smad through their MH2 domains (Fig. 3.3.1). These heteromers are thought to consist of two R-Smads and one Co-Smad (Qin et al., 2001). This heterotrimeric complex then translocates to the nucleus to regulate target gene expression (Fig. 3.3.1) (Miyazawa et al., 2002). This nuclear translocation may be mediated by a nuclear import activity of Smads, evidence of which have previously been reported for Smad2 (Xu et al., 2000), Smad3 (Xiao et al., 2000a, Xiao et al., 2000b), and Smad4 (Xiao et al., 2003). Additionally, Smad4 has a functional leucine-rich nuclear export sequence, known as CRM1 (Fornerod et al., 1997), that ensures cytoplasmic location of unstimulated Smad4. Heteromeric complex formation of Smad4 with R-Smads has been shown to inactivate this sequence to allow nuclear translocation (Watanabe et al., 2000, Pierreux et al., 2000). The nucleocytoplasmic shuttling of Smad proteins has been well characterised in a recent review (Hill, 2009).

3.3.3 Inhibitory Smads

The I-Smads, Smad6 and Smad7, physically interact with activated BMPRI via their MH2 domains, and compete with R-Smads for activation, thus inhibiting R-Smad signalling (Fig. 3.3.1) (Imamura et al., 1997, Hayashi et al., 1997, Heldin et al.,

1997, Souchelnytskyi et al., 1998). This receptor binding of I-Smads is supported by interactions between their MH2 domain and their N-terminal (Hanyu et al., 2001). I-Smads also bind to phosphorylated R-Smads, and prevent R-Smad-Co-Smad complex formation thus inhibiting Smad signalling (Fig. 3.3.1) (Hata et al., 1998, Murakami et al., 2003). Smad6 has been shown to preferentially inhibit the BR-Smads (Ishisaki et al., 1999), while Smad7 inhibits both BMP and TGFβ signalling (Mochizuki et al., 2004). I-Smads are important in the regulation of BR-Smad signalling, and it has recently been shown that Smad6 is essential to limit BMP signalling and thus facilitate proper cartilage development (Estrada et al., 2011). The inhibitory function of I-Smads is negatively regulated by specific cytoplasmic molecules. For example, AMSH (associated molecule with the SH3 domain of STAM) directly binds Smad6 upon BMP stimulation antagonizing its inhibitory effects by preventing the interaction of Smad6 with BMPRIs and BR-Smads (Itoh et al., 2001). Furthermore, ubiquitin-dependent degradation of Smad7 is induced by Arkadia, a RING type E3 ligase (Koinuma et al., 2003), and by Jab1/GCN5, a component of the COP9 signalosome complex (Kim et al., 2004), resulting in the enhancement of BR-Smad signalling. Conversely, Jab1/GCN5 has also been shown to induce the degradation of Smad4 (Wan et al., 2002), which antagonizes BMP function. The conditions under which Jab1/GCN5 promotes and antagonizes BR-Smad signalling are not known.

3.3.4 Interactions of Smad1, Smad5 and Smad8 with cytoplasmic molecules:

The interactions of BR-Smads with other cytoplasmic molecules has not been fully determined, and is significantly less characterised than that for the non BR-Smads. It is important to understand such interactions to gain an insight into how BR-Smads function and interact with intracellular signalling pathways. At the cell membrane, Smad1 has been shown to interact with the cytoplasmic domain of the CD44 receptor (Peterson et al., 2004), which may function to link inactive BR-Smads with the BMP receptor complex and thus enable its activation. Smad1 antagonistic effector (SANE) interacts with the MH2 domain of BR-Smads and with the BMPR1a and BMPR1b receptors (Raju et al., 2003). Surprisingly, SANE does not function to present BR-Smads to the type I receptor, but instead appears to antagonize BR-Smad signalling by inhibiting the phosphorylation of BR-Smads, and

blocking their nuclear translocation (Raju et al., 2003). The inner nuclear membrane protein XMAN1 interacts with Smad1, Smad5 and Smad8 via its C-terminal region to antagonize BMP-Smad signalling in the *Xenopus* (Osada et al., 2003). Protein phosphatase 1 dephosphorylates TGFβ type I receptors, via an interaction with Smad7 (Shi et al., 2004b), and has been shown to negatively regulate the phosphorylation of BMPRI receptors in *Drosophila* (Bennett and Alphey, 2002). Homologous to E6-AP carboxyl terminus (HECT) type E3 ligases, Smurf1 and Smurf2, interact with BR-Smads to induce their degradation (Zhu et al., 1999, Lin et al., 2000, Zhang et al., 2001, Ying et al., 2003b), and thus inactivation. The WW domains of Smurfs recognise the PPXY motifs in the linker regions of target Smads, and this interaction is key in Smurf-induced ubiquination of Smads (Sangadala et al., 2007, Chong et al., 2010). The *Drosophila* homolog DSmurf restricts BR-Smad function during *Drosophila* embryogenesis (Podos et al., 2001). Smurfs also interact with Smad7 to induce its translocation into the nucleus, its ubiquination, and also to use Smad7 as an adaptor molecule to facilitate their degradation of the TGFβ receptor (Kavsak et al., 2000, Ebisawa et al., 2001). Interestingly, Smurf1 has been demonstrated to enhance Smad6 function in a transgenic overexpression study in mice, in which Smad6 was overexpressed with or without Smurf1 and the effect on ossification was compared (Horiki et al., 2004). Additionally, Smurf1 overexpression in mouse airway epithelial cells reduced the expression of Smad1 and Smad5 proteins (Shi et al., 2004a). This negative effect of Smurf1 on Smad1 and Smad5, and its positive effect on Smad6, suggests a selective antagonism of Smurf1 on BR-Smad signalling. In support of this, Smad1, Smad5 and Smad6 are targets of Smurf1-mediated ubiquination (Sangadala et al., 2007). Another E3 ligase, CHIP, recruits Smad1 and Smad5 from the R-Smad-Co-Smad complex and induces ubiquitin-dependent degradation of Smad1, Smad5 and Smad4, and also inhibits BR-Smad induced transcription (Li et al., 2004, Wang et al., 2011). Tob (transducer of ErbB2), a member of the antiproliferative gene family, associates with I-Smads to inhibit BR-Smad signalling by interacting with activated BMPRIs (Yoshida et al., 2003). Tob inhibits BR-Smad signalling in osteoblasts by directly interacting with Smad1, Smad5 or Smad8 to repress their activity (Yoshida et al., 2000). The c-Ski oncogene interacts with the L3 loop of Smad4 through its I (Interacting) loop region to inhibit BR-Smad signalling (Wang et al., 2000, Wu et al., 2002, Takeda et al.,

2004). Similarly, DACH1 is a nuclear protein distantly related to Ski that interacts with Smad4 to antagonize its function (Wu et al., 2003), and may also inhibit BMP signalling. Smad1 and Smad5 interact with the scaffolding protein filamin, which enhances their signalling, possibly by providing a pseudo-tract for intracellular Smad movement (Sasaki et al., 2001). Smad1 can directly interact with proteins, such as Par3 and Dishevelled-1, that function in epithelial cell polarity, suggesting a role for BR-Smads in cell polarity (Warner et al., 2003). Bat3 (HLA-B-associated transcript 3) negatively regulates BR-Smad signalling by interacting with the nuclear phosphatase SCP2, and inducing BR-Smad dephosphorylation by SCP2 (Goto et al., 2011).

3.4 BMP-Smad 1/5/8 signalling in nervous system development

The expression profiles of Smad proteins during NS development has yet to be fully characterised, with most studies focusing on Smad expression in a defined region of the CNS or PNS. However, it is important to note that the BR-Smads, Smad 1/5/8, do not share identical expression patterns in the developing NS. For example, in the basal forebrain, Smad1 mRNA is highly expressed at E14 and P8 but to a lesser extent in the adult and perinatally, Smad5 expression is consistent throughout development, and Smad8 mRNA expression is absent early at E14 but increases thereafter with age (Lopez-Coviella et al., 2006). Herein we discuss the role of Smad 1/5/8 signalling in the development of the NS.

In addition to well-characterised roles in bone and cartilage development (Nishimura et al., 2012, Yoon and Lyons, 2004), BMP-Smad 1/5/8 signalling also instructs key developmental events during the development of the NS. Paradoxically, despite a role for BMP-Smad signalling in key neurodevelopmental events, the repression of BMP-Smad signalling is firstly required for the primary neurodevelopmental event, neural induction (Fig. 3.4.1a) (Smith and Harland, 1992, Smith et al., 1993, Spemann and Mangold, 1924, Liu and Niswander, 2005). BMP-Smad signalling on the ventral side of the embryo allows the formation of epidermal ectoderm, while dorsally-expressed BMP antagonists induce formation of neural tissue through the blockade of BMP-Smad signalling (Lamb et al., 1993, Wilson and Hemmati-Brivanlou, 1995, Sasai et al., 1995, Hemmati-Brivanlou and Melton, 1997).

3.4.1 BMP-Smad 1/5/8 signalling in neural induction

In the gastrula, neural tissue develops from the dorso-medial region of the ectoderm, while the remainder continues as epidermal (non-neural) ectoderm. This inductive role of BMP-Smad signalling became apparent following experiments involving the transplantation of dorsal embryonic tissue (the Spemann organizer) (Spemann and Mangold, 1924) to the ventral side of the early embryo, which caused the suppression of non-neural ectoderm and the formation of a secondary neural axis, and the subsequent identification of noggin as the dorsalizing factor of the Spemann organizer (Smith and Harland, 1992, Smith et al., 1993). Noggin is a BMP antagonist and, along with another BMP antagonist that is expressed by the Spemann organizer chordin, it binds BMPs in the extracellular space to inhibit BMPR activation (Sasai et al., 1994, Piccolo et al., 1996, Zimmerman et al., 1996). The Spemann organizer forms in the dorsal mesoderm in response to factors secreted from a nodal-producing signalling centre (Nieuwkoop center) located in the adjacent presumptive dorsal endoderm (Nieuwkoop, 1967, Agius et al., 2000). The establishment of this Nieuwkoop centre depends on the dorsal accumulation of the Wnt effector, β-catenin, in the early blastula (Larabell et al., 1997).

BMPs, which are expressed on the ventral side of the embryo, allow the formation of epidermal ectoderm in the Xenopus, while the dorsally-expressed noggin and chordin induce formation of neural tissue through the blockade of BMP-Smad signalling (Lamb et al., 1993, Wilson and Hemmati-Brivanlou, 1995, Sasai et al., 1995, Hemmati-Brivanlou and Melton, 1997). Interestingly, prior to gastrulation, BMP-activated Smad5 has recently been shown to antagonize ectopic primitive streak production on the anterior side of the embryo through an unusual interaction with Smad2 (Pereira et al., 2012), an R-Smad for TGFβ family members. In the Xenopus, overexpression of BMP mutants that block BMP-Smad signalling leads to neural induction, while knockdown of BMP antagonists causes loss of neural tissue (Hawley et al., 1995, Oelgeschlager et al., 2003, Kuroda et al., 2004, Khokha et al., 2005). Similarly in zebrafish, BMP or Smad mutations cause ventral defects, such as loss of the ventral tail fin, with strongly dorsalised embryos, while mutation of chordin results in neural defects (Kishimoto et al., 1997, Hild et al., 1999, Dick et al., 2000). In the mouse, combined mutation of both noggin and chordin results in severe prosencephalon defects (Bachiller et al., 2000), suggesting a vital role for BMP antagonism in forebrain development. The Xenopus Smad-interacting protein-1

(Sip1) gene induces a neural fate by repressing BMP-Smad signalling, and is thus important in neural induction (Nitta et al., 2004, van Grunsven et al., 2007, Lerchner et al., 2000). In support of this role, the knockdown of Sip-1 in zebrafish results in axial and neural patterning defects (Delalande et al., 2008). It is clear from these studies that the inhibition of BMP-Smad signalling is required for the induction of the neuroepithelium, the CNS primordium. As mentioned above, BMPs expressed in the ectoderm allow the formation of epidermal ectoderm, while BMP antagonists, such as noggin and chordin, which are expressed in the Spemann organizer, induce neural tissue formation (Fig. 3.4.2a) (Harland, 2000, Levine and Brivanlou, 2007, Rogers et al., 2009). Despite the inhibition of BMP-Smad signalling being sufficient to induce neural tissue in animal cap ectodermal explants (Hawley et al., 1995), it is not sufficient to induce neural tissue in the ventral, epidermal ectoderm of the embryo. For example, the inhibition of BMP-Smad signalling by overexpression of Smad6 in the ventral ectoderm represses the formation of epidermal ectoderm, but fails to induce the expression of neural markers (Delaune et al., 2005, Chang and Harland, 2007). However, inhibition of BMP-Smad signalling inhibition by dual suppression of Smad1 and Smad2 is sufficient to induce neural markers in the ventral ectoderm (Chang and Harland, 2007), suggesting a possible role for the inhibition of TGFβ signalling in neural induction as Smad2 is the transcriptional effector of the TGFβs. Interestingly, through working initially and mainly with chick embryos, FGF signalling has also been shown to be required for neural induction, as it sensitizes the epiblast (pre-gastrulation) to BMP antagonism by repressing BMP expression (Wilson et al., 2000, Streit et al., 2000, Delaune et al., 2005). Furthermore, a truncation of the FGF receptor has been shown to block neural induction in the Xenopus (Launay et al., 1996). FGF has been shown to positively regulate neural induction by inhibiting Smad1 transcriptional activity through phosphorylation of Smad1 in the linker region (Pera et al., 2003), an interaction that may occur at various stages throughout the development of the CNS. BMP-Smad signalling interacts with FGF and Wnt signalling in the development of a variety of neural populations, such as spinal cord (SC) neuronal populations and neural crest cells (NCCs) (LaBonne and Bronner-Fraser, 1998, Monsoro-Burq et al., 2005, Liu and Niswander, 2005), and a role for Wnt signalling in neural induction has also been suggested. Indeed, through the sequential mitogen-activated protein kinase (MAPK) and GSK3β mediated phosphorylation of the Smad1 linker region which

results in Smad1 degradation, BMP-Smad signalling integrates with FGF-MAPK and Wnt-GSK3β signalling pathways during neural development (Fuentealba et al., 2007, Eivers et al., 2008). This mechanism allows the early embryo to seamlessly integrate dorsal-ventral (BMPs) and anterior-posterior (Wnts) patterning. The Wnt family of proteins were identified when Wnt1 (originally called Int-1), was identified as a signalling molecule involved in the development of mammary tumours (Nusse and Varmus, 1982). Several years later, Wnt1 and Wingless, its Drosophila orthologue, emerged as key morphogens that regulate the embryonic body plan (Baker, 1988, McMahon and Bradley, 1990). It is now well established that Wnt proteins are important mediators of intracellular communication, and that signalling by members of the Wnt family of molecules is crucial for normal embryonic development in various systems including the nervous system where they have been shown to regulate diverse cellular processes, including cell proliferation and fate, cell polarity and movement, and programmed cell death (Ciani and Salinas, 2005).

Wnt signalling has been shown to induce neural markers and inhibit BMP-signalling in the early gastrula (Baker et al., 1999). Despite this finding, a direct role for Wnts in neural induction has yet to be shown, and the overexpression of a dominant-negative Wnt transcriptional effector (TCF3) or receptor (Fzd8) does not alter noggin induction of neural markers (Rogers et al., 2008). Alternatively, and like BMP-signalling, the inhibition of Wnt signalling may be required for neural induction, with Wnts being shown to be expressed in the ventral, but not dorsal, ectoderm in the amphibian gastrula (Christian et al., 1991). In support of this suggestion, the induction of neural markers through BMP-Smad signalling inhibition is inhibited by the overexpression of dominant active β-catenin in ectodermal explants (Heeg-Truesdell and LaBonne, 2006), and by the overexpression of Wnts in the chick embryo (Wilson et al., 2001). Interestingly, Wilson et al. (2001) suggest that this inhibition of neural induction is due to the continued Wnt signalling blocking the response of the epiblast to FGF signalling, which permits BMP-signalling to induce an epidermal fate. This is not surprising considering FGF-MAPK inhibition of BMP-Smad signalling requires its cooperative phosphorylation of the Smad1 linker region with GSK3β (Fuentealba et al., 2007), and canonical Wnt signalling inhibits GSK3β (Logan and Nusse, 2004).

Msx1 is a BMP-Smad signalling target gene (Maeda et al., 1997, Tucker et al., 1998, Ishimura et al., 2000, Yamamoto et al., 2000, Tribulo et al., 2003) and is

an important factor in the determination of epidermis versus neural tissue. The injection of Msx1 mRNA inhibits noggin-induced neurulation, while a dominant-negative form of Msx1, named as such because it is capable of inducing a partial secondary axis in ventral blastomeres (Takeda et al., 2000), directly induces the expression of the neural marker N-CAM in animal cap explants (Ishimura et al., 2000). Msx1 function is required for ventralisation by BMP-Smad signalling, as demonstrated by Msx1 knockdown (Yamamoto et al., 2000), and has been proposed to act as a mediator of BMP-Smad signalling in epidermal induction and in the inhibition of neural differentiation (Suzuki et al., 1997). In the Xenopus, a Smad-interacting protein XMAN1 induces anterior neural markers through the blockade of Msx1 expression, by inhibiting BMP-Smad signalling (Osada et al., 2003). During neural induction, genes involved in the stabilisation of a neural fate are induced in response to inhibition of BMP-Smad signalling, such as sox3 (Rogers et al., 2008), sox11 (Hyodo-Miura et al., 2002), Geminin (Kroll, 2007), and Zic3 (Nakata et al., 1998). It has been suggested that such genes achieve neural fate stabilization through the modification of signalling pathways involved in the induction of a neural fate. For example, Geminin and sox3 have both been shown to antagonize the BMP-Smad signalling pathway (Kroll et al., 1998, Rogers et al., 2008), sox11 antagonizes the Wnt signalling pathway (Hyodo-Miura et al., 2002), and FoxD5 reduces the nuclear accumulation of phosphorylated Smad 1/5/8 (Yan et al., 2009). During this process of neurulation, BMP-Smad signalling actively instructs the development of a neural population which arises at the border between the epidermis and neural plate, known as the neural crest.

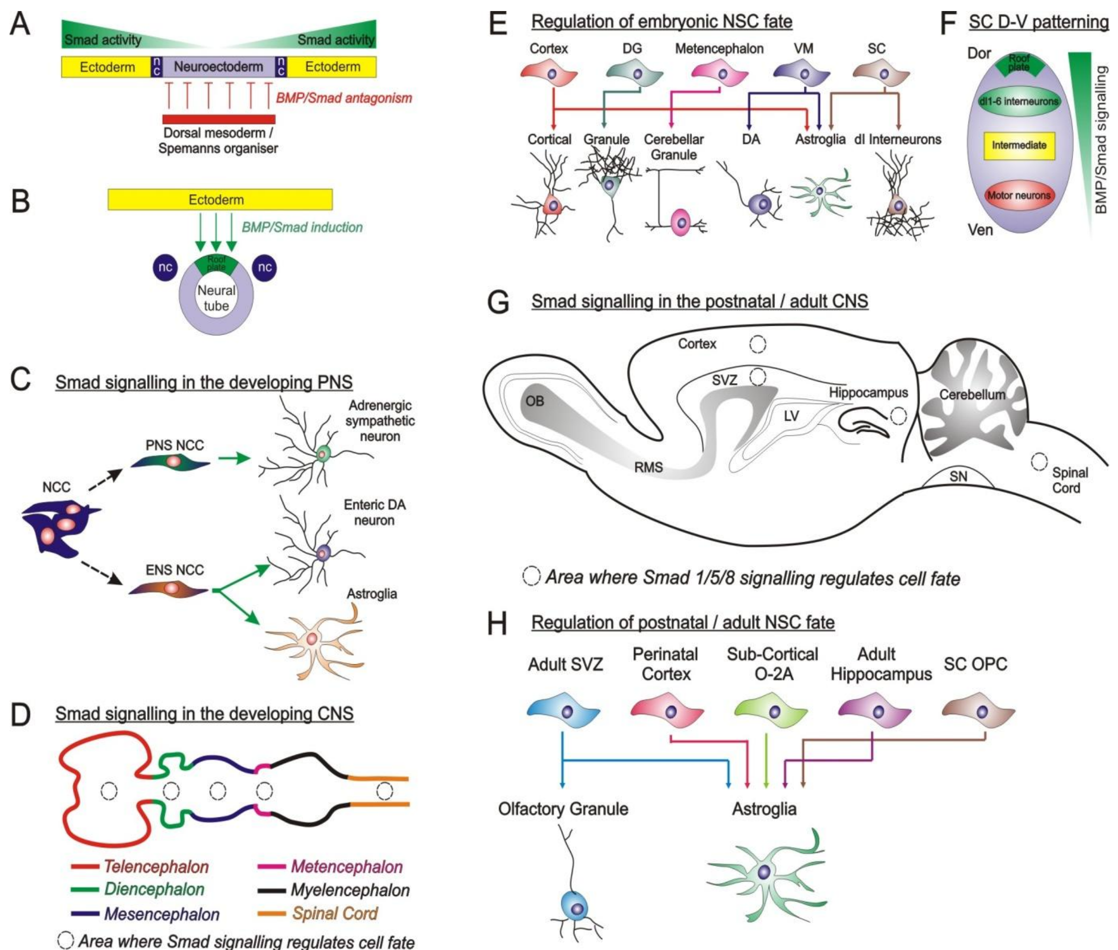


Figure 3.4.1: BMP-Smad 1/5/8 signalling in the development of the NS.

(a) BMP antagonists (red arrows) arising from Spemann's organizer inhibit BMP-Smad 1/5/8 signalling in the overlying dorsal ectoderm to induce a neural fate. An intermediate level of BMP-Smad signalling induces the formation of the neural crest at the border between the epidermis and neural plate. (b) BMP-Smad 1/5/8 signalling (green arrows) arising from the epidermal ectoderm induces the formation of the roof plate at the dorsal midline of the neural tube. Formation of the neural tube releases the NCCs. (c) PNS NCCs are induced to differentiate into adrenergic sympathetic neurons by BMP-Smad signalling (green arrow). ENS NCCs are induced to differentiate into enteric DA neuron and astroglia. (e) The neural fates (indicated by arrows; neuron unless stated), induced by Smad 1/5/8 signalling, of embryonic NSCs from different CNS regions. (f) Schematic representing the dorso-ventral patterning of the SC. The green shape represents the gradient of BMP-Smad 1/5/8 signalling strength which patterns the formation of the dorsal SC. (h) The neural fates (indicated by arrows; neuron unless stated), induced by BMP-Smad 1/5/8 signalling, of postnatal/adult NSCs from different CNS regions.

3.4.2 BMP-Smad 1/5/8 signalling in NCC development

NCCs give rise to a variety of cell populations in the PNS, as well as skeletal elements of the head (Farlie et al., 2004). BMP-Smad 1/5/8 signalling, emanating from the epidermal ectoderm and under negative regulation from the neural plate, plays an important role in the generation of NCCs (Fig. 3.4.1a). This is illustrated by the fact that active Smad 1/5/8 signalling, in response to BMPs either exogenously applied or emanating from epidermal ectoderm, is necessary for the generation of NCCs in a variety of *in vitro* and *in vivo* models (Moury and Jacobson, 1989, Moury and Jacobson, 1990, Selleck and Bronner-Fraser, 1995, Dickinson et al., 1995, Liem et al., 1995). Additionally, in the anterior ectoderm, the development of the ectodermal placodes, from the pre-placodal region between the neural plate and neural crest, requires attenuated BMP-Smad signalling (Streit, 2004, Litsiou et al., 2005). These placodes contribute to the formation of the cranial sensory NS and the special sense organs.

In terms of NCC development, the *zebrafish* BMP mutants, *swirl* (BMP2b) and *snailhouse* (BMP7), as well as the Smad 5 mutant *somitabun* (*sbn*), display alterations in neural crest formation (Nguyen et al., 2000). Specifically, trunk NCCs failed to form in these mutants, showing that BMP-Smad signalling is crucial for NCC generation. However, in an earlier study by this group the *swirl* mutant, which is the most severely dorsalised mutant of the three (discussed later) (Kishimoto et al., 1997), displayed a severe reduction in laterally-derived cranial NCCs, demonstrating that BMP2b-mediated Smad signalling is essential for cranial neural crest specification (Nguyen et al., 1998). In contrast, Nguyen et al. (1998) also showed that the *snailhouse* and *sbn* mutants exhibited an expansion of these cranial NCCs (Nguyen et al., 1998). However, the neural defects of the *snailhouse* and *sbn* hypomorphic allele mutants are more severe caudally (Nguyen et al., 1998, Mullins et al., 1996), and therefore the expression of BMP7 and Smad5 may not be diminished cranially to the same extent as it is caudally. In support of this suggestion, the addition of Smad5 morpholinos to the *sbn* mutant resulted in additional cranial defects, such as a compressed anterior/head region, which was attributed to the total loss of Smad5 activity, unlike with the hypomorphic *sbn* allele (Lele et al., 2001). Based on the opposing effects that these mutants had on cranial neural crest development, Nguyen et al. (1998) hypothesized that an intermediate level BMP-Smad signalling is involved in neural crest specification. These findings

have largely been supported by studies in mice showing that Smad4 knockdown in mouse NCCs causes the downregulation of genes critical to NCC development and results in the loss of NCC derivatives at the mid-gestational stage, coupled with alterations in cell fate specification, such as sensory neuronal fate acquisition in the trigeminal ganglia (Ko et al., 2007, Buchmann-Moller et al., 2009, Nie et al., 2008).

As aforementioned, Sip1 has been shown to induce a neural fate by repressing BMP-Smad signalling during neural induction (Nitta et al., 2004, van Grunsven et al., 2007, Lerchner et al., 2000). In zebrafish, the knockdown of two orthologues of Sip1 results in a loss of vagal/post-otic NCC derivatives due to an interference with Sip1-mediated negative regulation of BMP-Smad 1/5/8 signalling (Delalande et al., 2008). Similarly, the knockdown of Zfhx1b (the gene that encodes Sip1) in the NCCs of mice results in craniofacial and gastrointestinal malformations that resemble those found in patients with Mowat-Wilson syndrome (craniofacial dismorphology with Hirschsprung disease), which further demonstrates a role for an intermediate, regulated level of BMP-Smad signalling in NCC development (Van de Putte et al., 2007). This is not surprising considering the location at which NCCs develop, that is at the border of the BMP-Smad-induced epidermis and the BMP-antagonised neural plate. In support of Van de Putte et al. (2007), loss-of-function mutations in Sip1 deregulate BMP-Smad signalling to cause abnormal development of neural and NCC structures, resulting in some of the dysmorphic features of Hirschsprung disease, in particular defects of the enteric NS (ENS) such as aganglionosis of the distal colon (Cacheux et al., 2001, Wakamatsu et al., 2001). This finding reflects the fact that NCCs give rise to the ENS.

Studies using neural crest stem cells have shown that BMP-Smad signalling antagonizes Wnt-induced sensory neurogenesis of NCCs, while BMP-Smad signalling functions cooperatively with Wnt signalling to suppress differentiation and maintain multipotency of these stem cells (Lee et al., 2004, Kleber et al., 2005). BMP-Smad signalling interacts with Wnt and FGF signalling in the development of a variety of neural populations (LaBonne and Bronner-Fraser, 1998, Monsoro-Burq et al., 2005, Liu and Niswander, 2005). Indeed, through the sequential MAP kinase (MAPK)- and GSK3β-mediated phosphorylation of the Smad1 linker region, which results in Smad1 degradation, BMP-Smad signalling integrates with FGF-MAPK and WNT-GSK3β signalling pathways during neural development (Fuentealba et al., 2007, Eivers et al., 2008).

Collectively these data have shown that Smad-signalling is required for the NCC generation and cell fate choice during development. However, the involvement of Smad 1/5/8 in this process is often inferred given the involvement of BMPs, and an analysis of neural crest developmental and differentiation in NCC-specific Smad 1/5/8 conditional knockout mouse would be beneficial. Direct assessment of Smad 1/5/8 transcriptional activity *in vivo* is now possible using a mouse line expressing GFP under the control of a BMP-response element (BRE), thus allowing direct assessment of BMP-Smad transcriptional activity *in vivo* during NCC development (Monterio et al. 2008). Aside from NCC induction, BMP-Smad signalling also promotes neural crest migration (Sela-Donenfeld and Kalcheim, 1999), induces differentiation of adrenergic sympathetic neurons (Fig. 3.4.1c) (Varley and Maxwell, 1996) and mediates neural crest apoptosis (Graham et al., 1994). In agreement with much of these findings, intense BMP-Smad dependent transcriptional activity has been found in these regions during mouse development (Monterio et al. 2008), directly supporting a role for Smad 1/5/8 signalling in these processes.

3.4.3 BMP-Smad 1/5/8 signalling in the patterning of the dorsal SC

3.4.3.1 Dorso-ventral gradient of BMP-Smad signalling in the SC

BMPs act over the distance between the roof plate and intermediate region of the SC to pattern the dorsal SC, with the concentration of active BMP proteins (which decreases ventrally from the roof plate) being crucial for this patterning process (Liu and Niswander, 2005). Roof plate-derived BMPs achieve their inductive effects both locally, via direct cell-cell communication, and over a long-range, via BMP-binding proteins which establish diffusible BMP gradients. In Drosophila, BMPs (Dpp) interact with chordin (Sog) to form a hetero-complex which blocks BMP function (Biehs et al., 1996, Piccolo et al., 1996). BMP can be released from this complex by the action of Tolloid, a zinc metalloprotease (Marques et al., 1997). The dynamics of this complex formation and dissociation partly determines the dorso-ventral gradient of Dpp signalling, which results in a dose-dependent induction of dorsal and intermediate cell types in the neural tube (Nunes da Fonseca et al., 2010, Shimmi and O'Connor, 2003).

3.4.3.2 Patterning of the dorsal SC neuronal populations

BMP-Smad 1/5/8 signalling has been extensively studied in the patterning of the dorsal SC (Fig. 3.4.1f). The roof plate and the overlying epidermal ectoderm are rich sources of BMPs (Fig. 3.4.1b) (Liem et al., 1995, Lee et al., 1998, Lee and Jessell, 1999), and a high degree of Smad 1/5/8 transcriptional activity has been found in this region *in vivo*, in studies using mice that express GFP under the control of the BRE sequence (Monterio et al. 2008).

In the dorsal SC there are 6 discrete parallel layers of dorsal interneuronal (dI) populations, termed dI1-6 interneurons, that differentiate at progressively more ventral positions, with dI1-dI5 interneurons functioning in somatosensation, and dI6 interneurons contributing to the locomotor circuitry (Goulding, 2009). The first demonstration of the involvement of roof plate-derived BMPs in dorsal SC patterning arose when dI1A interneurons were lost in GDF7 null mice (Lee et al., 1998). Subsequently, ablation of the roof plate in mice resulted in the absence of dorsal SC interneuronal populations (Lee et al., 2000, Millonig et al., 2000), whereas addition of BMPs induced a dorsal SC cell phenotype in chick neural explants (Liem et al., 1997). These inductive effects have been shown to be dependent on both BMPRIa and BMPRIb (Timmer et al., 2002), specifically with BMPRIa promoting the proliferation of dorsal SC interneuron precursors, and BMPRIb promoting their neuronal differentiation (Panchision et al., 2001). In agreement with these findings, there was a significant loss of the most dorsal of the six interneuronal populations in BMPRIa/BMPRIb double knockout mice (Wine-Lee et al., 2004) which was also seen through forced expression of noggin, or through Smad4 knockdown in the chick embryo (Chesnutt et al., 2004). Similarly new data have shown that BMP7, Smad1 and Smad5 are all required for the generation of dI1, dI3 and dI5 interneuronal populations in mice (Le Dreau et al., 2012).

3.4.3.3 BMP-Smad and Wnt interactions in the patterning of the dorsal SC

To generate these classes of dorsal interneurons, BMP-Smad 1/5/8 cooperates with Wnt-β-catenin signalling to control the expression of Olig3, a transcription factor that is essential for the generation of dI1-dI3 interneurons (Muller et al., 2005), with BMP-Smad 1/5/8 acting upstream of Wnt-β-catenin (Zechner et al., 2007). It has been proposed that Wnts are responsible for the proliferation of BMP-specified dorsal interneuronal progenitors in the SC (Chesnutt et al. 2004), a suggestion

supported by the finding that Wnt signalling promotes progression from G_1 to S and inhibits cell cycle exit in the neural tube (Megason and McMahon, 2002). Furthermore, β-catenin knockout inhibits neural stem cell (NSC) proliferation (Zechner et al., 2003), while overexpression of constitutively-active β-catenin promotes NSC proliferation (Chenn and Walsh, 2002), in the developing mouse CNS. In addition to this, Smad6 inhibition of both BMP-Smad 1/5/8 signalling and Wnt-β-catenin signalling promotes the transition of neural progenitors from a proliferative state to a differentiating state in the chick dorsal SC (Xie et al., 2011). The other inhibitory Smad, Smad7, is expressed in newly differentiating neurons in the intermediate SC and, when ectopically expressed dorsally in the chick SC, blocks the acquisition of the dorsal interneuron dI1 and dI3 fates and results in a dorsal expansion of dI4–dI6 fates (Hazen et al., 2011). Hazen et al. (2011) showed the inhibition of BR-Smad activity by Smad7, and suggest that Smad7 functions to attenuate BMP-Smad induction of more dorsal fates to allow the generation of intermediate cell types in the SC. Interestingly, possibly contradictory to the findings of Xie et al. (2011), a model for inhibitory cross-regulation of BMP-Smad signalling and Wnt signalling was recently proposed, in which proliferation-inducing Wnt-signalling and differentiation-inducing BMP-Smad signalling inhibit one another to maintain slow-cycling, undifferentiated neural progenitors in the developing dorsal SC (Ille et al., 2007). Perhaps Smad6 functions to allow dorsal interneurons to exit this progenitor state and complete differentiation. Collectively these findings suggest that BMP-Smad signalling and Wnt-β-catenin signalling function cooperatively during dorsal spinal cord neurogenesis, with BMP-Smad important in the specification of neural fates and Wnt-β-catenin signalling functioning in appropriate proliferation of these BMP-specified dorsal interneuronal precursors. In light of contrasting suggestions of Ille et al. (2007) and Zechner et al. (2007), it remains to be determined whether their functional cooperation is mediated through a direct downstream positive regulation of one another, or indeed whether they have an inhibitory cross-regulatory relationship during SC development.

3.4.3.4 BMP-Smad signalling in the generation of intermediately-located SC neuronal populations

In zebrafish *swirl*, *snailhouse* and *sbn* mutants, there is an increase in the intermediately-located $Lim1^+$ interneurons (Nguyen et al., 2000). However, further

reductions in BMP-Smad signalling by administration of chordin to *swirl* embryos caused a decrease in these cells (Nguyen et al., 2000). These contrasting findings suggest that an intermediate level of BMP-Smad signalling is required to establish the correct number of $Lim1^{+}$ interneurons. Indeed, Chesnutt et al. (2004) have shown that BMP signalling positively regulates the expression of Wnts the dorsal SC, which is supported by the finding that double knockout of BMPR1a and BMPR1b causes a reduction in the expression of Wnts in the mouse SC (Wine-Lee et al., 2004). Perhaps the reduction in BMP-Smad signalling in the BMP mutants allows the expansion of more ventral cell types, causing an increase in $Lim1^{+}$ interneurons. However, the further reduction in BMP signalling by chordin administration may negatively affect Wnt expression, resulting in the inhibition of the proliferative effect of Wnts on SC interneurons, thus leading to a decrease in $Lim1^{+}$ interneurons. Similarly, in the dorsal SC of the chick embryo, strong activation of BMP-Smad signalling decreases the intermediately-located neurogenin 1-expressing cells (dI2), while weak activation causes the ventral expansion of neurogenin 1-expressing cells (Timmer et al., 2002). These results support the concept that distinct levels of BMP-Smad 1/5/8 signalling are required for the development of different dorsal and intermediate interneuronal populations in the dorsal SC, and that mechanisms must be in place to ensure the tight control of the levels of BMP-Smad signalling during each distinct developmental programme. Furthermore, the dorsal-ventral gradient of BMP-Smad 1/5/8 signalling in the SC is also important in the specification of ventral SC motor neurons, with its active repression being required for their induction (McMahon et al., 1998, Liem et al., 2000). In support of this, Sip1 has recently been implicated as a novel regulator of SC motor neuron diversification, with Sip1 playing an important role in visceral motor neuron differentiation (Roy et al., 2012). In addition to the roles described above, BMP-Smad signalling has been shown to play important roles in the patterning of the ventral SC, dorsal SC neuronal axonal guidance, forebrain development, and cerebellar granule neuron development (Furuta et al., 1997, Alder et al., 1999, Liu and Niswander, 2005).

Table 3.4.1: Neural fate induction by BMP-Smad signalling during development

Neural Precursor	Differentiated Cell Type	Reference(s)
Embryonic:		
PNS NCC	Adrenergic sympathetic neuron	(Varley and Maxwell, 1996, Varley et al., 1998, Reissmann et al., 1996, Wu and Howard, 2001)
ENS NCC	Enteric DA neuron	(Chalazonitis et al., 2004, Chalazonitis et al., 2008)
ENS NCC	Astroglia	(Chalazonitis et al., 2011)
VM NPs	VM DA neuron	(O'Keeffe et al., 2004a, Krieglstein et al., 1995b, Wood et al., 2005)
VM NPs	Astroglia	(O'Keeffe et al., 2004a, Krieglstein et al., 1995b, Wood et al., 2005)
Metencephalic NSC	Cerebellar granule neuron	(Alder et al., 1999, Qin et al., 2006)
Cortical NSC	Cortical neuron	(Li et al., 1998, Yung et al., 2002, Sun et al., 2010, Mehler et al., 2000)
Cortical NSC	Astroglia	(Mehler et al., 2000)
Dorsal telencephalic NSC	Dentate gyrus granule neuron	(Caronia et al., 2010)
Septal NSC	Basal forebrain cholinergic neuron	(Lopez-Coviella et al., 2000, Lopez-Coviella et al., 2005, Cho et al., 2008)
SVZ NSC	Stellate, non-neurogenic astroglia	(Bonaguidi et al., 2005)
Dorsal SC NSC	Dorsal spinal cord interneuron	(Le Dreau et al., 2012, Xie et al., 2011, Hazen et al., 2012)
Postnatal / Adult:		
Perinatal cortical NSC	Astroglia	(Mehler et al., 2000)
Postnatal subcortical O-2A progenitor cell	Astroglia	(Mabie et al., 1997)
Adult SVZ NSC	Olfactory granule neuron	(Colak et al., 2008)
Adult SVZ NSC	Astroglia	(Lim et al., 2000, Ciceroni et al., 2010, Cate et al., 2010)
Adult Hippocampal NSC	Astroglia	(Brederlau et al., 2004)
Adult SC OPC	Astroglia	(Cheng et al., 2007)

3.5 BMP-Smad 1/5/8 signalling in neuronal and glial development

3.5.1 BMP-Smad 1/5/8 signalling in neurogenesis

Smad 1/5/8 signalling in response to the BMP family of proteins is vital in several aspects of nervous system development, such as its inductive and patterning roles which have been outlined above. However, in addition to its role in regional specification, BMP-Smad signalling also has direct effects on the development of neuronal and non-neuronal cell populations from neural progenitor cells (see table 3.4.1; Fig. 3.4.1e, h).

3.5.1.1 BMP-Smad 1/5/8 signalling in neuronal development in the PNS

In the developing PNS, BMP-Smad signalling instructs neuronal differentiation from PNS NCCs via the induction of Mash1, a neuron-selective transcription factor (Groves and Anderson, 1996, Shah et al., 1996). Conversely, BMP-Smad signalling has also been shown to inhibit neuronal differentiation from CNS NSCs through the degradation of Mash1, resulting in the inhibition of neurogenesis (Shou et al., 1999). Such ambiguity may reflect intrinsic differences between PNS NCCs and CNS NSCs, with development of the former from the ectoderm requiring BMP-Smad signalling, and the generation CNS NSCs from the ectoderm being dependent upon the inhibition of BMP-Smad signalling. As mentioned in section 3.4.2, BMP-Smad signalling induces the differentiation of adrenergic sympathetic neurons from avian trunk NCCs (Fig. 3.4.1c), with the BMPRIa receptor particularly important in this process (Varley and Maxwell, 1996, Varley et al., 1998). Similarly, BMP-Smad signalling produced *in vitro* by dorsal aorta explant-derived BMPs has been shown to induce sympathetic differentiation from quail NCC cultures (Reissmann et al., 1996). The role of Mash1 in BMP-Smad-induced sympathetic neuronal differentiation was not assessed in these studies, but considering its identification in the Shah et al. (1996) study described above, it is likely to contribute to this neuronal specification. The catecholaminergic differentiation of avian NCCs was also shown to be mediated by BMP-Smad signalling *in vitro* (Wu and Howard, 2001). Wu and Howard (2001) demonstrated that Smad1 induced the expression of dHAND, a DNA binding protein required for the differentiation of catecholaminergic neurons. Collectively, these studies show that BMP-Smad signalling plays an important role in neuronal differentiation in the PNS. In addition to its inductive role in sympathetic

neuronal development, BMP-Smad signalling also promotes NGF-dependent dendritic outgrowth from sympathetic neurons (Lein et al., 1995). In the enteric nervous system, which is part of the PNS located in the wall of the gastrointestinal tract and is also derived from NCCs, BMP-Smad signalling induces the differentiation of trkC-expressing dopaminergic neurons (Fig. 3.4.1c) (Chalazonitis et al., 2004, Chalazonitis et al., 2008).

3.5.1.2 BMP-Smad 1/5/8 signalling in neuronal fate induction in the CNS

Similar to its role in the PNS, BMP-Smad 1/5/8 signalling is directly involved in the neurogenesis of various CNS neural populations. GDF5-Smad signalling induces an increase in DA neurons in E14 rat VM cultures (Fig. 3.4.1e) (Krieglstein et al., 1995b, O'Keeffe et al., 2004a, Wood et al., 2005, Clayton and Sullivan, 2007, O'Sullivan et al., 2010), while not increasing the total number of neurons (O'Keeffe et al., 2004a). Similarly, Smad 1/5/8 signalling in response to BMPs increases the numbers of DA neurons in E14 rat VM cultures (Jordan et al., 1997). This role of BMP-Smad signalling in VM DA neurogenesis is of interest to the field of Parkinson's disease research, a disorder in which VM DA neurons progressively degenerate (Lees et al., 2009, Toulouse and Sullivan, 2008), particularly for cell based therapies in which factors which promote the generation of VM DA neurons are in demand. In neural tissue cultured from E8 mouse VM/ventral metencephalon region, BMP-Smad signalling induced early markers of cerebellar granule progenitors (Fig. 3.4.1e), including Math1 and En1/En2 (Alder et al., 1999). When these Smad 1/5/8-induced cells were subsequently transplanted into the early postnatal cerebellum, they formed mature granule neurons (Alder et al., 1999). Furthermore, double knockdown of BMPRIa and BMPRIb results in a dramatic reduction in the number of cerebellar granule neurons in mice, with a concurrent downregulation of molecular markers of granule cell specification (Qin et al., 2006). BMP-Smad signalling is thus an important regulator of cerebellar granule neuron generation, with both BMPRIa and BMPRIb required for their specification. In support of a role for BMP-Smad 1/5/8 signalling in cerebellar development, the knockout of the transcription factor Zfp423 in mice, which binds to Smad1/Smad4 in response to BMP signalling (Hata et al., 2000), results in an underdeveloped cerebellum (small cerebellar hemispheres and severe reduction in vermis size) (Warming et al., 2006). In ventricular zone (VZ) neocortical neuroepithelial cell

cultures, BMP-Smad signalling was reported to induce neuronal differentiation (Fig. 3.4.1e) (Li et al., 1998). Similarly, noggin-regulated BMP-Smad signalling was shown to be involved in the elaboration of cortical GABAergic neurons from migrating ventral forebrain progenitors (Yung et al., 2002). This finding suggests that an intermediate, modulated level of BMP-Smad signalling plays a role in GABAergic neuronal differentiation in the cortex. Li et al. (1998) also showed that Smad 1/5/8 signalling in response to BMPRIa is required for neural precursors to differentiate and migrate away from the VZ in cortical explants. In support of this, BMP-Smad signalling, specifically via Smad1, has been shown to control neuronal migration and neurite outgrowth in the embryonic rodent cortex by suppressing the transcription of CRMP2, with Smad1 and Smad4 being demonstrated to bind to the CRMP2 promoter in the neocortex (Sun et al., 2010). BMPRIa-Smad1 dependent BMP signalling is therefore important for cortical neuronal migration and differentiation. In E14 murine septal cultures, BMP-Smad signalling was shown to induce both a cholinergic phenotype and the expression of a number of genes belonging to the transcriptome of basal forebrain cholinergic neurons, suggesting a role for BMP-Smad 1/5/8 signalling in the development of these neurons (Fig. 3.4.1e) (Lopez-Coviella et al., 2000, Lopez-Coviella et al., 2005). A transcriptional co-activator of BMP-Smad signalling, known as Smad-interacting zinc finger protein, was shown to be required for this BMP-Smad signalling-dependent induction of a cholinergic phenotype in E13.5 murine septal cultures (Cho et al., 2008). In mice that were deficient in BMPRIa and BMPRIb in the dorsal telencephalon, there was a decreased production of dentate gyrus (DG) granule neurons at peak DG neurogenesis and throughout life, showing a role for BMP-Smad signalling in DG granule cell neurogenesis (Fig. 3.4.1e) (Caronia et al., 2010). The resulting hippocampal defects led to fear-related behavioural deficits, demonstrating the functional importance of BMP-Smad-regulated DG neurogenesis. Using shRNA knockdown at the time of neurogenesis, Le Dreau et al. (2012) further demonstrated a role for Smad1 and Smad5 in primary neurogenesis. This study showed that BMP7-stimulated Smad1 and Smad5 signalling was required for the generation of dI1, dI3 and dI5 interneuronal populations in the chick dorsal SC (Fig. 3.4.1e, f) (Le Dreau et al., 2012). Surprisingly, Smad6 inhibition of BMP-Smad 1/5/8 signalling, and of Wnt-β-catenin signalling, was shown to promote neuronal differentiation in the intermediate zone of the chick dorsal SC (Xie et al., 2011). This result does not

preclude an involvement of BMP-Smad and Wnt-β-catenin signalling in the neuronal differentiation of dorsal SC neurons, but rather reflects a role for Smad6 in directing BMP-specified NPs to exit the cell cycle and terminally differentiate.

In addition to a neuronal inductive role in the dorsal SC, Smad1-dependent BMP signalling has been shown to govern axonal growth in the dorsal root ganglion (DRG), with the reactivation of Smad1 signalling in adult DRG resulting in sensory axon regeneration in a mouse model of SC injury (Parikh et al., 2011). In support of this, Smad6 has been shown to potently block dI1 axon outgrowth in the chick SC (Hazen et al., 2011). Furthermore, BMP-Smad signalling in the dorsal SC acts as a chemorepellent that orients the commissural axons of dI1 interneurons so that they grow ventrally (Butler and Dodd, 2003, Dent et al., 2011), and also acts to regulate the growth rate of these axons as they extend through the SC (Phan et al., 2010). This chemorepellent role of BMP-Smad signalling was recently demonstrated to be mediated by the BMPRIb (Yamauchi et al., 2008). A more recent paper by Hazen et al. has suggested that Smad1 and Smad5 confer diverse functions during the development of the dorsal SC. Knockdown experiments demonstrated that Smad1 is critical for the regulation of dI1 axonal growth while Smad5 is required for the specification of dI1 and dI3 interneuronal populations (Hazen et al., 2012). This is an important finding as it suggests that the various BR-Smads have distinct functions in the developing SC, whereas previous studies suggested that these BR-Smads function redundantly during the development of the nervous system (Arnold et al., 2006, Le Dreau et al., 2012). Parikh et al. (2011) also showed that inhibition of BMP-Smad 1/5/8 signalling using dorsomorphin, a small molecular inhibitor of BMPRI (Yu et al., 2008), negatively affects neurite outgrowth of E18.5 mouse hippocampal neurons. Perhaps this role of BMP-Smad signalling in the establishment of hippocampal neuronal projections contributes to the fear-related behavioural deficits caused by BMPRIa and BMPRIb conditional double mutation outlined above. Additionally, BMP-Smad signalling has been shown to induce neurite outgrowth from E14 rat VM DA neurons *in vitro* (O'Keeffe et al., 2004b, Reiriz et al., 1999), although the direct involvement of Smad 1/5/8 in this process remains to be demonstrated. BMP-Smad signalling therefore not only regulates neuronal specification, but also promotes neuronal differentiation and maturation in several regions of the embryonic CNS.

Furthermore, BMP-Smad signalling is actively involved in neuronal differentiation during post-natal development. BMP-Smad signalling is required for the initiation of neurogenesis in adult mouse subventricular zone (SVZ) NSCs (Fig. 3.4.1h) and the concurrent suppression of an oligodendroglial fate, since Smad4 knockdown or noggin infusion results in severe impairment of neurogenesis and subsequent differentiation of oligodendrocytes (Colak et al., 2008). Indeed, these effects could be contributed to altered TGFβ signalling, as Smad4 also mediates this pathway. However, Colak et al. (2008) showed that this Smad4-mediated neurogenic role is BMP-specific through the knockdown of the TGFβ type II receptor, in addition to the above mentioned demonstration that blockade of BMP signalling through noggin infusion phenocopied the Smad4-related defects. Conversely, it was recently shown that LRP2-mediated catabolism of BMP4 is required for neurogenesis in the adult mouse, since increases in BMP-Smad 1/5/8 signalling as a result of LRP2 knockdown coincides with reduced neurogenesis (Gajera et al., 2010). Similarly, increased BMP signalling was shown to potently inhibit neurogenesis of adult mouse SVZ NSCs *in vitro* and *in vivo*, while noggin promoted neurogenesis (Lim et al., 2000). These contradicting results may reflect a potential need for a regulated, intermediate level of BMP-Smad signalling to allow adult SVZ neurogenesis. Furthermore, BMP-Smad signalling regulators, such as noggin and LRP2, may indeed be involved in refining Smad 1/5/8 signalling to promote/allow neurogenesis. Indeed, noggin-regulated BMP-Smad signalling plays a role in embryonic cortical GABAergic neurogenesis (Yung et al., 2002), while regulated levels of BMP-Smad 1/5/8 signalling is required for the appropriate generation of intermediately-located interneuronal populations in the development of the SC (Timmer et al., 2002, Nguyen et al., 2000).

3.5.1.3 BMP-Smad 1/5/8 signalling in the neuronal differentiation of neural cell lines

In agreement with their roles in inducing neuronal differentiation in the PNS, BMP-Smad signalling has also been shown to induce neuronal differentiation in a sympathetic nervous system-derived cell line, known as the PC-12 cell line (Paralkar et al., 1992). Conversely, in a sympathoadrenal progenitor cell line, BMP-Smad signalling was shown to induce apoptosis, which could be rescued by the addition of growth factors such as NGF (Song et al., 1998). BMP-Smad signalling thus induces

dependence on exogenous growth factors for survival in these cells. This finding is surprising, considering the well established neurotrophic properties of BMPs, however it may suggest a role for BMP-Smad signalling in sympathetic target innervation. In the human neuroblastoma SH-SY5Y cell line, BMP signalling induces neuronal differentiation through a BMPRI-Smad 1/5/8 mediated pathway (Toulouse et al., 2012, Nakamura et al., 2003, Hegarty et al., 2013b). Likewise, in the mouse neuroblastoma-derived cell line, Neuro2a, BMP-stimulated phospho-Smad 1/5/8 nuclear translocation induces neuronal differentiation through a bi-phasic regulation of Id protein expression, and subsequent upregulation of the neural-specific transcriptional factors Dlx2, Brn3a, and NeuroD6 (Du and Yip, 2010). The use of noggin or Smad1 siRNA prevented this Smad-mediated regulation of Id protein expression. Thus, BMP-Smad signalling is also capable of inducing neuronal differentiation in neural cell lines.

3.5.2 BMP-Smad 1/5/8 signalling in gliogenesis

Despite its role in promoting neuronal differentiation from various neural precursor populations, Smad 1/5/8 signalling has also been shown by many studies to induce an astrocytic lineage. For example, in serum-free mouse embryonic cell cultures, BMP-Smad signalling induces the generation of glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes, while concomitantly inhibiting cellular proliferation (D'Alessandro and Wang, 1994a, D'Alessandro et al., 1994b). Similarly, in embryonic mouse SVZ multipotent progenitors, BMP-Smad 1/5/8 signalling induces the generation of GFAP-expressing astrocytes (Fig. 2e), with concurrent suppression of neuronal and oligodendroglial cell fates (Gross et al., 1996). Knowledge on the astroglial inductive effect of BMP-Smad signalling in the embryonic SVZ was recently refined to show that a particular subtype of GFAP-expressing cells are generated in response to Smad 1/5/8 activation. Specifically, stellate, post-mitotic, non-neurogenic GFAP-expressing cells, representative of mature astrocytes, are generated from mouse embryonic SVZ progenitor cells in response to BMP-Smad signalling. Conversely, leukemia inhibitory factor (LIF) signalling gives rise to bipolar/tripolar, self-renewing, neurogenic GFAP-expressing progenitors, representative of radial glial SVZ stem cells (Bonaguidi et al., 2005). However, these studies did not assess Smad activity, and thus further characterisation experiments would be required to conclusively attribute these effects

to the canonical Smad 1/5/8 signalling pathway. In a more recent study on E14 dorsal SC cultures, the prevention of BMP-induced Smad1 transcriptional activity by FGF-MAPK signalling promoted OPC generation from NSCs through the upregulation of olig2, with Smad1/Smad4 being shown to associate with the olig2 promoter (Bilican et al., 2008). This interaction of the Smad1/Smad4 complex with the olig2 promoter may therefore result in transcriptional repression. Despite inducing a neuronal lineage in NCCs, BMP-promoted nuclear translocation of phospho-Smad 1/5/8 induced glial differentiation in the NCC population that gives rise to the ENS (Fig. 3.4.1c) (Chalazonitis et al., 2011). As mentioned in section 3.5.1.1, BMP-Smad signalling is known to induce DA neurons from these NCCs (Chalazonitis et al., 2004, Chalazonitis et al., 2008), however it has been proposed that BMP-Smad signalling is accountable for the responsiveness of these enteric NCCs firstly to GDNF-induced neurogenesis, and later to glial growth factor (GGF)-2-induced gliogenesis (Chalazonitis et al., 2011, Chalazonitis and Kessler, 2012). Similarly, GDF5-Smad signalling increases the number of astrocytes in E14 rat VM cultures (Fig. 3.4.1e), while concomitantly increasing the numbers of DA neurons (Krieglstein et al., 1995b, O'Keeffe et al., 2004a, Wood et al., 2005). Despite inherent differences between CNS NSCs and PNS NCCs, perhaps there is a similar mechanism, to that proposed by Chalazonitis and colleagues for enteric NCCs, to explain the dual inductive role of GDF5-Smad signalling in E14 rat VM NSCs. In support of such a suggestion, GDNF is an important neurotrophic factor for the DA neurons of the VM, and may thus work cooperatively with BMPs in this population also (Peterson and Nutt, 2008, Toulouse and Sullivan, 2008).

In addition to its glial-inducing effects on embryonic pluripotent progenitors, BMP-Smad 1/5/8 signalling has been demonstrated to play a direct role in adult gliogenesis. BMP-Smad signalling induces postnatal subcortical bipotent oligodendroglial-astroglial (O-2A) progenitor cells to differentiate into astrocytes (Fig. 3.4.1h), while concomitantly suppressing oligodendroglial differentiation, in a dose-dependent manner (Mabie et al., 1997). Although the presence of type I and type II BMPRs on these cells was demonstrated, the activation of canonical Smad 1/5/8 pathway was not confirmed in this study (Mabie et al., 1997). The response of neural progenitors to BMP-Smad signalling appears to be temporally dependent. For example, cultures of cortical VZ neural progenitors have been shown to respond differentially to BMP-Smad signalling, depending on their ontogenic stage. At E13,

BMP-Smad signalling promotes cell death and inhibits proliferation of early VZ progenitors, while at E16, the response to BMP signalling is concentration-dependent with either enhancement of neuronal and astroglial elaboration (at 1-10ng/ml) or potentiation of cell death (at 100ng/ml) (Mehler et al., 2000). It would be interesting to determine whether activation of different signalling pathways contributes to these divergent effects. Indeed, particularly high BMP levels may act via pathways that are independent of Smad 1/5/8 (Rajan et al., 2003, Nohe et al., 2004). Mehler et al. (2000) showed that BMP-Smad signalling enhances the generation of astroglia during the perinatal period of cortical gliogenesis (Fig. 3.4.1h). Interestingly, the inhibitory effect of BMP-Smad signalling on oligodendroglial generation occured at all stages (Mehler et al., 2000). Similarly in adult rat SC oligodendrocyte precursor cell (OPC) cultures, shown to express the BMPRs, BMP-Smad signalling promotes astrocytic differentiation in a dose-dependent manner (Fig. 3.4.1h), with concurrent suppression of oligodendrocyte differentiation, by increasing Id4 expression and decreasing the expression of olig1 and olig2 (Cheng et al., 2007). In support of this finding, the Smad1/Smad4 complex has been suggested to interact with the olig2 promoter to repress olig2 expression (Bilican et al., 2008). A recent paper has identified Sip1 as an important regulator of oligodendrocyte differentiation and myelination. Sip1 represses BMP-Smad signalling, via a dual mechanism involving direct antagonism of Smad 1/5/8 and induction of Smad7 expression, to promote oligodendrocyte differentiation in the CNS (Weng et al., 2012). Cheng et al. (2007) propose that the blocking of BMP-Smad signalling combined with olig1/2 overexpression could potentially enhance endogenous remyelination in CNS demyelination disorders. In support of this suggestion, local increases in BMPs at the site of demyelination upregulates gliosis, with these astrocytes displaying increased phospho-Smad 1/5/8 signalling (Fuller et al., 2007). Furthermore, BMP-induced increases in the expression of chondroitin sulphate proteoglycans, such as neurocan and aggrecan, were also observed which could inhibit remyelination (Fuller et al., 2007). However, astrocytes generated in response to BMP-Smad signalling support axonal growth and regeneration of host sensory neurons when transplanted into a lesioned dorsal column of the SC (Haas et al., 2012). Cultured adult hippocampal NSCs were shown to be induced to an astroglial lineage in response to BMP signalling through the BMPRIb receptor (Fig. 3.4.1h) (Brederlau et al., 2004). In addition to this, a recent study modulated BMP-

Smad signalling to alter the numbers of astrocytes and oligodendrocytes in the adult SVZ during cuprizone-induced demyelination (Cate et al., 2010). Components of the BMP-signalling pathway, including BMPRs and Smad 1/5/8, were shown to be up-regulated during cuprizone-induced demyelination, along with an increase in astrocytes *in vivo*. The reduction of Smad 1/5/8 activation by intraventricular infusion of noggin resulted in a decrease in the numbers of astrocytes, and an increase in the number of oligodendrocytes in the adult SVZ during cuprizone-induced demyelination (Cate et al., 2010). Similarly, activation of the mGlu3 receptors in post-natal mouse SVZ NSCs attenuates the astroglial-promoting effect of phosphorylated Smad 1/5/8, via the mitogen-activated protein kinase pathway, which suggests a role for glutamate in SVZ NSC differentiation (Ciceroni et al., 2010). This further demonstrates the negative regulation of phospho-Smad 1/5/8 signalling by the MAPK pathway. In support of the role of BMP-Smad signalling in adult SVZ astrogliogenesis, Lim et al. (2000) also showed that BMP signalling induces an astroglial lineage in adult SVZ NSCs. Furthermore, it has been shown that mouse brain endothelial cells, which lie close to adult SVZ NSC, are the source of BMP-Smad signalling which regulates the development of these NSCs (Mathieu et al., 2008). The transgenic overexpression of BMP4 in mice resulted in an increase in astrocytes in multiple brain regions, with a concurrent decrease in oligodendrocytes, suggesting that BMP-Smad signalling is a likely mediator of astrocyte development *in vivo* (Gomes et al., 2003). The adult SVZ is now widely accepted to be a major site of neurogenesis (Tarabykin et al., 2001, Nieto et al., 2004, Noctor et al., 2004, Zecevic et al., 2005, Pontious et al., 2008). However, the effect of BMP-Smad signalling on SVZ neurogenesis remains unclear. The majority of evidence suggests that Smad 1/5/8 signalling results in astroglial differentiation, however Colak et al. (2008) showed that BMP-Smad signalling was required to initiate the neurogenic lineage in the adult SVZ. These results may not be as contradictory as they appear, and it may well be the case that both findings are directly related and even support one another. Perhaps in adult SVZ NSCs, BMP-Smad signalling acts to induce radial glial-like neuronal progenitors, with astrocytic characteristics, that subsequently generate the granule neurons which migrate to the olfactory bulb. The role of such 'astroglial' progenitors in neurogenesis has been well described in a recent review (Kriegstein and Alvarez-Buylla, 2009).

3.5.3 BMP-Smad 1/5/8 signalling in neural stem/progenitor cells

It is clear that BMP-Smad 1/5/8 signalling can induce a neuronal or astrocytic fate, or possibly even both, in neural precursors, but the factors which determine each of these fates are unknown. In the chick SC, BMP-Smad signalling, possibly mediated via the BMPRIb receptor, promotes neuronal specification rather than astrocytic specification in the dorsal-most progenitors at E5 (Agius et al., 2010). However, this study reported the opposite effect at E6, when BMP-Smad signalling promoted astrocyte development (Fig. 3.4.1e), rather than completely preventing it as it did at E5. This complete reversal of the neurogenic action was suggested to be due to an upregulation of BMPRIa receptors (Agius et al., 2010). It is thus likely that these differential inductive effects of Smad 1/5/8 signalling not only depend on the specific ligand-receptor combination, but are also temporally dependent, an example of which can be seen in the Mehler at al. (2000) paper discussed above. Furthermore, in the hippocampus, BMP-Smad signalling induces granule neuron generation pre-natally (Caronia et al., 2010), and astroglial differentiation during adulthood (Brederlau et al., 2004). This is not surprising, considering that primary neurogenesis precedes gliogenesis during CNS development. Logically, cell identity is a determining factor, with PNS NCCs being induced towards a neuronal fate and CNS embryonic SVZ precursors being induced to an astrocytic one during pre-natal nervous system development.

It is important to note that through the induction of differentiation, Smad 1/5/8 signalling negatively regulates the proliferation of NSCs. In rat NSC cultures derived from either the cortex or SC at E13.5 BMPs induced NSC growth arrest and GFAP expression through Smad signalling, however FGF2 prevented BMP-Smad-induced terminal astrocytic differentiation to preserve NSC potency in a dormant state (retain nestin expression but do not proliferate) (Sun et al., 2011). This effect is likely to reflect FGF-MAPK-induced repression of Smad 1/5/8 signalling. Similarly, BMP-Smad signalling negatively regulates NSC proliferation in the adult hippocampus (Bonaguidi et al., 2008). Smad 1/5/8 signalling in response to BMPRIa receptor activation by BMPs decreased proliferation of cultured adult hippocampal NSCs, while maintaining them in an undifferentiated state (Mira et al., 2010). Mira and co-workers also showed that knockout of either Smad4 or BMPRIa in hippocampal NSCs results in a transient increase in proliferation, followed by a reduction in the generation of neural precursors, demonstrating that Smad 1/5/8

signalling regulates NSC quiescence/proliferation and prevents the loss of NSC activity, which supports continuous neurogenesis, in the adult hippocampus. Taken together with the results of the Sun et al. study, the above findings support a role for Smad 1/5/8 signalling in the regulation of NSC proliferation and differentiation. Interestingly, this regulation of NSC proliferation in the hippocampus by BMP-Smad signalling has been suggested to be pertinent to the effects of exercise on adult hippocampal neurogenesis in mice, that is, exercise induced downregulation of BMP-Smad signalling results in enhanced neurogenesis (Gobeske et al., 2009). The Mira et al. study (2010) also supports the suggestion that BMP-Smad signalling induces the differentiation of adult SVZ NSCs into radial glial progenitors, which subsequently give rise to olfactory interneurons.

In mouse embryonic stem cells, BMP-Smad signalling promotes the self-renewal, and inhibits the differentiation through the induction of Id proteins (Ying et al., 2003a). This result is interesting considering the effects of BMP-Smad signalling to promote differentiation of NSCs. These contrasting results are likely to reflect the difference between embryonic stem cells and stem cells that are restricted (through inhibition of BMP-Smad signalling) to a neural fate.

3.6 Smad 1/5/8 signalling knockdown during nervous system development

The knockdown of the components of the Smad signalling pathway have given insights into their roles during the development of the NS (see table 3.6.1). It has proven difficult to determine the effects of global deletion of Smad 1/5/8 on nervous system development, as the Smad1 mutant mice die at E10.5 due to defects in allantois formation (Lechleider et al., 2001, Tremblay et al., 2001), and Smad5 mutant mice die at E10.5 due to angiogenic failure and other defects (Chang et al., 1999, Yang et al., 1999). Chang el al. (1999) did show a failure of cranial neural tube closure in Smad5 mutant mice at E9.5, which resulted in exencephaly, demonstrating the importance of Smad5-mediated signalling in cranial neural tube development. Heterozygous Smad1 mutant mice, and mice homozygous for a hypomorphic allele of Smad8, show midbrain and hindbrain reductions (gross reduction in anatomical size) at E11.5 (Hester et al., 2005). This is not surprising due to the role of BMP-Smad 1/5/8 signalling in cerebellar development (discussed above). However in

contrast to this, Smad8 null mutant mice are viable and fertile, with no discernible abnormalities (Arnold et al., 2006). Thus, Smad8 seems to play a nonessential role in development (mice develop without defects), and so the abnormalities reported by Hester et al. (2005) may be due to the neomycin protein affecting expression of neighbouring genes. It is more likely that the role of Smad8 is redundant; further investigation is required to determine the specific roles of Smad8 in development. When Arnold et al. (2006) crossed Smad8 mutant mice with heterozygous or homozygous Smad1 or Smad5 null alleles, there were no noticeable developmental disturbances in the Smad8$^{-/-}$:Smad1/5$^{+/-}$ mice, and the abnormalities associated with Smad1/Smad5 null mice were not exacerbated by the absence of Smad8. However, Smad1 and Smad5 double heterozygous mutant mice displayed striking phenotypic similarity to Smad1 and Smad5 mutant embryos and died at E10.5, suggesting that Smad1 and Smad5 function cooperatively in response to BMPs during development (Arnold et al., 2006). Indeed, although not related to nervous system development, Smad1 has been suggested to compensate for Smad5 loss in the angiogenic endothelium (Umans et al., 2007), while a triple conditional knockout study in mice reported that Smad1, Smad5 and Smad8 function redundantly in Mullerian duct regression (Orvis et al., 2008). Despite the limited information ascertained from studies involving complete knockdown of Smad1 and Smad5 in mice (due to mid-gestation embryonic lethality), the *sbn* mutant zebrafish allows the identification of severe neurodevelopmental defects caused by null mutations of Smad5 (Hild et al., 1999). The *sbn* mutant embryos are strongly dorsalised, displaying a complete loss of ventral and posterior structures, as well as a ventral expansion of dorsal structures such as the neuroectoderm and somites. This dorsalisation demonstrates the importance of BMP-Smad 1/5/8 signalling for appropriate development of the nervous system. In support of this role, these neurodevelopmental defects of the *sbn* mutant can be largely rescued by overexpression of BMPs, a constitutively active form of the BMPRIb, or Smad1 (Nguyen et al., 1998). The somites of *sbn* mutants do not completely circulise, as the *swirl* (BMP) mutant embryo does (Kishimoto et al., 1997). Furthermore, *sbn* mutants survive segmentation unlike the slightly more severely dorsalised *swirl* mutant. At a later stage, the *sbn* embryo is characterised by loss of the tail and a ‘snailshell-like’ winding up of the trunk (Lele et al., 2001). Lele et al. (2001) produced a more strongly dorsalised zebrafish mutant embryo following further knockdown of Smad5 using morpholino injection. These embryos displayed

additional features, such as a compressed anterior/head region, which was attributed to the total loss of Smad5 activity, unlike with the hypomorphic *sbn* allele. This finding is consistent with the cranial defects of Smad5 mutant mice described by Chang et al. (1999). The Smad 5 *sbn* mutant also displays NCC deficits, such as diminished trunk NCC formation, showing that BMP-activated Smad5 signalling is crucial for NCC generation (Nguyen et al., 2000). The conditional knockout of Smad1 and Smad5 in the mouse neural tube, demonstrated that these BR-Smads have distinct functions in the developing SC with Smad1 critical for the regulation of dI1 axonal growth and Smad5 required for the specification of dI1 and dI3 interneuronal populations (Hazen et al., 2012). This finding is surprising considering the wealth of evidence that suggests functional redundancy amoung Smad 1, 5 and 8 (Le Dreau et al., 2012, Arnold et al., 2006, Umans et al., 2007, Orvis et al., 2008). The conditional knockout of Smad4 in mouse NCC leads to the downregulation of genes critical for NCC development, such as the BMP target gene Msx1, and results in defective NCC derivatives which lead to mid-gestation death (Nie et al., 2008). In a similar study that focused on cranial NCC development, the conditional knockdown of Smad4 in NCC showed that BMP-Smad signalling is required for the fate specification of cranial NCC (Ko et al., 2007). It is important to note that the defects observed in these Smad4 mutants may be as a result of altered TGFβ signalling, and thus these studies demonstrate the importance of Smad4 in mediating the activities of BMPs, and/or TGFβs, in NCC development.

Due to the difficulty in generating viable Smad1/5/8 mutants, a number of studies have used RNA interference (RNAi) to determine the effects of the loss of Smad 1/5/8 signalling on the development of the nervous system, specifically in relation to the development of the dorsal SC. The knockdown of Smad1 in E12.5 DRG neurons by siRNA results in the inhibition of axonal growth capacity in these neurons, an effect which was rescued by an RNAi-resistant Smad1 construct (Parikh et al., 2011). Furthermore, cultured DRG neurons from Smad1 conditional knockout mice have a markedly decreased capacity to initiate or maintain axon extension (Parikh et al., 2011). A similar study that used RNAi to knockdown Smad1, and subsequently performed rescue experiments using an RNAi-resistant Smad1 construct, showed that Smad1 is required for axonal growth of cultured adult DRG neurons (Zou et al., 2009a). These studies demonstrate the importance of Smad1 in DRG neuronal differentiation, specifically axonal outgrowth. In contrast to the

Hazen et al. (2012) study mentioned above, *in vivo* shRNA knockdown experiments showed that BMP7, Smad1 and Smad5 are all required for the generation of dI1, dI3 and dI5 interneuronal populations in the chick (Le Dreau et al., 2012). Smad8 has also been shown to be required for the generation of dI1 interneurons, suggesting that Smad8 may not be completely functionally redundant during NS development (Le Dreau et al., 2012). Furthermore, in support of the idea of functional redundancy between Smad1 and Smad5, overexpression of a pseudo-phosphorylated mutant version of Smad1 rescued Smad5 loss of function, while pseudo-phosphorylated Smad5 overexpression rescued the Smad1 shRNA phenotype (Le Dreau et al., 2012). In light of the conflicting reports discussed in this review, whether Smad1 and Smad5 function redundantly or have unique but complementary roles during spinal cord neurogenesis will be an interesting question for future research, as well as their mechanisms of action during this process. The inhibition of BMP-Smad 1/5/8 signalling in the dorsal SC by reducing the expression of Smad4 using siRNA, results in the loss of most of the dI1 interneuronal population, and the dorsal expansion of dI2-4 interneurons in the chick embryo (Chesnutt et al., 2004). These studies demonstrate that Smad1, Smad4 and Smad5 are essential for the generation of the dorsal SC interneuronal populations. The Hazen et al. (2012) study suggests that Smad1 is not required, however these contradictory results may reflect subtle differences between mouse and chick SC patterning. Indeed, the disruption of Smad1 and/or Smad5 expression in the chick dorsal SC by RNAi showed that Smad1 can partially compensate for the loss of Smad5 in the chick (Hazen et al., 2012).

The knockdown studies discussed above provide further evidence for the importance of BMP-Smad 1/5/8 signalling in neural induction, neural crest development and dorsal SC development. Future studies involving the conditional knockout of Smad1, Smad5 and/or Smad8 or the use of RNAi for these BR-Smads in the developing nervous system, followed by phenotypic analysis of various regions of the CNS and PNS will provide further information regarding the roles of Smad 1/5/8 signalling in nervous system development. Despite the fact that the Smad 1/5/8 signalling pathway is the canonical signalling pathway of the BMP family, it cannot be assumed that the effects which BMPs exert on neural cells are mediated by these transcription factors. Knockout studies similar to the ones described above should be conducted, to conclusively verify the involvement of Smads 1/5/8 in mediating these effects.

Table 3.6.1: Effects of Smad 1/5/8 knockdown on nervous system development

Mutation(s)	Effect(s) on NS development	Reference(s)
Smad1:		
Smad1 +/-	midbrain/hindbrain reductions (anatomical size)	(Hester et al., 2005)
Smad1$^{flox/-}$; Wnt1 Cre	decreased axonal growth of cultured adult DRG from these conditional mutant mice	(Parikh et al., 2011)
Brn4::Cre; Smad1$^{flox/flox}$	reduction in axonal growth of dI1 interneurons of the dorsal spinal cord	(Hazen et al., 2012)
Smad1 siRNA	inhibition of the axonal growth of cultured adult DRG neurons	(Zou et al., 2009a)
Smad1 siRNA	inhibition of the axonal growth of cultured E12.5 DRG neurons	(Parikh et al., 2011)
Smad1 shRNA	reduced generation of dI1,dI3 and dI5 interneurons of the dorsal spinal cord	(Le Dreau et al., 2012)
Smad5:		
Smad5 -/-	exencephaly (failure cranial neural tube closure)	(Chang et al., 1999)
Smad5 -/- (*sbn* mutant)	ventral expansion of neuroectoderm and somites	(Lele et al., 2001)
Smad5 -/- (*sbn*)	trunk NCC deficiencies	(Nguyen et al., 2000)
Brn4::Cre; Smad5$^{flox/flox}$	reduction in dI1 and dI3 interneurons	(Hazen et al., 2012)
Smad5 shRNA	reduction in dI1,dI3, dI5 dorsal SC interneurons	(Le Dreau et al., 2012)
Smad8:		
Smad8 -/- (hypomorphic)	midbrain/hindbrain reductions (anatomical size)	(Hester et al., 2005)
Smad8 shRNA	reduced generation of dI1 interneurons of the dorsal spinal cord	(Le Dreau et al., 2012)
Smad4:		
Wnt1-Cre; Smad4	defective cranial NCC derivatives	(Ko et al., 2007)
Wnt1-Cre; Smad4$^{loxp/loxp}$	defective NCC derivatives	(Nie et al., 2008)
Smad4 siRNA	reduced generation of dI1, and dorsal expansion of dI2-4, dorsal SC interneurons	(Chesnutt et al., 2004)

3.7 The TGFβ superfamily in midbrain DA neuronal development

BMPs constitute the largest subfamily of the TGFβ superfamily, which also include the TGFβs, activin, GDNF, and GDF subfamilies (Bottner et al., 2000, Sullivan and O'Keeffe, 2005). As aforementioned for BMPs, TGFβ superfamily members are dimeric, structurally-conserved proteins, that have pleiotropic functions in vitro and in vivo (Massague and Wotton, 2000). The TGFβs elicit their cellular responses in a similar fashion to that described for BMPs in section 3.3, with TGFβs signalling via the type I receptors, ALK1, ALK2, ALK4 and ALK5, and the R-Smads, Smad2 and Smad3 (Nohe et al., 2004, Bragdon et al., 2011, Sieber et al., 2009, Miyazono et al., 2001).

There is a wealth of evidence suggesting that TGFβ superfamily members regulate midbrain DA neuronal development. However, the majority of this research has focused on their survival-promoting abilities, rather than potential roles in development, with the goal of using these factors therapeutically for PD. Such research is best highlighted by that carried out on the GDNF family (see table 3.7.1), two of which have been used in clinical trials (Peterson and Nutt, 2008, Sullivan and Toulouse, 2011), and this TGFβ subfamily will therefore be discussed first in this review.

3.7.1 The GDNF family

The GDNF family is composed of four members – GDNF, neurturin, persephin and artemin. GDNF, the prototypical member of this subfamily, was isolated from a glial cell line following the demonstration of its neurotrophic effects on cultured DA neurons (Lin et al., 1993). GDNF has consistently been shown to promote the survival, and reduce apoptosis, of cultured embryonic VM DA neurons (Lin et al., 1993, Krieglstein et al., 1995a, Widmer et al., 2000, Clarkson et al., 1995, Clarkson et al., 1997, Sawada et al., 2000). A vitamin D3 metabolite, calcitriol, has recently been shown to promote the survival of midbrain DA neurons *in vitro* through the upregulation of endogenous GDNF (Orme et al., 2013). Interestingly, a single dose of GDNF selectively enhances the survival of A9 DA neurons, while only repeated exposure of this factor increases the survival of A10 cells in E14 VM cultures (Borgal et al., 2007), suggesting that nigrostriatal DA neurons are more sensitive to the effects of GDNF. GDNF has consistently been shown to improve the survival of

embryonic DA neurons in VM transplants to the adult rodent striatum (Apostolides et al., 1998, Espejo et al., 2000, Granholm et al., 1997, Yurek, 1998, Sullivan et al., 1998a). Of more physiological relevance, GDNF has also been shown to inhibit the apoptotic death of postnatal midbrain DA neurons *in vitro* (Burke, 2003). Furthermore, two *in vivo* studies demonstrated that GDNF functions to promote the survival of midbrain DA neurons during their period of naturally-occurring cell death (Kholodilov et al., 2004, Granholm et al., 2000). These findings suggest that GDNF functions to regulate the survival of VM DA neurons during their development, particularly as a target-derived neurotrophic factor. In support of such a suggestion, GDNF is expressed in the developing and adult rat midbrain and striatum (Choi-Lundberg and Bohn, 1995, Gavin et al., 2013), which indicates functioning by endogenous GDNF. Interestingly, the activation of the dopamine D2 receptor has recently been shown to upregulate GDNF expression in the postnatal rat midbrain (Ahmadiantehrani and Ron, 2013), suggesting that activity-dependent GDNF neurotrophic support is important during nigrostriatal pathway development. GDNF has been shown to protect VM DA neurons from the DA toxins, 1-methyl-4-phenylpyridinium ion (MPP+) and 6-hydroxydopamine (6-OHDA), *in vitro* (Eggert et al., 1999, Hou et al., 1996), and in animal models of PD (Tomac et al., 1995, Gash et al., 1996, Connor et al., 2001, Kozlowski et al., 2000, Date et al., 1998, Kordower et al., 2000). Interestingly, the survival-promoting effect of GDNF in these models is significantly greater in younger aged rats (early post-natal) (Fox et al., 2001), with the greatest effect seen in 3-month-old rats at a time when the nigrostriatal pathway is still developing (Van den Heuvel and Pasterkamp, 2008). These findings led to clinical trials which delivered GDNF into the striatum of PD patients, but these had varying degrees of success (Gill et al., 2003, Slevin et al., 2005, Lang et al., 2006). A more developmentally-relevant protective role of GDNF was recently demonstrated, in a study showing that GDNF protected cultured VM DA neurons from lipopolysaccharide-induced degeneration, a model of neuroinflammation (Xing et al., 2010). This suggests that GDNF maintains DA neuronal integrity in occasions of increased brain inflammation. Furthermore, inflammation has consistently been suggested to contribute to the pathogenesis of PD (Collins et al., 2012, Nolan et al., 2013, Hirsch et al., 2012).

There is evidence to suggest that GDNF may play a role in inducing a DA phenotype during midbrain DA neurogenesis. A recent paper showed that GDNF is

capable of inducing the expression of both Nurr1 and Pitx3, two key genes in DA specification (Hegarty et al., 2013c), in neural precursors isolated from the VM during the time of DA neurogenesis (Lei et al., 2011). Similarly, in cultures of E12 rat VM neural precursors, GDNF significantly increased the number of cells expressing both Nurr1 and Pitx3 (Roussa and Krieglstein, 2004a). In support of these findings, GDNF has been shown to induce Pitx3 expression in the murine VM, with Pitx3 mediating GDNF-induced BDNF expression in A9 nigrostriatal DA neurons (Peng et al., 2011). This GDNF-Pitx3-BDNF feed-forward regulation may explain the specific sensitivity of the A9 subgroup of midbrain DA neurons to GDNF, with Pitx3 also being crucial for the survival and maintenance of nigrostriatal DA neurons (Smidt et al., 2004, Nunes et al., 2003, Hwang et al., 2003, van den Munckhof et al., 2003). Interestingly, Nurr1 has been shown to regulate the expression of the GDNF receptor, cRet, the expression of which is lost in Nurr1-deficient animals (Castillo et al., 1998). This suggests that GDNF and Nurr1 may function in an autoregulatory loop during VM DA neurogenesis, a mechanism which is not uncommon during this developmental process (Hegarty et al., 2013c). GDNF has been shown to induce expression of the dopamine synthetic enzyme, TH, in fetal human and rat cortical cultures (Theofilopoulos et al., 2001), which suggests it may play a similar role during VM DA neurogenesis. In support of such a proposal, Pitx3 has been suggested to induce TH expression in nigrostriatal DA neurons (Hegarty et al., 2013c), which indicates that Pitx3 may mediate GDNF-induced TH expression in a similar fashion to its mediation of GDNF-induced BDNF expression. Despite these findings, and the proposed roles for GDNF in the induction of a DA phenotype, GDNF null mice display no severe defects in midbrain DA neurons during embryonic development (Moore et al., 1996, Pichel et al., 1996, Sanchez et al., 1996) (see table 3.7.3), demonstrating that GDNF is not essential for VM DA neurogenesis. However, these mice die perinatally (Moore et al., 1996, Pichel et al., 1996, Sanchez et al., 1996), prohibiting the investigation of GDNF deficits on postnatal nigrostriatal pathway development. Granholm et al. (2000) circumvented this issue by transplanting GDNF null VM tissue into the adult wild-type mouse brain to demonstrate that GDNF is required for the postnatal survival and neuritic growth of midbrain DA neurons (Granholm et al., 2000).

GDNF has been shown to induce neurite growth from cultured rat VM DA neurons at E14 (Widmer et al., 2000, Lin et al., 1993), the time-point at which

midbrain DA neurons are extending their axons from the VM *in vivo* (Gates et al., 2004, Nakamura et al., 2000). These data suggest that GDNF may regulate DA axogenesis in the VM. This neurite growth-promoting role of GDNF on midbrain DA neurons was also demonstrated postnatally *in vivo* (Kholodilov et al., 2004, Granholm et al., 2000), suggesting that GDNF may function in the neurite arborisation of DA axons once they have reached their targets, in which GDNF is expressed (Choi-Lundberg and Bohn, 1995, Gavin et al., 2013). In support of such a role, GDNF administration to the adult rat striatum following intrastriatal 6-OHDA lesion causes the re-innervation of the striatum by midbrain DA afferents (Rosenblad et al., 1998). Similarly, GDNF is required for the spouting of nigrostriatal fibers following striatal injury (Batchelor et al., 2000), while GDNF has also been shown to increase the neurite growth from DA neurons in VM transplants to the striatum (Espejo et al., 2000). Furthermore, Bourque and Trudeau have shown that GDNF enhances the synaptogenesis of cultured postnatal midbrain DA neurons (Bourque and Trudeau, 2000). In normal adult rats, a single injection of GDNF into either the substantia nigra or striatum significantly increases the levels of DA and its metabolites in the striatum and midbrain (Martin et al., 1996), which may relect the findings of Bourque and Trudeau (Bourque and Trudeau, 2000). These findings suggest a role for GDNF in the maintenance of midbrain DA neurons in adulthood. Indeed, chronic striatal administration of GDNF in aged monkeys has a long-lasting protective action on nigrostriatal DA neurons (Maswood et al., 2002). Furthermore, $GDNF^{+/-}$ heterozygous mice show an accelerated decline of nigrostriatal DA neurons during aging, which leads to functional motor deficits (Boger et al., 2006) (see table 3.7.3).

A naturally-occurring analog of GDNF, neurturin, has been shown to be equally as a potent as GDNF at promoting the survival of midbrain DA neurons in vitro (Horger et al., 1998, Akerud et al., 1999). However, neurturin was found not to share the neuritogenic effects of GDNF (Akerud et al., 1999). These findings suggest that neurturin shares the roles of GDNF in the survival and maintenance of VM DA neurons during embryonic and postnatal development, which is supported by the expression of neurturin in the VM and striatum during development (Horger et al., 1998). Like GDNF, neuturin protects VM DA neurons in animal models of PD (Tseng et al., 1998, Hoane et al., 1999, Oiwa et al., 2002, Kordower et al., 2006, Herzog et al., 2007), and is now in clinical trials (Marks et al., 2008, Marks et al.,

2010, Bartus et al., 2013). The other two members of the GDNF family, persephin and artemin, have also been shown to promote the survival of midbrain DA neurons *in vitro* (Milbrandt et al., 1998, Baloh et al., 1998) and *in vivo* (Akerud et al., 2002, Cass et al., 2006), suggesting that the GDNF family may share protective roles in the developing nigrostriatal system. However, GDNF and neurturin have been demonstrated to have differential effects on VM DA neurons *in vivo* (Hoane et al., 1999). In contrast to the findings of Akerud at al. (Akerud et al., 1999), neurturin, as well as persephin and artemin, have recently been shown to promote the neurite growth of cultured midbrain DA neurons (Zihlmann et al., 2005). These findings suggest roles for these factors in the formation of the nigrostriatal pathway during development. However, mice with null mutations of neurturin (Heuckeroth et al., 1999), persephin (Tomac et al., 2002) or artemin (Honma et al., 2002) are viable and lack severe deficits in midbrain DA neurons (see table 3.7.3). These GDNF family ligands are therefore not essential for the development of the nigrostriatal system. However, it may be the case that in the absence of one of these GDNF family ligands, the other family members compensate functionally during midbrain DA development. The phenotypic analysis of double or treble mutants of the GDNF family ligands would address this possibility of functional redundancy.

Table 3.7.1: Roles of GDNF family in the development of midbrain DA neurons

Effects on midbrain DA neurons	Reference(s)
GDNF	
***in vitro*:**	
promotes survival and reduces apoptosis of embryonic DA neurons	(Lin et al., 1993, Krieglstein et al., 1995a, Widmer et al., 2000, Clarkson et al., 1995, Clarkson et al., 1997, Sawada et al., 2000, Orme et al., 2013)
promotes survival and reduces apoptosis of postnatal DA neurons	(Burke, 2003)
protects DA neurons from MPP+ and 6-OHDA	(Eggert et al., 1999, Hou et al., 1996)
protects DA neurons from lipopolysaccharide-induced neurotoxicity	(Xing et al., 2010)
induces Nurr1 and Pitx3 expression in VM neural precursors	(Lei et al., 2011, Roussa and Krieglstein, 2004a, Peng et al., 2011)

induces TH expression	(Theofilopoulos et al., 2001)
induces neurite growth of embryonic DA neurons	(Widmer et al., 2000, Lin et al., 1993)
enhances synaptogenesis of postnatal DA neurons	(Bourque and Trudeau, 2000)
***in vivo*:**	
promotes survival of embryonic DA neurons in VM transplants to adult striatum	(Apostolides et al., 1998, Espejo et al., 2000, Granholm et al., 1997, Yurek, 1998, Sullivan et al., 1998a)
promotes survival of postnatal midbrain DA neurons	(Kholodilov et al., 2004, Granholm et al., 2000)
promotes survival of adult DA neurons in animal models of PD	(Tomac et al., 1995, Gash et al., 1996, Connor et al., 2001, Kozlowski et al., 2000, Date et al., 1998, Kordower et al., 2000, Fox et al., 2001)
induces neurite growth of postnatal midbrain DA neurons	(Kholodilov et al., 2004, Granholm et al., 2000)
induces re-innervation of the lesioned striatum by midbrain DA afferents	(Rosenblad et al., 1998, Batchelor et al., 2000)
increases neurite growth from DA neurons in VM transplants to adult striatum	(Espejo et al., 2000)
increases levels of DA and its metabolites in the striatum and midbrain	(Martin et al., 1996)
long-lasting protective action on nigrostriatal DA neurons during aging	(Maswood et al., 2002)
Neurturin, Persephin and Artemin	
***in vitro*:**	
promote survival of embryonic DA neurons	(Horger et al., 1998, Akerud et al., 1999, Milbrandt et al., 1998, Baloh et al., 1998)
promote neurite growth of embryonic DA neurons	(Zihlmann et al., 2005)
***in vivo*:**	
promote survival of adult DA neurons in animal models of PD	(Tseng et al., 1998, Hoane et al., 1999, Oiwa et al., 2002, Kordower et al., 2006, Herzog et al., 2007, Akerud et al., 2002, Cass et al., 2006)

3.7.2 TGFβs

TGFβs have been shown to be essential co-factors for the neuroprotective effects of GDNF on midbrain DA neurons. The application of antibodies neutralizing TGFβ isoforms abolishes the survival-promoting effects of GDNF on midbrain DA neurons *in vitro* (Krieglstein et al., 1998) and *in vivo* (Schober et al., 2007), suggesting that the effects of GDNF are dependent on TGFβs. Is has been reported that TGFβ is required for the recruitment of the GDNF receptor, GFRα1, to the plasma membrane in primary neuron cultures (Peterziel et al., 2002), which may explain the requirement for TGFβ in the DA neurotrophic effects of GDNF. The cooperative functioning of TGFβ and GDNF has been highlighted *in vivo* by their co-storage in the secretory vesicles of a model neuron, the chromaffin cell, and the co-localisation of their receptors on GDNF-responsive neuronal populations (Krieglstein et al., 1998). TGFβs are known to be expressed in the floor plate and notochord during development (Unsicker et al., 1996, Flanders et al., 1991). They have been shown to be expressed in the ventral midbrain during DA neurogenesis (Krieglstein and Unsicker, 1994, Farkas et al., 2003), and their expression significantly increases in the striatum following MPP+ lesion (Schober et al., 2007), suggesting physiological roles for these factors, both during DA development and in response to neurotoxic insult. Indeed, TGFβs have been shown to have survival-promoting and neuroprotective effects (against MPP+) on cultured midbrain DA neurons; these effects are not mediated by astroglia or by increases in cell proliferation (Krieglstein and Unsicker, 1994, Krieglstein et al., 1995a). Furthermore, there is evidence to suggest that TGFβs regulate the neurite growth of midbrain DA neurons (Knoferle et al., 2010). It is unclear whether TGFβs achieve these effects directly, or function to sensitise midbrain DA neurons to the survival- and growth-promoting effects of endogenous GDNF.

TGFβs have been identified as important mediators in the induction of midbrain DA neurons (see table 3.7.2). It has been consistently shown that treatment with TGFβs increases the numbers of DA neurons in cultures of rodent VM precursors, through the induction of a DA phenotype in these cells (Farkas et al., 2003, Roussa et al., 2006). Furthermore, Farkas et al. (2003) showed that reduction of endogenous TGF-β *in vivo*, by the use of TGFβ-neutralizing antibodies, suppresses the differentiation of midbrain DA neurons in the chick embryo (Farkas

et al., 2003). VM astrocytes have been shown to induce DA neurogenesis in rat VM precursors by releasing high levels of the TGFβ3 isoform *in vitro* (Li et al., 2009). A similar role for VM astrocytes has previously been shown through their secretion of Wnts (Castelo-Branco et al., 2006), which are critical inducers of DA neurogenesis (Hegarty et al., 2013c). Interestingly, the inductive effects of TGFβs were shown to be dependent on Shh (Farkas et al., 2003), another factor vital for DA induction (Hegarty et al., 2013c). Likewise, Shh was shown to be incapable of inducing a DA phenotype in the absence of TGFβ (Farkas et al., 2003), suggesting that these factors function cooperatively to induce a DA phenotype during midbrain DA neurogenesis. GDNF has been shown to potentiate the DA-inductive effects of TGFβ on cultured VM neural precursors, however was not capable of rescuing the inductive defects resulting from the neutralization of endogenous TGFβs (Roussa et al., 2008). However, GDNF did rescue the TGFβ neutralization-dependent loss of differentiated midbrain DA neurons (Roussa et al., 2008), supporting its role as a factor which maintains these neurons, and as a facilitator of TGFβ-induced survival-promoting effects. Another GDNF family ligand, persephin, has been shown to enhance the inductive abilitites of TGFβ *in vitro*, with these TGFβ /persephin-induced DA neurons having increased resistance to MPP+ compared to untreated cultures of VM DA neurons (Roussa et al., 2008). In contradiction to these data proposing a role for TGFβs in induction of midbrain DA neurons, mice with null mutations of the TGFβ1, TGFβ2 or TGFβ3 isoforms die perinatally/shortly after birth, with no severe deficits in midbrain DA neurons (Sanford et al., 1997, Kaartinen et al., 1995, Shull et al., 1992). Furthermore, the double knockout of both TGFβ2 and GDNF did not result in a loss of midbrain DA neurons at E14.5 (Roussa et al., 2008) or E18.5 (Rahhal et al., 2009) (see table 3.7.3), indicating that the cooperative functioning of TGFβ2 and GDNF is not essential for DA induction *in vivo*. Roussa et al. (2008) also reported no midbrain DA deficiencies at E14.5 in $TGF\beta2^{+/-}/GDNF^{-/-}$ or $TGF\beta2^{-/-}/GDNF^{+/-}$ mice (Roussa et al., 2008) (see table 3.7.3). A likely explanation for such observations is that the TGFβ isoforms may compensate for the loss of each other. Indeed, the double knockout of TGFβ2 and TGFβ3 resulted in a significant reduction of midbrain DA neurons at E14.5 (Roussa et al., 2006). Roussa et al. (2006) then compared mice carrying one allele of TGFβ2 ($TGF\beta2^{+/-}/TGF\beta3^{-/-}$) or TGFβ3 ($TGF\beta2^{-/-}/TGF\beta3^{+/-}$) to demonstrate that the TGFβ2 isoform is more

important for the induction of midbrain DA neuronal population than TGFβ3 (Roussa et al., 2006) (see table 3.7.3). These data suggest that TGFβs function to induce a DA phenotype during midbrain DA neurogenesis, and that these isoforms can functionally compensate for one another.

Table 3.7.2: Roles of TGFβs in the development of midbrain DA neurons

Effects on midbrain DA neurons	Reference(s)
***in vitro*:**	
promote survival of embryonic DA neurons	(Krieglstein and Unsicker, 1994, Krieglstein et al., 1995a)
protect DA neurons from MPP+	(Krieglstein and Unsicker, 1994, Krieglstein et al., 1995a)
regulate neurite growth of embryonic DA neurons	(Knoferle et al., 2010)
increase numbers of DA neurons through the induction of a DA phenotypic	(Farkas et al., 2003, Roussa et al., 2006, Li et al., 2009)
***in vivo*:**	
induce differentiation of DA neurons	(Farkas et al., 2003)

Due to the perinatal lethality of TGFβ null mice, it is difficult to determine the functions of TGFβs during the postnatal development of the nigrostriatal pathway. Despite this, recent studies have found that TGFβ2 heterozygous mice have a reduction in midbrain DA neurons and striatal dopamine at 6 weeks of age (Andrews et al., 2006), which is similar to the nigrostriatal deficits that progress with age demonstrated in GDNF heterozygous mice (Boger et al., 2006) . Furthermore, the null mutation of Smad3, a crucial mediator of TGFβ signalling, resulted in a loss of nigrostriatal neurons between birth and 2-3 months of age in mice (Tapia-Gonzalez et al., 2011) (see table 3.7.3). These studies imply that TGFβs function to protect and maintain midbrain DA neurons in adulthood, as suggested above for GDNF. However, in contrast to the single haploinsufficiencies just described, a more recent study showed that the combined haploinsufficiency of TGFβ2 and GDNF has no impact of the survival of midbrain DA neurons during normal aging (Heermann et al., 2010) (see table 3.7.3). These contradicting findings led Heermann et al. (2010) to suggest that balanced TGFβ2 and GDNF levels are important for the maintenance of midbrain DA neurons in adulthood (Heermann et al., 2010).

Interestingly, a study which re-examined the TGFβ3 null mutant showed that, despite no deficiencies at E12.5, these mutants had a significant reduction of midbrain DA neurons at postnatal day (P)0 (Zhang et al., 2007) (see table 3.7.3), a time-point when neurotrophic support is vital for the survival of midbrain DA neurons due to naturally-occuring cell death (Hegarty et al., 2013c). This study again suggests that TGFβs may function redundantly in DA induction, but indicates that TGFβ3 is required for the maintenance and survival of midbrain DA neurons. Zhang et al. (2007) also showed that the transcriptional cofactor homeodomain interacting protein kinase 2 (HIPK2) is required for the TGFβ-mediated survival of mouse DA neurons (Zhang et al., 2007). This study demonstrates the importance of analysing mutants at several developmental time-points, especially at those times which are crucial to the developmental program. Perhaps the TGFβ2 and GDNF single and/or double-mutants should be re-examined in a similar fashion.

Table 3.7.3: Genetic mutations of TGFβ superfamily members and their effects on the development of midbrain DA neurons

Mutation(s)	Effect(s) on DA development	Reference(s)
GDNF -/-	no severe defects in DA neurons during embryonic development	(Moore et al., 1996, Pichel et al., 1996, Sanchez et al., 1996)
GDNF -/-	null VM transplants to adult striatum have reduced DA neuron numbers and fiber outgrowth	(Granholm et al., 2000)
GDNF +/-	accelerated decline of DA neurons during aging	(Boger et al., 2006)
Neurturin -/-	no severe defects in DA neurons	(Heuckeroth et al., 1999)
Persephin -/-	no severe defects in DA neurons	(Tomac et al., 2002)
Artemin -/-	no severe defects in DA neurons	(Honma et al., 2002)
GDNF -/- : **TGFβ2** -/-	no loss of midbrain DA neurons at E14.5 or E18.5	(Roussa et al., 2008, Rahhal et al., 2009)
GDNF +/- : **TGFβ2** -/-	no loss of midbrain DA neurons at E14.5	(Roussa et al., 2008)
GDNF -/- : **TGFβ2** +/-	no loss of midbrain DA neurons at E14.5	(Roussa et al., 2008)
TGFβ1 -/-	no severe defects in DA neurons during embryonic development	(Shull et al., 1992)
TGFβ2 -/-	no severe defects in DA neurons during	(Sanford et al., 1997)

	embryonic development	
TGFβ3 -/-	no severe defects in DA neurons during embryonic development	(Kaartinen et al., 1995)
TGFβ2 -/- : **TGFβ3** -/-	significant reduction of DA neurons at E14.5	(Roussa et al., 2006)
TGFβ2 +/- : **TGFβ3** -/-	significant reduction of DA neurons at E14.5 (less severe than double knockout)	(Roussa et al., 2006)
TGFβ2 -/- : **TGFβ3** +/-	significant reduction of DA neurons at E14.5 (less severe than TGFβ2 +/- : TGFβ3 -/- knockout)	(Roussa et al., 2006)
TGFβ2 +/-	reduction in DA neurons and striatal dopamine at 6 weeks of age	(Andrews et al., 2006)
Smad3 -/-	loss of nigrostriatal neurons between birth and 2-3 months of age	(Tapia-Gonzalez et al., 2011)
GDNF +/- : **TGFβ2** +/-	no deficits in DA neurons during normal aging	(Heermann et al., 2010)
TGFβ3 -/-	significant reduction of DA neurons at P0	(Zhang et al., 2007)
BMPRII -/-	reduction of DA neurons and striatal innervation in adulthood	(Chou et al., 2008a)
BMP7 +/-	increased sensitivity of adult DA neurons to methamphetamine toxicity	(Chou et al., 2008b)

3.8 The BMP family in midbrain DA neuronal development

The BMP family consists of at least 20 phylogenetically-conserved growth factors, including GDFs such as GDF5 (Kawabata et al., 1998). BMPs have been shown to function in many crucial aspects of nervous system development, including neural induction, neural crest development, dorsal spinal cord patterning and the fate specification of a number of neural populations (Hegarty et al., 2013a). In this part of the literature review, the current evidence supporting a role for BMPs in the development of midbrain DA neurons will be discussed (see tables 3.8.1 and 3.8.2).

3.8.1 GDFs

GDF5 is the family member whose roles have been best characterised in terms of DA development (see table 3.8.1). Like GDNF, this factor is under investigation for its therapeutic potential in PD (Sullivan and Toulouse, 2011). GDF5 is expressed in the developing and adult rat VM and striatum (Krieglstein et al., 1995b, O'Keeffe et

al., 2004b, Storm et al., 1994, Gavin et al., 2013). Its midbrain expression profile proposes roles for GDF5 in nigrostriatal development. GDF5 protein expression begins in the rat VM on E12 (when early progenitors of DA neurons are present), reaches a peak on E14 (the day that DA neurons undergo terminal differentiation), before decreasing with age to reach its lowest levels around the perinatal period, and then increasing in the postnatal period to reach maximal expression levels (O'Keeffe et al., 2004b). These data suggest that GDF5 is involved in the differentiation of VM precursors into DA neurons, and the maintenance of these neurons in adulthood. Krieglstein et al. were the first to examine the effects of GDF5 on DA neurons, and found that GDF5 increased the number of DA neurons in cultures of E14 rat VM (Krieglstein et al., 1995b). Other studies agreed with these findings, showing similar neurotrophic effects of GDF5 on VM cultures (O'Keeffe et al., 2004a, Wood et al., 2005, Clayton and Sullivan, 2007, O'Sullivan et al., 2010), and showed that GDF5 did not induce an increase in the total number of neurons in E14 rat VM cultures (O'Keeffe et al., 2004a), indicating a selective effect on DA neurons. It remains unclear whether GDF5 elicits its increases in midbrain DA neurons through the induction of a DA phenotype or by promoting their survival. The latter appears to be more likely as GDF5 has also been shown to protect cultured VM DA neurons from MPP+ (Krieglstein et al., 1995b), 6-OHDA (O'Sullivan et al., 2010), and free radical doners (Lingor et al., 1999). The numbers of astrocytes in E14 VM cultures dramatically increases following GDF5 treatment (Krieglstein et al., 1995b, O'Keeffe et al., 2004a, Wood et al., 2005), suggesting that GDF5 may have an indirect neurotrophic action, possibly by stimulating the production of glial-derived growth factor(s) production, such as GDNF, that may function in the neurotrophic response (Sullivan and O'Keeffe, 2005). Conversely, the neurotrophic effects of GDF5 on midbrain DA neurons have been shown to be unaltered in glial-depleted cultures (Wood et al., 2005). Furthermore, Wood et al. also showed an additive neurotrophic effect of GDF5 and GDNF on cutured embryonic DA neurons (Wood et al., 2005), indicating that GDF5 acts independently from GDNF, and that these factors may act on separate subpopulations of DA neurons. The GDF5-induced increase in midbrain DA neurons *in vitro* has been suggested to be mediated by BMPRIb, as application of GDF5 at the time of plating, when BMPRIb is expressed, increases DA neuronal number, whereas application after six days *in vitro*, when this receptor is no longer expressed, has no effect (O'Keeffe et al., 2004a). In support of

this data, the neurotrophic effects of GDF5 were recently demonstrated to be mediated by the BMPRIb in a model of human DA neurons, SH-SH5Y cells (Hegarty et al., 2013b). These findings are not surprising considering that BMPRIb is the preferential type I receptor of GDF5 (Nishitoh et al., 1996). Another study demonstrated that GDF5 exerts greater effects on cultures prepared from the lateral VM (Clayton and Sullivan, 2007), which corresponds to the A9 nigrostriatal subgroup of DA neurons (Abeliovich and Hammond, 2007), suggesting a selective effect of GDF5 on nigral DA neurons. In this study it was proposed that the increase in midbrain DA neurons was due to the induction of a DA phenotype in progenitor cells, rather than promotion of cell survival, and it was also showed that BMPRIb expression was higher in the lateral VM compared to the medial region (Clayton and Sullivan, 2007). In support of the suggested DA inductive role of GDF5, experiments carried out by the authors on E12 rat VM cultures treated with GDF5 suggests that the increase in DA neurons observed was due to the induction of TH expression in uncommitted NPs (Hegarty et al., unpublished data). Similarly, the present authors have also demonstrated that GDF5 induces TH expression in neurons derived from E14 rat VM NPs (Hegarty et al., unpublished data). Based on these data, and on the midbrain expression profile of GDF5, it is likely that GDF5 functions in the transition of VM DA NPs into TH-expressing DA neurons.

The effects of GDF5 to increase the numbers of midbrain DA neurons *in vitro* must also be considered in terms of survival-promoting effects, especially with respect to their neuroprotective effects from DA toxins *in vitro*. Indeed, *in vivo* studies have shown that GDF5 protects the adult rat nigrostriatal pathway against DA neuronal death induced by 6-OHDA (Sullivan et al., 1997, Sullivan et al., 1999, Hurley et al., 2004, Costello et al., 2012). A more developmentally-relevant survival-promoting effect was demonstrated when GDF5 was shown to enhance the survival of embryonic rat VM transplants *in vivo*, to the same extent as GDNF (Sullivan et al., 1998b). Furthermore, GDF5-overexpressing E13 VM transplants significantly restored function in 6-OHDA-lesioned adult rats, with the exogenous GDF5 being suggested to increase the survival of the remaining host DA neurons, as well as the transplanted DA neurons (O'Sullivan et al., 2010). Similarly, a continuous supply of GDF5, through the striatal transplantation of GDF5-overexpressing CHO cells *in vivo*, protects adult nigrostriatal DA neurons and increases the survival of transplanted embryonic VM DA neurons in the 6-OHDA-lesioned rat model of PD

(Costello et al., 2012). These data propose a role for GDF5 as a factor which promotes the survival of embryonic DA neurons during their generation, which would correlate with the peak of GDF5 expression at E14 (O'Keeffe et al., 2004b). Furthermore, the second peak of expression in the adult suggests a role for GDF5 in the maintenance of VM DA neurons during adulthood, which is supported by the survival-promoting effects of GDF5 on adult VM DA neurons *in vivo* discussed above. A similar study for GDF5 to the one carried out by Burke et al. (2003) on cultured postnatal DA neurons would address this. Moreover, studies on GDF5 null mice, such as the $GDF5^{bp}$ mouse (Storm et al., 1994), to examine midbrain DA neuronal number and striatal innervation at various stages of embryonic and postnatal development, will be critical to determine the *in vivo* role(s) of GDF5 in nigrostriatal system development. One study showed that adult mice with null mutations in the BMPRII, the type II receptor of GDF5, displayed significantly decreased numbers of nigrostriatal DA neurons (Chou et al., 2008a) (see table 3.7.3). However, caution must be employed when inferring the relevance of this study to the roles of GDF5 specifically, as several members of the BMP family acts via this type II receptor.

GDF5 has also been consistently shown to promote the neurite growth of cultured E14 rat VM DA neurons (O'Keeffe et al., 2004a, Clayton and Sullivan, 2007, Hegarty et al., 2014a). Crucially, this time-point reflects the peak period of DA axogenesis (Gates et al., 2004, Nakamura et al., 2000), as well as of GDF5 VM expression (O'Keeffe et al., 2004b), suggesting that GDF5 functions in this process. Furthermore, an *in vivo* study has suggested that exogenous GDF5 increases the neurite outgrowth of host nigrostriatal DA innervation, as well as transplanted embryonic VM DA neurons, in 6-OHDA-lesioned adult rats (O'Sullivan et al., 2010, Costello et al., 2012, Hurley et al., 2004). The neurite growth-promoting effects of GDF5 on VM DA neurons have recently been shown to be mediated by BMPRIb activation of Smad 1/5/8 signalling (Hegarty et al., 2014a, Hegarty et al., 2013b), which most likely requires BMPRII. Indeed, in adult BMPRII null mice there is a deficit in nigrostriatal innervation (Chou et al., 2008a) (see table 3.7.3). It has also been demonstrated that the neurite growth-promoting effects of GDF5 were not mediated by GDNF (Hegarty et al., 2014a), through the blockade of its heparan-dependent signalling (Barnett et al., 2002, Iwase et al., 2005, Orme et al., 2013). Taken together with the survival-promoting effects of GDF5 discussed above, as

well as its postnatal striatal expression (O'Keeffe et al., 2004b, Gavin et al., 2013), these data propose a role for GDF5 as a target-derived neurotrophic factor which regulates the survival and growth of DA neurons innervating the striatum. The postnatal/*in vivo* experiments outlined above would also address this potential role. Another GDF, GDF15, has been shown to promote the survival of control and iron-intoxicated E14 VM DA neurons *in vitro* (Strelau et al., 2000), suggesting that GDF15 may contribute to DA neuronal survival during development. Strelau et al. also demonstrated that GDF5 promotes the survival of adult VM DA neurons *in vivo* using the 6-OHDA-lesioned adult rat model (Strelau et al., 2000). The role of other GDF family members in midbrain DA development has yet to be demonstrated, although one study has reported that GDF6 does not have neurotrophic effects on cultured VM DA neurons (Brederlau et al., 2002).

Table 3.8.1: Roles of GDF family in the development of midbrain DA neurons

Effects on midbrain DA neurons	**Reference(s)**
GDF5	
***in vitro*:**	
increases numbers of embryonic DA neurons	(O'Keeffe et al., 2004a, Wood et al., 2005, Clayton and Sullivan, 2007, O'Sullivan et al., 2010, Krieglstein et al., 1995b)
promotes survival of embryonic DA neurons	(O'Keeffe et al., 2004a, Wood et al., 2005, Clayton and Sullivan, 2007, O'Sullivan et al., 2010, Krieglstein et al., 1995b)
protects DA neurons from MPP+, 6-OHDA, and free radical doners	(Krieglstein et al., 1995b, O'Sullivan et al., 2010, Lingor et al., 1999)
induces TH expression in VM precursors	in preparation
regulates neurite growth of embryonic DA neurons	(O'Keeffe et al., 2004a, Clayton and Sullivan, 2007, Hegarty et al., 2014a)
***in vivo*:**	
promotes survival of adult DA neurons in animal models of PD	(Sullivan et al., 1997, Sullivan et al., 1999, Hurley et al., 2004, O'Sullivan et al., 2010, Costello et al., 2012)
promotes survival of embryonic DA neurons in VM transplants to adult striatum	(Sullivan et al., 1998b, O'Sullivan et al., 2010, Costello et al., 2012)
induces re-innervation of the lesioned striatum by midbrain DA afferents	(O'Sullivan et al., 2010, Costello et al., 2012, Hurley et al., 2004)

increases neurite growth from DA neurons in VM transplants to adult striatum	(O'Sullivan et al., 2010, Costello et al., 2012)
GDF15	
promotes survival of control and iron-intoxicated embryonic DA neurons *in vitro*	(Strelau et al., 2000)
promotes survival of adult DA neurons *in vivo* following 6-OHDA lesion	(Strelau et al., 2000)

3.8.2 BMPs

The most compelling evidence for a role(s) for BMPs in the development of midbrain DA neurons (see table 3.8.2) can be seen in the phenotype of the BMPRII null mouse, which has a reduction of nigrostriatal neurons and striatal DA innervation at adulthood (Chou et al., 2008a) (see table 3.7.3). However, whether this loss of midbrain DA neurons and striatal innervation results from a failure in DA neuronal development, or from a later degenerative process, remains to be determined. A detailed analysis of the numbers of DA neurons present in the midbrain, as well as striatal innervation, of BMPRII null mice at multiple stages during embryonic and post-natal development is required to address this question. Furthermore, mice with null mutations (or mutations which permit postnatal investigation) in specific BMP family members should undergo a similar analysis to determine which factors are directly involved.

BMPs have been shown to be expressed in the nigrostriatal system of the developing and adult brain (Jordan et al., 1997, Soderstrom and Ebendal, 1999, Chen et al., 2003). The first report of a role for BMPs in midbrain DA neuronal development was provided by the Krieglstein research group, who investigated the neurotrophic effects of several BMPs on cultured E14 rat VM DA neurons (Jordan et al., 1997). Jordan et al. showed that BMP 2, 4, 6, 7 and 12 promote the survival of cultured DA neurons, with BMP6 and BMP12 showing similar efficacy to GDNF (Jordan et al., 1997). In a similar study, BMP5, 6 and 7, but not BMP3, significantly increased the numbers of DA neurons in embryonic VM cultures (Brederlau et al., 2002). Conversely, Brederlau et al. (2002) showed no effect for GDF5 on midbrain DA neurons (Brederlau et al., 2002). This was likely due to the fact that in this study treatments were carried out at 6DIV, at the time when BMPRIb has been shown to

be downregulated in culture (O'Keeffe et al., 2004a). BMP2 has also been demonstrated to increase the numbers of midbrain DA neurons, and promote their neurite growth, *in vitro* (Reiriz et al., 1999). Similarly, BMP2 was recently shown to promote the neurite growth of cultured midbrain DA neurons via a BMPRIb-Smad mediated mechanism (Hegarty et al., 2014a). These data suggest a role for BMP2 in the generation and growth of embryonic midbrain DA neurons. In support of this suggestion, BMP2 has also been shown to promote the survival and neurite growth of embryonic DA neurons in rat VM transplants grafted into the 6-OHDA-lesioned striatum (Espejo et al., 1999). Interestingly, the neurotrophic effects of BMP2 on midbrain DA neurons were initially suggested to be mediated by astrocytes (Jordan et al., 1997), however Reiriz et al. (1999) demonstrated BMP2-induced increases in DA neurons in glial-depleted VM cultures (Reiriz et al., 1999). Furthermore, BMP2 was shown to have neurotrophic effects in cultures of SH-SH5Y cells, a cell line model of human DA neurons, which do not contain glial cells (Hegarty et al., 2013b). Again it is unclear whether BMPs increase DA neuron numbers in culture through induction of neural precursors and/or promoting the survival of existing neurons, however Reiriz et al. (1999) did report that BMP2 did not increase the proliferation of DA neurons (Reiriz et al., 1999). One BMP family member, BMP7, has been shown to induce DA neuronal differentiation from embryonic rat VM neural precursors (Lee et al., 2003). This BMP was also identified in the Jordan et al. (1997) and Brederlau et al. (2002) studies as a DA neurotrophic factor. Indeed, BMP7 has been shown to promote the survival of the adult nigrostriatal pathway *in vivo* against DA toxins (Harvey et al., 2004, Chou et al., 2008b) (see table 3.8.2), suggesting that BMP7 may maintain this pathway in adulthood and in response to insult. In support of this suggestion, a recent study comparing the expression levels of BMPs in multiple brain regions at various time-points in adulthood reported uniquely high levels of BMP7 expression in the ventral midbrain, when compared to other BMPs and other brain regions (Chen et al., 2003). Taken together, these preliminary BMP studies suggest roles for BMPs in the induction, neuronal differentiation and survival of midbrain DA neurons. Further studies, such as those described for BMP(R) mutants above, should be carried out to investigate the *in vivo* roles of BMPs in DA neuronal development.

Table 3.8.2: Roles of BMP family in the development of midbrain DA neurons

Effects on midbrain DA neurons	Reference(s)
BMP2	
***in vitro*:**	
increases numbers of embryonic DA neurons	(Reiriz et al., 1999)
promotes survival of embryonic DA neurons	(Jordan et al., 1997)
regulates neurite growth of embryonic DA neurons	(Reiriz et al., 1999, Hegarty et al., 2014a)
***in vivo*:**	
promotes survival of embryonic DA neurons in VM transplants to the adult striatum	(Espejo et al., 1999)
increases neurite growth from DA neurons in VM transplants to the adult striatum	(Espejo et al., 1999)
BMP7	
***in vitro*:**	
increases numbers of embryonic DA neurons	(Brederlau et al., 2002)
promotes survival of embryonic DA neurons	(Jordan et al., 1997)
induces DA neuronal differentiation from embryonic rat VM neural precursors	(Lee et al., 2003)
***in vivo*:**	
promotes survival of the adult nigrostriatal pathway against DA toxins	(Harvey et al., 2004, Chou et al., 2008b)
BMP 4, 5, 6 and 12	
increase numbers of embryonic DA neurons *in vitro*	(Jordan et al., 1997, Brederlau et al., 2002)

3.9 Conclusion and aims of present study

The two most promising PD therapies involve (1) the application of neurotrophic factors to support the remaining DA neurons and protect them against the ongoing disease process, and (2) the transplantation of stem cell-derived midbrain DA neurons to replace those that have degenerated. In order to optimize these potential therapies, an understanding of the developmental program that regulates the development of midbrain DA neurons is vital, as it would facilitate both the

generation of transplantable DA neurons from stem cells, and the identification of developmentally-relevant neurotrophic factors.

This introduction thus firstly described the molecular mechanisms which are known to regulate VM DA neurogenesis. The 'normal' developmental program that regulates VM DA neurogenesis was outlined, including the cellular and molecular determinants involved in their regional specification, induction, differentiation and maturation. It is clear that DA neurogenesis involves a complex developmental program, which is complicated further by the fact that VM DA neurons are not a homogenous population. Midbrain DA neurons are now known to arise from floor plate radial glial-like NPs in response to specification by FGF8, Shh and Wnt1. This discovery has important implications for the specific isolation of VM DA NPs for use in cell replacement therapies. Additionally, following extensive research in recent years, Wnt1 is now accepted as an extrinsic instructive factor for VM DA neurons, along with Shh and FGF8, which has added another dimension to the developmental program of VM DA neurogenesis. Thus, other potential candidates should be investigated in the same way for their participation in VM DA neuronal specification, differentiation and neurite growth. The discovery of a number of new candidate transcription factors, for example Oc1/2/3, highlights that there are likely to be other, as yet unidentified, molecular pathways involved in regulating VM DA neurogenesis. Furthermore, new relationships are being uncovered between the transcription factors and molecular pathways that are well known to play key roles in DA development. These findings highlight that there is still a significant challenge remaining to understand the complexities of the dynamic molecular interactions between the known genetic networks involved in VM DA neurogenesis.

Some of the most promising candidate signalling proteins to be potentially involved in the development of nigrostriatal DA neurons belong to the TGFβ superfamily. BMPs signal via a canonical Smad signalling pathway involving two types of BMPRs, three R-Smads, Smad 1/5/8, and the co-Smad, Smad4. Before assessing the known literature on the involvement of this family in VM DA neuronal development, the present thesis reviewed the roles played by canonical BMP-Smad signalling in the development of the NS. Regulated Smad signalling is involved in the generation of the PNS primordium (the neural crest), while its inhibition is required for the formation of the CNS primordium (the neural plate). Following the

generation of these NS primordia, BMP-Smad signalling continues to regulate their further development, the most characterised example being in the patterning of the dorsal SC. Canonical BMP signalling is involved in the induction of both neuronal and glial fates from NSCs/NPs in a variety of CNS regions, such as the cortex, hippocampus, midbrain, hindbrain and SC. The mechanisms by which BMP-Smad signalling achieves the induction of both neuronal and glial phenotypes, as well as the induction of a variety of differentiation processes within specific neuronal populations, is unknown, however it is likely to be dependent on spatial and temporal regulation. Elucidating the various receptor combinations, cytosolic interactions, transcriptional effectors, and/or target genes that mediate these dual-inductive effects of canonical BMP-Smad 1/5/8 is crucial for a comprehensive understanding of the roles it plays in neural development.

This literature review then specifically focused on the roles that TGFβ superfamily members play in midbrain DA development. It was clear that many of these members influence various key steps of this developmental process, including the induction, differentiation, target innervation and survival of VM DA neurons. GDNF, in particular, has been shown to be a multi-step regulator of nigrostriatal system development, but BMP family members, such as GDF5 and BMP2, possess similar potential to that of GDNF. Extensive research is required before these factors can be confidently integrated into the VM DA neurogenesis developmental program. The roles of these TGFβ superfamily members in each step of this embryonic and postnatal developmental process, as well as the mechanisms regulating their effects, should thus be thoroughly investigated. This body of work focused on BMP2 and GDF5, which may contribute to the induction, differentiation and survival of midbrain DA neurons. Indeed, many studies have demonstrated their potent neurotrophic effects on VM DA neurons *in vitro* and *in vivo*. Despite these studies, the molecular and cellular mechanisms mediating their effects on DA neuronal development and survival are unknown. It is essential to understand these mechanisms if BMP2 and GDF5 are to be used in a clinical context for the treatment of PD. In an attempt to address this, the present thesis set out to examine the hypothesis that canonical Smad 1/5/8 signalling mediates the effects of BMP2 and GDF5 on the development of VM DA neurons. This hypothesis will be tested in both primary cultures of embryonic rat VM, and in a cell line model of human DA neurons, SH-SH5Y cells, by activating, modulating or inhibiting various components

of the BMP-Smad 1/5/8 signalling pathway. A role for BMP-Smad signalling in the induction of a DA phenotype will then be investigated in E14 rat VM NSC cultures. Following these *in vitro* investigations, the ability of GDF5 to activate canonical Smad signalling in thr rat VM *in vivo* will be assessed. The knowledge gained from these studies will provide insights into the involvement of BMP-Smad 1/5/8 signalling in the induction, differentiation and survival of VM DA neurons.

4. Materials and Methods

4.0 Declaration

All procedures were carried out accurately, and without bias, to the highest possible standards following appropriate training.

4.1 Cell culture

All procedures were carried out in a sterile laminar flow Class II Microflow Biological Safety Cabinet under aseptic conditions. All materials used were either tissue culture grade or had been sterilised by autoclaving at 121˚C for 20 min, or by passing through a 0.20 µm filter (Corning).

4.1.1 Poly-D lysine coating of culture plates

Poly-D lysine solution (Sigma) was added to the desired amount of wells of a 24-well tissue culture plate (VWR) or a 6-/96-well tissue culture plate (Sarstedt) and left for 20 minutes in sterile conditions. Poly-D lysine was re-used (up to 3 times) or aspirated, and the wells were washed 3 times with autoclaved water, after which they were left to dry.

4.1.2 Harvesting of the VM

Time-mated Sprague-Dawley rats (Biological Services Unit, UCC) were placed in a bell jar within a Class 1 flow cabinet (Bassaire Model O3HB) and anaesthetised by inhalational isoflurane (Abbott Laboratories Ltd.). The pregnant rats were decapitated quickly using a guillotine. E12 or E14 embryos were removed by laparotomy using a blunt forceps and large scissors, and were subsequently immersed in Hank's Balanced Salt Solution (HBSS) (with sodium bicarbonate and without phenol red, Ca^{2+} or Mg^{2+}; Sigma H6645) in a 90 mm Petri dish (Fannin Healthcare) and kept on ice. All procedures were carried out with approval of the Animal Experimental Ethics Committee of University College Cork. Further dissections were carried out using a dissection microscope (Leica Wild M8) in a fume hood (Brassaire). Embryos were removed from their sacs using a scissors, curved forceps and a tweezers (World Precision Instruments), and placed in fresh HBSS in a 90 mm Petri dish placed on ice. Embryos undergoing dissection were transferred to the lid of a 90 mm Petri dish a quarter filled with HBSS. The

mesencephalon was dissected out by making an incision at the midbrain-hindbrain boundary, and at the forebrain-midbrain boundary using a dissecting scissors and a curved forceps (World Precision Instruments). The dorsal mesencephalon was then cut along the midline, and the mesencephalon was opened/ flattened out to show the VM in the centre. A cut at a point between the lateral to medial VM was then made on one side. The meninges were removed, and a similar incision was subsequently made on the other side of the midline to leave the VM. Cranial and caudal cuts were then made to the medial VM to ensure no forebrain/ hindbrain tissue was used. Dissected VMs were stored in a 20 ml sterilin tube in HBSS on ice until all the embryos were dissected. Once the dissections were completed, tissue culture was carried out immediately.

4.1.3 VM NSC proliferation as neurospheres

The E14 (or E12) VM tissue was centrifuged at 1,100 rpm for 5 min (4 min for E12). The supernatant was removed, and the tissue was enzymatically dissociated by addition of 3ml of 0.1% Trypsin (Sigma) to the sterilin tube which was incubated at 37°C and 5% CO_2 for 5 min (3 min for E12). 500µl of fetal calf serum (FCS) (Sigma) was then added to neutralise the Trypsin, and the tissue was triturated using a pipette tip and a syringe (BD Plastipak™) and needle (Sterican). The VM tissue was subsequently centrifuged at 1,100 rpm for 5 min (4 min for E12). The supernatant was removed and the cell pellet was resuspended in 1 ml of Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM F-12) (Sigma) containing 100 nM L-Glutamine (Sigma), 6 mg/ml D-Glucose (Sigma), 100 U/ml Penicillin (Sigma), 10 µg/ml Streptomycin (Sigma), with 2% B-27 supplement (Invitrogen), 20 ng/ml FGF (Millipore) and 20 ng/ml of epidermal growth factor (EGF) (Sigma), referred to as B-27 expansion media (all culture medias warmed to 37°C before use). A 1:10 dilution of the cell suspension was then made using the B-27 Expansion Media for cell counting. 10 µl of the diluted cell suspension was added to a haemocytometer (Marenfield Superior), and 5 grids were counted. The total number of cells was calculated using the following formula:

$$\text{Cells/ml} = \frac{\text{Number of Cells counted in 5 Fields}}{5} \times \text{Dilution Factor} \times 10000$$

The cell suspension was added to a T25 flask (VWR 734-2311) at a density of ~2 x 10^6 cells or to a 24-well tissue culture plate (VWR) at a density of 5 x 10^5/well for

E14 VM cells (for GDF5 and BMP2 treatment), along with 10 ml of B-27 expansion media (1 ml for each well), and the flask/plate was incubated at 37℃ and 5% CO_2 (ThermoForma Series II, Thermo Electron Corporation) for 7 (one passage), 14 (two passages) or 21 (three passages) days *in vitro* (DIV). For each passage, neurospheres were dissociated into a single cell suspension and reseeded in flask for expansion. At 1 DIV (of each passage), the VM NSC were supplemented with 20 ng/ml of EGF and FGF. Half of the media was replaced every 2-3 days. For the treatment of E14 VM neurospheres with recombinant human GDF5 (Biopharm GmbH, Germany) and recombinant human BMP2 (R&D Systems), GDF5 or BMP2 were added directly to the media at a concentration of 20 ng/ml every 2 days (from 0 DIV), and the neurospheres were imaged using phase-contrast microscopy at 2, 4 and 7 DIV.

4.1.4 Differentiation of VM NSCs

E14 (or E12) VM NSC neurospheres were transferred to a 20ml sterilin tube and centrifuged at 1,100 rpm for 5 min. The supernatant was removed, and the cells were enzymatically dissociated by addition of 2 ml of 0.1% Trypsin to the sterilin tube which was incubated at 3℃ and 5% CO_2 for 5 min. 500 µl of FCS was then added to neutralise the Trypsin, and the tissue was centrifuged at 1,100 rpm for 5 min. The supernatant was removed and the cell pellet was resuspended in 1ml of DMEM F-12 containing 100 nM L-Glutamine, 6 mg/ml D-Glucose, 100 U/ml Penicillin, 10 µg/ml Streptomycin, with 2% B-27 supplement, and 1% FCS, referred to as differentiation media. The VM NSC suspension was then triturated using a syringe and needle. A 1:10 dilution of the cell suspension was then made with the differentiation media for cell counting. 10 µl of the diluted cell suspension was added to a haemocytometer, and 5 grids were counted. The total number of cells was calculated using the above formula. To allow the cells to adhere to the wells, 50 µl of a cell suspension (diluted in differentiation media to give 50,000 cells/ml) was added to each well of a poly-D lysine coated 24-well plate (~1 x 10^6 cells/ml for 6-well plate), and incubated for 1h at 37℃ and 5% CO_2. The wells were then 'flooded' with 500 µl of differentiation media (2 ml for 6-well plate), and incubated at 37℃ and 5% CO_2 for a period of 7, 14, 21 or 28 DIV. Half of the media was replaced every 2-3 days. Differentiating VM NSCs were treated, as indicated, with 20 ng/ml of GDF5 or BMP2.

4.1.5 Primary cultures of the E14 rat VM

For the preparation E14 rat VM cultures, dissected VM tissue was dissociated into a single cell suspension as per section 4.1.3, however the VM cells were resuspended in 1 ml of differentiation media. E14 VM cells were then plated on poly-D lysine-coated 24-well plates at a density of 5 x 10^4 cells per well in 500 µl of differentiation media, and incubated at 37˚C with 5% CO_2. Cells were treated 200 ng/ml of GDF5 or BMP2, and pre-treated (30 min prior to GDF5 or BMP2 application) with 1 µg/ml of Dorsomorphin (Sigma) or 200 ng/ml of Noggin (R&D Systems).

4.1.6 5-bromo-2'-deoxyuridine application to E14 VM cells

5-bromo-2'-deoxyuridine (BrdU) (Sigma), which was re-constituted in sterile water, was added to E14 VM cells at a concentration of 0.2 µM during the expansion of neurospheres at 5 DIV, and was supplemented every 3 DIV during differentiation.

4.1.7 Harvesting and dissociation of the SH-SY5Y cell line

Prior to use, SH-SY5Y cells were stored in liquid nitrogen in a vial of freezing medium consisting of 90% FCS and 10% Dimethyl Sulfoxide (Sigma). Cells were thawed, and then added to a 20 ml sterilin tube before being centrifuged at 1,100 rpm for 5 min. The supernatant was removed, and the cells were re-suspended in 1ml of DMEM F-12 containing 10% FCS, 100nM L-Glutamine, 100 U/ml Penicillin, 10 µg/ml Streptomycin, referred to as SH-SY5Y growth media. The cells were added to a T25 flask, along with 10 mls of SH-SY5Y growth media, and were incubated 37℃ and 5% CO_2 until ~80% confluency was reached.

SH-SY5Y growth media was removed from the flask, and cells were washed in 1ml of HBSS to remove any residual media. The cells were enzymatically dissociated in 0.2% Trypsin by incubating at 37℃ and 5% CO_2 for 5 min. 1 ml of SH-SY5Y growth media was then added to neutralise the Trypsin, and the cells were triturated using a plugged flame polished Pasteur pipette (Sarstedt) attached to a pipette pump. A 1:10 dilution of the cell suspension was then made using the SH-SY5Y growth media for cell counting. The total number of cells was calculated using the above formula. SH-SY5Y cells were added to a T25 flask, along with 10mls of SH-SY5Y growth media, and were incubated 37℃ and 5% CO_2 until ~80% confluency was reached. For experimentations, 500 µl of a SH-SY5Y cell

suspension (or 2 ml for 6-well plate), diluted in growth media to give the desired cells/ml value, was added to each well of an un-coated 6-well (RT-PCR) or 24-well (Immunocytochemistry) plate. Cells were treated with 200 ng/ml of GDF5 or BMP2, and pre-treated (30 min prior to GDF5 or BMP2 application) with 1 μg/ml of Dorsomorphin, 200 ng/ml of Noggin, 40 μm of Dynasore (Sigma) or 0.3 U/ml of Heparinase III (R&D Systems). For the neurite growth assay, cells were treated daily for 4 DIV (after 1DIV), and, where indicated, labelled with the vital fluorescent dye calcein-AM (1:500; Invitrogen) by incubation at 37℃ for 30 min. To test Smad pathway activation, cells were treated for 0, 5, 15, 30, 60 or 120 min (after 1DIV). To assess the endocytosis inhibition efficiency of dynasore, SHSH5Y cells were incubated in 30 μg/ml of Alexa594-transferrin (Invitrogen), as previously described in a separate study (Heining et al., 2011).

4.1.8 Fixation, blocking and immunocytochemical staining of cultured cells

The 24 well plates were removed from the incubator and medium was aspirated slowly. 500 μl of ice-cold methanol (-20℃: Reagecon) was added to VM cells, and 500 μl of 4% paraformaldehyde (PFA) (4℃) was added to SH-SY5Y cells, before the plates were incubated at -20℃ (RTemp for SH-SY5Ys) for 10 min. Following fixation, the cells were washed three times in 10mM phosphate buffered saline (PBS) containing 0.02% Triton X-100 (PBS-T) for 5 min. The cells were then incubated in 500 μl of 5% bovine serum albumin (BSA) (Sigma) in PBS at RTemp for 1 h. After blocking, the 5% BSA in PBS was removed and the cells were incubated in 250 μl of the desired primary antibodies, which were diluted in 1% BSA in PBS, at 4℃ overnight. The primary antibodies used were: mouse anti-βIII-tubulin (1:300; Medical Supply), rabbit anti-βIII-tubulin (1:300; Millipore) rabbit anti-tyrosine hydroxylase (1:300; Millipore), mouse anti-Sox2 (1:100; R&D Systems), mouse anti-Nestin (1:400; Millipore), mouse anti-Vimentin (1:200; Sigma), mouse anti-glial fibrillary acidic protein (GFAP) (1:300; Sigma), mouse anti-Myelin Basic Protein (MBP) (1:300; Millipore), mouse anti-β-actin (1:200; Sigma), rabbit anti-Smad 1/5/8 (1:200; Santa Cruz), rabbit anti-phopsho-Smad 1/5/8 (1:200; Cell Signalling), rabbit anti-Smad4 (1:100; Millipore), mouse anti-BMPRIa (1:200; R&D Systems), mouse anti-BMPRIb (1:200; R&D Systems), mouse anti-BMPRII (1:200; R&D Systems), phospho-p38 (1:50; Cell Signalling), phospho-JNK

(1:50; Cell Signalling), phospho-Erk (1:50; Cell Signalling) and mouse anti-BrdU (1:4; Millipore). For the mouse anti-BrdU primary antibody, which was diluted in PBS, 130 µl was added to the 24-well plate before incubation at RTemp for 1 h on the shaker. This was carried out after overnight incubation with another primary antibody and three 5 min PBS-T washes for double immunostaining. Following primary antibody application, the primary antibodies were removed and the cells were washed three times in PBS-T for 5 min. The cells were then incubated in 250 µl of Alexa Fluor 488 or 594, or both for double immunostaining, conjugated secondary antibodies (1:500, Invitrogen) reactive to the species of the primary antibodies and diluted in 1% BSA in PBS, at RTemp for 2 h in the dark. The secondary antibodies were removed, and three 5 min PBS-T washes were carried out before the cells were incubated in 300 µl of 4'-6-Diamidino-2-phenylindole (DAPI) (1:3000, Sigma), diluted in PBS, for 5 min at RTemp in the dark. The cells received a final three 5 min washes in PBS, and were stored in PBS at 4℃ in the dark until imaged on an inverted fluorescent microscope (FV1000, Olympus). Negative controls in which the primary antibody was omitted were also prepared.

4.1.9 MTT assay

0.5 mg/ml Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma) solution was prepared by dissolving in HBSS. The media was removed from the cells and 300 µl of MTT solution was added per well. The cells were then incubated with MTT solution for ~4 h at 37℃ and 5% CO_2. The MTT solution was carefully removed, and 100 µl of DMSO was added to each well to lyse the cells. 75 µl of the DMSO solution was pipetted into a 96 well plate, and the absorbance of each sample was determined using a plate reader (Tecan sunrise) at A600.

4.2 Transfection of cultured cells

4.2.1 Electroporation of SH-SH5Y cells and E14 VM cells

Electroporation of cells was carried out using the Neon^{TM} Transfection System (Invitrogen). For freshly-dissected E14 VM cells and cultured SH-SY5Y cells, cell suspensions were prepared for counting as described in sections 4.1.3 and 4.1.6 respectively. However, the resuspension media was antibiotic-free. Following cell counting, the required volume of cells to give 200,000 cells per well was centrifuged

at 1,100 rpm for 5 min. The cell pellet was washed twice with PBS (without $CaCl_2$ and $MgCl_2$) (Sigma), and then resuspended in the required amount of resuspension buffer (12 µl per transfection/plasmid). 0.5 µg of pcDNA3.1-GFP plasmid and 1 µg of desired plasmid DNA (1 µM for siSip1) was added to the resuspended cells. 10 µl of the cell/plasmid DNA mixture was then aspirated into a gold Neon™ Tip using the Neon™ Pipette. This tip, containing the cell/plasmid DNA mixture, was placed into a cuvette, containing 3 ml of the Electrolytic Buffer, in the Neon™ Pipette Station. E14 VM cells were transfected at a voltage of 1100 V (1200 V for SH-SY5Y cells), a width of 30 ms (20 ms for SH-SY5Y cells), and 2 pulses (3 pulses for SH-SY5Y cells). The cells were then added to 50 µl (per well) of antibiotic-free media, and this cell suspension was added to the required number of wells of a 24-well plate (poly-D lysine coated for E14 VM cells). E14 VM cells were allowed to adhere to the surface of the wells for 2 h in the incubator at 37℃ and 5% CO_2. The cells were then incubated with 500 µl of their respected media (outlined above), and incubated at 37℃ and 5% CO_2 for a defined period.

4.3 Analysis of neuronal complexity

4.3.1 E14 VM neuronal complexity analysis

The total neurite length of individual E14 VM neurons, which were either electroporated or immunostained for TH, was measured using Sholl analysis as previously described (Gutierrez and Davies, 2007, Collins et al., 2013). For the analysis of electroporated E14 VM DA neurons, traces of GFP^+/TH^+ neurons were carried out using the CorelDRAW x4 software and analysed as previously described (O'Keeffe et al., 2004a). Briefly, neurite length (NL) was calculated using the following formula; $NL = \alpha \times T \times (\pi/2)$, where α is the number of times the neurite intersects the grid lines, and T is the distance between the gridlines on the magnified image (taking into account the magnification factor). VM neurons with intact processes were analysed from 50 random fields per condition, where any neuron with a process that was ~1.5 times the somal length was deemed an intact process.

4.3.2 SH-SH5Y cell neurite length analysis

20 microscopic fields were randomly selected for each experiment, and photographed using an Olympus IX70 inverted microscope. All cells in each

photograph were measured. The length of the neuritic arborisation was estimated using standard stereological procedures (Mayhew, 1992). A line grid was superimposed on the microscopic images and the number of times each neurite intersected the grid was recorded. The neurite length was calculated using the formula; $NL = \alpha \times T \times (\pi/2)$ as described.

4.4 Polymerase chain reaction (PCR)

4.4.1 RNA isolation

An RNeasy Mini Kit (50) (Qiagen) was used to isolate RNA from tissues. For BMPR developmental expression profile study (see Chapter 3), the VM and striatum from E14 to P90 rats were dissected and, following the extraction of total RNA, semi-quantitative RT-PCR (both described below) for a variety of genes involved in DA development and maintenance (TH, Nurr1, Lmx1b, and Pitx3) (Hegarty et al., 2013c) was performed on the midbrain samples to confirm accuracy of the dissection at each age (Fig. 6.4.1a and data not shown).

700 µl of RLT buffer and 7 µl of β-mercaptoethanol (Sigma) were added to > 2×10^6 SHSY5Y cells (350 µl RLT Buffer and 3.5 µl β-ME were added to < 2×10^6 cells, or fresh E14 rat tissue) in a 1.5 ml eppendorf (Sarstedt). A needle and syringe were used to homogenize the tissue, and the tube was centrifuged for 3 min at 11,000 rpm. The supernatant was transferred to another tube where an equal volume of 70% ethanol (J.T Baker) was added. This mixture was placed in a spin cup and spun for 15 s at 11,000 rpm. The flow through was discarded and the DNA was digested using an Rnase-Free Dnase Set (Qiagen). The membrane of the spin cub was washed in 700 µl RPE buffer and spun at 11,000 rpm. Flow through was discarded. RNA was eluted in 50 µl of nuclease-free water (Ambion) by spinning it for 1 min at 11,000 rpm. RNA concentration was maximized by passing the elute through the membrane a second time. RNA concentration in ng/µl was determined by using a Spectrophotometer (ND1000; NanoDrop Technologies, Inc).

4.4.2 cDNA synthesis

cDNA synthesis was performed using an ImProm-II Reverse Transcription System (Promega). A volume of the sample that contained 1 µg of RNA (or the highest concentration possible between 150-1000 ng) was calculated, and a RNA mix was

prepared using this volume, 0.5 µl oligo dTs, 0.5 µl random primers and nuclease-free water to make up a total volume of 11.5 µl. A negative control RNA mix was also prepared for each sample. The tubes underwent 65℃ for 5 min in a Polymerase Chain Reaction (PCR) machine (Bio-Rad MJ Mini^{TM} Personal Thermal Cycler). A mastermix containing 4 µl 5X buffer, 2 µl $MgCl_2$, 1 µl dNTPs and 0.5 µl Rnasin inhibitor was made up for each tube, and was added along with 1 µl of Reverse Transcriptase enzyme to each tube. The negative controls received 1 µl of nuclease-free water instead of RT enzyme. The tubes then underwent 37℃ for 90 min before dropping to 4℃. cDNA samples were stored at 4℃ for 1-2 days (-20℃ for longer storage) and subsequently used in Reverse Transcriptase-PCR (RT-PCR).

4.4.3 RT-PCR

RT-PCR was carried out using a GoTaq Flexi DNA Polymerase system (Promega). 20.75µl of mastermix containing 5µl 5X PCR buffer, 1.5mM $MgCl_2$, 1.25mM PCR dNTPs and 10.25µl nuclease-free water was added to each tube. 2µl of cDNA sample, 2µl of the desired sense (F) and anti-sense (R) primer mix (10µl of F, 10µl of R, and 80µl nuclease-free water) and 0.25µl of Taq polymerase were also added. The RT negative cDNA samples were used as the negative control. The PCR conditions for the primers used are outlined in table 4.3.1. PCR products were stored at 4℃ for 1-2 days and at -20℃ for longer storage.

4.4.4 Running RT-PCR products in an agarose gel

PCR products were run on a 2.5% agarose gel (2.5g agarose (Sigma) dissolved in 100ml 1X TAE buffer). 5µl of safe view (NBS biologicals) was added to the molten gel, before it was poured into the gel tray to set for 30 min. The gel tray was lowered into the gel rig (Peqlab biotechnologie GmbH) and immersed in 1X TAE. 100 bp ladder (7µl; New England Biolabs) and 7µl of each sample were loaded into the wells (created by a comb in the gel), and the gel was run at 100 V for 1-1.5 hrs using a Bio-Rad Power Pac 200. The gel was imaged with a UV transluminator with UVI Pro software.

Table 4.4.1 PCR Primers

Gene	Species	Primer Sequence	Annealing Temp (°C)	Cycle Number	Product Size (bp)
GAPDH	Rn	**F:** 5'-TGGCACAGTCAAGGCTGAGA-3' **R:** 5'-CTTCTGAGTGGCAGTGATGG-3'	55	25	388
BMPRIa	Rn	**F:** 5'-GGAGGAATCGTGGAGGAATATC-3' **R:** 5'-CATACGCAAAGAACAGCATGTC-3'	55.1	34	464
BMPRIb	Hs	**F:** 5'-GCAGCACAGACGGATATTGT-3' **R:** 5'-TTTCATGCCTCATCAACACT-3'	53	30	630
BMPRII	Hs	**F:** 5'-GCTTCGCAGAATCAAGAACG-3' **R:** 5'GTGGACTGAGTGGTGTTGTG-3'	57	34	349
Smad1	Rn	**F:** 5'-AGTGACAGCAGCATCTTCGTGC-3' **R:** 5'-CGGGTGTATCTCAATCCAGCAG-3'	57	34	276
Smad4	Hs	**F:** 5'-AAGGTGAAGGTGATGTTTG-3' **R:** 5'-GAGCTATTCCACCTACTGAT-3'	56	30	264
Smad5	Rn	**F:** 5'-GGAGGAGTTGGAGAAAGCCTTG-3' **R:** 5'-GGGAGTTGGGATATGTGCTGC-3'	57	34	470
Smad8	Rn	**F:** 5-GTATCATCGCCAGGATGTCA-3' **R:** 5'-TGTGGGGAGCCCATCTGAGT-3'	60	40	104
Sip1	Rn	**F:** 5'-CGCTTGACATCACTGAAGGA-3' **R:** 5'-CTTGCCACACTCTGTGCATT-3'	55	28	224
Lmx1b	Rn	**F:** 5'-CGTGAGCCCGGATGAGTCTGA-3' **R:** 5'-AGGGGTCGCTGCTTCCGTAGG-3'	63.2	32	485
Msx1	Rn	**F:** 5'-GGAGGCCGAGTTGGAGAAGTTGAA-3' **R:**5'-AGAAGGGGTCGGAAGAGGGAGGAG-3'	62.1	32	405
Pitx3	Rn	**F:** 5'-GCAGTAATTCACAGCCTCTCTGG-3' **R:** 5'-GTCCCTGTTCCTGGCCTTAGT-3'	58.8	32	193
Nurr1	Rn	**F:** 5'-CTCTCTCCCGCCTTTCACTCTTCT-3' **R:** 5'-ATTTCGGCGGCGCTTATCCA-3'	60.9	32	434
TH	Rn	**F:** 5'-TGTCACGTCCCCAAGGTTCAT-3' **R:** 5'-GGGCAGGCCGGGTCTCTAAGT-3'	60	32	275
βIII-tubulin	Rn	**F:** 5'-TCACAAGTATGTGCCCAGAGCCATT-3' **R:**5'-GCCTGAATAGGTGTCCAAAGGCCCC-3'	56.8	32	92
GFAP	Rn	**F:** 5'-ACATCGAGATCGCCACCTAC-3' **R:** 5'-ACATCACATCCTTGTGCTCC-3'	54.3	30	219

4.4.5 Quantitative real-time PCR (RT-QPCR)

Midbrain and striatum samples were disrupted and homogenised in 1ml of QIAzol Lysis Reagent (Qiagen). After the addition of 200 µl chloroform, homogenates were separated into aqueous and organic phases by centrifugation at 13,000 rpm for 15 min. The upper aqueous phase was mixed with an equal volume of 70% ethanol, to precipitate the RNA, then transferred to an RNeasy Mini spin column placed in a 2 ml collection tube. Total RNA was purified using the Qiagen RNeasy Lipid Tissue Mini extraction kit and RNase-free DNase set, according to the manufacturer's

instructions. Following purification, total RNA was reverse transcribed using Stratascript reverse transcriptase (Agilent Technologies), for 1 h at 45°C, in a 30 µl reaction according to the manufacturer's instructions.

In order to amplify cDNAs encoding the normalising reference genes, GAPDH, succinate dehydrogenase complex, subunit A (SDHA), and ubiquitin C (UBQC), 2.5µl of cDNA was amplified in a 25 µl PCR reaction containing 1X FastStart Universal SYBR Green Master Mix (Rox) (Roche) and 150 nM forward and reverse primers. In the case of amplifying cDNAs encoding TH, BMPRIb and BMPRII, 2 µl of cDNA was amplified in a 20 µl PCR reaction containing 1X of Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies), 150 nM each forward and reverse primers and 300 nM cDNA specific FAM/BHQ1 dual-labelled hybridization probe (Eurofins), and 3 nM ROX reference dye.

Quantitative real-time PCR amplification was performed using the Stratagene MX3000P thermal cycler. GAPDH, SDHA, and UBQC quantitative real-time PCR amplification products were verified as being correct by melting curve analysis (melting temperatures 83.5°C, 80°C and 85°C, respectively) of the completed PCR reaction. The initial quantities of each cDNA in each PCR reaction was determined by comparison to a standard curve incorporated into the PCR run and constructed from serial dilutions of cDNA reverse transcribed from RNA extracted from P11 striatum and midbrain samples. Values for each gene of interest were normalised to the geometric mean of the three reference genes.

Primer and probe sequences for amplification of each cDNA are listed in table 4.3.2. Cycling parameters for GAPDH, SHDA, and UBQC were 10 min at 95°C followed by 40 cycles of: 95°C for 30 s; 55°C for 1 min; 72°C for 1 min. Cycling parameters for TH, BMPRIb, and BMPRII were 3 min at 95°C followed by 45 cycles of: 95°C for 13 s and 60°C for 30 s.

Table 4.4.2 RT-QPCR Primers and Probes

Gene	Forward primer	Reverse primer	Hybridisation probe
UBQC	5'-ctttgtgaagaccctgac-3'	5'-ccttctggatgttgtagtc-3'	-
SDHA	5'-gctctttcctacccgctcac-3'	5'-gtgtcatagaaatgccatctccag-3'	-
GAPDH	5'-gccttccgtgttcctacc-3'	5'-tagccatattcattgtcatacca-3'	-
TH	5'-tgtcacgtccccaaggttcat-3'	5'-gggcaggccgggtctctaagt-3'	5'-FAM-AAGCACGGTGAACCAATTCC-BHQ1 -3'
BMPRII	5'-gcttcgcagaatcaagaacg-3'	5'-gctaatacagaaaccgatg-3'	5'-FAM-CACCTCCTGATACAACACCAC TC-BHQ1 -3'
BMPRIb	5'-acacgcccatccctcatcaga-3'	5'-acgccactttcccatccacact-3'	5'-FAM-TCACCACGGAGGAAGCCA-BHQ1 -3'

4.5 Western Blotting

4.5.1 Protein extraction from fresh tissue and cultured cells

For cultured cells, the 24-well plates were removed from the incubator, and the medium was aspirated slowly. 150 µl of RIPA lysis buffer consisting of 145.5 µl RIPA buffer (50mM Tris-HCL, 150 mM NaCl, 2 mM EDTA, 0.5% Na-Deoxycholate, 0.1% SDS and 1% Triton X), 1.5 µl of phenylmethylsulfonyl fluoride, 1.5 µl sodium orthovanadate and 1.5 µl of sodium fluoride was added to each well to lyse the cultured cells. The plate was incubated on ice for 1 h and the bottom of each well was then scraped to lift off the adhering cells. The cell suspension was transferred into a 0.5 ml eppendorf tube, which was then centrifuged at 10,000 for 10 min. ~130 µl of the supernatant (containing the protein) was removed and pipetted into a 0.5 ml eppendorf tube. The protein was stored at -20℃.

For freshly dissected rat tissue, the tissue was weighed before a volume of RIPA buffer, containing 1% phenylmethylsulfonyl fluoride, sodium orthovanadate and sodium fluoride, ten times the tissue weight was added to lyse the tissue. The 1.5 ml eppendorf tube containing the tissue and lysis buffer was incubated on ice for 2 h, before the tissue was homogenised using a hand-held dounce homogeniser . The lysed and homogenised tissue was then centrifuged at 10,000 for 10 min. The supernatant was pipetted into a new eppendorf tube and stored at -20℃.

4.5.2 Bradford assay

BSA proteins standards (0 µg/ml – 30 µg/ml) were prepared, and the protein samples were diluted (1:100 in distilled water). 100 µl of each standard and diluted sample was added in triplicate to their designated wells. Bradford reagent (Bio-Rad) was diluted (1:5 in distilled water) and 100 µl of this was added to every well so that there was a total of 200 µl in each well. The plate was read in a plate reader @ A600. The average of the triplicates was calculated and the average blank was subtracted from all the other average values. Protein standard points were plotted on a log graph (Absorbance vs Protein Concentration) and the line of best fit was drawn using GraphPad Prism 5. The sample concentrations in µg/ml were interpolated from the graph using the average sample absobance values. This concentration was multiplied by 100 (dilution factor) to get the true concentration of the samples. The sample with the least amount of protein was chosen, and the concentration of this protein in 10 µl

was calculated. The volume of all other samples containing this concentration of protein was then calculated.

4.5.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blotting

A 4% sodium dodecyl sulphate (SDS) –polyacrylamide stacking gel cast above a 12% resolving gel was used to separate proteins based on molecular weight. Samples were prepared by adding a chosen concentration of protein to an equal volume of sample buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 30% Glycerol, 0.02% Bromophenol Blue) and boiled for 5 min. The total volume of each sample was loaded into the gel alongside 5 µl of molecular weight protein standards (Bio-Rad). Electophoresis was initiated by applying 150V for 60 min to the running apparatus (Bio-Rad) containing Tris-glycine running buffer with SDS (Sigma). The resolving gel was then placed in a transfer sandwich with two fibre pads, two filter papers and a piece of nitrocellulose membrane cut to size. Electrophoretic transfer to the membrane was carried out at 100V for 60 min in a trough of transfer buffer (5.85 g Tris-Base, 2.93 g glycine and 50ml methanol in 1 L distilled water). After the transfer, the membrane was incubated in a 5% blocking solution of dried milk (Marvel original dried skimmed milk) for 60 min on a rocker (~55 oscillations/min) at RTemp. The membrane was washed briefly in tris-buffered saline (TBS) containing 0.05% Tween (TBS-T) (Fisher Scientific) before primary antibody application. The primary antibody used was rabbit anti-phospho-Smad 1/5/8 (1:1000) or mouse anti-β-actin (1:10,000) diluted in 1% BSA in TBS-T, and the membrane was incubated with this overnight at 4℃. The blot underwent three washes in TBS-T for 10 min, and was then incubated for 60 min on a rocker (~55 oscillations/min) at RTemp with anti-rabbit *horseradish peroxidise*-conjugated secondary antibody. The blot was washed twice in TBS-T for 10 min and then washed in TBS once for 10 min. The proteins on the blot were detected using an ECL Plus Western Blotting Detection System (GE Healthcare) and the blot was then developed using an AGFA CP1000 developer.

4.6 Preparation of Smad siRNA expression vectors

4.6.1 Hairpin siRNA oligonucleotide preparation

The Smad4 21 nucleotide small interfering RNA (siRNA) target sequences were chosen based on homology between the mouse, rat and human mRNA sequences, and a GC content of 30-50%. This target sequence was then used as the basis to design the 55 nucleotide siRNA template oligonucleotide. Three different siRNA were prepared for each Smad protein (see table 4.5.1). The oligonucleotides were synthesised by biomers.net. The Smad4.3 siRNA was identified as the most efficient at Smad4 knockdown (Fig. 5.4.7b), and was subsequently used experimentally (see Chapter 5 and 6).

Table 4.6.1 Smad4 siRNA Oligonucleotides

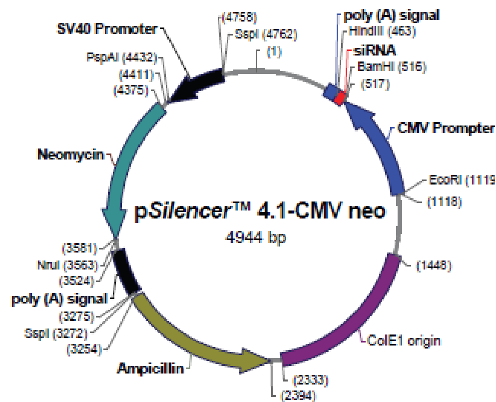
siRNA oligonucleotides:	Sequence:
Smad4 .1 Forward:	5'-GATCC AGGATTTCCTCATGTGATC TTCAAGAGA GATCACATGAGGAAATCCTTT A-3'
Smad4 .1 Reverse:	5'-AGCTT AAAGGATTTCCTCATGTGATC TCTCTTGAA GATCACATGAGGAAATCCT G-3'
Smad4 .2 Forward:	5'-GATCC ACACACCTAATTTGCCTCA TTCAAGAGA TGAGGCAAATTAGGTGTGTAT A-3'
Smad4 .2 Reverse:	5'-AGCTT ATACACACCTAATTTGCCTCA TCTCTTGAA TGAGGCAAATTAGGTGTGT G-3'
Smad4 .3 Forward:	5'-GATCC GGGTCAACTCTCCAATGTC TTCAAGAGA GACATTGGAGAGTTGACCCAA A-3'
Smad4 .3 Reverse:	5'-AGCTT TTGGGTCAACTCTCCAATGTC TCTCTTGAA GACATTGGAGAGTTGACCC G-3'

4.6.2 Cloning hairpin siRNA insert into pSilencer 4.1-CMV vector

The oligonucleotides were diluted to a concentration of 1 µg/µl (based on concentrations given in biomers.net data sheet) in 1X Tris EDTA (Sigma). The sense and antisense siRNA template oligonucleotides were annealed by addition of 2 µl of the sense and 2 µl of the antisense oligonucleotide to 46µl of the 1X DNA Annealing solution (Ambion), and heating this mixture at 90℃ for 3 min before being incubated at 37℃ for 1hr. 5 µl of the annealed siRNA oligonucleotide was diluted in 45 µl of nuclease-free water for a final concentration of 8 ng/µl. 1 µl of the diluted annealed siRNA insert was added to 6 µl nuclease-free water, 1 µl 10X T4 DNA Ligase Buffer (Ambion), 1 µl T4 DNA Ligase (5U/µl; Fermentas), and 1 µl pSilencer 4.1-CMV vector (Ambion) (see Fig. 4.5.1) and this mixture was incubated

overnight at 4℃ for the ligation of the annealed siRNA insert into the pSilencer 4.1-CMV vector. A minus-insert negative control was also prepared by adding 1 µl of 1X DNA Annealing solution in place of the siRNA insert.

Figure 4.6.1 pSilencer 4.1-CMV Neo Vector Map



4.7 Bacterial transformations and plasmid purification

4.7.1 Bacterial transformations

XL-1 blue bacteria were transformed with 25 ng/µl of each of the Smad siRNA expression vectors, and the minus-insert negative control vector. The bacteria and plasmid mixture was incubated on ice for 1 h before being heated at 41℃ for ~70 s. The mixture was replaced on ice, and 450 µl of LB Broth (Sigma) was added to each bacteria and plasmid mixture. The mixture was then incubated in a shaking incubator (~220 oscillations/min; Shel Lab) for 1 h at 37℃. The bacteria and plasmid mixture was spread on a LB Agar (Sigma) 96 mm petri dish containing 50 µg/ml ampicillin (Sigma), and incubated upside-down at 37℃ overnight.

4.7.2 Isolation and culture of transformed bacteria

Isolate a single colony of transformed bacteria from the LB Agar dish (4 bacteria per vector transformation) using a pipette tip, and drop this transformed colony into a sterile 500 ml conical flask (VWR International) of 100 ml of LB Broth containing 50 µl/ml ampicillin. Incubate the flasks in a shaking incubator (~100 oscillations/min) at 37℃ overnight.

4.7.3 Plasmid DNA purification

Plasmid purification was carried out using a Plasmid Midi Kit (Qiagen). Harvest bacterial cells by centrifugation at 6,000 x g for 15 min at 4℃. The bacterial pellet

was re-suspended and lysed using the buffers provided. Lysis buffer was left on no longer than 5 minutes before the addition of the neutralisation buffer to precipitate out the genomic DNA, proteins and cell debris. Mixing was carried out to completely neutralise the solution and effectively precipitate ot SDS. This mixture was incubated on ice for 15 min before centrifugation at 10,000 x g for 1 h at 4℃. 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 elution buffer. 3.5 ml of isopropanol (Sigma) was added to precipitate the DNA, and the precipitate was centrifuged at 15,000 x g for 30 min at 4℃. The supernatant was decanted and the pellet was washed with 2 ml of 70% ethanol. The pellet was re-dissolved in 200 µl of nuclease-free water, and DNA concentration in ng/µl was determined by using a Spectrophotometer. The Smad4 siRNA plasmid DNA samples were sequenced by Eurofins to investigate for any mutations in the sequence.

4.8 *In vivo* GDF5 study

GDF5 was applied to the VM of adult male rats to assess the ability of GDF5 to activate canonical Smad 1/5/8 signalling in DA neurons *in vivo*.

4.8.1 Stereotactic surgery

Stereotactic surgery was conducted under isofluorane anaesthesia (5% in oxygen for induction; 2% in oxygen for maintenance) in a stereotaxic frame with the nose bar set at -2.3 mm. An incision was made through the skin over the skull and the skull was exposed. Following the location of bregma, the stereotaxic arm holding the injection cannula was adjusted to the coordinates of the target SNpc and a drill was used to expose dura over the injection site. The injection needle (diameter 0.13 mm; Hamilton) was connected to a 10 µl microsyringe (Hamilton). The Hamilton syringe was depressed manually to allow the appropriate volume of solution to be delivered. GDF5 was applied to the right SNpc at a final concentration of 10 µg, by injecting 3 µl of 3.33 µg/µl GDF5 (dissolved in 0.01% ascorbate saline) at 1 µl/min, with 2 min for diffusion, at the stereotaxic coordinates AP -5.3, ML ±2.2 (from bregma) and DV -7.2 below dura. Sham surgery was performed by injecting saline in place of GDF5.

Following injection, the incision was sutured and the rats were allowed to recover for 2 before undergoing western blotting.

4.8.2 Postmortem Analysis

For immunohistochemical analysis, the rats were sacrificed by a lethal dose of Euthatal (150 mg/kg sodium pentobarbitone, i.p.) and perfused intracardially with 100 ml of 10 mM PBS, followed immediately by 200 ml of 4% PFA. The brains were removed and placed in 4% PFA. They were then cryoprotected in 30% sucrose, snap frozen in isopentane that was chilled by liquid nitrogen, and subsequently stored at -80°C. The brains were sectioned at 15 µm intervals using a Leica CM1900 cryostat, and each section was placed on a gelatine-coated slide before being stored at 80°C. The desired sections were incubated in blocking solution (10% normal horse serum in 10 mM PBS; 50 µl per section) for 1 h at RTemp. The sections were subsequently incubated in phopsho-Smad 1/5/8 (1:50) and/or TH (1:400) overnight at 4°C. Tissue was then washed in 10 mM PBS (3 x 5 min) and incubated in the dark with Alexa Fluor 488- and/or 594-conjugated secondary antibodies (1:1000) for 1.5 h at RTemp. After washing (10 mM PBS 3 x 5 min), sections were cover-slipped in mounting medium (PVA-DABCO) and visualised using an upright fluorescence microscope (IX70, Olympus).

For western blotting, adult VM and striatum were dissected. Following quick decapitation of the non-anaesthetised rats using a guillotine, the brain was removed and placed on the lid of a glass petri dish filled with ice. Using a curved forceps and blade, the left and right hemispheres were separated. The striatum was then exposed by peeling back the cortex and white matter, before being pinched out using the forceps. For the VM, the hindbrain was removed first. The tissue dorsal and lateral to the VM was then removed to leave the VM region. The tissue then underwent western blotting as described in section 4.5.

4.9 Statistical analysis

Unpaired Student's t-test or one-way ANOVA with a *post hoc* Tukey's test was performed, as appropriate, to determine significant differences between groups. Results were expressed as means with SEM and deemed significant when $p < 0.05$.

5. BMP2 and GDF5 induce neuronal differentiation through a Smad-dependant pathway in a model of human midbrain DA neurons.

5.0 Aims

- Verify SH-SH5Y cells as a suitable cell line to investigate BMP signalling via the canonical Smad 1/5/8 signalling pathway.
- Demonstrate neurotrophic effects of BMP2 and GDF5 in SH-SY5Y cells.
- Identify the receptors and intracellular signalling pathway(s) that mediate the neurotrophic effects of BMP2 and GDF5.

5.1 Abstract

Parkinson's disease is the second most common neurodegenerative disease, and is characterized by the progressive degeneration of the nigrostriatal DA system. Current treatments are symptomatic, and do not protect against the DA neuronal loss. One of the most promising treatment approaches is the application of neurotrophic factors to rescue the remaining population of nigrostriatal DA neurons. Therefore, the identification of new neurotrophic factors for midbrain DA neurons, and the subsequent elucidation of the molecular basis of their effects, is important. Two related members of the BMP family, BMP2 and GDF5, have been shown to have neurotrophic effects on midbrain DA neurons both *in vitro* and *in vivo*. However, the molecular (signalling pathway(s)) and cellular (direct neuronal or indirect via glial cells) mechanisms of their effects remain to be elucidated. Using the SH-SH5Y human neuronal cell line, as a model of human midbrain DA neurons, we have shown that GDF5 and BMP2 induce neurite outgrowth via a direct mechanism. Furthermore, we demonstrate that these effects are dependent on BMP type I receptor activation of the canonical Smad 1/5/8 signalling.

5.2 Introduction

PD is the second most common neurodegenerative disease and is characterized by motor symptoms, including bradykinesia, akinesia and resting tremor. The pathological hallmark of the disease is the progressive degeneration of DA neurons that project from the midbrain to the striatum. Despite fifty years of investigation, the mainstay of treatment is symptomatic, involving exogenous L-dopa or DA receptor agonists, but these treatments do not protect against the DA neuronal loss which continues unabated (Toulouse and Sullivan, 2008). A large variety of experimental treatment strategies have been proposed, but one promising approach is neurotrophic factor therapy. This involves the addition of neurotrophic factors to the brain to rescue the remaining DA neurons (Sullivan and Toulouse, 2011). An intensive research effort has identified GDNF as a potent DA neurotrophic factor (Lin et al., 1993). GDNF promotes the survival of midbrain DA neurons *in vitro* and *in vivo* but, despite initial successes in open-label clinical trials (Gill et al., 2003, Patel et al., 2005, Slevin et al., 2005), a double-blind placebo-controlled clinical trial showed no beneficial effect of GDNF administration to the striatum (Lang et al., 2006). Thus, it is important that new neurotrophic factors are identified and that the molecular basis of their effects on midbrain DA neurons are elucidated.

GDNF is a member of the TGFβ superfamily which is a large family of structurally-related molecules that are grouped into subfamilies based on sequence similarities. These subfamilies include the GDNF family, the BMPs, GDFs and others (Miyazono et al., 2001). Members of the BMP and GDF families have been shown to play diverse roles in the development and function in a variety of tissues, but in particular they play critical roles in skeletal development (Miyazono et al., 2010, Xiao et al., 2007). In recent years, members of the BMP and GDF families have been shown to play key roles as neurotrophic factors that regulate the development of the nervous system, as well as its maintenance in adulthood (Hegarty et al., 2014c, Liu and Niswander, 2005). Two of the most extensively-studied members of these families are GDF5 and BMP2. Both of these factors possess the characteristic cysteine-knot motif, a structural hallmark of members of the TGFβ superfamily, and share 52% sequence similarity (Sullivan and O'Keeffe, 2005).

GDF5 expression in developing rat VM correlates with the development of midbrain DA neurons (O’Keeffe et al., 2004b). It promotes the survival and growth

of these neurons both *in vitro* (Krieglstein et al., 1995b, O'Keeffe et al., 2004b, Wood et al., 2005) and *in vivo* (Costello et al., 2012, Hurley et al., 2004, Sullivan et al., 1997, Sullivan et al., 1999, Sullivan et al., 1998b). Similarly, BMP2 promotes the survival and growth of midbrain DA neurons *in vitro* (Reiriz et al., 1999, Jordan et al., 1997) and *in vivo* (Espejo et al., 1999). Despite these findings, the molecular mechanisms that mediate the neurotrophic effects of GDF5 and BMP2 on midbrain DA neurons are unknown. Furthermore, it is unclear whether BMP2 and GDF5 have direct effects on VM DA neurons. Indeed, it has been suggested that the DA neurotrophic effects of BMP2 and GDF5 may be indirectly mediated by glial cells (Sullivan and O'Keeffe, 2005), due to the concomitant increase in astrocytes in GDF5- and BMP2-treated E14 rat VM cultures (O'Keeffe et al., 2004b, Krieglstein et al., 1995b, Reiriz et al., 1999).

During skeletal development, GDF5 and BMP2 are known to act through a canonical pathway involving the activation of two cell-surface serine/threonine kinase receptors, type I and type II BMPRs (Miyazono et al., 2010, Sieber et al., 2009). Upon ligand binding, the constitutively-active BMPRII transphosphorylates the cytoplasmic domain of the BMPRI (BMPRIa or BMPRIb) which, through a series of protein-protein interactions, phosphorylates Smad proteins that translocate to the nucleus and modulate gene transcription. In recent years, both GDF5 and BMP2 have also been shown to signal via non-canonical, Smad-independent pathways, which involve the activation of a variety of intracellular pathways, including ERK, JNK and p38 MAPK, depending on the cellular context (Derynck and Zhang, 2003, Moustakas and Heldin, 2005). In the present study, SH-SY5Y neuroblastoma cells, which are widely-used models of human DA neurons and are a pure neuronal population, were used to investigate the molecular (signalling pathway(s)) and cellular (direct neuronal or indirect via glial cells) mechanisms mediating the neurotrophic effects of GDF5 and BMP2.

5.3 Results

5.3.1 BMP2 and GDF5 induce the neuronal differentiation in SH-SH5Y cells

Firstly, the expression of BMPRs and Smad proteins in the SH-SY5Y cell line was examined. To do this, SH-SY5Y cells were cultured for three days before being fixed and processed for immunocytochemistry. Alternatively, RNA was prepared from cultured cells for RT-PCR analysis of gene expression. RT-PCR analysis showed that the SH-SY5Y cell line expresses mRNAs for the BMPRs, BMPRII and BMPRIb (Fig. 5.4.1A) along with the receptor-regulated Smads, Smad1, Smad5 and Smad8 (Smad 1/5/8), and the common-mediator Smad, Smad4 (Fig. 5.4.1A). Immunocyochemistry showed strong expression at the protein level of BMPRII and BMPRIb (Fig. 5.4.1B). Similarly, immunocytochemistry for Smads 1/5/8 revealed that these proteins were strongly expressed, displaying a predominantly cytoplasmic distribution in unstimulated cells (Fig. 5.4.1C). Negative controls were performed for all immunocytochemical analyses to confirm the specificity of the primary antibodies (data not shown).

In cultures of E14 rat VM, GDF5 and BMP2 have been shown to induce differentiation of DA neurons, as evident from the increased morphological arborisation of treated cells (O'Keeffe et al., 2004b, Reiriz et al., 1999). To directly compare the effects of GDF5 and BMP2 on neuronal differentiation, using similar morphological parameters, the phenotypic effects of GDF5 and BMP2 on SH-SY5Y cells were assessed using a MTT assay. A decrease in the MTT absorbance in this assay may be indicative of an increase in cellular differentiation. SH-SY5Y cells were treated with 200 ng/ml of BMP2 or GDF5 daily, before a MTT assay was performed on 1 day *in vitro* (DIV), 2 DIV and 4 DIV. Both BMP2 and GDF5 significantly reduced, to a similar extent, the MTT absorbance at 4 DIV compared to the untreated control (Fig. 5.4.1D). These data suggest that BMP2 and GDF5 may promote the differentiation of SH-SY5Y cells.

To more directly assess differentiation, a morphological assessment of the neurite complexity in GDF5 and BMP2-treated SH-SY5Y cells was performed. SH-SY5Y cells were treated with BMP2 or GDF5 daily before being immunocytochemically stained for β-actin at 4 DIV, to allow visualisation of the cytoskeleton. The total neurite length was then measured using a modified line intercept method (Mayhew, 1992). Treatment with either BMP2 or GDF5 for 4DIV

resulted in a significant increase in the total length of neurites when compared to untreated controls (Fig. 5.4.1E, F). There was no significant difference in the number of cells analysed between the groups (data not shown). These data show that, similar to primary cultures of the E14 rat midbrain (O'Keeffe et al., 2004b, Reiriz et al., 1999), BMP2 and GDF5 induce neuronal differentiation in SH-SY5Y cells.

5.3.2 BMP2 and GDF5 activate canonical Smad 1/5/8 signalling in SH-SY5Y cells

To determine the molecular basis of this neurotrophic effect, the temporal kinetics of the activation of the canonical (Smad 1/5/8) and non-canonical (MAPK) signalling pathways by BMP2 and GDF5 were examined. SH-SY5Y cells were treated with BMP2 or GDF5 at 6 different time points (0, 5, 15, 30, 60, 120 min), and were then immunocytochemically stained for phospho-Smad 1/5/8, phospho-p38, phospho-JNK, and phospho-Erk. Densitometric analysis of the nuclear levels of phospho-Smad 1/5/8 showed that both BMP2 and GDF5 significantly increased the amount of nuclear phospho-Smad 1/5/8, although with different temporal profiles, compared to the untreated control (0 min). BMP2 treatment increased nuclear phospho-Smad 1/5/8 levels at all time points examined (Fig. 5.4.2A, B), whereas an increase in nuclear phospho-Smad was not detected until one hour post-GDF5 treatment (Fig. 5.4.2C, D). Both BMP2 and GDF5 reduced the basal (0 min) signalling levels of the p38, JNK and ERK MAPK pathways, as determined by densitometry of their phosphorylated forms at the different treatment time points (Fig. 5.4.3). These data suggest that the effects of BMP2 and GDF5 on the differentiation of SH-SY5Y cells (Fig. 5.4.1) may be mediated through the activation of the canonical Smad 1/5/8 signalling pathway.

5.3.3 Dorsomorphin prevents BMP2- and GDF5-induced neuronal differentiation and Smad activation in SH-SY5Y cells

To explore this premise, dorsomorphin, a small molecular inhibitor of BMPRI (Yu et al., 2008), was used to determine whether the effects of BMP2 and GDF5 were mediated by the BMPRI. To determine a working concentration of dorsomorphin, an array of concentrations was used, ranging from 100 ng/ml to 200 μg/ml. Dorsomorphin concentrations above 2 μg/ml caused non-selective SH-SY5Y cell death after 1 DIV (Fig. 5.4.4A). At concentrations of 1 μg/ml and lower, SH-SY5Y

cells were unaffected by daily dorsomorphin treatments for up to 4 DIV (the duration of BMP2 and GDF5 treatment in neurite growth assay), with no observable change in their cellular morphology compared to the control (Fig. 5.4.4B). An MTT assay performed at 4 DIV confirmed that daily treatments with 1 µg/ml of dorsomorphin did not significantly affect the viability of SH-SY5Y cells (Fig. 5.4.4C).

Pre-treatment of SH-SY5Y cells with 1 µg/ml of dorsomorphin completely prevented the BMP2- and GDF5-induced decreases in MTT absorbance at 4DIV (Fig. 5.4.5A). Similarly, when SH-SY5Y cells were pre-treated with dorsomorphin, BMP2 and GDF5 failed to induce any significant increase in the total neurite length compared to the untreated control group (Fig. 5.4.5B, C). There was no significant difference in the number of cells analysed between the groups (data not shown). To determine if dorsomorphin inhibited BMP2- and GDF5-induced Smad activation (Fig. 5.4.2), SH-SY5Y cells were pre-treated with dorsomorphin prior to the addition of BMP2 and GDF5, and the levels of nuclear phospho-Smad 1/5/8 were assessed and compared to non-dorsomorphin-treated, BMP2- and GDF5-treated controls. Dorsomorphin completely prevented the BMP2- (Fig. 5.4.5D) and GDF5- (Fig. 5.4.5E) induced activation of the Smad 1/5/8 signalling pathway. These data suggest that the phenotypic effects of BMP2 and GDF5 on neuronal differentiation may be directly mediated through the BMPR-dependent canonical Smad 1/5/8 pathway.

5.3.4 Canonical BMPR-Smad activation induces neuronal differentiation in SH-SY5Y cells.

It is well established that BMP2 can signal through both BMPRIa and BMPRIb, whereas GDF5 predominantly signals through BMPRIb (Nishitoh et al., 1996). This suggests that BMP2 and GDF5 may signal through BMPRIb to induce differentiation. To examine this, we transfected SH-SY5Y cells with a constitutively active BMPRIb (caBMPRIb) plasmid, which induced a significant increase in total neurite length compared to cells transfected with the relevant control plasmid (Fig. 5.4.6A, B). In agreement with this finding, caBMPRIb-transfected cells had significantly increased levels of nuclear phospho-Smad 1/5/8 when compared to controls (Fig. 5.4.6C, D). Importantly, cells transfected with a control plasmid displayed the same level of nuclear phospho-Smad 1/5/8 as non-transfected neurons, indicating that the transfection procedure did not alter the relative activation of this pathway (Fig. 5.4.6C, D).

To determine a functional link between BMPRIb-induced Smad activation and SH-SY5Y differentiation, siRNA against the co-Smad, Smad4, was developed. The association of phosphorylated Smad 1/5/8 proteins with Smad4 following BMPRIb activation is required for the effects of Smad 1/5/8 on gene transcription (Fig. 5.4.7A). Firstly, SH-SY5Y cells were transfected with Smad4 siRNA, with a transfection efficiency of 25%. When Smad4 expression was analysed by RT-PCR in these cultures, there was a clear reduction in Smad4 mRNA expression in Smad4 siRNA transfected cells (Fig. 5.4.7B). To explore this at the protein level, immunocytochemistry was used to examine Smad4 expression in individual cells transfected with Smad4 siRNA. Smad4 siRNA results in a sustained and consistent knockdown of Smad4 protein in Smad4 siRNA-transfected cells (Fig. 5.4.7C). To determine if modulation of Smad4 affected the differentiation of these cells, total neurite length of cells transfected with Smad4 siRNA or with Smad4 overexpression vectors was measured. In agreement with the dorsomorphin data (Fig. 5.4.5), modulation of Smad4 expression did not affect total neurite length in transfected SH-SH5Y cells (Fig. 5.4.7D). When SH-SY5Y cells were co-transfected with the caBMPRIb and Smad4 siRNA, Smad4 siRNA completely prevented the caBMPRIb-mediated significant increase in average neurite length in transfected cells (Fig. 5.4.7E, F). These data suggest that BMPR-mediated canonical Smad 1/5/8 signalling mediates the neurotrophic effects of BMP2 and GDF5. To explore this directly, SH-SY5Y cells were transfected with Smad4 siRNA and treated with BMP2 and GDF5. It was found that, in cells expressing Smad4 siRNA, BMP2 and GDF5 did not promote neurite outgrowth (Fig. 5.4.8A, B). Collectively, these data show that activation of canonical BMPRIb-Smad 1/5/8 signalling by BMP2 and GDF5 can induce neuronal differentiation.

5.4 Figures and Figure Legends

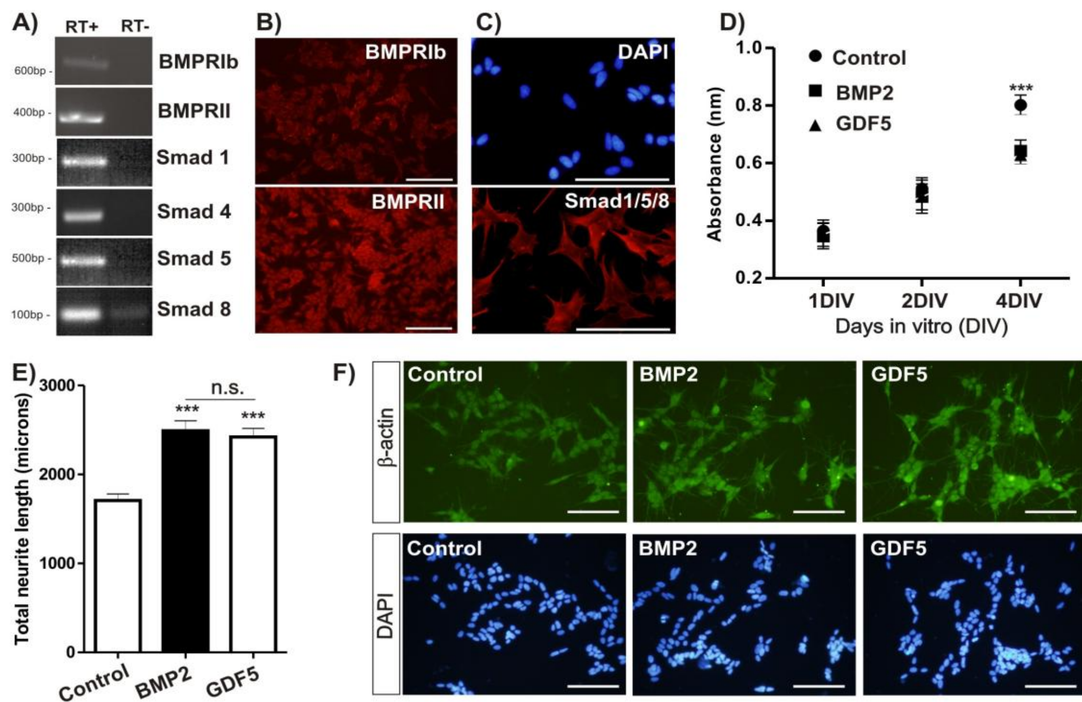


Figure 5.4.1: BMP2 and GDF5 induce neuronal differentiation in SH-SY5Y cells

(A) RT-PCR analysis of BMPR1b and BMPRII, and of Smads 1, 4, 5, and 8 in SH-SY5Y cells. A 100bp ladder was used to determine the size for each PCR product, and RT-PCR of GAPDH was used as a positive control (not shown). Representative photomicrographs of SH-SY5Y cells immunocytochemically stained for the BMPRs, (B) BMPRIb and BMPRII, or (C) Smads 1/5/8. (D) MTT assay of BMP2- and GDF5-treated (daily) SH-SY5Y cells at 1, 2 and 4 DIV, as indicated (*** $P < 0.001$ vs BMP2/GDF5 4 DIV; ANOVA with post-hoc Tukey's test; 4 groups for each treatment per experiment. Number of experimental repetitions (N) = 4). (E) Length of total neurites of BMP2- and GDF5-treated (daily for 4 DIV) SH-SY5Y cells, as indicated (*** $P < 0.001$ vs control; ANOVA with post-hoc Tukey's test; 20 images analysed for each group per experiment. N = 3). Data are expressed as mean ± SEM. (F) Representative photomicrographs of control, BMP2- and GDF5-treated SH-SY5Y cells, as indicated, immunocytochemically stained for β-actin and counterstained with DAPI. Scale bar = 100 μm.

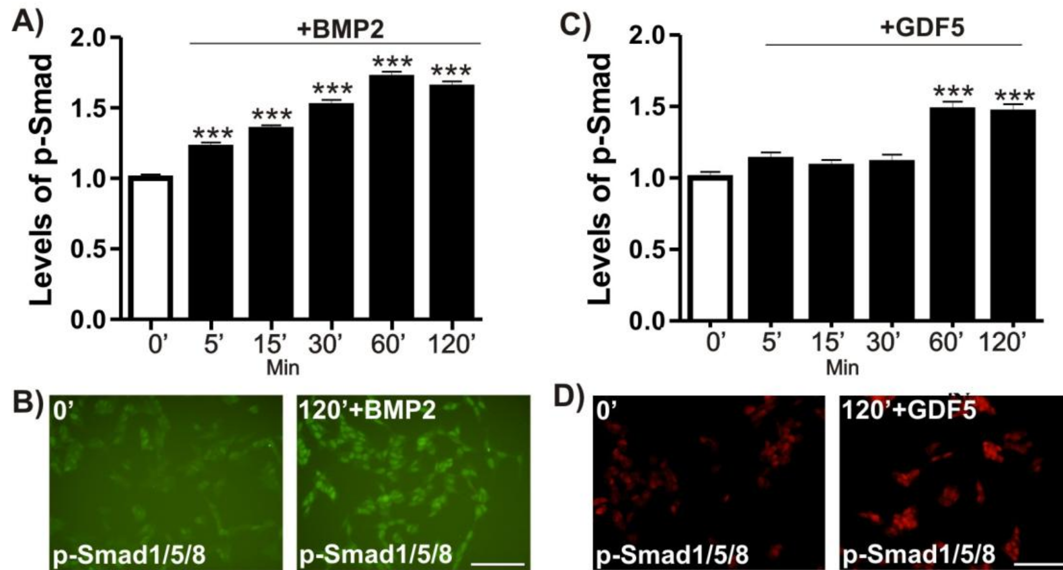


Figure 5.4.2: BMP2 and GDF5 activate the Smad 1/5/8 signalling pathway in SH-SY5Y cells

Relative immunofluorescence intensity of (A) BMP2- and (C) GDF5-treated SH-SY5Y cells, at 0 (untreated control), 5, 15, 30, 60 and 120 min, expressing phospho-Smad 1/5/8 as determined by densitometry (Image J) (*** $P < 0.001$ vs 0 min; One-way ANOVA and post hoc Tukey's test; 50 cells for each group per experiment. N = 3). Data are expressed as mean ± SEM. Representative photomicrographs of (B) BMP2- and (D) GDF5-treated SH-SY5Y cells immunocytochemically stained for phospho-Smad 1/5/8 at 0 and 120 min. Scale bar = 100 μm.

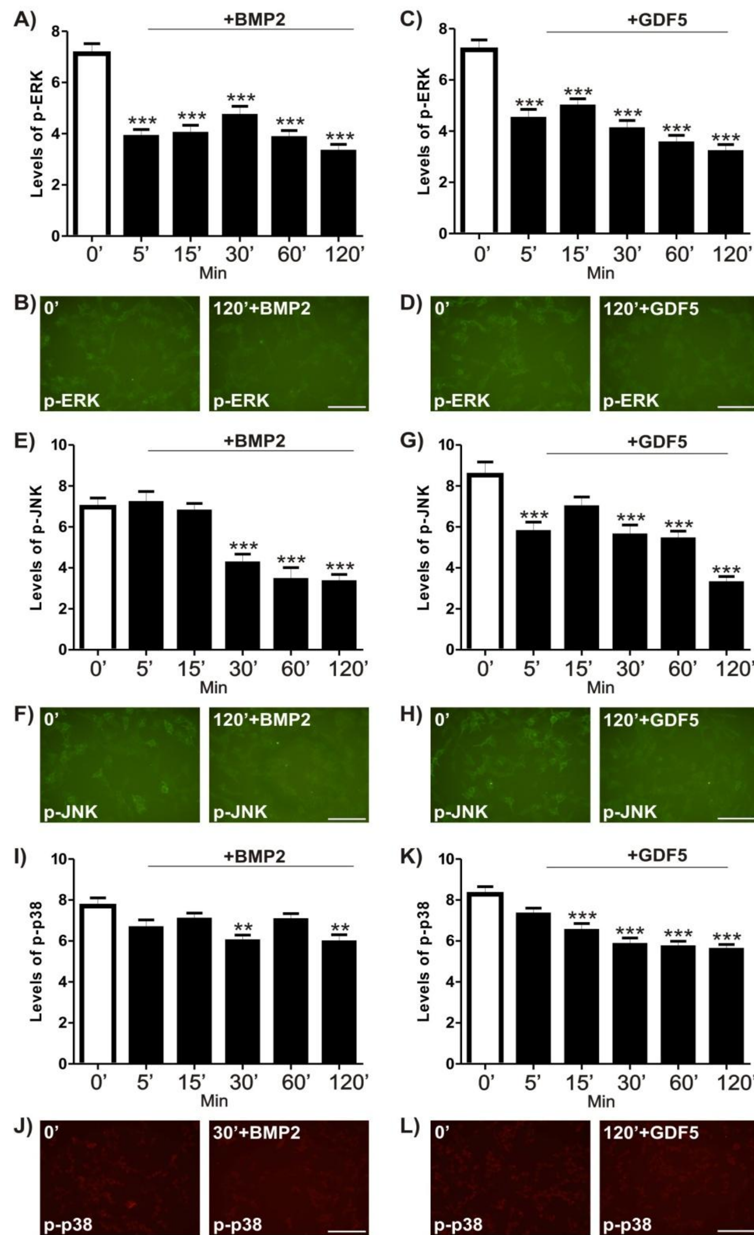


Figure 5.4.3: **BMP2 and GDF5 inhibit MAPK signalling in SH-SY5Y cells.** Relative immunofluorescence intensity of BMP2- and GDF5-treated SH-SY5Y cells, at 0 (untreated control), 5, 15, 30, 60 and 120 min, expressing (A and C) phospho-ERK, (E and G) phospho-JNK and (I and K) phospho-p38, as indicated (*** $P < 0.001$ vs 0 min; One-way ANOVA and post hoc Tukey's test; 50 cells for each group per experiment. N = 3). Data are expressed as mean ± SEM. Scale bar = 100 μm. Representative photomicrographs of BMP2- and GDF5-treated SH-SY5Y cells immunocytochemically stained for (B and D) phospho-ERK, (F and H) phosho-JNK and (J and L) phospho-p38 at 0 and 30/120 min. Scale bar = 100 μm.

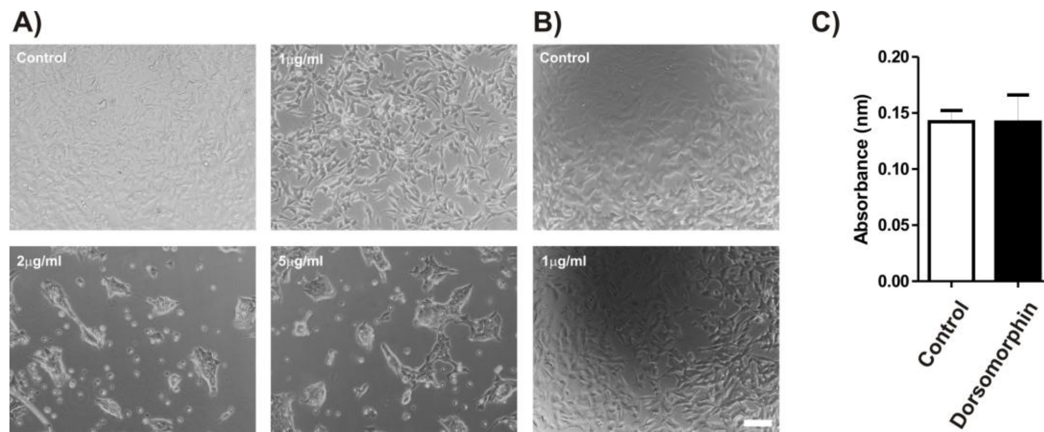


Figure 5.4.4: Working concentrations of dorsomorphin do not affect cell viability of SH-SY5Y cells

Representative phase contrast photomicrographs of dorsomorphin-treated SH-SY5Y cells at (A) 1 DIV and (B) 4 DIV. Scale bar = 100 µm. (C) MTT assay of dorsomorphin-treated (daily) SH-SY5Y cells after 4 DIV. No significant difference ($P < 0.05$; Student's *t*-test; 4 measurements for each group per experiment. N=4) was observed. Data are expressed as mean ± SEM.

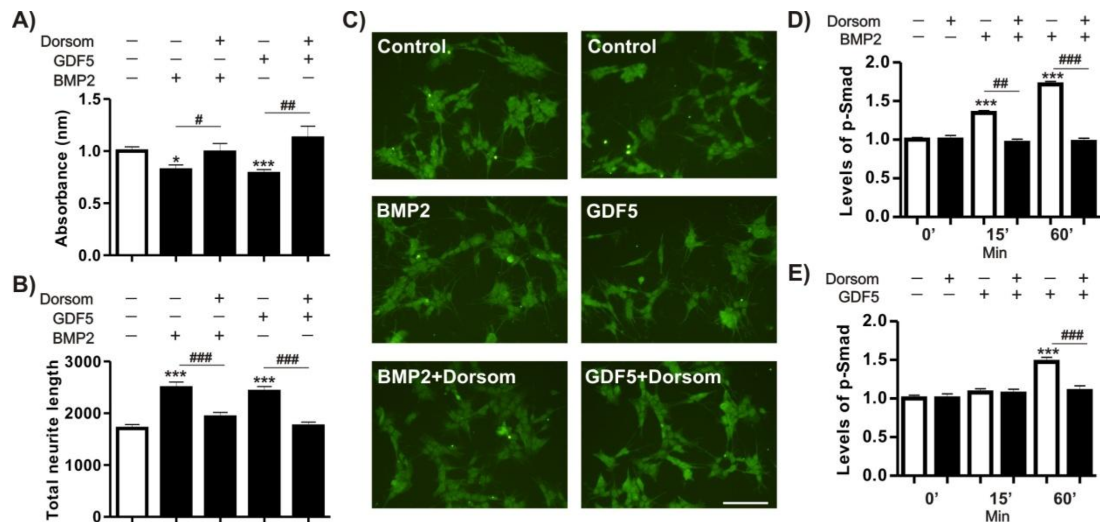


Figure 5.4.5: Dorsomorphin prevents BMP2- and GDF5-induced neuronal differentiation of SH-SY5Y cells

(A) MTT assay to assess cellular respiration of dorsomorphin pre-treated and/or BMP2- and GDF5-, treated (daily for 4DIV) SH-SY5Y cells, as indicated (*** $P < 0.001$, * $P < 0.05$ vs control; # $P < 0.05$, ## $P < 0.01$ vs BMP2/GDF5; ANOVA with post-hoc Tukey's test; 4 measurements for each group per experiment. N = 4). (B) Total neuritic length of dorsomorphin pre-treated and/or BMP2- and GDF5-treated (daily for 4DIV) SH-SY5Y cells, as indicated (*** $P < 0.001$ vs control; ### $P < 0.001$ vs BMP2/GDF5; ANOVA with post-hoc Tukey's test; 20 images analysed for each group per experiment. N = 3). (C) Representative photomicrographs of dorsomorphin pre-treated and BMP2- and GDF5-treated SH-SY5Y cells, as indicated, immunocytochemically stained for β-actin. Scale bar = 100µm. Relative immunofluorescence intensity of dorsomorphin pre-treated, and/or (D) BMP2- and (E) GDF5-treated SH-SH5Y cells, at 0 (control), 15 and 60 min, expressing phospho-Smad 1/5/8 as determined by densitometry (Image J), as indicated (*** $P < 0.001$ vs 0 min; ## $P < 0.01$, ### $P < 0.001$ vs BMP2/GDF5; One-way ANOVA and post hoc Tukey's test; 50 cells for each group per experiment. N = 3). Data are expressed as mean ± SEM.

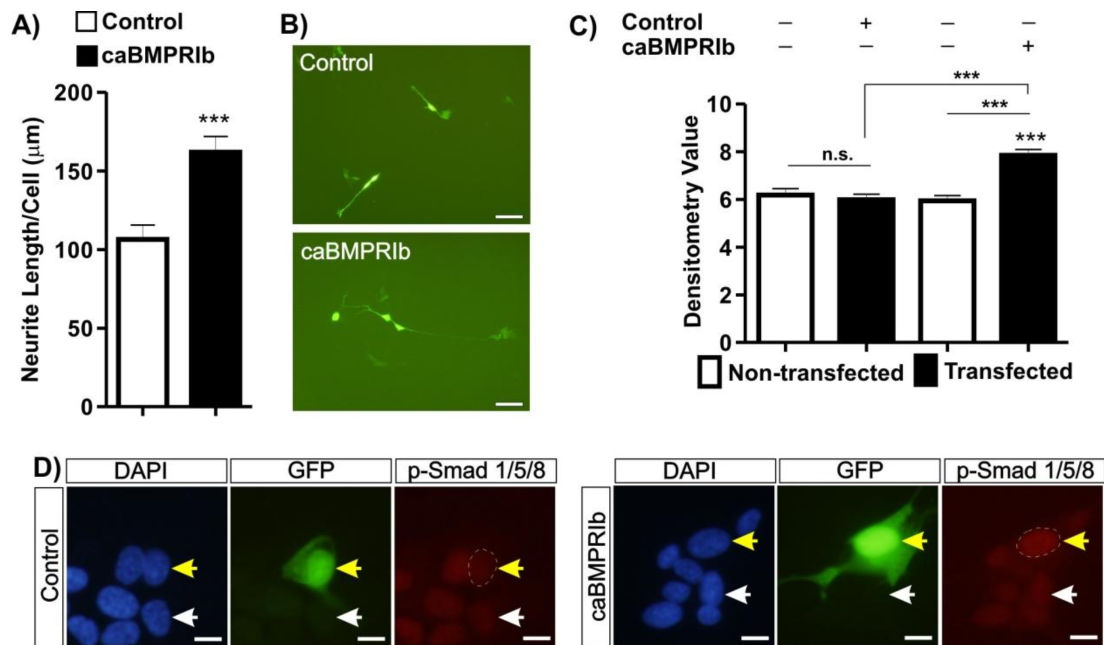


Figure 5.4.6: Activated BMPRIb induces neuronal differentiation and Smad 1/5/8 signalling in SH-SY5Y cells.

A) Neurite length of caBMPRIb-transfected SH-SY5Y cells, as indicated (*** $P < 0.001$ vs control; ANOVA with post-hoc Tukey's test; 40 cells for each group per experiment. N = 3). (B) Representative photomicrographs of control plasmid- and caBMPRIb plasmid-transfected SH-SY5Y cells expressing GFP. Scale bar = 25 μm. (C) Relative immunofluorescence intensity of caBMPRIb-transfected SH-SY5Y cells expressing phospho-Smad 1/5/8 as determined by densitometry (Image J), as indicated (*** $P < 0.001$ vs 0 min; One-way ANOVA and post hoc Tukey's test; 50 cells for each group per experiment. N = 3). Data are expressed as mean ± SEM. (D) Representative photomicrographs of control plasmid- and caBMPRIb plasmid-transfected (yellow arrows), and non-transfected (white arrows) SH-SY5Y cells immunocytochemically stained for phospho-Smad 1/5/8 and counterstained with DAPI. Scale bar = 25 μm.

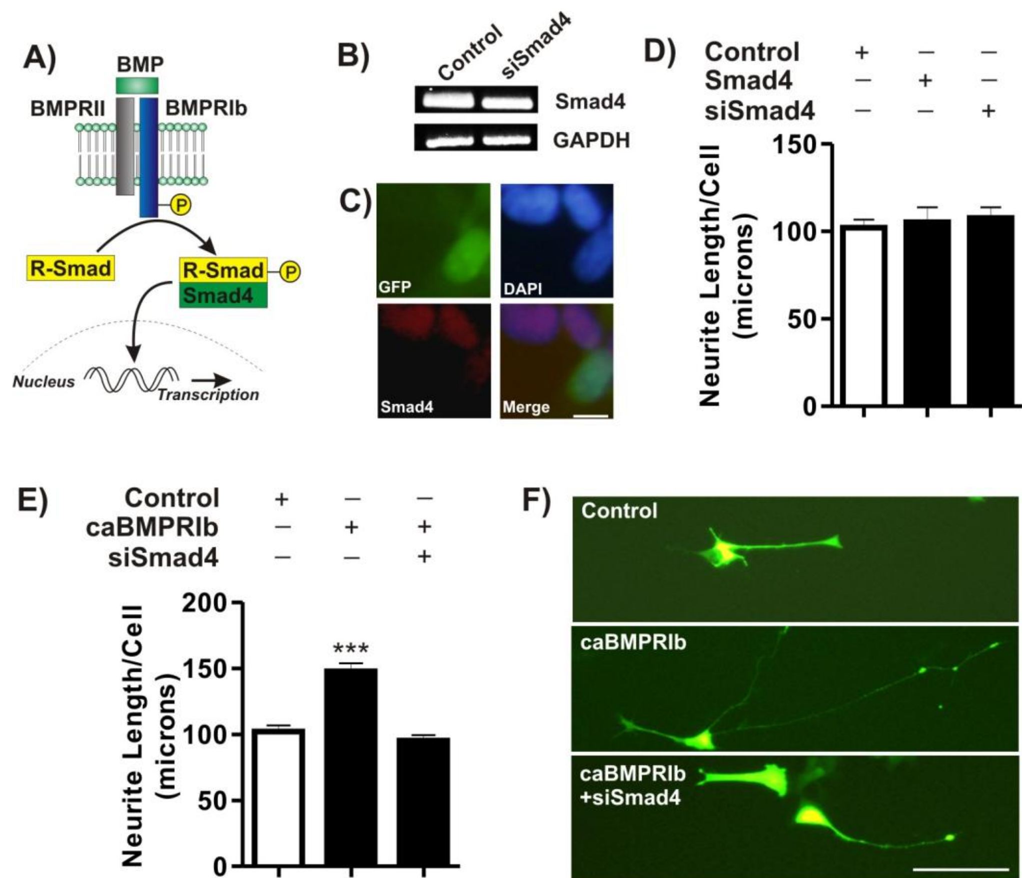


Figure 5.4.7: Inhibition of nuclear translocation of phosphorylated Smad 1/5/8 prevents BMPRIb-mediated induction of SHSY5Y neuronal differentiation.

(A) Graphical representation of the role of Smad4 in canonical BMP-Smad 1/5/8 signalling. (B) RT-PCR analysis of Smad4 and GAPDH (positive control) in siSmad4 (Smad4 siRNA)-transfected SH-SY5Y cells. (C) Representative photomicrographs of siSmad4-transfected SH-SY5Y cells immunocytochemically stained for Smad4 and counterstained with DAPI. Scale bar = 25 μm (D) Neurite length of Smad4- or siSmad4-transfected SH-SY5Y cells. No significant difference ($P < 0.05$; ANOVA with post-hoc Tukey's test; 40 cells for each group per experiment. N = 3) was observed between the groups. (E) Neurite length of caBMPRIb- and/or siSmad4-transfected SH-SY5Y cells, as indicated (*** $P < 0.001$ vs control; ANOVA with post-hoc Tukey's test; 40 cells for each group per experiment. N = 3). Data are expressed as mean ± SEM. (F) Representative photomicrographs of caBMPRIb- and/or siSmad4-transfected SH-SY5Y cells expressing GFP. Scale bar = 50 μm.

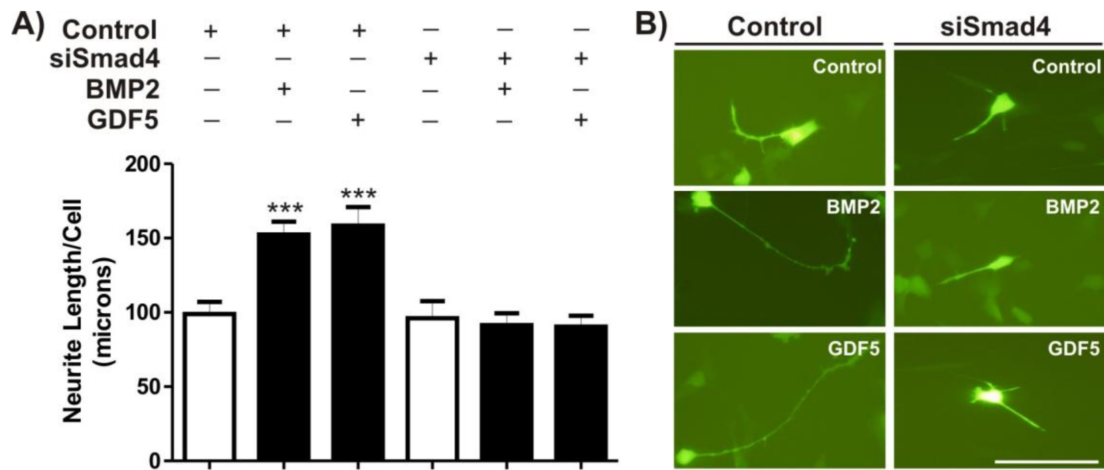


Figure 5.4.8: Inhibition of nuclear translocation of activated Smad 1/5/8 prevents BMP2- and GDF5-mediated induction of SHSY5Y neurite outgrowth. (A) Neurite length of control plasmid- and siSmad4-transfected SH-SY5Y cells with or without BMP2 or GDF5 treatment, as indicated (*** $P < 0.001$ vs control; ANOVA with post-hoc Tukey's test; 40 cells for each group per experiment. N = 3). Data are expressed as mean ± SEM. (B) Representative photomicrographs of control plasmid- and siSmad4-transfected, GFP-expressing, SH-SY5Y cells with or without BMP2 or GDF5 treatment. Scale bar = 100 μm.

5.5 Discussion

The neurotrophic effects of BMP2 (Espejo et al., 1999, Reiriz et al., 1999, Jordan et al., 1997) and in particular GDF5 (Costello et al., 2012, Hurley et al., 2004, Krieglstein et al., 1995b, O'Keeffe et al., 2004b, Sullivan et al., 1999, Wood et al., 2005), in particular, have been well documented in primary VM neural cultures and in animal models of Parkinson's disease. However, the downstream molecular mechanisms that mediate the neurotrophic effects of GDF5 and BMP2 on VM DA neurons are unknown. In an attempt to define these mechanisms, the present study used the SH-SY5Y neuroblastoma cell line, which has been used as a model of human VM DA neurons (Toulouse et al., 2012, Xie et al., 2010) and have been shown to be capable of differentiation into DA neurons (Gomez-Santos et al., 2002, McMillan et al., 2007, Presgraves et al., 2004, Xie et al., 2010).

This study initially characterised SH-SY5Y cells with regards to the expression of BMPRs and Smad transcription factors. It was confirmed that both type I and type II BMPRs are expressed on SH-SY5Y cells. In addition, it was shown that the principal signal transduction machinery for BMPs, Smad proteins 1, 5 and 8, are present in SH-SY5Y cells (including in their activated form). In addition, the presence of Smad4, which is required for the nuclear translocation of Smads 1/5/8, was verified. These results demonstrate that SH-SY5Y cells have the machinery to carry out canonical Smad 1/5/8 signalling in response to BMPs.

A common neurotrophic effect of BMP2 and GDF5 on VM DA neurons *in vitro* is the induction of neurite outgrowth (O'Keeffe et al., 2004b, Reiriz et al., 1999). This study has demonstrated that both BMP2 and GDF5 induce the neuronal differentiation of SH-SY5Y cells. BMP2- and GDF5-induced neurite extension and growth arrest in proliferating SH-SY5Y cells, which is consistent with previous results shown for BMP2 (Nakamura et al., 2003) and GDF5 (Toulouse et al., 2012) in SH-SY5Y cells. It has been proposed that BMP2 and GDF5 exert their neurotrophic effects on DA neurons indirectly through an action on glial cells that are present in mixed neural cultures of E14 rat VM (Sullivan and O'Keeffe, 2005), due to the concomitant increase in astrocytes in GDF5- and BMP2-treated E14 rat VM cultures (O'Keeffe et al., 2004b, Krieglstein et al., 1995b, Reiriz et al., 1999). However, the present study shows that the neurotrophic effects of GDF5 and BMP2 are mediated directly on neuronal-like cells, since there are no other cell types

present in SH-SY5Y cell cultures. This is in agreement with previous evidence showing that GDF5 still exerts its survival-promoting neurotrophic effects in glial-depleted VM cultures (Wood et al., 2005). A similar approach was used to demonstrate that the neurotrophic effects of BMP2 on striatal neurons were as a result of direct neuronal action (Gratacos et al., 2001).

In order to examine the mechanism of this neuronal action of GDF5 and BMP2, a small molecular inhibitor of BMPRI, termed dorsomorphin (Yu et al., 2008), was used. This experiment showed that the neurotrophic effects of BMP2 and GDF5 are dependent upon BMPRI activation. Dorsomorphin prevented BMP2- and GDF5-induced neuronal differentiation, and activation of Smad 1/5/8 signalling, in SH-SY5Y cells. This finding is similar to that of Parikh et al. (2011), who showed that dorsomorphin-induced inhibition of BMP7 signalling negatively affects BMP-induced neurite outgrowth of E18.5 mouse hippocampal neurons. The present study also showed that BMP2 and GDF5 activate Smad 1/5/8 signalling to the same extent, but that the kinetics of this Smad activation differed between the two ligands. BMP2 treatment resulted in nuclear translocation of activated Smad proteins from 5 min onwards, whereas following GDF5 application this translocation was not seen until after an hour. The reason for this difference in kinetics is unclear, as BMP2 and GDF5 share the same binding site on BMPRIb (Nishitoh et al., 1996). Similar results were reported by (Drevelle et al., 2013), who found that BMP2 caused Smad 1/5/8 phosphorylation within 30 min in cultured preosteoblasts, while GDF2 required 4 h to induce the same effect. The precise mechanism of these differing rates of activation and its possible functional significance is unknown.

A study using fluorescent biosensors for direct visualization of Smad1 and Smad4 proteins demonstrated that a delay of 2-5 min occurred between BMP4 (also known as BMP2b) activation of the BMPRs and subsequent Smad1 phosphorylation in mammalian cells (Gromova et al., 2007). This is consistent with our findings for BMP2 (also known as BMP2a), which showed Smad 1/5/8 activation from 5 min. Gromova et al. (2007) described Smad1 phosphorylation as the rate-limiting step of canonical BMP-Smad signalling. Because Smad1 phosphorylation is carried out by the kinase domain of BMP-activated BMPRI, it is likely that the time delay in Smad phosphorylation is determined by the BMPRI. Furthermore, in canonical BMP-Smad 1/5/8 signalling, BMPs bind to a pre-formed complex of BMPRI and BMPRII (Gilboa et al., 2000, Nohe et al., 2002). Therefore, BMPR heteromerization does not

contribute to the delay in BMPRI phosphorylation of Smads 1/5/8 in response to BMP binding. BMP2 can signal through both BMPRIa and BMPRIb, whereas GDF5 predominantly signals through BMPRIb (Nishitoh et al., 1996). It is possible that BMPRIa activation by BMP2 accounts for the distinct temporal profiles of Smad activation between BMP2 and GDF5. Indeed, SH-SY5Y cells have been reported to express BMPRIa (Toulouse et al., 2012) as well as BMPRIb, as shown here. Furthermore, another GDF, GDF2, preferentially signals via activin receptor-like kinase 1 (David et al., 2007), which may be the reason for the difference between its Smad 1/5/8 phosphorylation kinetics and those of BMP2. In light of these findings, it is possible that different BMPRI have distinct temporal profiles of Smad 1/5/8 phosphorylation, which would explain the different kinetics of Smad phosphorylation observed after treatment with various BMPs.

In addition to demonstrating canonical Smad signalling activation by BMP2 and GDF5, this study also showed that BMP2 and GDF5 reduce the basal signalling of the phospho-ERK, phospho-JNK and phospho-p38 MAPK pathways in SH-SH5Y cells. Thus, non-canonical BMP signalling pathways appear not to contribute to the neurite outgrowth-promoting effects of BMP2 and GDF5. Interestingly, activation of p38 MAPK signalling in VM DA neurons is known to inhibit neurite outgrowth *in vitro* (Collins et al., 2013). Thus, such inhibition of p38 phosphorylation by BMP2 and GDF5 may provide a permissive environment for optimal neurite outgrowth. The inverse regulation of Smad and MAPK pathways is in agreement with previous findings showing that MAPK signalling negatively regulates Smad 1/5/8 signalling by inducing Smad1 degradation (Fuentealba et al., 2007, Eivers et al., 2008). Therefore, negative regulation of MAPK pathway signalling by BMP2 and GDF5 may contribute to canonical Smad signalling-mediated neurite outgrowth, by preventing MAPK-induced Smad1 degradation. Conversely, BMP2 has been shown to induce neuronal differentiation of the PC12 cell line via activation of the p38 MAPK pathway (Iwasaki et al., 1996, Iwasaki et al., 1999). Furthermore, dorsomorphin has been shown to induce neurite outgrowth of PC12 cells via the ERK MAPK pathway (Kudo et al., 2011), whereas the current study found dorsomorphin to have no morphological affects on SH-SH5Y cells. The present study demonstrates that BMP2 and GDF5 promote neurite growth through activation of canonical Smad 1/5/8 signalling in SH-SY5Y cells. These contrasting results may

reflect inherent differences between SH-SY5Y neuroblastoma cells and PC12 cells, which arise from adrenal gland chromaffin cells.

The present findings show that the BMP2- and GDF5-induced neuronal differentiation of SH-SY5Y cells is mediated through BMPRIb. This reflects that fact that BMPRIb is the preferential BMPRI activated by GDF5. BMP2 induced the neuronal differentiation of SH-SY5Y cells to the same extent as GDF5, and thus likely acted via the BMPRIb also. The fact that caBMPRIb mimics the effects of BMP2 and GDF5 strongly supports this possibility.

This study has not only identified the BMPRI subtype that is most likely responsible for mediating BMP2- and GDF5-induced Smad signalling and neurite extension, but has also demonstarted that the transcriptional activity of Smad 1/5/8 is required for this BMP-induced neuronal differentiation. The inhibition of the nuclear translocation of the Smad 1/5/8 transcription factors, using a siRNA to target Smad4, prevented the SH-SY5Y neurite outgrowth induced by caBMPRIb, and by BMP2 and GDF5.

The current study adds new evidence to the growing body of work that suggests that BMP-Smad 1/5/8 signalling plays a key role in the neurite extension of a number of neural populations. For example, BMPs are widely expressed in the dorsal spinal cord, which houses a BMP signalling centre, known as the roof plate (Liem et al., 1995, Lee et al., 1998, Lee and Jessell, 1999). There is a wealth of evidence demonstrating that BMP signalling, particularly via Smad1, regulates neurite outgrowth in this region, including that of dorsal root ganglion neurons and dI1 interneurons (Parikh et al., 2011, Hazen et al., 2012, Hazen et al., 2011, Phan et al., 2010). GDF5 and BMP2 have both been shown to be expressed in the developing VM during DA neurogenesis (Jordan et al., 1997, O'Keeffe et al., 2004b), and thus may perform roles in the differentiation of VM DA neurons similar to those of BMPs in the dorsal spinal cord. Such a suggestion is plausible especially when one considers the evidence gained from previous studies of their DA neurotrophic effects. Monteiro et al. used Smad 1/5/8 reporter mice to demonstrate BMP-Smad-dependent transcriptional activity in the VM region during DA neurogenesis at E10.5 (Monteiro et al., 2008). It is thus possible that BMP2- and GDF5-induced Smad activation regulates VM DA neurite outgrowth at this developmental stage, as VM DA neurons begin to extend their axons dorsally at this time point (Nakamura et al.,

2000), as is the case for BMPs in the dorsal spinal cord (Chizhikov and Millen, 2005, Ulloa and Briscoe, 2007).

Cell replacement therapy is one of the most promising therapies for the treatment of PD (Orlacchio et al., 2010, Bonnamain et al., 2012, De Feo et al., 2012, Toulouse and Sullivan, 2008, Hedlund and Perlmann, 2009). Considering the importance of establishing functional connections following the striatal transplantation of VM DA neurons, factors which promote their neurite outgrowth are being considered as adjuncts to this potential therapy. GDF5 and BMP2 are potential candidates for such a role, as both have been shown to promote the survival of VM DA neurons (O'Keeffe et al., 2004b, Reiriz et al., 1999, Jordan et al., 1997, Sullivan et al., 1997). The present study has, for the first time, demonstrated that the downstream molecular mechanisms mediating the direct neurotrophic effects of GDF5 and BMP2 are dependent upon BMPRI-mediated activation of canonical Smad 1/5/8 signalling.

6. Canonical BMP-Smad signalling promotes neurite growth in embryonic rat midbrain DA neurons.

6.0 Aims

- Investigate the expression of the BMP receptors, BMPRIb and BMPRII, during nigrostriatal pathway development in the developing and adult rat brain.
- Demonstrate the neurite growth-promoting effects of BMP2 and GDF5 in E14 rat VM DA neurons.
- Identify the receptors and intracellular signalling pathway(s) that mediate the neurite growth-promoting effects of BMP2 and GDF5 in VM DA neurons.

6.1 Abstract

VM DA neurons project to the dorsal striatum via the nigrostriatal pathway to regulate voluntary movements, and their loss causes the motor dysfunction of PD. Despite recent progress in the understanding of VM DA neurogenesis, the factors regulating nigrostriatal pathway development remain largely unknown.

The BMP family of proteins regulates neurite growth in the developing NS, and may contribute to nigrostriatal pathway development. Two related members of this family, BMP2 and GDF5, have neurotrophic effects, including the promotion of neurite growth, on cultured VM DA neurons. However, the molecular mechanisms regulating their effects on DA neurons are unknown. By characterising the temporal expression profiles of endogenous BMPR in the developing and adult rat VM and striatum, this study identified a potential involvement of BMP2 and GDF5 in the regulation of nigrostriatal pathway development. Furthermore, through the use of noggin, dorsomorphin and BMPR/Smad plasmids, this study demonstrated that GDF5- and BMP2-induced neurite outgrowth from cultured VM DA neurons is dependent on BMP type I receptor activation of the Smad 1/5/8 signalling pathway.

6.2 Introduction

In the CNS, more than three-quarters of all DA neurons are found in the VM (Blum, 1998, German et al., 1983, Pakkenberg et al., 1991). These are subdivided into three distinct clusters, termed the A8, A9 and A10 groups of VM DA neurons. The A9 group of VM DA neurons, located in the SNpc, project to the dorso-lateral striatum via the nigrostriatal pathway (Dahlstroem and Fuxe, 1964, Bjorklund and Dunnett, 2007). These A9 DA neurons, and their striatal targets, are part of the basal ganglia circuitry that regulates the control of voluntary movement. Their functional importance is highlighted by the neurodegenerative disorder PD, the primary neuropathological signature of which is the loss of these neurons and their striatal projections, which results in the motor deficits that are characteristic of this disease (Toulouse and Sullivan, 2008, Lees et al., 2009).

During embryonic development, A9 DA neurons are generated in the VM under the influence of two key signalling centres, the isthmus and the floor plate (Hynes et al., 1995a, Crossley and Martin, 1995, Liu and Joyner, 2001). Much work in recent decades has focused on elucidating the molecular circuitry that is involved in the generation of A9 VM DA neurons (Hegarty et al., 2013c), however the molecular mechanisms that regulate the growth and guidance of the axonal projections of these DA neurons to their appropriate target regions in the striatum are less well understood.

During nervous system development, VM DA neurons extend their axons towards the telencephalon via the medial forebrain bundle in response to extrinsic directional cues (both chemo-attractive and -repulsive) from the caudal brainstem, midbrain, diencephalon, striatum and cortex (Gates et al., 2004, Nakamura et al., 2000). Despite the paucity of studies identifying the regulatory molecules involved in the formation of DA projections, a number of molecules have been implicated. Cell-surface ephrins and their Eph receptor tyrosine kinases, which are important in axonal guidance (Egea and Klein, 2007), have been shown to play roles in target innervation by nigrostriatal axons (Sieber et al., 2004, Halladay et al., 2004, Van den Heuvel and Pasterkamp, 2008, Calo et al., 2005, Yue et al., 1999, Cooper et al., 2009). Similarly, netrin signalling via the DCC receptor, which is known to actively regulate axonal growth (Round and Stein, 2007), has been strongly implicated in the formation of the VM DA circuitry (Xu et al., 2010, Flores et al., 2005, Manitt et al.,

2011, Lin et al., 2005, Sgado et al., 2012, Vitalis et al., 2000). Additionally, signalling between Slits and their Robo receptors (Bagri et al., 2002, Dugan et al., 2011, Lin et al., 2005, Lopez-Bendito et al., 2007), and by semaphorins (Hernandez-Montiel et al., 2008, Torre et al., 2010, Tamariz et al., 2010, Kolk et al., 2009), has been shown to regulate the formation of DA projections from the VM to the striatum. These identified molecules are well-established regulators of axonal growth and guidance in other regions of the nervous system. It is thus likely that further candidate molecules with similar functions in other areas of the NS may contribute to the regulation of DA axonal growth. One candidate group of molecules are the BMPs (Zou and Lyuksyutova, 2007, Bovolenta, 2005).

BMPs are regulators of axonal growth in a number of neuronal populations, with this role best characterised in the dorsal SC (Parikh et al., 2011, Hazen et al., 2012, Lein et al., 1995, Hegarty et al., 2013a, Gratacos et al., 2002). The two members of the BMP family of proteins that are of particular interest to this study, BMP2 and its related molecule GDF5, have been shown to regulate neurite growth in the dorsal spinal cord (Parikh et al., 2011, Hazen et al., 2012, Hazen et al., 2011, Phan et al., 2010, Niere et al., 2006). GDF5 and BMP2 activate a canonical signalling pathway involving two types of serine/threonine kinase receptors, type I and type II BMPRs (ten Dijke et al., 1994, Koenig et al., 1994, Yamashita et al., 1996, Shi and Massague, 2003). Upon ligand binding, the constitutively-active BMPRII transphosphorylates the cytoplasmic domain of the BMPRI (BMPRIa or BMPRIb), causing phosphorylation of the receptor-regulated Smads, Smads 1/5/8, by the activated BMPRI. The activated Smads 1/5/8 then form a heterocomplex with the co-Smad, Smad4, which mediates their nuclear translocation to allow the Smad transcription factors to modulate target gene expression (Miyazono et al., 2010, Sieber et al., 2009).

BMP2 and GDF5 are expressed in the developing rat VM during the period of DA axogenesis, suggesting that they may play a role in this process (O'Keeffe et al., 2004b, Jordan et al., 1997). In support of this suggestion, both GDF5 and BMP2 have been shown to promote the survival of (O'Keeffe et al., 2004a, Reiriz et al., 1999, Jordan et al., 1997, Sullivan et al., 1997), and induce neurite growth of (O'Keeffe et al., 2004a, Reiriz et al., 1999), rat VM DA neurons *in vitro*. Despite these studies, the expression patterns of the BMPRs in the VM and the target striatum during nigrostriatal pathway development are unknown. Furthermore, the

mechanisms by which GDF5 and BMP2 mediate their neurite growth-promoting effects on VM DA neurons remains to be determined. However, these effects have recently been proposed to occur via the canonical Smad signalling pathway in a cell line model of DA neurons (Chapter 2) (Hegarty et al., 2013b).

To address the gaps in our current knowledge of BMP-mediated DA neuronal growth, this study examined the expression of BMPRs over the developmental period between E14 and P90, since the generation and maturation of nigrostriatal dopaminergic neurons, the invasion and arborisation of their striatal targets, and the refinement of these connections, occurs over this time-period (Van den Heuvel and Pasterkamp, 2008). Furthermore, the molecular mechanisms by which BMP2 and GDF5 regulate axonal growth of VM DA neurons were investigated.

6.3 Results

6.3.1 BMPRs are expressed in the rat VM and striatum during embryonic and postnatal development

If GDF5- and BMP2-induced Smad signalling promotes the neurite growth of VM DA neurons, then the BMP receptors, BMPRII and BMPRIb, should be expressed in the VM and possibly the striatum, during the period of DA axogenesis. To examine this, RT-QPCR was used to quantify the expression levels of TH, BMPRII, and BMPRIb transcripts in the VM and striatum during embryonic and postnatal development, having confirmed the accuracy of the VM dissections by examining DA gene expression at each age (Fig. 6.4.1a-d). In the VM, TH mRNA levels are highest from E14 to P1 (Fig. 6.4.1b). A significant drop in TH transcript levels occurs between P1 and P11, after which a lower level of expression of TH mRNA remains stable through to adulthood (P90) (Fig. 6.4.1b). In the striatum, TH mRNA levels are significantly lower than those in the midbrain throughout the developmental period studied (Fig. 6.4.1b).

BMPRII mRNA levels are relatively stable throughout development in the VM (Fig. 6.4.1c), while in the developing striatum BMPRII mRNA levels increase 1.5-fold between E14 and P1. Between P1 and P31, the level of BMPRII transcripts expressed in the striatum falls almost 3-fold and this lower expression level is maintained through to adulthood (Fig. 6.4.1c). BMPRII mRNA levels in P90 midbrain are similar to those in P90 striatum (Fig. 6.4.1c). In the midbrain, BMPRIb mRNA levels increase 3-fold between E14 and P1, and thereafter remain unchanged until adulthood (Fig. 6.4.1d). In the developing striatum, BMPRIb mRNA levels increase by 2-fold between E14 and P1, before increasing a further 2-fold between P1 and P60 (Fig. 6.4.1d). BMPRIb striatal mRNA levels remain relatively steady thereafter through to P90, and are comparable to that of the adult midbrain at this time point (Fig. 6.4.1d, e). The expression levels of BMPRII and BMPRIb transcripts in the adult midbrain (P31-P90) are very similar. Indeed, RT-PCR and *in situ* hybridization showed that BMPRII and BMPRIb are strongly expressed in the adult rodent SNpc (Fig 6.4.1e-j). Furthermore, approximately 75% of DA neurons in the adult rat midbrain expressed BMPRII and BMPRIb (Fig. 6.4.1k, l).

Since the initial phase of DA axogenesis begins at E11 in the rat (Gates et al. 2004; Nakamura et al. 2000), this study also showed that BMPRII and BMPRIb are

expressed in the developing rat VM from E11 to E14 VM (Fig. 6.4.2a). Western blotting and immunocytochemistry was then used to confirm that the effector part of the BMP receptor complex, the BMPRIb protein, is expressed in the rat VM during this developmental period (Fig. 6.4.2b, c). To determine if these receptors are expressed on DA neurons, immunocytochemical analysis was used to confirm protein expression of BMPRII and BMPRIb on TH-positive neurons in E14 rat VM cultures (Fig. 6.4.2d, e and data not shown). The co-localisation of BMPRII- and BMPRIb-immunostaining with TH-immunostaining indicates that these receptors are expressed by DA neurons, although there is also expression of these BMPRs on TH-negative, non-DA cells (Fig. 6.4.2d, e).

6.3.2 BMP2 and GDF5 promote neurite growth and activate canonical Smad signalling in VM DA neurons

Following the characterisation of BMPR expression in the VM and striatum during development, this study next assessed the effects of BMP2 and GDF5 on neurite outgrowth from cultured E14 VM DA neurons. Treatment with 200 ng/ml of BMP2 or GDF5 for 4 DIV resulted in a significant increase in the neurite length of TH-positive neurons in E14 VM cultures, when compared to controls (Fig. 6.4.3a, b).

BMPs are well-known activators of a canonical signalling pathway involving activation of Smad 1/5/8 (Miyazono et al., 2010, Sieber et al., 2009). Densitometric analysis of the nuclear levels of phospho-Smad 1/5/8 showed that both BMP2 and GDF5 significantly increase the amount of phospho-Smad 1/5/8 in the nucleus of TH-positive DA neurons at 30 and 60 min, compared to the untreated controls (0 min) (Fig. 6.4.3c-e). To determine whether this effect of GDF5 and BMP2 on Smad phosphorylation was specific to DA neurons, nuclear phospho-Smad levels were also measured in TH-negative cells. BMP2 did not induce Smad phosphorylation in TH-negative cells at any time-point examined (Fig. 6.4.3c). Although GDF5 did not activate Smad phosphorylation in TH-negative cells at 30 min, it did so at 60 min (Fig. 6.4.3d). Using SH-SH5Y cells as a model of human DA neurons, BMP2 and GDF5 were both shown to significantly increase Smad-mediated transcriptional activity (as measured by the relative levels of GFP expression) at 2 DIV in SH-SH5Y cells transfected with a Smad reporter plasmid (GFP under the control of a Smad responsive element) (Fig. 6.4.3f, g). The ability of GDF5 to activate Smad signalling in the VM was then assessed *in vivo*. Intracerebral administration of 10 µg

of GDF5 to the adult rat SNpc (right) significantly increased phospho-Smad levels after 2 h when compared to the contralateral SNpc (left), as determined by western blotting (Fig. 6.4.3h). Collectively, these data show that BMP2 and GDF5 promote neurite growth from DA neurons in E14 VM cultures and activate the canonical Smad signalling pathway in these neurons.

6.3.3 BMPR inhibitors prevent BMP2- and GDF5-induced neurite outgrowth in VM DA neurons

To explore the possibility that the effects of BMP2 and GDF5 on neurite outgrowth from E14 VM DA neurons are mediated through BMPR-dependent activation of the canonical Smad 1/5/8 pathway, two approaches were employed to inhibit BMP-BMPR signalling. Firstly noggin, an extracellular inhibitor of BMPs which blocks their binding epitopes for BMPRs (Groppe et al., 2002, Smith and Harland, 1992), was used. Secondly dorsomorphin, a small molecular inhibitor of BMPRI (Yu et al., 2008), was used. It has previously been shown that dorsomorphin is an effective inhibitor of BMP2 and GDF5 signalling in SH-SY5Y cells (Hegarty et al., 2013b). Pretreatment with either 200 ng/ml of noggin or 1 μg/ml of dorsomorphin prevented BMP2- and GDF5-induced neurite growth in SH-SY5Y cells at 4DIV (Fig. 6.4.4). Similarly, the pretreatment of E14 VM cultures with noggin or dorsomorphin completely prevented the BMP2- and GDF5-induced increases in the neurite length of TH-positive cells at 4 DIV (Fig. 6.4.5a, b). Taken together, these data show that the neurite growth-promoting effects of BMP2 and GDF5 on VM DA neurons are directly mediated through a BMPR-dependent pathway.

6.3.4 Canonical BMPR-Smad activation promotes neurite outgrowth in VM DA neurons

It is well established that BMP2 can signal through both BMPRIa and BMPRIb, whereas GDF5 predominantly signals through BMPRIb (Nishitoh et al., 1996), which suggests that BMP2 and GDF5 may signal through BMPRIb to exert their neurotrophic effects on VM DA neurons. To test this possibility, E14 VM neurons were trasfected with a caBMPRIb plasmid, and neurite growth of the neurons was assessed and compared to that in cultures transfected with a control plasmid. Transfection of E14 VM neurons with the caBMPRIb plasmid induced a significant increase in their neurite length at 3 DIV, but not 1 DIV, when compared to cells

transfected with the relevant control plasmid (Fig. 6.4.6a, c). Importantly, electroporation of E14 VM neurons with a wild-type BMPRIb plasmid did not result in significant increases in neurite length (data not shown), demonstrating the importance of the activation of the BMPR for this effect.

To determine a functional link between BMPRIb-induced neurite growth and Smad 1/5/8 signalling, an siRNA that targets the co-Smad Smad4, which has been shown to be effective in inhibiting BMP2 and GDF5 signalling (Chapter 2) (Hegarty et al., 2013b), was used. Formation of a complex between phosphorylated Smad 1/5/8 and Smad4 following BMPRIb activation is required for the nuclear translocation of activated Smad 1/5/8, and thus their regulation of target gene expression (Miyazono et al., 2010, Sieber et al., 2009). To determine if modulation of Smad4 expression affected the growth of E14 VM neurons, the neurite length of cells transfected with Smad4 siRNA or with Smad4 overexpression vectors was measured. Modulation of Smad4 expression did not affect the neurite length of transfected E14 VM neurons (data not shown). When E14 VM cells were co-transfected with the caBMPRIb and Smad4 siRNA, Smad4 siRNA significantly reduced the caBMPRIb promotion of E14 VM neuronal growth (Fig. 6.4.6b, c). These data show that activation of the Smad signalling pathway by BMPRIb mimics the neurite growth promoting effects of BMP2 and GDF5 in E14 VM neurons. To ensure this effect was specific to DA neurons, we immunostained the electroporated neurons at 3 DIV for TH. This allowed the identification of TH-positive/ GFP-positive DA neurons, indicating that they were transfected (Fig. 6.4.7a, b). Traces of the TH-positive/ GFP-positive DA neurons were prepared for analysis of neuronal growth (Fig. 6.4.7c), which showed that DA neurons expressing caBMPRIb were significantly larger their control counterparts (Fig. 6.4.7d). Finally, to further demonstrate the requirement of the BMPRIb for the neurite growth-promoting effects of the BMP ligands, an siRNA against BMPRIb was employed, which induced efficient BMPRIb knockdown (Fig. 6.4.7e). The ability of GDF5 to promote growth in cells transfected with either a control siRNA or the BMPRIb siRNA was then investigated. GDF5 promoted a significant increase in neurite length in cells expressing the control siRNA, whereas this effect was lost in cell expressing the BMPRIb siRNA (Fig. 6.4.7f, g). Taken together, these data show that activation of canonical BMP-BMPRIb-Smad 1/5/8 signalling promotes neurite outgrowth in VM DA neurons.

6.4 Figures and Figure Legends

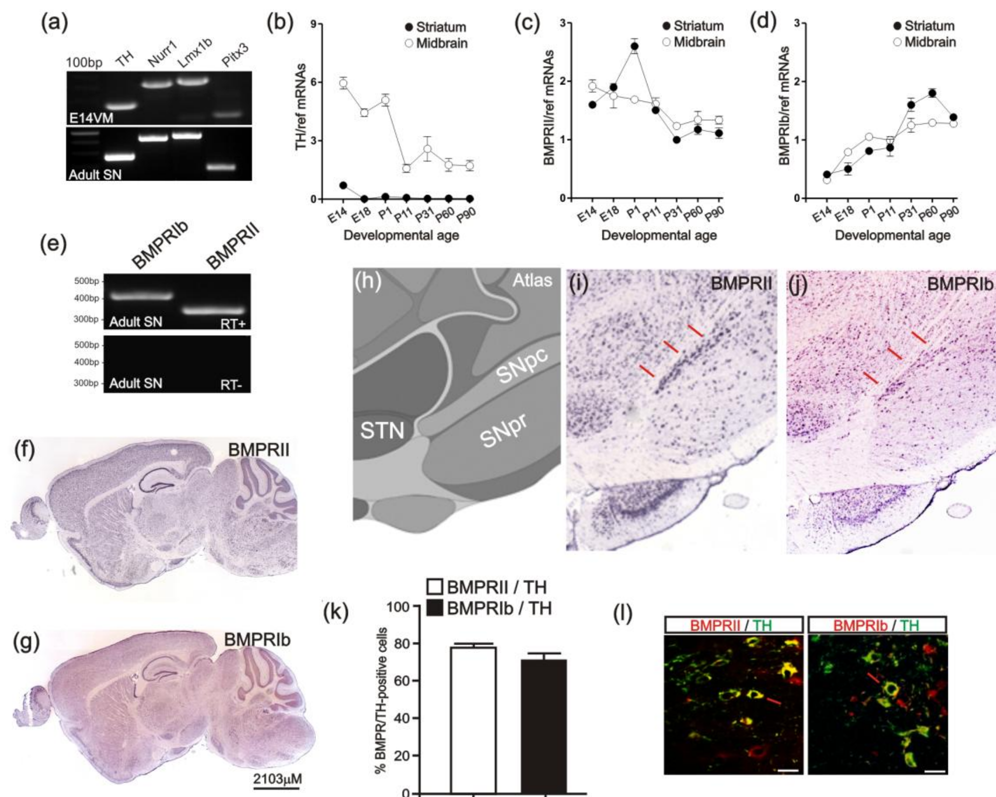


Figure 6.4.1: BMP receptors are expressed in the rat midbrain and striatum during embryonic and postnatal development.

(a) RT-PCR of TH, Nurr1, Lmx1b, and Pitx3 in E14 and adult rat VM (SN = substantia nigra). (b-d) Quantitative RT-QPCR data showing the levels of (b) TH, (c) BMPRII and (c) BMPRIb mRNA in the developing midbrain and striatum, from E14 to P90, relative to the levels of the reference mRNAs GAPDH, SDHA, and UBQC. Each data point represents pooled data from four samples from three separate litters/animals, and all data are presented as the mean ± SEM. (e) RT-PCR showing expression of BMPRII and BMPRIb in the adult rat SN. (f, g) *In situ* hybridization images taken from the Allen Developing Brain Atlas (©(Allen) Developing Mouse Brain Atlas, 2012) showing BMPRII and BMPRIb expression (purple colour) in sagittal sections of the P56 adult mouse brain. (h) Atlas showing the major nuclei in the midbrain region, including the SNpc, substantia nigra *pars reticulate* and subthalamic nucleus. *In situ* hybridization images of this region showing strong expression of (i) BMPRII and (j) BMPRIb in the SNpc (red arrows). Scale bar = 2103 μm. (k) Quantification of the percentage of DA neurons in the adult rat SNpc expressing BMPR-II and BMPR-Ib. (l) Photomicrographs showing immunostaining for BMPRII and BMPRIb co-expressed with TH in the adult rat SNpc.

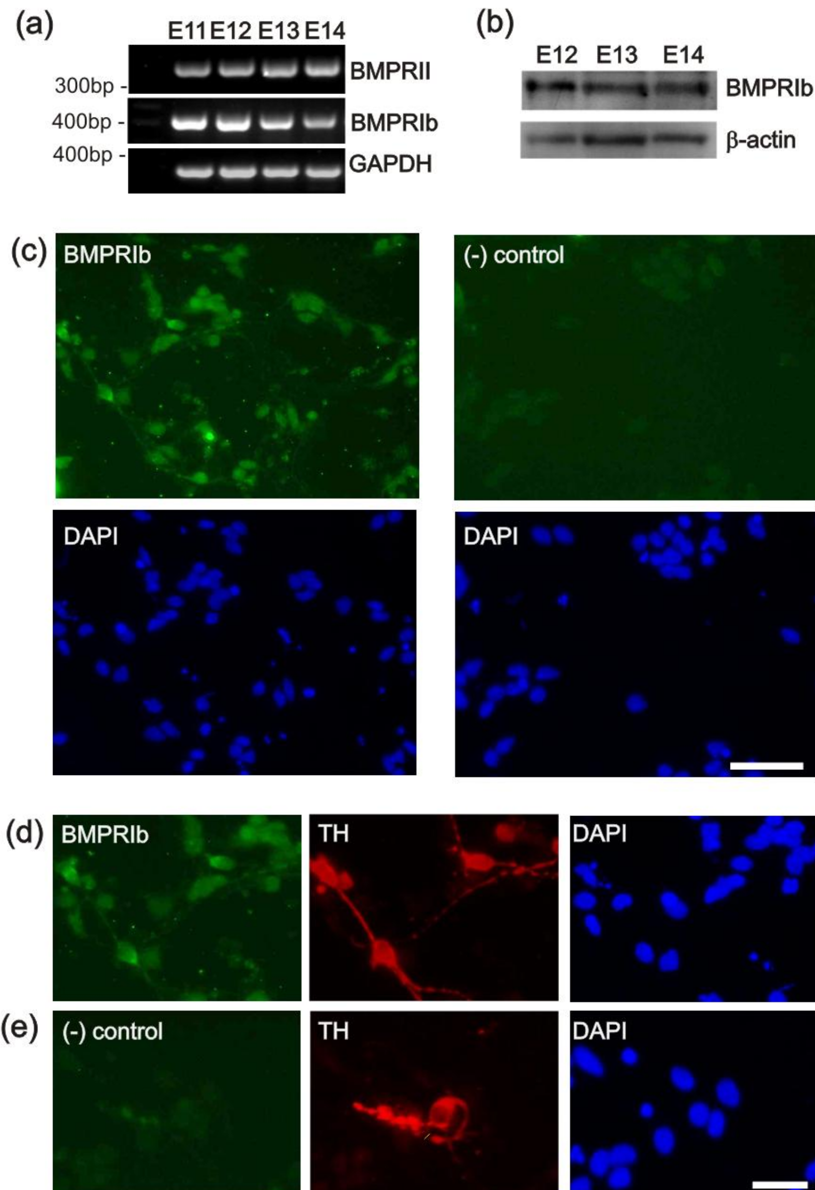


Figure 6.4.2: BMPRs are expressed on midbrain DA neurons during the peak period of DA axogenesis.

(a) RT-PCR of BMPRII, BMPRIb and GAPDH in E11 to E14 rat VM. (b) Western blotting showing BMPRIb protein expression in the developing rat VM. (c) Photomicrographs showing immunostaining for BMPRIb co-expressed with DAPI and the relevant negative controls ((-) control) in cultures of the E14 rat VM after 24 h *in vitro*. Photomicrographs showing immunostaining for (d) BMPRIb with (e) being the negative control, co-stained with DAPI and TH, in cultures of E14 rat VM after 24 h *in vitro*. Scale bar = 50 µm.

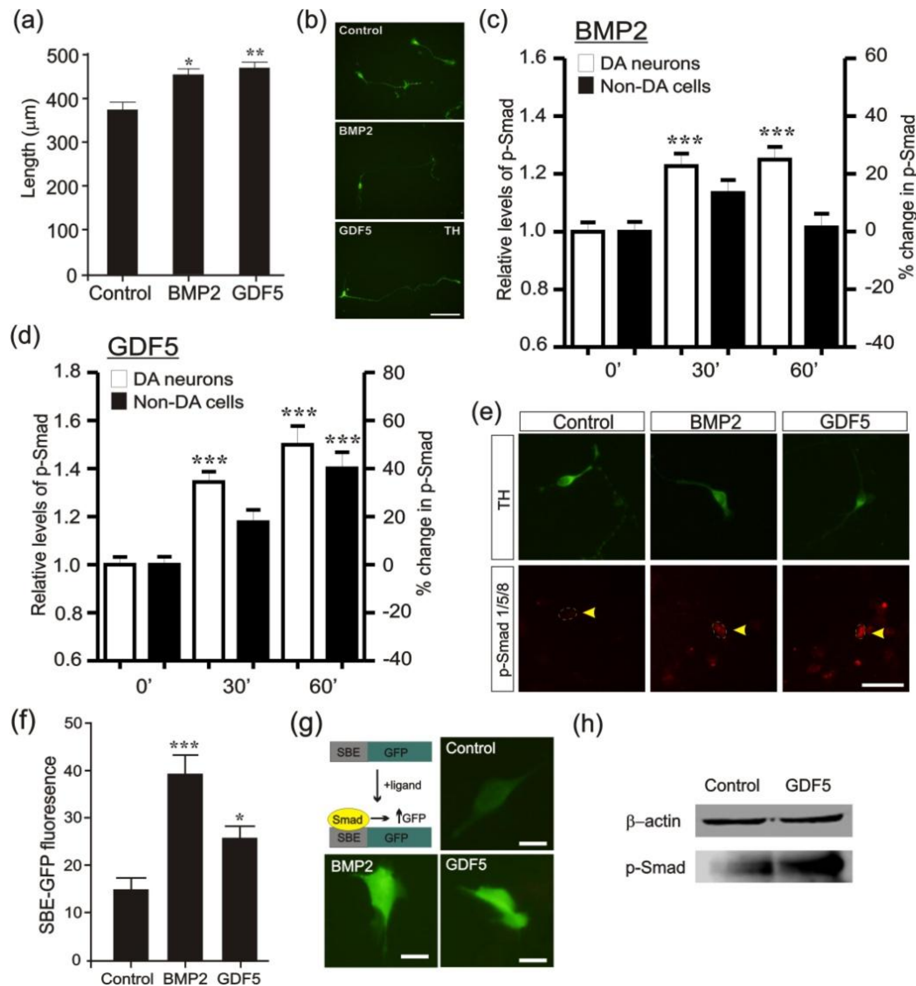


Figure 6.4.3: BMP2 and GDF5 promote neurite growth and activate canonical Smad signalling in cultured rat VM DA neurons.

(a) Total neurite length of BMP2- and GDF5-treated DA neurons in cultures of E14 rat VM. (b) Representative photomicrographs of BMP2- and GDF5-treated DA neurons in cultures of E14 rat VM at 4DIV, immunocytochemically stained for TH. Scale bar = 100 µm. Densitometric analysis of phospho-Smad 1/5/8 in (c) BMP2- and (d) GDF5-treated DA neurons and non-DA neurons in E14 rat VM cultures at 0 (control), 30 and 60 min. (e) Representative photomicrographs showing immunostaining of phospho-Smad 1/5/8 (yellow arrow heads) and TH in E14 rat VM cultures treated BMP2 and GDF5 for 60min. Scale bar = 100 µm. (f) Smad-dependent transcriptional activity in BMP2- and GDF5-treated SH-SY5Y cells 48 h after transfection with a Smad-GFP reporter. (g) Representative photomicrographs showing Smad-GFP reporter in BMP2- and GDF5- treated SH-SH5Y cells. Scale bar = 10 µm. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control; ANOVA with post-hoc Tukey's test; 50 cells analysed per group per experiment; N = 3). (h) Western blots showing phospho-Smad 1/5/8 protein expression in the GDF5-treated adult rat VM.

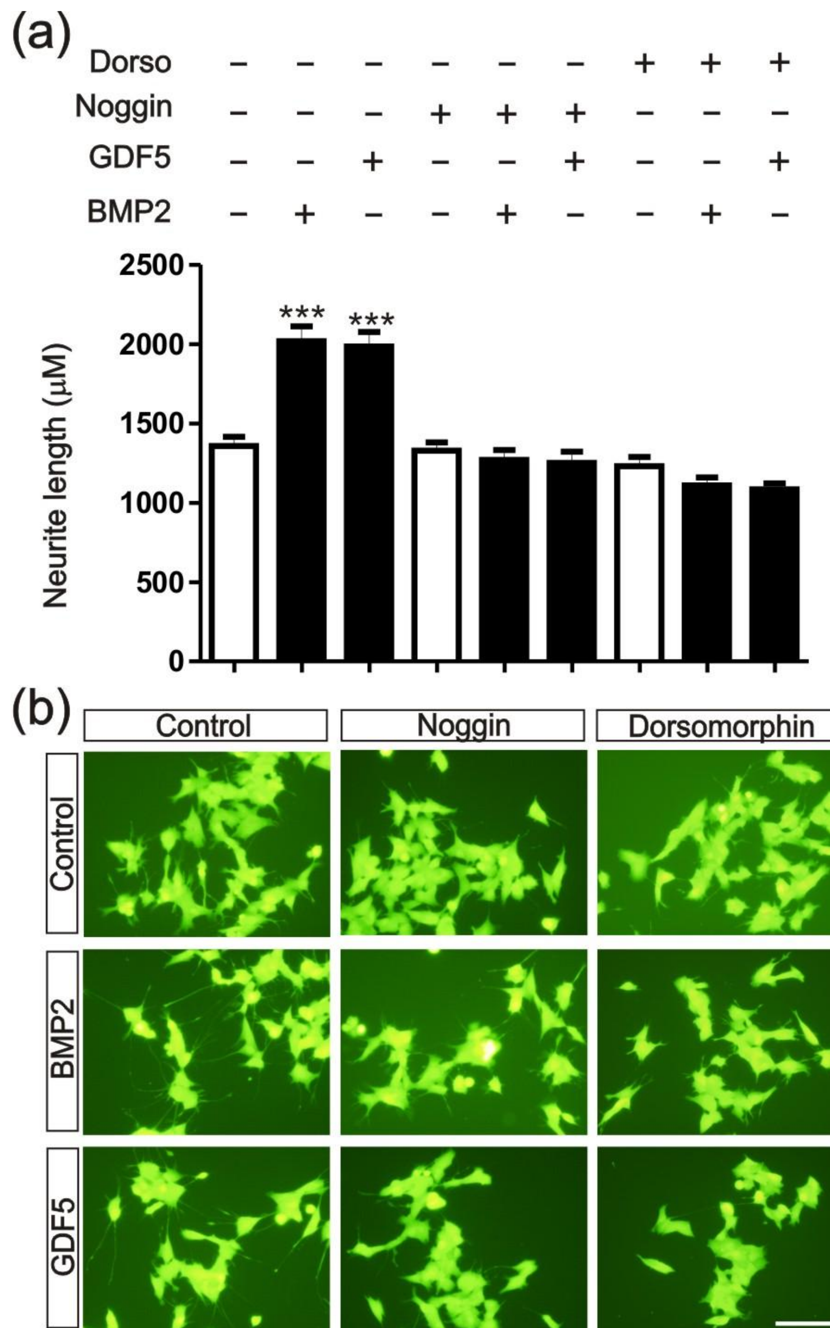


Figure 6.4.4: Noggin and dorsomorphin prevent the promotion of SH-SH5Y neurite growth by BMP2 and GDF5.

(a) Total neurite length of noggin- or dorsomorphin-pre-treated and/or BMP2- or GDF5-treated (daily for 4 DIV) SH-SY5Y cells, as indicated (*** $P < 0.001$ vs control; ANOVA with post-hoc Tukey's test; 20 images analysed for each group per experiment; N = 3). Data are expressed as mean ± SEM. (b) Representative photomicrographs of noggin-pre-treated and/or BMP2- or GDF5-treated SH-SY5Y cells, as indicated, immunocytochemically stained for β-actin. Scale bar = 100 μm.

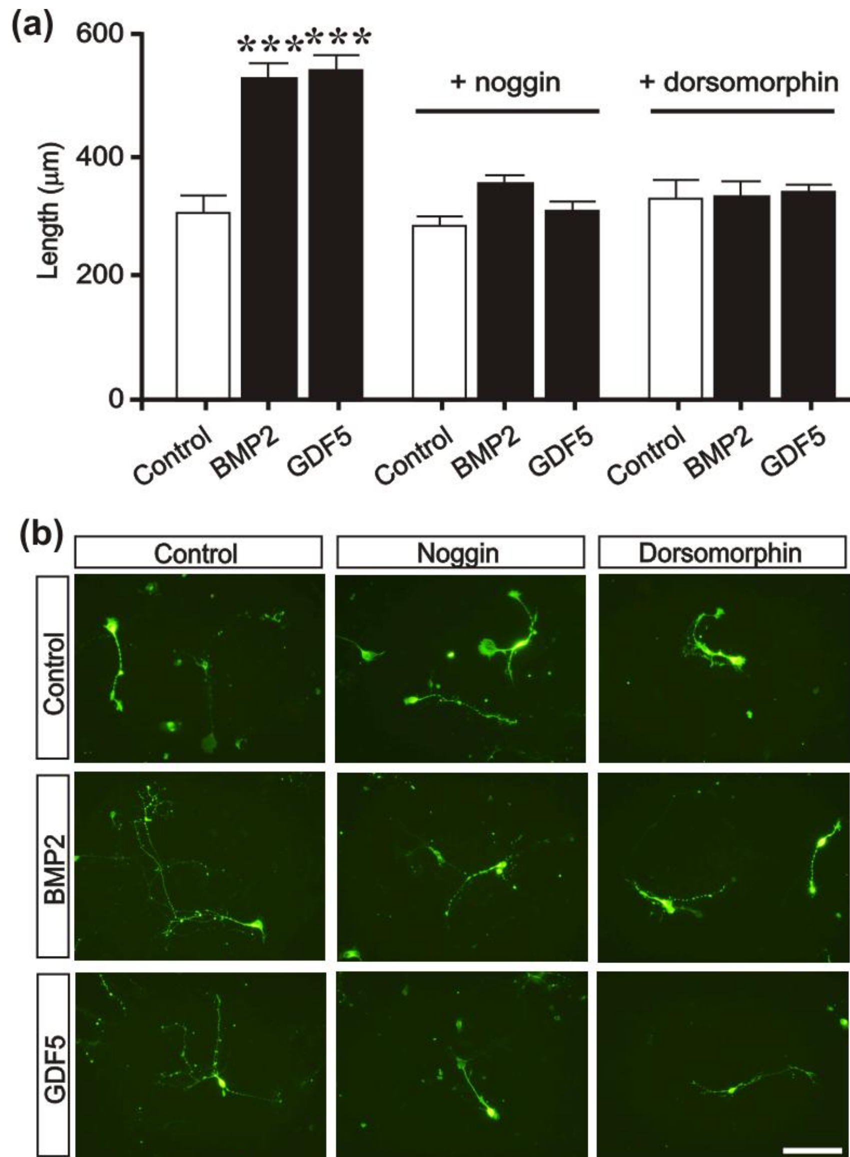


Figure 6.4.5: Noggin and dorsomorphin prevent the promotion of DA neurite growth by BMP2 and GDF5.

(a) Total neurite length of noggin- or dorsomorphin-pre-treated and/or BMP2- or GDF5-treated (daily for 4 DIV) DA neurons in E14 rat VM cultures, as indicated (*** $P < 0.001$ vs control; ANOVA with post-hoc Tukey's test; 50 TH-positive neurons analysed for each group per experiment; $N = 3$). (b) Representative photomicrographs of noggin- and dorsomorphin-pre-treated and/or BMP2- or GDF5-treated DA neurons in E14 rat VM cultures, immunocytochemically stained for TH. Scale bar = 100 µm. Data are expressed as mean ± SEM.

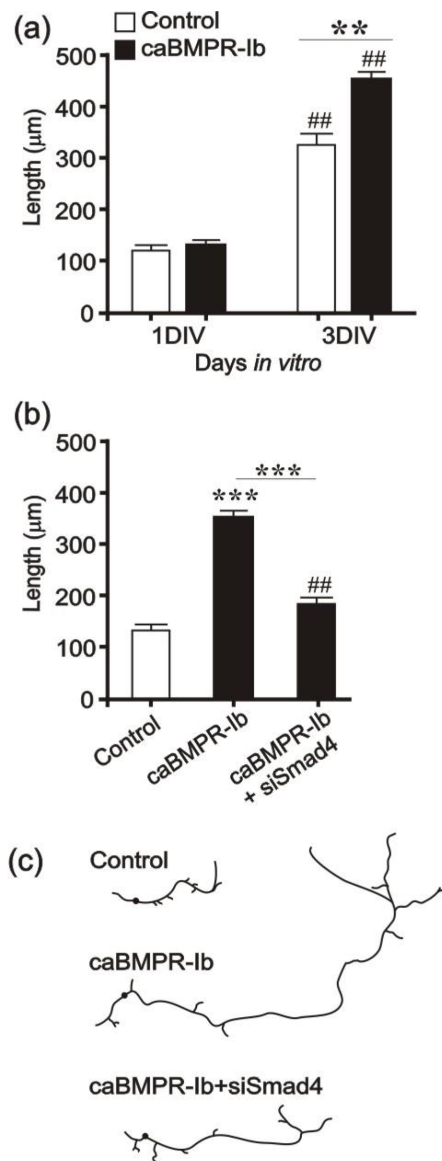


Figure 6.4.6: Overexpression of constitutively-active BMPRIb promotes neurite outgrowth in cultured rat VM DA neurons.

(a) Neurite length of control- or caBMPRIb-transfected neurons in E14 rat VM cultures at 1 and 3 DIV, as indicated (** $P < 0.01$ vs control at 3 DIV; ## $P < 0.01$ 3 DIV v 1 DIV; ANOVA with post-hoc Tukey's; 40 cells for each group per experiment; N = 3 experiments). (b) Neurite length of control- or caBMPRIb-transfected neurons and/or co-transfected with a Smad4 siRNA expression vector in E14 rat VM cultures at 3 DIV, as indicated (*** $P < 0.001$, ## $P < 0.01$ vs control at 3 DIV; ANOVA with post-hoc Tukey's; 40 cells for each group per experiment; N = 3 experiments). (c) Representative line drawing of neurons from each of these groups at 3 DIV. All data are presented as mean ± SEM.

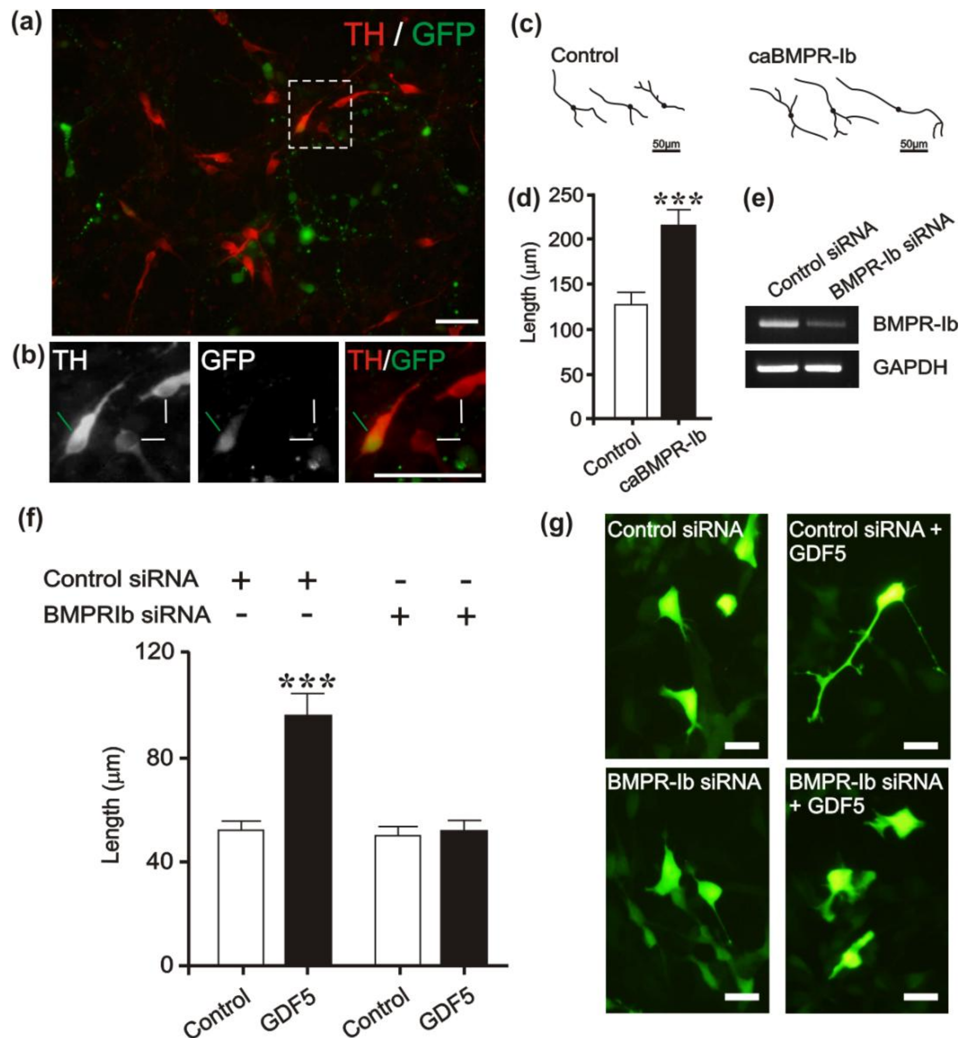


Figure 6.4.7: Overexpression of constitutively-active BMPRIb promotes neurite outgrowth in cultured rat VM DA neurons.

(a) Photomicrograph of an E14 rat VM culture transfected with ca-BMPRIb (GFP-positive) at the time of plating, and immunocytochemically stained for TH at 3 DIV. (b) Higher magnification of the dashed area in (a), showing co-localisation of TH and GFP used to identified transfected DA neurons. (c) Representative line drawing of control- or caBMPRIb-transfected DA neurons at 3DIV. Scale bar = 50 µm. (d) Neurite length of control- or caBMPRIb-transfected DA neurons in E14 rat VM cultures at 3 DIV, as indicated. (e) RT-PCR showing BMPRIb mRNA expression in SHSY5Y cells at 24h following transfection with either a control or BMPRIb siRNA. (f) Neurite length and (g) representative photomicrographs of control siRNA and BMPRIb siRNA transfected SH-SY5Y cells with or without GDF5 treatment, as indicated. (*** $P < 0.001$, vs control at 3 DIV; ANOVA with post-hoc Tukey's; 30 cells for each group per experiment; N = 3 experiments). All data are presented as mean ± SEM.

6.5 Discussion

Understanding the molecular signals that regulate the development of DA neurons is crucial for advancing cell replacement therapy for PD (Toulouse and Sullivan, 2008, Lees et al., 2009). While much progress has been made in understanding the signals that control DA neuron development, less is known about the molecules that promote the growth of DA neurites, which is crucial for functional integration of transplanted cells into the host parenchyma. Some molecules, such as Ephs and netrin1, have been identified as regulators of nigrostriatal pathway development in recent years (Hegarty et al., 2013a, Van den Heuvel and Pasterkamp, 2008). In an attempt to identify new candidate molecules and signalling pathways that may be involved in nigrostriatal development, this study focused on two BMPs, GDF5 and BMP2, since both of these factors have been implicated in axonal growth in other NS populations (Parikh et al., 2011, Hazen et al., 2012, Hazen et al., 2011, Phan et al., 2010, Niere et al., 2006, Lein et al., 1995, Hegarty et al., 2013a) and have been shown to have neurotrophic effects on VM DA neurons, specifically survival- and neurite growth-promoting effects (O'Keeffe et al., 2004a, Reiriz et al., 1999, Jordan et al., 1997, Sullivan et al., 1997, Hegarty et al., 2014c). Despite these studies, the downstream molecular mechanisms that mediate the effects of GDF5 and BMP2 on VM DA neurons are unknown. The present study aimed to define these molecular mechanisms, and to investigate the potential of BMP2 and GDF5 as regulators of nigrostriatal development.

To investigate this proposed role of BMP2 and GDF5 in the neurite growth of DA neurons, this study first characterised the temporal expression profiles of their receptors in the rat VM and striatum during embryonic and postnatal development. In the rat, the axons of the DA neurons in the VM extend towards the forebrain via the medial forebrain bundle from E13, and progressively innervate the striatum shortly thereafter, reaching the dorsal striatum around E20 (Gates et al., 2004, Nakamura et al., 2000, Specht et al., 1981a, Specht et al., 1981b, Verney, 1999, Voorn et al., 1988). In the first three post natal weeks, striatal innervation becomes more extensive, while naturally-occurring cell death refines these connections (Jackson-Lewis et al., 2000, Oo and Burke, 1997, Burke, 2003, Hegarty et al., 2013a, Van den Heuvel and Pasterkamp, 2008). This study found that BMPRII and BMPRIb were expressed at steady levels in the VM throughout embryonic

development (from E14) and into adulthood (until at least P90), with strong expression levels being detected in the P56 SNpc also. Crucially, the expression of these BMPRs, both of which are required for canonical BMP-Smad signalling (Miyazono et al., 2010, Sieber et al., 2009), in the VM from E14 onwards correlates with the timing of the generation of nigrostriatal projections. These data suggest that BMPs, such as BMP2 and GDF5 which are expressed in the developing and adult VM and striatum (Krieglstein et al., 1995b, O'Keeffe et al., 2004b, Storm et al., 1994, Chen et al., 2003, Jordan et al., 1997, Soderstrom and Ebendal, 1999, Hegarty et al., 2014c), may regulate the establishment of nigrostriatal projections from VM DA neurons. In support of this suggestion, the present study has demonstrated that both BMP2 and GDF5 promote neurite outgrowth from E14 VM neurons in culture. BMP2 and GDF5 may also act to orientate the axons of VM DA neurons away from the VM, since other BMPs, such as BMP7 and GDF7, have been shown to direct the orientation of the commissural axons of dorsal SC interneurons, an action which requires BMPRIb (Butler and Dodd, 2003, Dent et al., 2011, Phan et al., 2010, Yamauchi et al., 2008, Wen et al., 2007). The sustained expression of BMPRs in the VM during adulthood suggests that they may function in the maintenance of DA neurons, with both BMP2 and GDF5 being shown to promote the survival of VM DA neurons *in vitro* (O'Keeffe et al., 2004a, Wood et al., 2005, Reiriz et al., 1999, Jordan et al., 1997) and *in vivo* (Sullivan et al., 1997, Sullivan et al., 1999, Hurley et al., 2004, O'Sullivan et al., 2010, Sullivan et al., 1998b, Espejo et al., 1999). This study also demonstrated the expression of these BMPRs from E11 to E14 in the rat VM, further supporting their role in DA axogenesis, but also suggesting that BMPs may function in promoting the adoption of a DA phenotype during DA neurogenesis, which also occurs during this period (Lumsden and Krumlauf, 1996, Lauder and Bloom, 1974, Gates et al., 2006, Hegarty et al., 2013c). In agreement with this proposal, BMP-BMPR-Smad-dependent transcriptional activity is found in the VM region during DA neurogenesis at E10.5 in mice (Monteiro et al., 2008), which corresponds to the time of DA axon extension. BMP-Smad signalling may therefore concomitantly contribute to VM DA neuronal specification and their subsequent neurite outgrowth, which is the case for BMPs in the dorsal SC (Chizhikov and Millen, 2005, Ulloa and Briscoe, 2007).

In the rodent striatum there is a peak of BMPRII mRNA expression at P11, during the time period (P0-P20) when naturally-occurring cell death because of

limitations in the availability of target-derived neurotrophic factors (Jackson-Lewis et al., 2000, Oo and Burke, 1997, Burke, 2003, Van den Heuvel and Pasterkamp, 2008). Similarly, BMPRIb is also expressed at relatively high levels in the early postnatal (P1 to P11) rat striatum. These data suggest that BMP2 and GDF5 may function as target-derived neurotrophic factors for VM DA neurons at this stage of development. Indeed, both factors have been shown to promote the survival of VM DA neurons (O'Keeffe et al., 2004a, Wood et al., 2005, Reiriz et al., 1999, Jordan et al., 1997, Sullivan et al., 1997, Sullivan et al., 1999, Hurley et al., 2004, O'Sullivan et al., 2010, Sullivan et al., 1998b, Espejo et al., 1999). Furthermore, BMPRII null mice display reductions in nigrostriatal neurons, and in striatal DA innervation, in adulthood (Chou et al., 2008a), which is likely due to deficient neurotrophic support during the postnatal developmental period. There is a peak of BMPRIb expression during adulthood in the striatum which may point towards the aforementioned potential role of BMPs in the maintenance of VM DA neurons. Furthermore, it may suggest that BMPRIb functions in promoting the arborisation of DA axons which survive the period of naturally-occurring cell death. The sustained expression of BMPRs in the adult rat brain (up to P90) demonstrated in this study suggests a role for BMP2 and GDF5 in the maintenance of the nigrostriatal system during adulthood. In support of this role, BMPs (including BMP2) and BMPRs have been shown to be expressed in the midbrain and striatum from 6-24 months in the adult rat (Chen et al., 2003). Furthermore, in animal models of PD, exogenous GDF5 delivery into the nigrostriatal pathway has potent survival-promoting effects on adult nigral DA neurons (Sullivan et al., 1997, Sullivan et al., 1999, Hurley et al., 2004, O'Sullivan et al., 2010, Sullivan and Toulouse, 2011). Disruption to the normal expression of BMPRs may thus render nigrostriatal DA neurons more vulnerable to degeneration, and increase the risk of the development of PD. The phenotype of the BMPRII null mouse supports this suggestion, while haploinsufficiency of other TGFβ superfamily members, such as GDNF and TGFβ2, causes an accelerated decline of midbrain DA neurons during normal aging (Boger et al., 2006, Andrews et al., 2006). Interestingly, after a 6-hydroxydopamine (6-OHDA)-induced lesion of the adult rat nigrostriatal pathway, BMPRs were significantly downregulated in the nigra, but upregulated in the striatum (Chen et al., 2003). These findings likely reflect loss of BMPR expression by nigral DA neurons which are destroyed by 6-OHDA, and a potential compensatory mechanism by the striatum to restore BMP-

mediated survival-promoting effects on innervating VM DA neurons through upregulation of BMPR expression. The BMPR expression in the developing striatum may also reflect autocrine or paracrine trophic influences on cells within the striatum, since the BMPs have been shown to play roles in striatal neuronal development (Gratacos et al., 2002, Gratacos et al., 2001).

The present study found that TH mRNA levels in the VM are maximal at E14, which is expected since this is the time-point at which the greatest amount of post-mitotic DA neurons are present in the VM (Lumsden and Krumlauf, 1996, Lauder and Bloom, 1974, Gates et al., 2006). There was a subsequent significant decline in TH expression from birth onwards, reaching the lowest levels at P11, which correlates with the onset of programmed cell death for nigrostriatal DA neurons. TH mRNA expression was found to remain stable in the adult VM, reflecting the established population of A9 DA neurons.

Following the demonstration of the expression of BMPRs in the VM and striatal regions during embryonic and post-natal development, it was next demonstrated that BMPRs are expressed on both DA and non-DA cells in E14 rat VM cultures. This indicates that BMP2 and GDF5 may act in either autocrine or paracrine manners to exert neurotrophic effects on DA neurons. Immunocytochemical staining for phospho-Smad 1/5/8 showed that both DA and non-DA cells express these transcription factors, and the nuclear location of phospho-Smad 1/5/8 indicated that these VM cells also express Smad4, which is required for the nuclear translocation of Smad 1/5/8 following their activation. These results demonstrate that VM DA neurons have the machinery to carry out canonical Smad 1/5/8 signalling in response to BMPs.

The current study has demonstrated that both BMP2 and GDF5 induce the neurite outgrowth of E14 VM DA neurons, which is consistent with previous studies on BMP2 (Reiriz et al., 1999) and GDF5 (O'Keeffe et al., 2004a) in rat VM cultures. The molecular mechanisms mediating this neurite growth-promoting effect were then assessed. BMP2 and GDF5 were both shown to activate canonical Smad 1/5/8 in VM DA neurons, as demonstrated by nuclear accumulation of phosphorylated Smad 1/5/8. Furthermore, GDF5 was shown to activate Smad 1/5/8 signalling in the adult rat VM *in vivo*. Interestingly GDF5, but not BMP2, activated Smad 1/5/8 signalling in non-DA cells. This finding is not surprising considering that the numbers of astrocytes are dramatically increased in GDF5-treated E14 rat VM

cultures (Krieglstein et al., 1995b, O'Keeffe et al., 2004a, Wood et al., 2005). It has been suggesting that GDF5 may have an indirect neurotrophic action on VM DA neurons, possibly by stimulating the production of glial-derived growth factor(s), such as GDNF, that might be involved in the neurotrophic response (Sullivan and O'Keeffe, 2005). Conversely, Wood et al. showed that inhibition of the GDF5-induced increase in astrocytes did not prevent the neurotrophic effects of GDF5 on DA neurons in E14 rat VM cultures (Wood et al., 2005), suggesting that GDF5 has a direct neuronal action. Similarly, Reiriz et al. (1999) used the gliotoxin α-aminoadipic acid to demonstrate that the neurotrophic effects of BMP2 on E14 rat VM DA neurons were not mediated by astrocytes. These data, along with the present finding that BMP2 specifically activates Smad signalling in VM DA neurons, suggest that BMP2 and GDF5 act directly on DA neurons to induce axonal growth. The neurotrophic and gliogenic effects of GDF5 in VM cultures may thus be independent of one another. Similarly, BMP-Smad signalling has previously been shown to have such a dual-inductive role in enteric neural crest cells (Chalazonitis et al., 2004, Chalazonitis et al., 2011, Chalazonitis and Kessler, 2012). Collectively, these data suggest that canonical Smad signalling mediates the neurotrophic effects of BMP2 and GDF5 on VM DA neurons.

To explore this premise further, the effects of BMP2 and GDF5 were assessed following inhibition of their binding to BMPRs. BMPR activation by BMP2 and GDF5 was blocked by using noggin, an extracellular inhibitor of BMPs which blocks their binding epitopes for BMPRs (Groppe et al., 2002, Smith and Harland, 1992), or dorsomorphin, a small molecular inhibitor of BMPRI (Yu et al., 2008). Pretreatment with either noggin or dorsomorphin inhibited the neurite growth-promoting effects of BMP2 and GDF5 on E14 VM DA neurons. Noggin and dorsomorphin have both previously been used to prevent BMP-induced neurite outgrowth in other neuronal populations (Parikh et al., 2011, Li and LoTurco, 2000), and the current study also demonstrated their inhibition of BMP-induced neurite growth in SH-SH5Y cells. BMPR-activation is therefore crucial to BMP-induced axonal growth from VM DA neurons. BMP2 can signal through both BMPRIa and BMPRIb, whereas GDF5 predominantly signals through BMPRIb (Nishitoh et al., 1996), suggesting that BMPRIb is responsible for mediating the neurotrophic effects of BMP2 and GDF5. To test this hypothesis, E14 VM cultures were electroporated with a caBMPRIb, which has been previously shown to activate the Smad 1/5/8

signalling pathway (Chapter 2) (Hegarty et al., 2013b). E14 VM neurons expressing the caBMPRIb were significantly larger than those transfected with the control plasmid, suggesting that BMP2 and GDF5 act via BMPRIb to induce neurite extension. These findings are in agreement with a previous study in SH-SH5Y cells, a model of human DA neurons (Chapter 2) (Hegarty et al., 2013b). Furthermore, the application of GDF5 at the time of plating, when BMPR1b is expressed, results in neurotrophic effects on VM DA neurons, whereas application after six days *in vitro*, when the BMPRIb is no longer expressed, has no effect (O'Keeffe et al., 2004a). The present study demonstrated that the transcriptional activity of Smad 1/5/8 is required for this BMP-induced neurite growth of VM neurons. The inhibition of the nuclear translocation of the Smad 1/5/8 transcription factors, using a siRNA to target Smad4, significantly inhibited neurite outgrowth of E14 VM neurons induced by caBMPRIb. Finally, this study confirmed that the neurite growth-promoting effects of the caBMPRIb are specific to VM DA neurons. The caBMPRIb therefore mimics the effects of BMP2 and GDF5 on E14 VM DA neurons. Furthermore, siRNA knockdown of the BMPRIb also prevented GDF5-induced neurite growth in SH-SH5Y cells. Collectively, these data show that BMPRIb-activation of Smad 1/5/8 is the mechanism by which BMP2 and GDF5 promote the neurite growth of VM DA neurons.

This study has identified BMP2 and GDF5 as *bona fide* candidates for regulators of nigrostriatal pathway development. The expression profiles of their BMPRs in the VM and striatum, and their neurotrophic effects on cultured VM DA neurons, suggest roles for BMP2 and GDF5 in the extension/projection of DA axons from the developing VM. They may act as target-derived neurotrophic factors for innervating nigrostriatal fibres, and/or as factors which maintain the integrity of nigrostriatal projections during adulthood. However, the analysis of mice with deficiencies in GDF5 and/or BMP2 will be essential to further establish these factors as regulators of nigrostriatal pathway development. It is not unlikely for these morphogens to play multiple roles during nigrostriatal system development, since locally-expressed factors are employed throughout NS development to regulate multiple steps of particular developmental processes, with temporally-regulated functions. A relevant example of this is seen during chick dorsal SC development, in which BMP-Smad signalling promotes neuronal specification rather than astrocytic specification at E5, but at E6 has the opposite effect (Agius et al., 2010). The present

study has thus contributed to the growing body of knowledge regarding the development of the A9 pathway. A detailed, well-characterised understanding of nigrostriatal pathway development is vital, to provide important information regarding developmental abnormalities or age-related defects that may lead to the progressive degeneration of this pathway in PD. Furthermore, cell replacement therapy is one of the most promising therapies for the treatment of PD (Orlacchio et al., 2010, Bonnamain et al., 2012, De Feo et al., 2012, Toulouse and Sullivan, 2008, Hedlund and Perlmann, 2009). Due to the importance of the establishment of functional connections by transplanted DA cells in the host striatum, factors which promote neurite outgrowth are being considered as adjuncts to transplantation therapy. GDF5 and BMP2 are thus ideal candidates to be used as growth-promoting factors, with their survival-promoting effects on VM DA neurons being beneficial also. The present study has, for the first time, demonstrated that the downstream molecular mechanisms mediating the neurite outgrowth-promoting effects of GDF5 and BMP2 in VM DA neurons are dependent, at least in part, upon BMPRIb-mediated activation of canonical Smad 1/5/8 signalling.

7. Molecular mechanisms regulating BMP-Smad signalling-dependent neurite growth in a model of human midbrain DA neurons.

7.0 Aims

- Investigate the mechanism(s) by which canonical BMP-Smad 1/5/8 signalling induces neuronal differentiation in SH-SH5Y cells.
- Identify potential regulators of BMP2- and GDF5-induced Smad signalling, and neurite growth, in SH-SY5Y cells.

7.1 Abstract

Two members of the BMP family of proteins, BMP2 and GDF5, have emerged as factors which regulate the neurite growth of VM DA neurons *in vitro* and *in vivo*, and this neurotrophic effect has now been shown to be dependent on BMPRIb activation of canonical Smad signalling. However, the precise intracellular cascades that regulate BMP-Smad-induced neurite growth, and the downstream molecular changes that mediate this effect, are still unknown. To examine this further, the present study firstly examined the role of endocytosis in BMP2- and GDF5-induced Smad signalling and neurite growth in SH-SH5Y cells, a model of human midbrain DA neurons. BMP2-, but not GDF5-induced, Smad signalling and neurite growth was shown to be regulated by dynamin-dependent endocytosis. This study subsequently demonstrated that BMP2 and GDF5 do not require GDNF signalling to promote neurite growth. Finally, Sip1, which is an important negative modulator of BMP-Smad signalling during NS development, was identified as a novel regulator of neurite growth in SH-SH5Y cells. Sip1 likely acts to repress BMP-Smad-driven neurite growth.

7.2 Introduction

BMP2 and GDF5 are two well-characterised members of the BMP family with regards to NS development (Sullivan and O'Keeffe, 2005, Hegarty et al., 2014c). Prior to this work, although many studies had shown that both have potent neurotrophic effects on VM DA neurons *in vitro* (O'Keeffe et al., 2004b, Jordan et al., 1997, O'Keeffe et al., 2004a, Krieglstein et al., 1995b, Wood et al., 2005) and *in vivo* (Costello et al., 2012, Hurley et al., 2004, Sullivan et al., 1997, Sullivan et al., 1999, Sullivan et al., 1998b, Espejo et al., 1999), the molecular mechanisms mediating these DA neurotrophic effects were unknown. Using the SHSY5Y cell line (Chapter 2) and E14 rat VM primary cultures (Chapter 3) as models of midbrain DA neurons, BMP2 and GDF5 were shown to promote DA neuronal growth by acting through their canonical signalling pathway (Hegarty et al., 2014a, Hegarty et al., 2013b). In this pathway, BMPs bind to a complex of BMPRI and BMPRII. BMPRI subsequently phosphorylates Smad 1/5/8 that translocate to the nucleus, following Smad4 binding, to modulate target gene expression (Miyazono et al., 2010, Sieber et al., 2009). Specifically, BMP2 and GDF5 were shown to promote neurite growth through BMPRIb-dependent stimulation of Smad 1/5/8 nuclear translocation (Hegarty et al., 2014a, Hegarty et al., 2013b). However, the precise intracellular cascades that regulate this BMP-Smad driven process and the downstream molecular changes that promote neurite growth are still unknown. To examine this further, the present series of experiments examined; 1) the role of endocytosis in canonical BMP-Smad signalling, and its promotion of neurite growth, 2) Sip1 regulation of BMP2- and GDF5-induced neurite growth, and 3) the involvement of GDNF in the neurite growth-promoting effects of BMP2 and GDF5.

Endocytosis of transmembrane receptors, such as BMPRs, regulates their availability at the cell membrane, and also attenuates their signal transduction (Sorkin and von Zastrow, 2009). Receptor endocytosis occurs via either a clathrin-mediated or caveolae-mediated mechanism, both of which are dependent on dynamin (Heymann and Hinshaw, 2009, Le Roy and Wrana, 2005). In clathrin-mediated endocytosis, clathrin-coated pits pinch off the membrane, while caveolae-mediated endocytosis employs membrane invaginations containing caveolin. The internalized receptors are recycled to the plasma membrane, degraded in lysosomes, or alternatively use the endosome as a signalling platform, in which downstream

components are presented for further activation (Le Roy and Wrana, 2005). BMPRs initiate distinct intracellular cascades are depending on their endocytic route, membrane localization and mode of oligomerization (Hartung et al., 2006). Hartung et al. (2006) showed that the phosphorylation of Smad 1/5/8 by BMPRI is induced at the plasma membrane, while continuation of Smad signalling can occur, via endosomes, following clathrin-mediated endocytosis of the BMPRs. Both Smad-dependent and -independent signalling pathways require clathrin- and caveolae-mediated endocytosis to exert transcriptional activity (Hartung et al., 2006, Bragdon et al., 2009, Saldanha et al., 2013, Bonor et al., 2012, Alborzinia et al., 2013). Furthermore, inhibition of endocytosis has been shown to affect the differentiation of osteoblasts, suggesting that endocytosis plays a functional role in differentiation (Heining et al., 2011, Rauch et al., 2002). There have been a paucity of studies examining the role of endocytosis in mediating the effects BMP-Smad signalling in neurons. To address this, this study investigated the role of dynamin-dependent endocytosis in canonical BMP-Smad signalling in the SH-SH5Y neuronal cell line.

As aforementioned, the regulators and downstream effectors of BMP-Smad signalling induction of neurite growth are unknown. To address this, this study focused on Sip1, a negative regulator of BMP-Smad signalling (Verschueren et al., 1999, Postigo et al., 2003). Sip1 has recently emerged as a factor that contributes to the induction of a VM DA phenotype in human pluripotent stem cells (Cai et al., 2013), which suggests that Sip1 may play a role in regulating BMP2- and GDF5-induced DA neuronal growth. Indeed, knockdown studies have shown that Sip1 is required for BMP-Smad signalling-regulation of the development of the CNS and PNS primordia (Nitta et al., 2004, van Grunsven et al., 2007, Lerchner et al., 2000, Delalande et al., 2008, Van de Putte et al., 2007, Cacheux et al., 2001, Wakamatsu et al., 2001, Hegarty et al., 2013a). Furthermore, Sip1 regulation of BMP-Smad signalling has been shown to be involved in the development of SC motor neurons (Roy et al., 2012), and to mediate CNS myelination (Weng et al., 2012). GDNF is the best characterized neurotrophic factor for midbrain DA neurons (Peterson and Nutt, 2008, Hegarty et al., 2014c, Sullivan and Toulouse, 2011), and has been suggested to facilitate the DA neurotrophic effects of BMP2 and GDF5 (Sullivan and O'Keeffe, 2005, Wood et al., 2005). Thus, the present study also investigates the potential involvement of GDNF signalling in the neurite growth-promoting effects of BMP2 and GDF5 in SH-SH5Y cells.

7.3 Results

7.3.1 The kinetics and amplitude of BMP-induced Smad 1/5/8 signalling in SH-SH5Y cells are regulated by dynamin-dependent endocytosis

BMP signalling has been shown to be regulated by the oligomerization and membrane localization of BMPRs, while the propagation of intracellular signalling is under the control of various endocytic routes (Nohe et al., 2002, Hartung et al., 2006, Heining et al., 2011, Rauch et al., 2002, Bragdon et al., 2009, Saldanha et al., 2013, Alborzinia et al., 2013, Bonor et al., 2012). To examine whether BMP-Smad signalling is regulated by endocytosis in the nervous system, this study assessed the effect of inhibiting endocytosis on BMP2- and GDF5-induced Smad signalling in the SH-SH5Y neuroblastoma cell line. To do this, the small molecule inhibitor dynasore, which specifically interferes with dynamin-dependent endocytosis by reversibly blocking the GTPase activity of dynamin (Macia et al., 2006), was used. Dynamin is crucial for the fission of vesicles prior to their release from the plasma membrane, and is required for both clathrin-mediated and caveolae-mediated endocytosis (Heymann and Hinshaw, 2009, Le Roy and Wrana, 2005).

To test the ability of dynasore to inhibit endocytosis in SH-SH5Y cells, uptake of fluorescently-labelled transferrin by SH-SH5Y cells was examined as a measurement of endocytosis (Ehrlich et al., 2004). Uptake of 30 μg/ml of Alexa594-transferrin was potently inhibited in SH-SY5Y cells following treatment of the cells with 40 μm of dynasore for 2 h (Fig. 7.4.1a, b). Following this demonstration of the ability of dynasore to efficiently inhibit endocytosis in SH-SH5Y cells, the present study investigated the effect that inhibition of dynamin-dependent endocytosis has on the temporal kinetics of Smad signalling in the SH-SH5Y neuronal cell line. It has previously been shown that both BMP2 and GDF5 activate Smad signalling in SH-SY5Y cells, albeit with different temporal profiles of activation (Chapter 2) (Hegarty et al., 2013b). This study examined the activation of Smad signalling at 5, 30 and 60 min following treatment with 200 ng/ml of these BMPs, and concomitantly investigated whether dynasore pretreatment had an effect on BMP2- and GDF5-induced Smad signalling. Densitometric analysis of the nuclear levels of phospho-Smad 1/5/8 showed that BMP2 significantly increased the levels of nuclear phospho-Smad 1/5/8 at all time-points examined (Fig. 7.4.2c, d), while GDF5 significantly increased nuclear phospho-Smad levels at 30 and 60 min (Fig. 7.4.2e,

f). Additionally, phospho-Smad levels increased between each treatment time-point. Treatment with dynasore 30 min prior to BMP2 application delayed Smad signalling activation, so that significant increases in nuclear phospho-Smad levels were detected only after 30 min, rather than from 5 min onwards (Fig. 7.4.2c, d). Furthermore, dynasore pre-treatment significantly reduced the magnitude of BMP2-induced Smad signalling at 60 min (Fig. 7.4.2c). Inhibition of dynamin-dependent endocytosis did not significantly delay or reduce GDF5-induced Smad signalling at any of the time-points examined (Fig. 7.4.2e, f). Taken together, these data demonstrate that activation of Smad signalling by BMP2, but not GDF5, is regulated by dynamin-dependent endocytosis.

7.3.2 BMP2-induced neurite outgrowth of SH-SH5Y cells is significantly reduced by short-term inhibition of dynamin-dependent endocytosis

BMP2 and GDF5 have previously been shown to directly induce neurite outgrowth of SH-SH5Y cells via a BMPR-Smad dependent pathway (Chapter 2) (Hegarty et al., 2013b). Considering that inhibition of dynamin-dependent endocytosis affects the kinetics and magnitude of BMP2-induced Smad 1/5/8 signalling, this study examined the consequences of this on BMP-induced neurite growth. SH-SY5Y cells were treated with BMP2 or GDF5 daily before being stained with the vital fluorescent dye calcein at 4 DIV, to allow visualisation of the cytoskeleton. To inhibit dynamin-dependent endocytosis, dynasore was added 30 min prior to BMP application and for just the initial 4 h of BMP-stimulation, as long-term treatment over several days caused cell death (data not shown). The total neurite length was then measured using a modified line intercept method (Mayhew, 1992). Daily treatment with either BMP2 or GDF5 for 4 DIV resulted in a significant increase in the total length of neurites (Fig. 7.4.2). Similarly, SH-SY5Y cells treated with dynasore and BMP2 or GDF5, had significantly longer neurites when compared to the control (Fig. 7.4.2). Dynasore application significantly reduced BMP2-induced, but not GDF5-induced neurite outgrowth of SH-SH5Y cells (Fig. 7.4.2a-d). These data suggest that dynamin-dependent endocytosis is required during the initial phase of BMP2 stimulation for its maximal induction of SH-SH5Y neurite outgrowth.

7.3.3 Neurite growth-induction of BMP2 and GDF5 in SH-SH5Y cells is not dependent on GDNF signalling

It has been suggested that the neurotrophic effects of GDF5 and BMP2 on VM DA neurons may be mediated indirectly through the action of GDNF (Wood et al., 2005, Sullivan and O'Keeffe, 2005). To test this possibility, the present study, using SH-SH5Y cells as a model of human DA neurons, adopted a similar approach to the recent Orme et al. (2013) study, which inhibited GDNF's heparan sulphate-dependent signalling to prevent its DA neurotrophic effects (Barnett et al., 2002, Iwase et al., 2005, Orme et al., 2013). Pretreatment of SH-SH5Y cells with 0.3 U/ml of heparinase III (Barnett et al., 2002, Iwase et al., 2005, Orme et al., 2013) did not affect BMP2- or GDF5-induced neurite growth in SH-SY5Y cells (Fig. 7.4.3). These findings suggest that the direct neurotrophic effects of BMP2 and GDF5 on SH-SH5Y cells are not dependent on GDNF signalling.

7.3.4 Sip1 knockdown promotes neurite outgrowth in SH-SH5Y cells and in E14 rat VM cultures.

Sip1 is a negative regulator of Smad signalling in response to BMPs, which plays an essential part in BMP-Smad signalling during NS development (Hegarty et al., 2013a), and has been implicated in VM DA neurogenesis (Cai et al., 2013). Before assessing its role in BMP-Smad-induced neurite growth, the current study analysed Sip1 mRNA expression in response to BMP2 and GDF5 treatment for 0.5, 1, 4, 8 and 24 h in SH-SH5Y cells (Fig. 7.4.4a, b). Sip1 expression increased following BMP2 or GDF5 treatment, before returning to basal levels, albeit with different temporal profiles for BMP2 and GDF5 (Fig. 7.4.4a, b). This transient increase ceased after 1 h following BMP2 treatment (Fig. 7.4.4a), but decreased after 4 h following GDF5 treatment (Fig. 7.4.4b). Furthermore, the basal Sip1 expression levels remained until at least 24 hrs after BMP2 treatment (Fig. 7.4.4a), but from 4 to 24 h after GDF5 treatment (Fig. 7.4.4b). The transient increase of Sip1 expression following Smad activation is typical of negative regulators in response to the activation of the signalling pathway which they regulate.

Given that Sip1 is a negative regulator of Smad signalling, and that canonical BMP-Smad signalling drives neurite growth in SH-SY5Y cells and E14 rat VM DA neurons (Chapter 2 and 3) (Hegarty et al., 2014a, Hegarty et al., 2013b, O'Keeffe et al., 2004a, Reiriz et al., 1999, Nakamura et al., 2003, Toulouse et al., 2012), this

study next investigated the effect of Sip1 knockdown on the neurite growth within these two models of midbrain DA neurons. To do this, SH-SY5Y cells were transfected with Sip1 siRNA, with a transfection efficiency of 25%. There was a clear reduction in Sip1 mRNA expression in Sip1 siRNA-transfected cells, compared to control siRNA-transfected and untransfected SH-SH5Y cells (Fig. 7.4.4c). SH-SY5Y cells were then transfected with this Sip1 siRNA, which induced a significant increase in total neurite length compared to cells transfected with the relevant control siRNA (Fig. 7.4.4d, e). Furthermore, SH-SH5Y cells with Sip1 knockdown had significantly larger neurites than BMP2- and GDF5-treated SH-SH5Y cells transfected with the control siRNA (Fig. 7.4.4d, e). Finally, BMP2 and GDF5 treatment did not significantly attenuate the neurite growth promoting effects of Sip1 knockdown. This suggests that Sip1 is the principal regulator of SH-SH5Y neurite growth. E14 rat VM neurons were then transfected with the Sip1 siRNA, and neurite growth was assessed and compared to that in cultures transfected with the control siRNA. Electroporation of E14 VM neurons with the Sip1 siRNA induced a significant increase in their neurite length at 3 DIV (Fig. 7.4.4f, g).

The present study then assessed the *in vivo* expression of Sip1 during midbrain development. *In situ* hybridization showed that Sip1 is strongly expressed in the E11.5 mouse VM (Fig 7.4.5a, c), at a time when VM NPs are undergoing specification into DA neurons (Lumsden and Krumlauf, 1996, Lauder and Bloom, 1974, Specht et al., 1981a, Specht et al., 1981b, Hegarty et al., 2013c). Sip1 expression was significantly lower in the E13.5 mouse VM (Fig 7.4.5b, c), and continued to decrease in the midbrain until P4 (Fig 7.4.5c), a period which corresponds to the development of axonal projections from VM DA neurons to the striatum (Gates et al., 2004, Nakamura et al., 2000, Van den Heuvel and Pasterkamp, 2008). Collectively these data suggest that Sip1 may be a novel regulator of the axonal outgrowth of VM DA neurons.

7.4 Figures and Figure Legends

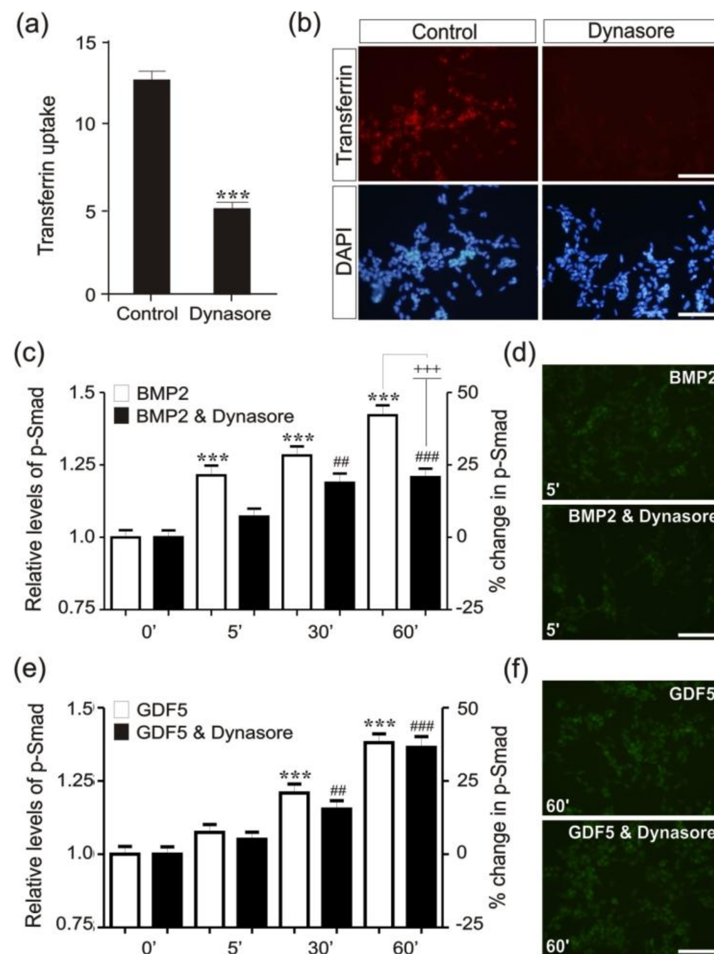


Figure 7.4.1: Inhibition of dynamin-dependent endocytosis delays and reduces BMP2-, but not GDF5-, induced Smad 1/5/8 signalling in SH-SY5Y cells.

(a) Relative immunofluorescence intensity of Alexa594-transferrin in dynasore-treated SH-SY5Y cells, as determined by densitometry (*** $P < 0.001$ vs control; Student's *t*-test; 20 fields for each group per experiment. N = 3). Data are expressed as mean ± SEM. (b) Representative photomicrographs of Alexa594-transferrin immunofluorescence in control and dynasore-treated SH-SY5Y cells. Scale bar = 100µm. Relative intensity of phospho-Smad immunofluorescence in dynasore-pretreated and/or (c) BMP2- or (e) GDF5-treated SH-SY5Y cells, at 0 (control), 5, 30, and 60 min, as indicated (*** $P < 0.001$ vs 0 min; ## $P < 0.01$ ### $P < 0.001$ vs 0 min (dynasore); +++ $P < 0.001$; One-way ANOVA and post hoc Tukey's test; 50 cells for each group per experiment. N = 3). Data are expressed as mean ± SEM. Representative photomicrographs of dynasore-pretreated and/or (d) BMP2- or (f) GDF5-treated, SH-SY5Y cells immunocytochemically stained for phospho-Smad 1/5/8 at (d) 5 and (f) 60 min. Scale bar = 100µm.

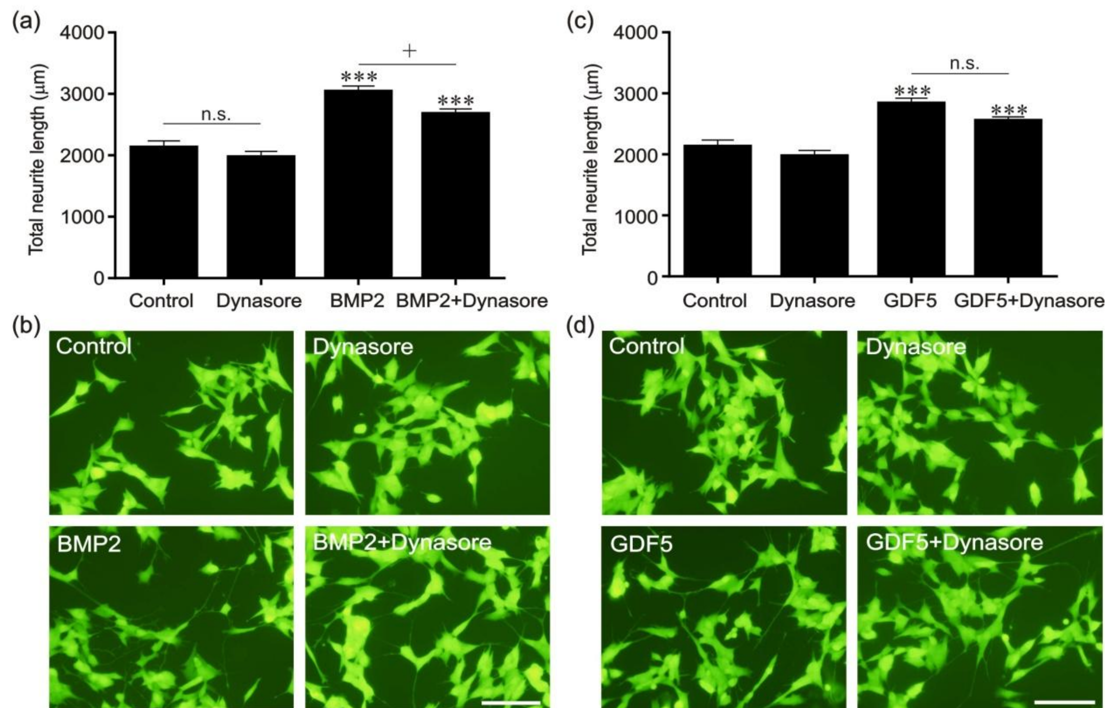


Figure 7.4.2: Inhibition of dynamin-dependent endocytosis reduces the promotion of SH-SH5Y neurite outgrowth by BMP2, but not by GDF5.

Total neurite length of dynasore-pre-treated and/or (a) BMP2- or (c) GDF5-treated SH-SY5Y cells, as indicated (*** $P < 0.001$ vs control; + $P < 0.05$ vs BMP2; ANOVA with post-hoc Tukey's test; 20 images analysed for each group per experiment; N = 3 experiments). Data are expressed as mean ± SEM. (b) Representative photomicrographs of dynasore pre-treated and/or (b) BMP2- or (d) GDF5-treated SH-SY5Y cells stained with the vital fluorescent dye calcein. Scale bar = 100 μm.

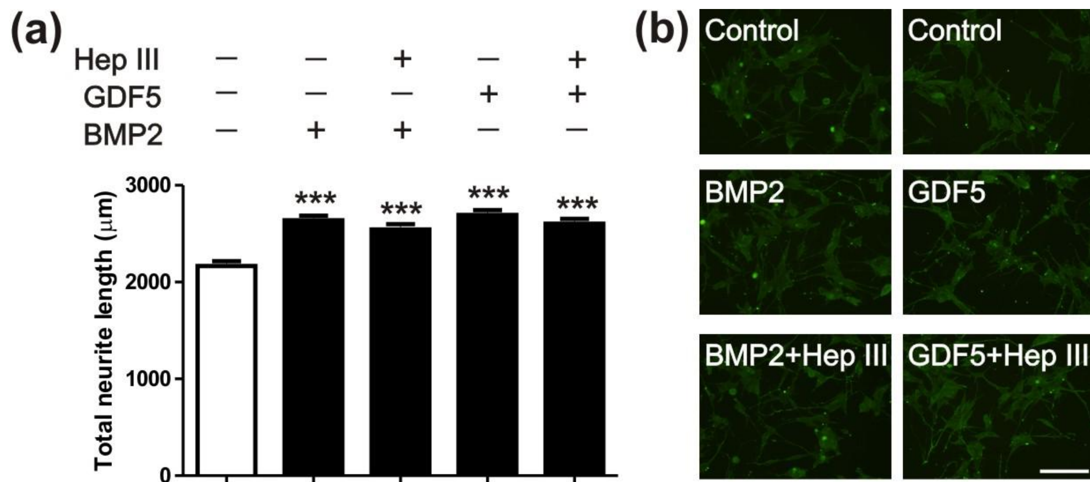


Figure 7.4.3: Heparinase III does not affect the induction of SH-SH5Y neurite growth by BMP2 and GDF5.

(a) Total neurite length of heparinase III-pre-treated and/or BMP2- or GDF5-treated (daily for 4 DIV) SH-SY5Y cells, as indicated (*** $P < 0.001$ vs control; ANOVA with post-hoc Tukey's test; 20 images analysed for each group per experiment; N = 3 experiments). Data are expressed as mean ± SEM. (b) Representative photomicrographs of heparinase III pre-treated and/or BMP2- or GDF5-treated SH-SY5Y cells stained with the vital fluorescent dye calcein. Scale bar = 100 μm.

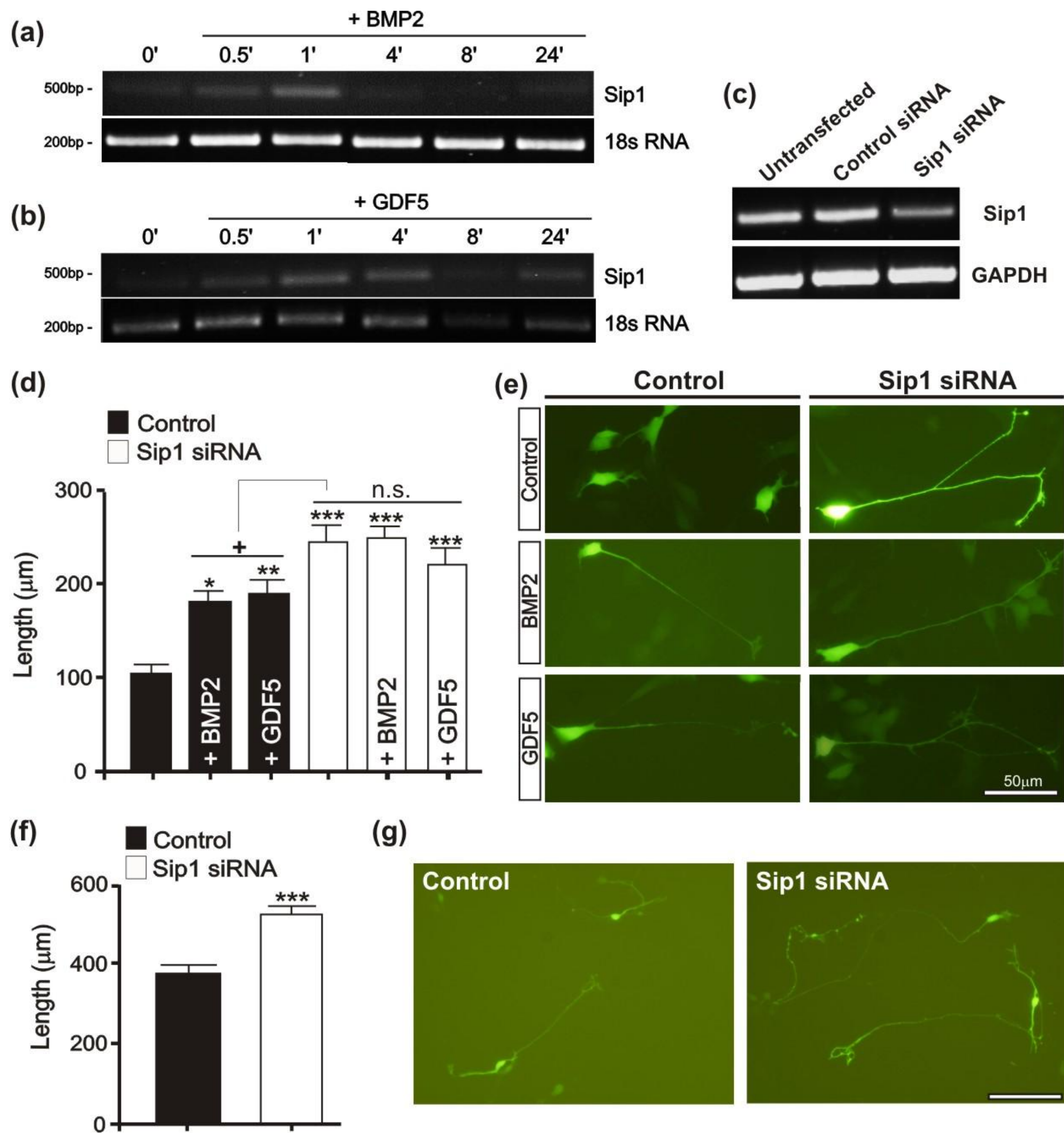


Figure 7.4.4: Sip1 knockdown significantly increases neurite growth in SH-SH5Y cells and in E14 rat VM cultures.

RT-PCR analysis of Sip1 and 18sRNA/GAPDH in SH-SY5Y cells treated with (a) BMP2 or (b) GDF5 for 0.5, 1, 4, 8 and 24 h, or (c) transfected with Sip1 or control siRNA. Neurite length of control plasmid- and Sip1 siRNA-transfected (d) SH-SY5Y cells or (f) E14 rat VM neurons with or without BMP2 or GDF5 treatment, as indicated (* $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$ vs control (untreated); + $P < 0.05$; (d) ANOVA with post-hoc Tukey's test or (f) Student's *t*-test ; 40 cells for each group per experiment. N = 3). Data are expressed as mean ± SEM. Representative photomicrographs of control plasmid- and Sip1 siRNA-transfected, GFP-expressing, (e) SH-SY5Y cells or (g) E14 rat VM neurons with or without BMP2 or GDF5 treatment, as indicated. Scale bar = (e) 50 µm or (g) 100 µm.

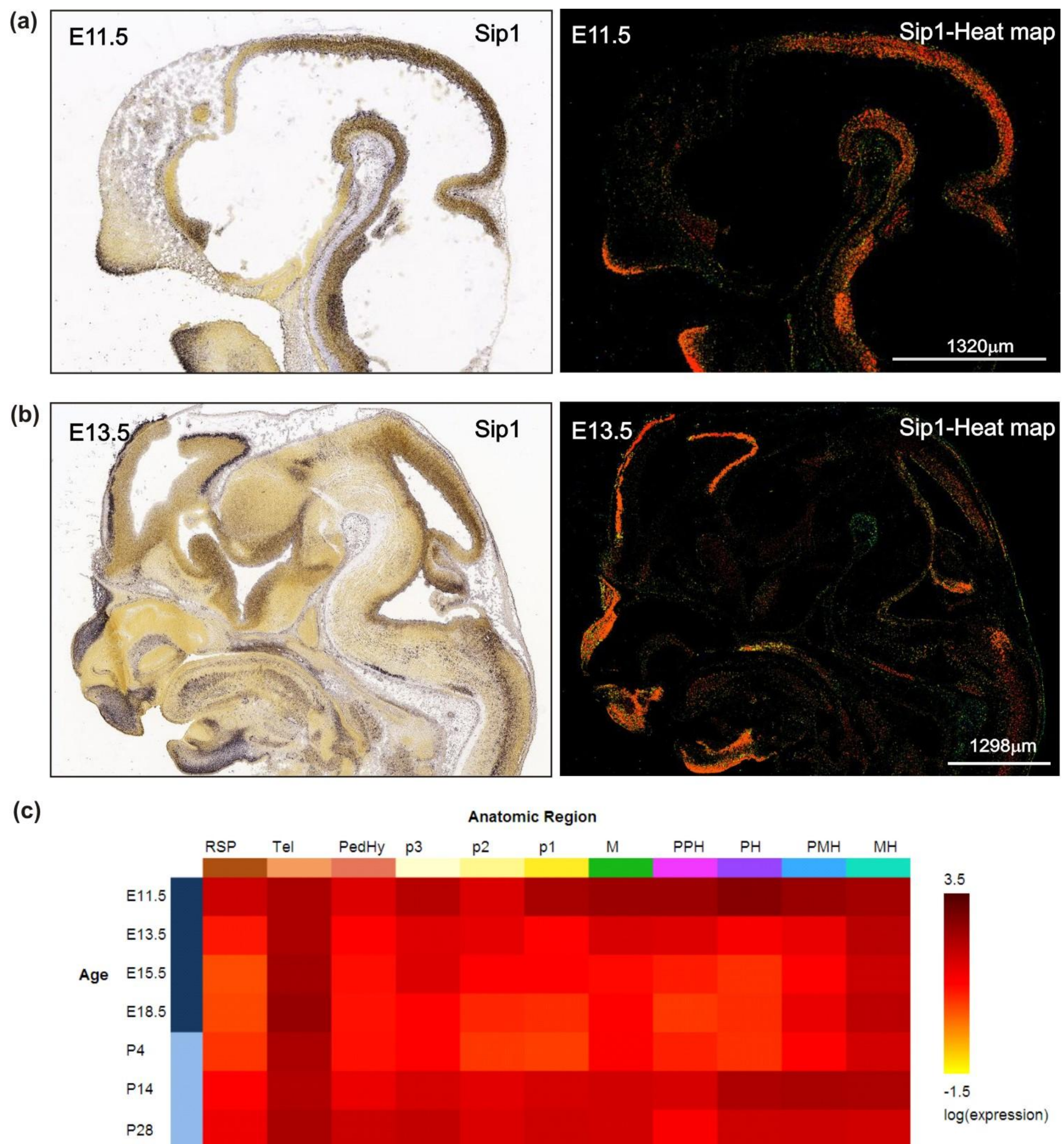


Figure 7.4.5: *In vivo* Sip1 expression in the mouse VM during embryonic and postnatal development.

In situ hybridization images taken from the Allen Developing Brain Atlas (©(Allen) Developing Mouse Brain Atlas, 2012) showing Sip1 expression (purple staining or heat signal) in saggital sections of the (a) E11.5 or (b) E13.5 mouse ventral midbrain. Scale bar = 1298 µm. (c) Grid heat map, taken from the Allen Developing Brain Atlas (©(Allen) Developing Mouse Brain Atlas, 2012), of the devloping mouse brain from E11.5 to P28 showing Sip1 expression in various brain regions (M = midbrain).

7.5 Discussion

BMP2 and GDF5 have emerged as factors which regulate the neurite growth of VM DA neurons *in vitro* and *in vivo* (O'Keeffe et al., 2004a, Clayton and Sullivan, 2007, Hegarty et al., 2014a, O'Sullivan et al., 2010, Costello et al., 2012, Hurley et al., 2004, Reiriz et al., 1999, Espejo et al., 1999, Hegarty et al., 2014c), and this effect has now been shown to be dependent on BMPRIb activation of canonical Smad signalling (Chapter 2 and 3) (Hegarty et al., 2014a, Hegarty et al., 2013b). The present study aimed to build on these findings through the investigation of the precise intracellular cascades that regulate BMP-Smad induced neurite growth, and the determination of the downstream molecular changes that mediate this effect, in the SH-SH5Y cell line model of human VM DA neurons.

This study first investigated the role played by endocytosis plays in regulating BMP2- and GDF5-induced Smad signalling and neurite growth. Endocytosis of transmembrane receptors, which occurs via a clathrin- or caveolin-mediated mechanism, regulates membrane availability of receptors, and provides a signalling platform, via endosomes, that attenuates signal propagation (Sorkin and von Zastrow, 2009). Despite a paucity of studies which have investigated the role played by endocytosis in BMP-Smad signalling and its effects, particularly in the NS, it has been shown that canonical BMP-Smad signalling is regulated by the oligomerization and membrane localization of type I and type II BMPRs, while the subsequent propagation of intracellular Smad signalling is controlled by various endocytic routes (Nohe et al., 2002, Hartung et al., 2006, Heining et al., 2011, Rauch et al., 2002, Bragdon et al., 2009, Saldanha et al., 2013, Alborzinia et al., 2013, Bonor et al., 2012). As aforementioned, clathrin-mediated and caveolae-mediated endocytosis are dependent on dynamin (Heymann and Hinshaw, 2009, Le Roy and Wrana, 2005). The current study inhibited dynamin-dependent endocytosis using dynasore (Macia et al., 2006), an approach which was validated by measuring tranferrin uptake. This study showed that Smad 1/5/8 signalling is still activated by BMP2 and GDF5 when dynamin-dependent endocytosis is inhibited. However, dynasore treatment delayed the onset and reduced the levels of BMP2-, but not GDF5-, induced phospho-Smad 1/5/8 activation. These data suggest that efficient phosphorylation and/or nuclear translocation of Smad 1/5/8, in response to BMP2, is regulated by dynamin-dependent endocytosis in SH-SH5Y cells. In support of these

findings, Heining et al. (2011) showed that inhibition of endocytosis delays and reduces BMP2-induced Smad phosphorylation in the C2C12 mouse mesenchymal cell line (Heining et al., 2011). It is important to note that the time-points examined in the present study may have precluded the detection of dynasore-induced alterations in Smad phosphorylation by GDF5 at later time-points, as well as any alterations in initial Smad activation. Dynamin is crucial for fission of vesicles prior to their release from the plasma membrane (Heymann and Hinshaw, 2009, Le Roy and Wrana, 2005), which means that dynasore prevents the detachment of vesicles from membranes and leads to an accumulation of vesicles at the membrane. Such a disturbance to the localization of BMPRs may contribute to the delay and reduction in BMP2-induced Smad 1/5/8 phosphorylation. However, it is unclear whether inhibition of dynamin-dependent endocytosis delays Smad 1/5/8 nuclear translocation or phosphorylation, or both. Likewise, it is unclear whether the dynasore-induced reduction in the nuclear accumulation of phospho-Smad 1/5/8 induced by BMP2 is due to a reduction in the levels of Smad phosphorylation at the membrane, or to a disruption of the translocation of phosphorylated Smads into the nucleus. To address this, the mechanisms which regulate BMP2-induced Smad 1/5/8-BMPRI dissociation following phosphorylation, and the involvement of endocytosis in this process, must be understood. However, Hartung et al. (2006) have shown that the phosphorylation of Smad 1/5/8 by BMPRI is induced at the plasma membrane, suggesting that Smad phosphorylation may be endocytosis-independent.

The current study subsequently investigated whether dynamin-dependent endocytosis regulates BMP2- and GDF5-induced neurite growth. Inhibition of dynamin-dependent endocytosis significantly reduced BMP2-, but not GDF5-, induced neurite outgrowth of SH-SH5Y cells. This result is not surprising, considering that inhibition of dynamin-dependent endocytosis affects the kinetics and magnitude of BMP2-, but not GDF5-, induced Smad 1/5/8 signalling, which is known to drive SH-SH5Y neurite growth (Chapter 2) (Hegarty et al., 2013b). Collectively, these data suggest a differential requirement for dynamin-dependent endocytosis in BMP2- and GDF5-induced neurite growth in SH-SH5Y cells.

Thus, BMP2-, but not GDF5-induced, effects on neurite outgrowth appear to be regulated by endocytosis, despite the fact that both factors signal via BMPRs. Indeed, only BMP2 and BMP4 (also known as BMP2b) have been shown to be

internalised by endocytosis in C2C12 cells (Alborzinia et al., 2013, Kelley et al., 2009, von Einem et al., 2011, Pi et al., 2012), while GDF5 has yet to be shown to undergo endocytosis. Interestingly, endocytosis of BMP2-BMPR occurs immediately after formation of the ligand-receptor complex, with no further increase in BMP2-BMPR endocytosis occurring until after 30 min (Alborzinia et al., 2013). This is due to an initial BMPR saturation by the ligand, which is overcome following BMPR recycling to the plasma membrane (Alborzinia et al., 2013). Hartung et al. (2006) showed that the phosphorylation of Smad 1/5/8 by BMPRI is induced at the plasma membrane, while propogation of Smad signalling can occur following clathrin-mediated endocytosis of the BMPRs, via endosomes. In this study, BMP2-induced Smad signalling levels increased from 5 to 30 min, and from 30 to 60 min. Taking the findings of Hartung et al. (2006) and Alborzinia et al. (2013) into account, perhaps plasma membrane-Smad phosphorylation accounts for the 5 min signalling levels, while the significant increase at 30 min is due to continuation of Smad signalling that occurs following endocytosis of the BMP2-BMPR complex. The increase in BMP2-BMPR endocytosis after 30 min of treatment may then contribute to the further increase in BMP2-induced nuclear phosho-Smad 1/5/8 levels at 60 min. In this case, perhaps the dual-temporal inhibition of endocytosis by dynasore, first at 0 min (which delays Smad signalling) and then again at 30 min, causes the significant reduction in the magnitude of Smad signalling levels at 60 min following BMP2 treatment in SH-SH5Y cells. Moreover, Alborzinia et al. (2013) demonstrated that dorsomorphin, a small molecular inhibitor of BMPRI (Yu et al., 2008), significantly reduces BMP2-BMPR endocytosis for up to 8 h. Perhaps this contributes to dorsomorphin-induced inhibition of BMP2-induced activation of the Smad 1/5/8 signalling pathway at 15 and 60 min in SH-SH5Y cells, as was observed in a previous study (Chapter 2) (Hegarty et al., 2013b).

BMP2 can signal through both BMPRIa and BMPRIb, whereas GDF5 predominantly signals through BMPRIb (Nishitoh et al., 1996). In light of these findings, it may be the case that these BMPRI subtypes are differentially regulated by endocytosis, which has been shown to be the case for other receptor subtypes (Stanasila et al., 2008). It is thus possible that Smad signalling in response to the BMPRIa undergoes endocytic regulation, while BMPRIb-Smad signalling does not (or at least does not to the same extent). In support of this suggestion, the BMPRIa has consistently been shown to undergo endocytosis (Alborzinia et al., 2013, Bonor

et al., 2012, Saldanha et al., 2013), but this remains to be demonstrated for BMPRIb. Therefore, in this case, inhibition of dynamin-dependent endocytosis would inhibit BMP2-BMPRIa Smad signalling, while BMP2/GDF5-BMPRIb Smad signalling would remain largely unaffected.

Dynamin-dependent endocytosis appears to be required during the initial phase of BMP2 stimulation for its maximal induction of SH-SH5Y neurite outgrowth. In support of this finding, BMP2-induced osteoblastic differentiation of C2C12 cells was inhibited following inhibition of dynamin-dependent endocytosis (Heining et al., 2011). Another study, however, showed that epigenetic inhibition of BMP endocytosis resulted in a ten-fold increase in the speed of BMP2-induced Smad nuclear translocation, which enhanced osteoblastic differentiation of C2C12 cells (Rauch et al., 2002). These contradicting results may reflect the different techniques used to inhibit endocytosis, with Rauch et al. (2002) employing chemical and mechanical methods. In the Heining et al. (2011) study, two classes of BMP-induced genes, termed endocytosis-dependent and endocytosis-independent genes, were indentified. Given the fact that BMP2 and GDF5 both induced neurite growth of SH-SH5Y cells, despite the inhibition of endocytosis, it is likely that the genes which mediate the SH-SH5Y neurite outgrowth are endocytosis-independent. In this case, and together with the findings of Chapter 2 (Hegarty et al., 2013b), BMP2 and GDF5 induction of neurite growth may be BMPRIb- and Smad 1/5/8-dependent, but endocytosis-independent. This suggestion supports the aforementioned hypothesis that BMPRIb-Smad signalling does not undergo endocytic regulation. Furthermore, within this proposed mechanism of BMP-induced SH-SH5Y neurite growth, the significant reduction of BMP2-induced neurite outgrowth, induced by short-term inhibition of endocytosis, would reflect the dynasore-induced reduction in the magnitude of BMP2-induced Smad signalling.

Alternatively, perhaps BMP2 and GDF5 induce a different combination of genes to instruct neurite elongation of SH-SH5Y cells. In this case, perhaps a proportion of these genes downstream of BMP2 are endocytosis-dependent, and the prevention of their induction in response to BMP2 by dynasore accounts for the reduction in growth. Additionally, taking the above suggestion into account, it may also be the case that BMP2-BMPRIa-Smad signalling induces the expression of these endocytosis-dependent neurite growth genes. In support of this suggestion, Heining et al. (2011) demonstrated that a combination of endocytosis-dependent and

endocytosis-independent genes are required for BMP2-induced osteoblastic differentiation, with dynasore inhibition of BMP2-induced expression of the endocytosis-dependent genes preventing the completion of osteoblastic differentiation. The expression of endocytosis-dependent and endocytosis-independent genes may thus be regulated by BMPRIa and BMPRIb signalling, respectively. Whole genome expression profiling of SH-SH5Y cells following BMP2 and/or dynasore treatment would be required to address this possibility.

It has been suggested that BMP2 and GDF5 may have an indirect neurotrophic action on VM DA neurons, possibly by stimulating the production of glial-derived growth factor(s), such as GDNF, that subsequently mediate the neurotrophic response (Sullivan and O'Keeffe, 2005). This is supported by the concomitant increase of astrocytes in GDF5- and BMP2-treated E14 rat VM cultures (O'Keeffe et al., 2004b, Krieglstein et al., 1995b, Reiriz et al., 1999). However, the neurotrophic effects of BMP2 and GDF5 on DA neurons were unaltered in glial-depleted E14 rat VM cultures (Wood et al., 2005, Reiriz et al., 1999), and GDF5 and BMP2 have direct neurotrophic effects on SH-SH5Y cells (Chapter 2) (Hegarty et al., 2013b), suggesting that glial-derived factors may not be involved. However, this does not preclude the involvement of GDNF signalling in the mediation of these BMP-induced effects. Indeed, BMP-Smad signalling has been shown to increase the responsiveness of enteric NCCs to the neurotrophic effects of GDNF (Chalazonitis et al., 2011, Chalazonitis and Kessler, 2012). To test this possibility, this study investigated whether GDF5 and BMP2 were capable of promoting neurite growth in the absence of GDNF signalling. Heparinase III-mediated inhibition of GDNF signalling (Barnett et al., 2002, Iwase et al., 2005, Orme et al., 2013) did not affect GDF5- or BMP2-induced SH-SH5Y neurite growth, suggesting that GDF5 and BMP2 do not require GDNF for their growth-promoting effects. GDNF and BMPs may thus signal independently to exert their DA neurotrophic effects. In the context of neurotrophic therapy for PD, perhaps GDNF could be co-applied with GDF5 and/or BMP2 to give synergistic neurotrophic support. In support of this suggestion, co-treatment with GDF5 and GDNF has been shown to have additive neurotrophic effects on cultured E14 VM DA neurons (Wood et al., 2005).

The regulators and downstream effectors of BMP-Smad signalling-induced neurite growth are unknown. To address this, the current study firstly assessed the role of endocytosis-regulation in this process, and then determined the involvement

of GDNF. The final part of this study focused on Sip1, a member of the ZEB family of zinc finger proteins that negatively regulates BMP-Smad signalling (Verschueren et al., 1999, Postigo et al., 2003), chosen due to its recent emergence as a factor that contributes to the induction of a VM DA phenotype in stem cells (Cai et al., 2013). Taken together, these findings suggest that Sip1 may play a role in VM DA neuronal growth. In support of this theory, Sip1 that is essential in the mediation of the roles that BMP-Smad signalling has during NS development (Nitta et al., 2004, van Grunsven et al., 2007, Lerchner et al., 2000, Delalande et al., 2008, Van de Putte et al., 2007, Cacheux et al., 2001, Wakamatsu et al., 2001, Hegarty et al., 2013a).

Before the investigation of Sip1 regulation of BMP-Smad-induced neurite growth, the current study analysed Sip1 expression in SH-SY5Y cells in response to BMP2 and GDF5 signalling. Sip1 expression transiently increased following treatment with either BMP2 or GDF5, before returning to basal levels, which is typical of the feedback regulation of negative regulators in biochemical pathways (Kaern et al., 2005, Maithreye et al., 2008). The period of Sip1 downregulation was more prolonged in response to BMP2 than to GDF5, which may reflect endocytosis-induced, endosomal-propogated BMP2-, but not GDF5-induced, Smad signalling.

This study then demonstrated that knockdown of Sip1 promotes neurite growth of SH-SH5Y cells. Furthermore, Sip1 siRNA-transfected SH-SH5Y cells were shown to have significantly longer neurites than BMP2- and GDF5-treated SH-SH5Y cells. These findings demonstrate that Sip1 is a novel regulator of neurite growth. These data suggest that Sip1, a known regulator of BMP-Smad signalling (Verschueren et al., 1999, Postigo et al., 2003), may negatively regulate BMP2- and GDF5-induced neurite growth.

BMP2 and GDF5 treatment did not significantly attenuate the neurite growth-promoting effects of Sip1 knockdown, suggesting that Sip1 is a principal regulator of neurite growth in SH-SY5Y cells. The finding that Sip1 knockdown alone is sufficient to induce SH-SH5Y neurite growth, suggests that active BMP-Smad signalling may not be required for the neurite growth induced by the Sip1 siRNA. Indeed, Sip1 has been shown to act via BMP-independent mechanisms during NS development (van Grunsven et al., 2007).

In SH-SH5Y cell cultures, there is endogenous BMP-Smad signalling, which is demonstrated by the basal/control (0 min) levels of phosho-Smad signalling in this study. Smad signalling and Smad-dependent gene transcription, in the absence of

exogenous BMPs, has also been demonstrated in SH-SH5Y cells in previous studies (Hegarty et al., 2014a, Hegarty et al., 2013b). Furthermore, endogenous BMP-Smad signalling is known to occur in other cell lines (Shepherd et al., 2008, Theriault and Nachtigal, 2011, Herrera et al., 2009). This basal level of endogenous BMP-Smad signalling is insufficient to induce the neurite growth of SH-SH5Y cells, which is possibly due to Sip1-mediated growth inhibition. In the case of exogenous BMP2- and GDF5-induced SH-SH5Y neurite growth, perhaps the significantly greater Smad signalling levels, demonstrated in this and previous studies (Chapter 2 and 3) (Hegarty et al., 2014a, Hegarty et al., 2013b), overcome Sip1 negative regulation to achieve neurite growth-promotion. However, when Sip1 is knocked down in SH-SH5Y cells, perhaps endogenous BMP-Smad signalling is capable of inducing neurite growth. Thus, endogenous BMP-Smad signalling may therefore be responsible for the neurite growth-promotion that is seen in Sip1 siRNA-transfected SH-SH5Y cells. The measurement of Smad-dependent gene transcription, and transfection with Smad4 dominant negative plasmids, following Sip1 knockdown would help to address this.

As aforementioned, neurite growth following Sip1 knockdown, with or without exogenous BMP2 or GDF5, is significantly greater than that induced in response to exogenous BMP-Smad signalling alone, suggesting that this is under Sip1 negative regulation. It is important to note, however, that the growth levels induced by Sip1 knockdown may be at a maximal level for this neuronal population, due to the removal of this (potential) principal regulator of SH-SH5Y neurite growth. This may explain why this maximal growth of SH-SH5Y cells (with Sip1 knockdown) is unaltered by addition of exogeneous BMPs. In comparison, BMP2 and GDF5-treatment alone may give regulated levels of neurite growth. This finding also suggests that BMP-Smad signalling and Sip1 appear to be on the same signalling axis for neurite-growth promotion. If BMP-Smad signalling acted separately to Sip1, an additive growth effect would have been expected. Taken together, these findings demonstrate that Sip1 is a novel regulator of SH-SY5Y neurite growth. However, the mechanism of Sip1 action, and whether it acts independently of BMP-Smad signalling, remains unclear. The two most likely modes of Sip1 action are: 1) Sip1 acts independently to inhibit SH-SH5Y neurite growth, and then is downregulated by the BMP-Smad (and potentially other) signalling pathway for neurite growth-promotion, and 2) Sip1 negatively regulates

BMP-Smad signalling-driven neurite growth, which is achieved when Smad signalling levels are sufficient to overcome Sip1 inhibition. Future experiments in which endogenous BMP-Smad signalling is inhibited in SH-SH5Y cells with Sip1 knockdown should address this.

The present study then assessed whether Sip1 plays a similar role in the neurite growth of VM DA neurons. Indeed, E14 VM neurons electroporated with the Sip1 siRNA had significantly longer neurites than those transfected with the control plasmid. Sip1 may therefore be a novel regulator of midbrain DA neuronal growth. The analysis of TH-positive, Sip1 siRNA-electroporated VM neurons would conclusively show that the growth-promotion is specific to VA DA neurons. To further explore this possible role of Sip1, the present study assessed the *in vivo* expression of Sip1, using expression profiles taken from the Allen Developing Brain Atlas (©(Allen) Developing Mouse Brain Atlas, 2012), in the midbrain during the development of VM DA neurons and their striatal projections, a process which occurs over a developmental period between E10 and P20 in rodents (Van den Heuvel and Pasterkamp, 2008, Hegarty et al., 2013c). Sip1 is strongly expressed in the E11.5 mouse VM, at a time when VM NPs are undergoing specification into DA neurons (Lumsden and Krumlauf, 1996, Lauder and Bloom, 1974, Specht et al., 1981a, Specht et al., 1981b, Hegarty et al., 2013c). This is not surprising considering the recent discovery that Sip1 actively regulates the induction of a VM DA phenotype in stem cells (Cai et al., 2013). Sip1 expression is significantly lower at E13.5, and continues to decrease until P4 in the VM, a period which corresponds to the development of axonal projections from VM DA neurons to the striatum (Gates et al., 2004, Nakamura et al., 2000, Van den Heuvel and Pasterkamp, 2008). This reduced expression profile supports the suggestion that Sip1 is a novel regulator of VM DA neurite growth, as a reduction in Sip1 expression would facilitate neurite growth. Furthermore, BMP2 and GDF5 have been suggested to actively contribute to the development of nigrostriatal projections (Hegarty et al., 2014a, Hegarty et al., 2014c); perhaps the reduction in midbrain Sip1 expression permits this potential action of these BMPs. Interestingly, Sip1 expression increases after birth, suggesting that Sip1 may be actively involved in the later stages of nigrostriatal pathway development. Indeed, Sip1 regulation of BMP-Smad signalling has been shown to mediate CNS myelination (Weng et al., 2012), which is known to occur from birth (de Graaf-Peters and Hadders-Algra, 2006). Furthermore, BMP2 and GDF5 have

also been suggested to act as target-derived neurotrophic factors during the naturally-occurring cell death period of nigrostriatal pathway development (Hegarty et al., 2014c, Sullivan and O'Keeffe, 2005, Sullivan and Toulouse, 2011), which occurs in the first 3 postnatal weeks of rodent development (Jackson-Lewis et al., 2000, Oo and Burke, 1997, Burke, 2003, Van den Heuvel and Pasterkamp, 2008). Perhaps Sip1 plays a regulatory role in this neurotrophic effect of BMP2 and GDF5 also. Studies assessing the survival-promoting effects of BMP2 and GDF5 in VM DA neurons with Sip1 knockdown would begin to address this. Taken together, these data suggest that Sip1 may play diverse roles throughout the development of the DA nigrostriatal pathway. Firstly, it is involved in the induction of a DA phenotype, then in the establishment of striatal projections, and finally in the myelination/survival of this pathway. A detailed analysis of the numbers of DA neurons present in the midbrain, as well as striatal innervation, of Sip1 null mice at multiple stages during embryonic and post-natal development is required to examine these possibilities. Furthermore, targeting Sip1 expression in stem cell sources may provide a mechanism for inducing both DA specification and subsequent neuronal growth, which are both required if stem cell-derived VM DA neurons are to successfully engraft to the PD striatum (Orlacchio et al., 2010, Bonnamain et al., 2012, De Feo et al., 2012, Toulouse and Sullivan, 2008, Hedlund and Perlmann, 2009). Indeed, Sip1 modulation has already been shown to contribute to DA specification in stem cells (Cai et al., 2013).

8. VM NSCs have delayed neurogenic potential *in vitro*, and GDF5 and BMP2 differentially regulate their differentiation.

8.0 Aims

- To assess the neurogenic potential of E14 rat VM NSCs, by characterising their *in vitro* development into their post-mitotic progeny.
- To examine the effects of GDF5 and BMP2 on the differentiation of E14 VM NSCs *in vitro*, focusing particularly on DA differentiation.

8.1 Abstract

NSCs have been the focus of an intensive effort to direct their differentiation *in vitro* towards desired neuronal phenotypes for cell replacement therapies. It is thought that NSCs derived from older embryos have limited neurogenic capacity and are restricted towards an astroglial fate. This idea is largely based on studies that typically analysed NSC-derived progeny following one week of *in vitro* differentiation. In this report, the neurogenic capacity of older VM NSCs was assessed. When the older NSCs were differentiated for three weeks, there were significant increases in the numbers of newly-born neurons at 14 and 21 days, as assessed by BrdU incorporation. Therefore this study demonstrates that older NSCs retain significantly more neurogenic potential than was previously thought. The ability of GDF5 and BMP2 to induce these VM NSCs towards DA neuronal differentiation was subsequently investigated. Both GDF5 and BMP2 induce the differentiation of VM NSCs in a similar fashion, but differentially induced a DA phenotype in VM NSC-derived neurons. These data have implications for NSC preparatory protocols and for the choice of donor age for cell transplantation studies. They also contribute to our understanding of NSC behaviour *in vitro* and identify BMP2 and GDF5 as potential regulators of midbrain DA neuronal differentiation.

8.2 Introduction

In recent years, NSCs and other stem cell types have been the focus of much research aimed at directing their differentiation *in vitro*, firstly into neurons and secondly into a committed VM DA phenotype, for use in transplantation approaches in PD (Orlacchio et al., 2010, Bonnamain et al., 2012, De Feo et al., 2012). The most relevant source of NSCs for the generation of VM DA neurons are those isolated from the VM during the period of DA neurogenesis, which occurs between E11 and E14 in the developing rat VM *in vivo* (Gates et al., 2006, Lauder and Bloom, 1974, Altman and Bayer, 1981). Understanding the *in vitro* development of these NSCs is crucial for the choice of donor ages from which to culture VM NSCs. This study thus focused on E12 and E14 rat VM NSCs.

NSCs can be isolated from multiple regions of the embryonic brain, and their numbers expanded *in vitro* as free-floating aggregates termed "neurospheres" when grown in the presence of the mitogens, EGF and FGF2 (Reynolds and Weiss, 1992, Deleyrolle and Reynolds, 2009, Kitchens et al., 1994, Rietze and Reynolds, 2006). The proliferating NSCs then spontaneously differentiate into neurons and glia upon mitogen withdrawl (Reynolds and Weiss, 1992, Deleyrolle and Reynolds, 2009, Rietze and Reynolds, 2006). It has been suggested that the age of the donor embryo from which NSCs are initially isolated is a critical determinant of subsequent neuronal differentiation *in vitro*, as NSCs derived from younger donors gave rise to more neurons than those derived from older donors (O'Keeffe and Sullivan, 2005, Gates et al., 2006). These studies have suggested that NSCs from older donor embryos are more restricted towards an astroglial fate.

Neurosphere studies typically determine their NSC-derived progeny by assessing the numbers of neurons and glia generated following differentiation for one week *in vitro* (Ostenfeld et al., 2002, O'Keeffe and Sullivan, 2005, Jensen et al., 2011, Spitere et al., 2008, Roybon et al., 2005). In this report, by assessing the neuronal progeny for longer differentiation periods, we show that older NSCs retain significantly more neurogenic potential than previously thought, and suggest that care should be taken when interpreting *in vitro* studies that use GFAP as a marker of "differentiated" astrocytes.

If the use of NSCs for CRT in PD is to become a reality, then it is crucial that the signalling molecules and pathways which direct VM NSCs to become mature

DA neurons are elucidated. There has been significant advances in our understanding of VM DA neurogenesis in recent years (Hegarty et al., 2013c), however further research is necessary to identify novel, as well as to further characterise existing, signalling pathways that contribute to DA development.

VM DA neurons are generated under the influence of two major signalling centres, the isthmus and the floor plate (Ye et al., 1998, Hegarty et al., 2013c). A number of previous studies have used agents, such as neurotrophic factors, ascorbic acid, cAMP or cytokines (Jin et al., 2005, Maciaczyk et al., 2008, Sanchez-Pernaute et al., 2001, Storch et al., 2001), to induce a DA phenotype in VM NSCs. Although such methods do achieve the ultimate goal of DA neuron generation, a more recent set of studies used developmentally-appropriate VM-specific signalling factors, such as WNTs, FGFs and Shh (Ye et al., 1998, Castelo-Branco et al., 2003, Hegarty et al., 2013c) to achieve DA neurogenesis from VM NSCs (Parish et al., 2008, Ribeiro et al., 2012). This approach is more likely to yield *bona fide* VM DA neurons from NSCs, but depends on identifying factors which instruct DA differentiation *in vivo*.

Interestingly, TGFβ superfamily members have been shown to play inductive roles during DA neurogenesis (Farkas et al., 2003, Roussa et al., 2006, Roussa et al., 2009, Hegarty et al., 2014c). The BMP family is a sub-family of the TGF-β superfamily which plays multiple roles during nervous system development (Hegarty et al., 2013a, Bragdon et al., 2011), and various BMP family members have been demonstrated to function in the development and survival of VM DA neurons (Brederlau et al., 2002, Jordan et al., 1997, Krieglstein et al., 1995b, O'Keeffe et al., 2004a, Hegarty et al., 2014c). Two of the most promising BMP candidates to be involved in DA neurogenesis are GDF5 and BMP2, which are expressed in the developing rat VM during the period of DA development (Krieglstein et al., 1995b, O'Keeffe et al., 2004b, Storm et al., 1994, Chen et al., 2003, Jordan et al., 1997, Soderstrom and Ebendal, 1999, Hegarty et al., 2014c), suggesting that they may play a role in this process. However, it is unknown whether these factors are capable of inducing DA differentiation. In the present study, we assessed the ability of GDF5 and BMP2 to induce a DA phenotype in rat VM NSCs *in vitro*, and showed that GDF5 may function to induce the transition of VM DA NPs into mature DA neurons.

8.3 Results

8.3.1 Effects of gestational age and passage number on VM-derived NSCs *in vitro*

Firstly, E12 and E14 rat VM NSCs which had been expanded for 7 DIV (passage 1), 14 DIV (passage 2) or 21 DIV (passage 3) as free-floating neuropsheres were allowed to differentiate after mitogen withdrawal for 7 days of differentiation (DD) (Fig. 8.4.1a), before being immunocytochemically stained for markers of neurons/neuronal precursors (βIII-tubulin) or of astrocytes/glial precursors (GFAP) (Fig. 8.4.1c, e). Further characterisation of later passages was not possible due to a marked increase in cell death and lack of neurosphere formation following 28 DIV (passage 4) (data not shown). Following proliferation, >90% of these cells expressed the NSC markers, Sox2 and nestin (not shown). Following 7 DD, the percentages of βIII tubulin-positive neurons and GFAP-positive astrocytes relative to the total cell number were determined (Fig. 8.4.1b, d).

Passage 1 VM NSCs, isolated at E12 or E14, generated a significantly higher percentage of βIII tubulin-positive neurons then did either passage 2 or 3 VM NSCs (Fig. 8.4.1b). Passage 2 or 3 VM NSCs, isolated at E12 or E14, generated a significantly higher percentage of GFAP-positive astrocytes in comparison to passage 1 VM NSCs (Fig. 8.4.1d). Therefore, VM NSCs of early passages are more neurogenic, and less gliogenic, than those of older passages, irrespective of the age of the donor embryo.

In all passages examined, the 7DD progeny of VM NSCs isolated at E12 generate a significantly higher percentage of βIII tubulin-positive neurons in comparison to VM NSCs isolated at E14 (Fig. 8.4.1b). E14 VM NSCs generated a significantly higher percentage of GFAP-positive astrocytes at 7DD in comparison to E12 VM NSCs, at all passages examined (Fig. 8.4.1d). Therefore, VM NSCs isolated from younger embryos are more neurogenic and less gliogenic, than those derived from older embryos, irrespective of passage number, when their progeny are differentiated for 7DIV. These data suggest that gestational age and prolonged *in vitro* propagation influences the proportion of NSC-derived cells which are specified to a neuronal or glial lineage.

8.3.2 Assessment of E14 VM NSC differentiation for longer periods *in vitro*

Despite the fact neurogenesis increases between E12 and E14 in the developing rat VM *in vivo* (Gates et al., 2006, Lauder and Bloom, 1974, Altman and Bayer, 1981), E14 VM-derived NSCs appear to have a diminished neurogenic capacity when compared to their E12 counterparts *in vitro* (Fig. 8.4.1b). The data obtained suggest that E14 VM NSCs are significantly more restricted towards a glial lineage than are E12 NSCs (Fig. 8.4.1d), which could explain this apparent decreased neurogenesis by E14 VM NSCs. However, when nestin expression was examined at 7DD, it was found that ~80% of the total cell population remained nestin-positive in these cultures (Fig. 8.4.2a). These cells thus have the potential to differentiate further. Many of the nestin-positive NSCs were GFAP-positive at 7 DD (Fig. 8.4.2a, b), and also stained positively for other NSC markers, such as Sox2 (Fig. 8.4.2c) and vimentin (Fig. 8.4.2d).

To further examine the neurogenic capacity of E14 VM NSCs, these NSCs were differentiated for a further two weeks to allow them to complete their terminal differentiation, with the numbers of neurons and astrocytes being assessed at 14 DD and 21 DD. Characterisation of cell phenotypes at 7 DD, 14 DD and 21 DD in E14 VM NSC cultures, showed that the numbers of nestin-positive cells and GFAP-positive cells significantly decreased at 14 DD compared to 7 DD, and at 21 DD when compared to 14 DD and to 7 DD (Fig. 8.4.2e). The percentage of βIII-tubulin-positive cells (Fig. 8.4.2g) and MBP-positive cells (Fig. 8.4.3) significantly increased at 14 DD compared to 7 DD, and at 21 DD compared to 14 and to 7 DD.

At 7 DD, the GFAP-positive cells have a morphology typical of protoplasmic astrocytes, hereafter referred to as a protoplasmic morphology (Fig. 8.4.2b, f), which was similar to that of the nestin-, Sox2- and vimentin positive NSCs (Fig. 8.4.2b-d). However, at 14 and 21 DD, GFAP-positive cells had a stellate morphology, typical of differentiated astrocytes (Fig. 8.4.2f). These stellate GFAP-positive cells did not express nestin, as demonstrated by the absence of nestin expression at 21 DD, when these stellate astrocytes occupied the largest proportion of the total cell population (Fig. 8.4.2e, f). By 14 DD the cultured cells had grouped into clusters, which were absent at 7 DD, and therefore must have been generated during the second week of differentiation (Fig. 8.4.4a). These cell clusters contained large numbers of newly-born neurons at 14 DD (Fig. 8.4.4a, b). By 21 DD, the cell population consisted of post-mitotic neurons (~31%) (Fig. 8.4.2g), astrocytes (~40%) (Fig. 8.4.2e) and

oligodendrocytes (~28%) (Fig. 8.4.3), with few nestin-positive cells remaining (~2%) (Fig. 8.4.2e), indicating that the E14 VM NSCs had completed their differentiation. The total population of differentiated neural cells was thus accounted for at 21 DD. It is important to note that at all differentiation time-points examined, no TH-positive neurons were observed (data not shown). Additionally, the neurons in this study were viable and healthy, with no adverse effects on neurons being observed during the three week differentiation protocol, as determined through analysis of the numbers, apoptosis (pyknotic nuclei) and neurite length of cultured neurons. Neuronal numbers and neurite length continued to increase throughout the duration of the experiment, while less than 1% of neurons displayed signs of apoptosis at any time point examined (data not shown).

To determine whether the neurons found in the E14 rat VM NSC cultures were derived from NSCs or were post-mitotic neurons which had been present in the culture since the tissue was harvested from the animal, BrdU was applied to the cultures (Fig. 8.4.4c). BrdU is a thymidine analogue that is incorporated into the nucleus during the S phase of cell division. 0.2 μM of BrdU was used due to its neurotoxicity at higher concentrations (Caldwell et al., 2005), however, due to this low concentration, supplementation of BrdU was required every 3 DIV to ensure its detection (Fig. 8.4.4c). BrdU applied to NSC cultures would only be incorporated into nuclei of proliferating cells. Thus, subsequent detection of BrdU in post-mitotic neurons indicates that these cells differentiated from the proliferating VM NSCs. The addition of 0.2 μM BrdU to the differentiating E14 VM NSCs labeled the βIII-tubulin-positive neurons which were grouped in clusters at 14 DD (Fig. 8.4.4e). However, the βIII-tubulin-positive neurons found at 7 DD were BrdU negative (Fig. 8.4.4d). Collectively, these data demonstrate that E14 VM NSCs have the capacity to generate large numbers of newly-born neurons when allowed to fully differentiate *in vitro*.

8.3.3 Effects of GDF5 and BMP2 on the proliferation of E14 VM NSCs

In order to determine whether BMP2 and GDF5 are capable of inducing the differentiation of VM NSCs, the effects of these factors on the proliferation of E14 VM NSCs were assessed. E14 VM NSCs, in the presence of mitogens, proliferated to form neurospheres which significantly increased in volume over time *in vitro* (Fig. 8.4.5a, b). The volume of the neurospheres directly reflects the proliferation of VM

NSCs, therefore a reduction in neurosphere volume corresponds to inhibition of NSC proliferation. The volumes of the neurospheres at 2, 4 and 7 DIV were compared following treatment with either 20 ng/ml of BMP2 or GDF5 (Fig. 8.4.5c-e). There was no significant difference in the volume of E14 VM neurospheres at 2 DIV following treatment (Fig. 8.4.5c). However at 4 DIV and 7 DIV, the volumes of E14 VM neurospheres in BMP2- and GDF5-treated cultures were significantly lower than those in controls (Fig. 8.4.5d, e). This inhibition of VM NSC proliferation by BMP2 and GDF5 may be due to the induction of differentiation or the promotion of cell death, with the former more likely due to the nature of BMPs (Hegarty et al., 2013a).

8.3.4 Effects of GDF5 and BMP2 on the differentiation of E14 VM NSCs

The first indication that BMP2 and GDF5 induced the differentiation of proliferating E14 rat VM NSCs was the finding that BMP2- and GDF5-treated neurospheres had 'plated down' after 7 DIV, indicating differentiation of the NSCs, while the significantly larger control neurospheres remained free-floating (Fig. 8.4.6a). These plated-down neurospheres were surrounded by a 'halo' of differentiating VM cells (Fig. 8.4.6a). RT-PCR analysis of the BMP2- and GDF5-treated E14 VM NSCs at 7 DIV revealed that both BMP2 and GDF5 induced an upregulation of GFAP in these neurospheres, with no change in βIII-tubulin expression observed (Fig. 8.4.6b). Neurosphere-expanded E14 VM NSCs have been shown to express GFAP (Fig. 8.4.6b). These data suggest that BMP2 and GDF5 induced the glial differentiation of the VM NSCs (Fig. 8.4.6c, d). However, GFAP-positive cells in E14 rat VM NSC cultures may be capable of neuronal differentiation (Section 8.3.2) (Hegarty et al., 2014b). Therefore, this induction of GFAP expression does not preclude the possibility that these factors induce DA differentiation in VM NSCs, with GFAP induction being a potential intermediate stage of this process (Fig. 8.4.6c, d).

To determine the capability of BMP2 and GDF5 to induce a DA neuronal fate in E14 rat VM NSCs, BMP2 or GDF5 was added to differentiating cultures of E14 rat VM NSCs. The finding that there were no TH-positive DA neurons at 7, 14 or 21 DD (not shown), demonstrated that these VM NSCs did not undergo DA neuronal differentiation. BMP2 or GDF5 was added to the cultures, every 2 DIV from 21 DD, before assessing DA differentiation at 28 DD, to test if these factors could induce a DA phenotype in the VM NSC-derived neurons present at 21 DD.

GDF5, but not BMP2, induced TH expression in VM NSC-derived neurons (Fig. 8.4.6e, f). This was demonstrated by RT-PCR and immunocytochemical analysis for TH in BMP2- and GDF5-treated VM NSC cultures at 28 DD (Fig. 8.4.6c, d). Control and BMP2-treated E14 VM cultures showed no TH expression (Fig. 8.4.6e, f). RT-PCR for genes required for DA differentiation, such as Msx1, Lmx1b, Nurr1 and Pitx3 (Hegarty et al., 2013c), showed that both BMP2 and GDF5 induced Nurr1 expression, but only BMP2 induced Msx1, Lmx1b and Pitx3 expression (Fig. 8.4.6c, d). These data demonstrate that BMP2 and GDF5 differentially regulate the induction of a DA phenotype in VM NSC-derived neurons.

8.4 Figures and Figure Legends

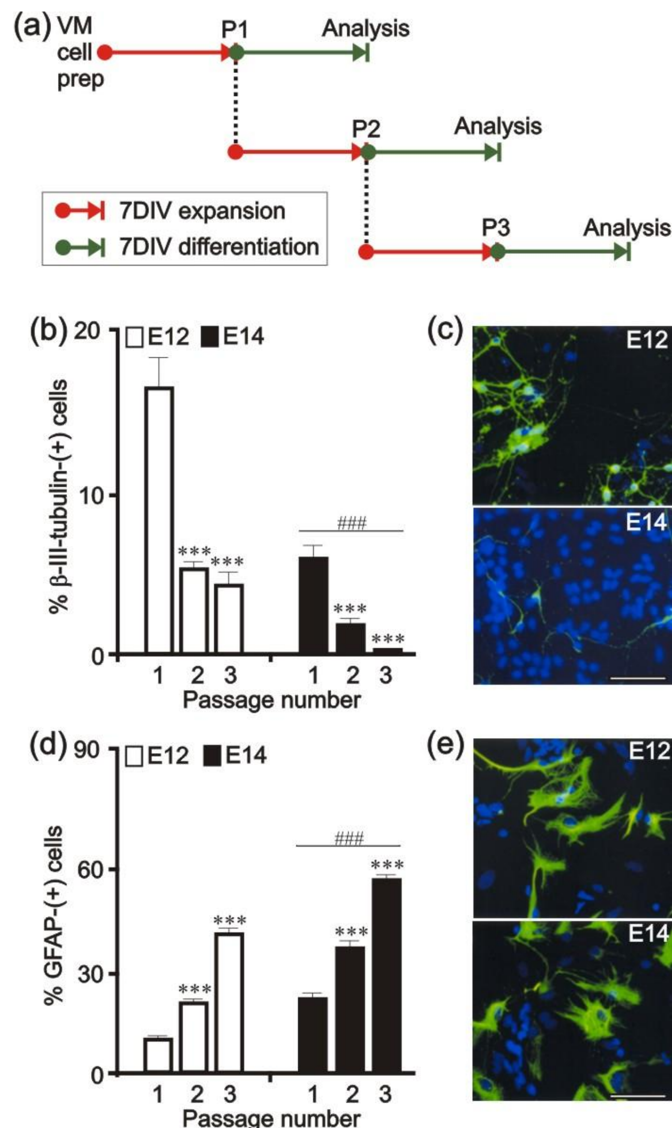


Figure 8.4.1: Characterisation of neurogenesis and gliogenesis in cultures of VM NSCs of various gestational ages and passage numbers.

(a) Schematic representation of the passaging protocol for E12 and E14 VM NSC neurosphere cultures. Graphical representation of the mean numbers (expressed as a percentage of total cells) of (b) βIII-tubulin-positive cells and (d) GFAP-positive cells following 7 DD, in neurosphere cultures of E12 or E14 VM NSCs which were passaged once, twice or three times before differentation, as indicated (*** $P < 0.001$ vs Passage 1; ### $P < 0.001$ vs E12, ANOVA with post-hoc Tuckey's test). Data are expressed as mean $\pm$ SEM, n = 60 fields. Representative photomicrographs of cultures of passage 1 VM NSCs isolated at E12 or E14, differentiated for 7 DIV and immunocytochemically stained for and (c) βIII-tubulin or (e) GFAP, and counterstained with DAPI. Scale bar = 100μm.

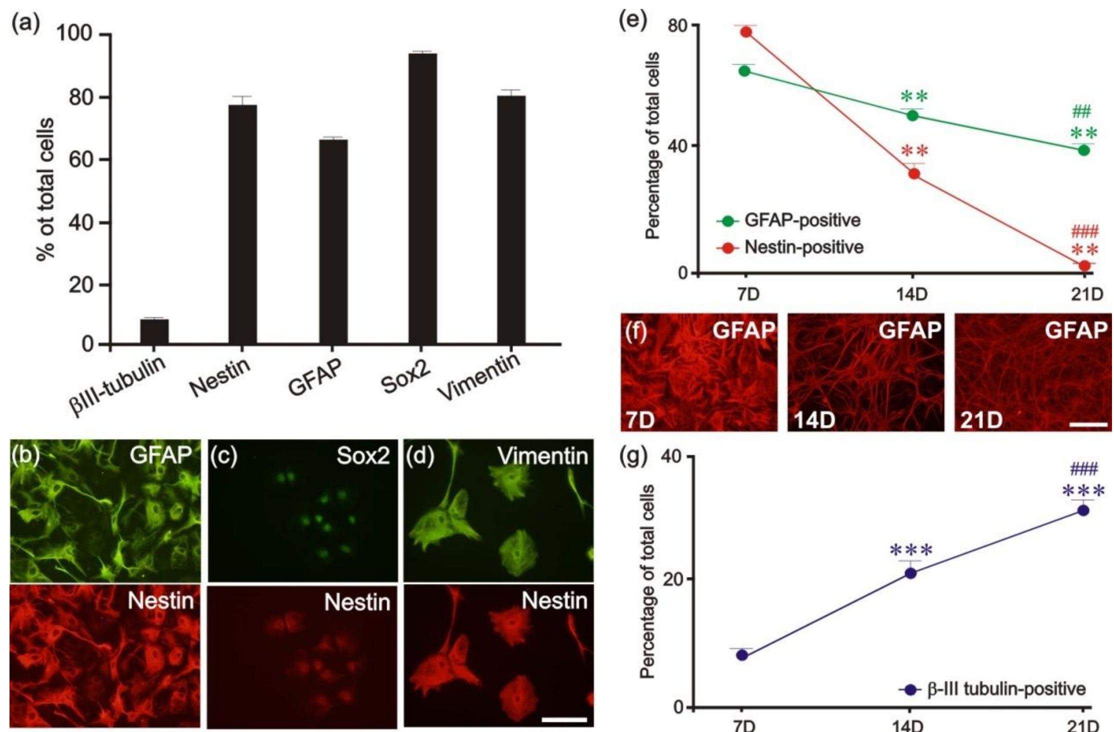


Figure 8.4.2: Characterisation of cell phenotypes in E14 rat VM NSC cultures after 7, 14, and 21 DD.

(a) Graphical representation of the mean numbers (expressed as a percentage of total cells) of βIII-tubulin-, nestin-, GFAP-, Sox2- and vimentin-positive cells following 7 DD generated from E14 VM NSCs after 7 DIV expansion. Representative photomicrographs of E14 rat VM NSCs cultures after 7 DIV expansion and 7 DD, immunocytochemically stained for (b) GFAP (c) Sox2, (d) vimentin or (b-d) nestin. Scale bar = 100μm. Graphical representation of the number of (e) nestin-, (e) GFAP-, and (g) βIII-tubulin-positive cells (expressed as a percentage of total cells) in E14 rat VM NSCs cultures after 7 DIV expansion and 7, 14 or 21 DD, as indicated (** $P < 0.01$, *** $P < 0.001$ vs 7 DD, ## $P < 0.01$, ### $P < 0.001$ vs 14 DD; ANOVA with post-hoc Tuckey's test). Data are expressed as mean $\pm$ SEM, n = 60 fields. (f) Representative photomicrographs of E14 rat VM NSCs cultures after 7 DIV expansion and 7, 14 or 21 DD, immunocytochemically stained for GFAP. Scale bar = 100μm.

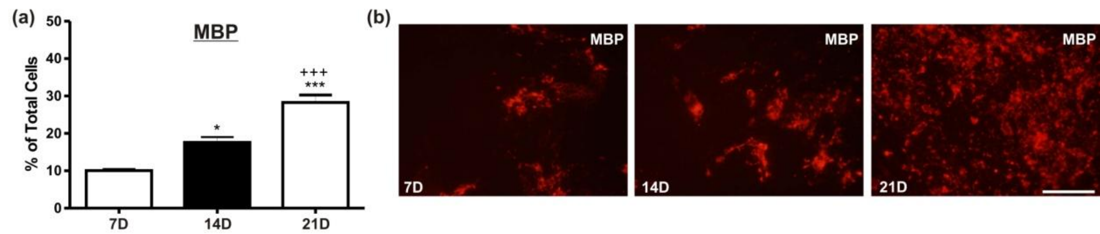


Figure 8.4.3: Characterisation of cell phenotypes in E14 rat VM NSC cultures after 7, 14, and 21 DD.

(a) Graphical representation of the number of MBP-positive cells (expressed as a percentage of total cells) in E14 rat VM NSCs cultures after 7 DIV expansion and 7, 14 or 21 DD, as indicated (* $P < 0.05$, *** $P < 0.001$ vs 7 DD, +++ $P < 0.001$ vs 14 DD; ANOVA with post-hoc Tuckey's test). Data are expressed as mean $\pm$ SEM, n = 60 fields. (b) E14 rat VM NSCs cultures after 7 DIV expansion and 7, 14 or 21 DD, immunocytochemically stained for MBP. Scale bar = 100µm.

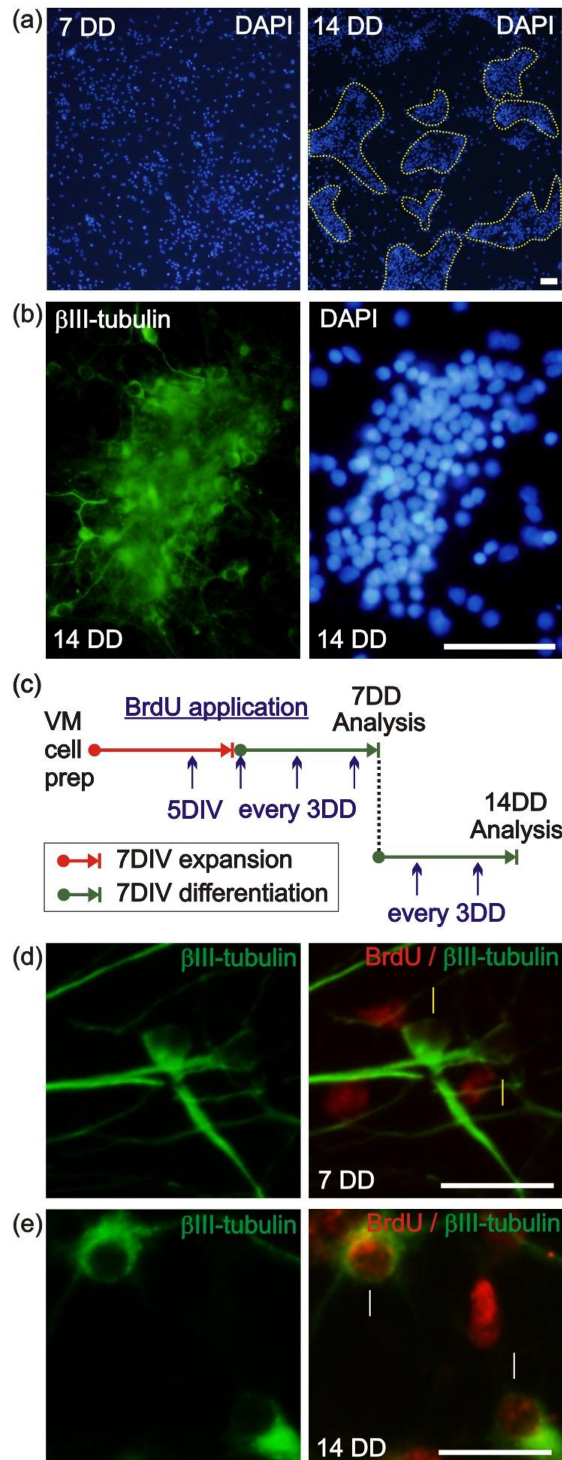


Figure 8.4.4: E14 VM NSC neurogenesis during second two weeks of differentiation.

Representative photomicrographs of E14 rat VM NSCs cultures after 7 DIV expansion and 7 DD or 14 DD, immunocytochemically stained for (b, d and e) βIII-tubulin and/or (d-e) BrdU, and counterstained with (a-b) DAPI. Scale bar = 100µm (a), 50µm (b) and 25µm (d-e). (c) Schematic representation of the BrdU application protocol for E14 VM NSC neurosphere cultures.

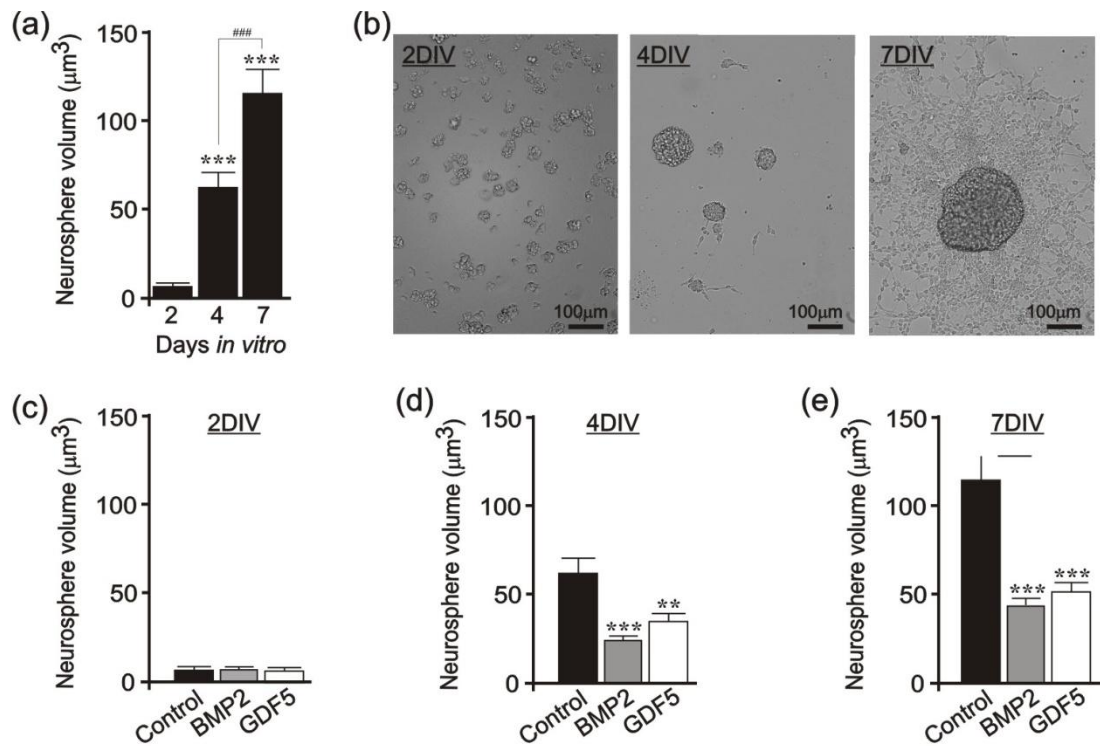


Figure 8.4.5: Effect of BMP2 and GDF5 on the proliferation of E14 VM NSCs. (a) Volume of E14 rat VM neurospheres after 2, 4 or 7 DIV expansion as indicated (*** $P < 0.001$ vs 2 DIV; ANOVA with post-hoc Tukey's test). (b) Representative phase contrast photomicrographs at 2, 4 and 7 DIV of E14 rat VM neurospheres. Scale bar = 100µm. Volume after (c) 2, (d) 4 or (e) 7 DIV expansion of control, BMP2- or GDF5-treated E14 rat VM neurospheres, as indicated (** $P < 0.01$, *** $P < 0.001$ vs control; ANOVA with post-hoc Tukey's test).

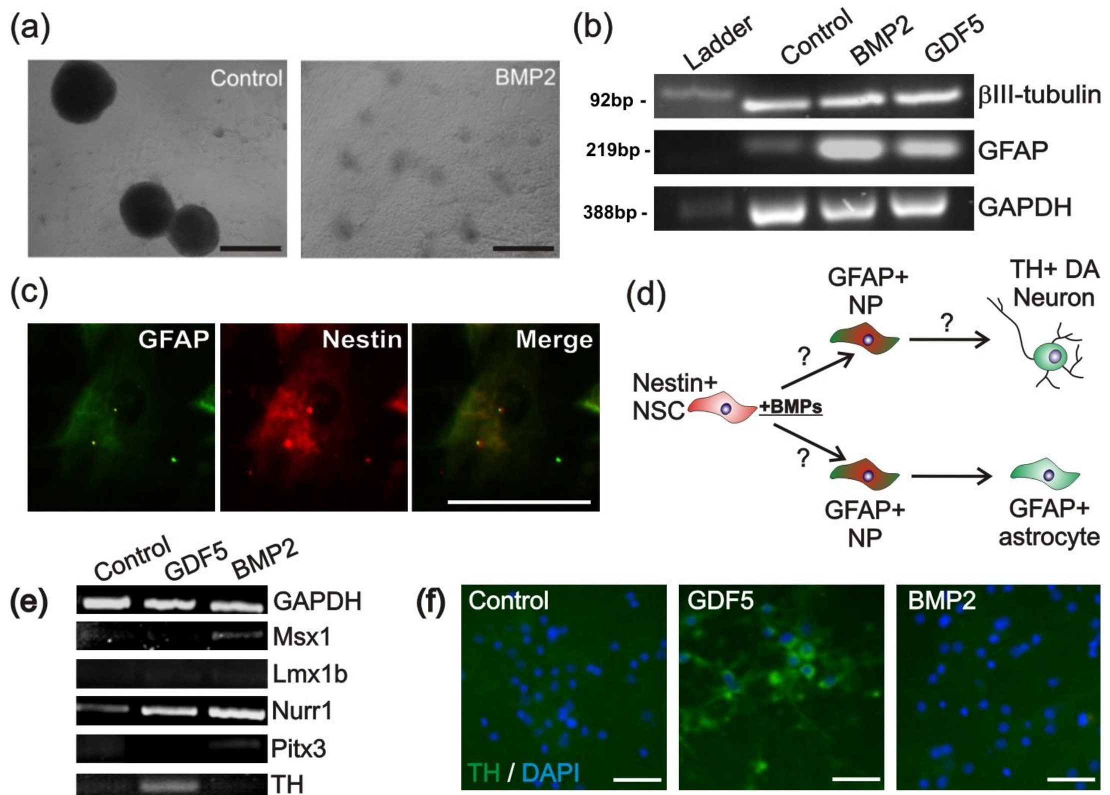


Figure 8.4.6: Effect of BMP2 and GDF5 on the differentiation of E14 VM NSCs. (a) Representative phase contrast photomicrographs at 7 DIV of control and BMP2-treated E14 rat VM neurospheres. Scale bar = 100µm. (b) RT-PCR analysis for βIII-tubulin and GFAP of control, BMP2- and GDF5-treated E14 rat VM neurospheres at 7 DIV. (c) Representative photomicrographs of E14 rat VM NSCs cultures after 7 DIV expansion and 7 DD, immunocytochemically stained for GFAP and/or nestin. Scale bar = 50µm. (d) Schematic diagram of the potential lineages of BMP-treated VM NSCs. E14 rat VM NSC cultures after 7 DIV expansion and 28 DD, treated from 21DD with BMP2 or GDF5, (e) RT-PCR-analysed for Msx1, Lmx1b, Nurr1, Pitx3 and TH or (f) immunocytochemically stained for TH, and counterstained with DAPI. For RT-PCR, a 100bp ladder was used to determine the size for each PCR product, and GAPDH was used as a control. Scale bar = 100µm.

8.5 Discussion

8.5.1 Assessment of the neurogenic potential of E14 VM NSCs

This study describes the differentiation of E14 rat VM NSCs which have been expanded and differentiated for various periods *in vitro*. Such an approach has allowed a re-evaluation of the developmental potential of cultured VM NSCs. The assessment of the differentiated progeny of VM NSCs isolated at E12 or E14, and expanded for 7, 14 or 21 DIV, demonstrated that VM NSCs of older ages and later passages are more glial-restricted than those of younger age and lower passages, which were more restricted towards a neuronal fate. These findings mirror the *in vivo* developmental precept that primary neurogenesis precedes gliogenesis.

The yield of approximately 8% of neurons following the expansion and differentiation (both for 7 DIV) of E14 rat VM NSCs is consistent with previous studies (O'Keeffe and Sullivan, 2005, Ostenfeld et al., 2002). However, the fact that ~80% of the total cell population were nestin-positive neural precursors (NPs) after 7 DD demonstrates that, at this time point, the E14 rat VM NSCs have the potential to undergo further differentiation. The subsequent differentiation of E14 VM NSCs for a further two weeks confirmed that these cells retained the ability to differentiate into neurons, despite their apparent astroglial restriction, and that the E14 VM NSCs completed their differentiation by 21 days. With the majority of previous studies employing one week of differentiation protocols to evaluate the numbers of differentiated NSC progeny, it is possible that the neurogenic potential of these cells have been underestimated (Ostenfeld et al., 2002, O'Keeffe and Sullivan, 2005, Jensen et al., 2011, Spitere et al., 2008, Roybon et al., 2005).

Following one week of differentiation, most cells in the E14 VM NSC cultures were GFAP- and nestin-positive, which would suggest that these cells are committed towards an astrocytic lineage. The propensity of NSCs within neurospheres to differentiate primarily into glial cells has been described by many groups (O'Keeffe and Sullivan, 2005, Ostenfeld et al., 2002, Moses et al., 2006, Mokry et al., 2005, Westerlund et al., 2003). Indeed, it has been proposed that the environment within the neurosphere prohibits NSCs from following a neuronal lineage, while promoting the amplification of glial precursors (Baizabal et al., 2012). The findings of this study following one week of differentiation supports the above suggestion, and thus it should not be surprising that there is limited neurogenesis of neurosphere-

expanded E14 VM NSCs after one week of differentiation. However, the significant increase in neurons generated during the second and third weeks of differentiation may modify the previous perception of the glial commitment of neurosphere-expanded NSCs. The three-fold increase in βIII-tubulin-positive cells between 7DD and 21DD is due to the presence of clusters of large numbers of newly-born neurons. These cell clusters, which are absent at 7 DD and which develop during the second week of differentiation, must be generated from the NPs present at 7 DD, which are largely GFAP- and nestin-positive. It is thus likely that these GFAP-positive NPs become neurogenic, and generate newly-born neurons, during the second and third weeks of *in vitro* differentiation in E14 VM NSC cultures. However, such conclusions cannot be conclusively drawn without real-time monitoring of marker-labelled E14 VM NSCs as they differentiate in culture. Nevertheless, in support of this theory, GFAP has previously been shown to label neuronal precursors (Casper and McCarthy, 2006, Ganat et al., 2006, Silbereis et al., 2010). Furthermore, cultured rat GFAP-positive NPs have been shown to be capable of generating both neurons and glia (Itoh et al., 2006). In fact, the separate identity of NSCs and astroglial cells is regularly challenged in the literature, with some authors suggesting that they are in fact the same cell type (Kriegstein and Alvarez-Buylla, 2009). Thus, in agreement with previous studies, VM NSCs are largely glial-committed (express GFAP) after one week of differentiation, however a proportion of these GFAP-positive cells may be intermediate neuronal precursors.

The GFAP-positive cells present after one week of differentiation expressed nestin, and had a protoplasmic morphology, which was similar to that of the nestin/Sox2/vimentin-positive NSCs, suggesting that these GFAP-positive cells at 7 DD were NSCs, rather than astrocytes. The GFAP/nestin-positive cells differentiated during the second two weeks, as evidenced by a reduction in the numbers of GFAP- and nestin-double labeled cells after 14 and 21 DD. GFAP-positive cells at 7 DD ceased to express nestin and adopted a differentiated, stellate morphology during the second two weeks of differentiation. There were no GFAP- and nestin-double labeled cells present at 21DD. The cessation of proliferation of GFAP-positive cells, coinciding with significantly increases in the numbers of βIII-tubulin-positive cells and MBP-positive cells, accounted for GFAP-positive cells occupying a significantly lower percentage of the total cell population at 14 DD and 21 DD than at 7DD. Although GFAP-positive cells occupied the largest proportion of the total cell

population at all differentiation time points examined, the neurogenic capacity of neurosphere-derived NSCs was significantly greater than previously considered, given the significant increase in the numbers of newly-born neurons during the second and third weeks of differentiation. It is important to note, however, that no TH-positive DA neurons were generated from the VM NSCs in this study, in agreement with a previous study on E14 rat VM NSC cultures (O'Keeffe and Sullivan, 2005). The lack of DA neurogenesis from VM NSCs, cells which would normally generate DA neurons *in vivo*, likely reflects the absence *in vitro* of important developmental guidance cues for DA neurogenesis, described in a recent review (Hegarty et al., 2013c). This is an important issue to consider when using neurosphere cultures of any isolated NSC population to generate specific neuronal populations.

The present investigation of the origin of the neurons in E14 rat VM cultures provides insights into the source of neurons found in NSC differentiation studies. The identification of the cluster-located neurons as BrdU-positive at 14DD showed that these cells are derived from the cultured E14 VM NSCs. In contrast, the neurons at 7DD were not BrdU-labelled, and thus were likely to have been present as post-mitotic neurons in the donor tissue at the time of harvesting, and to have subsequently persisted in culture. In support of this, neurospheres prepared from E13.5 mouse VM, which is at a similar developmental stage as E14 rat VM, have been shown to contain differentiated neurons before mitogen withdrawal (Khaing and Roberts, 2009). Collectively these data suggest that the vast majority of neurons present after one week of differentiation are not progeny of the E14 VM NSCs, but mature neurons that had been present in the source tissue. The vast majority of cells expressed GFAP following 7DD. However, as this study has shown, these GFAP-positive NPs may have the capacity for both gliogenesis and neurogenesis. These findings highlight the importance of using the presence of newly-born neurons to assess the true neurogenic potential of VM NSCs, and show that care must be taken in using GFAP as an "astrocytic" marker, especially at earlier stages of NSC differentiation.

The isolation, expansion and differentiation of NSCs is a useful technique to develop a detailed, well-characterised understanding of the development of NSCs into NPs, which in turn can generate post-mitotic neurons or glia. The current study has demonstrated that neurosphere-expanded E14 rat VM NSCs require three weeks

to complete differentiation into their progeny, with neurogenesis proceeding during the second two weeks. This method of NSC culture results in at least a three-fold increase in the yield of newly-born neurons over conventional methods. This study suggests that the apparent glial restriction (as evidenced by GFAP expression) of older NSCs may be a necessary intermediate developmental stage during their neurogenesis. This has important implications for the choice of age of donor tissue for the use of NSCs for transplantation studies. Indeed, tissue-specific NSCs should be isolated during the developmental time-window which corresponds to the neurogenesis of the neuronal subtype of interest, for example from E11-E14 for rat VM DA neurogenesis (Gates et al., 2006, Lauder and Bloom, 1974, Altman and Bayer, 1981). Based on the perceived limited neurogenic capacity of older E14 VM NSCs, younger VM NSCs have been suggested as a more appropriate stem cell source (Gates et al., 2006). However, this study has demonstrated that NSCs derived from E14 embryos, which give larger numbers of NSCs and are less labour intensive for NSC isolation, have significant neurogenic capacity.

8.5.2 Examination of the effects of BMP2 and GDF5 on the differentiation of E14 VM NSCs

In the second part of this study, the effects of BMP2 and GDF5 on the development of E14 rat VM NSCs *in vitro* was assessed, with the particular goal of determining their ability to induce DA neuronal differentiation in these cells. The volume of E14 VM neurospheres was shown to significantly reduce over time in culture as a result of BMP2 and GDF5 treatment. As neurosphere volume is directly related to the proliferation of NSCs, this suggests that BMP2 and GDF5 negatively regulate the proliferation of VM NSCs. Indeed, BMP2 and GDF5 were shown to induce the expression of GFAP in this population of NSCs, suggesting that BMP2 and GDF5 inhibit the proliferation of VM NSCs through the induction of glial differentiation. In support of this suggestion, BMPs have regularly been shown to inhibit the proliferation of NSCs isolated from various NSC populations, such as the embryonic cortex (Mehler et al., 2000, Sun et al., 2011), SVZ (Bonaguidi et al., 2005), midbrain (O'Keeffe et al., 2004a, Reiriz et al., 1999), neural crest (Chalazonitis et al., 2011) and SC (Sun et al., 2011), and adult hippocampus (Brederlau et al., 2004) and SVZ (Ciceroni et al., 2010, Lim et al., 2000), through the induction of the differentiation of GFAP-positive cells. However, the induction of GFAP expression in these NSCs

does not necessarily mean that they have terminally differentiated into post-mitotic astrocytes. Indeed, BMPs negatively regulate the proliferation of cultured adult hippocampal NSCs while maintaining an undifferentiated state (Mira et al., 2010). The induction of GFAP-expressing cells in these NSCs by BMPs may thus be an intermediate stage in their neurogenesis (Lim et al., 2000, Ciceroni et al., 2010, Colak et al., 2008), as has been suggested to occur in the adult SVZ (Hegarty et al., 2013a). This is a distinct possibility for cultured E14 VM NSCs also, with this study suggesting that the GFAP-positive NSCs that are present at 7DD give rise to significant numbers of newly-born neurons when differentiated for a further two weeks *in vitro*. At the current time, it is difficult to conclusively state whether BMP2 and GDF5 induce gliogenesis or neurogenesis, while it is even possible that they are inducing both. The induction of GFAP-expression in proliferating VM NSCs by BMP2 and GDF5 may represent two things: 1) induction of a commitment to an astroglial lieage, or 2) induction of the differentiation of proliferating NSCs to a GFAP-positive NP cell stage, which subsequently contributes to neurogenesis. These possibilities do not have to be mutually exclusively, and may be intrinsically linked. For example, it is possible that the final stages of DA induction may be timed to coincide with the initial stages of astrocytic differentiation in the VM. Indeed, astrocytes secrete a variety of known molecular factors that promote DA neuronal survival and growth (Li et al., 2009, Castelo-Branco et al., 2006), suggesting that this possibility is a plausible one. These suggestions are supported by data showing a dual neurogenic and gliogenic role for BMPs in a number of NP populations (Agius et al., 2010, Chalazonitis et al., 2011, Chalazonitis and Kessler, 2012, Hegarty et al., 2013a, Krieglstein et al., 1995b, O'Keeffe et al., 2004a, Wood et al., 2005, Reiriz et al., 1999). In terms of DA neurogenesis, GFAP-positive glial precursors have been identified as the NPs of the DA neurons of the VM *in vivo* (Bonilla et al., 2008, Hebsgaard et al., 2009). Therefore, the GFAP-positive NSCs in this study may share similarities to these DA NPs. Indeed, the VM NSCs were isolated from the same region as the VM DA NPs, at the time when these NPs are undergoing DA neurogenesis. However, no TH-positive DA neurons were generated in this study, therefore caution must be taken when comparing the *bona fide* VM DA NPs *in vivo* with the GFAP-positive NSCs in this study.

To investigate the potential DA-inductive roles of BMP2 and GDF5, their effect on the DA differentiation of E14 rat VM NSCs were determined. It has been

previously proposed that NSCs derived from the VM should retain region-specific gene expression (Kim et al., 2006), however, the RT-PCR results in this study demonstrated little or no expression of Msx1 and Lmx1b, two region-specific genes important in DA development (Alavian et al., 2008, Andersson et al., 2006, Hegarty et al., 2013c), in differentiated VM NSCs, suggesting that the expression of important genes required for DA development have been downregulated in E14 VM NSCs in culture. The lack of DA neurogenesis of these NSCs *in vitro* supports this suggestion, and is likely explained by the absence of region-specific signalling factors.

In the present study, GDF5, but not BMP2, was shown to induce TH expression in neurons derived from E14 VM NSCs. In addition to inducing TH expression, GDF5 also induced Nurr1 expression in differentiating E14 rat VM NSCs. GDF5 has been shown to reach its highest levels of expression during the final period of VM DA neurogenesis (O'Keeffe et al., 2004b), and has consistently been shown to increase the numbers of DA neurons in E14 VM cultures (O'Keeffe et al., 2004a, Wood et al., 2005, Clayton and Sullivan, 2007, O'Sullivan et al., 2010, Krieglstein et al., 1995b). Taken together with the findings of this study, it appears that GDF5 may play a role in the final transition of NPs into differentiated DA neurons. In support of this, GDF5 was shown not to increase the expression of Msx1 and Lmx1b, two genes expressed in VM DA NPs during the early stages of DA neurogenesis (Hegarty et al., 2013c). BMP2 was shown to increase the expression of important genes in the generation of DA NPs in the VM, such as Msx1, Lmx1b, Nurr1 and Pitx3 (Alavian et al., 2008, Andersson et al., 2006, Hegarty et al., 2013c), suggesting that BMP2 may be involved in the early stages of VM DA neurogenesis. Perhaps BMP2 and GDF5 act sequentially during DA neurogenesis, with BMP2 acting first to induce Msx1, Lmx1b, Nurr1 and Pitx3 expression in VM NPs, and GDF5 then acting to induce the expression of Nurr1 and TH, thus promoting the differentiation of BMP2-induced DA NPs into mature midbrain DA neurons.

DA neurons derived from NSCs have been proposed as an alternative cell source for transplantation approaches in PD. As aforementioned, *in vitro* differentiation of DA neurons from VM NSCs has been achieved using both non-region specific factors, such as neurotrophic factors, ascorbic acid, cAMP or cytokines (Jin et al., 2005, Maciaczyk et al., 2008, Sanchez-Pernaute et al., 2001, Storch et al., 2001) and, more appropriately, region-specific factors such as WNTs,

FGFs and Shh (Parish et al., 2008, Ribeiro et al., 2012). The second part of this study has demonstrated that GDF5 is capable of inducing a DA phenotype in VM NSCs, and likely plays a role in the transition of NPs into differentiated DA neurons. Furthermore, BMP2 was suggested to be involved in the early inductive stages of DA neurogenesis. Taken together with their well-characterised neurotrophic effects, such as neurite-outgrowth and survival promoting effects, on VM DA neurons (Costello et al., 2012, Hurley et al., 2004, O'Keeffe et al., 2004a, O' Sullivan et al., 2010, Sullivan et al., 1997, Sullivan et al., 1998b, Sullivan and O'Keeffe, 2005, Hegarty et al., 2014a, Reiriz et al., 1999, Jordan et al., 1997, Espejo et al., 1999), GDF5 and BMP2 are ideal candidates to be used as adjuncts to the use of stem cells in CRT, as they promote the induction, neurite growth and survival of midbrain DA neurons. The examination of the effects of knockout of BMP2 or GDF5 on VM DA neurogenesis will be crucial to the establishment of BMP2 and GDF5 as novel regulators of these processes.

9. Summary and Final Discussion

The present thesis focused on the roles played by the TGFβ superfamily members, BMP2 and GDF5, in the regulation of neurite growth of midbrain DA neurons. These BMPs have similar neurotrophic actions to GDNF (Sullivan and Toulouse, 2011, Peterson and Nutt, 2008), on VM DA neurons, both *in vitro* (O'Keeffe et al., 2004b, Jordan et al., 1997, O'Keeffe et al., 2004a, Krieglstein et al., 1995b, Wood et al., 2005) and *in vivo* (Costello et al., 2012, Hurley et al., 2004, Sullivan et al., 1997, Sullivan et al., 1999, Sullivan et al., 1998b, Espejo et al., 1999). Despite this, the molecular and cellular mechanisms mediating their effects on DA neurons were unknown. It is essential to understand these mechanisms if BMP2 and GDF5 are ever to be used in a clinical context for the treatment of PD. In an attempt to address this, this thesis examined the hypothesis that 'canonical Smad 1/5/8 signalling mediates the effects of BMP2 and GDF5 on the development of VM DA neurons'.

Firstly, the roles of canonical (Smad-dependent) and non-canonical (Smad-independent) signalling pathways in mediating the neurite growth-promoting effects of BMP2 and GDF5, on both the SHSY5Y cell line (Chapter 2) and E14 rat VM primary cultures (Chapter 3), were examined. BMP2 and GDF5 both induced neurite growth in SH-SH5Y cells, which were shown to express the components of the canonical Smad 1/5/8 signalling pathway. The responses of the canonical Smad 1/5/8 pathway and the non-canonical MAPK pathways to BMP2 and GDF5 were assessed in a time-course experiment. BMP2 and GDF5 both activated Smad 1/5/8 signalling to the same extent, but with different temporal kinetics. Conversely, BMP2 and GDF5 both reduced the basal signalling of the non-canonical pathways involving phospho-ERK, phospho-JNK and phospho-p38 MAPK, in SH-SH5Y cells. These data suggested that BMP2 and GDF5 activate canonical Smad signalling, and not the MAPK pathways, to induce neurite growth of SH-SH5Y cells. This study then showed that BMPRI was required for BMP2- and GDF5-induced Smad signalling and neurite growth, and that BMPRIb mediates the growth-promoting effects of BMP2 and GDF5. The neurite growth-promoting effects were dependent upon Smad transcriptional activity, as the knockdown of Smad4 prevented these effects. Thus, this study demonstrated that the downstream molecular mechanisms mediating the direct neurotrophic effects of GDF5 and BMP2 are dependent upon BMPRI-mediated activation of the canonical Smad 1/5/8 signalling pathway in

SHSY5Y cells. These findings were later confirmed in DA neurons within primary cultures of E14 rat VM. Furthermore, the expression of BMPRIb and BMPRII, both of which are required for canonical BMP-Smad signalling, were found to be expressed in the rat midbrain and striatum *in vivo* (from E11) and into adulthood (until at least P90) during the period of nigrostriatal pathway development, which occurs between E11 and P90 (Van den Heuvel and Pasterkamp, 2008). Furthermore, the present study showed that activation of these receptors by injection of GDF5 to the adult midbrain results in Smad phosphorylation *in vivo*.

As aforementioned, the elucidation of the various receptor combinations, cytosolic interactions, transcriptional effectors, and/or target genes that mediate the multiple-inductive effects of BMPs is crucial for a comprehensive understanding of the roles played by these family members in neural development. The present work has focused on the roles of BMP2 and GDF5 in VM DA development, with the goal of integrating these factors into the molecular framework of VM DA neurogenesis in order to contribute to the current understanding of this process. To this point, this thesis has specifically shown that BMP2 and GDF5 promote neurite growth through BMPRIb-dependent stimulation of Smad 1/5/8 nuclear translocation in two models of VM DA neurons (Hegarty et al., 2014a, Hegarty et al., 2013b). However, the precise intracellular cascades that regulate BMP-Smad-driven neurite growth, and the downstream molecular changes that mediate this process, were still unclear. To investigate this further, the following were examined: 1) the role of endocytosis in canonical BMP-Smad signalling, and its promotion of neurite growth, 2) the regulation of BMP2- and GDF5-induced neurite growth by Sip1, and 3) the requirement of GDNF for the neurite growth-promoting effects of BMP2 and GDF5.

Firstly, by examining Smad 1/5/8 phosphorylation over time, Smad 1/5/8 signalling was shown to be activated by BMP2 and GDF5 when dynamin-dependent endocytosis was inhibited. However, inhibition of dynamin-dependent endocytosis using dynasore resulted with a delayed and reduced level of BMP2-, but not of GDF5-, induced phospho-Smad 1/5/8 activation, suggesting that efficient BMP2-Smad signalling is dependent on endocytosis in SH-SH5Y cells. Inhibition of dynamin-dependent endocytosis did not prevent BMP2- or GDF5-induced neurite growth, demonstrating that BMP-induced neurite growth is endocytosis-independent. However, dynasore pre-treatment significantly reduced BMP2-, but not GDF5-, induced neurite outgrowth of SH-SH5Y cells, which was most likely due to the

effects of dynamin-dependent endocytosis on the kinetics and magnitude of BMP2-induced Smad 1/5/8 signalling. Thus, there may be a differential requirement of dynamin-dependent endocytosis for BMP2- and GDF5-induced neurite growth in SH-SH5Y cells. As BMP2 can signal through both BMPRIa and BMPRIb, whereas GDF5 predominantly signals through BMPRIb (Nishitoh et al., 1996), this suggests the BMP2-BMPRIa complex is regulated by endocytosis, while Smad-signalling induced by the BMP2/GDF5-BMPRIb complex is not (or at least not to the same extent). In support of this, BMP2 and BMPRIa undergo endocytosis (Alborzinia et al., 2013, Bonor et al., 2012, Saldanha et al., 2013, Kelley et al., 2009, von Einem et al., 2011, Pi et al., 2012), while this not been demonstrated for GDF5 or BMPRIb. Hartung et al. (2006) showed that the phosphorylation of Smad 1/5/8 by BMPRI is induced at the plasma membrane, while continuation of Smad signalling can occur following clathrin-mediated endocytosis of the BMPRs. Thus, endocytosis by BMP2-BMPRIa may account for the faster kinetics of BMP2-induced Smad signalling. This work has shown that BMPRIb-induced Smad signalling mediates the neurite growth-promoting effects of BMP2 and GDF5. In this context, perhaps the endocytosis-induced inhibition of BMP2-promoted SH-SH5Y neurite growth results from the reduced magnitude of BMP2-Smad signalling. Alternatively, perhaps BMP2 induces a combination of endocytosis-dependent genes, via BMPRIa, and endocytosis-independent genes, via BMPRIb, to instruct neurite outgrowth, and the inhibition of endocytosis thus attenuates growth promotion (Fig. 9.1), as endocytosis-inhibited BMP2 still induces significant SH-SH5Y neurite growth. Taken together, these studies show that GDF5 and BMP2 induce neurite growth via a BMPRIb-dependent, endocytosis-independent, pathway which requires Smad 1/5/8 transcriptional activity (Fig. 9.1a).

The next part of this study addressed the possibility that GDF5 and BMP2 act indirectly on VM DA neurons, by stimulating the production of glial-derived growth factor(s), such as GDNF. Despite the neurotrophic effects of BMP2 and GDF5 on DA neurons being unaltered in glial-depleted E14 rat VM cultures (Wood et al., 2005, Reiriz et al., 1999), these studies did not preclude the involvement of GDNF signalling, since GDNF could be released by DA neurons themselves via an autocrine mechanism. In the present study, inhibition of GDNF signalling did not affect GDF5- or BMP2-induced SH-SH5Y neurite growth, suggesting that GDF5 and BMP2 do not require GDNF for their growth-promoting effects.

The final part of this study focused on Sip1, a negative regulator of BMP-Smad signalling (Verschueren et al., 1999, Postigo et al., 2003), due to its recent emergence as a factor that contributes to the induction of a VM DA phenotype in stem cells (Cai et al., 2013). These two functions of Sip1 suggested that Sip1 may regulate the potential roles of BMP2 and GDF5 in VM DA neurogenesis. It was found that BMP2 and GDF5 directly regulated Sip1 expression (Fig. 9.1). Sip1 expression transiently increased before returning to basal levels, which is typical of the negative feedback regulation (Kaern et al., 2005, Maithreye et al., 2008). BMP2-induced down-regulation of Sip1 was more prolonged, which may be due to BMP2-BMPRIa-induced, endosomal-propogated Smad signalling.

Sip1 knock-down in SH-SH5Y cells significantly increased neurite growth over that induced by BMP2 and GDF5 alone. These findings demonstrated that Sip1 is a novel regulator of neurite growth. As it can regulate BMP-Smad signalling (Verschueren et al., 1999, Postigo et al., 2003), Sip1 may negatively regulate BMP2- and GDF5-induced neurite growth (Fig. 9.1), which is achieved when Smad signalling levels are sufficient to overcome Sip1-mediated growth inhibition. As Sip1 knockdown alone is sufficient to induce neurite growth, this suggests that Sip1 may act independently to inhibit neurite growth, and is then downregulated by the BMP-Smad (and potentially other) growth-promoting signals (Fig. 9.1). Conversely, Sip1 knockdown may “release” endogenous BMP-Smad signalling to promote neurite growth which is seen in Sip1 siRNA-transfected cells.

Finally, this study showed that Sip1 expression was strong during VM DA neuronal specification, decreased during the development of axonal projections from VM DA neurons to the striatum, before increasing again after birth during maintenance and myelination of the nigrostriatal pathway. This expression profile reflects the functions demonstrated for Sip1 in VM DA specification (Cai et al., 2013), neurite growth (herein), and CNS myelination (Weng et al., 2012). Similarly, BMP2 and GDF5 have been suggested to regulate the induction, differentiation, neurite growth, survival and maintenance of VM DA neurons (Hegarty et al., 2014c). Considering the likely role of Sip1 in the regulation of BMP2- and GDF5-promoted neurite growth, perhaps Sip1 is the primary downstream regulator of the proposed roles of BMP2- and GDF5-induced Smad signalling in VM DA development.

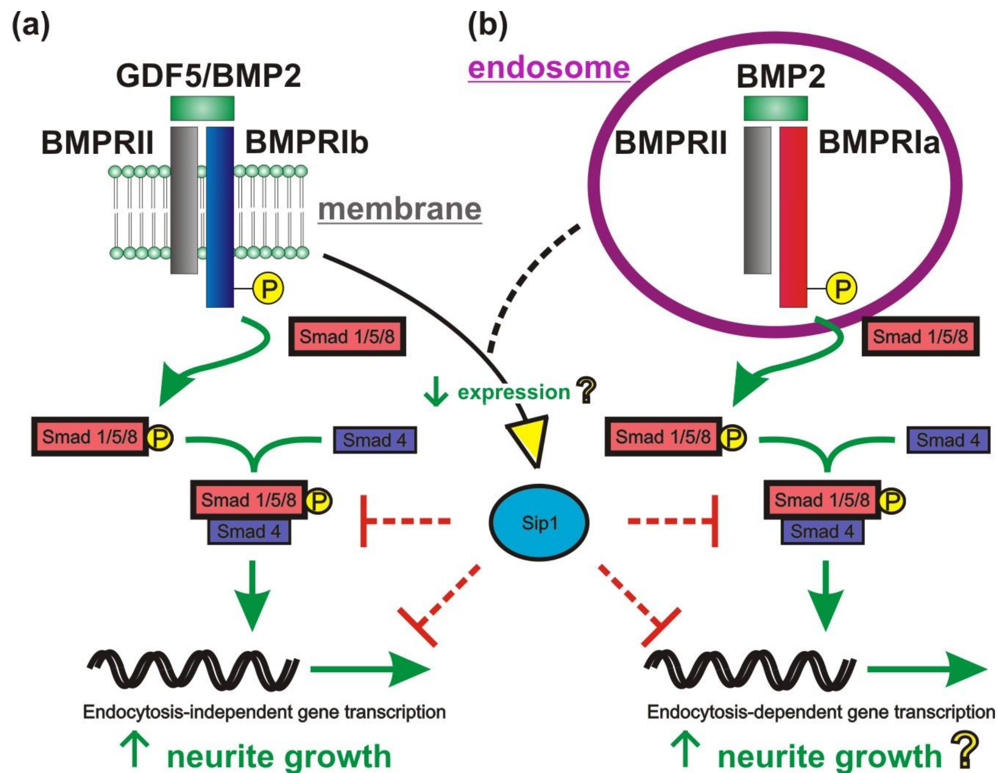


Figure 9.1: Molecular pathways involved in BMP2- and GDF5-induced neurite growth.

(a) Schematic of the mechanisms involved in BMPRIb-Smad-dependent, endocytosis-independent, BMP2- and GDF5-induced neurite growth. GDF5/BMP2-activated BMPRIb phophoryates Smad 1/5/8, which translocate to the nucleus to induce neurite growth, likely through endocytosis-independent genes. (b) Schematic of the mechanisms involved in endosomal-propogated Smad signalling following endocytosis of BMP2-activated BMPRIa. The BMP2-BMPRIa complex uses the endosome to further increase Smad signalling and to regulate the expression of endocytosis-dependent genes, which may be involved in BMP2-, but not GDF5-induced, neurite growth. Sip1 negatively regulates neurite growth, either by the inhibition of BMP-Smad-driven neurite growth or by independently inhibiting the expression of genes which promote neurite growth. BMP2 and GDF5 regulate Sip1 expression, however it is unclear whether BMP2 and GDF5 downregulate Sip1 to achieve their growth-promotion, or whether endocytosis-mediated BMP2 signalling contributes to its regulation of Sip1 expression.

In the final part of this thesis, we examined the differentiation of E14 rat VM NSCs *in vitro* and determined whether BMP2 and GDF5 could modify the proliferation and differentiation of these cells. The assessment of the differentiated progeny of VM NSCs isolated at E12 or E14 demonstrated that VM NSCs of older ages and later passages appear to be more glial-restricted than those of younger ages and lower passage number. However, despite the apparent glial-restriction of E14 rat VM NSCs, significant increases in newly-born (BrdU-positive), NSC-derived neurons were found during the second and third weeks of differentiation. In fact, the neurons detected at 7DD were BrdU-negative, and thus were likely to have been present as post-mitotic neurons in the donor tissue at the time of harvesting. The three-fold increase in βIII-tubulin-positive cells between 7DD and 21DD was due to the presence of clusters of large numbers of newly-born neurons at 14DD and 21DD. These neurons are likely generated from the nestin-, Sox2-, vimentin- and GFAP-positive NSCs, which had a protoplasmic morphology, at 7DD. Indeed, nestin-, Sox2-, vimentin- and GFAP-positive NSCs with a radial morphology were intermingled with the neurons in these clusters at 14DD (Fig. 9.2). Thus, it is likely that these neurogenic radial glial-like cells undergo asymmetrical neurogenesis to produce significant amounts of βIII-tubulin-positive cells during the second two weeks of differentiation (Fig. 9.2). Additionally, a small proportion of nestin-positive NSCs co-express βIII-tubulin at 7DD, which explains the slight difference in nestin- and GFAP-positive cells at this time-point (Fig. 9.3). These nestin-/βIII-tubulin-positive NPs appear to undergo symmetrical division(s) to generate newly-born neurons during the second two weeks of differentiation (Fig. 9.3). These mitotic neuronal precursors undergo gradual morphological reorganisation from a protoplasmic NSC into a bipolar neuron (Fig. 9.3). Nestin expression ceases once this bipolar neuronal morphology has been adopted, and neuronal differentiation has been completed (Fig. 9.3). However, due to the limited numbers of these neuronal precursors, symmetrical neurogenesis does not account for the significant increases in neurons at 14DD and 21DD. Real-time analysis of nestin- and βIII-tubulin-labelled E14 rat VM NSCs during their *in vitro* development is required to confirm the occurrence of the suggested symmetrical and asymmetrical neurogenesis, as these suggestions are based on inferences from information obtained at fixed time-points during VM NSC differentiation.

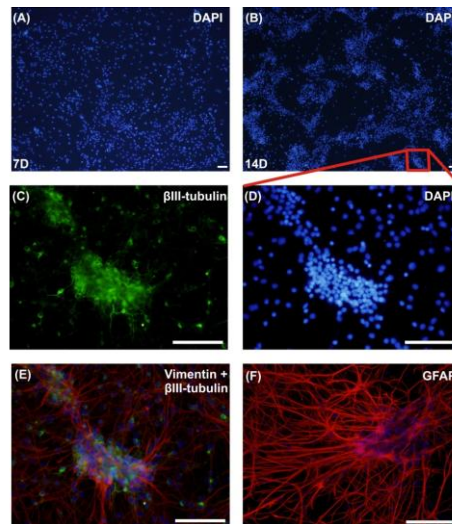


Figure 9.2: Radial glial-like NPs generate neurons in E14 VM NSC cultures. Representative photomicrographs showing representative images of E14 rat VM NSCs cultures after 7DIV expansion and 7DD (A) or 14DD (B-F), immunocytochemically stained for DAPI, vimentin (E), GFAP (F) and/or βIII-tubulin (C and E). Radial glial-like NPs (E and F) generate neurons (C and E) during the second week of differentiation. The proliferation and asymmentrical neurogenesis of radial glial-like NPs causes the formation of clusters of cells by 14DD (B) which are not present at 7DD (A). Scale bar = 100µm.

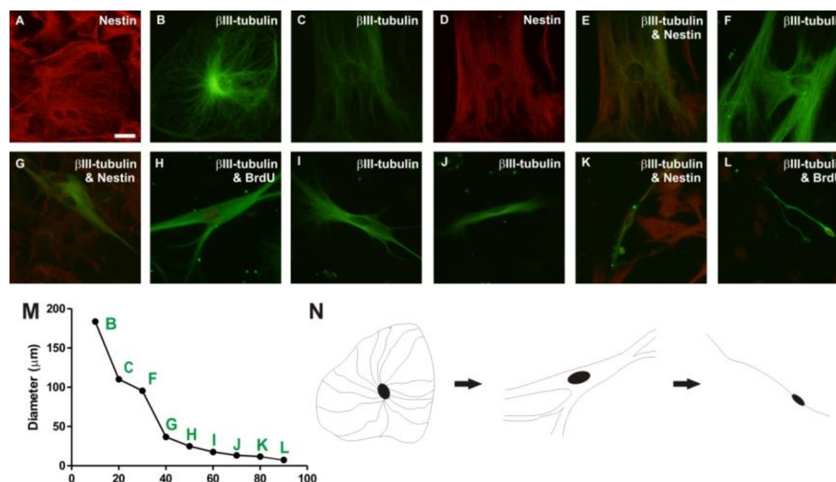


Figure 9.3: Symmetrical neurogenesis, with morphological reorganisation, of neuronal precursors in E14 VM NSC cultures. E14 rat VM NSCs cultures after 7DIV expansion and 7 or 14 DD, immunocytochemically stained for nestin (A, D, E, H and K), βIII-tubulin (B-L) and/or BrdU (H and L). Scale bar = 25µm. (M) Graphical representation (M) and line drawings (N) of the morphological reorganisation of the βIII-tubulin-positive neuronal precursors in images B to L.

The GFAP/nestin-NSCs differentiated during the second two weeks, resulting in a significant reduction in the numbers of GFAP- and nestin-double labeled cells after 14 and 21 DD. GFAP-positive cells at 7 DD ceased to express nestin and adopted a differentiated, stellate morphology during the second two weeks of differentiation. There were no TH-positive DA neurons generated from the VM NSCs in this study, most likely due to the absence *in vitro* of important developmental guidance cues for DA neurogenesis. This study demonstrated that older NSCs retain significantly more neurogenic potential than was previously thought, and that this neurogenesis is likely to be mediated by GFAP-positive NPs.

In the second part of this study, the effects of BMP2 and GDF5 on the development of E14 rat VM NSCs *in vitro* was assessed, with the particular goal of determining their ability to induce DA neuronal differentiation in these cells. BMP2 and GDF5 were shown to significantly reduce the volume of E14 VM neurospheres over time in culture. This apparent negative regulation of VM NSC proliferation was due to the induction of GFAP expression, suggesting that BMP2 and GDF5 inhibit the proliferation of VM NSCs through the induction of glial differentiation. The induction of GFAP-expression in proliferating VM NSCs by BMP2 and GDF5 may represent two things: 1) induction of a commitment to an astroglial lineage, or 2) induction of the differentiation of proliferating NSCs to a GFAP-positive NP cell stage, which subsequently contributes to neurogenesis.

In the final part of this thesis, the DA-inductive ability of BMP2 and GDF5 in E14 rat VM NSCs was assessed. GDF5, but not BMP2, was shown to induce TH expression in neurons derived from E14 VM NSCs, while GDF5 also induced Nurr1 expression. BMP2 was shown to increase the expression of important genes in the generation of DA NPs in the VM, such as Msx1, Lmx1b, Nurr1 and Pitx3 (Alavian et al., 2008, Andersson et al., 2006, Hegarty et al., 2013c) in these E14 VM NSC-derived neurons. Taken together, these data suggest that BMP2 and GDF5 act sequentially during DA neurogenesis, with BMP2 acting first to induce Msx1, Lmx1b, Nurr1 and Pitx3 expression in VM NPs, and GDF5 then acting to induce the expression of Nurr1 and TH, thus promoting the differentiation of BMP2-induced DA NPs into post-mitotic midbrain DA neurons.

Taken together, the experiments of this thesis identify GDF5 and BMP2 as novel regulators of midbrain DA neuronal induction and differentiation, and demonstrate that their effects on DA neurons are mediated by canonical BMP-

BMPR-Smad signalling. However, while this work proposes that BMP2 and GDF5 play roles in the developing VM, they are best recognised for the role in induction of a dorsal identify in the developing NS (Hegarty et al., 2014c, Liu and Niswander, 2005). As such, the following mechanism is now proposed to potentially explain the present findings, in relation to the established roles of BMPs in the NS (Fig. 9.4).

The first sign of the induction of a DA fate in the VM is the expression of Lmx1a and Msx1 at around E8 in the mouse; these factors act as key determinants of midbrain DA neurons (Andersson et al., 2006, Alavian et al., 2008, Hegarty et al., 2013c). Both of these genes are induced by BMP signalling during NS development (Maeda et al., 1997, Tribulo et al., 2003, Chizhikov and Millen, 2004, Liu et al., 2004), while this study has shown that BMP2 induces the expression of these genes in cultured E14 rat VM NSCs. Furthermore, FGF, Shh and WNT signalling have all been shown to play critical roles in the development of DA neurons in the VM (Roussa and Krieglstein, 2004b, Alavian et al., 2008, Joksimovic et al., 2009b). An interaction between BMP-Smad signalling and FGF, Shh and WNT signalling has been demonstrated in the induction, specification and development of a variety of neural populations, such as SC neuronal populations and NCCs, the relevant aspects of which have been detailed in the introduction (LaBonne and Bronner-Fraser, 1998, Monsoro-Burq et al., 2005, Liu and Niswander, 2005, Eivers et al., 2008, Fuentealba et al., 2007). However, a number of recent studies have provided substantial evidence that VM DA neurons arise from floor plate DA neural progenitors (Hegarty et al., 2013c, Ono et al., 2007, Bonilla et al., 2008, Hebsgaard et al., 2009). This suggests that BMP signalling is not involved in DA development, since the Shh-expressing floor plate is the opposing signalling centre to the BMP-expressing roof plate along the dorsoventral axis of the neural tube (Altmann and Brivanlou, 2001, Ulloa and Briscoe, 2007). It may be the case that BMP signalling acts dorsally and that Shh signalling acts ventrally along the length of the neural tube to induce various neuronal phenotypes, with these signals intersecting intermediately. However, the VM floor plate is different to its caudal counterparts. The floor plate was thought to consist of specialised non-neurogenic glial type cells that ventralize the neural tube, mainly by secreting Shh (Jessell, 2000, Placzek and Briscoe, 2005, Fuccillo et al., 2006). This currently remains true for the neural tube caudal to the midbrain, with the hindbrain floor plate being shown to be non-neurogenic (Joksimovic et al., 2009b). As afore-mentioned, VM floor plate cells are now known

to be neurogenic, and to specifically give rise to DA neurons (Hegarty et al., 2013c, Ono et al., 2007, Bonilla et al., 2008, Hebsgaard et al., 2009). The suggestion that BMP signalling may be involved in this process arises from the finding that WNT antagonism of Shh signalling facilitates VM floor plate neurogenesis, and that Shh inhibits this neurogenesis (Joksimovic et al., 2009b). WNTs are expressed in the midbrain floor plate and are vital for VM DA neurogenesis (Echelard et al., 1994, Castelo-Branco et al., 2003, Zervas et al., 2004, Prakash et al., 2006, Hegarty et al., 2013c). BMP-Smad 1/5/8 signalling positively regulates WNT expression in the spinal cord (Chesnutt et al., 2004, Wine-Lee et al., 2004), and may continue this role in the VM floor plate. In support of this, BMPs, GDF5 and GDF15 have all been shown to be expressed in the mesencephalic floor plate during DA neurogenesis (Jordan et al., 1997, Strelau et al., 2000, O'Keeffe et al., 2004b, Soderstrom and Ebendal, 1999). During development, Lmx1a expression caudal to the midbrain is restricted to the roof plate and the cerebellum (Failli et al., 2002, Chizhikov et al., 2010, Griesel et al., 2011). BMPs induce the expression of Lmx1a and other co-factors that regulate the induction of the roof plate in the dorsal neural tube (Chizhikov and Millen, 2004) and determine the fate of cerebellar granule neurons (Alder et al., 1999, Qin et al., 2006). These findings again demonstrate that Lmx1a is a key mediator of BMP signalling. Rostral to the pons in the mid-gestation mouse embryo, Lmx1a expression becomes ventralized, with its expression found in the VM and the basal plates of the diencephalon (Failli et al., 2002). This ventral midbrain expression of Lmx1a is accompanied, and possibly preceded, by BMP expression, as mentioned above. Furthermore, BMPs have been reported to act in a dual role with Shh to induce ventral cell types in the diencephalon (Dale et al., 1999). Indeed, a regulated level of BMP signalling is involved in the neurogenesis of Shh-responsive ventral cell types in the forebrain (Yung et al., 2002). It is not unlikely to suggest that this ventralization of dorsal signalling/expression is due to the formation of the pontine flexure which causes the ventral displacement of the BMP-expressing/signalling alar plates in the metencephalic region. In support of this theory, the pontine flexure forms before DA neurogenesis in the VM (Wallace and Lauder, 1983).

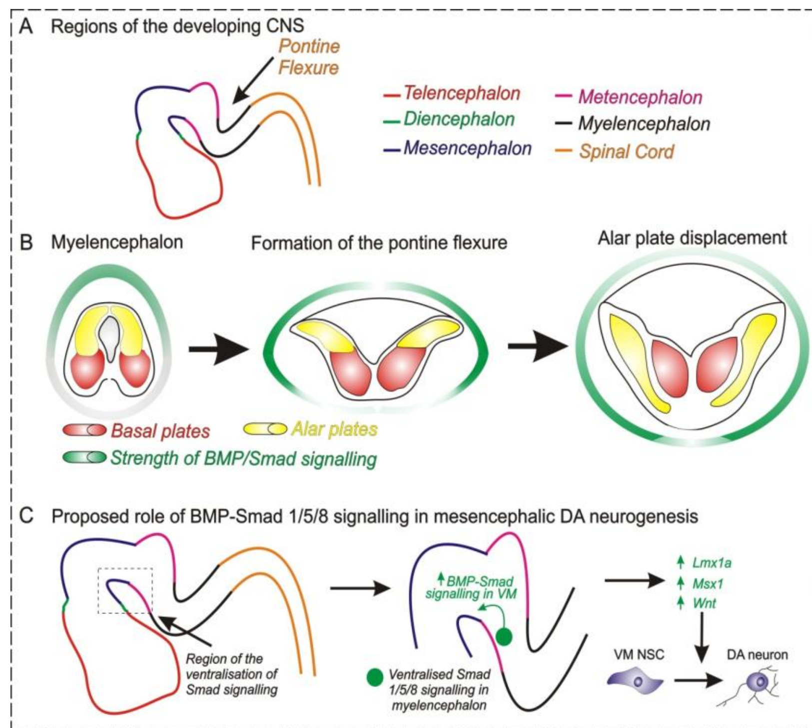


Figure 9.4 Proposed role of canonical BMP-Smad Signalling in the development of VM DA Neurons.

(A) Regions of the developing CNS. (B) Graphical representation of the morphological changes of the myelencephalon due to pontine flexure formation. The alar plates are displaced ventrally, resulting in ventralisation of BMP-Smad signalling. (C) Graphical representation of the proposed role for BMP-Smad signalling in VM DA neurogenesis. Ventralised BMP-Smad signalling in the myelencephalon induces BMP-Smad signalling in the adjacent VM. BMP-Smad signalling subsequently induces Lmx1a, Msx1 and WNT expression in the VM, which results in VM DA neurogenesis.

FGF signalling from the isthmus organizer may fit into this proposed role of BMP signalling in VM DA neurogenesis, by acting to repress BMP-Smad signalling to refine its effects. FGF plays such a role during neural induction (Wilson et al., 2000, Streit et al., 2000, Pera et al., 2003), and intermediate levels of BMPs are required for the proper development of specific ‘intermediate’ (i.e. not directly in contact with the roof plate) neuronal phenotypes, as discussed in a recent review (Hegarty et al., 2013a). Furthermore, an intermediate level of BMP-Smad signalling has been shown to be necessary for the induction of Msx1 (Tribulo et al., 2003), one of the two key determinants of midbrain DA neurons, along with Lmx1a (Andersson

et al., 2006); FGF has also been shown to induce Msx1 expression in the neural crest (Monsoro-Burq et al., 2005). Furthermore, engrailed1/engrailed2 expression are vital for VM DA neurogenesis (Hegarty et al., 2013c), and BMP signalling has been shown to induce the expression of these genes in VM cultures (Alder et al., 1999). In support of a proposed role of Smad 1/5/8 signalling in VM DA neuronal development, BMP signalling increases the numbers of DA neurons in embryonic rat VM cultures (Krieglstein et al., 1995b, Jordan et al., 1997, O'Keeffe et al., 2004a, Reiriz et al., 1999, Brederlau et al., 2002, Lee et al., 2003). Furthermore, this study suggests that the BMPs, BMP2 and GDF5, may act sequentially during the generation of VM DA neurons. Additionally, based on data obtained in this thesis, BMP2- and GDF5-induced Smad signalling may function in the development of the striatal projections of VM DA neurons, coinciding with their roles in DA neuronal generation.

10. Conclusions and Future Perspectives

This thesis has shown that BMP2- and GDF5-induced neurite growth of VM DA neurons is BMPRIb-dependent and requires Smad 1/5/8 transcriptional regulation. Additionally, the BMP2- and GDF5-mediated neurite growth-promoting effects are direct, and independent of GDNF signalling. In terms of DA neuronal growth, Sip1 has been identified as a novel negative regulator of this process, and likely acts through inhibition of BMP-Smad-driven neurite growth. These findings represent significant contributions to the present understanding of the molecular and cellular mechanisms by which these BMPs function during midbrain DA development, as well as in the NS in general. Through the comparison of the effects of BMP2 and GDF5, this study has demonstrated that their diverse effects, within the same cell populations, are potentially mediated by different subtypes of the BMPRI. Perhaps temporal and spatial regulation of the expression of the BMPRIa and BMPRIb is integral to the varying effects of these BMPs during NS development. In terms of DA neuronal induction, BMP2 and GDF5 appear to act sequentially in this process, with BMP2 inducing a DA phenotype in VM NPs, and GDF5 inducing the transition of VM DA NPs into midbrain DA neurons. The expression profiles of BMPRIb, BMPRII and Sip1 correlate with the proposed roles of BMP2 and GDF5 as multi-step regulators of VM DA development. Taken together, this thesis has identified

GDF5 and BMP2 as potential regulators of midbrain DA neuronal induction, differentiation and survival, and has demonstrated that their effects on DA neurons are mediated by canonical BMPR-Smad signalling.

However, despite these findings regarding BMP2 and GDF5-mediated regulation of VM DA development, further experimentation is required to conclusively show their participation *in vivo*. To build on the *in vitro* characterisations performed in this study, which also need further experimentation as described in each chapter throughout, future work should assess mice with homozygous and/or heterozygous mutations in GDF5, BMP2, BMPRIb, Smad 1/4/5/8 and/or Sip1. Crucially, a detailed analysis of the numbers of DA neurons present in the midbrain, as well as striatal innervation, at multiple stages during embryonic and post-natal development is required to address whether canonical BMP-signalling is involved in these developmental processes during VM DA development *in vivo*. For example, the BMPRII null mouse has a reduction of nigrostriatal neurons and of striatal DA innervation in adulthood (Chou et al., 2008a), but it is unclear whether this results from a failure in DA neuronal development, or from a later degenerative process. Analysis of mutant mice at each developmental stage of this embryonic and postnatal process would address this. Furthermore, the Smad 1/5/8 reporter mouse (Monteiro et al., 2008) could be used to demonstrate BMP-Smad-dependent transcriptional activity in the VM region, and the crossing of the BMP/BMPR/Smad/Sip1 mutants with this reporter mouse would allow investigation of the impact of the loss of one of these BMP-Smad pathway components on transcriptional activity *in vivo* during VM DA development.

From a clinical perspective, cell replacement therapy is one of the most promising therapies for the treatment of PD (Orlacchio et al., 2010, Bonnamain et al., 2012, De Feo et al., 2012, Toulouse and Sullivan, 2008, Hedlund and Perlmann, 2009). Considering the importance of the establishment of functional connections by transplanted DA cells in the host striatum, factors which promote DA neurite outgrowth are being considered as adjuncts to this potential therapy. GDF5 and BMP2 are thus ideal candidates for such a role, as both have been shown to promote the survival of VM DA neurons (O'Keeffe et al., 2004b, Reiriz et al., 1999, Jordan et al., 1997, Sullivan et al., 1997). These two functions of BMP2 and GDF5 in neurite growth-promotion and DA neuronal survival make these BMPs ideal neurotrophic factors for nigrostriatal DA neurons, especially considering that the expression

profiles of BMPRIb and BMPRII propose that BMP2 and GDF5 play such a function during normal development. The induction of relevant DA genes by BMP2 and GDF5 has implications for the specification of stem cell sources towards VM DA phenotypes prior to transplantation. Furthermore, targeting Sip1 expression in stem cell sources may provide a mechanism for the dual-induction of both DA specification and their subsequent neuronal growth. Indeed, Sip1 modulation has already been shown to contribute to DA specification in stem cells (Cai et al., 2013), while the present study modulated Sip1 expression to induce DA neurite growth. In terms of stem cell sources, the current study has demonstrated that neurosphere-expanded E14 rat VM NSCs require three weeks to complete differentiation into their progeny, with neurogenesis proceeding during the second two weeks via GFAP-expressing NPs. This has important implications for the choice of age of donor tissue for the use of NSCs for transplantation studies, as well as the identity of neuronal precursors *in vitro*.

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ORIGINAL PAPER

Canonical BMP–Smad Signalling Promotes Neurite Growth in Rat Midbrain Dopaminergic Neurons

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Abstract Ventral midbrain (VM) dopaminergic (DA) neurons project to the dorsal striatum via the nigrostriatal pathway to regulate voluntary movements, and their loss leads to the motor dysfunction seen in Parkinson's disease (PD). Despite recent progress in the understanding of VM DA neurogenesis, the factors regulating nigrostriatal pathway development remain largely unknown. The bone morphogenetic protein (BMP) family regulates neurite growth in the developing nervous system and may contribute to nigrostriatal pathway development. Two related members of this family, BMP2 and growth differentiation factor (GDF)5, have neurotrophic effects, including promotion of neurite growth, on cultured VM DA neurons. However, the molecular mechanisms regulating their effects on DA neurons are unknown. By characterising the temporal expression profiles of endogenous BMP receptors (BMPRs) in the developing and adult rat VM and striatum, this study identified BMP2 and GDF5 as potential regulators of nigrostriatal pathway development. Furthermore, through the use of noggin, dorsomorphin and BMPR/Smad plasmids, this study demonstrated that GDF5- and BMP2-induced neurite outgrowth from cultured VM DA neurons is dependent on BMP type I receptor activation of the Smad 1/5/8 signalling pathway.

Keywords BMP2 · GDF5 · Ventral midbrain · Dopaminergic neurons · Neurite growth · BMP receptor · Smad signalling

Abbreviations

6-OHDA	6-Hydroxydopamine
BMP(s)	Bone morphogenetic protein(s)
BMPR(s)	Bone morphogenetic protein receptor(s)
caBMPRIb	Constitutively active BMPRIb
CNS	Central nervous system
DA	Dopaminergic/dopamine
DIV	Day(s) in vitro
E	Embryonic day
FCS	Foetal calf serum
GDF(s)	Growth differentiation factor(s)
GDNF	Glial cell line-derived neurotrophic factor
N	Number of repetitions
P	Post-natal day
PBS	Phosphate-buffered saline
PD	Parkinson's disease
RT-QPCR	Quantitative real-time PCR
RT-PCR	Reverse transcriptase-polymerase chain reaction
SC	Spinal cord

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SNpc	Substantia nigra *pars compacta*
TGF	Transforming growth factor
TH	Tyrosine hydroxlase
VM	Ventral midbrain/mesencephalon

Introduction

In the central nervous system (CNS), more than three-quarters of all DA neurons are found in the VM (Blum 1998; German et al. 1983; Pakkenberg et al. 1991). These are subdivided into three distinct clusters, termed the A8, A9 and A10 groups of VM DA neurons. The A9 group of VM DA neurons, located in the substantia nigra *pars compacta* (SNpc), projects to the dorsolateral striatum via the nigrostriatal pathway (Dahlstroem and Fuxe 1964; Bjorklund and Dunnett 2007). These A9 DA neurons, and their striatal targets, are part of the basal ganglia circuitry that regulates the control of voluntary movement. Their functional importance is highlighted by the neurodegenerative disorder PD, the primary neuropathological signature of which is the loss of these neurons and their striatal projections, which results in the motor deficits that are the characteristic of this disease (Toulouse and Sullivan 2008; Lees et al. 2009).

During embryonic development, A9 DA neurons are generated in the VM under the influence of two key signalling centres, the isthmus and the floor plate (Hynes et al. 1995; Crossley and Martin 1995; Liu and Joyner 2001). Much work in recent decades has focused on elucidating the molecular circuitry that is involved in the generation of A9 VM DA neurons (Hegarty et al. 2013c); however, the molecular mechanisms that regulate the growth and guidance of the axonal projections of these DA neurons to their appropriate target regions in the striatum are less well understood.

VM DA neurons extend their axons towards the telencephalon via the medial forebrain bundle in response to extrinsic directional cues (both chemo-attractive and chemo-repulsive) from the caudal brain stem, midbrain, diencephalon, striatum and cortex (Gates et al. 2004; Nakamura et al. 2000). Despite the paucity of studies identifying the regulatory molecules involved in the formation of DA projections, a number of molecules have been implicated. Cell surface ephrins and their Eph receptor tyrosine kinases, which are important in axonal guidance (Egea and Klein 2007), have been shown to play roles in target innervation by nigrostriatal axons (Sieber et al. 2004; Halladay et al. 2004; Van den Heuvel and Pasterkamp 2008; Calo et al. 2005; Yue et al. 1999; Cooper et al. 2009). Similarly, netrin signalling via the deleted colorectal cancer (DCC) receptor, which is known to actively regulate axonal growth (Round and Stein 2007), has been strongly implicated in the formation of the VM DA circuitry (Xu et al. 2010; Flores et al. 2005; Manitt et al. 2011; Lin et al. 2005; Sgado et al. 2012; Vitalis et al. 2000). Additionally, signalling between Slits and their Robo receptors (Bagri et al. 2002; Dugan et al. 2011; Lin et al. 2005; Lopez-Bendito et al. 2007), and by semaphorins (Hernandez-Montiel et al. 2008; Torre et al. 2010; Tamariz et al. 2010; Kolk et al. 2009), has been shown to regulate the formation of DA projections from the VM to the striatum. These identified molecules are well-established regulators of axonal growth and guidance in other regions of the nervous system. It is thus likely that further candidate molecules with similar functions in other areas of the NS may contribute to the regulation of DA axonal growth. One candidate group of molecules is the BMP family (Zou and Lyuksyutova 2007; Bovolenta 2005).

BMPs are regulators of axonal growth in a number of neuronal populations, with this role best characterised in the dorsal spinal cord (SC) (Parikh et al. 2011; Hazen et al. 2012; Lein et al. 1995; Hegarty et al. 2013a; Gratacos et al. 2002). The two members of the BMP family of proteins, BMP2 and a related molecule GDF5, have been shown to regulate neurite growth in the dorsal SC (Parikh et al. 2011; Hazen et al. 2011, 2012; Phan et al. 2010; Niere et al. 2006). GDF5 and BMP2 both activate a canonical signalling pathway involving two types of serine/threonine kinase receptors, type I and type II BMPRs (ten Dijke et al. 1994; Koenig et al. 1994; Yamashita et al. 1996; Shi and Massague 2003). Upon ligand binding, the constitutively active BMPRII transphosphorylates the cytoplasmic domain of the BMPRI (BMPRIa or BMPRIb), causing phosphorylation of the receptor-regulated Smads, Smads 1/5/8, by the activated BMPRI. The phosphorylated Smads 1/5/8 then form a heterocomplex with the co-Smad, Smad4, which mediates their nuclear translocation to allow modulation of target gene expression (Miyazono et al. 2010; Sieber et al. 2009).

BMP2 and GDF5 are expressed in the developing rat VM during the period of DA axogenesis, suggesting that they may play a role in this process (Krieglstein et al. 1995; O'Keeffe et al. 2004b; Storm et al. 1994; Chen et al. 2003; Jordan et al. 1997; Soderstrom and Ebendal 1999; Hegarty et al. 2014). In support of such a suggestion, both GDF5 and BMP2 have been shown to promote the survival of rat VM DA neurons (O'Keeffe et al. 2004a; Reiriz et al. 1999; Jordan et al. 1997; Sullivan et al. 1997) and induce neurite growth of rat VM DA neurons in vitro (O'Keeffe et al. 2004a; Reiriz et al. 1999). Despite these studies, the expression patterns of the BMP receptors (BMPRs) in the VM and the target striatum during nigrostriatal pathway development are unknown. Furthermore, the mechanisms

by which GDF5 and BMP2 mediate their neurite growth-promoting effects on VM DA neurons remain to be determined. However, these effects have recently been proposed to occur via the canonical Smad signalling pathway in a cell line model of dopaminergic neurons (Hegarty et al. 2013b).

To address the gaps in our current knowledge of BMP-mediated DA neuronal growth, this study examined the expression of BMP receptors over the developmental period between embryonic day (E) 14 and post-natal day (P) 90 in rats, since the generation and maturation of nigrostriatal dopaminergic neurons, the invasion and arborisation of their striatal targets, and the refinement of these connections occur over this time period (Van den Heuvel and Pasterkamp 2008). Furthermore, the molecular mechanisms by which BMP2 and GDF5 regulate axonal growth of VM DA neurons were investigated.

Materials and Methods

Cell Culture

For the preparation of E14 rat VM cultures, E14 embryos were obtained by laparotomy from date-mated female Sprague–Dawley rats following decapitation under terminal anaesthesia induced by the inhalation of isoflurane (Isoflo®). Dissected VM tissue was centrifuged at 1,100 rpm for 5 min at 4 °C. The tissue pellet was incubated in a 0.1 % trypsin–Hank's balanced salt solution for 5 min, at 37 °C with 5 % CO_2. Foetal calf serum (FCS) was then added to the tissue followed by centrifugation at 1,100 rpm for 5 min at 4 °C. The resulting cell pellet was resuspended in 1 ml of differentiation media (Dulbecco's modified Eagle's medium/F12, 33 mM D-glucose, 1 % L-glutamine, 1 % FCS, supplemented with 2 % B27) using a P1000 Gilson pipette and carefully triturated using a sterile plugged flame-polished Pasteur pipette, followed by a 25-gauge needle and syringe, ensuring not to add air bubbles into the cell suspension. Cell density was estimated using a haemocytometer. Cells were plated on poly-D-lysine (Sigma)-coated 24-well tissue culture plates at a density of 5×10^4 cells per well in 500 µl of differentiation media at 37 °C with 5 % CO_2. SH-SY5Y cells were used as a model of human DA neurons in this study, and their cell culture was performed as previously outlined (Hegarty et al. 2013b).

Cells were treated with 200 ng/ml of GDF5 (kindly provided by Biopharm GmbH) or recombinant human BMP2 (R&D Systems) and pre-treated (30 min prior to GDF5 or BMP2 application) with 1 µg/ml of Dorsomorphin (Sigma), 200 ng/ml of Noggin (R&D Systems), or 0.3 U/ml of Heparinase III (R&D Systems). For the neurite growth assay, cells were treated daily for 4 DIV. To test Smad pathway activation, cells were treated for 0, 30 or 120 min.

Electroporation of E14 Rat VM Cells

Electroporation of E14 VM cells was carried out using the Neon™ Transfection System (Invitrogen). E14 VM cell suspensions were prepared for counting (as outlined above), and the required volume of cells to give 200,000 cells per well was centrifuged at 4 °C at 1,100 rpm for 5 min. The cell pellet was washed twice with 10 mM phosphate-buffered saline (PBS) (without $CaCl_2$ and $MgCl_2$) (Sigma) and then resuspended in the required amount of the manufacturers resuspension buffer (12 µl per transfection/plasmid) (Invitrogen). About 0.5 µg of a GFP plasmid, 1 µg of plasmid DNA (caBMPRIb and/or Smad4 siRNA vector (Hegarty et al. 2013b)) and/or 1 µM of desired siRNA (Control or BMPRIb; Life Technologies) were added to the resuspended cells. About 10 µl of the cell/plasmid mixture was then electroporated according to the manufacturer's protocol under specific parameters (1,100 V; 30 ms; 2 pulses).

Immunocytochemistry

Cultures were fixed for 10 min using 100 % ice-cold methanol. Following 3 washes in 10 mM PBS-T (0.02 % Triton X-100 in 10 mM PBS) for permeabilization, cultures were incubated in blocking solution (5 % bovine serum albumin) for 1 h at room temperature. Cultures were subsequently incubated in the following antibodies: BMPRII (1:200; R&D Systems), BMPRIb (1:200; R&D Systems), phopsho-Smad 1/5/8 (1:200; Cell Signalling), tyrosine hydroxylase (TH; 1:200; mouse monoclonal; Millipore, or 1:300; rabbit polyclonal; Millipore) and β-actin (1:200; Sigma) diluted in 1 % bovine serum albumin in 10 mM PBS at 4 °C overnight. Following 3 × 5 min washes in PBS-T, cells were incubated in Alexa Fluor 488- and/or 594-conjugated secondary antibodies (1:500; Invitrogen) reactive to the species of the primary antibodies and diluted in 1 % bovine serum albumin in 10 mM PBS, at room temperature for 2 h in the dark. Cultures were counterstained with bisbenzimide (1:1,000 in 10 mM PBS; Sigma). Negative controls in which the primary antibody was omitted were also prepared (not shown). Cells were imaged under an Olympus IX70 inverted microscope fitted with an Olympus DP70 camera and AnalysisD™ software. For densitometric analysis, the fluorescence intensity of individual cells stained for phospho-Smad 1/5/8 was measured using ImageJ analysis software (Rasband, WJ, http://rsb.info.nih.gov/ij/). The relative fluorescence intensity was

calculated for each individual cell after subtraction of the background noise.

Immunohistochemistry

Four adult (8- to 12-week old) female Sprague–Dawley rats were killed by terminal anaesthesia (150 mg/kg sodium pentobarbitone, i.p.) and perfused intracardially with 100 ml of 10 mM PBS, pH 7.4, containing 500 Units of heparin sulphate, followed immediately by 200 ml of freshly prepared 4 % ice-cold paraformaldehyde in PBS. The brains were removed and placed in 4 % paraformaldehyde overnight, cryoprotected in 30 % sucrose in PBS, and then snap-frozen in isopentane on liquid nitrogen.

Three pairs of coronal cryosections (15 µm; Cryostat manufacturer: Leica—model CM1900) were collected at each of three levels through the midbrain (AP −4.8, −5.6, −6.4 relative to bregma; (Paxinos and Watson 1988)). The sections were mounted on gelatine-coated slides and then stained immunocytochemically for TH and/or BMPRIb or BMPRII. Firstly, endogenous peroxidase was inactivated by incubation in 20 % methanol, 0.2 % Triton X-100, 1.5 % hydrogen peroxide in 10 mM PBS for 10 min. Sections were incubated in blocking solution (3 % normal goat serum, 0.2 % Triton X-100 in 10 mM PBS) for 1 h at room temperature and then in a solution (1:1,000) of antiserum to TH (rabbit) and/or BMPRIb or BMPRII (mouse) overnight at 4 °C. After four washes in 0.02 % Triton X-100 in 10 mM PBS for 10 min each, sections were incubated in Alexa Fluor 488- and/or 594-conjugated secondary antibodies (1:500) reactive to the species of the primary antibodies diluted in 10 mM PBS, at room temperature for 2 h in the dark. Sections were then coverslipped in PVA-DABCO before fluorescent imaging.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The VM and striatum from E14 to P90 rats were dissected, and following the extraction and purification of total RNA, semi-quantitative RT-PCR for a variety of genes involved in DA development and maintenance (TH, Nurr1, Lmx1b and Pitx3) (Hegarty et al. 2013c) was performed on the midbrain samples to confirm the accuracy of the dissection at each age. RT-PCR was also performed on E11–E14 rat VM tissue for BMPRII and BMPRIb, as well as on SH-SH5Y cells for BMPRIb.

Dissected embryonic and adult tissue in ice-cold Hank's balanced salt solution was centrifuged at 500 rpm for 2 min, the supernatant discarded and the tissue pellet stored immediately at −80 °C until RNA extraction. Cultured SH-SH5Y cells ($\sim 1 \times 10^6$ cells) were centrifuged at 10,000 rpm for 10 min before storage/usage. RNA was isolated using an RNeasy mini extraction kit (Qiagen). An ImProm-II Reverse Transcription System (Promega) was used to synthesise cDNA using 1 µg of RNA in an 11.5 µl reaction volume for 90 min at 37 °C. Amplification was carried out using a GoTaq Flexi DNA Polymerase system (Promega) as per the manufacturer's instructions. Each reaction mixture consisted of 2 µl cDNA, 2 µl forward and reverse primer mix, 5X PCR buffer, 1.5 mM $MgCl_2$, 1.25 mM PCR dNTPs, 0.25 µl Taq polymerase and made up to a total volume of 25 µl with nuclease-free water. Forward and reverse primer pairs for TH (275 bp), Nurr1 (434 bp), Lmx1b (485 bp), Pitx3 (193 bp), BMPRIb (425 bp), BMPRII (349 bp) and GAPDH (388 bp) are listed in Supplementary Fig. 1.

Quantitative Real-Time PCR (RT-QPCR)

Midbrain and striatum samples were disrupted and homogenised in 1 ml of QIAzol Lysis Reagent (Qiagen). After the addition of 200 µl chloroform, homogenates were separated into aqueous and organic phases by centrifugation at 13,000 rpm for 15 min. The upper aqueous phase was mixed with an equal volume of 70 % ethanol, to precipitate the RNA, and then transferred to an RNeasy Mini spin column placed in a 2-ml collection tube. Total RNA was purified using the Qiagen RNeasy Lipid Tissue Mini extraction kit and RNase-free DNase set, according to the manufacturer's instructions. Following purification, total RNA was reverse transcribed using Stratascript reverse transcriptase (Agilent Technologies), for 1 h at 45 °C, in a 30 µl reaction according to the manufacturer's instructions.

In order to amplify cDNAs encoding the normalising reference genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), succinate dehydrogenase complex, subunit A (SDHA) and ubiquitin C (UBQC), 2.5 µl of cDNA was amplified in a 25 µl PCR containing 1X FastStart Universal SYBR Green Master Mix (Rox) (Roche) and 150 nM forward and reverse primers. In the case of amplifying cDNAs encoding TH, BMPRIb and BMPRII, 2 µl of cDNA was amplified in a 20 µl PCR containing 1X of Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies), 150 nM each forward and reverse primers and 300 nM cDNA-specific FAM/BHQ1 dual-labelled hybridization probe (Eurofins), and 3 nM ROX reference dye.

Quantitative real-time PCR amplification was performed using the Stratagene MX3000P thermal cycler. GAPDH, SDHA and UBQC quantitative real-time PCR amplification products were verified as being correct by melting curve analysis (melting temperatures 83.5, 80 and 85 °C, respectively) of the completed PCR. The initial quantities of each cDNA in each PCR were determined by comparison to a standard curve incorporated into the PCR run and

constructed from serial dilutions of cDNA reverse transcribed from RNA extracted from P11 striatum and midbrain samples. Values for each gene of interest were normalised to the geometric mean of the three reference genes.

Primer and probe sequences for the amplification of each cDNA are listed in Supplementary Fig. 1. Cycling parameters for GAPDH, SDHA and UBQC were 10 min at 95 °C followed by 40 cycles of 95 °C for 30 s; 55 °C for 1 min; 72 °C for 1 min. Cycling parameters for TH, BMPRIb and BMPRII were 3 min at 95 °C followed by 45 cycles of 95 °C for 13 s and 60 °C for 30 s.

Analysis of Neuronal Complexity

The total neurite length of individual E14 VM neurons was measured at 1 and 3 DIV using Sholl analysis as previously described (Gutierrez and Davies 2007; Collins et al. 2013). Traces of GFP^{+}/TH^{+} neurons were carried out using the CorelDRAW ×4 software and analysed as previously described (O'Keeffe et al. 2004a). Briefly, neurite length (NL) was calculated using the following formula: $NL = \alpha \times T \times (\pi/2)$, where α is the number of times the neurite intersects the grid lines, and T is the distance between the gridlines on the magnified image (taking into account the magnification factor). VM neurons with intact processes were analysed from 20 random fields per condition, where any neuron with a process that was at least one and half times the length of the soma was determined as an intact process (which precludes the analysis of apoptotic neurons). For SH-SY5Y cells, cellular morphology was assessed as previously described (Hegarty et al. 2013b).

Western Blotting

Western blotting was carried out as previously described (Crampton et al. 2012). The cells were lysed in RIPA buffer, and insoluble debris was removed by centrifugation. Samples were run on an agarose gel and transferred to nitrocellulose membranes using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, CA, USA). The membranes were incubated with primary antibodies against BMPRIb (1:1,000) or β-actin (1:1,000) overnight at 4 °C, washed, incubated with horseradish peroxidase-labelled anti-rabbit IgG (1:2,000; Promega), washed and developed with ECL-Plus (Amersham).

Statistical Analysis

Unpaired Student's *t* test or one-way ANOVA with a post hoc Tukey's test was performed, as appropriate, to determine significant differences between groups. Results were expressed as means with SEM and deemed significant when $p < 0.05$.

Results

BMPRs are Expressed in the Rat VM and Striatum During Embryonic and Post-natal Development

If BMP–Smad signalling promotes the neurite growth of VM DA neurons, then the BMP receptors, BMPRII and BMPRIb, should be expressed in the VM and possibly the striatum, during the period of DA axogenesis. To examine this, RT-QPCR was used to quantify the expression levels of TH, BMPRII and BMPRIb transcripts in the VM and striatum during embryonic and post-natal development, having confirmed the accuracy of the VM dissections by examining DA gene expression at each age (Fig. 1a–d). In the VM, TH mRNA levels are highest from E14 to P1 (Fig. 1b). A significant drop in TH transcript levels occurs between P1 and P11, after which the expression of TH mRNA remains stable through to adulthood (P90) (Fig. 1b). In the striatum, TH mRNA levels are significantly lower than those in the midbrain throughout the developmental period studied (Fig. 1b).

BMPRII mRNA levels are relatively stable throughout development in the VM (Fig. 1c), while in the developing striatum BMPRII mRNA levels increase 1.5-fold between E14 and P1. Between P1 and P31, the level of BMPRII transcripts expressed in the striatum falls almost threefold and this lower expression level is maintained through to adulthood (Fig. 1c). BMPRII mRNA levels in P90 midbrain are similar to those in P90 striatum (Fig. 1c). In the midbrain, BMPRIb mRNA levels increase threefold between E14 and P1, and thereafter remain unchanged until adulthood (Fig. 1d). In the developing striatum, BMPRIb mRNA levels increase by twofold between E14 and P1, before increasing a further twofold between P1 and P60 (Fig. 1d). BMPRIb striatal mRNA levels remain relatively steady thereafter through to P90 and are comparable to that of the adult midbrain at this time point (Fig. 1d). The expression levels of BMPRII and BMPRIb transcripts in the adult midbrain (P31–P90) are very similar. Indeed, RT-PCR and in situ hybridization showed that BMPRII and BMPRIb are strongly expressed in the adult rodent SNpc (Fig. 1e–j). Furthermore, approximately 75 % of DA neurons in the adult rat midbrain expressed BMPRII and BMPRIb (Fig. 1k, l).

Since the initial phase of DA axogenesis begins at E11 in the rat (Gates et al. 2004; Nakamura et al. 2000), this study also showed that BMPRII and BMPRIb are expressed in the developing rat VM from E11 to E14 VM (Fig. 2a). Western blotting and immunocytochemistry were then used to confirm that the effector part of the BMP receptor complex, the BMPRIb protein, is expressed in the rat VM during this developmental period (Fig. 2b, c). To determine whether these receptors are expressed on DA

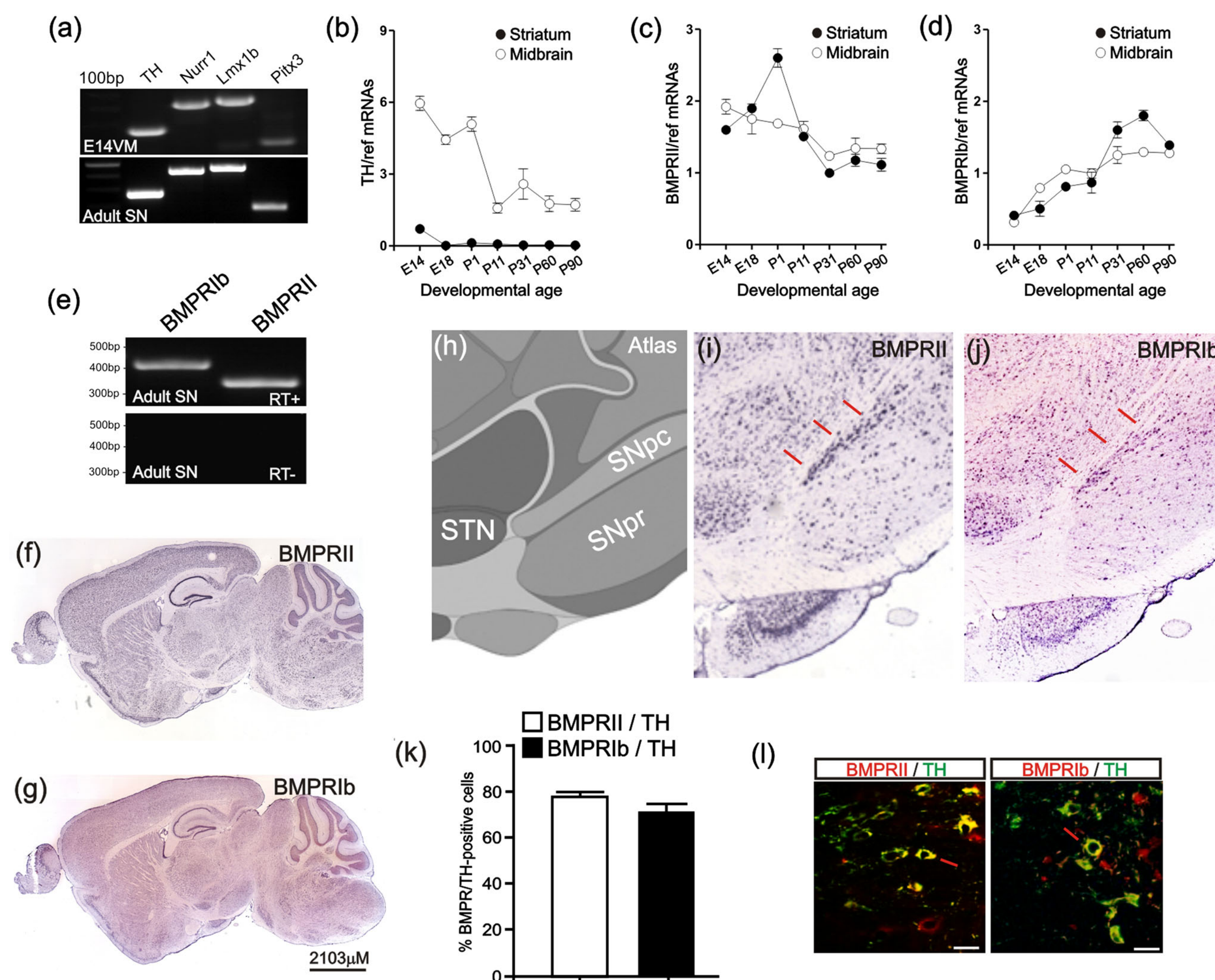

Fig. 1 BMP receptors are expressed in the midbrain and striatum during embryonic and post-natal development. **a** RT-PCR of TH, Nurr1, Lmx1b, and Pitx3 in E14 and adult rat VM (SN = substantia nigra). **b–d** Quantitative RT-QPCR data showing the levels of (**b**) TH, (**c**) BMPRII and (**c**) BMPRIb mRNA in the developing midbrain and striatum, from E14 to P90, relative to the levels of the reference mRNAs GAPDH, SDHA and UBQC. Each data point represents pooled data from four samples from three separate litters/animals, and all data are presented as the mean ± SEM. **e** RT-PCR showing the expression of BMPRII and BMPRIb in the adult rat SN. **f**, **g** In situ hybridization images taken from the Allen Developing Brain Atlas (©(Allen) Developing Mouse Brain Atlas, 2012) showing BMPRII and BMPRIb expression (purple colour) in sagittal sections of the P56 adult mouse brain. **h** Atlas showing the major nuclei in the midbrain region, including the SNpc, substantia nigra *pars reticulate* (SNpr) and subthalamic nucleus (STN). Corresponding in situ hybridization images of this region showing strong expression of **i** BMPRII and **j** BMPRIb in the SNpc (identified by *red arrows*). *Scale bar* = 2103 μm. **k** Quantification of the percentage of DA neurons in the adult rat SN expressing BMPRII and BMPRIb. **l** Photomicrographs showing immunostaining for BMPRII and BMPRIb co-expressed with TH in the adult rat SNpc

neurons, immunocytochemical analysis was used to confirm protein expression of BMPRII and BMPRIb on TH-positive neurons in E14 rat VM cultures (Fig. 2d, e and data not shown). The co-localisation of BMPRII and BMPRIb immunostaining with TH immunostaining indicates that these receptors are expressed by DA neurons, although there is also expression of these BMPRs on TH-negative, non-DA cells (Fig. 2d, e).

BMP2 and GDF5 Promote Neurite Growth and Activate Canonical Smad Signalling in VM DA Neurons

Following the characterisation of BMPR expression in the VM and striatum during development, we next assessed the effects of BMP2 and GDF5 on the promotion of neurite growth from cultured E14 VM DA neurons. Treatment

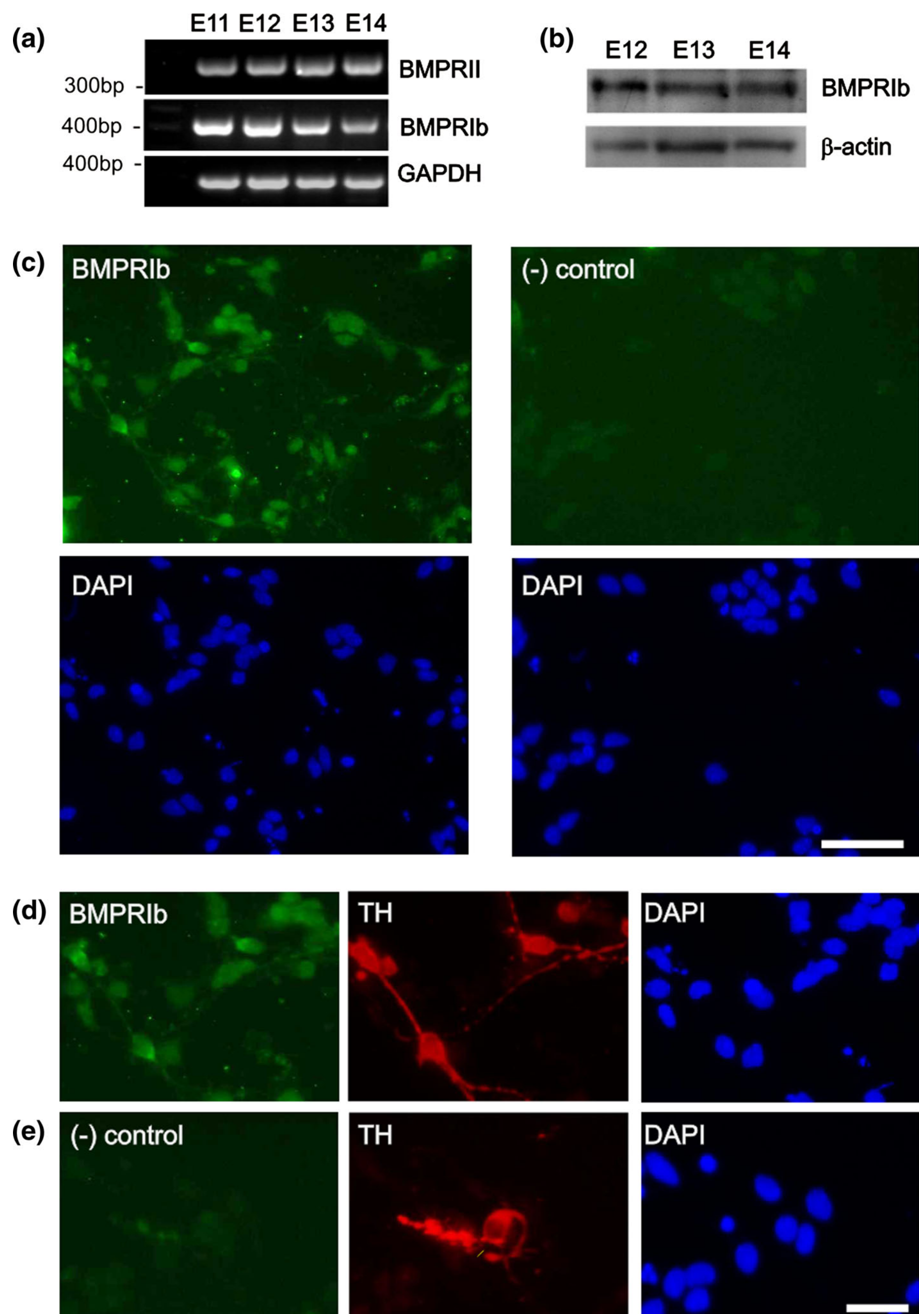

Fig. 2 BMPRs are expressed on DA neurons during the peak period of DA axogenesis. **a** RT-PCR of BMPRII, BMPRIb and GAPDH in E11 to E14 rat VM. **b** Western blotting showing BMPRIb protein expression in the developing rat VM. **c** Photomicrographs showing immunostaining for BMPRIb co-expressed with DAPI and the relevant negative controls ((−) control) in cultures of the E14 rat VM after 24 h in vitro. Photomicrographs showing immunostaining for **d** BMPRIb with **e** being the negative control, co-stained with DAPI and TH, in cultures of E14 rat VM after 24 h in vitro. *Scale bar* = 50 µm

with either BMP2 or GDF5 for 4 DIV resulted in a significant increase in the neurite length of TH-positive neurons in E14 VM cultures, when compared to untreated controls (Fig. 3a, b).

BMPs are well-known activators of a canonical signalling pathway involving activation of Smad 1/5/8 (Miyazono et al. 2010; Sieber et al. 2009). Densitometric analysis of the nuclear levels of phospho-Smad 1/5/8 showed that both BMP2 and GDF5 significantly increased the amount of phospho-Smad 1/5/8 in the nucleus of TH-positive DA neurons at 30 and 120 min, compared to the untreated controls (0 min) (Fig. 3c–e). To determine whether this effect of GDF5 and BMP2 on Smad phosphorylation was specific to DA neurons, nuclear phospho-Smad levels were also measured in TH-negative cells. BMP2 did not induce Smad phosphorylation in these cells at any time point examined (Fig. 3c). Although GDF5 did not activate Smad phosphorylation in TH-negative cells at 30 min, it did so at 120 min (Fig. 3d). Using SH-SH5Y cells as a model of human DA neurons (Hegarty et al. 2013b), BMP2 and GDF5 were both shown to significantly increase Smad-mediated transcriptional activity (as measured by the

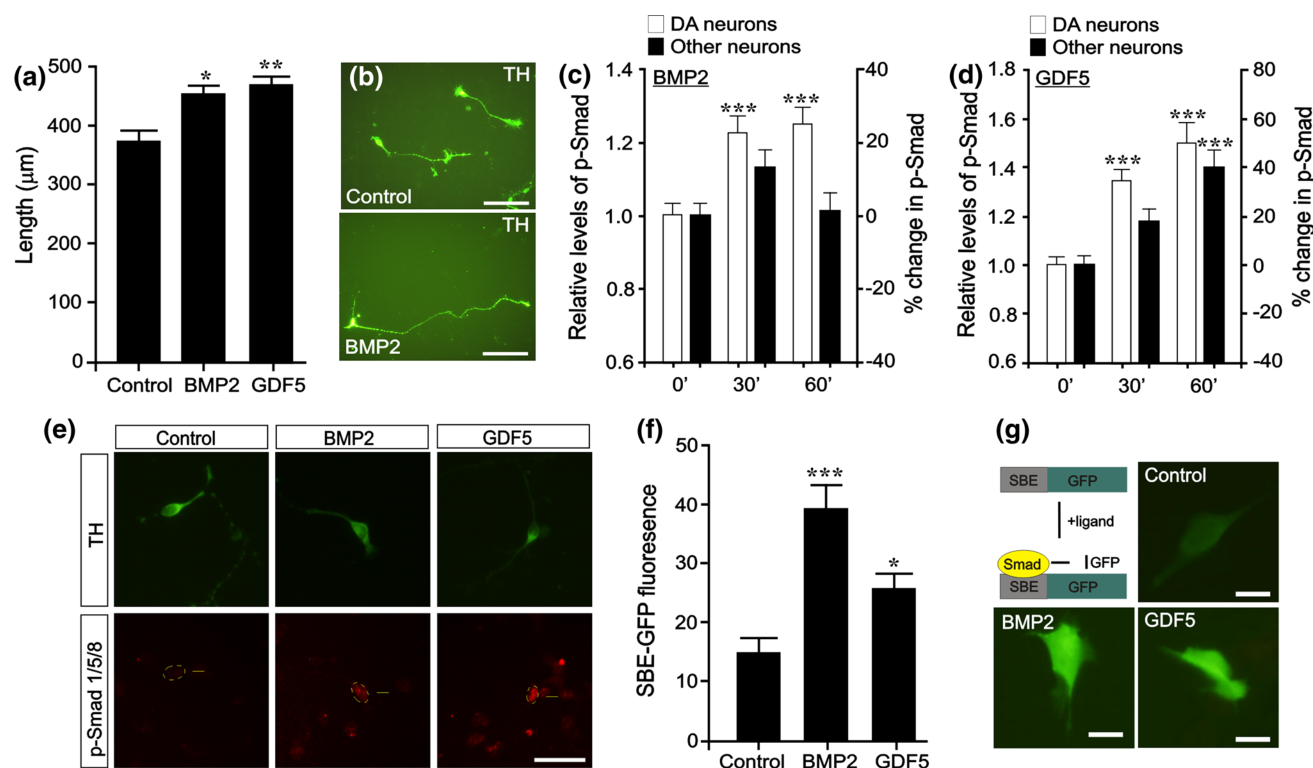

Fig. 3 BMP2 and GDF5 promote neurite growth and activate canonical Smad signalling in cultured DA neurons. **a** Total neurite length of BMP2- and GDF5-treated (10 ng/ml daily for 4 DIV) DA neurons in cultures of E14 rat VM. **b** Representative photomicrographs of control and BMP2-treated DA neurons in cultures of E14 rat VM at 4DIV, immunocytochemically stained for TH. *Scale bar* = 100 µm. **c**, **d** Densitometric analysis of phospho-Smad 1/5/8 in **c** BMP2- and **d** GDF5-treated DA neurons and non-DA neurons in E14 rat VM cultures at 0 (control), 30 and 60 min, as indicated. **e** Representative photomicrographs of phospho-Smad 1/5/8 immunostaining (*yellow arrow heads*), co-localised with TH immunostaining, in DA neurons treated BMP2 or GDF5 for 60 min in E14 rat VM cultures. *Scale bar* = 100 µm. **f** Smad-dependent transcriptional activity in BMP2- and GDF5-treated SH-SY5Y cells 48 h after transfection with a Smad-GFP reporter. **g** Photomicrographs showing increased Smad-GFP reporter fluorescence in SH-SH5Y cells treated with BMP2 or GDF5 for 2DIV. *Scale bar* = 10 µm. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control; ANOVA with post hoc Tukey's test; 50 cells analysed per group per experiment; $N = 3$ experiments)

relative levels of GFP expression) at 2 DIV in SH-SH5Y cells transfected with a Smad reporter plasmid (GFP under the control of a Smad responsive element), compared to the control (Fig. 3f, g). Collectively, these data show that BMP2 and GDF5 promote neurite growth from DA neurons in E14 VM cultures and activate the canonical Smad signalling pathway in these neurons.

BMPR Inhibitors Prevent BMP2- and GDF5-Induced Neurite Outgrowth in VM DA Neurons

To explore the possibility that the effects of BMP2 and GDF5 on the neurite outgrowth from E14 VM DA neurons are mediated through BMPR-dependent activation of the canonical Smad 1/5/8 pathway, two approaches were employed to inhibit BMP–BMPR signalling. Firstly noggin, an extracellular inhibitor of BMPs, which blocks their binding epitopes for BMPRs (Groppe et al. 2002; Smith and Harland 1992), and secondly dorsomorphin, a small molecular inhibitor of BMPRI (Yu et al. 2008), were used. It has previously been shown that dorsomorphin is an effective inhibitor of BMP2 and GDF5 signalling in SH-SY5Y cells (Hegarty et al. 2013b). The ability of noggin to inhibit BMP2 and GDF5 in these cells was assessed first, and pre-treatment with either noggin or dorsomorphin prevented BMP2- and GDF5-induced neurite growth in SH-SY5Y cells (Supplementary Fig. 2). Similarly, the pre-treatment of E14 VM cultures with noggin or dorsomorphin completely prevented the BMP2- and GDF5-induced increases in the neurite length of TH-positive cells at 4 DIV (Fig. 4a, b). It has been suggested that the neurotrophic effects of GDF5 on DA neurons may be mediated indirectly through the action of glial cell line-derived neurotrophic factor (GDNF) (Sullivan and O'Keeffe 2005). To test this possibility, we adopted a similar approach to Orme et al. (2013) who prevented the DA neurotrophic effects of GDNF by blocking its heparan sulphate-dependent signalling (Barnett et al. 2002; Iwase et al. 2005; Orme et al.

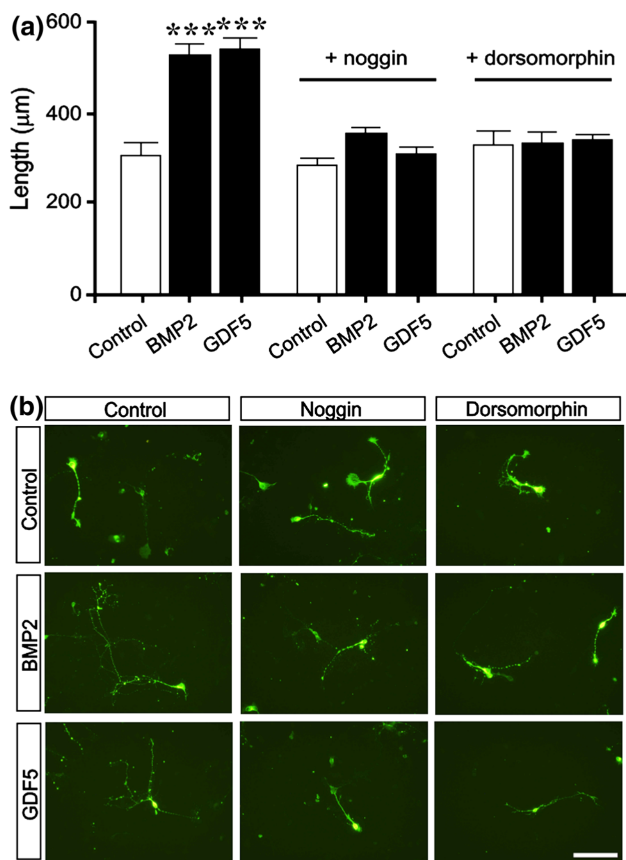

Fig. 4 Noggin and dorsomorphin prevent the promotion of DA neurite growth by BMP2 and GDF5. **a** Total neurite length of noggin- or dorsomorphin-pre-treated and/or BMP2- or GDF5-treated (daily for 4 DIV) DA neurons in E14 rat VM cultures, as indicated (*** $P < 0.001$ vs. control; ANOVA with post hoc Tukey's test; 50 TH-positive neurons analysed for each group per experiment; $N = 3$ experiments). **b** Representative photomicrographs of noggin- and dorsomorphin-pre-treated and/or BMP2- or GDF5-treated DA neurons in E14 rat VM cultures, immunocytochemically stained for TH. *Scale bar* = 100 µm. Data are expressed as mean ± SEM

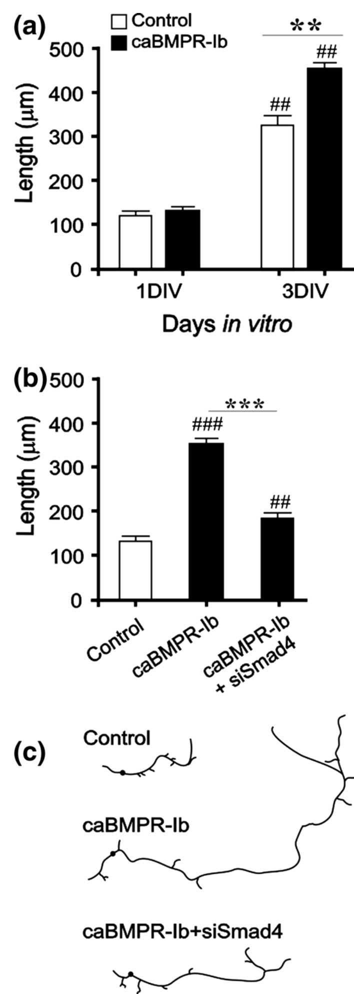

Fig. 5 Overexpression of constitutively active BMPRIb promotes neurite outgrowth in cultured VM neurons. **a** Neurite length of control- or caBMPRIb-transfected neurons in E14 rat VM cultures at 1 and 3 DIV, as indicated (** $P < 0.01$; ## $P < 0.01$ vs. 1 DIV; ANOVA with post hoc Tukey's; 40 cells for each group per experiment; $N = 3$ experiments). **b** Neurite length of control- or caBMPRIb-transfected neurons and/or co-transfected with a Smad4 siRNA expression vector in E14 rat VM cultures at 3 DIV, as indicated (*** $P < 0.001$; ## $P < 0.01$, ## $P < 0.001$ vs. control; ANOVA with post hoc Tukey's; 40 cells for each group per experiment; $N = 3$ experiments). **c** Representative line drawing of neurons from each of these groups at 3 DIV. All data are presented as mean ± SEM

2013). The pre-treatment of SH-SH5Y cells with Heparinase III did not affect BMP2- and GDF5-induced neurite growth in SH-SY5Y cells (Supplementary Fig. 3). Collectively, these data show that the neurite growth-promoting effects of BMP2 and GDF5 on VM DA neurons are directly mediated through a BMPR-dependent pathway.

Canonical BMPR–Smad Activation Promotes Neurite Outgrowth in VM DA Neurons

It is well established that BMP2 can signal through both BMPRIa and BMPRIb, whereas GDF5 predominantly signals through BMPRIb (Nishitoh et al. 1996), which suggests that BMP2 and GDF5 may signal through BMPRIb to exert their neurotrophic effects on VM DA neurons. To test this possibility, E14 VM neurons were transfected with a constitutively active BMPRIb (caBMPRIb) plasmid, and the neurite growth of the neurons was assessed and compared to that of neurons transfected with a control plasmid. Transfection of E14 VM neurons with the caBMPRIb plasmid induced a significant increase in their neurite length at 3 DIV, but not 1 DIV, when compared to cells transfected with the relevant control plasmid (Fig. 5a, c). Importantly, electroporation of E14 VM neurons with a wild-type BMPRIb plasmid did not result in significant increases in neurite length (data not shown), demonstrating

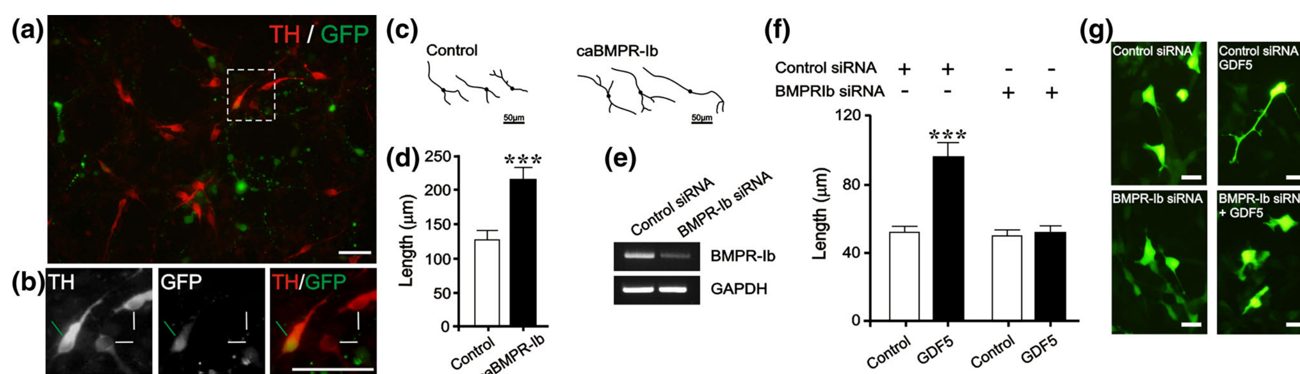

Fig. 6 Overexpression of constitutively active BMPRIb promotes neurite outgrowth in cultured DA neurons. **a** Photomicrograph of an E14 rat VM culture transfected with ca-BMPRIb (GFP-positive) at the time of plating, and immunocytochemically stained for TH at 3 DIV. **b** Higher magnification of the dashed area in (**a**), showing co-localisation of TH and GFP to identify transfected DA neurons. **c** Representative line drawing of control- or caBMPRIb-transfected DA neurons at 3DIV. *Scale bar* = 50 μm. **d** Neurite length of control- or caBMPRIb-transfected DA neurons in E14 rat VM cultures at 3 DIV, as indicated. **e** RT-PCR showing BMPRIb mRNA expression in SHSY5Y cells at 24 h following transfection with either a control or BMPRIb siRNA. **f** Neurite length and **g** representative photomicrographs of control siRNA and BMPRIb siRNA transfected SH-SY5Y cells with or without GDF5 treatment, as indicated. (***$P < 0.001$, vs. control; ANOVA with post hoc Tukey's; 30 cells for each group per experiment; $N = 3$ experiments). All data are presented as mean ± SEM

the importance of the activation of the BMPR for this effect.

To determine a functional link between BMPRIb-induced neurite growth and Smad 1/5/8 signalling, an siRNA that targets the co-Smad Smad4, which has been shown to be effective in inhibiting BMP2 and GDF5 signalling (Hegarty et al. 2013b), was used. The complex of phosphorylated Smad 1/5/8 with Smad4 following BMPRIb activation is required for the nuclear translocation of activated Smad 1/5/8 and thus their regulation of target gene expression (Miyazono et al. 2010; Sieber et al. 2009). To determine whether modulation of Smad4 expression affected the growth of E14 VM neurons, the neurite length of cells transfected with Smad4 siRNA or with Smad4 overexpression vectors was measured. Modulation of Smad4 expression did not affect the neurite length of transfected E14 VM neurons (data not shown). When E14 VM cells were co-transfected with the caBMPRIb and Smad4 siRNA, Smad4 siRNA significantly reduced the caBMPRIb promotion of E14 VM neuronal growth (Fig. 5b, c). These data show that the activation of the Smad signalling pathway by BMPRIb mimics the neurite growth-promoting effects of BMP2 and GDF5 in E14 VM neurons. To ensure that this effect was specific to DA neurons, we immunostained the electroporated neurons at 3 DIV for TH. This allowed the identification of TH-positive/GFP-positive DA neurons, indicating that they were transfected (Fig. 6a, b). Traces of the TH-positive/GFP-positive DA neurons were prepared for the analysis of neuronal growth (Fig. 6c), which showed that DA neurons expressing caBMPRIb had significantly longer neurites than their control counterparts (Fig. 6d). Finally, to further demonstrate the requirement of the BMPRIb for the neurite growth-promoting effects of the BMP ligands, an siRNA against BMPRIb was employed, which induced efficient BMPRIb knockdown (Fig. 6e). The ability of GDF5 to promote growth in cells transfected with either a control siRNA or the BMPRIb siRNA was then investigated. GDF5 promoted a significant increase in neurite length in cells expressing the control siRNA, whereas this effect was lost in cells expressing the BMPRIb siRNA (Fig. 6f, g). Taken together, these data show that the activation of canonical BMP–BMPRIb–Smad 1/5/8 signalling promotes neurite outgrowth in VM DA neurons.

Discussion

Understanding the molecular signals that regulate the development of DA neurons is crucial for advancing cell replacement therapy for PD (Toulouse and Sullivan 2008; Lees et al. 2009). While much progress has been made in understanding the signals that control DA neuron development, less is known about the molecules that promote the growth of DA neurites, which is crucial for the functional integration of transplanted cells into the host parenchyma. However, some molecules, such as Ephs and netrin1, have been identified as regulators of nigrostriatal pathway development in recent years (Hegarty et al. 2013a; Van den Heuvel and Pasterkamp 2008). In an attempt to identify new candidate molecules and signalling pathways that may be involved in nigrostriatal development, this study focused on two BMPs, GDF5 and BMP2, since both of these factors have been implicated in axonal growth in

other NS populations (Parikh et al. 2011; Hazen et al. 2011, 2012; Phan et al. 2010; Niere et al. 2006; Lein et al. 1995; Hegarty et al. 2013a) and have been shown to have neurotrophic effects on VM DA neurons, specifically survival- and neurite growth-promoting effects (O'Keeffe et al. 2004a; Reiriz et al. 1999; Jordan et al. 1997; Sullivan et al. 1997; Hegarty et al. 2014). Despite these studies, the downstream molecular mechanisms that mediate the effects of GDF5 and BMP2 on VM DA neurons are unknown. The present study thus aimed to define these molecular mechanisms and to investigate the potential of BMP2 and GDF5 as regulators of nigrostriatal development.

To investigate this proposed role of BMP2 and GDF5 in the neurite growth of DA neurons, this study first characterised the temporal expression profiles of their receptors in the rat VM and striatum during embryonic and post-natal development. In the rat, the axons of the DA neurons in the VM extend towards the forebrain via the medial forebrain bundle from E13, and progressively innervate the striatum shortly thereafter, reaching the dorsal striatum around E20 (Gates et al. 2004; Nakamura et al. 2000; Specht et al. 1981a, b; Verney 1999; Voorn et al. 1988). In the first three post-natal weeks, striatal innervation becomes more extensive, while naturally occurring cell death refines these connections (Jackson-Lewis et al. 2000; Oo and Burke 1997; Burke 2003; Hegarty et al. 2013a; Van den Heuvel and Pasterkamp 2008). This study found that BMPRII and BMPRIb were expressed at steady levels in the VM throughout embryonic development (from E14) and into adulthood (until at least P90), with strong expression levels being detected on DA neurons in the P56 SNpc. Crucially, the expression of these BMPRs, both of which are required for canonical BMP–Smad signalling (Miyazono et al. 2010; Sieber et al. 2009), in the VM from E14 onwards correlates with the timing of the generation of nigrostriatal projections. These data suggest that BMPs, such as BMP2 and GDF5 that are expressed in the developing and adult VM and striatum (Krieglstein et al. 1995; O'Keeffe et al. 2004b; Storm et al. 1994; Chen et al. 2003; Jordan et al. 1997; Soderstrom and Ebendal 1999; Hegarty et al. 2014), may regulate the establishment of nigrostriatal projections from VM DA neurons. In support of this suggestion, the present study has demonstrated that both BMP2 and GDF5 promote neurite outgrowth from E14 VM neurons in culture. BMP2 and GDF5 may also act to orientate the axons of VM DA neurons away from the VM, since other BMPs, such as BMP7 and GDF7, have been shown to orient the commissural axons of dorsal SC interneurons via BMPRIb (Butler and Dodd 2003; Dent et al. 2011; Phan et al. 2010; Yamauchi et al. 2008; Wen et al. 2007). The sustained expression of BMPRs in the VM during adulthood suggests that they may function in the maintenance of DA neurons, with both BMP2 and GDF5 being shown to promote the survival of VM DA neurons in vitro (O'Keeffe et al. 2004a; Wood et al. 2005; Reiriz et al. 1999; Jordan et al. 1997) and in vivo (Sullivan et al. 1997, 1998, 1999; Hurley et al. 2004; O'Sullivan et al. 2010; Espejo et al. 1999). This study also demonstrated the expression of these BMPRs from E11 to E14 in the rat VM, further supporting their role in DA axogenesis, but also suggesting that BMPs may function in adoption of a DA phenotype during DA neurogenesis, which occurs during this period (Lumsden and Krumlauf 1996; Lauder and Bloom 1974; Gates et al. 2006; Hegarty et al. 2013c). In agreement with this proposal, BMP–BMPR–Smad-dependent transcriptional activity is found in the VM region during DA neurogenesis at E10.5 in mice (Monteiro et al. 2008), which also corresponds to the time of DA axon extension. BMP–Smad signalling may therefore concomitantly contribute to VM DA neuronal specification and their subsequent neurite outgrowth, which is the case for BMPs in the dorsal SC (Chizhikov and Millen 2005; Ulloa and Briscoe 2007).

In the striatum, there is a peak of BMPRII mRNA expression at P11, during the time period (P0–P20) when naturally occurring cell death is occurring due to limitations in the availability of target-derived neurotrophic factors (Jackson-Lewis et al. 2000; Oo and Burke 1997; Burke 2003; Van den Heuvel and Pasterkamp 2008). Similarly, BMPRIb is also expressed at relatively high levels in the early post-natal (P1 to P11) striatum. These data suggest that BMP2 and GDF5 may function as target-derived neurotrophic factors for VM DA neurons at this stage of development. Indeed, both factors have been shown to promote the survival of VM DA neurons (O'Keeffe et al. 2004a; Wood et al. 2005; Reiriz et al. 1999; Jordan et al. 1997; Sullivan et al. 1997, 1998, 1999; Hurley et al. 2004; O'Sullivan et al. 2010; Espejo et al. 1999). Furthermore, BMPRII null mice have reductions in nigrostriatal neurons, and striatal DA innervation, when examined in adulthood (Chou et al. 2008), which is likely due to deficient neurotrophic support during this post-natal developmental period. There is a peak of BMPRIb expression during adulthood in the striatum, which may point towards the aforementioned potential role of BMPs in the maintenance of VM DA neurons. Furthermore, it may suggest that BMPRIb functions in promoting the arborisation of DA axons that survive the period of naturally occurring cell death. The sustained expression of BMPRs in the adult rat brain (up to P90) demonstrated in this study suggests a role for BMP2 and GDF5 in the maintenance of the nigrostriatal system during adulthood. In support of this role, BMPs (including BMP2) and BMPRs have been shown to be expressed in the midbrain and striatum from 6–24 months in the adult rat (Chen et al. 2003). Furthermore, in animal models of PD, exogenous GDF5 delivery into the

nigrostriatal pathway has potent survival-promoting effects on adult nigral DA neurons (Sullivan et al. 1997, 1999; Hurley et al. 2004; O'Sullivan et al. 2010; Sullivan and Toulouse 2011). Disruption to the normal expression of BMPRs may thus render nigrostriatal DA neurons more vulnerable to degeneration and increase the risk of the development of PD. The phenotype of the BMPRII null mouse supports this suggestion, while haploinsufficiency of other transforming growth factor (TGF)β superfamily members, such as GDNF and TGFβ2, causes an accelerated decline of midbrain DA neurons during normal ageing (Boger et al. 2006; Andrews et al. 2006). Interestingly, after a 6-hydroxydopamine (6-OHDA)-induced lesion of the adult rat nigrostriatal pathway, BMPRs were significantly downregulated in the nigra, but upregulated in the striatum (Chen et al. 2003). These findings likely reflect the loss of BMPR expression by nigral DA neurons, which are destroyed by 6-OHDA, and a potential compensatory mechanism by the striatum to restore BMP-mediated survival-promoting effects on innervating VM DA neurons through upregulation of BMPR expression. The BMPR expression in the developing striatum may also reflect autocrine or paracrine trophic influences on cells within the striatum, since the BMPs have been shown to play roles in striatal neuronal development (Gratacos et al. 2001, 2002).

The present study found that TH mRNA levels in the VM are maximal at E14, which is expected as this is the time point at which the greatest amount of post-mitotic DA neurons are present in the VM (Lumsden and Krumlauf 1996; Lauder and Bloom 1974; Gates et al. 2006). There was a subsequent significant decline in TH expression from birth onwards, reaching the lowest levels at P11, which correlates with the onset of programmed cell death for nigrostriatal DA neurons. TH mRNA expression was found to remain stable in the adult VM, reflecting the established population of A9 DA neurons.

Following the demonstration of the expression of BMPRs in the VM and striatal regions during embryonic and post-natal development, we next demonstrated that BMPRs are expressed on both DA and non-DA cells in E14 rat VM cultures, indicating that BMP2 and GDF5 may act in either an autocrine or paracrine manner to exert neurotrophic effects on DA neurons. Immunocytochemical staining for phospho-Smad 1/5/8 showed that both DA and non-DA cells express these transcription factors, and the nuclear location of phospho-Smad 1/5/8 indicated that these VM cells also express Smad4, which is required for the nuclear translocation of Smad 1/5/8 following their activation. These results demonstrate that VM DA neurons have the machinery to carry out canonical Smad 1/5/8 signalling in response to BMPs.

The current study has demonstrated that both BMP2 and GDF5 induce the neurite outgrowth of E14 VM DA neurons, which is consistent with previous studies on BMP2 (Reiriz et al. 1999) and GDF5 (O'Keeffe et al. 2004a) in rat VM cultures. The molecular mechanisms mediating this neurite growth-promoting effect were then assessed. BMP2 and GDF5 were both shown to activate canonical Smad 1/5/8 in VM DA neurons, as demonstrated by nuclear accumulation of phosphorylated Smad 1/5/8. Interestingly, GDF5, but not BMP2, activated Smad 1/5/8 signalling in non-DA cells. This finding is not surprising considering that the numbers of astrocytes are dramatically increased in GDF5-treated E14 rat VM cultures (Krieglstein et al. 1995; O'Keeffe et al. 2004a; Wood et al. 2005). It has thus been suggested that GDF5 may have an indirect neurotrophic action on VM DA neurons, possibly by stimulating glial-derived growth factor(s) production, such as GDNF, that might be involved in the neurotrophic response (Sullivan and O'Keeffe 2005). To test this possibility, this study investigated whether GDF5 and BMP2 were capable of promoting neurite growth in the absence of heparan sulphate-dependent GDNF signalling and showed that GDF5 and BMP2 did not require GDNF for this effect. Similarly, Wood et al. (2005) showed that the inhibition of the GDF5-induced increase in astrocytes does not prevent the neurotrophic effects of GDF5 on DA neurons in E14 rat VM cultures, suggesting that GDF5 has a direct neuronal action. Similarly, Reiriz et al. (1999) used the gliotoxin α-aminoadipic acid to demonstrate that the neurotrophic effects of BMP2 on E14 rat VM DA neurons were not mediated by astrocytes. These data, along with the present finding that BMP2 specifically activates Smad signalling in VM DA neurons, suggest that BMP2 and GDF5 act directly on DA neurons to induce axonal growth. The neurotrophic and gliogenic effects of GDF5 in VM cultures may thus be independent of one another. Similarly, BMP–Smad signalling has previously been shown to have such a dual-inductive role in enteric neural crest cells (Chalazonitis et al. 2004, 2011; Chalazonitis and Kessler 2012). Collectively, these data suggest that canonical Smad signalling mediates the neurotrophic effects of BMP2 and GDF5 on VM DA neurons.

To explore this premise further, the effects of BMP2 and GDF5 were assessed following the inhibition of their binding to BMPRs. BMPR activation by BMP2 and GDF5 was blocked by using noggin, an extracellular inhibitor of BMPs, which blocks their binding epitopes for BMPRs (Groppe et al. 2002; Smith and Harland 1992), or dorsomorphin, a small molecular inhibitor of BMPRI (Yu et al. 2008). Pre-treatment with either noggin or dorsomorphin inhibited the neurite growth-promoting effects of BMP2 and GDF5 on E14 VM DA neurons. Noggin and dorsomorphin have both previously been used to prevent BMP-induced neurite outgrowth in other neuronal populations (Parikh et al. 2011; Li and LoTurco 2000), and the current

study also demonstrated their inhibition of BMP-induced neurite growth in SH-SH5Y cells. BMPR activation is therefore crucial to BMP-induced axonal growth from VM DA neurons. BMP2 can signal through both BMPRIa and BMPRIb, whereas GDF5 predominantly signals through BMPRIb (Nishitoh et al. 1996), suggesting that BMPRIb is responsible for mediating the neurotrophic effects of BMP2 and GDF5. To test this hypothesis, E14 VM cultures were electroporated with a constitutively active BMPRIb, which has been previously shown to activate the Smad 1/5/8 signalling pathway (Hegarty et al. 2013b). E14 VM neurons expressing the caBMPRIb were significantly larger than those transfected with the control plasmid, suggesting that BMP2 and GDF5 activate BMPRIb to induce neurite extension. These findings are in agreement with a previous study in SH-SH5Y cells, a model of human DA neurons (Hegarty et al. 2013b). Furthermore, the application of GDF5 at the time of plating, when BMPR1b is expressed, results in neurotrophic effects on VM DA neurons; however, application after six days in vitro, when the BMPRIb is no longer expressed, has no effect (O'Keeffe et al. 2004a). The present study next demonstrated that the transcriptional activity of Smad 1/5/8 is required for this BMP-induced neurite growth of VM neurons. The inhibition of the nuclear translocation of the Smad 1/5/8 transcription factors, using siRNA to target Smad4, significantly inhibited neurite outgrowth of E14 VM neurons induced by caBMPRIb. Finally, this study confirmed that the neurite growth-promoting effects of the caBMPRIb are specific for VM DA neurons. The caBMPRIb therefore mimics the effects of BMP2 and GDF5 on E14 VM DA neurons. Furthermore, siRNA knockdown of the BMPRIb also prevented GDF5-induced neurite growth in SH-SH5Y cells. Collectively, these data show that BMPRIb activation of Smad 1/5/8 is the mechanism by which these BMPs promote the neurite growth of VM DA neurons.

This study has identified BMP2 and GDF5 as bona fide candidates to be regulators of nigrostriatal pathway development. The expression profiles of their BMPRs in the VM and striatum, and their neurotrophic effects on cultured VM DA neurons, propose roles for BMP2 and GDF5 in the extension/projection of DA axons from the VM. They may act as target-derived neurotrophic factors for innervating nigrostriatal fibres, and/or as factors that maintain the integrity of nigrostriatal projections during adulthood. However, the analysis of mice with deficiencies in GDF5 and/or BMP2 is essential to further establish these factors as regulators of nigrostriatal pathway development. It is not unlikely that these morphogens may play multiple roles during nigrostriatal system development, since locally expressed factors are employed throughout NS development to regulate multiple steps of particular developmental processes, with temporally regulated functions. A relevant example of this is seen during chick dorsal SC development, in which BMP–Smad signalling promotes neuronal specification rather than astrocytic specification at E5, but at E6 has the opposite effect (Agius et al. 2010). The present study has thus contributed to the growing body of knowledge regarding the development of the A9 pathway. A detailed, well-characterised understanding of nigrostriatal pathway development is vital, to provide important information regarding developmental abnormalities or age-related defects that may lead to the progressive degeneration of this pathway in PD. Furthermore, cell replacement therapy is one of the most promising therapies for the treatment for PD (Orlacchio et al. 2010; Bonnamain et al. 2012; De Feo et al. 2012; Toulouse and Sullivan 2008; Hedlund and Perlmann 2009). Due to the importance of the establishment of functional connections by transplanted DA cells in the host striatum, factors that promote neurite outgrowth are being considered as adjuncts to transplantation therapy. GDF5 and BMP2 are thus ideal candidates to be used as growth-promoting factors, with their survival-promoting effects on VM DA neurons being beneficial also. The present study has, for the first time, demonstrated that the downstream molecular mechanisms mediating the neurite outgrowth-promoting effects of GDF5 in VM DA neurons are dependent, upon BMPRIb-mediated activation of canonical Smad 1/5/8 signalling.

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Conflict of interest The authors declare that there are no conflicts of interest.

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Roles for the TGFβ Superfamily in the Development and Survival of Midbrain Dopaminergic Neurons

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Abstract The adult midbrain contains 75 % of all dopaminergic neurons in the CNS. Within the midbrain, these neurons are divided into three anatomically and functionally distinct clusters termed A8, A9 and A10. The A9 group plays a functionally non-redundant role in the control of voluntary movement, which is highlighted by the motor syndrome that results from their progressive degeneration in the neurodegenerative disorder, Parkinson's disease. Despite 50 years of investigation, treatment for Parkinson's disease remains symptomatic, but an intensive research effort has proposed delivering neurotrophic factors to the brain to protect the remaining dopaminergic neurons, or using these neurotrophic factors to differentiate dopaminergic neurons from stem cell sources for cell transplantation. Most neurotrophic factors studied in this context have been members of the transforming growth factor β (TGFβ) superfamily. In recent years, an intensive research effort has focused on understanding the function of these proteins in midbrain dopaminergic neuron development and their role in the molecular architecture that regulates the development of this brain region, with the goal of applying this knowledge to develop novel therapies for Parkinson's disease. In this review, the current evidence showing that TGFβ superfamily members play critical roles in the regulation of midbrain dopaminergic neuron induction, differentiation, target innervation and survival during embryonic and postnatal development is analysed, and the implications of these findings are discussed.

Keywords TGFβ superfamily · BMP family · Midbrain dopaminergic neurons · Development · Neurogenesis

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Abbreviations

6-OHDA	6-Hydroxydopamine
ALK	Activin receptor-like kinases
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
BMPR	BMP receptors
Co-Smads	Common mediator Smads
DA	Dopaminergic/dopamine
E	Embryonic day
FGF	Fibroblast growth factor
GDF	Growth/differentiation factor
GDNF	Glial cell line-derived neurotrophic factor
I-Smads	Inhibitory Smads
MPP+	1-Methyl-4-phenylpyridinium ion
P	Postnatal day
PD	Parkinson's disease
R-Smads	Receptor-regulated Smads
Shh	Sonic hedgehog
TGFβ	Transforming growth factor β
TH	Tyrosine hydroxylase
VM	Ventral midbrain/mesencephalon

Introduction

Discrete populations of dopaminergic neurons in the ventral midbrain are crucial to the normal functioning of the human brain. Three functionally and anatomically distinct clusters of dopaminergic (DA) neurons, termed the A8, A9 and A10 groups, arise from the ventral midbrain/mesencephalon (VM) floor plate during embryonic development [1]. The A9 cluster of DA neurons, which gives rise to the substantia

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nigra pars compacta, projects to the dorsal striatum via the nigrostriatal pathway to regulate voluntary motor control, while the A8 and A10 groups, which develop into the ventral tegmental area and the retrorubal field, respectively, innervate the ventral striatum and prefrontal cortex via the mesocorticolimbic system to regulate emotion and reward [2, 3]. The importance of these VM DA populations is highlighted in disorders in which their neurotransmission is altered, impaired or gradually lost. The progressive degeneration of A9 DA neurons results in the motor syndrome of Parkinson's disease (PD) [4, 5], while defective functioning of the mesocorticolimbic system has been shown to contribute to the development of schizophrenia, drug addiction and depression [6, 7].

PD is the second most common neurodegenerative disorder, affecting 0.5 to 1 % of the population aged 65–69 years of age and 1 to 3 % of the population over 80 years of age [8]. Many strategies have been proposed to halt or even reverse the DA neuronal loss in PD, and one proposed strategy that has received intense focus in recent years is the application of neurotrophic factors to protect the remaining DA neurons. Since the discovery of the best-known DA neurotrophic factor, glial cell line-derived neurotrophic factor (GDNF), in 1993, it is now known that many DA neurotrophic factors are members of the transforming growth factor β (TGFβ) superfamily of proteins. While some members of this family of proteins, including GDNF, have been used in clinical trials in PD, work in recent years has established that they also play key roles in DA neuron development. The clinical use of these factors in PD has been extensively discussed in recent reviews [9, 10]. In this review, the available evidence that suggests that members of the TGFβ superfamily are critical regulators of midbrain DA neuron induction, differentiation, target innervation and survival during embryonic and postnatal development will be described.

The TGFβ Superfamily

The TGFβ superfamily is grouped into subfamilies based on sequence homology, which include the TGFβ, activin, GDNF, growth/differentiation factor (GDF) and bone morphogenetic protein (BMP) subfamilies [11, 12]. TGFβ superfamily members are dimeric, structurally conserved proteins, that have pleiotropic functions in vitro and in vivo [13]. They elicit their cellular responses by binding to two distinct types of serine/threonine kinase cell surface receptors, both of which are required for signal transduction [14, 15]. There are seven known type I receptors, the activin receptor-like kinases (ALK) 1–7, and five type II receptors. Upon ligand binding, the constitutively active type II receptor phosphorylates the type I receptor. The activated type I receptor subsequently phosphorylates receptor-regulated Smads (R-Smads), which then form a heterocomplex with the common mediator Smad (co-Smad) that mediates Smad nuclear translocation to allow the Smad transcription factors to regulate target gene expression. TGFβs, including TGFβ1–3, GDNF, activin and nodal, signal via ALK1, ALK2, ALK4, ALK5 and ALK7, while BMPs signal via ALK1, ALK3 (also known as BMPRIa) and ALK6 (also known as BMPRIb) [16-20].

The major signalling molecules acting downstream from the serine/threonine kinase receptors are the Smad proteins [21]. The R-Smads for the BMP family are Smad1, Smad5 and Smad8, while Smad2 and Smad3 mainly mediate the effects of TGFβs [20, 21]; however, TGFβ signalling via ALK1 is known to activate Smad1 [22]. The co-Smad, Smad 4, is a shared component of the two Smad signalling pathways. Similarly, the inhibitory Smads (I-Smads), Smad6 and Smad7, negatively regulate R-Smad/co-Smad signalling of both pathways [23-25].

The TGFβ Superfamily in Midbrain DA Neuronal Development

There is a wealth of evidence suggesting that TGFβ superfamily members regulate midbrain DA neuronal development. However, the majority of this research has focused on their survival-promoting abilities, rather than potential roles in development, with the goal of using these factors therapeutically for PD. Such research is best highlighted by that carried out on the GDNF family (Table 1), two of which have been used in clinical trials [9, 10], and this TGFβ subfamily will therefore be discussed first in this review.

The GDNF Family

The GDNF family is composed of four members—GDNF, neurturin, persephin and artemin. GDNF, the prototypical member of this subfamily, was isolated from a glial cell line following the demonstration of its neurotrophic effects on cultured DA neurons [26]. GDNF has consistently been shown to promote the survival, and reduce apoptosis, of cultured embryonic VM DA neurons [26-31]. A vitamin D_3 metabolite, calcitriol, has recently been shown to promote the survival of midbrain DA neurons in vitro through the upregulation of endogenous GDNF [32]. Interestingly, a single dose of GDNF selectively enhances the survival of A9 DA neurons, while only repeated exposure of this factor increases the survival of A10 cells in embryonic day (E)14 VM cultures [33], suggesting that nigrostriatal DA neurons are more sensitive to the effects of GDNF. GDNF has consistently been shown to improve the survival of embryonic DA neurons in VM transplants to the adult rodent striatum [34-39]. Of more physiological relevance, GDNF has also been shown to inhibit the apoptotic death of postnatal midbrain DA neurons in vitro

Table 1 Effects of GDNF family on midbrain DA neurons

Effect	Reference(s)
GDNF	
In vitro	
Promotes survival and reduces apoptosis of embryonic DA neurons	[26-32]
Promotes survival and reduces apoptosis of postnatal DA neurons	[39]
Protects DA neurons from MPP + and 6-OHDA	[45, 46]
Protects DA neurons from lipopolysaccharide-induced neurotoxicity	[58]
Induces Nurr1 and Pitx3 expression in VM neural precursors	[63-65]
Induces TH expression	[71]
Induces neurite growth of embryonic DA neurons	[26, 28]
Enhances synaptogenesis of postnatal DA neurons	[79]
In vivo	
Promotes survival of embryonic DA neurons in VM transplants to adult striatum	[34-39]
Promotes survival of postnatal midbrain DA neurons	[40, 41]
Promotes survival of adult DA neurons in animal models of PD	[47-53]
Induces neurite growth of postnatal midbrain DA neurons	[40, 41]
Induces re-innervation of the lesioned striatum by midbrain DA afferents	[77, 78]
Increases neurite growth from DA neurons in VM transplants to adult striatum	[35]
Increases levels of DA and its metabolites in the striatum and midbrain	[80]
Long-lasting protective action on nigrostriatal DA neurons during aging	[81]
Neurturin, persephin and artemin	
In vitro	
Promote survival of embryonic DA neurons	[83, 84, 93, 94]
Promote neurite growth of embryonic DA neurons	[97]
In vivo	
Promote survival of adult DA neurons in animal models of PD	[85-89, 95, 96]

[39]. Furthermore, two in vivo studies demonstrated that GDNF functions to promote the survival of midbrain DA neurons during their period of naturally occurring cell death [40, 41]. These findings suggest that GDNF functions to regulate the survival of VM DA neurons during their development, particularly as a target-derived neurotrophic factor. In support of such a suggestion, GDNF is expressed in the developing and adult rat midbrain and striatum [42, 43], which indicates functioning by endogenous GDNF. Interestingly, the activation of the dopamine D2 receptor has recently been shown to upregulate GDNF expression in the postnatal rat midbrain [44], suggesting that activity-dependent GDNF neurotrophic support is important during nigrostriatal pathway development. GDNF has been shown to protect VM DA neurons from the DA toxins, 1-methyl-4-phenylpyridinium ion (MPP+) and 6-hydroxydopamine (6-OHDA), in vitro [45, 46], and in animal models of PD [47-52]. Interestingly, the survival-promoting effect of GDNF in these models is significantly greater in younger rats (3 and 18 months) when compared to older rats (24 months) [53], with the greatest effect seen in 3-month-old rats at a time when the nigrostriatal pathway is still developing [54]. These findings led to clinical trials which delivered GDNF into the striatum of PD patients, but these had varying degrees of success [55-57]. A more developmentally relevant protective role of GDNF was recently demonstrated, in a study showing that GDNF protected cultured VM DA neurons from lipopolysaccharide-induced degeneration, a model of neuroinflammation [58]. This suggests that GDNF maintains DA neuronal integrity in occasions of increased brain inflammation. Furthermore, inflammation has consistently been suggested to contribute to the pathogenesis of PD [59-61].

There is evidence to suggest that GDNF may play a role in inducing a DA phenotype during midbrain DA neurogenesis. A recent paper showed that GDNF is capable of inducing the expression of both Nurr1 and Pitx3, two key genes in DA specification [62], in neural precursors isolated from the VM during the time of DA neurogenesis [63]. Similarly, in cultures of E12 rat VM neural precursors, GDNF significantly increased the number of cells expressing both Nurr1 and Pitx3 [64]. In support of these findings, GDNF has been shown to induce Pitx3 expression in the murine VM, with Pitx3 mediating GDNF-induced brain-derived neurotrophic factor (BDNF) expression in A9 nigrostriatal DA neurons [65]. This GDNF-Pitx3-BDNF feed-forward regulation may explain the specific sensitivity of the A9 subgroup of midbrain DA neurons to GDNF, with Pitx3 also being crucial for the survival and maintenance of nigrostriatal DA neurons [66-69]. Interestingly, Nurr1 has been shown to regulate the expression of the GDNF receptor, cRet, the expression of which is lost in Nurr1-deficient animals [70]. This suggests that GDNF and Nurr1 may function in an autoregulatory loop during VM DA neurogenesis, a mechanism which is not uncommon during this developmental process [62]. GDNF has been shown to induce expression of the dopamine synthetic enzyme, tyrosine hydroxylase (TH), in fetal human and rat cortical cultures [71], which suggests that it may play a similar role during VM DA neurogenesis. In support of such a proposal, Pitx3 has been suggested to induce TH expression in nigrostriatal DA neurons [62], which indicates that Pitx3 may mediate GDNF-induced TH expression in a similar fashion to its mediation of GDNF-induced BDNF expression. Despite these findings, and the proposed roles for GDNF in the induction of a DA phenotype, GDNF null mice display no severe defects in midbrain DA neurons during embryonic development [72-74] (Table 5), demonstrating that GDNF is not essential for VM DA neurogenesis. However, these mice die perinatally [72-74], prohibiting the investigation of GDNF deficits on

postnatal nigrostriatal pathway development. Granholm et al. [41] circumvented this issue by transplanting GDNF null VM tissue into the adult wild-type mouse brain to demonstrate that GDNF is required for the postnatal survival and neuritic growth of midbrain DA neurons.

GDNF has been shown to induce neurite growth from cultured rat VM DA neurons at E14 [26, 28], the time point at which midbrain DA neurons are extending their axons from the VM in vivo [75, 76]. These data suggest that GDNF may regulate DA axogenesis in the VM. This neurite growth-promoting role of GDNF on midbrain DA neurons was also demonstrated postnatally in vivo [40, 41], suggesting that GDNF may function in the neurite arborisation of DA axons once they have reached their targets, in which GDNF is expressed [42, 43]. In support of such a role, GDNF administration to the adult rat striatum following intrastriatal 6-OHDA lesion causes the re-innervation of the striatum by midbrain DA afferents [77]. Similarly, GDNF is required for the sprouting of nigrostriatal fibers following striatal injury [78], while GDNF has also been shown to increase neurite growth from DA neurons in VM transplants to the striatum [35]. Furthermore, Bourque and Trudeau [79] have shown that GDNF enhances the synaptogenesis of cultured postnatal midbrain DA neurons. In normal adult rats, a single injection of GDNF into either the substantia nigra or striatum significantly increases the levels of DA and its metabolites in the striatum and midbrain [80], which may reflect the findings of Bourque and Trudeau [79]. These findings suggest a role for GDNF in the maintenance of midbrain DA neurons in adulthood. Indeed, chronic striatal administration of GDNF in aged monkeys has a long-lasting protective action on nigrostriatal DA neurons [81]. Furthermore, $GDNF^{+/-}$ heterozygous mice show an accelerated decline of nigrostriatal DA neurons during aging, which leads to functional motor deficits [82] (Table 5).

A naturally occurring analog of GDNF, neurturin, has been shown to be equally as a potent as GDNF at promoting the survival of midbrain DA neurons in vitro [83, 84]. However, neurturin was found not to share the neuritogenic effects of GDNF [84]. These findings suggest that neurturin shares the roles of GDNF in the survival and maintenance of VM DA neurons during embryonic and postnatal development, which is supported by the expression of neurturin in the VM and striatum during development [83]. Like GDNF, neurturin protects VM DA neurons in animal models of PD [85-89] and is now in clinical trials [90-92]. The other two members of the GDNF family, persephin and artemin, have also been shown to promote the survival of midbrain DA neurons in vitro [93, 94] and in vivo [95, 96], suggesting that the GDNF family may share protective roles in the developing nigrostriatal system. However, GDNF and neurturin have been demonstrated to have differential effects on VM DA neurons in vivo [86]. In contrast to the findings of Akerud at al. [84], neurturin, as well as persephin and artemin, has recently been shown to promote the neurite growth of cultured midbrain DA neurons [97]. These findings suggest roles for these factors in the formation of the nigrostriatal pathway during development. However, mice with null mutations of neurturin [98], persephin [99] or artemin [100] are viable and lack severe deficits in midbrain DA neurons (Table 5). These GDNF family ligands are therefore not essential for the development of the nigrostriatal system. However, it may be the case that in the absence of one of these GDNF family ligands, the other family members compensate functionally during midbrain DA development. The phenotypic analysis of double or treble mutants of the GDNF family ligands would address this possibility of functional redundancy.

TGFβs

The TGFβs, which include TGFβ1, TGFβ2 and TGFβ3 isoforms, have been shown to be essential co-factors for the neuroprotective effects of GDNF on midbrain DA neurons. The application of antibodies neutralizing TGFβ isoforms abolishes the survival-promoting effects of GDNF on midbrain DA neurons in vitro [101] and in vivo [102], suggesting that the effects of GDNF are dependent on TGFβs. It has been reported that TGFβ is required for the recruitment of the GDNF receptor, GFRα1, to the plasma membrane in primary neuron cultures [103], which may explain the requirement for TGFβ in the DA neurotrophic effects of GDNF. The cooperative functioning of TGFβ and GDNF has been highlighted in vivo by their co-storage in the secretory vesicles of a model neuron, the chromaffin cell, and the co-localisation of their receptors on GDNF-responsive neuronal populations [101]. TGFβs are known to be expressed in the floor plate and notochord during development [104, 105]. They have been shown to be expressed in the ventral midbrain during DA neurogenesis [106, 107], and their expression significantly increases in the striatum following MPP+lesion [102], suggesting physiological roles for these factors, both during DA development and in response to neurotoxic insult. Indeed, TGFβs have been shown to have survival-promoting and neuroprotective effects (against MPP+) on cultured midbrain DA neurons; these effects are not mediated by astroglia or by increases in cell proliferation [27, 106]. Furthermore, there is evidence to suggest that TGFβs, specifically TGFβ1, regulate the neurite growth of midbrain DA neurons [108]. It is unclear whether TGFβs achieve these effects directly, or function to sensitise midbrain DA neurons to the survival- and growth-promoting effects of endogenous GDNF.

TGFβs have been identified as important mediators in the induction of midbrain DA neurons (Table 2). It has been consistently shown that treatment with TGFβs increases the numbers of DA neurons in cultures of rodent VM precursors, through the induction of a DA phenotype in these cells [107,

Table 2 Effects of TGFβs on midbrain DA neurons

Effect	Reference(s)
In vitro	
Promote survival of embryonic DA neurons	[27, 106]
Protect DA neurons from MPP+	[27, 106]
Regulate neurite growth of embryonic DA neurons	[108]
Increase numbers of DA neurons through the induction of a DA phenotypic	[107, 109, 110]
In vivo	
Induce differentiation of DA neurons	[107]

109]. Furthermore, Farkas et al. [107] showed that reduction of endogenous TGF-β in vivo, by the use of TGFβ-neutralizing antibodies, suppresses the differentiation of midbrain DA neurons in the chick embryo. VM astrocytes have been shown to induce DA neurogenesis in rat VM precursors by releasing high levels of the TGFβ3 isoform in vitro [110]. A similar role for VM astrocytes has previously been shown through their secretion of Wnts [111], which are critical inducers of DA neurogenesis [62]. Interestingly, the inductive effects of TGFβs were shown to be dependent on Sonic hedgehog (Shh) [107], another factor vital for DA induction [62]. Likewise, Shh was shown to be incapable of inducing a DA phenotype in the absence of TGFβ [107], suggesting that these factors function cooperatively to induce a DA phenotype during midbrain DA neurogenesis. GDNF has been shown to potentiate the DA-inductive effects of TGFβ on cultured VM neural precursors; however, it was not capable of rescuing the inductive defects resulting from the neutralization of endogenous TGFβs [112]. However, GDNF did rescue the TGFβ neutralization-dependent loss of differentiated midbrain DA neurons [112], supporting its role as a factor which maintains these neurons, and as a facilitator of TGFβ-induced survival-promoting effects. Another GDNF family ligand, persephin, has been shown to enhance the inductive abilities of TGFβ in vitro, with these TGFβ/persephin-induced DA neurons having increased resistance to MPP+ compared to untreated cultures of VM DA neurons [112]. In contradiction to these data proposing a role for TGFβs in the induction of midbrain DA neurons, mice with TGFβ null mutations have not been reported to display severe deficits in midbrain DA neurons. However, mice with null mutations of TGFβ2 or TGFβ3 isoforms die perinatally, while the TGFβ1 null mutant dies shortly after birth [113-115]. The double knockout of both TGFβ2 and GDNF did not result in a loss of midbrain DA neurons at E14.5 [112] or E18.5 [116] (Table 5), indicating that the cooperative functioning of TGFβ2 and GDNF is not essential for DA induction in vivo. Furthermore, Roussa et al. [112] also reported no midbrain DA deficiencies at E14.5 in *TGFβ2*$^{+/-}$/*GDNF*$^{-/-}$ or *TGFβ2*$^{-/-}$/*GDNF*$^{+/-}$ mice (Table 5). A likely explanation for such observations is that the TGFβ isoforms may compensate for the loss of each other. Indeed, the double knockout of TGFβ2 and TGFβ3 resulted in a significant reduction of midbrain DA neurons at E14.5 [109]. Roussa et al. [109] then compared mice carrying one allele of TGFβ2 (*TGFβ2*$^{+/-}$/*TGFβ3*$^{-/-}$) or TGFβ3 (*TGFβ2*$^{-/-}$/*TGFβ3*$^{+/-}$) to demonstrate that the TGFβ2 isoform is more important for the induction of midbrain DA neuronal population than TGFβ3 (Table 5). These data suggest that TGFβs function to induce a DA phenotype during midbrain DA neurogenesis and that these isoforms can functionally compensate for one another.

Due to the death of TGFβ null mice at, or shortly after, birth, it is difficult to determine the functions of TGFβs during the postnatal development of the nigrostriatal pathway. Despite this, recent studies have found that TGFβ2 heterozygous mice have a reduction in midbrain DA neurons and striatal dopamine at 6 weeks of age [117], which is similar to the nigrostriatal deficits that progress with age demonstrated in GDNF heterozygous mice [82]. Furthermore, the null mutation of Smad3, a crucial mediator of TGFβ signalling, resulted in a loss of nigrostriatal neurons between birth and 2–3 months of age in mice [118] (Table 5). These studies imply that TGFβs function to protect and maintain midbrain DA neurons in adulthood, as suggested above for GDNF. However, in contrast to the single haploinsufficiencies just described, a more recent study showed that the combined haploinsufficiency of TGFβ2 and GDNF has no impact on the survival of midbrain DA neurons during normal aging [119] (Table 5). These contradicting findings led Heermann et al. [119] to suggest that balanced TGFβ2 and GDNF levels are important for the maintenance of midbrain DA neurons in adulthood. Interestingly, a study which re-examined the TGFβ3 null mutant showed that, despite no deficiencies at E12.5, these mutants had a significant reduction of midbrain DA neurons at postnatal day (P)0 [120] (Table 5), a time point when neurotrophic support is vital for the survival of midbrain DA neurons due to naturally occurring cell death [62]. This study again suggests that TGFβs may function redundantly in DA induction, but indicates that TGFβ3 is required for the maintenance and survival of midbrain DA neurons. Zhang et al. [120] also showed that the transcriptional cofactor homeodomain interacting protein kinase 2 (HIPK2) is required for the TGFβ-mediated survival of mouse DA neurons. This study demonstrates the importance of analysing mutants at several developmental time points, especially at those times which are crucial to the developmental program. Perhaps the TGFβ2 and GDNF single and/or double mutants should be re-examined in a similar fashion.

The BMP Family in Midbrain DA Neuronal Development

The BMPs constitute the largest subgroup of the TGFβ superfamily and consist of at least 20 phylogenetically

conserved growth factors, including GDFs such as GDF5 [121]. BMPs have been shown to function in many crucial aspects of nervous system development, including neural induction, neural crest development, dorsal spinal cord patterning and the fate specification of a number of neural populations [122]. In this review, the current evidence supporting a role for BMPs in the development of midbrain DA neurons will be discussed (Tables 3 and 4).

GDFs

GDF5 is the family member whose roles have been best characterised in terms of DA development (Table 3). Like GDNF, this factor is under investigation for its therapeutic potential in PD [9]. GDF5 is expressed in the developing and adult rat VM and striatum [43, 123-125]. Its midbrain expression profile proposes roles for GDF5 in nigrostriatal development. GDF5 protein expression begins in the rat VM on E12 (when early progenitors of DA neurons are present) and reaches a peak on E14 (the day that DA neurons undergo terminal differentiation), before decreasing with age to reach its lowest levels around the perinatal period, and then increasing in the postnatal period to reach maximal expression levels [124]. These data suggest that GDF5 is involved in the differentiation of VM precursors into DA neurons and the maintenance of these neurons in adulthood. Krieglstein et al. [123] were the first to examine the effects of GDF5 on DA neurons and found that GDF5 increased the number of DA neurons in cultures of E14 rat VM. Other studies agreed with these findings, showing similar neurotrophic effects of GDF5 on VM cultures [126-129], and showed that GDF5 did not induce an increase in the total number of neurons in E14 rat VM cultures [126], indicating a selective effect on DA neurons. It remains unclear whether GDF5 elicits its increases in midbrain DA neurons through the induction of a DA phenotype or by promoting their survival. The latter appears to be more likely as GDF5 has also been shown to protect cultured VM DA neurons from MPP+[123], 6-OHDA [129] and free radical donors [130]. The numbers of astrocytes in E14 VM cultures dramatically increase following GDF5 treatment [123, 126, 127], suggesting that GDF5 may have an indirect neurotrophic action, possibly by stimulating the production of glial-derived growth factor(s) production, such as GDNF, that may function in the neurotrophic response [12]. Conversely, the neurotrophic effects of GDF5 on midbrain DA neurons have been shown to be unaltered in glial-depleted cultures [127]. Furthermore, Wood et al. [127] also showed an additive neurotrophic effect of GDF5 and GDNF on cultured embryonic DA neurons, indicating that GDF5 acts independently from GDNF, and that these factors may act on separate subpopulations of DA neurons. The GDF5-induced increase in midbrain DA neurons in vitro has been suggested to be mediated by BMPRIb, as application of GDF5 at the time of

Table 3 Effects of GDF family on midbrain DA neurons

Effect	Reference(s)
GDF5	
In vitro	
Increases numbers of embryonic DA neurons	[123, 126-129]
Promotes survival of embryonic DA neurons	[123, 126-129]
Protects DA neurons from MPP+, 6-OHDA, and free radical donors	[123, 129, 130]
Induces TH expression in VM precursors	In preparation
Regulates neurite growth of embryonic DA neurons	[126, 128, 141]
In vivo	
Promotes survival of adult DA neurons in animal models of PD	[129, 135-139]
Promotes survival of embryonic DA neurons in VM transplants to adult striatum	[38, 129, 138]
Induces re-innervation of the lesioned striatum by midbrain DA afferents	[129, 137, 138]
Increases neurite growth from DA neurons in VM transplants to adult striatum	[38, 129, 138]
GDF15	
Promotes survival of control and iron-intoxicated embryonic DA neurons in vitro	[144]
Promotes survival of adult DA neurons in vivo following 6-OHDA lesion	[144]

Table 4 Effects BMP family on midbrain DA neurons

Effect	Reference(s)
BMP2	
In vitro	
Increases numbers of embryonic DA neurons	[149]
Promotes survival of embryonic DA neurons	[146]
Regulates neurite growth of embryonic DA neurons	[141, 149]
In vivo	
Promotes survival of embryonic DA neurons in VM transplants to the adult striatum	[150]
Increases neurite growth from DA neurons in VM transplants to the adult striatum	[150]
BMP7	
In vitro	
Increases numbers of embryonic DA neurons	[145]
Promotes survival of embryonic DA neurons	[146]
Induces DA neuronal differentiation from embryonic rat VM neural precursors	[151]
In vivo	
Promotes survival of the adult nigrostriatal pathway against DA toxins	[152, 153]
BMP4, BMP5, BMP6 and BMP12	
Increase numbers of embryonic DA neurons in vitro	[145, 146]

plating, when BMPRIb is expressed, increases DA neuronal number, whereas application after 6 days in vitro, when this receptor is no longer expressed, has no effect [131]. In support of this data, the neurotrophic effects of GDF5 were recently demonstrated to be mediated by the BMPRIb in a model of human DA neurons, SH-SH5Y cells [132]. These findings are not surprising considering that BMPRIb is the preferential type I receptor of GDF5 [133]. Another study demonstrated that GDF5 exerts greater effects on cultures prepared from the lateral VM [128], which corresponds to the A9 nigrostriatal subgroup of DA neurons [134], suggesting a selective effect of GDF5 on nigral DA neurons. In this study, it was proposed that the increase in midbrain DA neurons was due to the induction of a DA phenotype in progenitor cells, rather than promotion of cell survival, and it was also shown that BMPRIb expression was higher in the lateral VM compared to the medial region [128]. In support of the suggested DA-inductive role of GDF5, experiments carried out by the authors on E12 rat VM cultures treated with GDF5 suggest that the increase in DA neurons observed was due to the induction of TH expression in uncommitted neural progenitor cells (Hegarty et al., University College Cork, Ireland). Similarly, the present authors have also demonstrated that GDF5 induces TH expression in neurons derived from E14 rat VM neural precursors (Hegarty et al., University College Cork, Ireland). Based on these data, and on the midbrain expression profile of GDF5, it is likely that GDF5 functions in the transition of VM DA neural precursors into TH-expressing DA neurons.

The effects of GDF5 to increase the numbers of midbrain DA neurons in vitro must also be considered in terms of survival-promoting effects, especially with respect to their neuroprotective effects from DA toxins in vitro. Indeed, in vivo studies have shown that GDF5 protects the adult rat nigrostriatal pathway against DA neuronal death induced by 6-OHDA [135-139]. A more developmentally relevant survival-promoting effect was demonstrated when GDF5 was shown to enhance the survival of embryonic rat VM transplants in vivo, to the same extent as GDNF [38]. Furthermore, GDF5-overexpressing E13 VM transplants significantly restored function in 6-OHDA-lesioned adult rats, with the exogenous GDF5 being suggested to increase the survival of the remaining host DA neurons, as well as the transplanted DA neurons [129]. Similarly, a continuous supply of GDF5, through the striatal transplantation of GDF5-overexpressing CHO cells in vivo, protects adult nigrostriatal DA neurons and increases the survival of transplanted embryonic VM DA neurons in the 6-OHDA-lesioned rat model of PD [138]. These data propose a role for GDF5 as a factor which promotes the survival of embryonic DA neurons during their generation, which would correlate with the peak of GDF5 expression at E14 [124]. Furthermore, the second peak of expression in the adult suggests a role for GDF5 in the maintenance of VM DA neurons during adulthood, which is supported by the survival-promoting effects of GDF5 on adult VM DA neurons in vivo discussed above. A similar study for GDF5 to the one carried out by Burke et al. [39] on cultured postnatal DA neurons would address this. Moreover, studies on GDF5 null mice, such as the GDF5^{bp} mouse [125], to examine midbrain DA neuronal number and striatal innervation at various stages of embryonic and postnatal development, will be critical to determine the in vivo role(s) of GDF5 in nigrostriatal system development. One study showed that adult mice with null mutations in the BMPRII, the type II receptor of GDF5, displayed significantly decreased numbers of nigrostriatal DA neurons [140] (Table 5). However, caution must be employed when inferring the relevance of this study to the roles of GDF5 specifically, as several members of the BMP family act via this type II receptor.

GDF5 has also been consistently shown to promote the neurite growth of E14 rat midbrain DA neurons in vitro [126, 128, 141]. Crucially, this time point reflects the peak period of DA axogenesis [75, 76], as well as of GDF5 VM expression [124], suggesting that GDF5 functions in this process. Furthermore, an in vivo study has suggested that exogenous GDF5 increases the neurite outgrowth of host nigrostriatal DA innervations, as well as transplanted embryonic VM DA neurons, in 6-OHDA-lesioned adult rats [38, 129, 138]. The neurite growth-promoting effects of GDF5 on midbrain DA neurons have recently been shown to be mediated by BMPRIb activation of Smad 1/5/8 signalling [132, 141], which most likely requires BMPRII. Indeed, in adult BMPRII null mice, there is a deficit in nigrostriatal innervation [140] (Table 5). It has also been demonstrated that the neurite growth-promoting effects of GDF5 were not mediated by GDNF [141], through the blockade of its heparan-dependent signalling [32, 142, 143]. Taken together with the survival-promoting effects of GDF5 discussed above, as well as its postnatal striatal expression [43, 124], these data propose a role for GDF5 as a target-derived neurotrophic factor which regulates the survival and growth of DA neurons innervating the striatum. The postnatal/in vivo experiments outlined above would also address this potential role. Another GDF, GDF15, has been shown to promote the survival of control and iron-intoxicated E14 midbrain DA neurons in vitro [144], suggesting that GDF15 may contribute to DA neuronal survival during development. Strelau et al. also demonstrated that GDF5 promotes the survival of adult VM DA neurons in vivo using the 6-OHDA-lesioned adult rat model [144]. The role of other GDF family members in midbrain DA development has yet to be demonstrated, although one study has reported that GDF6 does not have neurotrophic effects on cultured VM DA neurons [145].

Table 5 Genetic mutations of TGFβ superfamily members and their effects on the development of midbrain DA neurons

Mutation(s)	Effect(s) on DA development	Reference(s)
$GDNF^{-/-}$	No severe defects in DA neurons during embryonic development	[72-74]
$GDNF^{-/-}$	Null VM transplants to adult striatum have reduced DA neuron numbers and fiber outgrowth	[41]
$GDNF^{+/-}$	Accelerated decline of DA neurons during aging	[82]
$Neurturin^{-/-}$	No severe defects in DA neurons	[98]
$Persephin^{-/-}$	No severe defects in DA neurons	[99]
$Artemin^{-/-}$	No severe defects in DA neurons	[100]
$GDNF^{-/-}$: $TGF\beta2^{-/-}$	No loss of midbrain DA neurons at E14.5 or E18.5	[112, 116]
$GDNF^{+/-}$: $TGF\beta2^{-/-}$	No loss of midbrain DA neurons at E14.5	[112]
$GDNF^{-/-}$: $TGF\beta2^{+/-}$	No loss of midbrain DA neurons at E14.5	[112]
$TGF\beta1^{-/-}$	No severe defects in DA neurons during embryonic development	[115]
$TGF\beta2^{-/-}$	No severe defects in DA neurons during embryonic development	[113]
$TGF\beta3^{-/-}$	No severe defects in DA neurons during embryonic development	[114]
$TGF\beta2^{-/-}$: $TGF\beta3^{-/-}$	Significant reduction of DA neurons at E14.5	[109]
$TGF\beta2^{+/-}$: $TGF\beta3^{-/-}$	Significant reduction of DA neurons at E14.5 (less severe than double knockout)	[109]
$TGF\beta2^{-/-}$: $TGF\beta3^{+/-}$	Significant reduction of DA neurons at E14.5 (less severe than $TGF\beta2^{+/-}$: $TGF\beta3^{-/-}$ knockout)	[109]
$TGF\beta2^{+/-}$	Reduction in DA neurons and striatal dopamine at 6 weeks of age	[117]
$Smad3^{-/-}$	Loss of nigrostriatal neurons between birth and 2–3 months of age	[118]
$GDNF^{+/-}$: $TGF\beta2^{+/-}$	No deficits in DA neurons during normal aging	[119]
$TGF\beta3^{-/-}$	Significant reduction of DA neurons at P0	[120]
$BMPRII^{-/-}$	Reduction of DA neurons and striatal innervation in adulthood	[140]
$BMP7^{+/-}$	Increased sensitivity of adult DA neurons to methamphetamine toxicity	[153]

BMPs

The most compelling evidence for a role(s) for BMPs in the development of midbrain DA neurons (Table 4) can be seen in the phenotype of the BMPRII null mouse, which has a reduction of nigrostriatal neurons and striatal DA innervation at adulthood [140] (Table 5). However, whether this loss of midbrain DA neurons and striatal innervation results from a failure in DA neuronal development, or from a later degenerative process, remains to be determined. A detailed analysis of the numbers of DA neurons present in the midbrain, as well as of striatal innervation, of BMPRII null mice at multiple stages during embryonic and post-natal development is required to address this question. Furthermore, mice with null mutations (or mutations which permit postnatal investigation) in specific BMP family members should undergo a similar analysis to determine which factors are directly involved.

BMPs have been shown to be expressed in the nigrostriatal system of the developing and adult brain [146-148]. The first report of a role for BMPs in midbrain DA neuronal development was provided by the Krieglstein research group, who investigated the neurotrophic effects of several BMPs on cultured E14 rat VM DA neurons [146]. Jordan et al. [146] showed that BMP2, BMP4, BMP6, BMP7 and BMP12 promote the survival of cultured DA neurons, with BMP6 and BMP12 showing similar efficacy to GDNF. In a similar study, BMP5, BMP6 and BMP7, but not BMP3, significantly increased the numbers of DA neurons in embryonic VM cultures [145]. Conversely, Brederlau et al. [145] showed no effect for GDF5 on midbrain DA neurons. This was likely due to the fact that in this study treatments were carried out at 6DIV, at the time when BMPRIb has been shown to be downregulated in culture [131]. BMP2 has also been demonstrated to increase the numbers of midbrain DA neurons and promote their neurite growth, in vitro [149]. Similarly, BMP2 was recently shown to promote the neurite growth of cultured midbrain DA neurons via a BMPRIb-Smad-mediated mechanism [141]. These data suggest a role for BMP2 in the generation and growth of embryonic midbrain DA neurons. In support of this suggestion, BMP2 has also been shown to promote the survival and neurite growth of embryonic DA neurons in rat VM transplants grafted into the 6-OHDA-lesioned striatum [150]. Interestingly, the neurotrophic effects of BMP2 on midbrain DA neurons were initially suggested to be mediated by astrocytes [146]; however, Reiriz et al. [149] demonstrated BMP2-induced increases in DA neurons in glial-depleted VM cultures. Furthermore, BMP2 was shown to have neurotrophic effects in cultures of SH-SH5Y cells, a cell line model of human DA neurons, which do not contain glial cells [132]. Again, it is unclear whether BMPs increase DA neuron numbers in culture through induction of neural precursors and/or promoting the survival of existing neurons; however, Reiriz et al. did report that BMP2 did not increase the proliferation of DA neurons [149]. One BMP family member, BMP7, has been shown to induce DA neuronal differentiation from embryonic rat VM neural precursors [151]. This BMP was also identified in the Jordan et al. [146] and Brederlau et al. [145] studies as a DA neurotrophic factor. Indeed, BMP7 has been shown to promote the survival of the adult nigrostriatal pathway in vivo against DA toxins [152, 153] (Table 4), suggesting that BMP7 may maintain this pathway in adulthood and in response to insult. In support of this suggestion, a recent study comparing the expression levels of BMPs in multiple brain regions at various time points in adulthood reported uniquely high levels of BMP7 expression in the ventral midbrain, when compared to other BMPs

and other brain regions [148]. Taken together, these preliminary BMP studies suggest roles for BMPs in the induction, neuronal differentiation and survival of midbrain DA neurons. Further studies, such as those described for BMP(R) mutants above, should be carried out to investigate the in vivo roles of BMPs in DA neuronal development.

Potential Mechanism of BMP Signalling in Midbrain DA Neuronal Development

Although a direct role for BMP signalling in the development of VM DA neurons has yet to be conclusively shown, there is some evidence for a role for BMPs in the induction of a DA fate in this region. The first sign of the induction of a DA fate in the VM is the expression of Lmx1a and Msx1 at around E8 in the mouse; these factors act as key determinants of midbrain DA neurons [62, 154, 155]. Both of these genes have been shown to be induced by BMP signalling during nervous system development [156-159]. Furthermore, fibroblast growth factor (FGF), Shh and WNT signalling have all been shown to play critical roles in the development of DA neurons in the VM [155, 160, 161]. An interaction between BMP-Smad signalling and FGF, Shh and WNT signalling has been demonstrated in the induction, specification and development of a variety of neural populations, such as spinal cord neuronal populations and neural crest cells, the relevant aspects of which have been mentioned above [162-166]. However, a number of recent studies have provided substantial evidence that VM DA neurons arise from floor plate DA neural progenitors [1, 62, 167, 168]. This suggests that BMP signalling is not involved in DA development, since the Shh-expressing floor plate is the opposing signalling centre to the BMP-expressing roof plate along the dorsoventral axis of the neural tube [169, 170]. It may be the case that BMP signalling acts dorsally and that Shh signalling acts ventrally along the length of the neural tube to induce various neuronal phenotypes, with these signals intersecting intermediately. However, the VM floor plate is different to its caudal counterparts. The floor plate was thought to consist of specialised non-neurogenic glial type cells that ventralize the neural tube, mainly by secreting Shh [171-173]. This currently remains true for the neural tube caudal to the midbrain, with the hindbrain floor plate being shown to be non-neurogenic [161]. As aforementioned, VM floor plate cells are now known to be neurogenic and to specifically give rise to DA neurons [1, 62, 167, 168]. The suggestion that BMP signalling may be involved in this process arises from the finding that WNT antagonism of Shh signalling facilitates VM floor plate neurogenesis and that Shh inhibits this neurogenesis [161]. WNTs are expressed in the midbrain floor plate and are vital for VM DA neurogenesis [62, 174-177]. BMP-Smad 1/5/8 signalling positively regulates WNT expression in the spinal cord [178, 179] and may continue this role in the VM floor plate. In support of this, BMPs, GDF5 and GDF15 have all been shown to be expressed in the mesencephalic floor plate during DA neurogenesis [124, 144, 146, 147]. During development, Lmx1a expression caudal to the midbrain is restricted to the roof plate and the cerebellum [180-182]. BMPs induce the expression of Lmx1a and other co-factors that regulate the induction of the roof plate in the dorsal neural tube [158] and determine the fate of cerebellar granule neurons [183, 184]. These findings again demonstrate that Lmx1a is a key mediator of BMP signalling. Rostral to the pons in the mid-gestation mouse embryo, Lmx1a expression becomes ventralized, with its expression found in the VM and the basal plates of the diencephalon [180]. This ventral midbrain expression of Lmx1a is accompanied, and possibly preceded, by BMP expression, as mentioned above. Furthermore, BMPs have been reported to act in a dual role with Shh to induce ventral cell types in the diencephalon [185]. Indeed, a regulated level of BMP signalling is involved in the neurogenesis of Shh-responsive ventral cell types in the forebrain [186].

It is not unlikely to suggest that this ventralization of dorsal signalling/expression is due to the formation of the pontine flexure which causes the ventral displacement of the BMP-expressing/signalling alar plates in the metencephalic region. In support of this theory, the pontine flexure forms before DA neurogenesis in the VM [187]. FGF signalling from the isthmus organizer may fit into this proposed role of BMP signalling in VM DA neurogenesis, by acting to repress BMP-Smad signalling to refine its effects. FGF plays such a role during neural induction [188-190], and intermediate levels of BMPs are required for the proper development of specific 'intermediate' (i.e. not directly in contact with the roof plate) neuronal phenotypes, as discussed in a recent review [122]. Furthermore, an intermediate level of BMP-Smad signalling has been shown to be necessary for the induction of Msx1 [157], one of the two key determinants of midbrain DA neurons, along with Lmx1a [154]; FGF has also been shown to induce Msx1 expression in the neural crest [163]. Furthermore, engrailed1/engrailed2 expressions are vital for VM DA neurogenesis [62], and BMP signalling has been shown to induce the expression of these genes in VM cultures [183]. In support of a proposed role of Smad 1/5/8 signalling in VM DA neuronal development, BMP signalling increases the numbers of DA neurons in embryonic rat VM cultures [123, 126, 145, 146, 149, 151].

Conclusion

The development of midbrain DA neurons is a complex process involving the interaction of various instructive signalling factors, a number of which remain to be identified. The identification and characterisation of new candidate factors

which contribute to the development of DA neurons is thus of paramount importance. This paper reviews the available data from studies which assessed roles for TGFβ superfamily members in midbrain DA development. It is clear that many of these members influence various key steps of this developmental process, including the induction, differentiation, target innervation and survival of DA neurons. GDNF, in particular, has been shown to be a multi-step regulator of nigrostriatal system development. However, the elucidation of these roles for GDNF reflects the focus upon this particular family member, based on its clinical potential as a neurotrophic factor for PD treatment. TGFβ/BMP family members, such as GDF5, have been shown to have similar potential to that of GDNF in the development of midbrain DA neurons, and the roles of these members in each step of this embryonic and postnatal developmental process should be investigated. Additionally, TGFβ superfamily members yet to be assessed in terms of DA development should also be examined. Despite the extensive research on GDNF, in particular, much of these studies focused on its survival-promoting abilities. Following the preliminary evidence of the roles of this factor in DA induction, axon extension, target innervation and maintenance, future studies should be employed to further establish the regulation of such processes by GDNF. A similar approach should be adopted for each TGFβ superfamily member demonstrated to regulate DA development, such as the TGFβs, GDFs and BMPs identified in this review.

Another important consideration when discussing the roles of TGFβ superfamily members in midbrain DA development is that the majority of these findings are based on in vitro data. Future studies should thus focus on demonstrating these roles in vivo, for example through the analysis of mice with deficiencies in specific TGFβ superfamily member(s). As outlined, knockout studies to date have not been as informative as was anticipated from the preliminary in vitro data. However, the perinatal lethality of a number of the null mutants, the restricted developmental time points examined and the functional redundancy between closely related family members have hindered these studies. Thus, future knockdown studies should examine mutants at developmental time points which correspond to each step of embryonic and postnatal nigrostriatal system development, as well as addressing potential functional redundancies.

The identification of TGFβ superfamily members as potential regulators of midbrain DA development is an important advancement in the current understanding of this developmental process. Such knowledge is vital if the induction of stem cells toward a DA phenotype for cell replacement therapy in PD is to become a realistic therapeutic strategy. It is also of great importance for the identification of potential developmental abnormalities that may contribute to the pathogenesis of PD.

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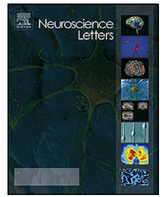

Ventral midbrain neural stem cells have delayed neurogenic potential *in vitro*

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HIGHLIGHTS

- VM NSCs from older embryos and later passages are more gliogenic (after 7DD).
- E14 VM NSC cultures have significant increases in neurons at 14DD and 21DD.
- E14 VM NSC cultures require 3 weeks to complete their differentiation.
- Neurons at 7DD in E14 VM NSC cultures are not NSC-derived (BrdU-negative).
- GFAP-positive cells at 7DD may be both neurogenic and gliogenic.

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ABSTRACT

Neural stem cells (NSCs) have been the focus of an intensive effort to direct their differentiation *in vitro* towards desired neuronal phenotypes for cell replacement therapies. It is thought that NSCs derived from older embryos have limited neurogenic capacity and are restricted towards an astroglial fate. This idea is largely based on studies that typically analysed NSC-derived progeny following one week of *in vitro* differentiation. In this report, the neurogenic capacity of older ventral midbrain (VM) NSCs was assessed. When the older NSCs were differentiated for three weeks, there were significant increases in the numbers of newly born neurons at 14 and 21 days, as assessed by 5-bromo-2′-deoxyuridine (BrdU) incorporation. Therefore this study demonstrates that older NSCs retain significantly more neurogenic potential than was previously thought. These data have implications for NSC preparatory protocols and the choice of donor age for cell transplantation studies, and contributes to the understanding of NSC behaviour *in vitro*.

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1. Introduction

In recent years, NSCs and other stem cell types have been the focus of much research aimed at directing their differentiation *in vitro*, firstly into neurons and secondly into a committed VM dopaminergic (DA) phenotype, for use in transplantation approaches in Parkinson's disease [3,6,20]. The most relevant source of NSCs for the generation of VM DA neurons are those isolated from the VM during the period of DA neurogenesis, which occurs between embryonic day (E) 11 and E14 in the developing rat VM *in vivo* [1,9,16]. Understanding the *in vitro* development of these NSCs is crucial for the choice of donor ages from which to culture VM NSCs. This study thus focused on E12 and E14 rat VM NSCs.

NSCs can be isolated from multiple regions of the embryonic brain, and their numbers expanded *in vitro* as free-floating aggregates termed "neurospheres" when grown in the presence of the mitogens, epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) [7,14,22,23]. The proliferating NSCs then spontaneously differentiate into neurons and glia upon mitogen withdrawal [7,22,23]. It has been suggested that the age of the donor embryo from which NSCs are initially isolated is a critical determinant of subsequent neuronal differentiation *in vitro*, as NSCs derived from younger donors gave rise to more neurons than those derived from older donors [9,19]. These studies have suggested that NSCs from older donor embryos are more restricted towards an astroglial fate.

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; CNS, central nervous system; DA, dopaminergic; DD, days of differentiation; DIV, days *in vitro*; E, embryonic day; EGF, epidermal growth factor; FGF2, fibroblast growth factor 2; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; NP(s), neural progenitor(s)/precursor(s); NSC(s), neuroepithelial/neural stem cell(s); PBS (-T), phosphate buffered saline (-Triton X); VM, ventral midbrain/mesencephalon; VZ, ventricular zone.

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Neurosphere studies typically determine their NSC-derived progeny by assessing the numbers of neurons and glia generated following differentiation for one week *in vitro* [12,19,21,24,26]. In this report, by assessing the neuronal progeny for longer differentiation periods, we show that older NSCs retain significantly more neurogenic potential than previously thought, and suggest that care should be taken when interpreting *in vitro* studies that use glial fibrillary acidic protein (GFAP) as a marker of "differentiated" astrocytes.

2. Materials and methods

2.1. Preparation of embryonic rat VM NSCs cultures

Cultures of E12/E14 Sprague-Dawley rat VM NPCs were prepared as previously described (O'Keeffe and Sullivan, 2005). 2×10^6 cells were grown in T-25 culture flasks in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12, 100 nM L-Glutamine, 6 mg/ml D-Glucose, 100 U/ml Penicillin, 10 μg/ml Streptomycin (Sigma), 2% B27 (Invitrogen), 20 ng/ml EGF (Sigma) and 20 ng/ml of FGF2 (Millipore) for 7 days *in vitro* (DIV). Neurospheres were enzymatically dissociated [19] and reseeded in flask for subsequent expansion. Also 5×10^4 cells per well in poly-D-lysine-coated 24-well tissue culture plate were allowed to differentiate for 7, 14 or 21 DIV in the medium above minus EGF/FGF, with the addition of 1% FCS. 0.2 μM of 5-bromo-2′-deoxyuridine (BrdU) (Sigma B5002) was added during expansion from 5 DIV, and supplemented every 3 DIV from the time of plating.

2.2. Immunocytochemistry

Cultures were fixed in ice-cold methanol for 10 min, washed in 10 mM phosphate buffered saline (PBS) containing 0.02% Triton X-100 (PBS-T), and incubated in blocking solution (5% bovine serum albumin (Sigma), 0.2% Triton X-100 in 10 mM PBS) for 1 h at room temperature. Cultures were incubated in the following antibodies: mouse anti-βIII-tubulin (1:300; Medical Supply), rabbit anti-βIII-tubulin (1:300; Millipore), mouse anti-nestin (1:400; Millipore), mouse anti-GFAP (1:300; Sigma), and mouse anti-BrdU (1:4; Millipore) diluted in 1% bovine serum albumin in 10 mM PBS at 4 °C overnight. Following washes in PBS-T, cells were incubated in the appropriate Alexa Fluor 488 and/or 594-conjugated secondary antibodies (1:500; Invitrogen) diluted in 1% bovine serum albumin in PBS, at room temperature for 2 h. Cultures were counterstained with DAPI or Sytox (1:1000; Invitrogen) and imaged using Olympus IX70 inverted microscope. The total number of cells (assessed by DAPI staining) and the numbers of each cell type were counted in each individual image [19].

2.3. Statistical analysis

Unpaired Student's *t*-test or one-way ANOVA with a *post hoc* Tukey's test were performed as appropriate to determine statistical significance. Results were expressed as means with SEM and considered to be significant when $p < 0.05$.

3. Results

3.1. Effect of gestational age and passage number on VM-derived NSCs in vitro

Firstly, E12 and E14 rat VM NSCs which had been expanded for 7 DIV (passage 1), 14 DIV (passage 2) or 21 DIV (passage 3) as free-floating neurospheres were allowed to differentiate after mitogen withdrawal for 7 days of differentiation (DD) (Fig. 1a), before being

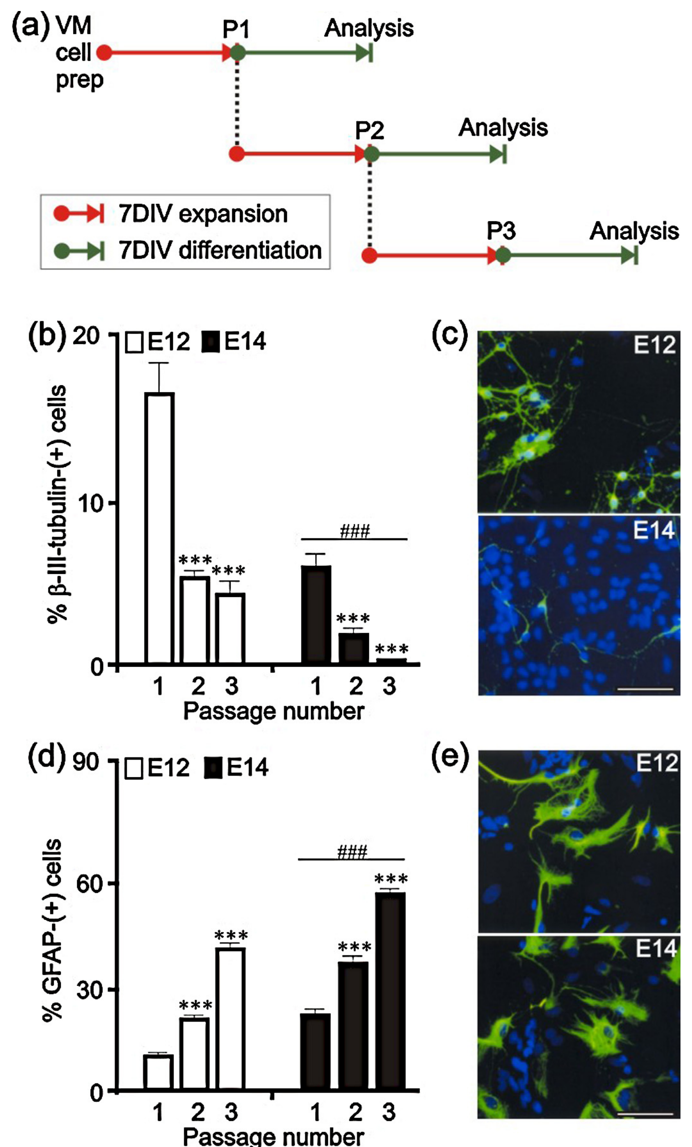

Fig. 1. Characterisation of neurogenesis and gliogenesis in cultures of VM NSCs of various gestational ages and passage numbers. (a) Schematic representation of the passaging protocol for E12 and E14 VM NSC neurosphere cultures. Graphical representation of the mean numbers (expressed as a percentage of total cells) of (b) βIII-tubulin-positive cells and (d) GFAP-positive cells following 7 DD, in neurosphere cultures of E12 or E14 VM NSCs which were passaged once, twice or three times before differentiation, as indicated (***$p < 0.001$ *vs* passage 1; ###$p < 0.001$ *vs* E12, ANOVA with *post hoc* Tukey's test). Data are expressed as mean ± SEM, $n = 60$ fields. Representative photomicrographs of cultures of passage 1 VM NSCs isolated at E12 or E14, differentiated for 7 DIV and immunocytochemically stained for and (c) βIII-tubulin or (e) GFAP, and counterstained with DAPI. Scale bar = 100 μm.

immunocytochemically stained for βIII-tubulin (neurons) or GFAP (astrocytes) (Fig. 1b–e). Later passages were not characterised due to a marked increase in cell death and lack of neurosphere formation following 28 DIV (data not shown). Passage 1 VM NSCs, isolated at E12 or E14, generated a significantly higher percentage of βIII tubulin-positive neurons than at passage 2 or 3 (Fig. 1b). Passage 2 or 3 VM NSCs, isolated at E12 or E14, generate a significantly higher percentage of GFAP-positive astrocytes in comparison to passage 1 VM NSCs (Fig. 1d). Therefore, VM NSCs of early passages are more neurogenic, and less gliogenic, than those of older passages, irrespective of the age of the donor embryo.

In all passages examined, the 7DD progeny of VM NSCs isolated at E12 generated a significantly higher percentage of βIII

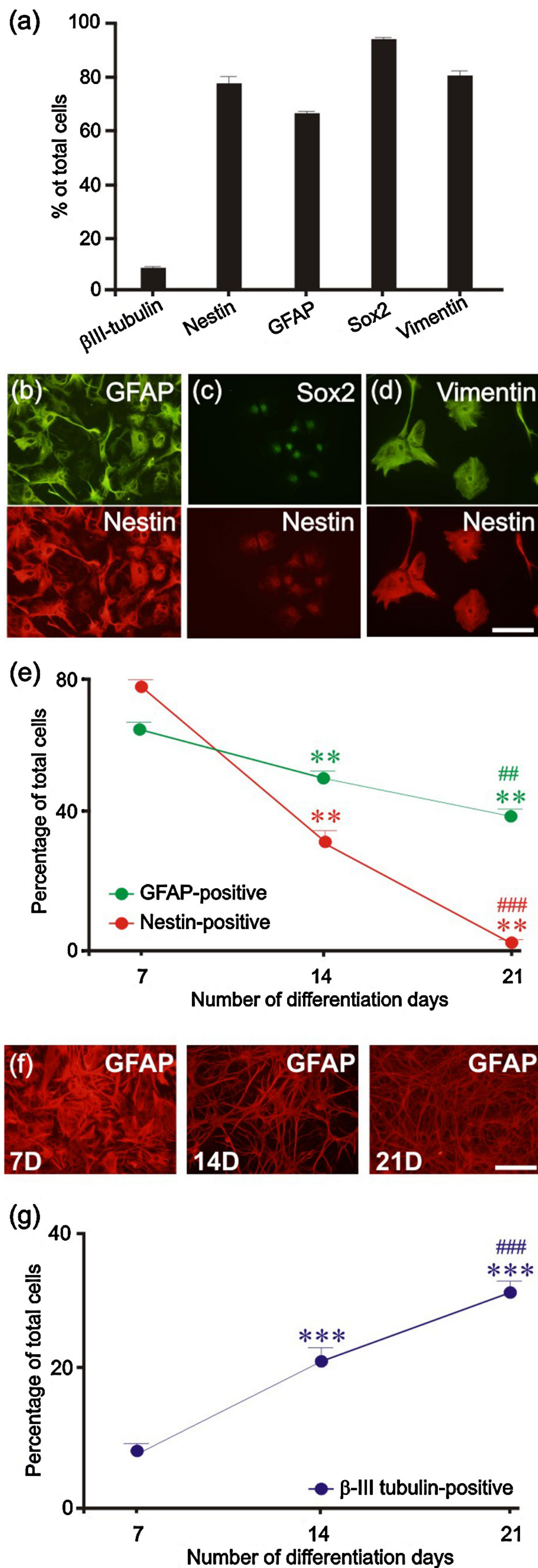

Fig. 2. Characterisation of cell phenotypes in E14 rat VM NSC cultures after 7, 14, and 21 DD. (a) Graphical representation of the mean numbers (expressed as a percentage of total cells) of βIII-tubulin-, nestin-, GFAP-, Sox2- and vimentin-positive cells following 7 DD generated from E14 VM NSCs after 7 DIV expansion. E14 rat VM NSCs cultures after 7 DIV expansion and 7 DD, immunocytochemically stained for (b) GFAP (c) Sox2, (d) vimentin or (b–d) nestin. Scale bar = 100 μm. Graphical representation of the number of (e) nestin-, (e) GFAP-, and (g) βIII-tubulin-positive cells (expressed as a percentage of total cells) in E14 rat VM NSCs cultures after 7 DIV expansion and 7, 14 or 21 DD, as indicated ($^{**}p < 0.01$, $^{***}p < 0.001$ *vs* 7 DD, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs* 14 DD; ANOVA with *post hoc* Tuckey's test). Data are expressed as mean ± SEM, $n = 60$ fields. (f) E14 rat VM NSCs cultures after 7 DIV expansion and 7, 14 or 21 DD, immunocytochemically stained for GFAP. Scale bar = 100 μm.

tubulin-positive neurons (Fig. 1b and c) and lower percentage of GFAP-positive astrocytes (Fig. 1d and e) at 7DD in comparison to E14 VM NSCs. These data suggest that VM NSCs isolated from younger embryos are more neurogenic and less gliogenic, than those derived from older embryos, irrespective of passage number. These data suggest that gestational age can influence the proportion neurons and glia derived from VM NSCs.

3.2. Assessment of E14 VM NSC differentiation for prolonged periods in vitro

Despite the fact neurogenesis increases between E12 and E14 in the developing rat VM *in vivo* [1,9,16], E14 VM NSCs have a diminished neurogenic capacity when compared to their E12 counterparts *in vitro* (Fig. 1b). These data suggest that E14 VM NSCs may be more glial restricted than E12 NSCs (Fig. 1d). However, when nestin expression was examined in cultures of E14VM NSCs at 7DD, 80% of the total cells were nestin-positive (Fig. 2a), suggesting the potential for further differentiation. Many of the nestin-positive NSCs were GFAP-positive at 7 DD (Fig. 2a and b), and also expressed other NSC markers, including Sox2 and vimentin (Fig. 2c and d).

To further examine this premise, these cells were differentiated for a further two weeks, and the numbers of neurons and astrocytes were assessed 14 and 21 days later. Interestingly, the numbers of nestin-positive and GFAP-positive cells decreased significantly from 7DD to 14DD to (Fig. 2e), where there was a significance increase in the percentage of βIII-tubulin-positive cells (Fig. 2g) and MBP-positive cells (data not shown).

At 7DD, the GFAP-positive cells have a morphology typical of protoplasmic astrocytes, hereafter referred to as a protoplasmic morphology (Fig. 2b and f), which was similar to that of the nestin-, Sox2- and vimentin-positive NSCs (Fig. 2b–d). However, at 14 and 21DD, the GFAP-positive cells have a more stellate morphology (Fig. 2f), typical of differentiated astrocytes. These stellate GFAP-positive cells did not express nestin, as demonstrated by the absence of nestin expression at 21DD when these stellate astrocytes occupied the largest proportion of the cell population (Fig. 2e and f). By 14DD cultured cells had grouped into clusters, which were absent at 7DD, and therefore are likely to have been generated during the second week of differentiation (Fig. 3a). These cell clusters contained large numbers of newly born neurons at 14DD (Fig. 3a and b). By 21DD, the cell population consisted of post-mitotic neurons (31%) (Fig. 2f), astrocytes (40%) (Fig. 2d) and oligodendrocytes (28%) (data not shown), with few nestin-positive cells remaining (2%) (Fig. 2e). The total population of differentiated neural cells was thus accounted for at 21 DD. No tyrosine hydroxylase-positive neurons were observed (data not shown). Additionally, no adverse effects on neuronal viability were observed during the 21DD as neuronal numbers and neurite length continued to increase throughout the duration of the experiment, while less than 1% of neurons displayed signs of apoptosis at any time point examined (data not shown).

To determine whether the neurons found in the E14 rat VM NSC cultures were derived from NSCs or were post-mitotic neurons, which had been present in the culture since the tissue was harvested from the animal, BrdU was applied to the cultures (Fig. 3c). BrdU is a thymidine analogue that is incorporated into the nucleus during the S phase of cell division. 0.2 μM of BrdU was used due to

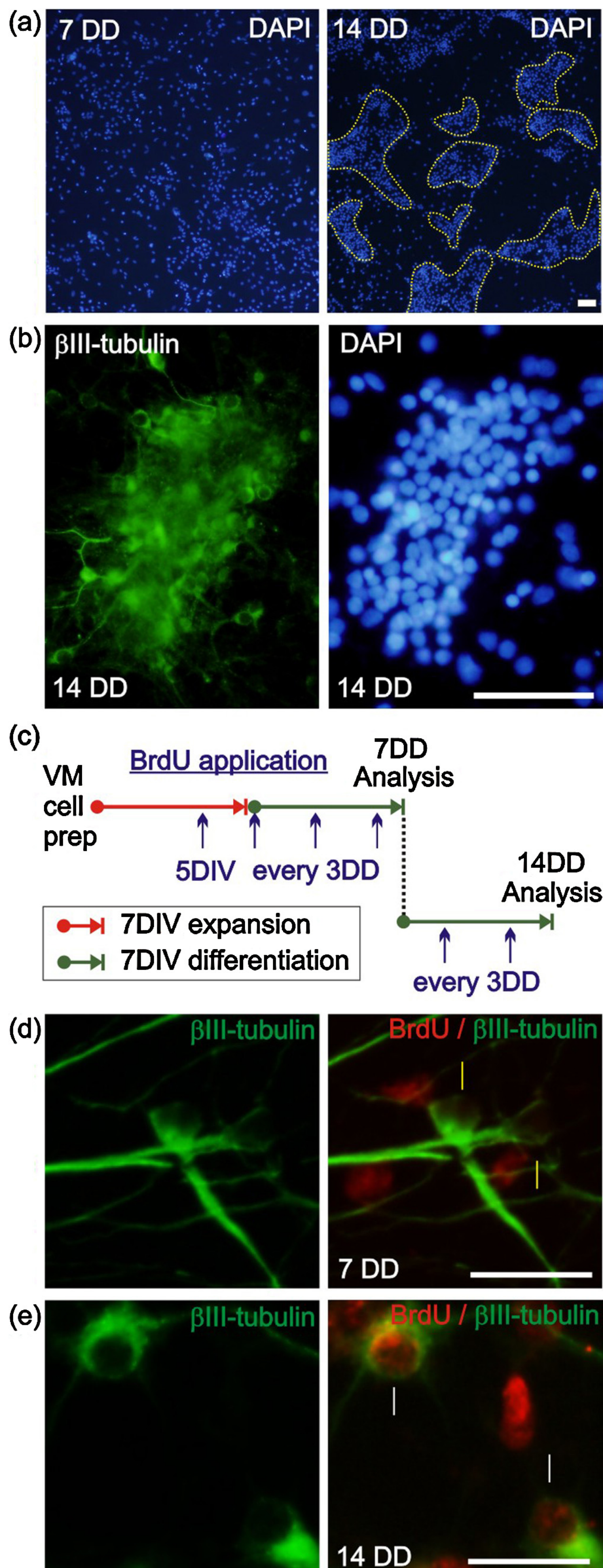

Fig. 3. E14 VM NSC neurogenesis during second two weeks of differentiation. Representative photomicrographs of E14 rat VM NSCs cultures after 7 DIV expansion and 7 DD or 14 DD, immunocytochemically stained for (b, d and e) βIII-tubulin and/or (d)–(e) BrdU, and counterstained with (a) and (b) DAPI. Scale bar = 100 μm (a), 50 μm (b) and 25 μm (d)–(e). (c) Schematic representation of the BrdU application protocol for E14 VM NSC neurosphere cultures.

its neurotoxicity at higher concentrations [4], however, due to this low concentration, supplementation of BrdU was required every 3 DIV to ensure its detection (Fig. 3c). BrdU applied to NSC cultures would only be incorporated into nuclei of proliferating cells. Thus, subsequent detection of BrdU in post-mitotic neurons indicates that these cells differentiated from the proliferating VM NSCs. The addition of 0.2 μM BrdU to the differentiating E14 VM NSCs labelled the βIII-tubulin-positive neurons, which were grouped in clusters at 14 DD (Fig. 3d). However, the βIII-tubulin-positive neurons found at 7 DD were BrdU negative (Fig. 3d). Collectively, these data demonstrate that E14 VM NSCs have the capacity to generate large numbers of newly born neurons when allowed to fully differentiate *in vitro*.

4. Discussion

This study describes the differentiation of E14 rat VM NSCs cultured for various periods *in vitro* to re-evaluate their neurogenic potential. The assessment of the differentiated progeny of VM NSCs isolated at E12 or E14, and expanded for 7, 14 or 21 DIV, demonstrated that VM NSCs of older ages and later passages are more glial-restricted than those of younger age and lower passages, which were more restricted towards a neuronal fate. These findings mirror the *in vivo* developmental precept that primary neurogenesis precedes gliogenesis.

The yield of approximately 8% of neurons following the expansion and differentiation (both for 7 DIV) of E14 rat VM NSCs is consistent with previous studies [19,21]. However, the fact that ~80% of the total cell population were nestin-positive neural precursors (NPs) after 7 DD demonstrates that, at this time point, E14 rat VM NSCs have the potential to undergo further differentiation. The subsequent differentiation of E14 VM NSCs for a further two weeks confirmed that these cells retained the ability to differentiate into neurons, despite their apparent astroglial restriction, and that the E14 VM NSCs completed their differentiation by 21 days. With the majority of previous studies employing one week of differentiation protocols to evaluate the numbers of differentiated NSC progeny, it is possible that the neurogenic potential of these cells have been underestimated [12,19,21,24,26].

Following 7DD, most cells in the E14 VM NSC cultures were GFAP- and nestin-positive, which would suggest that these cells are committed towards an astrocytic lineage. The propensity of NSCs within neurospheres to differentiate primarily into glial cells has been described by many groups [17–19,21,27]. Indeed, it has been proposed that the environment within the neurosphere prohibits NSCs from following a neuronal lineage, while promoting the amplification of glial precursors [2]. The findings of this study following 7DD supports this, and thus it should not be surprising that there is limited neurogenesis of neurosphere-expanded E14 VM NSCs after one week of differentiation. However, the significant increase in neurons generated during the second and third weeks of differentiation may modify the previous perception of the glial commitment of neurosphere-expanded NSCs. The three-fold increase in βIII-tubulin-positive cells between 7DD and 21DD is due to the presence of clusters of large numbers of newly born neurons. These cell clusters, which are absent at 7 DD and which develop during the second week of differentiation, must be generated from the NPs present at 7 DD, which are largely GFAP- and nestin-positive. It is thus likely that these GFAP-positive NPs become neurogenic, and generate newly born neurons, during the second and third weeks of *in vitro* differentiation in E14 VM NSC cultures. However, such conclusions cannot be conclusively drawn without real-time monitoring of marker-labelled E14 VM NSCs as they differentiate in culture. Nevertheless, in support of this theory, GFAP has previously been shown to label neuronal precursors

[5,8,25]. Furthermore, cultured rat GFAP-positive NPs have been shown to be capable of generating both neurons and glia [11]. In fact, the separate identity of NSCs and astroglial cells is regularly challenged in the literature, with some authors suggesting that they are in fact the same cell type [15]. Thus, in agreement with previous studies, the majority of VM NSCs express GFAP after one week of differentiation, however a proportion of these GFAP-positive cells may be intermediate neuronal precursors.

The GFAP-positive cells present after 7DD expressed nestin, and had a protoplasmic morphology, similar to that of the nestin/Sox2/vimentin-positive NSCs, suggesting that these GFAP-positive cells at 7 DD were NSCs, rather than astrocytes. There was a reduction in the numbers of these GFAP/nestin doubled labelled cells during the second two weeks, as evidenced by a reduction in their numbers after 14 and 21 DD. Specifically, GFAP-positive cells at 7 DD stopped expressing nestin and adopted a stellate morphology during the second two weeks of differentiation. There were no GFAP- and nestin-double labelled cells present at 21DD. The cessation apparent differentiation of these GFAP-positive cells, coincided with increases in the numbers of βIII-tubulin-positive cells, accounted for GFAP-positive cells occupying a significantly lower percentage of the total cell population at 14 DD and 21 DD than at 7DD. Although GFAP-positive cells occupied the largest proportion of the total cell population at all differentiation time points examined, the neurogenic capacity of neurosphere-derived NSCs was significantly greater than previously considered, given the significant increase in the numbers of newly born neurons during the second and third weeks of differentiation. No tyrosine hydroxylase-positive DA neurons were generated from the VM NSCs in this study, in agreement with a previous study on E14 rat VM NSC cultures [19]. The lack of DA neurogenesis from VM NSCs, cells, which would normally generate DA neurons *in vivo*, likely reflects the absence *in vitro* of important developmental guidance cues for DA neurogenesis [10]. This is an important issue to consider when using neurosphere cultures of any isolated NSC population to generate specific neuronal populations.

The present investigation of the origin of the neurons in E14 rat VM cultures provides insights into the source of neurons found in NSC differentiation studies. The identification of the cluster-located neurons as BrdU-positive at 14DD showed that these cells are derived from the cultured E14 VM NSCs. In contrast, the neurons at 7DD were not BrdU-labelled, and thus were likely to have been present as post-mitotic neurons in the donor tissue at the time of harvesting, and to have subsequently persisted in culture. In support of this, neurospheres prepared from E13.5 mouse VM, which is at a similar developmental stage as E14 rat VM, have been shown to contain differentiated neurons before mitogen withdrawal [13]. Collectively these data suggest that the vast majority of neurons present after one week of differentiation are not progeny of the E14 VM NSCs, but mature neurons that had been present in the source tissue. The vast majority of cells expressed GFAP following 7DD. However, as this study has shown, these GFAP-positive NPs may have the capacity for both gliogenesis and neurogenesis. These findings highlight the importance of using the presence of newly born neurons to assess the true neurogenic potential of VM NSCs, and show that care must be taken in using GFAP as an “astrocytic” marker, especially at earlier stages of NSC differentiation.

The study of NSCs is important to develop a detailed, well-characterised understanding of the development of NSCs into post-mitotic neurons or glia. We demonstrate E14 rat VM NSCs require three weeks to complete differentiation into their progeny, with neurogenesis proceeding during the second two weeks. This method of NSC culture results in at least a three-fold increase in the yield of newly born neurons over conventional methods. This study suggests that the apparent glial restriction (as evidenced by GFAP expression) of older NSCs may be an intermediate developmental stage during neurogenesis. This has implications for the choice of age of donor tissue for the use of NSCs for transplantation. Indeed, tissue-specific NSCs should be isolated during the developmental time-window which corresponds to the neurogenesis of the neuronal subtype of interest, for example from E11–E14 for rat VM DA neurogenesis [1,9,16]. Based on the perceived limited neurogenic capacity of older E14 VM NSCs, younger VM NSCs have been suggested as a more appropriate stem cell source [9]. However, this study has demonstrated that NSCs derived from E14 embryos, which give larger numbers of NSCs and are less labour intensive for NSC isolation, have significant neurogenic capacity.

Conflict of interest

None.

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BMP-Smad 1/5/8 signalling in the development of the nervous system

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ABSTRACT

The transcription factors, Smad1, Smad5 and Smad8, are the pivotal intracellular effectors of the bone morphogenetic protein (BMP) family of proteins. BMPs and their receptors are expressed in the nervous system (NS) throughout its development. This review focuses on the actions of Smad 1/5/8 in the developing NS.

The mechanisms by which these Smad proteins regulate the induction of the neuroectoderm, the central nervous system (CNS) primordium, and finally the neural crest, which gives rise to the peripheral nervous system (PNS), are reviewed herein. We describe how, following neural tube closure, the most dorsal aspect of the tube becomes a signalling centre for BMPs, which directs the pattern of the development of the dorsal spinal cord (SC), through the action of Smad1, Smad5 and Smad8. The direct effects of Smad 1/5/8 signalling on the development of neuronal and non-neuronal cells from various neural progenitor cell populations are then described. Finally, this review discusses the neurodevelopmental abnormalities associated with the knockdown of Smad 1/5/8.

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Abbreviations: ALK, Activin Receptor-like Kinases; BMP(s), Bone Morphogenetic Protein(s); BMPR, BMP receptor(s); CNS, Central Nervous System; Co-Smads, Common-mediator Smads; DA, Dopaminergic; DG, Dentate Gyrus; dI, Dorsal Interneuron; DRG, Dorsal Root Ganglion; E, Embryonic Day; FGF, Fibroblast Growth Factor; GDF(s), Growth/Differentiation Factor(s); GDNF, Glial Cell Line Derived Neurotrophic Factor; GFAP, Glial Fibrillary Acidic Protein; I-Smads, Inhibitory Smads; MAPK, MAP kinase; NCC(s), Neural Crest Cell(s); NS, Nervous System; NSC(s), Neuroepithelial/Neural stem cell(s); OPC, Oligodendrocyte Precursor Cell; PD, Parkinson's Disease; PNS, Peripheral Nervous System; RNAi, RNA Interference; R-Smads, Receptor-regulated Smads; RTK, Receptor Tyrosine Kinase; *sbn*, *somitabun*; SC, Spinal Cord; Shh, Sonic Hedgehog; Sip1, Smad-interacting Protein-1; Smad 1/5/8, Smad1, Smad5 and Smad8; SVZ, Subventricular Zone; TGFβ, Transforming Growth Factor β; VM, Ventral Midbrain/Mesencephalon; VZ, Ventricular Zone.

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1. Introduction

Smad transcription factors are the pivotal effectors of transforming growth factor β (TGFβ) family members, of which the vast majority are dimeric, structurally conserved proteins, that have pleiotropic functions *in vitro* and *in vivo* (Massague and Wotton, 2000). The bone morphogenetic proteins (BMPs) constitute the largest subgroup of the TGFβ superfamily, and consist of at least 20 growth factors (Kawabata et al., 1998). The structure, signalling, and functions of BMPs have been extensively reviewed (for reviews see Bragdon et al., 2011; Miyazono et al., 2010; Sieber et al., 2009: Xiao et al., 2007). This review describes the role of the BMP-activated Smad proteins in the development of the nervous system (NS).

Understanding the functional roles of Smad signalling requires a brief overview of how these proteins are activated. BMP signalling occurs through a canonical pathway, involving Smad activation, and non-canonical pathways which involve the activation of a variety of intracellular pathways, including mitogen activated protein kinases pathways. The non-canonical pathways activated by BMPs have been reviewed thoroughly elsewhere and will not be discussed further (for reviews see Derynck and Zhang, 2003; Moustakas and Heldin, 2005). In the canonical pathway, BMPs bind to two types of cell-surface serine/threonine kinase receptors (BMPR), and both of these are required for signal transduction (Shi and Massague, 2003; Yamashita et al., 1996). There are two known type I BMP receptors (BMPRI): activin receptor-like kinase (ALK) 3 (also known as BMPRIa) and ALK6 (also known as BMPRIb), with BMPs also capable of binding to the type I receptors ALK1 and ALK2. BMPs can bind to three type II receptors: BMP type II receptor (BMPRII), activin type IIa receptor and activin type IIb receptor (Bragdon et al., 2011; Nohe et al., 2004). Upon ligand binding, the constitutively active type II receptor transphosphorylates the intracellular domain of the BMPRI, which then recruits and phosphorylates Smad proteins (Miyazono et al., 2010). Smads are classified into three subclasses based on function: receptor-regulated Smads (R-Smads), common-mediator Smads (Co-Smads) and inhibitory Smads (I-Smads) (Heldin et al., 1997). The BMP R-Smads (Smad1, Smad5 and Smad8) are specifically phosphorylated by activated BMPR-Is, interact with co-Smad4 (Zhang et al., 1997) to form heterotrimeric complexes, and translocate into the nucleus to regulate gene expression. The I-Smads, Smad6 and Smad7, limit BMP-induced Smad signalling in two ways: (i) they physically interact with activated BMPRI and compete with R-Smads (Hayashi et al., 1997; Heldin et al., 1997; Imamura et al., 1997; Souchelnytskyi et al., 1998), and (ii) they bind to R-Smads and prevent R-Smad-Co-Smad complex formation (Hata et al., 1998: Murakami et al., 2003). Smad proteins are composed of highly conserved N-terminal Mad homology (MH) 1 domain and C-terminal MH2 domain, which are joined by a divergent proline-rich linker region of variable length that contains multiple phosphorylation sites, each of which allow specific cross-talk with specific signalling pathways (Heldin et al., 1997; Massague and Wotton, 2000; Shi and Massague, 2003). The MH2 domains of R-Smads are responsible for their direct interaction with type I receptor kinases, in which their C-terminal Ser-Ser-Val/Met-Ser (SSXS) motifs are phosphorlyated by the BMPRIs, and are also responsible for Smad complex formation (Miyazawa et al., 2002; Miyazono et al., 2005). The MH1 domain is the DNA-binding domain of R-Smads and Co-Smads, with I-Smads lacking this domain (Souchelnytskyi et al., 1998). In the absence of receptor activation, the MH1 and MH2 domains are physically associated with one another (Miyazawa et al., 2002; Miyazono et al., 2005). The BMP-Smad signalling pathway has been more comprehensively described elsewhere (Sieber et al., 2009) and we will focus on the function of this signalling pathway during neural development. It is important that whilst this review refers to most findings as BMP-Smad signalling, the vast majority of these results were obtained through studies focusing on Smad1 and/or Smad5, and not Smad8. The expression profiles of Smad proteins during NS development is yet to be fully characterised, with most studies focusing on Smad expression in a defined region of the CNS or PNS. However, it is important to note that the BR-Smads, Smad 1/5/8, do not share identical expression patterns in the developing NS. For example, in the basal forebrain, Smad1 mRNA is highly expressed at E14 and P8 but to a lesser extent in the adult and perinatally, Smad5 expression is consistent throughout development, and Smad8 mRNA expression is absent early at E14 but increases thereafter with age (Lopez-Coviella et al., 2006). For the remainder of this review we will focus on the role of Smad 1/5/8 signalling in the development of the NS.

2. Smad 1/5/8 signalling in nervous system development

In addition to well-characterised roles in bone and cartilage development (Nishimura et al., 2012; Yoon and Lyons, 2004), BMP-Smad 1/5/8 signalling also instructs key developmental events during the development of the NS. Paradoxically, despite a role for BMP-Smad signalling in key neurodevelopmental events, the repression of BMP-Smad signalling is firstly required for the primary neurodevelopmental event, neural induction (Fig. 1A) (Liu and Niswander, 2005; Smith and Harland, 1992; Smith et al., 1993: Spemann and Mangold, 1924). BMP-Smad signalling on the ventral side of the embryo allows the formation of epidermal ectoderm, while dorsally expressed BMP antagonists induce formation of neural tissue through the blockade of BMP-Smad signalling (Hemmati-Brivanlou and Melton, 1997; Lamb et al., 1993: Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995). During this process of neurulation, BMP-Smad signalling actively instructs the development of a neural population which arises at the border between the epidermis and neural plate, known as the neural crest.

2.1. Smad 1/5/8 signalling in neural crest cell development

Neural crest cells (NCCs) give rise to a variety of cell populations in the peripheral NS (PNS), as well as skeletal elements of the head (Farlie et al., 2004). BMP-Smad 1/5/8 signalling, emanating from the epidermal ectoderm and under negative regulation from the neural plate, plays an important role in the generation of NCCs (Fig. 1A). This is illustrated by the fact that active Smad 1/5/8 signalling, in response to BMPs either exogenously applied or emanating from epidermal ectoderm, is necessary for the generation of NCCs in a variety of *in vitro* and *in vivo* models (Dickinson et al., 1995; Liem et al., 1995; Moury and Jacobson, 1989; Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995). Additionally, in the anterior ectoderm, the development of the ectodermal placodes, from the pre-placodal region between the neural plate and neural crest, requires attenuated BMP-Smad signalling (Litsiou et al., 2005; Streit, 2004). These placodes contribute to the formation of the cranial sensory NS and the special sense organs.

In terms of NCC development, the *zebrafish* BMP mutants, *swirl* (BMP2b) and *snailhouse* (BMP7), as well as the Smad 5 mutant *somitabun* (*sbn*), display alterations in neural crest formation (Nguyen et al., 2000). Specifically, trunk NCCs failed to form in these mutants, showing that BMP-Smad signalling is crucial for NCC generation. However, in an earlier study by this group the *swirl* mutant, which is the most severely dorsalised mutant of the three (discussed later) (Kishimoto et al., 1997), displayed a severe reduction in laterally derived cranial NCCs, demonstrating that BMP2b-mediated Smad signalling is essential for cranial neural

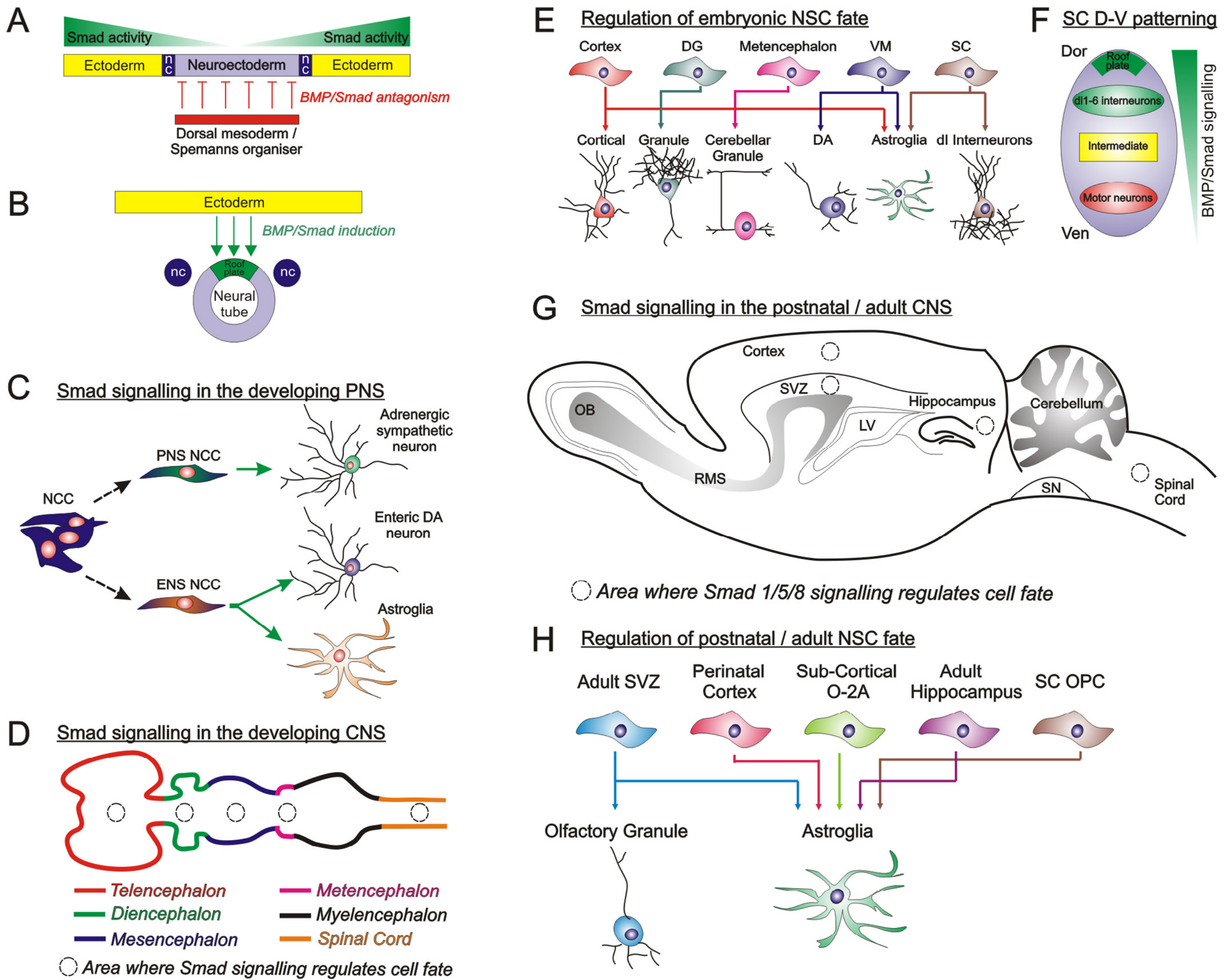

Fig. 1. Smad 1/5/8 signalling in the development of the NS. (A) BMP antagonists (red arrows) arising from Spemann's organiser inhibit BMP-Smad 1/5/8 signalling in the overlying dorsal ectoderm to induce a neural fate. An intermediate level of BMP-Smad signalling induces the formation of the neural crest at the border between the epidermis and neural plate. (B) BMP-Smad 1/5/8 signalling (green arrows) arising from the epidermal ectoderm induces the formation of the roof plate at the dorsal midline of the neural tube. Formation of the neural tube releases the NCCs. (C) PNS NCCs are induced to differentiate into adrenergic sympathetic neurons by BMP-Smad signalling (green arrow). Enteric nervous system NCCs are induced to differentiate into enteric DA neuron and astroglia. (E) The neural fates (indicated by arrows; neuron unless stated), induced by Smad 1/5/8 signalling, of embryonic NSCs from different CNS regions. (F) Schematic representing the dorso-ventral patterning of the SC. The green gradient represents the gradient of BMP-Smad 1/5/8 signalling strength which patterns the formation of the dorsal SC. (H) Describes the cell fate (indicated by arrows; neuron unless stated), induced by Smad 1/5/8 signalling in postnatal/adult NSCs from different regions of the CNS.

crest specification (Nguyen et al., 1998). In contrast, Nguyen et al. (1998) also showed that the *snailhouse* and *sbn* mutants exhibited an expansion of these cranial NCCs (Nguyen et al., 1998). However, the neural defects of the *snailhouse* and *sbn* hypomorphic allele mutants are more severe caudally (Mullins et al., 1996; Nguyen et al., 1998), and therefore the expression of BMP7 and Smad5 may not be diminished cranially to the same extent as it is caudally. In support of this suggestion, the addition of Smad5 morpholinos to the *sbn* mutant resulted in additional cranial defects, such as a compressed anterior/head region, which was attributed to the total loss of Smad5 activity, unlike with the hypomorphic *sbn* allele (Lele et al., 2001). Based on the opposing effects that these mutants had on cranial neural crest development, Nguyen et al. (1998) hypothesised that an intermediate level BMP-Smad signalling is involved in neural crest specification. These findings have largely been supported by studies in mice showing that Smad4 knockdown in mouse NCCs causes the downregulation of genes critical to NCC development and results in the loss of NCC derivatives at the mid-gestational stage, coupled with alterations in cell fate specification, such as sensory neuronal fate acquisition in the trigeminal ganglia (Buchmann-Moller et al., 2009; Ko et al., 2007; Nie et al., 2008).

Smad-interacting protein-1 (Sip1) represses Smad signalling in response to BMPs, and has been shown to induce a neural fate by repressing BMP-Smad signalling during neural induction (Lerchner et al., 2000; Nitta et al., 2004; Van Grunsven et al., 2007). In zebrafish, the knockdown of two orthologues of Sip1 results in a loss of vagal/post-otic NCC derivatives due to an interference with Sip1-mediated negative regulation of BMP-Smad 1/5/8 signalling (Delalande et al., 2008). Similarly, the knockdown of Zfhx1b (the gene that encodes Sip1) in the NCCs of mice results in craniofacial and gastrointestinal malformations that resemble those found in patients with Mowat-Wilson syndrome (craniofacial dismorphology with Hirschsprung disease), which further demonstrates a role for an intermediate, regulated level of BMP-Smad signalling in NCC development (Van De Putte et al., 2007). This is not surprising considering the location at

which NCCs develop, that is at the border of the BMP-Smad-induced epidermis and the BMP-antagonised neural plate. In support of Van De Putte et al. (2007), loss-of-function mutations in Sip1 deregulate BMP-Smad signalling to cause abnormal development of neural and NCC structures, resulting in some of the dysmorphic features of Hirschsprung disease, in particular defects of the enteric NS such as aganglionosis of the distal colon (Cacheux et al., 2001; Wakamatsu et al., 2001). This finding reflects the fact that NCCs give rise to the enteric NS.

Studies using neural crest stem cells have shown that BMP-Smad signalling antagonises Wnt-induced sensory neurogenesis of NCCs, while BMP-Smad signalling functions cooperatively with Wnt signalling to suppress differentiation and maintain multipotency of these stem cells (Kleber et al., 2005; Lee et al., 2004). BMP-Smad signalling interacts with WNT and FGF signalling in the development of a variety of neural populations (Labonne and Bronner-Fraser, 1998; Liu and Niswander, 2005: Monsoro-Burq et al., 2005). Indeed, through the sequential MAP kinase (MAPK)- and GSK3β-mediated phosphorylation of the Smad1 linker region, which results in Smad1 degradation, BMP-Smad signalling integrates with FGF-MAPK and WNT-GSK3β signalling pathways during neural development (Eivers et al., 2008; Fuentealba et al., 2007).

Collectively these data have shown that Smad-signalling is required for the NCC generation and cell fate choice during development. However, the involvement of Smad 1/5/8 in this process is often inferred given the involvement of BMPs, and an analysis of neural crest developmental and differentiation in NCC-specific Smad 1/5/8 conditional knockout mouse would be beneficial. Direct assessment of Smad 1/5/8 transcriptional activity *in vivo* is now possible using a mouse line expressing GFP under the control of a BMP-response element (BRE), thus allowing direct assessment of BMP-Smad transcriptional activity *in vivo* during NCC development (Monteiro et al., 2008). Aside from NCC induction, BMP-Smad signalling also promotes neural crest migration (Sela-Donenfeld and Kalcheim, 1999), induces differentiation of adrenergic sympathetic neurons (Varley and Maxwell, 1996) and mediates neural crest apoptosis (Graham et al., 1994). In agreement with much of these findings, intense BMP-Smad dependent transcriptional activity has been found in these regions during mouse development (Monteiro et al., 2008), directly supporting a role for Smad 1/5/8 signalling in these processes.

2.2. Smad 1/5/8 signalling in the patterning of the dorsal spinal cord (SC)

2.2.1. Dorso-ventral gradient of BMP-Smad signalling in the SC

BMPs act over the area between the roof plate and intermediate region of the SC to pattern the dorsal SC, with the concentration of active BMP proteins (which decreases ventrally from the roof plate) being crucial for this patterning process (Liu and Niswander, 2005). Roof plate-derived BMPs achieve their inductive effects both locally, via direct cell-cell communication, and over a long range, via BMP-binding proteins which establish diffusible BMP gradients. In Drosophila, BMPs (Dpp) interact with chordin (Sog) to form a hetero-complex which blocks BMP function (Biehs et al., 1996; Piccolo et al., 1996). BMP can be released from this complex by the action of Tolloid, a zinc metalloprotease (Marques et al., 1997). The dynamics of this complex formation and dissociation partly determines the dorso-ventral gradient of Dpp signalling, which results in a dose-dependent induction of dorsal and intermediate cell types in the neural tube (Nunes Da Fonseca et al., 2010; Shimmi and O'connor, 2003).

2.2.2. Patterning of the dorsal SC neuronal populations

BMP-Smad 1/5/8 signalling has been extensively studied in the patterning of the dorsal spinal cord (SC) (Fig. 1F). The roof plate and the overlying epidermal ectoderm are rich sources of BMPs (Fig. 1B) (Lee and Jessell, 1999; Lee et al., 1998; Liem et al., 1995), and a high degree of Smad 1/5/8 transcriptional activity has been found in this region *in vivo*, in studies using mice that express GFP under the control of the BRE sequence (Monteiro et al., 2008).

In the dorsal SC there are six discrete parallel layers of dorsal interneuronal (dI) populations, termed dI1-6 interneurons, that differentiate at progressively more ventral positions, with dI1–dI5 interneurons functioning in somatosensation, and dI6 interneurons contributing to the locomotor circuitry (Goulding, 2009). The first demonstration of the involvement of roof plate-derived BMPs in dorsal SC patterning arose when dorsal dI1A interneurons were lost in GDF7 null mice (Lee et al., 1998). Subsequently, ablation of the roof plate in mice resulted in the absence of dorsal SC interneuronal populations (Lee et al., 2000, Millonig et al., 2000), whereas addition of BMPs induced a dorsal SC cell phenotype in chick neural explants (Liem et al., 1997). These inductive effects have been shown to be dependent on both BMPR1a and BMPR1b (Timmer et al., 2002), specifically with BMPR1a promoting the proliferation of dorsal SC interneuron precursors, and BMPR1b promoting their neuronal differentiation (Panchision et al., 2001). In agreement with these findings, there was a significant loss of the most dorsal of the six interneuronal populations in BMPR1a/BMPRIb double knockout mice (Wine-Lee et al., 2004) which was also observed through forced expression of noggin, or through Smad4 knockdown in the chick embryo (Chesnutt et al., 2004). Similarly, new data have shown that BMP7, Smad1 and Smad5 are all required for the generation of dI1, dI3 and dI5 interneuronal populations in mice (Le Dreau et al., 2012), demonstrating that BMP-Smad signalling is critical for the patterning of the dorsal SC region.

2.2.3. BMP-Smad and WNT interactions in the patterning of the dorsal SC

To generate these classes of dorsal interneurons, BMP-Smad 1/5/8 cooperates with WNT-β-catenin signalling to control the expression of Olig3, a transcription factor that is essential for the generation of dI1-dI3 interneurons (Muller et al., 2005), with BMP-Smad 1/5/8 acting upstream of WNT-β-catenin (Zechner et al., 2007). It has been proposed that WNTs are responsible for the proliferation of BMP-specified dorsal interneuronal progenitors in the SC (Chesnutt et al., 2004), a suggestion supported by the finding that WNT signalling promotes progression from G_1 to S and inhibits cell cycle exit in the neural tube (Megason and Mcmahon, 2002). Furthermore, β-catenin knockout inhibits neural stem cell (NSC) proliferation (Zechner et al., 2003), while overexpression of constitutively active β-catenin promotes NSC proliferation (Chenn and Walsh, 2002), in the developing mouse CNS. In addition to this, Smad6 inhibition of both BMP-Smad 1/5/8 signalling and WNT-β-catenin signalling promotes the transition of neural progenitors from a proliferative state to a differentiating state in the chick dorsal SC (Xie et al., 2011). The other inhibitory Smad, Smad7, is expressed in newly differentiating neurons in the intermediate SC and, when ectopically expressed dorsally in the chick SC, blocks the acquisition of the dorsal interneuron dI1 and dI3 fates and results in a dorsal expansion of dI4–dI6 fates (Hazen et al., 2011). Hazen et al. (2011) showed the inhibition of BR-Smad activity by Smad7, and suggest that Smad7 functions to attenuate BMP-Smad induction of more dorsal fates to allow the generation of intermediate cell types in the SC. Interestingly, possibly contradictory to the findings of Xie et al. (2011), a model for inhibitory cross-regulation of BMP-Smad signalling and WNT signalling was recently proposed, in which proliferation-inducing WNT-signalling and differentiation-inducing BMP-Smad signalling inhibit

each another to maintain slow-cycling, undifferentiated neural progenitors in the developing dorsal SC (Ille et al., 2007). Perhaps Smad6 functions to allow dorsal interneurons to exit this progenitor state and complete differentiation. Collectively these findings suggest that BMP-Smad signalling and Wnt-β-catenin signalling function cooperatively during dorsal SC neurogenesis, with BMP-Smad important in the specification of neural fates and Wnt-β-catenin signalling functioning in appropriate proliferation of these BMP-specified dorsal interneuronal precursors. In light of contrasting suggestions of Ille et al. (2007) and Zechner et al. (2007), it remains to be determined whether their functional cooperation is mediated through a direct downstream positive regulation of one another, or indeed whether they have an inhibitory cross-regulatory relationship during SC development.

2.2.4. BMP-Smad signalling in the generation of intermediately located SC neuronal populations

In zebrafish *swirl*, *snailhouse* and *sbn* mutants, there is an increase in the intermediately located Lim1$^+$ interneurons (Nguyen et al., 2000). However, further reductions in BMP-Smad signalling by administration of chordin to *swirl* embryos caused a decrease in the number of these cells (Nguyen et al., 2000). These contrasting findings suggest that an intermediate level of BMP-Smad signalling is required to establish the correct number of Lim1$^+$ interneurons. Indeed, Chesnutt et al. (2004) have shown that BMP signalling positively regulates the expression of WNTs at the dorsal SC, which is supported by the finding that double knockout of BMPR1a and BMPR1b causes a reduction in the expression of WNTs in the mouse SC (Wine-Lee et al., 2004). Perhaps the reduction in BMP-Smad signalling in the BMP mutants allows the expansion of more ventral cell types, causing an increase in Lim1$^+$ interneurons. However, the further reduction in BMP signalling by chordin administration may negatively affect WNT expression, resulting in the inhibition of the proliferative effect of WNTs on SC interneurons, thus leading to a decrease in Lim1$^+$ interneurons. Similarly, in the dorsal SC of the chick embryo, strong activation of BMP-Smad signalling decreases the intermediately located neurogenin 1-expressing cells (dI2), while weak activation causes the ventral expansion of neurogenin 1-expressing cells (Timmer et al., 2002). These results, as well as the afore-mentioned Hazen et al. (2011) study, support the concept that distinct levels of BMP-Smad 1/5/8 signalling are required for the development of different dorsal and intermediate interneuronal populations in the dorsal SC, and that mechanisms must be in place to ensure the tight control of the levels of BMP-Smad signalling during each distinct developmental programme. Furthermore, the dorsal-ventral gradient of BMP-Smad 1/5/8 signalling in the SC is also important in the specification of ventral SC motor neurons, with its active repression being required for their induction (Liem et al., 2000; Mcmahon et al., 1998). In support of this, Sip1 has recently been implicated as a novel regulator of SC motor neuron diversification, with Sip1 playing an important role in visceral motor neuron differentiation (Roy et al., 2012). In addition to the roles described above, BMP-Smad signalling has been shown to play important roles in the patterning of the ventral SC, dorsal SC neuronal axonal guidance, forebrain development, and cerebellar granule neuron development (Alder et al., 1999; Furuta et al., 1997; Liu and Niswander, 2005).

3. Smad 1/5/8 signalling in neuronal and glial development

3.1. Smad 1/5/8 signalling in neurogenesis

Smad 1/5/8 signalling in response to the BMP family of proteins is vital in several aspects of NS development, such as its inductive and patterning roles which have been outlined above. However, in addition to its role in the regional specification, BMP-Smad signalling also has direct effects on the development of neuronal and non-neuronal cell populations from neural progenitor cells (see Table 1).

3.1.1. BMP-Smad 1/5/8 signalling in neuronal development in the PNS

In the developing PNS, BMP-Smad signalling instructs neuronal differentiation from PNS NCCs via the induction of Mash1, a neuron-specific transcription factor (Groves and Anderson, 1996; Shah et al., 1996). Conversely, BMP-Smad signalling has also been shown to inhibit neuronal differentiation from CNS NSCs through the degradation of Mash1, resulting in the inhibition of neurogenesis (Shou et al., 1999). Such ambiguity may reflect intrinsic differences between PNS NCCs and CNS NSCs, with development of the former from the ectoderm requiring BMP-Smad signalling, and the generation CNS NSCs from the ectoderm being dependent upon the inhibition of BMP-Smad signalling. As mentioned in Section 2.1, BMP-Smad signalling induces the differentiation of adrenergic

Table 1
Neural fate induction by BMP-Smad signalling during NS development.

Neural precursor	Differentiated neural cell type	Reference(s)
Embryonic		
Peripheral NS NCC	Adrenergic sympathetic neuron	Reissmann et al. (1996); Varley and Maxwell (1996); Varley et al. (1998); Wu and Howard (2001)
Enteric NS NCC	Enteric dopaminergic neuron	Chalazonitis et al. (2004); Chalazonitis et al. (2008)
Enteric NS NCC	Astroglia	Chalazonitis et al. (2011)
Ventral midbrain neural precursors	VM dopaminergic neuron	Jordan et al. (1997); Krieglstein et al. (1995); O'Keeffe et al. (2004)
Ventral midbrain neural precursors	Astroglia	Krieglstein et al. (1995); O'Keeffe et al. (2004); Wood et al. (2005)
Metencephalic NSC	Cerebellar granule neuron	Alder et al. (1999); Qin et al. (2006)
Cortical NSC	Cortical neuron	Li et al. (1998); Mehler et al. (2000); Sun et al. (2010); Yung et al. (2002)
Cortical NSC	Astroglia	Mehler et al. (2000)
Dorsal telencephalic NSC	Dentate gyrus granule neuron	Caronia et al. (2010)
Septal NSC	Basal forebrain cholinergic neuron	Cho et al. (2008); Lopez-Coviella et al. (2000); Lopez-Coviella et al. (2005)
SVZ NSC	Stellate, non-neurogenic astroglia	Bonaguidi et al. (2005)
Dorsal spinal cord NSC	Dorsal spinal cord interneuron	Hazen et al. (2012); Le Dreau et al. (2012); Xie et al. (2011)
Postnatal/adult		
Perinatal cortical NSC	Astroglia	Mehler et al. (2000)
Postnatal subcortical O-2A progenitor cell	Astroglia	Mabie et al. (1997)
Adult SVZ NSC	Olfactory granule neuron	Colak et al. (2008)
Adult SVZ NSC	Astroglia	Cate et al. (2010); Ciceroni et al. (2010); Lim et al. (2000)
Adult Hippocampal NSC	Astroglia	Brederlau et al. (2004)
Adult spinal cord OPC	Astroglia	Cheng et al. (2007)

sympathetic neurons from avian trunk NCCs, with the BMPR1a receptor particularly important in this process (Fig. 1C) (Varley and Maxwell, 1996; Varley et al., 1998). Similarly, BMP-Smad signalling produced *in vitro* by dorsal aorta explant-derived BMPs has been shown to induce sympathetic differentiation from quail NCC cultures (Reissmann et al., 1996). The role of Mash1 in BMP-Smad-induced sympathetic neuronal differentiation was not assessed in these studies, but considering its identification in the Shah et al. (1996) study described above, it is likely to contribute to this neuronal specification. The catecholaminergic differentiation of avian NCCs was also shown to be mediated by BMP-Smad signalling *in vitro* (Wu and Howard, 2001). Wu and Howard (2001) demonstrated that Smad1 induced the expression of dHAND, a DNA binding protein required for the differentiation of catecholaminergic neurons. Collectively, these studies show that BMP-Smad signalling plays an important role in neuronal differentiation in the PNS. In addition to its inductive role in sympathetic neuronal development, BMP-Smad signalling also promotes NGF-dependent dendritic outgrowth from sympathetic neurons (Lein et al., 1995). In the enteric NS, which is part of the PNS located in the wall of the gastrointestinal tract and is also derived from NCCs, BMP-Smad signalling induces the differentiation of trkC-expressing dopaminergic neurons (Fig. 1C) (Chalazonitis et al., 2004; Chalazonitis et al., 2008).

3.1.2. Smad 1/5/8 signalling in neuronal fate induction in the CNS

Similar to its role in the PNS, Smad 1/5/8 signalling is directly involved in the neurogenesis of various CNS neural populations. GDF5-Smad signalling induces an increase in DA neurons in E14 rat VM cultures (Fig. 1E) (Clayton and Sullivan, 2007; Krieglstein et al., 1995; O'Keeffe et al., 2004; O'sullivan et al., 2010; Wood et al., 2005), while not increasing the total number of neurons (O'Keeffe et al., 2004). Similarly, Smad 1/5/8 signalling in response to BMPs increases the numbers of DA neurons in E14 rat VM cultures (Jordan et al., 1997). This role of BMP-Smad signalling in VM DA neurogenesis is of interest to the field of Parkinson's disease research, a disorder in which VM DA neurons progressively degenerate (Lees et al., 2009; Toulouse and Sullivan, 2008), particularly for cell based therapies in which factors which promote the generation of VM DA neurons are in demand. In neural tissue cultured from E8 mouse VM/ventral metencephalon region, BMP-Smad signalling induced early markers of cerebellar granule progenitors (Fig. 1E), including Math1 and En1/En2 (Alder et al., 1999). When these Smad 1/5/8-induced cells were subsequently transplanted into the early postnatal cerebellum, they formed mature granule neurons (Alder et al., 1999). Furthermore, double knockdown of BMPRIa and BMPRIb results in a dramatic reduction in the number of cerebellar granule neurons in mice, with a concurrent downregulation of molecular markers of granule cell specification (Qin et al., 2006). BMP-Smad signalling is thus an important regulator of cerebellar granule neuron generation, with both BMPRIa and BMPRIb required for their specification. In support of a role for BMP-Smad 1/5/8 signalling in cerebellar development, the knockout of the transcription factor Zfp423 in mice, which binds to Smad1/Smad4 in response to BMP signalling (Hata et al., 2000), results in an underdeveloped cerebellum (small cerebellar hemispheres and severe reduction in vermis size) (Warming et al., 2006). In ventricular zone (VZ) neocortical neuroepithelial cell cultures, BMP-Smad signalling was reported to induce neuronal differentiation (Fig. 1E) (Li et al., 1998). Similarly, noggin-regulated BMP-Smad signalling was shown to be involved in the elaboration of cortical GABAergic neurons from migrating ventral forebrain progenitors (Yung et al., 2002). This finding suggests that an intermediate, modulated level of BMP-Smad signalling plays a role in GABAergic neuronal differentiation in the cortex. Li et al. (1998) also showed that Smad 1/5/8 signalling in response to BMPRIa is required for neural precursors to differentiate and migrate away from the VZ in cortical explants. In support of this, BMP-Smad signalling, specifically via Smad1, has been shown to control neuronal migration and neurite outgrowth in the embryonic rodent cortex by suppressing the transcription of CRMP2, with Smad1 and Smad4 being demonstrated to bind to the CRMP2 promoter in the neocortex (Sun et al., 2010). BMPRIa-Smad1 dependent BMP signalling is therefore important for cortical neuronal migration and differentiation. In E14 murine septal cultures, BMP-Smad signalling was shown to induce both a cholinergic phenotype and the expression of a number of genes belonging to the transcriptome of basal forebrain cholinergic neurons, suggesting a role for BMP-Smad 1/5/8 signalling in the development of these neurons (Fig. 1E) (Lopez-Coviella et al., 2000; Lopez-Coviella et al., 2005). A transcriptional co-activator of BMP-Smad signalling, known as Smad-interacting zinc finger protein, was shown to be required for this BMP-Smad signalling-dependent induction of a cholinergic phenotype in E13.5 murine septal cultures (Cho et al., 2008). In mice that were deficient in BMPR1a and BMPR1b in the dorsal telencephalon, there was a decreased production of dentate gyrus (DG) granule neurons at the peak of DG neurogenesis and throughout life, showing a role for BMP-Smad signalling in DG granule cell neurogenesis (Fig. 1E) (Caronia et al., 2010). The resulting hippocampal defects led to fear-related behavioural deficits, demonstrating the functional importance of BMP-Smad-regulated DG neurogenesis. Using shRNA knockdown at the time of neurogenesis, Le Dreau et al. (2012) further demonstrated a role for Smad1 and Smad5 in primary neurogenesis (generation of projection neurons). This study showed that BMP7-stimulated Smad1 and Smad5 signalling was required for the generation of dI1, dI3 and dI5 interneuronal populations in the chick dorsal SC (Fig. 1E and F) (Le Dreau et al., 2012). Surprisingly, Smad6 inhibition of BMP-Smad 1/5/8 signalling, and of WNT-β-catenin signalling, was shown to promote neuronal differentiation in the intermediate zone of the chick dorsal SC (Xie et al., 2011). This result does not preclude an involvement of BMP-Smad and WNT-β-catenin signalling in the neuronal differentiation of dorsal SC neurons, but rather reflects a role for Smad6 in directing BMP-specified neuronal progentiors to exit the cell cycle and terminally differentiate.

In addition to a neuronal inductive role in the dorsal SC, Smad1-dependent BMP signalling has been shown to regulate axonal growth in the dorsal root ganglion (DRG), with the reactivation of Smad1 signalling in adult DRG resulting in sensory axon regeneration in a mouse model of SC injury (Parikh et al., 2011). In support of this, the inhibitory Smad, Smad6, is a potent inhibitor of dI1 axon outgrowth in the chick SC (Hazen et al., 2011). Furthermore, BMP-Smad signalling in the dorsal SC acts as a chemorepellent that orients the commissural axons of dI1 interneurons, directing them ventrally (Butler and Dodd, 2003; Dent et al., 2011), and also acts to regulate the growth rate of these axons as they extend through the SC (Phan et al., 2010). This chemorepellent role of BMP-Smad signalling was recently demonstrated to be mediated by the BMPRIb (Yamauchi et al., 2008). A more recent paper by Hazen et al. has suggested that Smad1 and Smad5 confer diverse functions during the development of the dorsal SC. Knockdown experiments demonstrated that Smad1 is critical for the regulation of dI1 axonal growth while Smad5 is required for the specification of dI1 and dI3 interneuronal populations (Hazen et al., 2012). This is an important finding as it suggests that the various BR-Smads have distinct functions in the developing SC, whereas previous studies suggested that these BR-Smads function redundantly during the development of the NS (Arnold et al., 2006; Le Dreau et al., 2012). Parikh et al. (2011) also showed that inhibition of BMP-Smad 1/5/8 signalling using dorsomorphin, a small molecular inhibitor of

BMPRI (Yu et al., 2008), negatively affects neurite outgrowth of E18.5 mouse hippocampal neurons. Perhaps this role of BMP-Smad signalling in the establishment of hippocampal neuronal projections contributes to the fear-related behavioural deficits caused by BMPR1a and BMPR1b conditional double mutation outlined above. Additionally, BMP-Smad signalling has been shown to induce neurite outgrowth from E14 rat VM DA neurons *in vitro* (O'Keeffe et al., 2004; Reiriz et al., 1999), and recently the direct involvement of Smad 1/5/8 signalling in this process has been demonstrated in a model of human midbrain DA neurons (Hegarty et al., 2013). BMP-Smad signalling therefore not only regulates neuronal specification, but also promotes neuronal differentiation and maturation in several regions of the embryonic CNS.

Furthermore, BMP-Smad signalling is actively involved in neuronal differentiation during post-natal and adult life. BMP-Smad signalling is required for the initiation of neurogenesis in adult mouse subventricular zone (SVZ) NSCs (Fig. 1H) and the concurrent suppression of an oligodendroglial fate, since Smad4 knockdown or noggin infusion results in a significant decrease in neurogenesis and an increase in the differentiation of oligodendrocytes (Colak et al., 2008). As Smad4 knockdown or noggin infusion would also inhibit TGFβ signalling, it is possible that these effects were are a result of TGFβ inhibition, however Colak et al. (2008) showed that this Smad4-mediated neurogenic role is BMP-dependant as conditional deletion of the TGFβ type II receptor did not replicate the reduced neurogenesis and increase in olidodendrocyte differentiaton caused by Smad 4 knockdown or noggin infusion. In contrast to this, it was recently shown that LRP2-mediated catabolism of BMP4 is required for neurogenesis in the adult mouse, since increases in BMP-Smad 1/5/8 signalling as a result of LRP2 knockdown coincide with reduced neurogenesis (Gajera et al., 2010). Similarly, increased BMP signalling was shown to potently inhibit neurogenesis of adult mouse SVZ NSCs *in vitro* and *in vivo*, while noggin promoted neurogenesis (Lim et al., 2000). These contradicting results may reflect a potential need for a regulated, intermediate level of BMP-Smad signalling to allow adult SVZ neurogenesis. Furthermore, BMP-Smad signalling regulators, such as noggin and LRP2, may indeed be involved in refining Smad 1/5/8 signalling to promote/permit neurogenesis. Indeed, noggin-regulated BMP-Smad signalling plays a role in embryonic cortical GABAergic neurogenesis (Yung et al., 2002), while regulated levels of Smad 1/5/8 signalling is required for the appropriate generation of intermediately located interneuronal populations in the development of the SC (Nguyen et al., 2000, Timmer et al., 2002).

3.1.3. Smad 1/5/8 signalling in the neuronal differentiation of neural cell lines

In agreement with their roles in inducing neuronal differentiation in the PNS, BMP-Smad signalling has also been shown to induce neuronal differentiation in a sympathetic NS-derived cell line, known as the PC-12 cell line (Paralkar et al., 1992). Conversely, in a sympathoadrenal progenitor cell line, BMP-Smad signalling was shown to induce apoptosis, which could be rescued by the addition of growth factors such as NGF (Song et al., 1998). BMP-Smad signalling thus induces dependence on exogenous growth factors for survival in these cells. This finding is surprising, considering the well-established neurotrophic properties of BMPs, however it may suggest a role for BMP-Smad signalling in sympathetic target innervation. In the human neuroblastoma SH-SY5Y cell line, BMP signalling induces neuronal differentiation through a BMPRI-Smad 1/5/8 mediated pathway (Hegarty et al., 2013; Nakamura et al., 2003; Toulouse et al., 2012). Likewise, in the mouse neuroblastoma-derived cell line, Neuro2a, BMP-stimulated phospho-Smad 1/5/8 nuclear translocation induces neuronal differentiation through a bi-phasic regulation of Id protein expression, and subsequent upregulation of the neural-specific transcriptional factors Dlx2, Brn3a, and NeuroD6 (Du and Yip, 2010). The use of noggin or Smad1 siRNA prevented this Smad-mediated regulation of Id protein expression. Thus, BMP-Smad signalling is also capable of inducing neuronal differentiation in neural cell lines.

3.2. Smad 1/5/8 signalling in gliogenesis

Despite its role in promoting neuronal differentiation from various neural precursor populations, Smad 1/5/8 signalling has also been shown by many studies to promote NSC differentiation towards an astrocytic lineage. For example, in serum-free mouse embryonic cell cultures, BMP-Smad signalling induces the generation of glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes, while concomitantly inhibiting cellular proliferation (D'alessandro and Wang, 1994; D'alessandro et al., 1994). Similarly, in embryonic mouse SVZ multipotent progenitors, BMP-Smad 1/5/8 signalling induces the generation of GFAP-expressing astrocytes (Fig. 1E), with concurrent suppression of neuronal and oligodendroglial cell fates (Gross et al., 1996). Knowledge on the astroglial inductive effect of BMP-Smad signalling in the embryonic SVZ was recently refined to show that a particular subtype of GFAP-expressing cells are generated in response to Smad 1/5/8 activation. Specifically, stellate, post-mitotic, non-neurogenic GFAP-expressing cells, representative of mature astrocytes, are generated from mouse embryonic SVZ progenitor cells in response to BMP-Smad signalling (Bonaguidi et al., 2005). Conversely, leukemia inhibitory factor (LIF) signalling gives rise to bipolar/tripolar, self-renewing, neurogenic GFAP-expressing progenitors, representative of radial glial SVZ stem cells (Bonaguidi et al., 2005). However, these studies did not assess Smad activity, and thus further experiments are necessary to determine if these effects require canonical Smad 1/5/8 signalling. In a more recent study on E14 dorsal SC cultures, the prevention of BMP-induced Smad1 transcriptional activity by FGF-MAPK signalling promoted OPC generation from NSCs through the upregulation of olig2, with Smad1/Smad4 being shown to associate with the olig2 promoter (Bilican et al., 2008). This interaction of the Smad1/Smad4 complex with the olig2 promoter may therefore result in transcriptional repression. Despite inducing a neuronal lineage in NCCs, BMP-promoted nuclear translocation of phospho-Smad 1/5/8 induced glial differentiation in the NCC population that gives rise to the enteric NS (Fig. 1C) (Chalazonitis et al., 2011). As mentioned in Section 3.1.1, BMP-Smad signalling is known to induce DA neurons from these NCCs (Chalazonitis et al., 2004; Chalazonitis et al., 2008), however it has been proposed that BMP-Smad signalling determines the responsiveness of these enteric NCCs firstly to glial cell line derived neurotrophic factor (GDNF)-induced neurogenesis, and later to glial growth factor (GGF)-2-induced gliogenesis (Chalazonitis et al., 2011; Chalazonitis and Kessler, 2012). Similarly, GDF5-Smad signalling dramatically increases the number of astrocytes in E14 rat VM cultures (Fig. 1E), while concomitantly increasing the numbers of DA neurons (Krieglstein et al., 1995; O'Keeffe et al., 2004; Wood et al., 2005). Despite inherent differences between CNS NSCs and PNS NCCs, perhaps there is a similar mechanism, to that proposed by Chalazonitis and colleagues for enteric NCCs, to explain the dual inductive role of GDF5-Smad signalling in E14 rat VM NSCs. In support of such a suggestion, GDNF is an important neurotrophic factor for the DA neurons of the VM, and may thus work cooperatively with BMPs in this population also (Peterson and Nutt, 2008; Toulouse and Sullivan, 2008).

In addition to its glial-inducing effects on embryonic pluripotent progenitors, Smad 1/5/8 signalling has been demonstrated to

play a direct role in adult gliogenesis. BMP-Smad signalling induces postnatal subcortical bipotent oligodendroglial-astroglial (O-2A) progenitor cells to differentiate into astrocytes (Fig. 1H), while concomitantly suppressing oligodendroglial differentiation, in a dose-dependent manner (Mabie et al., 1997). Although the presence of type I and type II BMPRs on these cells was demonstrated, the activation of canonical Smad 1/5/8 pathway was not confirmed in this study (Mabie et al., 1997). The response of neural progenitors to BMP-Smad signalling appears to be temporally dependent. For example, cultures of cortical VZ neural progenitors have been shown to respond differentially to BMP-Smad signalling, depending on their ontogenic stage. At E13, BMP-Smad signalling promotes cell death and inhibits proliferation of early VZ progenitors, while at E16, the response to BMP signalling is concentration-dependent with either enhancement of neuronal and astroglial elaboration (at 1–10 ng/ml) or potentiation of cell death (at 100 ng/ml) (Mehler et al., 2000). It would be interesting to determine whether activation of different signalling pathways contributes to these divergent effects. Indeed, particularly high BMP levels may act via pathways that are independent of Smad 1/5/8 (Nohe et al., 2004; Rajan et al., 2003). Mehler et al. (2000) showed that BMP-Smad signalling enhances the generation of astroglia during the perinatal period of cortical gliogenesis (Fig. 1H). Interestingly, the inhibitory effect of BMP-Smad signalling on oligodendroglial generation occurred at all stages (Mehler et al., 2000). Similarly in adult rat SC oligodendrocyte precursor cell (OPC) cultures, shown to express the BMPRs, BMP-Smad signalling promotes astrocytic differentiation in a dose-dependent manner (Fig. 1H), with concurrent suppression of oligodendrocyte differentiation, by increasing Id4 expression and decreasing the expression of olig1 and olig2 (Cheng et al., 2007). In support of this finding, the Smad1/Smad4 complex has been suggested to interact with the olig2 promoter to repress olig2 expression (Bilican et al., 2008). A recent paper has identified Sip1 as an important regulator of oligodendrocyte differentiation and myelination. Sip1 represses BMP-Smad signalling, via a dual mechanism involving direct antagonism of Smad 1/5/8 and induction of Smad7 expression, to promote oligodendrocyte differentiation in the CNS (Weng et al., 2012). Cheng et al. (2007) propose that the blocking of BMP-Smad signalling combined with olig1/2 overexpression could potentially enhance endogenous remyelination in CNS demyelination disorders. In support of this suggestion, local increases in BMPs at the site of demyelination upregulates gliosis, with these astrocytes displaying increased phospho-Smad 1/5/8 signalling (Fuller et al., 2007). Furthermore, BMP-induced increases in the expression of chondroitin sulphate proteoglycans, such as neurocan and aggrecan, were also observed which could inhibit remyelination (Fuller et al., 2007). However, astrocytes generated in response to BMP-Smad signalling support axonal growth and regeneration of host sensory neurons when transplanted into a lesioned dorsal column of the SC (Haas et al., 2012). Cultured adult hippocampal NSCs were shown to be induced to an astroglial lineage in response to BMP signalling through the BMPR1b receptor (Fig. 1H) (Brederlau et al., 2004). In addition to this, a recent study modulated BMP-Smad signalling to alter the numbers of astrocytes and oligodendrocytes in the adult SVZ during cuprizone-induced demyelination (Cate et al., 2010). Components of the BMP-signalling pathway, including BMPRs and Smad 1/5/8, were shown to be up-regulated during cuprizone-induced demyelination, along with an increase in astrocytes *in vivo*. The reduction of Smad 1/5/8 activation by intraventricular infusion of noggin resulted in a decrease in the numbers of astrocytes, and an increase in the number of oligodendrocytes in the adult SVZ during cuprizone-induced demyelination (Cate et al., 2010). Similarly, activation of the mGlu3 receptors in post-natal mouse SVZ NSCs attenuates the astroglial-promoting effect of phosphorylated Smad 1/5/8, via the mitogen-activated protein kinase pathway, which suggests a role for glutamate in SVZ NSC differentiation (Ciceroni et al., 2010). This further demonstrates the negative regulation of phospho-Smad 1/5/8 signalling by the MAPK pathway. In support of the role of BMP-Smad signalling in adult SVZ astrogliogenesis, Lim et al. (2000) also showed that BMP signalling induces an astroglial lineage in adult SVZ NSCs. Furthermore, it has been shown that mouse brain endothelial cells, which lie close to adult SVZ NSC, are the source of BMP-Smad signalling which regulates the development of these NSCs (Mathieu et al., 2008). The transgenic overexpression of BMP4 in mice resulted in an increase in astrocytes in multiple brain regions, with a concurrent decrease in oligodendrocytes, suggesting that BMP-Smad signalling is a likely mediator of astrocyte development *in vivo* (Gomes et al., 2003). The adult SVZ is now widely accepted to be a major site of neurogenesis (Nieto et al., 2004; Noctor et al., 2004; Pontious et al., 2008; Tarabykin et al., 2001; Zecevic et al., 2005). However, the effect of BMP-Smad signalling on SVZ neurogenesis remains unclear. The majority of evidence suggests that Smad 1/5/8 signalling results in astroglial differentiation, however Colak et al. (2008) showed that BMP-Smad signalling was required to initiate the neurogenic lineage in the adult SVZ. These results may not be as contradictory as they appear, and it may well be the case that both findings are directly related and even support one another. Perhaps in adult SVZ NSCs, BMP-Smad signalling acts to induce radial glial-like neuronal progenitors, with astrocytic characteristics, that subsequently generate the granule neurons which migrate to the olfactory bulb. The role of such 'astroglial' progenitors in neurogenesis has been well described in a recent review (Kriegstein and Alvarez-Buylla, 2009).

3.3. Smad 1/5/8 signalling in neural stem/progenitor cells

It is clear that Smad 1/5/8 signalling can induce a neuronal or astrocytic fate, or possibly even both, in neural precursors, but the factors which determine each of these fates are unknown. In the chick SC, BMP-Smad signalling, possibly mediated via the BMPR1b receptor, promotes neuronal specification rather than astrocytic specification in the dorsal-most progenitors at E5 (Agius et al., 2010). However, this study reported the opposite effect at E6, when BMP-Smad signalling promoted astrocyte development (Fig. 1E), rather than completely preventing it as it did at E5. This complete reversal of the neurogenic action was suggested to be due to an upregulation of BMPR1a receptors (Agius et al., 2010). It is thus likely that these differential inductive effects of Smad 1/5/8 signalling not only depend on the specific ligand-receptor combination, but are also temporally dependent, an example of which be seen in the Mehler et al. (2000) paper discussed above. Furthermore, in the hippocampus, BMP-Smad signalling induces granule neuron generation pre-natally (Caronia et al., 2010), and astroglial differentiation during adulthood (Brederlau et al., 2004). This is not surprising, considering that primary neurogenesis precedes gliogenesis during CNS development. Logically, cell identity is a determining factor, with PNS NCCs being induced towards a neuronal fate and CNS embryonic SVZ precursors being induced to an astrocytic one during pre-natal NS development.

It is important to note that through the induction of differentiation, Smad 1/5/8 signalling negatively regulates the proliferation of NSCs. In rat NSC cultures derived from either the cortex or SC at E13.5 BMPs induced NSC growth arrest and GFAP expression through Smad signalling, however FGF2 prevented BMP-Smad-induced terminal astrocytic differentiation to preserve NSC potency in a dormant state (retain nestin expression but do not proliferate) (Sun et al., 2011). This effect is likely to reflect FGF-MAPK-induced repression of Smad 1/5/8 signalling. Similarly, BMP-Smad signalling negatively regulates NSC proliferation in the

adult hippocampus (Bonaguidi et al., 2008). Smad 1/5/8 signalling in response to BMPR1a receptor activation by BMPs decreased proliferation of cultured adult hippocampal NSCs, while maintaining them in an undifferentiated state (Mira et al., 2010). Mira and co-workers also showed that knockout of either Smad4 or BMPR1a in hippocampal NSCs results in a transient increase in proliferation, followed by a reduction in the generation of neural precursors, demonstrating that Smad 1/5/8 signalling regulates NSC quiescence/proliferation and prevents the loss of NSC activity, which supports continuous neurogenesis, in the adult hippocampus. Taken together with the results of the Sun et al. study, the above findings support a role for Smad 1/5/8 signalling in the regulation of NSC proliferation and differentiation. Interestingly, this regulation of NSC proliferation in the hippocampus by BMP-Smad signalling has been suggested to be pertinent to the effects of exercise on adult hippocampal neurogenesis in mice, that is, exercise induced downregulation of BMP-Smad signalling results in enhanced neurogenesis (Gobeske et al., 2009). The Mira et al. (2010) also supports the suggestion that BMP-Smad signalling induces the differentiation of adult SVZ NSCs into radial glial progenitors, which subsequently give rise to olfactory interneurons.

In mouse embryonic stem cells, BMP-Smad signalling promotes the self-renewal, and inhibits the differentiation through the induction of Id proteins (Ying et al., 2003). This result is interesting considering the effects of BMP-Smad signalling to promote differentiation of NSCs. These contrasting results are likely to reflect the difference between embryonic stem cells and stem cells that are restricted (through inhibition of BMP-Smad signalling) to a neural fate.

4. Smad 1/5/8 knockdown during nervous system development

It has proven difficult to determine the effect global deletion of Smad 1/5/8 on NS development, as the Smad1 mutant mice die at E10.5 due to defects in allantois formation (Lechleider et al., 2001; Tremblay et al., 2001), and Smad5 mutant mice die at E10.5 due to angiogenic failure and other defects (Chang et al., 1999; Yang et al., 1999). However, Chang et al. (1999) did show a failure of cranial neural tube closure in Smad5 mutant mice at E9.5 (see Table 2), which resulted in exencephaly, demonstrating the importance of Smad5-mediated signalling in cranial neural tube development. The study of non-null Smad mutants has highlighted the importance of BMP-Smad signalling in NS development (see Table 2). Heterozygous Smad1 mutant mice, and mice homozygous for a hypomorphic allele of Smad8, show midbrain and hindbrain reductions (gross reduction in anatomical size) at E11.5 (Hester et al., 2005). This is not surprising due to the role of BMP-Smad 1/5/8 signalling in cerebellar development (discussed above). However in contrast to this, Smad8 null mutant mice are viable and fertile, with no discernible abnormalities (Arnold et al., 2006). Thus, Smad8 seems to play a nonessential role in development (mice develop without defects), and so the abnormalities reported by Hester et al. (2005) may be due to the neomycin protein affecting expression of neighbouring genes. It is more likely that the role of Smad8 is redundant; further investigation is required to determine the specific roles of Smad8 in developmental. When Arnold et al. (2006) crossed Smad8 mutant mice with heterozygous or homozygous Smad1 or Smad5 null alleles, there were no noticeable developmental disturbances in the $Smad8^{-/-}$:$Smad1/5^{+/-}$ mice, and the abnormalities associated with Smad1/Smad5 null mice were not exacerbated by the absence of Smad8. However, Smad1 and Smad5 double heterozygous mutant mice displayed striking phenotypic similarity to Smad1 and Smad5 mutant embryos and died at E10.5, suggesting that Smad1 and Smad5 function cooperatively in response to BMPs during development (Arnold et al., 2006). Indeed, although not related to NS development, Smad1 has been suggested to compensate for Smad5 loss in the angiogenic endothelium (Umans et al., 2007), while a triple conditional knockout study in mice reported that Smad1, Smad5 and Smad8 function redundantly in Mullerian duct regression (Orvis et al., 2008). Despite the limited information ascertained from studies involving complete knockdown of Smad1 and Smad5 in mice (due to mid-gestation embryonic lethality), the *sbn* mutant zebrafish allows the identification of severe neurodevelopmental defects caused by null mutations of Smad5 (Hild et al., 1999). The *sbn* mutant embryos are strongly dorsalised, displaying a complete loss of ventral and posterior structures, as well as a ventral expansion of dorsal structures such as the neuroectoderm and somites. This dorsalisation demonstrates the importance of BMP-Smad 1/5/8 signalling for appropriate development of the NS. In support of this role, these neurodevelopmental defects of the *sbn* mutant can be largely rescued by overexpression of BMPs,

Table 2
The effects of Smad 1/5/8 knockdown on nervous system development.

Mutation(s)/knockdown	Effect(s) on NS development	Reference(s)
Smad1		
Smad1±	Midbrain/hindbrain reductions (anatomical size)	Hester et al. (2005)
$Smad1^{flox/-}$; Wnt1 Cre	Decreased axonal growth of cultured adult DRG from these conditional mutant mice	Parikh et al. (2011)
Brn4::Cre; $Smad1^{flox/flox}$	Reduction in axonal growth of dI1 interneurons of the dorsal spinal cord	Hazen et al. (2012)
Smad1 siRNA	Inhibition of the axonal growth of cultured adult DRG neurons	Zou et al. (2009)
Smad1 siRNA	Inhibition of the axonal growth of cultured E12.5 DRG neurons	Parikh et al. (2011)
Smad1 shRNA	Reduced generation of dI1,dI3 and dI5 interneurons of the dorsal spinal cord	Le Dreau et al. (2012)
Smad5		
$Smad5^{-/-}$	Exencephaly (failure cranial neural tube closure)	Chang et al. (1999)
$Smad5^{-/-}$ (*sbn* mutant)	Ventral expansion of neuroectoderm and somites	Lele et al. (2001)
$Smad5^{-/-}$ (*sbn*)	trunk NCC deficiencies	Nguyen et al. (2000)
Brn4::Cre; $Smad5^{flox/flox}$	Reduction in dI1 and dI3 interneurons of the dorsal spinal cord	Hazen et al. (2012)
Smad5 shRNA	Reduced generation of dI1,dI3 and dI5 interneurons of the dorsal spinal cord	Le Dreau et al. (2012)
Smad8		
Smad8 (hypomorphic)	Midbrain/hindbrain reductions (anatomical size)	Hester et al. (2005)
Smad8 shRNA	reduced generation of dI1 interneurons of the dorsal spinal cord	Le Dreau et al. (2012)
Smad4		
Wnt1-Cre; Smad4	Defective cranial NCC derivatives	Ko et al. (2007)
Wnt1-Cre; $Smad4^{loxp/loxp}$	Defective NCC derivatives	Nie et al. (2008)
Smad4 siRNA	reduced generation of dI1 interneurons of the dorsal spinal cord, and dorsal expansion of dI2-4 interneuronal populations	Chesnutt et al. (2004)

a constitutively active form of the BMPRIb, or Smad1 (Nguyen et al., 1998). The somites of *sbn* mutants do not completely circulise, as the *swirl* (BMP) mutant embryo does (Kishimoto et al., 1997). Furthermore, *sbn* mutants survive segmentation unlike the slightly more severely dorsalised *swirl* mutant. At a later stage, the *sbn* embryo is characterised by loss of the tail and a 'snailshell-like' winding up of the trunk (Lele et al., 2001). Lele et al. (2001) produced a more strongly dorsalised zebrafish mutant embryo following further knockdown of Smad5 using morpholino injection. These embryos displayed additional features, such as a compressed anterior/head region, which was attributed to the total loss of Smad5 activity, unlike with the hypomorphic *sbn* allele. This finding is consistent with the cranial defects of Smad5 mutant mice described by Chang et al. (1999). The Smad 5 *sbn* mutant also displays NCC deficits, such as diminished trunk NCC formation, showing that BMP-activated Smad5 signalling is crucial for NCC generation (Nguyen et al., 2000). The conditional knockout of Smad1 and Smad5 in the mouse neural tube, demonstrated that these BR-Smads have distinct functions in the developing SC with Smad1 critical for the regulation of dI1 axonal growth and Smad5 required for the specification of dI1 and dI3 interneuronal populations (Hazen et al., 2012). This finding is surprising considering the wealth of evidence that suggests functional redundancy among Smad 1, 5 and 8 (Arnold et al., 2006, Le Dreau et al., 2012, Orvis et al., 2008, Umans et al., 2007). The conditional knockout of Smad4 in mouse NCC leads to the downregulation of genes critical for NCC development, such as the BMP target gene Msx1, and results in defective NCC derivatives which lead to mid-gestation death (Nie et al., 2008). In a similar study that focused on cranial NCC development, the conditional knockdown of Smad4 in NCC showed that BMP-Smad signalling is required for the fate specification of cranial NCC (Ko et al., 2007). It is important to note that the defects observed in these Smad4 mutants may be as a result of altered TGFβ signalling, and thus these studies demonstrate the importance of Smad4 in mediating the activities of BMPs, and/or TGFβs, in NCC development.

Due to the difficulty in generating viable Smad1/5/8 mutants, a number of studies have used RNA interference (RNAi) to determine the effects of the loss of Smad 1/5/8 signalling on the development of the NS (see Table 2), specifically in relation to the development of the dorsal SC. The knockdown of Smad1 in E12.5 DRG neurons by siRNA results in the inhibition of axonal growth capacity in these neurons, an effect which was rescued by an RNAi-resistant Smad1 construct (Parikh et al., 2011). Furthermore, cultured DRG neurons from Smad1 conditional knockout mice have a markedly decreased capacity to initiate or maintain axon extension (Parikh et al., 2011). A similar study that used RNAi to knockdown Smad1, and subsequently performed rescue experiments using an RNAi-resistant Smad1 construct, showed that Smad1 is required for axonal growth of cultured adult DRG neurons (Zou et al., 2009). These studies demonstrate the importance of Smad1 in DRG neuronal differentiation, specifically axonal outgrowth. In contrast to the Hazen et al. (2012) study mentioned above, *in vivo* shRNA knockdown experiments showed that BMP7, Smad1 and Smad5 are all required for the generation of dI1, dI3 and dI5 interneuronal populations in the chick (Le Dreau et al., 2012). Smad8 has also been shown to be required for the generation of dI1 interneurons, suggesting that Smad8 may not be completely functionally redundant during NS development (Le Dreau et al., 2012). Furthermore, in support of the idea of functional redundancy between Smad1 and Smad5, overexpression of a pseudo-phosphorylated mutant version of Smad1 rescued Smad5 loss of function, while pseudo-phosphorylated Smad5 overexpression rescued the Smad1 shRNA phenotype (Le Dreau et al., 2012). In light of the conflicting reports discussed in this review, whether Smad1 and Smad5 function redundantly or have unique but complementary roles during SC neurogenesis will be an interesting question for future research, as well as their mechanisms of action during this process. The inhibition of BMP-Smad 1/5/8 signalling in the dorsal SC by reducing the expression of Smad4 using siRNA, results in the loss of most of the dI1 interneuronal population, and the dorsal expansion of dI2-4 interneurons in the chick embryo (Chesnutt et al., 2004). These studies demonstrate that Smad1, Smad4 and Smad5 are essential for the generation of the dorsal SC interneuronal populations. The Hazen et al. (2012) study suggests that Smad1 is not required; however, these contradictory results may reflect subtle differences between mouse and chick SC patterning. Indeed, the disruption of Smad1 and/or Smad5 expression in the chick dorsal SC by RNAi showed that Smad1 can partially compensate for the loss of Smad5 in the chick (Hazen et al., 2012).

The knockdown studies discussed above provide further evidence for the importance of BMP-Smad 1/5/8 signalling in neural induction, neural crest development and dorsal SC development. Future studies involving the conditional knockout of Smad1, Smad5 and/or Smad8 or the use of RNAi for these BR-Smads in the developing NS, followed by phenotypic analysis of various regions of the CNS and PNS will provide further information regarding the roles of Smad 1/5/8 signalling in NS development. Despite the fact that the Smad 1/5/8 signalling pathway is the canonical signalling pathway of the BMP family, it cannot be assumed that the effects which BMPs exert on neural cells are mediated by these transcription factors. Knockout studies similar to the ones described above should be conducted, to conclusively verify the involvement of Smads 1/5/8 in mediating these effects.

5. Conclusion

The signalling of Smads 1/5/8 in response to the BMP family of proteins is essential in the development of the NS. Regulated Smad signalling is involved in the generation of the PNS primordium (the neural crest), while its inhibition is required for the formation of the CNS primordium (the neural plate). Following the generation of these NS primordia, BMP-Smad signalling continues to regulate their further development. The most characterised example is in the patterning of the dorsal SC, where BMP-Smad signalling regulates neurite outgrowth. Smad 1/5/8 signalling is involved in the induction of both neuronal and glial fates from NSCs/neural precursors in a variety of CNS regions, such as the cortex, hippocampus, midbrain, hindbrain and SC. The mechanism by which BMP-Smad signalling achieves the induction of both neuronal and glial phenotypes is unknown, however it is likely to be dependent on spatial and temporal factors. Elucidating the various receptor combinations, cytosolic interactions, transcriptional effectors, and/or target genes that mediate this dual-inductive effect of Smad 1/5/8 is crucial for thorough understanding of the roles of BMP-Smad signalling in neural development. In the PNS, Smad 1/5/8 signalling is involved in the development of both the sympathetic and enteric NSs, in which it also mediates neurite outgrowth in the former and induces both neuronal and glial cell fates in the latter. It is clear that BMP-Smad 1/5/8 is a key regulator of neural development, however its role in the development of a large number of neuronal populations remains to be investigated. Such knowledge could provide important neurodevelopmental information that could be used in the treatment of neurological disorders.

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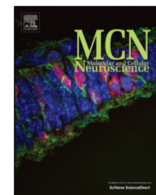

BMP2 and GDF5 induce neuronal differentiation through a Smad dependant pathway in a model of human midbrain dopaminergic neurons

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ABSTRACT

Parkinson's disease is the second most common neurodegenerative disease, and is characterised by the progressive degeneration of the nigrostriatal dopaminergic (DA) system. Current treatments are symptomatic, and do not protect against the DA neuronal loss. One of the most promising treatment approaches is the application of neurotrophic factors to rescue the remaining population of nigrostriatal DA neurons. Therefore, the identification of new neurotrophic factors for midbrain DA neurons, and the subsequent elucidation of the molecular bases of their effects, are important. Two related members of the bone morphogenetic protein (BMP) family, BMP2 and growth differentiation factor 5 (GDF5), have been shown to have neurotrophic effects on midbrain DA neurons both *in vitro* and *in vivo*. However, the molecular (signalling pathway(s)) and cellular (direct neuronal or indirect *via* glial cells) mechanisms of their effects remain to be elucidated. Using the SH-SH5Y human neuronal cell line, as a model of human midbrain DA neurons, we have shown that GDF5 and BMP2 induce neurite outgrowth *via* a direct mechanism. Furthermore, we demonstrate that these effects are dependent on BMP type I receptor activation of canonical Smad 1/5/8 signalling.
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Introduction

Parkinson's disease is the second most common neurodegenerative disease and is characterised by motor symptoms, including bradykinesia, akinesia and resting tremor. The pathological hallmark of the disease is the progressive degeneration of dopaminergic neurons that project from the midbrain to the striatum. Despite fifty years of investigation, the mainstay of treatment is symptomatic, involving exogenous L-dopa or dopamine receptor agonists, but these treatments do not protect against the dopaminergic neuronal loss which continues unabated (Toulouse and Sullivan, 2008). A large variety of experimental treatment strategies have been proposed, but one promising approach is neurotrophic factor therapy. This involves the addition of neurotrophic factors to the brain to rescue the remaining dopaminergic neurons (Sullivan and Toulouse, 2011). An intensive research effort has identified glial cell line-derived neurotrophic factor (GDNF) as a potent dopaminergic neurotrophic factor (Lin et al., 1993). GDNF promotes the survival of midbrain dopaminergic neurons *in vitro* and *in vivo* but, despite initial successes in open-label clinical trials (Gill et al., 2003; Patel et al., 2005; Slevin et al., 2005), a double-blind placebo-controlled clinical trial showed no beneficial effect of GDNF administration to the striatum (Lang et al., 2006). Thus, it is important that new neurotrophic factors are identified and that the molecular bases of their effects on midbrain dopaminergic neurons are elucidated.

GDNF is a member of the transforming growth factor (TGF)-β superfamily which is a large family of structurally-related molecules that are grouped into subfamilies based on sequence similarities. These subfamilies include the GDNF family itself, the bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs) and others (Miyazono et al., 2001). Members of the BMP and GDF families have been shown to play diverse roles in the development and function in a variety of tissues, but in particular they play critical roles in skeletal development (Miyazono et al., 2010; Xiao et al., 2007). In recent years, members of the BMP and GDF families have been shown to play key roles as neurotrophic factors that regulate the development of the nervous system and its maintenance in adulthood (Liu and Niswander, 2005). Two of the most extensively studied members of these families are GDF5 and BMP2. Both of these factors possess the characteristic cystine-knot motif, a structural hallmark of members of the TGF-β superfamily and share 52% sequence similarity (Sullivan and O'Keeffe, 2005).

GDF5 expression in developing rat ventral midbrain (VM) correlates with the development of midbrain DA neurons (O'Keeffe et al., 2004b). It promotes the survival and growth of these neurons both *in vitro* (Krieglstein et al., 1995; O'Keeffe et al., 2004a; Wood et al., 2005) and

Abbreviations: BMP(s), bone morphogenetic protein(s); BMPR(s), bone morphogenetic protein receptor(s); caBMPRIb, constitutively active BMPRIb; DA, dopaminergic/dopamine; DIV, day(s) *in vitro*; GDF(s), growth differentiation factor(s); GDNF, glial cell line-derived neurotrophic factor; MAPK, mitogen activated protein kinases; MTT, thiazolyl blue tetrazolium bromide; N, number of repetitions; PBS, phosphate buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; Smad 1/5/8, Smad1, Smad5 and Smad8; TGF, transforming growth factor; VM, ventral midbrain.

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in vivo (Costello et al., 2012; Hurley et al., 2004; Sullivan et al., 1997, 1998, 1999). Similarly, BMP2 promotes the survival and growth of midbrain dopaminergic neurons *in vitro* (Jordan et al., 1997; Reiriz et al., 1999) and *in vivo* (Espejo et al., 1999). Despite these findings, the molecular mechanisms that mediate the neurotrophic effects of GDF5 and BMP2 on midbrain dopaminergic neurons are unknown.

During skeletal development, GDF5 and BMP2 are known to act through a canonical pathway involving the activation of two cell-surface serine/threonine kinase receptors, type I and type II bone morphogenetic protein receptors (BMPRs) (Miyazono et al., 2010; Sieber et al., 2009). Upon ligand binding, the constitutively-active BMPRII transphosphorylates the cytoplasmic domain of the BMPRI (BMPRIa or BMPRIb) which through a series of protein–protein interactions phosphorylate Smad proteins that translocate to the nucleus and modulate gene transcription. In recent years, both GDF5 and BMP2 have also been shown to signal *via* non-canonical, pathways, which involve the activation of a variety of intracellular pathways, including ERK, JNK and p38 mitogen activated protein kinases (MAPK), depending on the cellular context (Derynck and Zhang, 2003; Moustakas and Heldin, 2005). In the present study SH-SY5Y neuroblastoma cells, widely used as models of human DA neurons, were used to investigate the molecular mechanisms mediating the neurotrophic effects of GDF5 and BMP2.

Results

BMP2 and GDF5 induce the neuronal differentiation in SH-SH5Y cells

Firstly the expression of BMPRs and Smad proteins in the SH-SY5Y cell line was examined. To do this, SH-SY5Y cells were cultured for three days before being fixed and processed for immunocytochemistry. Alternatively, RNA was prepared from cultured cells for RT-PCR analysis of gene expression. RT-PCR analysis showed that the SH-SY5Y cell line expresses mRNAs for the BMPRs, BMPR-II and BMPR-Ib (Fig. 1A) along with the receptor-regulated Smads, Smad1, Smad5 and Smad8 (Smad 1/5/8), and the common-mediator Smad, Smad4 (Fig. 1A). Immunocytochemistry showed strong expression at the protein level of BMPRII and BMPRIb (Fig. 1B). Similarly, immunocytochemistry for Smads 1/5/8 revealed that these proteins were strongly expressed, displaying a predominantly cytoplasmic distribution in unstimulated cells (Fig. 1C). Negative controls were performed for all immunocytochemical analyses to confirm the specificity of the primary antibodies (data not shown).

In cultures of E14 rat VM, GDF5 and BMP2 have been shown to induce differentiation of DA neurons, as evident from the increased morphological arborisation of treated cells (O'Keeffe et al., 2004a; Reiriz et al., 1999). To directly compare the effects of GDF5 and BMP2 on neuronal differentiation, using similar morphological parameters, the phenotypic effects of GDF5 and BMP2 on SH-SY5Y cells were assessed using a MTT assay. A decrease in the MTT absorbance in this assay may be indicative of an increase in differentiation. SH-SY5Y cells were treated with 200 ng/ml of BMP2 or GDF5 daily, before a MTT assay was performed on 1 day *in vitro* (DIV), 2 DIV and 4 DIV. Both BMP2 and GDF5 significantly reduced, to an identical extent, the MTT absorbance at 4 DIV compared to the untreated control (Fig. 1D). These data suggest that BMP2 and GDF5 may be promoting the differentiation of SH-SY5Y cells.

To more directly assess differentiation, a morphological assessment of the neurite complexity in GDF5 and BMP2-treated SH-SY5Y cells was performed. SH-SY5Y cells were treated with BMP2 or GDF5 daily before being immunocytochemically stained for β-actin at 4 DIV, to allow visualisation of the cytoskeleton. The total length of the neurites was then measured using a modified line intercept method (Mayhew, 1992). Treatment with either BMP2 or GDF5 for 4 DIV resulted in a significant increase in the total length of neurites when compared to untreated controls (Fig. 1E, F). There was no significant difference in the number of cells analysed between the groups (data not shown). These data show that similar to primary cultures of the E14 rat midbrain (O'Keeffe et al., 2004a; Reiriz et al., 1999), BMP2 and GDF5 induce neuronal differentiation in SH-SY5Y cells.

BMP2 and GDF5 activate canonical Smad 1/5/8 signalling in SH-SY5Y cells

To determine the molecular basis of this neurotrophic effect, the temporal kinetics of the activation of the canonical (Smad 1/5/8) and non-canonical (MAPK) signalling pathways by BMP2 and GDF5 were examined. SH-SY5Y cells were treated with BMP2 or GDF5 at 6 different time points (0, 5, 15, 30, 60, 120 min), and were then immunocytochemically stained for phospho-Smad 1/5/8, phospho-p38, phospho-JNK, and phospho-Erk. Densitometric analysis of the nuclear levels of phospho-Smad 1/5/8 showed that both BMP2 and GDF5 significantly increased the amount of nuclear phospho-Smad 1/5/8, although with different temporal profiles, compared to the untreated control (0 min). BMP2 increased nuclear phospho-Smad 1/5/8 levels at all time points examined (Fig. 2A, B), whereas an increase in nuclear phospho-Smad was not detected until 1 hour post-GDF5 treatment (Fig. 2C, D). Interestingly, both BMP2 and GDF5 reduced the basal level of all MAPK pathways examined (Supplementary Fig. 1). These data suggest that the effects of BMP2 and GDF5 on the differentiation of SH-SY5Y cells (Fig. 1) may be mediated through a BMPR-dependent activation of the canonical Smad 1/5/8 pathway.

Dorsomorphin prevents BMP2- and GDF5-induced neuronal differentiation and Smad activation in SHSY5Y cells

To explore this premise, dorsomorphin, a small molecular inhibitor of BMPRI (Yu et al., 2008), was used to determine whether the effects of BMP2 and GDF5 were mediated by the BMPRI. To determine a working concentration of dorsomorphin, an array of different concentrations was used, ranging from 100 ng/ml to 200 μg/ml. Dorsomorphin concentrations above 2 μg/ml caused non-selective SH-SY5Y cell death after 1 DIV (Supplementary Fig. 2A). At concentrations of 1 μg/ml and below, SH-SY5Y cells were unaffected by daily dorsomorphin treatments for up to 4 DIV (the duration of BMP2 and GDF5 treatment), with no observable change in their cellular morphology compared to the control (Supplementary Fig. 2B). An MTT assay performed at 4 DIV confirmed that daily treatments with 1 μg/ml of dorsomorphin did not significantly affect the viability of SH-SY5Y cells (Supplementary Fig. 2C).

Firstly, pre-treatment of SH-SY5Y cells with 1 μg/ml of dorsomorphin completely prevented the BMP2- and GDF5-induced decrease in MTT absorbance at 4 DIV (Fig. 3A). Similarly, when SH-SY5Y cells were pre-treated with dorsomorphin, BMP2 and GDF5 failed to induce any significant increase in the total neurite length compared to the untreated control group (Fig. 3B, C). There was no significant difference in the number of cells analysed between the groups (data not shown). To determine if dorsomorphin inhibited BMP2- and GDF5-induced Smad activation (Fig. 2), SH-SY5Y cells were pre-treated with dorsomorphin prior to the addition of BMP2 and GDF5, and the levels of nuclear phospho-Smad 1/5/8 were assessed and compared to non-dorsomorphin treated, BMP2- and GDF5-treated controls. Dorsomorphin completely prevented the BMP2- (Fig. 3D) and GDF5- (Fig. 3E) induced activation of the Smad 1/5/8 signalling pathway. These data suggest that the phenotypic effects of BMP2 and GDF5 on neuronal differentiation may be directly mediated through a BMPR-dependent canonical Smad 1/5/8 pathway.

Canonical BMPR-Smad activation induces neuronal differentiation in SH-SY5Y cells

It is well established that BMP2 can signal through both BMPRIa and BMPRIb, whereas GDF5 predominantly signals through BMPRIb

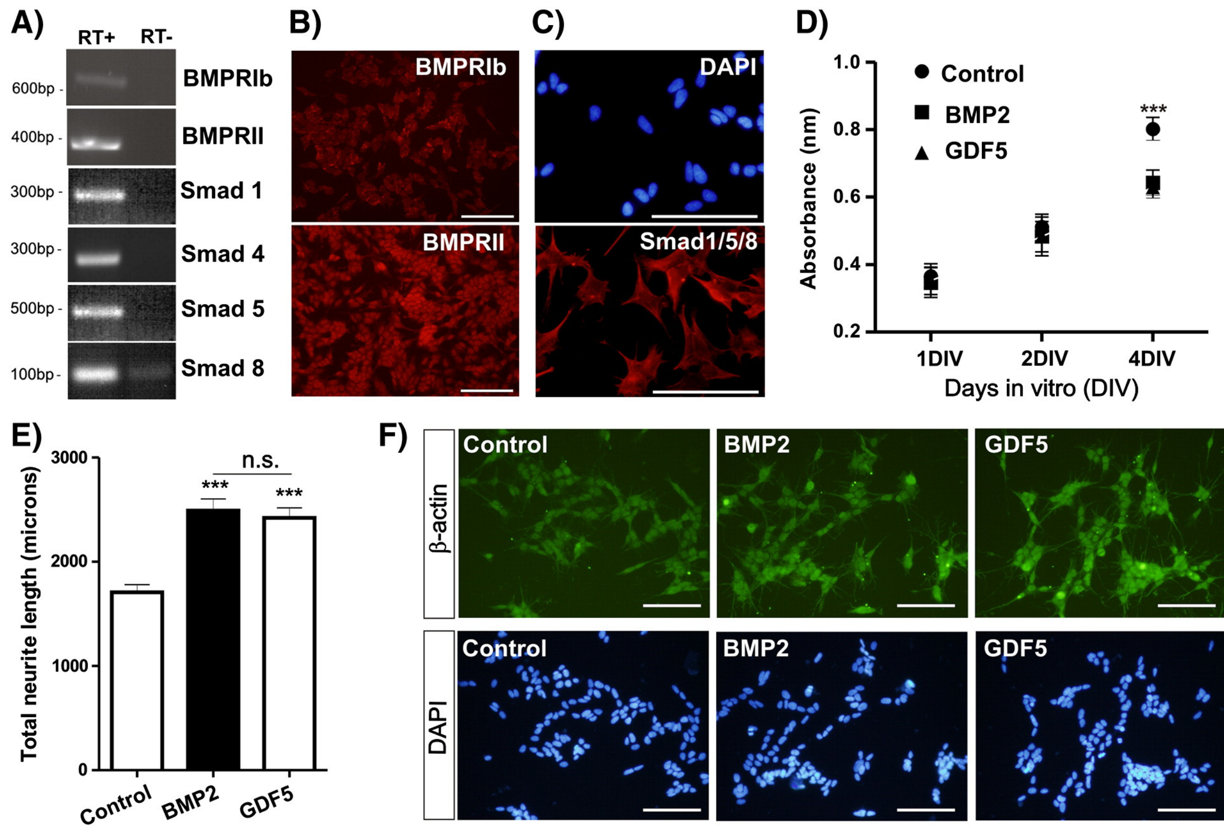

Fig. 1. BMP2 and GDF5 induce neuronal differentiation in SH-SY5Y cells. (A) RT-PCR analysis of the BMPRs, BMPR1b and BMPRII, and of Smads 1, 4, 5, and 8 in SH-SY5Y cells. A 100 bp ladder was used to determine the band size for each PCR product, and RT-PCR of GAPDH was used as a positive control (not shown). Representative photomicrographs of SH-SY5Y cells immunocytochemically stained for the BMPRs, (B) BMPRIb and BMPRII, or (C) Smad 1/5/8. (D) MTT assay of BMP2- and GDF5-treated (daily) SH-SY5Y cells at 1, 2 and 4 DIV, as indicated (*** $P < 0.001$ vs BMP2/GDF5 4DIV; ANOVA with post-hoc Tukey's test; 4 measurements for each group per experiment. Number of repetitions ($N = 4$). (E) Length of total neurites of BMP2- and GDF5-treated (daily for 4 DIV) SH-SY5Y cells, as indicated (*** $P < 0.001$ vs control; ANOVA with post-hoc Tukey's test; 20 images analysed for each group per experiment. $N = 3$). Data are expressed as mean ± SEM. (F) Representative photomicrographs of control, BMP2- and GDF5-treated SH-SY5Y cells, as indicated, immunocytochemically stained for β-actin and counterstained with DAPI. Negative controls in which the primary antibody was omitted were also prepared (not shown). Scale bar = 100 μm.

(Nishitoh et al., 1996). This suggests that BMP2 and GDF5 may signal through BMPRIb to induce differentiation. To examine this, we transfected SH-SY5Y cells with a constitutively active BMPRIb (caBMPRIb) plasmid, which induced a significant increase in total neurite length compared to cells transfected with the relevant control plasmid (Fig. 4A, B). In agreement with this finding, caBMPRIb transfected cells had significantly

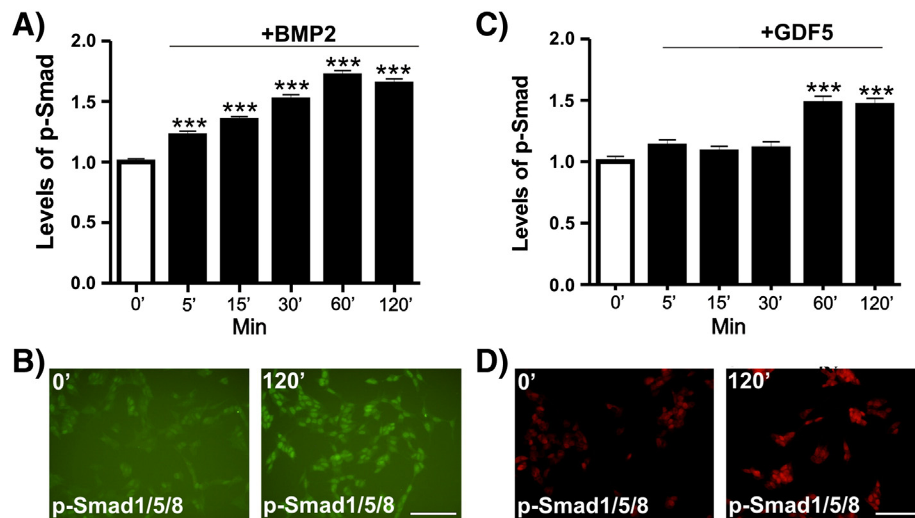

Fig. 2. BMP2 and GDF5 activate the Smad 1/5/8 signalling pathway in SH-SY5Y cells. The relative immunofluorescence intensity of (A) BMP2- and (C) GDF5-treated SH-SY5Y cells, at 0 (untreated control), 5, 15, 30, 60 and 120 min, expressing phospho-Smad 1/5/8 as determined by densitometry (Image J) (*** $P < 0.001$ vs 0 min; One-way ANOVA and post hoc Tukey's test; 50 cells for each group per experiment. $N = 3$). Data are expressed as mean ± SEM. Representative photomicrographs of (B) BMP2- and (D) GDF5-treated SH-SY5Y cells immunocytochemically stained for phospho-Smad 1/5/8 at 0 and 120 min. Negative controls in which the primary antibody was omitted were also prepared (not shown). Scale bar = 100 μm.

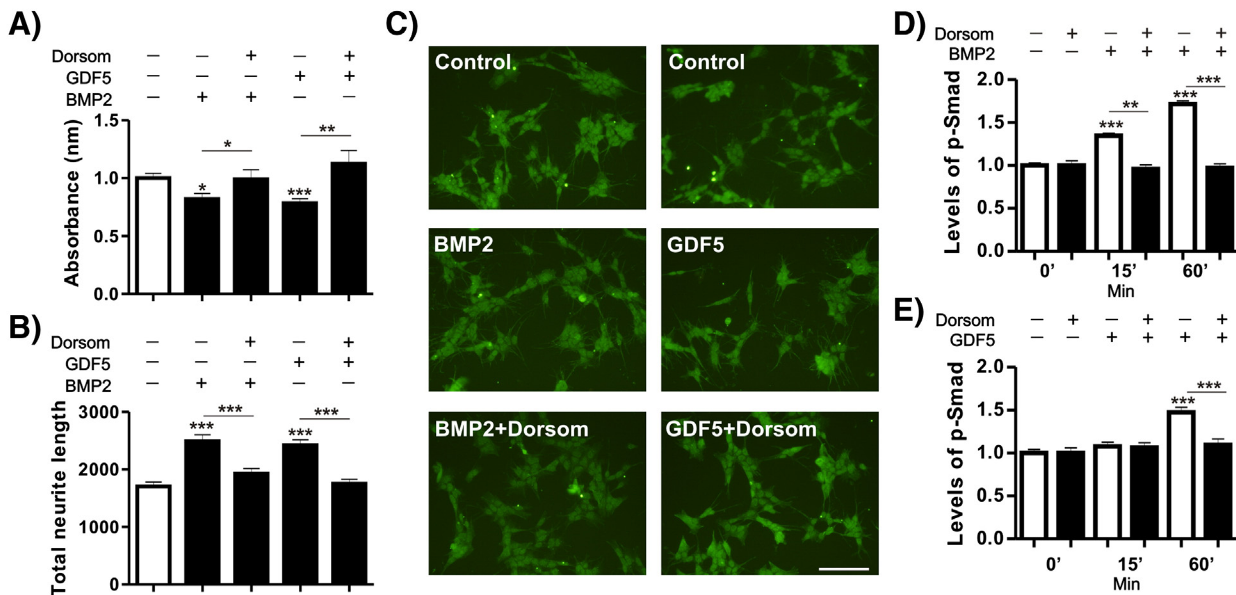

Fig. 3. Dorsomorphin prevents BMP2- and GDF5-induced neuronal differentiation of SH-SY5Y cells. (A) MTT assay to assess cellular respiration of dorsomorphin pre-treated and/or BMP2- and GDF5-, treated (daily for 4DIV) SH-SY5Y cells, as indicated (*** P < 0.001, * P < 0.05 vs control; ANOVA with post-hoc Tukey's test; 4 measurements for each group per experiment. N = 4). (B) Length of total neurites of dorsomorphin pre-treated and/or BMP2- and GDF5-treated (daily for 4DIV) SH-SY5Y cells, as indicated (*** P < 0.001 vs control; ANOVA with post-hoc Tukey's test; 20 images analysed for each group per experiment. N = 3). (C) Representative photomicrographs of dorsomorphin pre-treated and BMP2- and GDF5-treated SH-SY5Y cells, as indicated, immunocytochemically stained for β-actin. Negative controls in which the primary antibody was omitted were also prepared (not shown). Scale bar = 100 μm. The relative immunofluorescence intensity of dorsomorphin pre-treated, and/or (D) BMP2- and (E) GDF5-treated SH-SH5Y cells, at 0 (control), 15 and 60 min, expressing phospho-Smad 1/5/8 as determined by densitometry (Image J), as indicated (*** P < 0.001 vs 0 min; One-way ANOVA and post hoc Tukey's test; 50 cells for each group per experiment. N = 3). Data are expressed as mean ± SEM.

increased levels of nuclear phospho-Smad 1/5/8 when compared to controls (Fig. 4C, D). Importantly, cells transfected with a control plasmid displayed the same level of nuclear phospho-Smad 1/5/8 as non transfected neurons, indicating that the transfection procedure did not alter the relative activation of this pathway (Fig. 4C, D).

To determine a functional link between BMPRIb-induced Smad activation and SH-SY5Y differentiation, a siRNA against the co-Smad, Smad4, was developed. The association of phosphorylated Smad 1/5/8 proteins with Smad4 following BMPRIb activation is required for the effects of Smad 1/5/8 on gene transcription (Fig. 5A). Firstly, SH-SY5Y cells were transfected with Smad4 siRNA with a transfection efficiency of 25%. When we analysed Smad4 expression by RT-PCR in these cultures, there was a clear reduction in Smad4 mRNA expression in siRNA transfected cells (Fig. 5B). To explore this at the protein level, immunocytochemistry was used to examine Smad4 expression in individual cells transfected with Smad4 siRNA. Smad4 siRNA results in a sustained and consistent knockdown of Smad4 protein in Smad4 siRNA-transfected cells (Fig. 5C). To determine if modulation of Smad4 affected the differentiation of these cells, total neurite length of cells transfected with Smad4 siRNA or with Smad4 overexpression vectors was measured. In agreement with the dorsomorphin data (Fig. 3), modulation of Smad4 expression did not affect total neurite length per transfected SH-SH5Y cell (Fig. 5D). When SH-SY5Y cells were co-transfected with the caBMPRIb and Smad4 siRNA, Smad4 siRNA completely prevented the caBMPRIb-mediated significant increase in average neurite length per transfected cell (Fig. 5E, F). These data suggest that BMPR-mediated canonical Smad 1/5/8 signalling may mediate the neurotrophic effects of BMP2 and GDF5. To explore this directly, we transfected SH-SY5Y cells with Smad4 siRNA and treated them with BMP2 and GDF5. We found that in cells expressing Smad4 siRNA, BMP2 and GDF5 did not promote neurite outgrowth compared to relevant controls (Fig. 6A, B). Collectively these data show that activation of canonical BMPRIb-Smad 1/5/8 signalling by BMP2 and GDF5 can induce neuronal differentiation.

Discussion

The neurotrophic effects of BMP2 (Espejo et al., 1999; Jordan et al., 1997; Reiriz et al., 1999) and GDF5 (Costello et al., 2012; Hurley et al., 2004; Krieglstein et al., 1995; O'Keeffe et al., 2004a; Sullivan et al., 1999; Wood et al., 2005), in particular, have been well documented in primary VM neural cultures and in animal models of Parkinson's disease. However, the downstream molecular mechanisms that mediate the neurotrophic effects of GDF5 and BMP2 on VM DA neurons are unknown. In an attempt to define these molecular mechanisms, the present study used the SH-SY5Y neuroblastoma cell line, which has been used extensively as a model of human VM DA neurons (Toulouse et al., 2012; Xie et al., 2010) and has been shown to be capable of differentiation into DA neurons (Gomez-Santos et al., 2002; McMillan et al., 2007; Presgraves et al., 2004; Xie et al., 2010).

The SH-SY5Y cells were first characterised with regards to the expression of BMPRs and Smad transcription factors. This study confirmed that both type I and type II BMPRs are expressed on SH-SY5Y cells. In addition, it was shown that the principal signal transduction machinery for BMPs, Smad proteins 1, 5 and 8, is present in SH-SY5Y cells (including in their activated form) as well as Smad4 which is required for the nuclear translocation of Smads 1/5/8. These results demonstrate that SH-SY5Y cells have the machinery to carry out canonical Smad 1/5/8 signalling in response to BMPs.

A common neurotrophic effect of BMP2 and GDF5 on VM DA neurons *in vitro* is the induction of neurite outgrowth (O'Keeffe et al., 2004a; Reiriz et al., 1999). This study has demonstrated that both BMP2 and GDF5 induce the neuronal differentiation of SH-SY5Y cells. BMP2- and GDF5 induced neurite extension and growth arrest in proliferating SH-SY5Y cells, which is consistent with previous results shown for BMP2 (Nakamura et al., 2003) and GDF5 (Toulouse et al., 2012) in SH-SY5Y cells. It has been proposed that BMP2 and GDF5 exert their neurotrophic effects on DA neurons indirectly through an action on glial cells that are present in mixed neural cultures of E14 rat VM

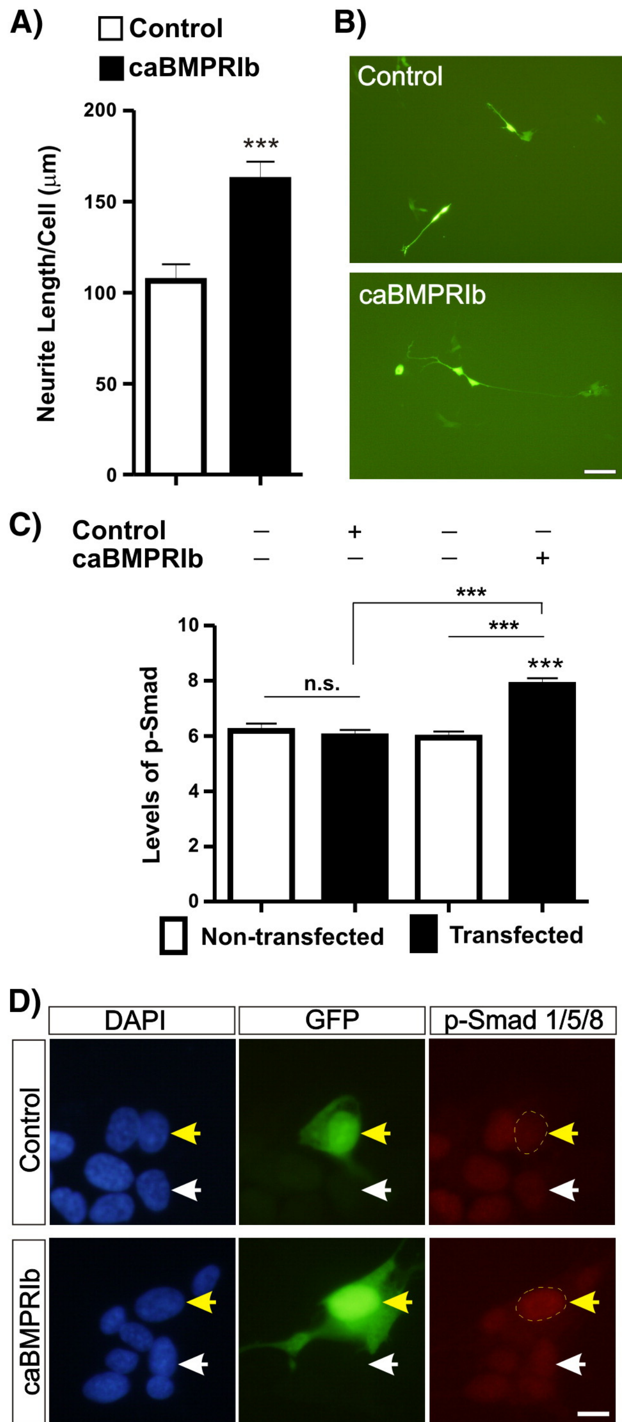

Fig. 4. Activated BMPRIb induces neuronal differentiation and Smad 1/5/8 signalling in SH-SY5Y cells. (A) Neurite length of caBMPRIb transfected SH-SY5Y cells, as indicated (*** $P < 0.001$ vs control; ANOVA with post-hoc Tukey's test; 40 cells for each group per experiment. $N = 3$). (B) Representative photomicrographs of control plasmid and caBMPRIb plasmid-transfected SH-SY5Y cells expressing GFP. Scale bar = 25 μm. (C) The relative immunofluorescence intensity of caBMPRIb-transfected SH-SY5Y cells expressing phospho-Smad 1/5/8 as determined by densitometry (Image J), as indicated (*** $P < 0.001$ vs 0 min; One-way ANOVA and post hoc Tukey's test; 50 cells for each group per experiment. $N = 3$). Data are expressed as mean ± SEM. (D) Representative photomicrographs of control plasmid and caBMPRIb plasmid-transfected (yellow arrows), and non-transfected (white arrows) SH-SY5Y cells immunocytochemically stained for phospho-Smad 1/5/8 and counterstained with DAPI. Negative controls in which the primary antibody was omitted were also prepared (not shown). Scale bar = 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Sullivan and O'Keeffe, 2005). However, the present study shows that the neurotrophic effects of GDF5 and BMP2 are mediated directly on neuronal-like cells, since there are no other cell types present in SH-SY5Y cell cultures. This is in agreement with previous evidence showing that GDF5 still exerts its survival-promoting neurotrophic effects in glial-depleted VM cultures (Wood et al., 2005). A similar approach was used to demonstrate that the neurotrophic effects of BMP2 on striatal neurons were as a result of direct neuronal action (Gratacos et al., 2001).

In order to examine the mechanism of this neuronal action of GDF5 and BMP2, dorsomorphin, a small molecular inhibitor of BMPRI (Yu et al., 2008), was used. This showed that the neurotrophic effects of BMP2 and GDF5 are dependent upon BMPRI activation. Dorsomorphin prevented BMP2- and GDF5-induced neuronal differentiation, and activation of Smad 1/5/8 signalling, in SH-SY5Y cells. This finding is similar to that of Parikh et al. (2011), who showed that inhibition of BMP7 signalling using dorsomorphin negatively affects BMP-induced neurite outgrowth of E18.5 mouse hippocampal neurons. The present study also shows that BMP2 and GDF5 activate Smad 1/5/8 signalling to the same extent, however the kinetics of this Smad activation differed between the two ligands. BMP2 resulted in nuclear translocation of activated Smad proteins from 5 min onwards, whereas following GDF5 this translocation was not seen until after an hour. The reason for this difference in kinetics is unclear, as BMP2 and GDF5 share the same binding site on BMPRIb (Nishitoh et al., 1996). Similar results were reported by Drevelle et al. (2013), who found that BMP2 caused Smad 1/5/8 phosphorylation within 30 min in cultured preosteoblasts, while GDF2 required 4 h to induce the same effect; the precise mechanism of these differing rates of activation and what, if any, is its functional significance is unknown.

A study using fluorescent biosensors for direct visualisation of Smad1 and Smad4 proteins demonstrated that a delay of 2–5 min occurred between BMP4 (also known as BMP2b) activation of the BMPRs and subsequent Smad1 phosphorylation in mammalian cells (Gromova et al., 2007). This is consistent with our findings for BMP2 (also known as BMP2a) showing Smad 1/5/8 activation from 5 min. Gromova et al. describe Smad1 phosphorylation as the rate-limiting step of canonical BMP-Smad signalling (Gromova et al., 2007). Because Smad1 phosphorylation is carried out by the kinase domain of BMP-activated BMPRI, it is likely that the time delay in Smad phosphorylation is determined by the BMPRI. Furthermore, in canonical BMP-Smad 1/5/8 signalling, BMPs bind to a pre-formed complex of BMPRI and BMPRII (Gilboa et al., 2000; Nohe et al., 2002). Therefore, BMPR heteromerization does not contribute to the delay in BMPRI phosphorylation of Smads 1/5/8. BMP2 can signal through both BMPRIa and BMPRIb, whereas GDF5 predominantly signals through BMPRIb (Nishitoh et al., 1996). It is possible that BMPRIa activation by BMP2 accounts for the distinct temporal profiles of Smad activation between BMP2 and GDF5. Indeed, SH-SY5Y cells have been reported to express BMPRIa (Toulouse et al., 2012) as well as BMPRIb, as shown here. Furthermore, GDF2 preferentially signals *via* activin receptor-like kinase 1 (David et al., 2007), which may be the reason for the difference between its Smad 1/5/8 phosphorylation kinetics and those of BMP2. In light of these findings, it is possible that different BMPRI have distinct temporal profiles of Smad 1/5/8 phosphorylation, which would explain the different kinetics of Smad phosphorylation observed after treatment with various BMPs.

In addition to demonstrating canonical Smad signalling activation by BMP2 and GDF5, this study also showed that BMP2 and GDF5 reduce the basal signalling of the phospho-ERK, phospho-JNK and phospho-p38 MAPK pathways in SH-SH5Y cells. Thus, non-canonical BMP signalling pathways would appear not to contribute to the neurite outgrowth promoting affects of BMP2 and GDF5. Interestingly, activation of p38 MAPK signalling in VM DA neurons is known to inhibit neurite outgrowth *in vitro* (Collins et al., 2013), as such inhibition of p38 phosphorylation by BMP2 and GDF5 may provide a permissive environment for optimal neurite outgrowth. The inverse regulation of Smad and MAPK pathways is in agreement with previous findings showing that MAPK signalling

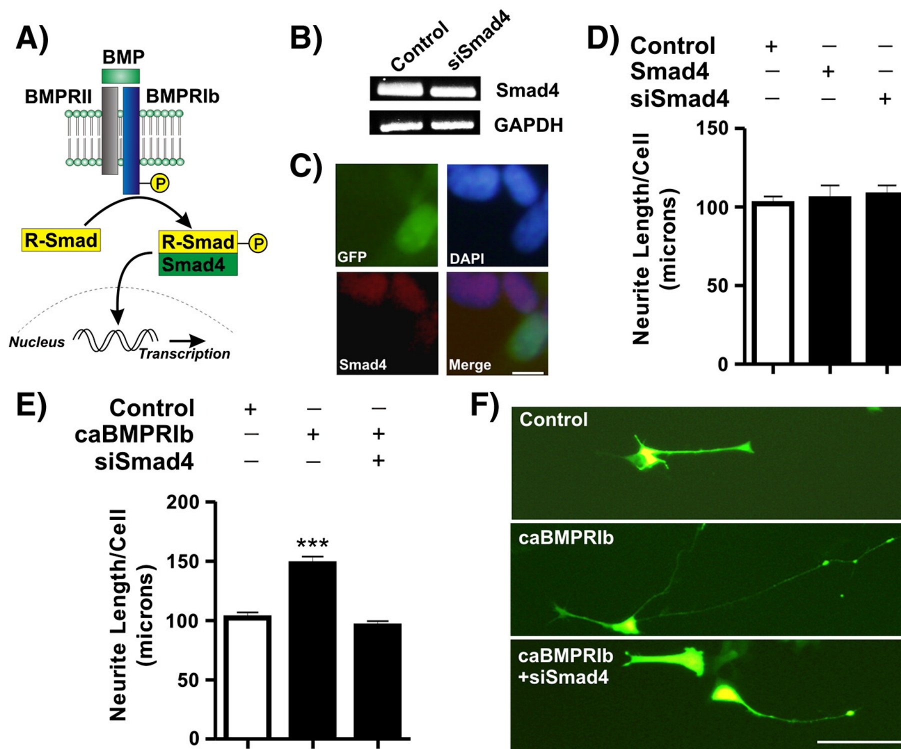

Fig. 5. Inhibition of the nuclear translocation of phosphorylated Smad 1/5/8 prevents BMPRIb-mediated induction of SHSY5Y neuronal differentiation. (A) Graphical representation of the role of Smad4 in canonical BMP-Smad 1/5/8 signalling. (B) RT-PCR analysis of Smad4 and GAPDH (positive control) in siSmad4 (Smad4 siRNA)-transfected SH-SY5Y cells. (C) Representative photomicrographs of siSmad4 transfected SH-SY5Y cells immunocytochemically stained for Smad4 and counterstained with DAPI. Negative controls in which the primary antibody was omitted were also prepared (not shown). Scale bar = 25 μm (D) Neurite length of Smad4- or siSmad4-transfected SH-SY5Y cells. No significant difference ($P < 0.05$; ANOVA with post-hoc Tukey's test; 40 cells for each group per experiment. N = 3) was observed between the groups. (E) Neurite length of caBMPRIb- and/or siSmad4-transfected SH-SY5Y cells, as indicated (*** $P < 0.001$ vs control; ANOVA with post-hoc Tukey's test; 40 cells for each group per experiment. N = 3). Data are expressed as mean ± SEM. (F) Representative photomicrographs of caBMPRIb- and/or siSmad4-transfected SH-SY5Y cells expressing GFP. Scale bar = 50 μm.

negatively regulates Smad 1/5/8 signalling by inducing Smad1 degradation (Eivers et al., 2008; Fuentealba et al., 2007). Therefore, negative regulation of MAPK pathway signalling by BMP2 and GDF5 may contribute to canonical Smad signalling-mediated neurite outgrowth, by preventing MAPK-induced Smad1 degradation. Conversely, BMP2 has been shown to induce neuronal differentiation of the PC12 cell line *via* activation of the p38 MAPK pathway (Iwasaki et al., 1996, 1999). Furthermore, dorsomorphin has been shown to induce neurite outgrowth of PC12 cells *via* the ERK MAPK pathway (Kudo et al., 2011), while this study found dorsomorphin to have no morphological affects on SH-SH5Y cells. The present study demonstrates that BMP2 and GDF5 promote neurite growth through activation of canonical Smad 1/5/8 signalling in SH-SY5Y cells. These contrasting results may reflect inherent differences between SH-SY5Y neuroblastoma cells and PC12 cells, which arise from adrenal gland chromaffin cells.

The present findings show that the BMP2- and GDF5-induced neuronal differentiation of SH-SY5Y cells is mediated through BMPRIb. Such a suggestion reflects the fact that BMPRIb is the preferential BMPRI activated by GDF5. BMP2 induced the neuronal differentiation of SH-SY5Y cells to the same extent as GDF5, and thus likely acted *via* the BMPRIb also. The fact that caBMPRIb mimics the effects of BMP2 and GDF5 strongly supports this possibility.

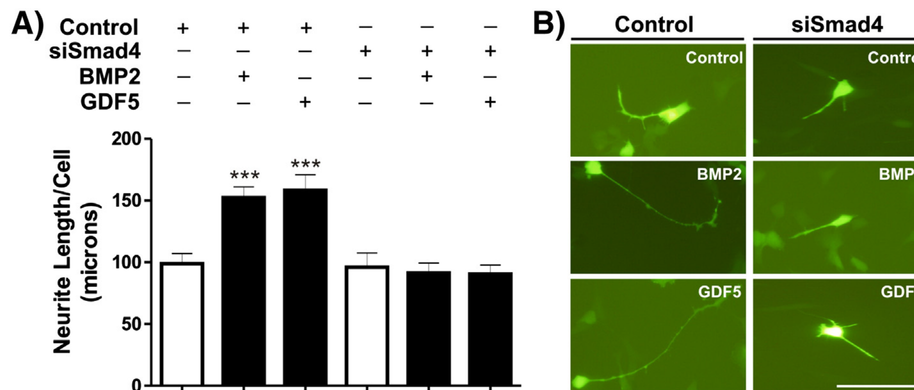

Fig. 6. Inhibition of the nuclear translocation of activated Smad 1/5/8 prevents BMP2 and GDF5 induction of SHSY5Y neurite outgrowth. (A) Neurite length of control plasmid- and siSmad4-transfected SH-SY5Y cells with or without BMP2 or GDF5 treatment, as indicated (*** $P < 0.001$ vs control; ANOVA with post-hoc Tukey's test; 40 cells for each group per experiment. N = 3). Data are expressed as mean ± SEM. (F) Representative photomicrographs of control plasmid- and siSmad4-transfected SH-SY5Y cells with or without BMP2 or GDF5 treatment expressing GFP. Scale bar = 100 μm.

This study has not only identified the BMPRI subtype that is most likely responsible for mediating BMP2- and GDF5-induced Smad signalling and neurite extension, but has also demonstrated that the transcriptional activity of Smad 1/5/8 is required for this BMP-induced neuronal differentiation. The inhibition of the nuclear translocation of the Smad 1/5/8 transcription factors, using a siRNA to target Smad4, prevented the SH-SY5Y neurite outgrowth induced by caBMPRIb, and by BMP2 and GDF5.

The current study adds new evidence to the growing body of work that suggests that BMP-Smad 1/5/8 signalling plays a key role in the neurite extension of a number of neural populations. For example, BMPs are widely expressed in the dorsal spinal cord, which houses a BMP signalling centre known, as the roof plate (Lee and Jessell, 1999; Lee et al., 1998; Liem et al., 1995). There is a wealth of evidence demonstrating that BMP signalling, including BMP2 and GDF5, regulates neurite outgrowth in this region, including that of dorsal root ganglion neurons and dI1 interneurons (Hazen et al., 2011, 2012; Parikh et al., 2011; Phan et al., 2010; Niere et al., 2006). GDF5 and BMP2 have both been shown to be expressed in the developing VM during DA neurogenesis (Jordan et al., 1997; O'Keeffe et al., 2004b), and thus may perform a role in the differentiation of VM DA neurons similar to that of BMPs in the dorsal spinal cord. Such a suggestion is plausible especially when one considers the evidence gained from previous studies of their DA neurotrophic effects. Furthermore, although not discussed in the paper, Monteiro et al. used Smad 1/5/8 reporter mice to demonstrate BMP-Smad-dependent transcriptional activity in the VM region during DA neurogenesis at E10.5 (Monteiro et al., 2008). It is thus possible that BMP2- and GDF5-induced Smad activation regulates VM DA neurite outgrowth at this developmental stage, as VM DA neurons begin to extend their axons dorsally at this time point (Nakamura et al., 2000), as is the case for BMPs in the dorsal spinal cord (Chizhikov and Millen, 2005; Ulloa and Briscoe, 2007).

Cell replacement therapy is one of the most promising therapies for the treatment of Parkinson's disease (Bonnamain et al., 2012; De Feo et al., 2012; Hedlund and Perlmann, 2009; Orlacchio et al., 2010; Toulouse and Sullivan, 2008). Considering the importance of establishing functional connections following the striatal transplantation of VM DA neurons, factors which promote their neurite outgrowth are being considered as adjuncts to this potential therapy. GDF5 and BMP2 would appear to be potential candidates for such a role, as both have been shown to promote the survival of VM DA neurons (Jordan et al., 1997; O'Keeffe et al., 2004a; Reiriz et al., 1999; Sullivan et al., 1997). The present study has, for the first time, demonstrated that the downstream molecular mechanisms mediating the direct neurotrophic effects of GDF5 and BMP2 are dependent upon BMPRI-mediated activation of canonical Smad 1/5/8 signalling.

Experimental methods

Cell culture

SH-SY5Y cells were maintained in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (Sigma), supplemented with 10% foetal calf serum (Sigma), 100 nM L-Glutamine (Sigma), 100 U/ml Penicillin (Sigma), 10 μg/ml Streptomycin (Sigma), in a humidified atmosphere containing 5% CO_2 at 37 °C. Where indicated, the cells were treated with 200 ng/ml of GDF5 (kindly provided by Biopharm GmbH) or recombinant human BMP2 (R&D Systems), and pre-treated (30 min prior to GDF5 or BMP2 application) with 1 μg/ml of Dorsomorphin (Sigma).

Electroporation of SH-SH5Y Cells

Electroporation of SH-SY5Y cells was carried out using the Neon™ Transfection System (Invitrogen). SH-SY5Y cell suspensions were prepared for counting, and the required volume of cells to give 100,000 cells per well were centrifuged at 4 °C at 1100 rpm for 5 min. The cell pellet was washed twice with 10 mM phosphate buffered saline (PBS) (without $CaCl_2$ and $MgCl_2$) (Sigma), and then resuspended in the required amount of resuspension buffer (12 μl per transfection/plasmid) (Invitrogen). 0.5 μg of a GFP plasmid and 1 μg of desired plasmid DNA were added to the resuspended cells. 10 μl of the cell/plasmid mixture was then electroporated according to the manufacturer's protocol under specific parameters (1200 V; 20 ms; 3 pulses).

Smad4 small interfering RNA (siRNA) construction

The target sequence (5′TTGGGTCAACTCTCCAATGTC′3) was chosen against Smad4, based on homology between the mouse, rat and human mRNA sequences, and a GC content of 30–50%. This sequence was used to design the 55 nucleotide siRNA template oligonucleotides according to the vector manufacturer's protocol, which were then cloned into the pSilencer 4.1-CMV vector (Ambion) according to the manufacturer's instructions.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA from SH-SY5Y cells was isolated using an RNeasy mini extraction kit (Qiagen). An ImProm-II Reverse Transcription System (Promega) was used to synthesise cDNA using a volume containing 1 μg of RNA in an 11.5 μl reaction for 90 min at 37 °C. Amplification was carried out using a GoTaq Flexi DNA Polymerase system (Promega) as per manufacturer's instructions. Each reaction mixture consisted of 2 μl cDNA, 2 μl forward and reverse primer mix, 5× PCR buffer, 1.5 mM $MgCl_2$, 1.25 mM PCR dNTPs, 0.25 μl Taq polymerase and made up to a total of 25 μl with nuclease-free water. Forward and reverse primer pairs, respectively, for RT-PCR were as follows: GCAGCACAGACGGATATTGT and TTTCATGCC TCATCAACACT for BMPRIb, GCTTCGCAGAATCAAGAACG and GTGGACTG AGTGGTGTTGTG for BMPRII, AGTGACAGCAGCATCTTCGTGC and CGGG TGTATCTCAATCCAGCAG for Smad1, AAGGTGAAGGTGATGTTTG and GA GCTATTCCACCTACTGAT for Smad4, GGAGGAGTTGGAGAAAGCCTTG and GGGAGTTGGGATATGTGCTGC for Smad5, and GTATCATCGCCAGGATG TCA and TGTGGGGAGCCCATCTGAGT for Smad8. The expected product sizes were 630 bp, 349 bp, 276 bp, 264 bp, 470 bp, and 104 bp respectively.

Immunocytochemistry

Cultures were fixed in 4% paraformaldehyde in PBS for 10 min. Following washes in 10 mM PBS containing 0.02% Triton X-100 (PBS-T in 10 mM PBS) for permeabilization, cultures were incubated in blocking solution (5% bovine serum albumin) for 1 h at room temperature. Cultures were subsequently incubated in the following antibodies: BMPRII (1:200; R&D Systems), BMPRIb (1:200; R&D Systems), Smad 1/5/8 (1:200; Cell Signalling), Smad4 (1:50; Millipore), phopsho-Smad 1/5/8 (1:200; Cell Signalling), phospho-p38 (1:50; Cell Signalling), phospho-JNK (1:50; Cell Signalling), phospho-Erk (1:50; Cell Signalling), and β-actin (1:200; Sigma) diluted in 1% bovine serum albumin in 10 mM PBS at 4 °C overnight. Following 3 × 5min washes in PBS-T, cells were incubated in Alexa Fluor 488- or 594-conjugated secondary antibodies (1:500; Invitrogen) reactive to the species of the primary antibodies and diluted in 1% bovine serum albumin in 10 mM PBS, at room temperature for 2 h in the dark. Cultures were counterstained with bisbenzimide (1:1000; in 10 mM PBS; Sigma). Cells were imaged under an Olympus IX70 inverted microscope fitted with an Olympus DP70 camera and AnalysisD™ software. The fluorescence intensity of individual cells stained for phospho-Smad 1/5/8 was measured using the Image J analysis software (Rasband, WJ, http://rsb.info.nih.gov/ij/). The relative fluorescence intensity was calculated as the intensity of each individual cell after subtraction of the background noise.

MTT assay

Thiazolyl Blue Tetrazolium Bromide (MTT) assays were performed to assess cell viability as previously described (Toulouse et al., 2012). MTT was added to cells at a concentration of 0.5 mg/ml.

Measurement of cellular morphology

20 microscopic fields were randomly selected for each experiment, and photographed using an Olympus IX70 inverted microscope. All cells in each photograph were measured. The length of the neuritic arborisation was estimated using standard stereological procedures (Mayhew, 1992). A line grid was superimposed on the microscopic images and the number of times each neurite intersected the grid was recorded. The neurite length was calculated using the following formula; $NL = \alpha \times T \times (\pi/2)$, where α is the number of times the neurite intersect the grid lines, and T is the distance between the gridlines on the magnified image (taking into account the magnification factor).

Statistical analysis

Unpaired Student's *t*-test or one-way ANOVA with a *post hoc* Tukey's test were performed, as appropriate, to determine significant differences between groups. Results were expressed as means with SEM and deemed significant when $P < 0.05$.

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Review

Midbrain dopaminergic neurons: A review of the molecular circuitry that regulates their development

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ABSTRACT

Dopaminergic (DA) neurons of the ventral midbrain (VM) play vital roles in the regulation of voluntary movement, emotion and reward. They are divided into the A8, A9 and A10 subgroups. The development of the A9 group of DA neurons is an area of intense investigation to aid the generation of these neurons from stem cell sources for cell transplantation approaches to Parkinson's disease (PD). This review discusses the molecular processes that are involved in the identity, specification, maturation, target innervation and survival of VM DA neurons during development. The complex molecular interactions of a number of genetic pathways are outlined, as well as recent advances in the mechanisms that regulate subset identity within the VM DA neuronal pool. A thorough understanding of the cellular and molecular mechanisms involved in the development of VM DA neurons will greatly facilitate the use of cell replacement therapy for the treatment of PD.

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Introduction

In the adult CNS, almost 75% of all dopaminergic neurons reside in the ventral midbrain (VM), with 400,000–600,000 found in the human VM and 20,000–30,000 in the mouse VM (Blum, 1998; German et al., 1983; Pakkenberg et al., 1991). During embryonic development, these DA neurons are generated in the floor plate region of the mesencephalon (Ono et al., 2007), and give rise to three distinct clusters of VM DA neurons which ultimately develop into anatomically and functionally distinct entities termed the A8, A9 and A10 groups. The A9 cluster gives rise to the substantia nigra pars compacta (SNc), whose neurons project to the dorsal striatum via the nigrostriatal pathway. These neurons and their striatal projections are required for the control of voluntary movement, and the loss of these neurons is the pathological hallmark of Parkinson's disease (PD), which is a neurodegenerative disorder characterised by impaired motor function (Lees et al., 2009; Toulouse and Sullivan, 2008). The other groups of DA neurons, the A10 and A8 clusters, develop into the ventral tegmental area (VTA) and the retrorubal field (RRF), respectively, whose neurons innervate the ventral striatum and the prefrontal cortex via the mesocorticolimbic system, and are involved in the regulation of emotion and reward (Tzschentke and Schmidt, 2000). Altered/defective neurotransmission of the mesocorticolimbic DA system has been associated with the development of schizophrenia, drug addiction and depression (Meyer-Lindenberg et al., 2002; Robinson and Berridge, 1993).

Interestingly, the A9 group of SNc DA neurons, which undergo progressive degeneration in PD, are particularly vulnerable to cell death in comparison to the other VM DA neuronal populations (Alavian et al., 2008; Betarbet et al., 2000; Farrer, 2006; McNaught et al., 2004). The anatomical, functional and apparent sensitivity differences between these three populations of VM DA neurons likely results from subtle developmental differences during their ontogeny. However, little is known regarding the molecular mechanisms that regulate the phenotypic and functional diversities between these VM DA neuronal populations. Given the involvement of A9 DA neurons in PD, an intensive research effort over the last five decades has focused on identifying the molecules and mechanisms that regulate their development. This information is vital to advance efforts to generate SNc DA neurons from stem cells for application in cell replacement therapy for PD. Through the mutation of specific genes, and the subsequent analysis of VM DA neurogenesis and development, a number of molecular pathways have been shown to play key roles in the

Abbreviations: A/P, anterior–posterior; BDNF, brain-derived neurotrophic factor; DA, dopaminergic/dopamine; DAT, dopamine transporter; D/V, dorso-ventral; E, embryonic day; *En1/2*, *Engrailed-1/2*; FGF8, fibroblast growth factor 8; Fzd, frizzled; GDNF, glial cell line-derived neurotrophic factor; MFB, medial forebrain bundle; NPs, neural progenitor(s)/precursor(s); NSCs, neuroepithelial/neural stem cells; P, postnatal day; PD, Parkinson's disease; RRF, retrorubal field; Shh, sonic hedgehog; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase; VM, ventral midbrain/mesencephalon; VTA, ventral tegmental area; VZ, ventricular zone.

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development of VM DA neurons. This review discusses the 'normal' developmental programme that regulates VM DA neurogenesis, including the cellular and molecular determinants involved in their regional specification, induction, differentiation and maturation.

Early patterning of the ventral mesencephalon

The first key steps in VM DA generation are the early patterning events which lead to the formation of the VM region. During gastrulation, the dorsal ectoderm is restricted towards a neural fate in response to signals arising from the Speamann organiser (Harland, 2000; Hemmati-Brivanlou and Melton, 1997; Liu and Niswander, 2005). The resulting neural plate is then subdivided into restricted domains and subsequently closes to form the neural tube, which is specified by graded signals along the anterior–posterior (A/P) and dorso-ventral (D/V) axes (Puelles, 2001; Simon et al., 1995; Ulloa and Briscoe, 2007). The development of the VM region relies on appropriate A/P and D/V patterns of gene expression which are regulated by signals arising from two key structures in the early embryo: the floor plate of the midbrain and the isthmus organiser. Organisation of the VM region is initiated upon formation of these signalling centres.

The floor plate is present along the length of the neural tube and secretes the sonic hedgehog (Shh) signalling protein from around embryonic day (E) 8.5 onwards in the mouse (Echelard et al., 1993; Ho and Scott, 2002; Hynes et al., 1995a). Interestingly, the spatiotemporal expression pattern of *Shh* in the VM has been shown to contribute to the diverse populations of VM DA neurons, with the 'early medial pool' giving rise primarily to VTA, and very few SNc, DA neurons and the 'later intermediate pool' giving rise to DA neurons of all three subgroups, but largely contributing to the SNc (Joksimovic et al., 2009a). In the floor plate, the bHLH (basic helix–loop–helix) transcription factor Hes1 (also expressed by the isthmus organiser) has been shown to suppress proneural gene expression and induce cell cycle exit (Baek et al., 2006; Ono et al., 2010). Null mutation of *Hes1* results in a transient increase in the number of VM DA neurons between E11.5 and E12.5, followed by a significant reduction in their number from E13.5, compared to the wild type (Kameda et al., 2011). Interestingly, another bHLH transcription factor expressed in the floor plate, Nato3, has been shown to repress *Hes1* expression, and mutation of *Nato3* has been shown to result in a reduction in the number of VM DA neurons generated due to unchecked Hes-1-mediated suppression of proneural genes and the induction of cell cycle arrest (Ono et al., 2010).

The isthmus organiser is a unique signalling centre that separates the midbrain from the hindbrain and is necessary for the development of both of these brain regions (Liu and Joyner, 2001; Rhinn and Brand, 2001). The correct positioning of the isthmus organiser at the midbrain-hindbrain boundary is dependent on the mutual repression of two opposing homeodomain transcription factors: *Otx2* and *Gbx2* (Martinez-Barbera et al., 2001). *Otx2* is expressed in the forebrain and midbrain of the developing anterior neural tube (Acampora et al., 1997; Matsuo et al., 1995; Simeone et al., 1992), while *Gbx2* is expressed more posteriorly in the anterior hindbrain (Wassarman et al., 1997). *Gbx2* expression at the posterior border limits *Otx2* expression which creates the sharp boundary between the midbrain and the hindbrain (Millet et al., 1999).

Fibroblast growth factor 8 (FGF8) is a diffusible factor secreted by the isthmus organiser (Rhinn and Brand, 2001), from around E8 until at least E12.5 in the mouse midbrain-hindbrain boundary (Crossley and Martin, 1995). Surprisingly, although *Otx2* and *Gbx2* are critical for the correct positing of the isthmus organiser, they are not required for the expression of *FGF8*, or for the induction of other isthmus organiser-genes, however they are essential for the correct positioning of the expression domains of these genes (Brodski et al., 2003; Liu and Joyner, 2001). This is highlighted by studies showing that if the position of the isthmus organiser is moved caudally as a result of ectopic *Otx2* expression in hindbrain, there is an increase in the number of VM DA neurons (Brodski et al., 2003). Similarly if its position is moved rostrally by depleting *Otx2* in the midbrain, there is a decrease in the number of VM DA neurons (Brodski et al., 2003), demonstrating the critical importance of isthmus organiser positioning for normal VM DA generation.

As *Otx2*- and *Gbx2*-dependent sharpening of the borders of the isthmus is occuring, a second group of transcription factors begin to be expressed in the isthmus organiser. These include the paired box gene *Pax2* (Urbanek et al., 1997), the lim-homeodomain factor *Lmx1b* (Adams et al., 2000; Smidt et al., 2000), the secreted glycoprotein *Wnt1* (Adams et al., 2000; Crossley and Martin, 1995, Davis and Joyner, 1988; Wilkinson et al., 1987), and E*ngrailed-1* (*En1*) (Davis and Joyner, 1988). Of these, *Pax2* is required for the induction of *FGF8* expression by the isthmus, whereas *Wnt1* and *En1* function cooperatively with *Otx2* and *Gbx2* to further refine the position of the expression domain of *FGF8* at the isthmus (Ye et al., 2001).

Shortly after the induction and positioning of *FGF8* expression, *Engrailed-2* (*En2*) and *Pax5* start to be expressed in the midbrain-hindbrain boundary. These genes play critical roles in the regional specification of the VM, and homozygous mutant mice null for *Otx2* (Acampora et al., 1995; Ang et al., 1996), *Wnt1* (McMahon and Bradley, 1990; Prakash et al., 2006), *Pax2* and *Pax5* (double mutant) (Schwarz et al., 1997), *En1* and *En2* (double mutant) (Liu and Joyner, 2001; Simon et al., 2001), or *Lmx1b* (Smidt et al., 2000) all display major VM defects, including partial or total loss of VM DA neurons (see Table 1).

Identity of ventral midbrain dopaminergic neural precursors

Once the appropriate patterning of the VM region has occurred, a developmental programme involving a sequential pattern of gene expression establishes the identity of VM DA neural precursors (NPs) that ultimately generate VM DA neurons (Fig. 1). The identity of these VM DA NPs has been the focus of intensive research in recent years, largely due to their potential to be used as a cell source to generate DA neurons for cell replacement therapy in PD (Kim, 2011; Morizane et al., 2008; Toulouse and Sullivan, 2008).

The origin of VM DA NPs has been debated for many years, with regions such as the diencephalon (Marin et al., 2005), isthmus (Marchand and Poirier, 1983) and VM basal plate (Hynes et al., 1995a, 1995b) emerging as potential candidates. Despite this research, the precise identity of VM DA NPs remained elusive until recently, when a study showed that floor plate cells in the murine VM become neurogenic and subsequently give rise to DA neurons (Ono et al., 2007). This discovery was surprising as the floor plate was thought to consist of specialised non-neurogenic glial type cells that were largely involved in ventralizing the neural tube, mainly by secreting Shh (Fuccillo et al., 2006; Jessell, 2000; Placzek and Briscoe, 2005). This role in ventralisation seems to remains the main function for floor plate cells caudal to the midbrain, as the hindbrain floor plate has been shown to be non-neurogenic (Joksimovic et al., 2009b; Ono et al., 2007). However, the VM floor plate is different to its caudal counterparts and attains neurogenic potential. Ono et al. (2007) demonstrated that *Otx2*, which is critical for the positioning of the isthmus organiser, is also essential for the neurogenic potential of VM floor

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Table 1
Genetic mutations affecting the development of VM DA neurons.

Mutation(s)	Effect(s) on DA development	Reference(s)
Otx2 –/–	Loss of VM DA neurons (midbrain absent)	Acampora et al. (1995), Ang et al. (1996)
Wnt1 –/–	Severe reduction in VM DA neurons generated	McMahon and Bradley (1990), Prakash et al. (2006)
Pax2 –/–: **Pax5** –/–	Loss of VM DA neurons (midbrain absent)	Schwarz et al. (1997)
En1 –/–: **En2** –/–	VM DA neurons lost by E14 via apoptosis	Alberi et al. (2004), Liu and Joyner (2001), Simon et al. (2001)
Lmx1b –/–	Substantial reduction in VM DA neurons generated, and disappearance of Pitx3⁻ VM DA neurons by birth	Deng et al. (2011), Smidt et al. (2000)
Hes1 –/–	Transient increase (~E12) in VM DA neurons, followed by their significant reduction (from E13.5). Dorsal migration and hindbrain invasion	Kameda et al. (2011)
Nato3 –/–	Reduction in VM DA neurons generated	Ono et al. (2010)
FGFR –/–	Deficit of VM DA neurons	Lahti et al. (2012), Saarimaki-Vire et al. (2007)
FGF2 –/–	Peri-/post-natal increase in VM DA neurons	Ratzka et al. (2012)
Lmx1a –/–	Substantial reduction in VM DA neurons generated	Deng et al. (2011), Ono et al. (2007)
En1$^{Cre/+}$; **Otx2**$^{flox/flox}$	Reduction in VM DA neurons due to failure of VM DA NP induction	Omodei et al. (2008), Puelles et al. (2004)
Ngn2 –/–	Reduction in mature VM DA neurons generated	Kele et al. (2006)
Oc1 –/–: **Oc2** –/–	Reduction in VM DA neurons generated	Chakrabarty et al. (2012)
Gli1 –/–: **Gli2** –/–	Deficient VM DA neurogenesis (more severe than Gli2 –/–)	Park et al. (2000)
Gli2 –/–	Deficient VM DA neurogenesis	Park et al. (2000)
Lmx1a –/–; **Shh**$^{Cre/+}$; **Lmx1b** –/–	Substantial reduction in VM DA neurons generated (more severe than Lmx1a –/–)	Yan et al. (2011)
Lmx1a –/–: **Lmx1b** +/–	Substantial reduction in the generation of VM DA neurons (more severe than Lmx1a –/–)	Deng et al. (2011)
Lrp6 –/–	Delayed differentiation of VM DA neurons	Castelo-Branco et al. (2010), Pinson et al. (2000)
Fzd3 –/–: **Fzd6** –/–	Deficient VM DA neurogenesis (severe midbrain defects)	Stuebner et al. (2010)
Fzd3 –/–	Transient reduction in VM DA neuron generated	Stuebner et al. (2010)
Wnt2 –/–	Reduction in VM DA neurons generated	Sousa et al. (2010)
β-catenin (Th-IRES-Cre; β-Ctn$^{Ex3/+}$ **mutant)**	Reduction in VM DA neurogenesis	Tang et al. (2009)
Dkk1 –/–	Severe loss of VM DA neurons	Ribeiro et al. (2011)
L1 –/–	Positional abnormalities of VM DA neurons	Demyanenko et al. (2001)
Reelin –/–	Failure of VM DA neuron lateral migration	Nishikawa et al. (2003)
DCC –/–	Aberrant VM DA neuron migration, dorsal shifting of ventral striatal DA projections, aberrant crossing of MFB fibres at caudal diencephalic midline, and reduction of prefrontal cortex DA innervation	Xu et al. (2010)
Ebf1 –/–	Impaired tangential migration of VM DA neurons	Yin et al. (2009)
Nurr1 –/–	Lack TH, AADC, VMAT2 and DAT expression in VM DA neurons, and their subsequently loss	Castillo et al. (1998), Filippi et al. (2007), Saucedo-Cardenas et al. (1998), Smits et al. (2003), Wallen et al. (1999)
Nurr1 –/– **(at late stage of DA dev.)**	VM DA neuron degeneration (snc more vulnerable)	Kadkhodaei et al. (2009)
FoxA2 –/–	Increase in numbers of Nurr1⁺ TH⁻ neurons in VM	Ferri et al. (2007)
FoxA2 +/–	Aged mice develop PD-like symptoms and pathologies	Kittappa et al. (2007)
Pitx3 –/–	Deficit of snc DA neurons from E12.5 (VTA largely unaffected)	Hwang et al. (2003), Nunes et al. (2003), Smidt et al. (2004), Van Den Munckhof et al. (2003)
En1 +/–: **En2** +/+	Progressive degeneration of VM DA neurons (8–24 weeks)	Sonnier et al. (2007)
En1 +/–: **En2** –/–	Progressive degeneration of VM DA neurons (more pronounced in snc)	Sgado et al. (2006), Sonnier et al. (2007)
Nkx2.1 –/–	Aberrant crossing of MFB fibres at caudal diencephalic midline	Kawano et al. (2003)
DCC +/–	Increased branching of VM DA fibres in prefrontal cortex	Manitt et al. (2011)
Pbx1a –/–	Partial misrouting of VM DA fibres	Sgado et al. (2012)
Pax6 –/–	Dorsal deflection of MFB fibres in the diencephalon	Vitalis et al. (2000)
Slit1 –/–: **Slit2** –/–	Aberrant crossing of MFB fibres at caudal diencephalic midline	Bagri et al. (2002), Dugan et al. (2011)
Robo1 –/–: **Robo2** –/–	Aberrant crossing of MFB fibres at caudal diencephalic midline, and abnormal dorsal trajectories of VM DA fibres	Dugan et al. (2011), Lopez-Bendito et al. (2007)

plate cells. This finding expands the importance of this gene in determining the overall structure of the VM region.

However, although the region from which VM DA NPs arise has been determined, the specific floor plate cell type which is a DA NP cell remains to be identified. Fate-mapping studies using a marker for radial glia-specific marker GLAST demonstrated that radial glial-like cells in the floor plate of the mouse VM were DA NPs (Bonilla et al., 2008). Shortly thereafter, a similar study using human VM tissue demonstrated that DA NPs in the VM floor plate showed radial glial characteristics, that is they expressed the radial glial markers, vimentin and BLBP, and displayed a radial morphology (Hebsgaard et al., 2009). VM DA neurons therefore arise from floor plate radial glial-like NPs. The radial-glial origin of DA neurons should not be considered peculiar due to its departure from the classical view of radial glia as a supportive glial cell type. Indeed, the separate identities of radial neuroectodermal stem cells and radial glial cells is regularly challenged in the literature, with some authors suggesting that

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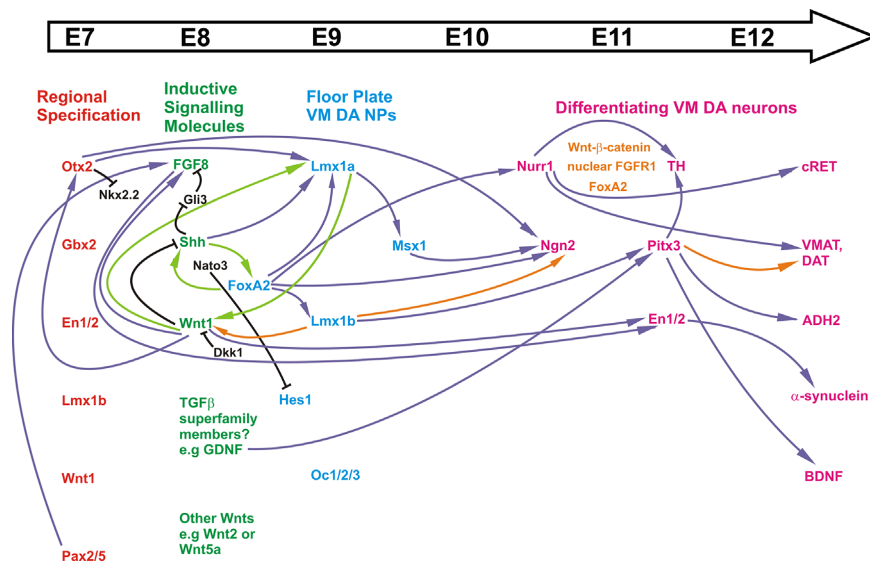

Fig. 1. Molecular factors involved in the development of VM DA neurons. *Molecular interactions in the genesis of VM DA neurons*: The sequence of appearance (see time-course arrow) of each of the factors involved in VM DA neuronal development, and their effects on each other. (Note: the molecules in black text are not shown at the time point at which they appear.) The arrows denote the effect on expression: purple = positive regulation, green = autoregulatory loop, orange = cooperative regulation, and black = negative regulation. The factors are colour-coded as per their role (listed above molecules). Otx2, Gbx2, En1/2, Lmx1b, Wnt1 and Pax2/5 play vital roles in the establishment of the midbrain/hindbrain region, including the isthmus organizer and VM floor plate, and the majority also play direct roles in VM DA neurogenesis. The diffusible signalling factors FGF8, Shh and Wnt1 induce VM DA neurogenesis in radial glial-like floor plate cells, through the induction of FoxA2, Lmx1a/1b, and Msx1 expression. Two autoregulatory loops, Shh–FoxA2 and Wnt1–Lmx1a, contribute to this process, with Hes1 being involved in the Shh–FoxA2 autoregulatory loop, and Lmx1b functioning cooperatively with Lmx1a. The expression of Nurr1 and Pitx3 promotes the differentiation of VM DA NPs into post-mitotic neurons. Nurr1 induces the expression of proteins that are key to the neurotransmitter phenotype of VM DA neurons. A number of factors facilitate the induction of TH by Nurr1 (listed in orange), and Pitx3 has been shown to cooperatively regulate a number of important genes involved in VM DA neurogenesis with Nurr1.

they are in fact the same cell type (Kriegstein and Alvarez-Buylla, 2009).

Induction of a dopaminergic phenotype in ventral midbrain neural precursors

While the floor plate and isthmus organiser are critical determinants of VM patterning, they are also crucial for the induction of a VM DA phenotype. Their role in induction of a DA phenotype is dependent upon the interaction of floor plate-secreted Shh and isthmus-secreted FGF8 (Hynes et al., 1997; Ye et al., 1998).

FGF8

The mechanism by which FGF8 regulates VM DA development is still under investigation, however a recent study suggested that FGF8 is required to induce the correct patterning of VM DA NPs, as the loss of FGFRs (FGF receptors) resulted in altered patterning of the VM and failure of VM DA neuron maturation, with the DA domain adopting diencephalic characteristics (Lahti et al., 2012). In support of this, a similar study that mutated the *FGFRs* reported a reduction in the generation of VM DA NPs, and a disturbance in the maturation of VM DA neurons (Saarimaki-Vire et al., 2007). Another FGF, FGF2, has been shown to function in the regulation of SNc DA NPs proliferation, and also in the developmental cell death of mature SNc DA neurons (Ratzka et al., 2012).

Sonic hedgehog (Shh) signalling and Lmx1 expression

The first sign of a DA phenotype in VM NPs is the initiation of expression of two key determinants of VM DA cell fate, the lim-homeodomain factor *Lmx1a* and the homeodomain transcription factor *Msx1*, at ~E9 in the mouse (Alavian et al., 2008). Shh induces the expression of *Lmx1a*, which subsequently induces the expression of its downstream effector Msx1 (Andersson et al., 2006). The overexpression of *Lmx1a* in the anterior VM results in the ectopic generation of DA neurons, while reduced expression results in a loss of VM DA neurons (Andersson et al., 2006). Additionally, null mutation of *Lmx1a* or the spontaneous mutation of *Lmx1a* in *dreher* mice results in substantial reductions in the numbers of VM DA neurons generated (Deng et al., 2011; Ono et al., 2007). *Lmx1a* expression is maintained in post-mitotic VM DA neurons until postnatal day (P) 180 in mice (Zou et al., 2009); however *Msx1* expression is confined to VM DA NPs (Andersson et al., 2006; Failli et al., 2002). This is surprising considering that *Lmx1a* is upstream of *Msx1*, suggesting that the post-mitotic repression of *Msx1* expression somehow overrides the inductive effect of Lmx1a. *Msx1* contributes to DA neurogenesis by inducing the expression of the proneural gene, *neurogenin* (*Ngn*) *2*, and thus neuronal differentiation. *Ngn2* expression in VM NPs also appears to be under the control of *Otx2* expression, as conditional *Otx2* mutant mice display a loss of *Ngn2* expression in DA NPs (Vernay et al., 2005). This finding is not surprising, considering that *Otx2* induces the expression of *Lmx1a* in VM floor plate cells, suggesting that the loss of *Ngn2* expression in the *Otx2* knockout mouse is due to a failure of *Lmx1a* induction and subsequently *Msx1* expression, rather than a direct effect on *Ngn2* expression, but this remains to be determined (Ono et al., 2007). This suggestion is supported by recent findings showing that in the absence of *Otx2*, VM NPs fail to activate the expression of *Lmx1a*, *Msx1* and *Ngn2*, and therefore largely fail to differentiate into VM DA neurons (Omodei et al., 2008).

Support for the role of Ngn2 in DA induction comes from studies showing that loss of Ngn2 in mice results in a severe reduction in the expression of post-mitotic VM DA markers *Nurr1* and *tyrosine hydroxylase* (*TH*) (discussed later), demonstrating its importance in the generation of mature VM DA neurons (Kele et al., 2006). However, the role of Ngn2 is likely to be generally proneural, rather than specific for VM DA neuronal differentiation.

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In support of this, overexpression of *Ngn2* induces neurogenesis but not a DA phenotype in cultured VM NPs (Kim et al., 2007). These data suggest the existence of a developmental programme consisting of an inductive effect of floorplate-secreted Shh on *Lmx1a* expression which subsequently induces the expression of *Msx1*, which in turn induces the expression of *Ngn2* which is required for neuronal differentiation of VM DA NPs. How and where *Otx2* fits into this cascade is unclear, but it is known that *Otx2* is required for the expression of *Lmx1a*. It has yet to be determined whether this is a parallel pathway cooperating with Shh or whether *Otx2* is a master regulator of Shh-induced *Lmx1a* expression. In support of a role of *Otx2* as a master regulator, studies involving the conditional knockout of *Otx2* in the midbrain have suggested that *Otx2* controls the fate of VM progenitors through the repression of *Nkx2.2* and maintenance of *Nkx6.1* expression (Puelles et al., 2004). FoxA2, involved in a feedback loop with Shh (discussed later), induces *Lmx1a* expression and also inhibits *Nkx2.2* (Lin et al., 2009). It is possible that *Otx2* functions via a similar pathway to FoxA2, or indeed that FoxA2 may function downstream of *Otx2* and Shh in the regulation of *Lmx1a* and *Nkx2.2* expression during VM DA neurogenesis.

Interestingly, a recent genome-wide gene expression profiling study has expanded the regulatory role of Lmx1a in this process by identifying novel transcription factors involved in the generation of the VM DA neuronal field. The Oc transcription factors, *Oc1*, *2* and *3*, display similar expression profiles to that of *Lmx1a* in the developing VM, and their loss resulted in diminished generation of VM DA neurons (Chakrabarty et al., 2012). Whether these Oc transcription factors are regulated by Lmx1a, which is plausible given their overlapping expression patterns, or whether they act in parallel to regulate neuronal differentiation in the VM, will be an important question for future research.

Similar to Lmx1a, the related protein Lmx1b has also been shown to promote VM DA neurogenesis (Deng et al., 2011; Lin et al., 2009; Yan et al., 2011). *Lmx1a* and *Lmx1b* are co-expressed in VM DA NPs, and have been shown to mediate the initial steps of NP DA specification (Andersson et al., 2006; Smidt et al., 2000). Furthermore, *Lmx1a* and *Lmx1b* are co-expressed in the P0 VM, suggesting that they may function in the maturation of VM DA neurons also (Zou et al., 2009). Similar to *Lmx1a*, *Lmx1b* can induce the ectopic production of VM DA neurons when ectopically expressed (Nakatani et al., 2010), and its loss results in a substantial reduction in the number of VM DA neurons (Deng et al., 2011; Smidt et al., 2000). A recent study using conditional knockout of *Lmx1a* and *Lmx1b* in mice demonstrated that Lmx1a and Lmx1b function cooperatively to regulate the proliferation of VM DA NPs and Ngn2 expression (Yan et al., 2011). This suggestion is supported by studies on *Lmx1a* null mice carrying one mutant *Lmx1b* allele (as double null mutations are embryonically lethal) which found that *Lmx1a* and *Lmx1b* function cooperatively in the generation of VM DA neurons. This study also showed that *Lmx1b* is involved in the generation of ocular motor neurons and red nucleus neurons in the VM (Deng et al., 2011) and it has been suggested that *Lmx1b* partially compensates for *Lmx1a* function in *dreher* mice, as only 46% of VM DA neurons are lost in these mutants (Ono et al., 2007). It will be important to understand whether Lmx1b exerts its effects in precisely the same way as Lmx1a, for example by modulating *Msx1* expression or that of *Oc1*, *2* and *3* (Chakrabarty et al., 2012).

Sonic hedgehog (Shh) signalling and FoxA expression

Floor plate-derived Shh has been shown to play a key role in induction of a DA phenotype by modulating the expression of the transcriptional regulator *FoxA2*, a well-known floor plate marker. The modulation of *FoxA2* expression is mediated by the downstream effector of Shh signalling, *Gli1* (Hynes et al., 1997). *Gli1* expression is upregulated by a related molecule Gli2 (activator) in response to Shh signalling, which is required for generation of VM DA neurons, while *Gli3* (repressor) is suppressed by Shh to allow the de-repression of *FGF8* expression (Blaess et al., 2006). *Gli2* homozygous null mutants demonstrate the importance of Gli2 in inducing ventral phenotypes, as these mice display clear deficits in VM DA neurogenesis (Park et al., 2000). Loss of both *Gli2* and *Gli1* resulted in a more severe phenotype (Park et al., 2000). *FoxA2*, along with *FoxA1*, is expressed in the VM and in differentiated DA neurons during development. Both have been shown to regulate the expression of *Ngn2* and to maintain the expression of *Lmx1a* and *Lmx1b*, which promotes VM DA neurogenesis (Bayly et al., 2012; Ferri et al., 2007; Lin et al., 2009). As aforementioned, Gli1 has been shown to induce the expression of *FoxA2* (Hynes et al., 1997), with *FoxA2* being reported as a downstream target of Shh signalling (Chung et al., 2009). However, *FoxA2* expression precedes that of *Shh* in the ventral neural tube and is proposed to regulate *Shh* expression (Echelard et al., 1993). These findings likely reflect a regulatory feedback loop between *Shh* and *FoxA2* expression, with Gli1 functioning downstream of Shh in this loop. In addition to Gli1, Nato3, a bHLH transcription factor that contributes to VM DA neurogenesis through the repression of *Hes1* (Ono et al., 2010), has been shown to integrate with the Shh–FoxA2 regulatory feedback loop in the SN4741 dopaminergic cell line (Nissim-Eliraz et al., in press). It has recently been suggested that Shh is necessary and sufficient for lateral floor plate generation, and necessary but not sufficient for medial floor plate generation, while FoxA2 is necessary and sufficient to specify the entire floor plate, acting through both Shh-dependent and independent mechanisms (Bayly et al., 2012). This induction of *FoxA2* expression by Shh has also been proposed to function cooperatively with Lmx1a and Lmx1b in the generation of DA neurons from VM floor plate NPs (Nakatani et al., 2010). This is not surprising, considering that Shh and FoxA2 positively regulate *Lmx1a* and *Lmx1b* expression. *FoxA2* mutant mice have a defective floor plate, as well as notochord, and die at E9.5 (Ang and Rossant, 1994; Sasaki and Hogan, 1994), which precludes examination of their role in DA induction. Given the recent identification of VM radial-glial progenitors, it will be interesting to use targeted strategies to conditionally remove *FoxA2* in the midbrain while preserving its expression in the floor plate (possibly through the use of *GLAST*-cre mice), and thus examine its inductive effect on *Shh* expression and its specific role in DA neurogenesis.

Wnt signalling

The Wnt family of secreted glycoproteins have become increasingly recognised as key regulators of DA neuron induction. *Wnt1* is expressed in the isthmus organiser, in an area rostral to *FGF8* at E9.5 in mice (Wilkinson et al., 1987), and is also expressed in the developing midbrain (Davis and Joyner, 1988; Wilkinson et al., 1987), along with other members of the Wnt family (Andersson et al., 2008; Parr et al., 1993; Rawal et al., 2006). In vitro, Wnt1 has been shown to regulate the proliferation of VM DA NPs and to increase the number of DA neurons generated from these cells. Wnt3a has been shown to enhance VM DA NP proliferation but to inhibit their terminal DA differentiation, whereas Wnt5a regulates the acquisition of a DA phenotype to increase DA neuronal numbers (Castelo-Branco et al., 2003). Wnt5a in particular has recently been demonstrated to play a role in the acquisition of a DA phenotype in VM DA NPs in vivo (Andersson et al., 2008). The effect of Wnt5a on DA differentiation has been suggested to be regulated by the Rac1 guanosine exchange factor, Tiam1 (Cajanek et al., 2013), and Wnt5a has been proposed to be an important mediator of the DA inductive activity of VM glia (Castelo-Branco

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et al., 2006). Another Wnt, Wnt2, has been implicated as a novel regulator of VM DA NP proliferation as *Wnt2* null mice displayed reductions in DA neurogenesis (Sousa et al., 2010).

Given that *Wnt1* is expressed in the isthmus and developing midbrain, it is perhaps not surprising that null mice displayed a loss of most of the midbrain and the DA neurons therein (McMahon and Bradley, 1990). Subsequently it was shown that although *Wnt1* null mice develop VM DA NPs, these NPs fail to proliferate and differentiate appropriately, and the few DA neurons that are generated are lost shortly thereafter (Prakash et al., 2006). This is in agreement with data describing Wnt1 as a key regulator of VM DA NPs proliferation and subsequent differentiation (Castelo-Branco et al., 2003). There is now a large body of evidence describing the key role of Wnt signalling in DA generation. Loss of the Wnt receptor *Lrp6* replicates some of the *Wnt1* developmental abnormalities (Castelo-Branco et al., 2010; Pinson et al., 2000). Similarly, loss of the Wnt receptors, *frizzled (Fzd) 3* and *Fzd6*, severely impairs midbrain morphogenesis (Stuebner et al., 2010). Interestingly, the null mutation of *Fzd3* results in a transient reduction in the numbers of VM DA neurons generated, similar to that seen in the *Lrp6* null mutant (Castelo-Branco et al., 2010; Stuebner et al., 2010). Furthermore, the specific inactivation of β-catenin, which mediates canonical Wnt signalling, mimics the midbrain-hindbrain deficits observed in *Wnt1* null mice (Brault et al., 2001; Chilov et al., 2010), suggesting that Wnt1 acts via β-catenin during midbrain-hindbrain development.

As a result of these studies, the molecular bases of Wnt-induced DA differentiation have been the focus of intensive research. Wnt1 has been shown to be essential for the maintenance of *En1* and *En2* expression (Danielian and McMahon, 1996; McGrew et al., 1999; McMahon et al., 1992), with *En1*/*En2* double knockout mice displaying a similar defective VM phenotype as the *Wnt1* null mutants (Simon et al., 2001), suggesting that the effects of loss of *Wnt1* may be due to a loss of *En* expression. This was subsequently confirmed when it was shown that *En1* was sufficient to rescue early midbrain deficits in *Wnt1* mutant mice (Danielian and McMahon, 1996). *En1* and *En2* are expressed in the ventral mesencephalon at the same time as *Wnt1* (~E8.0 in mice), however the overlapping expression domains of these three genes become restricted by E12 (Davis and Joyner, 1988). The expression of *Wnt1* in the *En1* expression domain (*En1*-*Wnt1* knock-in) causes a ventro-rostral and ventro-caudal expansion of *Wnt1* expression, which is usually restricted to the caudal VM (Danielian and McMahon, 1996), and results in an expansion of the most ventro-rostral DA cell group (Panhuysen et al., 2004). This cell group corresponds to the SNc, thus these studies demonstrate that this cell group is the most robustly influenced by Wnt1 signalling. *En1* expression is detectable in VM DA neurons from the time point at which they initiate their differentiation and persists into adulthood, while *En2* is only expressed in a subset of DA neurons (Simon et al., 2001; Zhong et al., 2010).

As mentioned earlier, *Wnt1* is expressed in the isthmus organiser, in an area rostral to where *FGF8* is expressed (Wilkinson et al., 1987). Interestingly, FGF8 signalling has also been shown to regulate *En1* expression in the developing VM (Lahti et al., 2012). It is tempting to speculate that this may be achieved through the induction of Wnt1. In support of this suggestion, a functional link between FGF8 and Wnt signalling has recently been described, where it was shown that Wnt-β-catenin signalling positively regulated *FGF8* expression in the midbrain-rhombomere1 region (Chilov et al., 2010). It is possible that this may be an autoregulatory loop, similar to that of Shh and FoxA2, with FGF 8 inducing the expression of *Wnt1* and subsequently *En1*/*En2* expression in the midbrain. Interestingly, new data have now shown that Lmx1a and Lmx1b function cooperatively to control the proliferation of VM DA NPs through the regulation of *Wnt1* expression (Yan et al., 2011).

A link between Shh and Wnt signalling has recently emerged with the proposal that canonical Wnt-β-catenin signalling is required to antagonise *Shh*, and that the subsequent reduced *Shh* levels allow the induction of VM DA NPs and the promotion of DA neurogenesis (Joksimovic et al., 2009b). The finding that Shh inhibits DA neurogenesis (Joksimovic et al., 2009b) is surprising, considering its well-established role in the induction of VM DA neurogenesis (Andersson et al., 2006; Blaess et al., 2006; Hynes et al., 1995a; Ye et al., 1998). However, Joksimovic et al., 2009a, 2009bsuggest that Shh is initially required for the early establishment of the VM DA NP pool, but that later it inhibits VM DA NP proliferation and neurogenesis. The current model suggests that once the Shh-induced VM DA NP pool has been established, Wnt-β-catenin signalling suppresses *Shh* levels in the VM to facilitate DA neurogenesis. Additionally Wnt signalling has been shown to induce *Otx2* and *Lmx1a* expression (Joksimovic et al., 2009b; Prakash et al., 2006). Furthermore, a Wnt1–Lmx1a autoregulatory loop has been identified which is proposed to regulate *Otx2* expression via β-catenin during VM DA neurogenesis (Chung et al., 2009). Interestingly, Otx2 has recently been suggested to regulate the proliferation of VM DA NPs via Wnt1 regulation (Omodei et al., 2008), suggesting a possible Otx2-Wnt1 regulatory feedback loop. In contrast to Joksimovic et al. (2009); Chung et al. (2009) suggested a mechanism by which the Wnt1–Lmx1a autoregulatory loop and a Shh–FoxA2 autoregulatory loop control VM DA neurogenesis synergistically. Despite this finding, a more recent paper described an antagonistic relationship between Wnt-β-catenin signalling and Shh signalling that is important in the progression of DA NPs into VM DA neurons (Tang et al., 2010), thus supporting the Joksimovic et al. (2009) theory. Furthermore, the stabilisation of β-catenin in VM NPs, by the inhibition of GSK3β, leads to an increase in DA differentiation (Castelo-Branco et al., 2004; Tang et al., 2009) and targeted deletion of *β-catenin* in VM NPs (Th-IRES-Cre; β-Ctn$^{Ex3/+}$ mutant) results in reduced VM DA neurogenesis (Tang et al., 2009). Surprisingly, mice with mutations in the Wnt/β-catenin inhibitor *Dkk1* actually have a reduction in VM DA neurons (Ribeiro et al., 2011). This is surprising, given that the stabilisation of β-catenin in VM NPs, through the inhibition of GSK3β, leads to an increase in DA differentiation (Castelo-Branco et al., 2004; Tang et al., 2009). Collectively these data largely support the theory that Wnt signalling is required for DA induction, but it is also clear that this is likely to involve a complex interplay with Shh and FGF8 signalling, and potentially other extrinsic signalling factors which have been suggested to induce VM DA neurogenesis, including TGFβs (Farkas et al., 2003; Roussa et al., 2009, 2006).

Development of post-mitotic ventral midbrain dopaminergic neurons

Once NPs of the VM floor plate are specified towards a DA phenotype, these DA NPs gradually become post-mitotic from E10-E14 in mice (E12–E16 in rats) (Lauder and Bloom, 1974; Lumsden and Krumlauf, 1996), with the greatest proportion of VM DA NPs undergoing their final division at E12 in the rat (Gates et al., 2006). The induction of *TH* expression, the rate-limiting enzyme for DA synthesis, is the first sign of the acquisition of the DA neuronal phenotype, and occurs shortly after the final mitosis of VM DA NPs while they are actively migrating to their final positions (Puelles and Verney, 1998, Specht et al., 1981a; Specht et al., 1981b). This process of migration of VM DA neurons from the floor plate ventricular zone to the presumptive VTA and SNc involves two steps: firstly, DA neurons migrate ventrally along tenascin-expressing radial glial processes which project to the pial surface, and secondly, once they have reached the basal part of the VM, they migrate laterally along

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tangentially orientated fibres to form the VTA and SNc (Kawano et al., 1995; Shults et al., 1990). These tangentially-orientated fibres express the neural cell adhesion molecule L1, while VM DA neurons express the chondroitin sulphate proteoglycan 6B4. A heterophilic interaction between L1 and 6B4 has been proposed to facilitate this process of lateral migration of VM DA neurons (Ohyama et al., 1998). There have been a variety of studies that show that this process of migration is crucial for the normal positioning of VM DA neurons. Specifically, VM DA neurons are abnormally located in *L1* knockout mice (Demyanenko et al., 2001). A role for Wnt signalling in this process has been implicated by a study showing that the targeted deletion of *β-catenin* in the VM disrupts the integrity of these radial glia, resulting in perturbed migration of VM DA neurons (Tang et al., 2009). It is unclear whether these migratory defects are secondary to a disrupted radial glial scaffold or whether Wnt signalling can also directly affect this process of migration. A number of other molecules involved in neuronal migration in the developing CNS also appear to be involved in the migration of VM DA neurons. These include the well-known migrational regulator *Reelin*, as VM DA neurons fail to migrate laterally to the SNc, in *reeler* (*reelin null*) mice (Nishikawa et al., 2003), and the netrin receptor, *DCC*, which is expressed by migrating VM DA neurons in mice, and its loss results in aberrant migration of these neurons (Xu et al., 2010). In terms of the molecular regulatory networks that control this migration, there have been a number of studies describing roles for Ebf1 (early B-cell factor 1) (Yin et al., 2009) and Hes1 (Kameda et al., 2011) in this process. A key goal for future research will be to understand the molecular networks that control this process of VM DA migration and how newly-identified molecules such as Ebf1 and Hes1 "fit" within this network. While much work has focused on identifying the molecular signals that are required for neuronal migration, it will also be important to understand what positional cues inhibit these processes so that VM DA neurons "know" when to stop.

Several transcription factors have been identified which are essential for the differentiation and subsequent long-term survival of VM DA neurons. These include Lmx1b, Nurr1, Pitx3, En1 and En2. Each of these factors are not individually capable of inducing a complete DA phenotype, suggesting that they function as part of a network (Fig. 1).

Lmx1b

The lim-homeodomain factor *Lmx1b* is broadly expressed in the presumptive midbrain before neural tube closure, and its expression becomes restricted to VM DA NPs at E10.5 in mice, where it is co-expressed with *Lmx1a* and *Msx1* (Andersson et al., 2006; Smidt et al., 2000). Surprisingly, *Lmx1b* expression disappears in the VM at around E11.5, but reappears at E16 in post-mitotic VM DA neurons. *Lmx1b* is subsequently co-expressed with *Pitx3* and *TH* into adulthood in the VM (Dai et al., 2008). Although loss of *Lmx1b* leads to a loss of VM DA neurons (Smidt et al., 2000), *Lmx1b* mutant mice express *Nurr1* and *TH* normally during early development, but fail to express *Pitx3*. These TH-positive VM neurons, which lack *Pitx3* expression, are lost by birth, suggesting a role for Lmx1b in the regulation of *Pitx3* expression and VM DA neuronal survival. Similarly in *Wnt1* null mice, the few TH-positive VM neurons generated lack *Pitx3* expression, and are subsequently lost before E12.5 (Prakash et al., 2006). A similar regulatory loop may exist between Wnt1 and Lmx1b, as Lmx1b induces and/or maintains the expression of *Wnt1*, an important extrinsic factor in VM DA neurogenesis (see above), around the midbrain-hindbrain boundary (Adams et al., 2000; Matsunaga et al., 2002). Wnt1 has also been proposed to act downstream of Lmx1b in the potential regulation of *Pitx3* expression (Prakash et al., 2006). This maintenance of *Wnt1* expression by Lmx1b may be important in the generation of post-mitotic DA neurons, as Wnt1 is required for the terminal differentiation of VM DA neurons at later stages of embryogenesis (Prakash et al., 2006).

Nurr1

Nurr1 is a member of the nuclear receptor superfamily of steroid-thyroid hormone-activated transcription factors (Law et al., 1992), which atypically lacks both a ligand cavity and a canonical coactivator-binding site (Wang et al., 2003). *Nurr1* is expressed in the VM from E10.5 in the mouse, as VM DA NPs begin to become post-mitotic, one day before the appearance of *TH* (Zetterstrom et al., 1996), and *Nurr1* expression is maintained into adulthood (Backman et al., 1999). *Nurr1* expression levels show a sharp peak between E13 and E15 in the rat, at a stage of development when most VM DA neurons are undergoing terminal differentiation (Volpicelli et al., 2004). VM DA neurons in *Nurr1*-deficient animals do not express *TH*, l-aromatic amino acid decarboxylase (*AADC*), the vesicle monoamine transporter 2 (*VMAT2*) or the dopamine transporter (*DAT*), all markers of a DA neuron that has acquired its DA neurotransmitter identity (Castillo et al., 1998; Filippi et al., 2007; Smits et al., 2003). Nurr1 has been shown to play a direct role in regulating the expression of these genes, and a number of well-established signalling pathways in VM DA development cooperate with Nurr1 to mediate this induction. Specifically, Nurr1 has been shown to induce *TH* expression by binding to a NRBE (NGFI-B response element) sequence in the *TH* promoter (Kim et al., 2003; Sakurada et al., 1999), and is known to induce *DAT* expression via an NRBE-independent mechanism (Sacchetti et al., 2001). These effects of Nurr1 on the expression of these genes are enhanced by Wnt-activated β-catenin, which has been shown to promote Nurr1-induced *TH* promoter activation, by interacting with Nurr1 at NRBEs, causing the dissociation of transcriptional co-repressors and recruitment of transcriptional co-activators (Kitagawa et al., 2007). Similarly, nuclear FGFR1 has been shown to cooperate with Nurr1 to promote activation of the *TH* promoter (Baron et al., 2012a). Collectively these data show that Nurr1 functions as a master regulator in the induction of the neurotransmitter phenotypic identity of VM DA neurons, and controls the expression of the molecules that regulate the synthesis, vesicle packaging, axonal transport and reuptake of DA.

Aside from this role in DA identity, Nurr1 has also been shown to be crucial for long-term VM DA neuron survival. In *Nurr1*-deficient animals, VM DA neurons adopt a correct ventral position and express the DA markers *Lmx1b*, *Pitx3* and *En1* (Saucedo-Cardenas et al., 1998; Wallen et al., 1999), demonstrating that *Nurr1* is not required for all aspects of VM DA specification and differentiation. However, these *Pitx3*-expressing VM DA neurons are lost in *Nurr1*-deficient animals during later development (Saucedo-Cardenas et al., 1998), suggesting a role for *Nurr1* in the survival and maintenance of VM DA neurons. In support of these findings, *Nurr1* is expressed throughout the life of VM DA neurons and its heterozygous mutation increases the vulnerability of VM DA neurons to the parkinsonian toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-HCl (MPTP) (Le et al., 1999). Furthermore, the conditional ablation of *Nurr1* at a late stage of VM DA neuron development or in the adult brain results in loss of striatal DA, loss of VM DA markers and DA neurodegeneration, with SNc DA neurons more vulnerable than those of the VTA (Kadkhodaei et al., 2009).

While Nurr1 has been shown to directly regulate the expression of *TH* and *DAT*, Nurr1 may promote VM DA neuron survival indirectly, by inducing the expression of genes essential for their survival. Nurr1 has been found to regulate the expression of the glial cell line-derived neurotrophic factor (GDNF) receptor, *cRet*, whose expression is lost in *Nurr1*-deficient animals (Castillo et al., 1998). GDNF is a well-known survival-promoting factor for VM DA

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neurons (Lin et al., 1993; Toulouse and Sullivan, 2008; Yasuhara et al., 2007). *Nurr1* has also been shown to regulate the expression of *VIP* (vasoactive intestinal peptide), which has been proposed to function in the survival of VM DA neurons (Luo et al., 2007). It remains to be determined whether the VM DA neuronal death in *Nurr1* null mutants is as a direct consequence of the absence of a Nurr1-mediated survival-promoting effect, and/or is induced by a lack of neurotransmission by these cells. Aside from its role in DA survival, Nurr1 has been suggested to play a role in target innervation by VM DA neurons (Wallen et al., 1999); however, this finding has been challenged (Witta et al., 2000). It will be important to assess the role of Nurr1 in striatal innervation in vivo using an approach where DA neurons can survive long term in the absence of Nurr1. Such a strategy has been employed successfully in the PNS, where Bax-deficient mice were used to analyse specifically the effects on a gene of interest on target innervation independent of this genes role in neuronal survival (Barker et al., 2001; Glebova and Ginty, 2004; Middleton and Davies, 2001).

The molecular mechanisms by which *Nurr1* expression is induced and regulated in the VM are largely unknown, but Nurr1 has been shown to function independently of FGF8 and Shh signalling (Sakurada et al., 1999). However, recent data suggests that FoxA1 and FoxA2 may be critical to *Nurr1* induction. Through the analysis of single and double mutants, *FoxA1* and *FoxA2* have been reported to regulate the expression of *Nurr1* in a dose-dependent manner, with a dramatic decrease in *Nurr1* expression observed in double mutants (Ferri et al., 2007). In addition to this, a gain-of-function study has demonstrated that *FoxA2* mediates *Nurr1* expression (Lee et al., 2010). Lee et al. (2010) further demonstrated that FoxA2 acts synergistically cooperates with Nurr1 during VM DA neurogenesis, with both factors binding to the *TH* promoter. In support of this, the loss of *FoxA2* resulted in an increase in the numbers of Nurr1-positive, TH-negative, cells in the VM (Ferri et al., 2007). These more recent data suggest that Nurr1 function may not be independent of Shh signalling, as *FoxA2* is a downstream target of Shh; however FoxA2 can function independently of Shh. Similar to Nurr1, FoxA2 appears to be involved in the survival and maintenance of VM DA neurons, as aged mice with a heterozygous mutation in *FoxA2* develop PD-like symptoms and pathologies (Kittappa et al., 2007), but this maybe due to altered FoxA2-induction of Nurr1 expression.

Pitx3

Pitx3 is a bicoid-related, homeodomain-containing transcription factor that is exclusively expressed in the mouse VM from E11.5, at a time when VM DA neurons are beginning to appear (Smidt et al., 1997). VM DA neurons only begin to express *Pitx3* when they arrive at their final ventral position, suggesting that Pitx3 is not involved in the early development or migration of VM DA neurons (Smidt et al., 2004). GDNF has been suggested to induce the expression of *Pitx3* in the VM (Lei et al., 2011; Peng et al., 2011) which is interesting as Nurr1 is known to regulate the expression of the GDNF receptor *cRet*, and is expressed before *Pitx3* in the VM. Nurr1 may therefore play an indirect, non-essential (*Pitx3* expression is retained in *Nurr1* null mutants) role in the induction of *Pitx3* expression. Pitx3 and Nurr1 have been shown to function cooperatively in the regulation of target genes involved in VM DA neurogenesis (discussed later) (Chakrabarty et al., 2012; Hwang et al., 2009; Jacobs et al., 2009a; Jacobs et al., 2009b). Pitx3 is co-expressed in the TH-positive neurons of the VM (Smidt et al., 1997; Van Den Munckhof et al., 2003; Zhao et al., 2004). In *aphakia* mice, which lack *Pitx3* expression due to deletions in the *Pitx3* gene, there is unaltered VM DA development until E12.5, at which time a deficit is observable in the lateral population of VM DA neurons which constitute the presumptive SNc (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; Van Den Munckhof et al., 2003). VTA DA neurons are largely unaffected in these mice. The specific absence of SNc DA neurons in *Pitx3* null (*aphakia*) mice results in a loss of nigrostriatal projections to the dorsal striatum (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; Van Den Munckhof et al., 2003), and suggests distinct developmental programmes for SNc and VTA DA neurons. Interestingly, it has been reported that lateral VM DA neurons express *Pitx3* prior to *TH*, while the medial VM DA neurons express *Pitx3* coincidently with *TH* (Maxwell et al., 2005). In addition to this, Pitx3 has been suggested to regulate *TH* expression (Cazorla et al., 2000; Lebel et al., 2001; Maxwell et al., 2005). Pitx3 may therefore be critical for the induction of *TH* expression in SNc DA neurons, but not those of the VTA. In support of this, the absence of *Pitx3* results in a failure of SNc DA neurons to express *TH*, while VTA neurons do so. Interestingly, *Pitx3* expression has been reported to be six times higher in VTA DA neurons then in those of the SNc (Korotkova et al., 2005). Perhaps this lower expression level of Pitx3 functions in the induction of *TH* expression in SNc DA neurons, while it may also contribute to their inherent sensitivity. In support of the proposed role for Pitx3 in *TH* expression, Pitx3 has been shown to contribute to the neurotransmitter phenotype of VM DA neurons by inducing the expression of *DAT* and *VMAT2* (Hwang et al., 2009). However, the loss of TH-positive neurons in the ventro-lateral VM is not due to the loss of TH mRNA expression, but to neuronal loss (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004). Perhaps the selective neurodegeneration of SNc DA neurons in *aphakia* mice is not as a result of a failure of DA neurogenesis in the absence of *Pitx3*, but may reflect the characteristic sensitivity of this VM population in comparison to those of the VTA. Indeed, the VTA DA neurons in *aphakia* mice display a normal DA phenotype (Smidt et al., 2004). A recent study has demonstrated that Pitx3 induces the expression of *brain-derived neurotrophic factor* (*BDNF*) in SNc DA neurons, which may be important in the survival of these neurons (Peng et al., 2011). Peng et al. (2011) showed that loss of *BDNF* expression correlates with the SNc neuronal loss in *Pitx3* null mice, and that BDNF treatment induces the survival of *Pitx3* (−/−) VM DA neurons and protects them against the dopaminergic neurotoxin 6-hydroxydopamine. Pitx3 may therefore be critical in the maintenance and survival of SNc DA neurons, acting via BDNF. Similarly, *BDNF* has been identified as a target gene of Nurr1 (Volpicelli et al., 2007).

Despite the lack of a direct role for Nurr1 in *Pitx3* expression, a recent set of studies has shown that Nurr1 regulates target gene expression cooperatively with Pitx3 during VM DA neurogenesis, with Pitx3 potentiating Nurr1 activity by releasing it from SMRT-mediated repression (Jacobs et al., 2009a, 2009b). Similarly, the same group demonstrated that Nurr1 and Pitx3 cooperatively regulate the expression of two cholinergic receptors, *Chrna3* and *Chrnb6*, which may play non-essential roles in VM DA neurogenesis (Chakrabarty et al., 2012). Furthermore, Pitx3 has been shown to induce the expression of the Nurr1-target genes, *VMAT2* and *DAT*, potentially in coordination with Nurr1 (Hwang et al., 2009), with Pitx3 also inducing the expression of aldehyde dehydrogenase 2 (*ADH2*), an enzyme which is highly expressed in SNc DA neurons (Chung et al., 2005). In support of this combinatorial function, Nurr1 and Pitx3 have been shown to cooperatively promote terminal maturation of VM DA neurons in stem cell cultures (Martinat et al., 2006) Collectively, these data suggest Nurr1 and Pitx3 may cooperate to promote VM DA survival and acquisition of a mature DA neurotransmitter phenotype by cooperatively regulating the expression of DA neurotrophic factors, *BDNF* and *GDNF*, and of genes involved in DA neurotransmission respectively.

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En1 and En2

En1 and *En2* are important in the formation of the isthmus organiser and in the generation of VM DA neurons (Liu and Joyner, 2001; Simon et al., 2001). Following their initial expression in the midbrain-hindbrain boundary (Davis and Joyner, 1988), VM DA neurons begin to express *En1* and *En2* between E11.5 and E14 in mice, and this expression is maintained into and throughout adulthood (Alberi et al., 2004). Interestingly, in *En1* and *En2* double mutants, VM DA neurons develop normally initially, but are lost by E14 due to caspase-dependent apoptosis, just after the expression of *En* begins in the wild type (Alberi et al., 2004; Simon et al., 2001). Alberi et al. (2004) demonstrated that *En1* and *En2* are required cell-autonomously in post-mitotic VM DA neurons to prevent apoptosis. However, further studies are required to ascertain that VM DA neuronal loss in the *En* double mutants is not as a result of the large midbrain/hindbrain deletion in these mice. Despite this possibility, these data strongly suggest a role for *En1* and *En2* in the maintenance and survival of VM DA neurons. Indeed, intermediate genotypes between wild type and double *En* mutants show varying degrees of VM DA neuronal deficiencies (Sgado et al., 2006; Simon et al., 2001; Sonnier et al., 2007), as has been well-described in recent reviews (Alavian et al., 2008; Alves Dos Santos and Smidt, 2011). The most notable phenotype was observed in *En1* (+/−)/*En2* (+/+) mutant mice, which display a progressive degeneration (between 8 and 24 weeks) of VM DA neurons that can be antagonised by recombinant En2 protein infusion (Sonnier et al., 2007). The progressive degeneration of VM DA neurons in *En1* heterozygotes (*En2* null background in Sgado et al. (2006) study) is more pronounced in the SNc and results in reduced striatal DA and motor deficits, as is characteristic of PD pathology (Sgado et al., 2006; Sonnier et al., 2007). These findings further support the theory that *En1*/*En2* function as important survival-promoting factors for VM DA neurons.

Diversity in genetic regulation of DA neuron development

As mentioned earlier, the molecular mechanisms controlling phenotypic and functional diversity between the various VM DA neuronal subpopulations remain poorly understood. However, recent work has implicated *Otx2* as a factor that may contribute to these distinct developmental pathways. *FoxA2*, *En1*, *Lmx1b*, *Nurr1* and *Pitx3* are ubiquitously expressed in post-mitotic VM DA neurons throughout life; however *Otx2* expression is restricted to VTA DA neurons in the adult brain (Di Salvio et al., 2010b). *Otx2* was shown to regulate subtype identity in the VTA by antagonising the expression of *Girk2* and *DAT*, and was also shown to antagonise the neurotoxic effect of the MPTP in these VTA neurons (Di Salvio et al., 2010a). Interestingly, ectopic *Otx2* expression also provides SNc neurons with neuroprotection to MPTP (Di Salvio et al., 2010a). This potential role of *Otx2* in VM DA neuronal subtype identity has been comprehensively described in a recent review (Simeone et al., 2011).

It is also necessary to mention that a proportion of DA neurons arise anterior to the VM in the diencephalon (Gonzalez et al., 1999. Lahti et al., 2012; Marin et al., 2005; Puelles and Verney, 1998; Smits et al., 2006; Verney, 1999; Verney et al., 2001. Vitalis et al., 2000), and develop earlier than those from the VM (Lahti et al., 2012; Marin et al., 2005). The diencephalic DA domain differs to that of the midbrain. The DA NPs in the diencephalon are intermingled with non-DA Pou4f1+FoxP1+ cells, they lack *Pitx3* and *DAT* expression, and lose *En1*/*2* expression by E9.5 (Lahti et al., 2012), unlike those in the midbrain (Alberi et al., 2004). FGF8 regulates the diverse identities of the DA neurons from the VM and caudal diencephalon. This anterior–posterior patterning by FGF8 suppresses diencephalic identity and maintains midbrain identity (Lahti et al., 2012; Scholpp et al., 2003). A study using zebrafish showed that Nodal signalling was required for the specification of ventral diencephalic and pretectal catecholaminergic neurons (Holzschuh et al., 2003). (Holzschuh et al., 2003) also demonstrated that FGF8 signalling was not required for the specification of these neurons but was important for their proliferation or survival, and that Shh signalling is required for pretectal DA development. Diencephalic DA neurons therefore seem to be subject to a different programme of neurogenesis than those of the VM. Genetic fate-mapping studies are needed to verify whether, or to what extent, these diencephalic DA neurons contribute to the DA subpopulations of the VM.

Establishment of dopaminergic projections from the ventral midbrain

Following their generation, post-mitotic VM DA neurons undergo functional maturation, which involves axonal pathfinding and synaptogenesis. Axons from VM DA neurons, which arise at E11 in mice (E13 in rat), initially project dorsally but then deflect ventro-rostrally towards the forebrain, in response to extrinsic directional cues in the dorsal midbrain and repulsive cues in the caudal brain stem (Gates et al., 2004; Nakamura et al., 2000). The reorientated VM DA neuronal axons then extend towards the telencephalon, through the diencephalon, via the medial forebrain bundle (MFB) which has been reported to have a chemo-attractive effect on these axons (Gates et al., 2004). A recent paper has suggested that Nurr1 regulates the axonal extension of VM DA neurons through the regulation of the expression of the axon genesis gene *Topoisomerase II*β (*TopII*β) (Heng et al., 2012). Furthermore, a study using retrograde labelling suggested that Nurr1 plays a role in target innervation by VM DA neurons (Wallen et al., 1999). However, as mentioned before, this finding has been challenged (Witta et al., 2000). Gates et al. (2004) also demonstrated that the thalamus prevents entry of VM DA axons through the action of contact-dependent inhibitors, which likely function to maintain the orientation of these axons in the MFB. Furthermore, *Nkx2.1* mutant mice display aberrant midline crossing of MFB fibres at the caudal diencephalon, suggesting that chemorepulsive factors involved in maintaining the ipsilateral trajectory of the MFB at the medial part of the caudal diencephalon are lost in this mutant (Kawano et al., 2003).

The VM DA neuronal axons run via the MFB into the telencephalon, where they terminate in the striatum and cerebral cortex (Specht et al., 1981a, 1981b; Zhao et al., 2004). In the case of the nigrostriatal pathway, chemoattraction from the striatum and chemorepulsion from the cortex have been suggested to facilitate appropriate striatal innervation by nigral DA neurons (Gates et al., 2004). The molecular signals that guide the axons of the various populations of VM DA neurons remain to be characterised; however a relatively recent review has comprehensively described the current understanding of the development of VM DA circuitry (Van Den Heuvel, Pasterkamp, 2008). Molecules which are known to be involved in the establishment of the VM DA circuit are illustrated in Fig. 2.

Despite the current paucity of studies determining the molecular basis of the formation of VM DA projections, several molecules have been implicated to play a role in this process. *EphrinB2* and its receptor *EphB1* have been shown to be expressed in a complementary pattern to facilitate nigro-striatal innervation, with *EphB1* expressed by VM DA neurons (with highest expression in the SNc) and *ephrinB2* expressed in the striatum (Yue et al., 1999). Cell-surface tethered ephrins, and their Eph receptor tyrosine kinases, are known to play important roles in axonal guidance (Egea and Klein, 2007). Furthermore, Yue et al. (1999)

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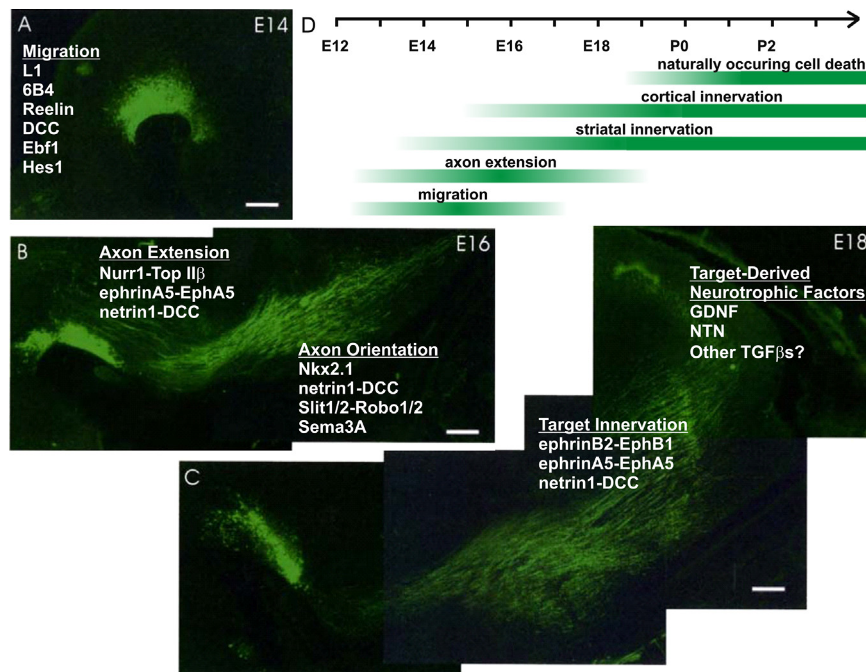

Fig. 2. Molecules involved in the formation of the nigrostriatal DA circuitry. *Development of the rat nigrostriatal pathway*: Representative photomicrographs showing cryosections through the developing rat nigrostriatal pathway at (A) E14, (B) E16 and (C) E18, immunostained for TH. Molecules involved in the migration (A), axon extension (B), axon orientation (B), target innervation (C) and survival (C) of VM DA neurons are labelled on the images where appropriate. Scale bar = 100 μm. (D) Graphical representation of the time-course of DA circuitry formation. VM DA neurons begin to migrate and extend axons at E13 in the rat. These DA fibres begin to reach the striatum by E14, and the cortex at E16. The innervation of these targets continues into the first week after birth. Naturally occurring cell death begins close to birth, reaching a peak at P2 and P14, before subsiding around P20.

showed that co-culture with ephrinB2-expressing NIH-3T3 cells reduced neurite outgrowth and induced death of SNc, but not VTA, DA neurons. These results suggest that the interaction between ephrinB2 and EphB1 in the striatum ensures that SNc DA neurons are confined to the dorsal striatum. Conversely, *EphB1* expression has been shown to disappear in the SNc from E18, and its null mutation resulted in no observable defects in the nigrostriatal pathway (Richards et al., 2007). These results challenge the role for EphB1 in the formation of the nigrostriatal pathway; however other Eph receptors may allow SNc DA axons to detect ephrinB2 in the striatum. In support of a role for ephrinB2 in the correct target innervation of nigral DA neurons, the application of ephrinB2 to VM cultures resulted in an upregulation of *Nurr1* (Calo et al., 2005). This action by ephrinB2 could function to support and maintain correctly-innervated DA neurons. However, this effect was suggested to be mediated by the EphB1 receptor (Calo et al., 2005). Other ephrins and Ephs have also been implicated in DA pathway formation. For example, studies on genetically-altered EphAs and ephrinAs have shown that these molecules are important in the formation of VM DA projections (Halladay et al., 2004; Sieber et al., 2004; Van Den Heuvel, Pasterkamp, 2008), with *ephrinA5* expression being reduced in the forebrain of *Nkx2.1* mutants (described above) (Marin et al., 2002). *EphrinA5* has been shown to be expressed in the developing telencephalon and striatum, in the vicinity of VM DA axons, and to have a repulsive effect on these axons, likely through the action of EphA5 (Deschamps et al., 2009). Conversely, another study has shown that ephrinA5-EphA5 signalling promotes DA axonal growth in vitro (Cooper et al., 2009). Perhaps ephrinA5 initially functions in the establishment of VM DA projections, but later functions to restrict these axons to their targets. Semaphorin signalling has also been proposed to function in VM DA axonal pathfinding (Hernandez-Montiel et al., 2008; Kolk et al., 2009, Tamariz et al., 2010; Torre et al., 2010), with a number of semaphorins and their receptors being expressed in VM DA neurons (Torre et al., 2010). Furthermore, *Sema3A* expression is reduced in *Nkx2.1* mutants (Kawano et al., 2003), and the expression of its co-receptor *Neuropilin1* has been shown to be regulated by Nurr1 in the developing midbrain (Hermanson et al., 2006).

The netrin receptor *DCC* has been demonstrated to play an important role in the formation of VM DA axonal projections. *DCC* is expressed in the VM and in cultured VM DA neurons, as well as in VM DA targets, such as the striatum and prefrontal cortex (Lin et al., 2005; Livesey and Hunt, 1997; Xu et al., 2010), while *netrin1* is expressed in a complimentary fashion in VM, striatal and cortical neurons (Hamasaki et al., 2001; Livesey and Hunt, 1997; Manitt et al., 2011). Studies of heterozygous and homozygous *DCC* mutants have provided insights into how netrin-DCC signalling may regulate the formation of VM DA neuronal projections (Flores et al., 2005; Xu et al., 2010). DA innervation of the dorsal striatum is not affected in heterozygous and homozygous *DCC* mutants, while the ventral striatal DA projections are aberrantly shifted to a more dorsal location in null mutants. The innervation of the prefrontal cortex by VM DA neurons is significantly reduced in null mutants, suggesting that *DCC* is an important mediator of VM DA axonal guidance. Xu et al. (2010) also propose that DCC signalling is an important negative regulator of DA axon arborisation, demonstrating that DA innervation is maintained/increased despite significant VM DA neuronal loss in *DCC* deficient animals. In support of this, analysis of heterozygous *DCC* mutants has shown that DCC can selectively influence the branching of VM DA fibres in the prefrontal cortex at puberty, with a significant increase in the number of TH-positive varicosities present post-puberty in these heterozygotes (Manitt et al., 2011). This proposed role of DCC contradicts previous reports which had suggested that DCC mediates netrin1-promotion of axonal outgrowth in VM DA neuronal cultures (Lin et al., 2005). However, these contrasting results may reflect differences between the responses of VM DA neurons in vitro and in vivo. Furthermore, DCC receptors are known to mediate both attraction and repulsion aspects of the

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axon growth-promoting effects of netrins (Round and Stein, 2007). The atypical homeoprotein Pbx1a has been shown to regulate the expression of *DCC* in VM DA neurons, and its deficiency results in partial misrouting of VM DA fibres (Sgado et al., 2012). Interestingly, *DCC* null mice also display aberrant midline crossing of MFB DA fibres at the caudal diencephalon, which is similar to that observed in *Nkx2.1* mutant mice (Xu et al., 2010), likely reflecting a loss of chemorepellant(s) at the ventral midline. In *Pax6* null mice, which display a ventro-dorsal expansion of netrin1 expression, VM DA axons within the MFB are deflected dorsally in the diencephalon, seemingly avoiding areas of ectopic *netrin1* expression (Vitalis et al., 2000). Netrin-DCC signalling may therefore act at the ventral midline of the diencephalon to repel VM DA projections, ensuring that they maintain their ipsilateral course in the MFB. Similarly, mice deficient in both *Slit1* and *Slit2* display abnormal ventral midline crossing of MFB fibres in the diencephalon, suggesting they may also act as ventral midline chemorepellants (Bagri et al., 2002; Dugan et al., 2011). In support of this theory, Slit2 repels VM DA neuronal axons and inhibits their growth in vitro (Dugan et al., 2011; Lin et al., 2005). *Nkx2.1* mutant mice display altered *Slit1* and *Slit2* expression, and a more severe phenotype than that of *Slit1/Slit2* double mutants (Marin et al., 2002). This suggests that *Nkx2.1* may regulate the expression of a number of important chemorepellants at the diencephalic ventral midline, such as *Slit1/Slit2*, *Sema3A*, *ephrinA5* and perhaps *netrin1*. However, *netrin1* expression is maintained in the subpallium of the *Nkx2.1* mutants (Marin et al., 2002), and has been reported to attract diencephalic (A11) DA neurons towards the ventral midline in the absence of repulsive Slit signals (Kastenhuber et al., 2009). Mice deficient in the Slit receptors, *Robo1* and *Robo2*, also display guidance errors in the MFB tract similar to those in the *Slit* double mutant (Dugan et al., 2011; Lopez-Bendito et al., 2007). However, Dugan et al. (2011) also demonstrated abnormal dorsal trajectories of VM DA fibres in *Robo1/2* knockout mice, which does not occur in the *Slit1/2* mutant, suggesting that these Robos also function Slit-independently. The expression patterns of Slits also propose a role for these molecules in VM DA axonal guidance, with *Slit1* expressed in the dorsal midbrain (Nakamura et al., 2000) and *Slit3* expressed in the caudal midbrain (Gates et al., 2004), suggesting that these Slits contribute to the ventro-rostral trajectory of VM DA fibres.

Upon innervation of their targets, the axons of VM DA neurons compete to establish functional synapses and survive. There are two peak postnatal periods of naturally-occurring cell death for VM DA neurons. Cell death begins close to birth, reaching an initial peak at P2, before a second peak of apoptosis occurs at P14, with this process largely subsiding around P20 in rodents (Burke, 2003; Jackson-Lewis et al., 2000; Oo and Burke, 1997). This programmed cell death pathway relies on the limited availability of target-derived neurotrophic factors (Burke, 2003), with striatal and prefrontal cortex tissue being shown to promote VM DA neuronal survival when co-cultured in vitro (Hoffmann et al., 1983). The most well-established target-derived neurotrophic factor for VM DA neurons is GDNF (Akerud et al., 1999; Beck et al., 1995; Burke, 2003; Costantini and Isacson, 2000; Gash et al., 1996; Lei et al., 2011; Lin et al., 1993; Redmond et al., 2009; Tomac et al., 1995; Wang et al., 2010). Another member of the GDNF protein family, neurturin (NTN), also acts as a neurotrophic factor for VM DA neurons (Akerud et al., 1999; Horger et al., 1998; Oiwa et al., 2002; Tseng et al., 1998; Zihlmann et al., 2005). Other neurotrophic factors identified for VM DA neurons include transforming growth factor βs (TGFβs) (Farkas et al., 2003), BDNF (Alonso-Vanegas et al., 1999) and growth/differentiation factor 5 (GDF5) (Costello et al., 2012; Hurley et al., 2004; O'Keeffe et al., 2004; O'Sullivan et al., 2010; Sullivan and O'keeffe 2005; Sullivan et al., 1997, 1998). Interestingly, FGF2 has recently been shown to act as a target-derived regulator of VM DA innervation (Baron et al., 2012b).

Concluding remarks and future perspectives

This paper reviews the recent data from molecular studies on VM DA development, and highlights a number of important genetic pathways involved during the neurogenesis of these cells. It is clear that this is a complex developmental programme, complicated further by the fact that VM DA neurons are not a homogenous population of neurons. However, insights are beginning to be made on the molecular mechanisms that may, in part, confer subtype identity within the VM DA circuit, such as those described for Shh, Pitx3 and Otx2. Despite these developments, future studies will be crucial to elucidate the molecular basis of these subtle developmental differences between A8, A9 and A10 DA neuronal identities.

VM DA neurons are now known to arise from floor plate radial glial-like NPs in response to specification by FGF8, Shh and Wnt1. This recent discovery is important if VM DA NPs are to be specifically isolated for use in cell replacement therapies. Additionally, Wnt1 is now accepted as an extrinsic factor for VM DA neurons, along with Shh and FGF8, which has added another dimension to the developmental programme of VM DA neurogenesis. A number of TGFβ superfamily members, and other Wnts, have also been implicated as instructive signalling molecules during VM DA neurogenesis. Similar to the recent studies carried out for Wnt1, these candidates should be investigated thoroughly for their participation in these developmental processes.

The discovery of a number of new candidate transcription factors, for example Oc1/2/3, highlights that there are likely to be other, as yet unidentified, molecular pathways involved in regulating VM DA neurogenesis. Furthermore, new relationships are being uncovered between the transcription factors and molecular pathways that are well-known to play key roles in DA development. For example, Nurr1 and Pitx3 were previously thought to function independently, however, recent data show that these key transcription factors function cooperatively. These findings highlight that there is still a significant challenge remaining to understand the complexities of the dynamic molecular interactions between the known genetic networks involved in VM DA neurogenesis.

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