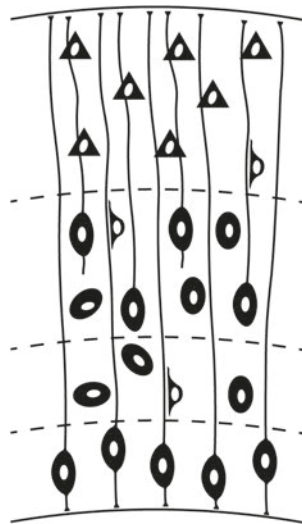


Investigating Brain Repair and Development Using Stem Cells

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Abstract

The directed differentiation of human pluripotent stem cells into neuronal subtypes has generated immense enthusiasm that they could be used therapeutically or to study development *in vitro*. Two areas of particular interest are the replacement of midbrain dopaminergic neurons that degenerate in Parkinson's disease and the ability to study the early development of the human cortex.

The loss of midbrain dopamine neurons in Parkinson's disease leads to a breakdown in basal ganglia circuitry and motor dysfunction. Previous clinical trials utilizing fetal dopamine tissue have provided proof-of-principle that transplantation of new dopamine neurons can relieve motor symptoms for up to two decades. However, the widespread use of fetal tissue in the clinic presents multiple ethical and logistical hurdles. As a result, the generation of human pluripotent stem cell-derived dopamine neurons has been an area of intense focus in recent years. Current protocols are not amenable to clinical translation and generate dopamine neurons that poorly reinnervate the striatum following transplantation. Here we develop a fully-defined protocol for midbrain dopamine generation and improve transplantation survival, plasticity and function via overexpression of glial cell line-derived neurotrophic factor.

In contrast to dopaminergic differentiation strategies, protocols for deriving neurons of the neocortex are relatively limited, generating heterogeneous populations of progenitors, neurons and glia. The human neocortex is arguably the most altered structure during mammalian evolution and underlies the cognitive abilities that define human intelligence. However, the majority of research into cortical development has been carried out in frog, chick or mouse models. Therefore, there is an unmet need to investigate how the relative complexity of the human cortex is developed. In particular, the intrinsic and extrinsic cues that control temporal development and drive the progressive generation of neuronal subtypes that form the six-layered mammalian neocortex remain unknown. Here we investigated the role of FGF-ERK signaling in the development of early-born, deep layer neurons of the neocortex in a reductionist pluripotent stem cell model. We find that FGF-ERK signaling, in part, modulates the timing and temporal progression of neocortical progenitors. Together, these studies advance the use of pluripotent stem cell tools to model neural development and to aid in neuroregeneration.

Declaration

This is to certify that:

i) this thesis comprises only my original work towards the PhD, except where otherwise indicated in the preface.

ii) due acknowledgement has been made in the text to all other material used.

iii) this thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Carlos Gantner

Preface

I acknowledge that the work presented in this thesis is a result of collaboration. My contribution is as follows:

Chapter 3 – Xeno-Free vmDA neurons from hPSC

Published. Efficiently specified ventral midbrain dopamine neurons from human pluripotent stem cells under xeno-free conditions restore motor deficits in parkinsonian rodents. *Stem Cells Translational Medicine* 2017; 6:937-948. The version presented here has been modified in response to thesis examiner's comments. >80%. Co-authored with Jonathan Niclis*, Walaa Alsanie, Stuart McDougall, Christopher Bye, Andrew Elefanty, Edouard Stanley, John Haynes, Colin Pouton, Lachlan Thompson and Clare Parish. JN and WA assisted with cell culture. SM conducted electrophysiological experiments. CB assisted with qPCR. AE, ES, JH and CP provided reagents. LT and CP provided funding and edited the manuscript. *Co-first author. See declaration and co-author forms. This chapter was the focus of a review by the Office of Research Ethics and Integrity. Their findings confirm this chapter's suitability for inclusion within the thesis. Their findings have been taken into account and are reflected in the revised version of the thesis.

Chapter 4 – GDNF improves DA graft function

A version of this chapter has been submitted for publication to *Cell Stem Cell*, October 2019. The version presented here has been modified in response to thesis examiner's comments. >90%. Co-authored with Jessica Kauhausen, Isabelle de Luzy, Jonathan Niclis, Vanessa Penna, Cameron Hunt, Christopher Bye, Charlotte Ermine, Colin Pouton, Deniz Kirik, Lachlan Thompson and Clare Parish. JK, IDL, VP, CB, CE provided technical assistance. CH, CP, DK and LT provided reagents. CP provided funding, contributed to experimental design and edited the manuscript.

Chapter 5 – hPSC modelling of cortical neurogenesis

At the time of thesis submission this paper is in preparation for journal submission. >80%. Co-authored with Cameron Hunt, Jonathan Niclis, Vanessa Penna, Lachlan Thompson and Clare Parish. CH and VP conducted qPCR experiments. JN assisted in experimental design. LT provided reagents. CP provided funding and edited the manuscript.

Appendix 1 – DA and non-DA graft innervation

Published. A PITX3-EGFP Reporter Line Reveals Connectivity of Dopamine and Non-dopamine Neuronal Subtypes in Grafts Generated from Human Embryonic Stem Cells. *Stem Cell Reports* 2017; 9:868-882. The version presented here has been modified in response to thesis examiner's comments. 40%. Conducted cell culture, animal husbandry and behavior and contributed to data collection and manuscript.

Appendix 2 – LMX1A sorting improves vmDA transplantation

A version of this chapter has been accepted for publication in October 2019 in the *Journal of Neuroscience*. The version presented here has been modified in response to thesis examiner's comments. 20%. Provided assistance with animal husbandry, behavior and data collection.

Appendix 3 – Human-specific transcriptional analysis

A version of this chapter has been accepted for publication in September 2019 in *Stem Cell Reports*. The version presented here has been modified in response to thesis examiner's comments. 10%. Conducted cell culture and contributed to data collection

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Table of Contents

Abstract	I
Declaration	II
Preface	III
Acknowledgements	IV
Contents	V
Abbreviations	X
Table of figures	XIII
Table of tables	XV

Chapter 1. Literature review	1
-------------------------------------	----------

1.1	Introduction	1
1.2	Cell replacement therapy for Parkinson's disease	1
1.2.1	Parkinson's disease	1
1.2.2	Fetal DA transplantation	3
1.2.3	Deriving DA neurons from hPSC	6
1.2.4	Strategies to improve DA transplantation	8
1.3	Development of the mammalian neocortex	10
1.3.1	Early forebrain specification	11
1.3.2	Neocortical progenitor diversity	13
1.3.3	Sequential specification of cortical projection neurons	15
1.3.4	Extrinsic regulation of corticogenesis	19
1.3.5	Stem cell modelling of neocortical development	20
1.4	Proposed research	23
1.4.1	Aims of this thesis	23
1.5	References	24

Chapter 2. Materials and Methods	38
---	-----------

2.1	Pluripotent stem cell maintenance	38
2.2	Neural differentiation	38
2.3	Animals and ethical approval	40
2.4	Stereotaxic surgery	40

2.5	Behavioural testing of motor function	42
2.5.1	Amphetamine rotation test	42
2.5.2	Cylinder test	43
2.6	Transcardial fixation and tissue processing	44
2.7	Immunohistochemistry	44
2.7.1	Chromogenic immunohistochemistry	44
2.7.2	Fluorescent immunohistochemistry	45
2.8	Microscopy and quantification	45
2.8.1	<i>In vitro</i> analysis of neural populations	45
2.8.2	Graft volume, phenotype and innervation analysis	45
2.9	Gene expression analysis	47
2.9.1	Real-time qPCR	48
2.9.2	RNA sequencing	48
2.10	Flow cytometry	48
2.11	Statistical analysis	50
2.12	References	50

Chapter 3. Xeno-free generation of functional midbrain dopamine neurons from human pluripotent stem cells **52**

3.1	Abstract	52
3.2	Introduction	53
3.3	Materials and methods	54
3.3.1	Maintenance of human pluripotent stem cells	55
3.3.2	Dopaminergic differentiation	55
3.3.3	Cryopreservation	56
3.3.4	Immunocytochemistry and cell quantification	56
3.3.5	Flow cytometry	57
3.3.6	Gene expression analysis	57
3.3.7	High-performance liquid chromatography	57
3.3.8	Electrophysiology	57
3.3.9	Surgical Procedures	58
3.4	Results	58

3.4.1	Establishment of a fully defined feeder-free, xeno-free vmDA differentiation protocol	59
3.4.2	Xeno-free conditions generate correctly specified, high-purity vmDA progenitors	59
3.4.3	Identification of mechanism underlying enhanced vmDA specification in xeno-free cultures	62
3.4.4	Xeno-free vmDA differentiation is amenable to scalability and cryopreservation of progenitors	63
3.4.5	Xeno-free cultures yield increased proportions of functionally mature DA neurons	65
3.4.6	Transplantation of xeno-free DA progenitors results in grafts with improved phenotype composition and restoration of motor deficits in parkinsonian rodents	68
3.5	Discussion	70
3.6	Acknowledgements	72
3.7	Author contributions	72
3.8	References	72
3.9	Supplementary Figures	75

Chapter 4. Viral delivery of GDNF Improves the functional integration of human pluripotent stem cell-derived dopamine grafts in rodent models of Parkinson's disease **83**

4.1	Abstract	83
4.2	Introduction	84
4.3	Materials and methods	85
4.3.1	Human ESC differentiation	86
4.3.2	Surgical procedures	86
4.3.3	Behavioural analysis	87
4.3.4	Immunohistochemistry	87
4.3.5	Microscopy and quantification	88
4.3.6	Gene expression analysis	88
4.3.7	High-performance liquid chromatography	88
4.3.8	Statistical analysis	89
4.4	Results	89

4.4.1	Validation of DA differentiation, GDNF expression and GDNF-induced motor recovery	89
4.4.2	Transplantation of PSC-derived DA precursors into a GDNF-overexpressing environment promotes graft survival but disrupts axonal targeting	91
4.4.3	Delayed GDNF delivery promotes recovery of motor function without increasing cell survival	95
4.4.4	Delayed delivery of GDNF modulates appropriate innervation of host targets from human stem cell-derived DA neurons	98
4.4.5	GDNF restores DA levels and modulates graft gene expression	100
4.5	Discussion	102
4.6	Acknowledgements	106
4.7	Author contributions	107
4.8	References	107
4.9	Supplementary Figures	111

Chapter 5. Manipulation of deep-layer neurogenesis in human pluripotent stem cell-derived cortical progenitors

		116
5.1	Abstract	116
5.2	Introduction	117
5.3	Materials and methods	119
5.3.1	hPSC maintenance and differentiation	119
5.3.2	Flow cytometry	120
5.3.3	Cumulative EdU cell cycle analysis	121
5.3.4	Gene expression analysis	121
5.3.5	Immunohistochemistry, Microscopy and Quantification	121
5.3.6	Electrophysiology	122
5.4	Results	122
5.4.1	PAX6 ⁺ cortical progenitors form both deep- and superficial-layer cortical neurons	122
5.4.2	Notch inhibition rapidly generates TBR1 ⁺ , early born cortical neurons	123
5.4.3	Loss of FGF signalling induces layer 5-like CTIP2 ⁺ neurogenesis	125

5.4.4	Aberrant FGF signalling retards normal cortical laminar fate and blocks pro-neural gene expression	127
5.4.5	Altered FGF signalling impacts cortical progenitor dynamics and cleavage plane orientation	129
5.4.6	Cell cycle analysis highlights early progenitor characteristics of FGF2 treated cultures	131
5.5	Discussion	133
5.6	Acknowledgements	136
5.7	Author contributions	136
5.8	References	136
5.9	Supplementary Figures	140
Chapter 6. Conclusions and future directions		143
6.1	Generating xenogeneic-free cultures for clinical translation	143
6.2	Employment of reporter cell lines for aiding cell characterisation, isolation and tracking	144
6.3	GDNF improves DA graft function	145
6.4	Future directions of hPSC-derived mDA research	147
6.5	Stem cell modelling of cortical neurogenesis	150
6.6	Future directions in cortical modelling	154
6.7	Concluding remarks	156
6.8	References	156
Appendix 1. A PITX3-GFP reporter line reveals connectivity of dopamine and non-dopamine neuronal subtypes in grafts generated from human embryonic stem cells		162
Appendix 2. Isolation of LMX1A ventral midbrain progenitors improves the safety and predictability of human pluripotent stem cell-derived transplants in parkinsonian rodents		186
Appendix 3. Xenograft-specific transcriptional profiling for the characterisation of human pluripotent stem cell-derived neural transplants in the rodent brain		209

Abbreviations

5-HT	Serotonin
6-OHDA	6-hydroxydopamine
A9/A10	Midbrain dopamine subtype A9/A10
AA	Ascorbic acid
AAV	Adeno-associated virus
aRG	Apical radial glia
ASCL1	Archaete-scute family transcription factor 1
BDNF	Brain derived neurotrophic factor
BMP	Bone morphogenetic protein
BP	Basal progenitor
bRG	Basal radial glia
BRN2	Brain 2
CALB	Calbindin 1
CNS	Central nervous system
CPN	Cortical projection neuron
CThPN	Corticothalamic projection neuron
CTIP2	Coup-TF-interacting protein 2
CUX1/2	Cut-like homeobox 1/2
CXCR4	C-X-C motif chemokine receptor 4
DA	Dopamine
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
dcAMP	Dibutyryl cyclic AMP
DIV	Days <i>in vitro</i>
DL	Deep layer
DL STR	Dorsolateral striatum
DLL	Delta-like canonical notch ligand
DLX1/2	Distal-less homeobox 1/2
EdU	5-ethynyl-2'-deoxyuridine
EMX2	Empty spiracles homeobox 2
EN1/2	Engrailed 1/2
ERK	Extraceullar signal-regulated kinase
FACS	Fluorescence-activated cell sorting

FEZF2	FEZ family zinc finger 2
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FOXA2	Forkhead box A2
FOXG1	Forkhead box G1
FOXP2	Forkhead box P2
GABA	Gamma-aminobutyric acid
GBX2	Gastrulation brain homeobox 2
GDNF	glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFRα1	GDNF family receptor alpha 1
GID	Graft-induced dyskinesia
GIRK2	G-protein-gated inwardly rectifying K channel subunit 2
GLI3	GLI family zinc finger 3
GW	Gestational week
HES1/5	Hairy and enhancer of split 1/5
hESC	Human embryonic stem cell
hPSC	Human pluripotent stem cell
IKNM	Interkinetic nuclear migration
IPC	Intermediate progenitor cell
iPSC	Induced pluripotent stem cell
ISVZ	Inner subventricular zone
JAG	Jagged
LHX2	Lim homeobox 2
LMX1A	LIM homeobox transcription factor 1A
MAPK	Mitogen-activated protein kinase
mDA	Midbrain dopamine
MEF	Mouse embryonic fibroblast
MEK	Mitogen-activated protein kinase kinase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSX1	Muscle segment homeobox homolog 1
NCAM	Neural cell adhesion molecule
NESC	Neuroepithelial stem cell
NGN2	Neurogenin 2

NICD	Notch intracellular domain
NKX2-1	NK2 Homeobox 1
NRTN	Neurturin
NURR1	Nuclear receptor subfamily 4 group A member 2
OSVZ	Outer subventricular zone
OTX2	Orthodenticle homeobox 2
PARD3	PAR3 family cell polarity regulator
PAX6	Paired box 6
PD	Parkinson's disease
PH3	Phosphohistone 3
PITX3	paired-like homeodomain 3
PM	Purmorphamine
RELN	Reelin
RET	Ret proto-oncogene
RG	Radial glia
ROCK1	Rho Kinase inhibitor Y-27632
RORβ	Retinoid-related orphan receptor β
SATB2	Special AT-rich sequence binding protein 2
SCPN	Subcerebral projection neuron
SHH	Sonic hedgehog
SOX1/2/4/5/11	Sry box 1/2/4/5/11
STR	Striatum
SVZ	Subventricular zone
TBR1/2	T-box brain protein 1/2
TGFβ	Transforming growth factor β
TH	Tyrosine hydroxylase
UL	Upper layer
VL STR	Ventrolateral striatum
vmDA	Ventral midbrain dopamine
VZ	Ventricular zone
WNT	Wingless-type MMTV integration site family
ZO1	Zona occludens 1

Table of Figures

Figure 1.1 Parkinson's disease.	2
Figure 1.2 Transplantation of fetal mDA neurons for PD.	4
Figure 1.3 Morphogenetic and transcriptional regulation of mDA development.	7
Figure 1.4 GDNF promotes survival and plasticity of fetal mDA grafts.	9
Figure 1.5 Anterior-posterior and dorsoventral patterning of the forebrain.	12
Figure 1.6 Diverse neocortical progenitors generate sequential cortical layers over time.	14
Figure 1.7 Projection patterns of cortical neurons.	16
Figure 1.8 A repression-derepression circuit governs laminar fate specification.	18
Figure 1.9 Comparison of <i>in vitro</i> stem cell models of corticogenesis.	21
Figure 2.1 DA and cortical neural differentiation protocols.	39
Figure 2.2 Unilateral 6-OHDA ablation of midbrain DA neurons.	42
Figure 2.3 Motor function following 6-OHDA lesioning or mDA transplantation.	43
Figure 3.1 Xeno-free vmDA differentiation to enriched ventral floorplate precursors interrogated with a LMX1A-GFP reporter line.	60
Figure 3.2 Differentiation of hPSCs under xeno-free conditions increases generation of <i>bina fide</i> vmDA neurons at D25.	62
Figure 3.3 Residual fibroblasts, at the initiation of vmDA differentiation, influence fate specification.	64
Figure 3.4 Scaled differentiation and cryopreservation of xeno-free vmDA precursors.	65
Figure 3.5 Maturation of hPSC under xeno-free conditions improved the yield of mature vmDA neurons and DA metabolism.	66
Figure 3.6 Transplantation of xeno-free vmDA progenitors improved the yield of correctly specified cells within grafts of parkinsonian rodents.	69

Figure 4.1 Exposure of hESC-derived mDA transplants to GDNF, from the time of implantation, improved motor deficits in Parkinsonian rats.	90
Figure 4.2 Implantation of hESC-derived mDA progenitors into a GDNF-rich environment promotes graft survival.	92
Figure 4.3 Early exposure of hESC-derived mDA transplants to GDNF impedes axonal sprouting and integration into the host brain.	93
Figure 4.4 Delayed exposure of hESC-derived mDA grafts to GDNF enhances functional recovery in parkinsonian rats, independent of cell survival.	96
Figure 4.5 Delayed exposure of the mDA graft to GDNF promoted DA innervation of the host tissue, activation of striatal neurons and upregulated DA biosynthesis.	99
Figure 4.6 Transcriptional profiling of mDA grafts in the presence and absence of GDNF identified known and novel DA homeostatic, synaptogenesis and axonal plasticity-related genes.	101
Figure 5.1 PAX6 ⁺ cortical progenitors form both DL and UL cortical neurons.	123
Figure 5.2 Notch inhibition of PAX6 ⁺ progenitors rapidly generates TBR1 ⁺ , early born cortical neurons.	124
Figure 5.3 Differential layer phenotype after FGF2 or MEK inhibition.	126
Figure 5.4 MEK inhibition accelerates the development of CTIP2-competent progenitors through activation of a pro-neural gene network.	128
Figure 5.5 Progenitor organisation and cleavage plane highlight immaturity of FGF2-treated progenitors.	130
Figure 5.6 Cell cycle analysis highlights early progenitor characteristics of FGF2-treated cultures.	132
Figure 6.1 Laminar consequences of FGF2 or MEK inhibition on human cortical progenitors <i>in vitro</i> .	153

Table of tables

Table 2.1 Rodent stereotaxic coordinates	41
Table 2.2 List of primary antibodies used in this study	46
Table 2.3 List of secondary antibodies used in this study	47
Table 2.4 List of primers used in this study	49
Table 5.1 Cell cycle analysis of cortical progenitors at DIV26 and DIV35 under basal, FGF2 or MEKi conditions	131

Chapter 1.

LITERATURE REVIEW

1.1 Introduction

How the complexity and incredible abilities of the human central nervous system (CNS) emerge during development is an enduring challenge in developmental biology. Studies in tractable vertebrate model organisms have defined the key molecular determinants of CNS specification conserved between phylogenetically diverse species. However, the rapid evolutionary expansion of the human CNS has led to human-specific characteristics, including structural diversification, neuronal and glial subtype specification and functional connectivity that are thought to underlie superior cognitive function (Lui et al., 2011; Smith and Dragunow, 2014; Suzuki and Vanderhaeghen, 2015). Thus, there is a need for models of development and disease that are better able to recapitulate human ontogeny.

To this end the past decade has seen an explosion of interest in human pluripotent stem cell (hPSC) models of development. hPSCs, derived either from the inner cell mass of the blastocyst (embryonic stem cells - hESC) (Thomson et al., 1998) or by reprogramming of somatic cells (induced pluripotent stem cells - iPSC) (Takahashi et al., 2007) are able to give rise to all cell types of the adult. Harnessing this ability through directed differentiation approaches provides a unique opportunity to directly study human tissue. The ability to derive region- and neurotransmitter-specific subpopulations *in vitro* relies on mimicking *in utero* developmental cascades (for review see Suzuki and Vanderhaeghen, 2015). This review will focus on two stem cell applications: (1) the derivation of midbrain DA (mDA) neurons from hPSC and their use in reversing the motor symptoms of Parkinson's disease and (2) the modelling of human cortical neurogenesis *in vitro*.

1.2 Cell replacement therapy for Parkinson's disease

1.2.1 Parkinson's disease

The defining features of Parkinson's disease (PD) are the progressive loss of mDA neurons in the substantia nigra pars compacta and the presence of neurons containing α -synuclein⁺ Lewy-bod-

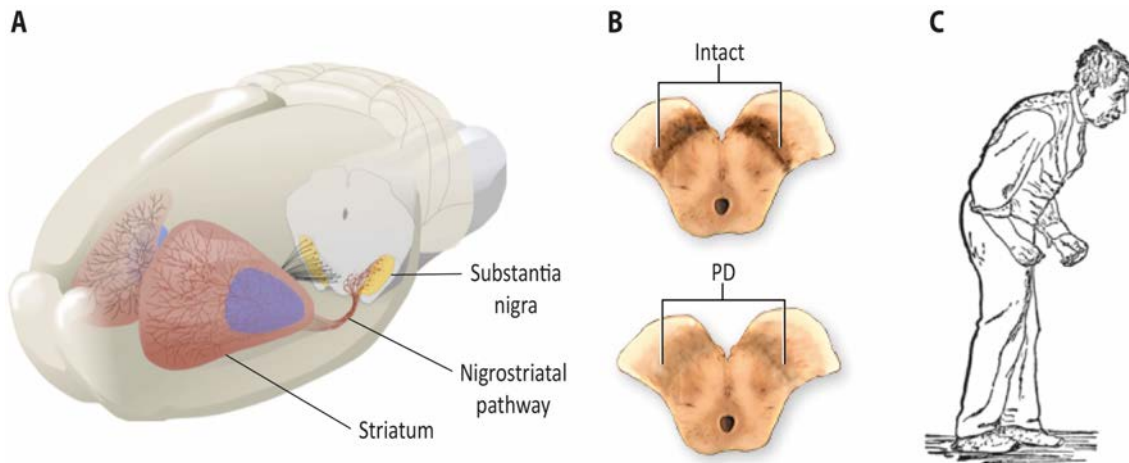


Figure 1.1 Parkinson's disease.

(A) Schematic of the nigrostriatal DA system in the mouse brain. DA neurons residing in the substantia nigra pars compacta project along the nigrostriatal pathway, innervating the striatum and playing a critical role in basal ganglia and movement circuitry. (B) In PD, pigmented DA neurons are lost in the substantia nigra pars compacta. (C) Loss of DA neurons results in stereotypical movement dysfunction, including akinesia, bradykinesia and tremor. Illustration courtesy of Bengt Mattson.

-ies (Braak et al., 2003; Spillantini et al., 1997). In healthy individuals these neurons project along the nigrostriatal pathway, innervating striatal and cortical targets involved in movement (Figure 1.1A). In PD patients, mDA cell loss results in a concomitant reduction in striatal DA release, basal ganglia dysfunction and canonical movement symptoms, including bradykinesia, akinesia, resting tremor and rigidity (Dauer and Przedborski, 2003) (Figure 1.1B and C). Motor dysfunction typically manifests once 70-80% of mDA neurons are lost (Ruberg et al., 1995). Approximately 1-2% of the population over 65 suffer from PD and its prevalence is expected to increase concurrently with longevity (Dorsey et al., 2018). Despite this, current pharmacological and surgical therapies display waning efficacy and do not treat the underlying pathology. Notably, the precursor in dopamine synthesis, L-DOPA, dopamine agonists and inhibitors of dopamine degradation (e.g monoamine oxidase inhibitors) can restore DA-neurotransmission by increasing its concentration in the striatum. However, efficacy decreases with disease progression and prolonged use can lead to significant dyskinesias (Zhang et al., 2013). In addition, deep brain stimulation can provide profound motor recovery but is associated with significant post-operative risk and cognitive dysfunction (Frank et al., 2007; Kleiner-Fisman et al., 2006; Voon et al., 2008). These inadequacies have led to widespread efforts to bring forward alternative approaches. One promising strategy is the direct replacement of lost neurons by transplantation of fetal- or hPSC-derived mDA neurons. This approach holds wide promise due to the unique pathophysiology of PD, namely, the loss of a single, anatomically defined population that underpins the motor symptoms of the disease. Of note, whilst we recognise that

PD involves multiple cell populations and symptoms, the scope of this thesis is limited to correcting mDA pathology.

1.2.2 Fetal DA transplantation

Pioneering studies in the first half of the 20th century demonstrated that rodent neural tissue could survive transplantation into the CNS (Clark, 1940; Dunn, 1917). However, it was commonly thought that axonal regeneration and growth in the mammalian CNS was limited. This idea was first challenged in a series of papers from Björklund and colleagues who were able to show neurotransmitter-specific reinnervation and integration following transplantation of fetal tissue (Björklund and Stenevi, 1977; Björklund et al., 1976). Taking advantage of the unilateral 6-hydroxydopamine (6-OHDA) lesion model of PD (Figure 1.2B), which recapitulates DA cell loss and associated motor dysfunction (Ungerstedt, 1968; Ungerstedt and Arbuthnott, 1970; Ungerstedt et al., 1974), two groups went on to demonstrate robust amelioration of motor recovery following transplantation of fetal-derived mDA tissue containing DA progenitors (Björklund and Stenevi, 1979; Björklund et al., 1981; Fray et al., 1983; Gage et al., 1983; Perlow et al., 1979) (Figure 1.2A-C). Critically, recovery directly correlated with dopaminergic striatal reinnervation, indicating that behavioural recovery was dependent on reforming nigrostriatal connections. Subsequent studies in rodents confirmed that human fetal, DA-rich midbrain tissue was also able to survive transplantation, innervate the denervated striatum, form appropriate synaptic connections and restore motor function (Brundin et al., 1986; Brundin et al., 1988; Clarke et al., 1988). These studies also identified the need for immunosuppression in xenografts as well as the optimal age of tissue (7-9 weeks).

The first open label clinical trial was rapidly carried out in Lund in 1987. In total, 18 patients received 3-8 unilateral grafts from 4-5 donor fetuses. In a landmark study, Lindvall et al. (1990) confirmed graft survival, functional DA release and clinical improvement 6 months after transplantation in the 3rd and 4th patients. mDA-rich grafts were able to survive and improve clinical outcomes for up to 24 years in the best cases, enabling some patients to discontinue L-DOPA medication (Kefalopoulou et al., 2014; Li et al., 2016; Piccini et al., 1999). The Lund trial's success, in conjunction with further studies demonstrating minor (Defer et al., 1996; Freed et al., 1992; Peschanski et al., 1994; Widner et al., 1992) and/or major clinical improvement (Mendez et al., 2000), led to two major NIH funded double-blind, placebo-controlled trials. In both, significant improvement was absent (Freed et al., 2001; Olanow et al., 2003). Crucially, these trials had varying immunosuppression, tissue preparation and primary

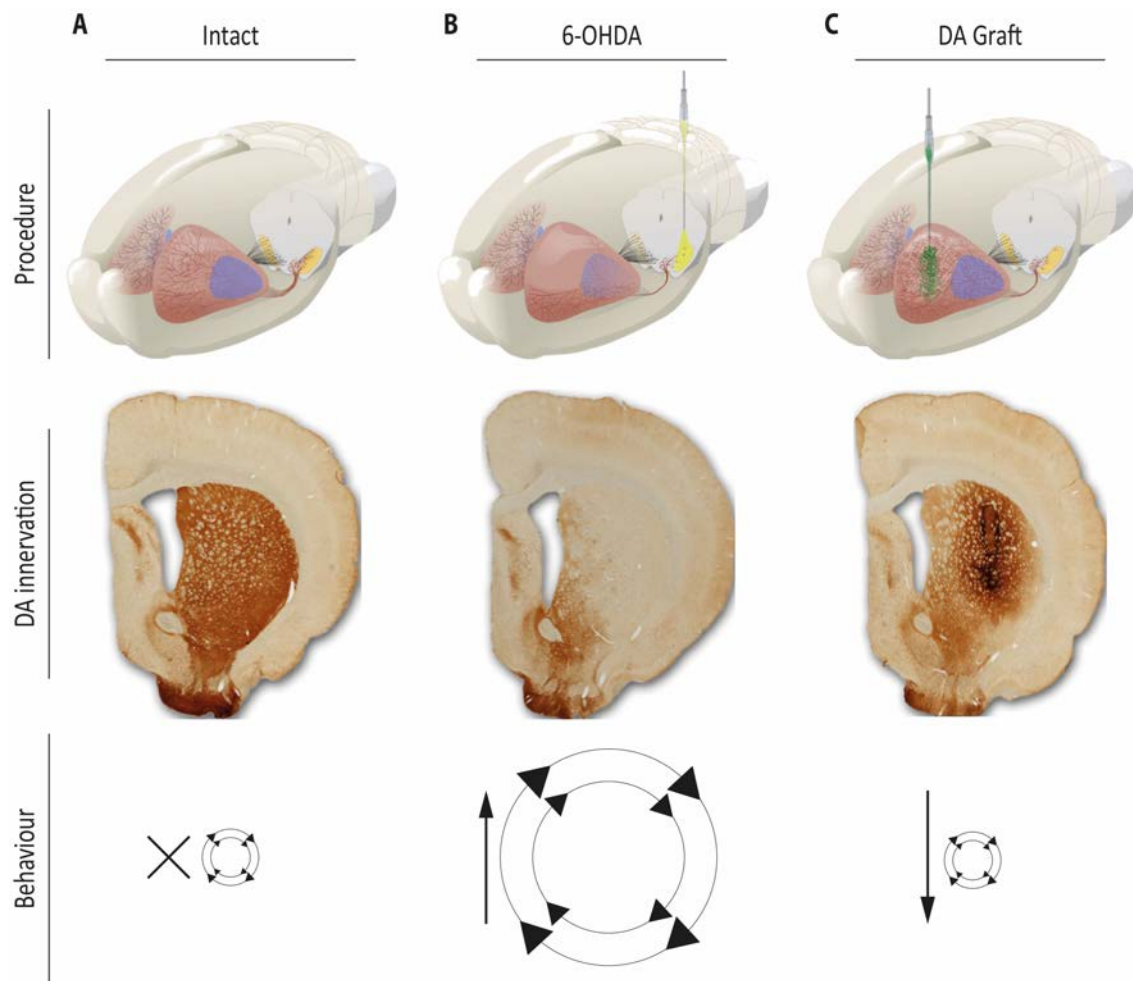


Figure 1.2 – Transplantation of fetal mDA neurons for PD.

(A) In the intact brain, DA neurons projecting from the substantia nigra innervate the striatum and rodents have no rotational bias in the amphetamine rotations test. (B) In the 6-OHDA model of mDA loss, the toxin is unilaterally injected into the substantia nigra (mice) or medial forebrain bundle (rats) to induce cell death in mDA neurons of the substantia nigra on one side of the brain. This leads to a unilateral loss of DA innervation in the striatum, hemispheric DA imbalance, basal ganglia dysfunction and consequent rotational behaviour after amphetamine administration. (C) mDA progenitors, dissected from ventral midbrain fetal tissue, can be transplanted ectopically into the striatum and reinnervate the host tissue. The graft releases DA, restoring DA balance and results in a reduction in the rotational bias of the animal. Illustration courtesy of Bengt Mattson.

end points to the initial Lund trial. Notably, immunosuppression was either not used or administered for only 6 months under the assumption that the brain was immune privileged. However, the decline in benefit from the graft beyond the period of immunosuppression suggests graft rejection (Olanow et al., 2003). Most discouraging, however, was the appearance of graft-induced dyskinesias (GID) in 15% and 54% of the two trials respectively (Freed et al., 2001; Olanow et al., 2003), which were subsequently observed in the Lund patients after retrospective analysis (Hagell et al., 2002). GID severity did not correlate with DA innervation, indicating that symptoms were unrelated to DA overgrowth or concentration (Hagell et al., 2002; Piccini et al., 2005). Instead, PET analysis and antagonist-induced reversal of GID have

implicated DA:5-HT imbalance and serotonergic hyperinnervation as causative, often as a result of poor dissection technique and tissue age (Politis et al., 2011; Politis et al., 2010).

While clinical trials using fetal midbrain have shown significant evidence of long-term graft function, clinical outcomes between and within each study were variable. Critical reappraisal of original data has allowed a greater understanding of the factors determining positive and/or negative clinical outcomes (for review see Barker et al., 2013). The important lessons are: (1) younger, less advanced patients with limited extrastriatal DA-denervation have better outcomes; (2) survival (>100,000 mDA neurons) and integration of the graft within the striatum is necessary for function; (3) immune suppression must be consistent and long-term (>6 months); (4) graft-function peaks after several years, therefore long-term observation is necessary; and (5) levodopa-induced dyskinesias at the time of surgery is predictive of the development of GID when serotonergic neurons are included within donor tissue preparations (Barker et al., 2013; Piccini et al., 2005; Winkler et al., 2005).

The surprising discovery of α -synuclein containing Lewy-bodies in post-mortem grafted tissue has led to a re-evaluation of PD pathophysiology. Additionally, there is evidence that host-to-graft disease transmission occurs at protracted periods after transplantation and that the rate of pathology spread is similar to endogenous mDA neurons (Kordower et al., 2008; Kurowska et al., 2011; Li et al., 2008; Li et al., 2016). One mechanism supported by both *in vitro* and *in vivo* evidence is that neurodegenerative disorders characterized by fibrillary protein aggregates such as Alzheimer's and Parkinson's diseases may result from altered protein-protein interaction in a prion-like manner (Brundin et al., 2010; Frost and Diamond, 2010; Olanow and Brundin, 2013). In one patient, graft-induced motor recovery deteriorated as the disease progressed and graft pathology increased (Li et al., 2016). Despite this, patients displaying host-to-graft pathology continued to show marked clinical improvement up to 20 years, highlighting the feasibility of this approach. Therefore, it is thought that while DA grafts do not reverse the extra-nigral pathology of PD they represent a valid and worthwhile intervention for the amelioration of DA-related motor symptoms. In light of the collective reassessment of the open and closed label trials, a new, highly controlled clinical trial using human fetal mDA tissue (TRANSEURO - <http://www.transeuro.org.uk>) has commenced, aiming to assimilate past lessons and develop a standardized clinical approach that will pave the way for stem cell therapies.

1.2.3 Deriving DA neurons from hPSC

Logistical considerations limit the widespread use of fetal tissue. Thus, hPSC-based approaches have generated extensive interest. However, the therapeutic use of stem cell-derived neurons has several challenges: (1) can *bona fide* DA neurons be generated; (2) can hPSC-derived neurons correct motor deficits with the same efficacy as fetal mDA tissue; and (3) do transplants pose a tumorigenic risk?

Necessary for the generation of mDA neurons from hPSC is an understanding of the cascade of events that lead to the birth of these cells during embryonic development. Synergistic sonic hedgehog (Shh), fibroblast growth factor (Fgf), bone morphogenetic protein (Bmp) and Wnt signalling gradients determine the rostrocaudal and dorsoventral axes of the prospective midbrain where midbrain DA neurons are born by inducing the intrinsic molecular determinants that drive mDA fate specification (Arenas et al., 2015; Dessaud et al., 2010; Joksimovic et al., 2009; Jovanovic et al., 2018; Placzek, 2005; Prakash et al., 2006; Prakash and Wurst, 2004). By E7.5 (in mice), orthodenticle homeobox 2 (Otx2) and gastrulation brain homeobox 2 (Gbx2), expressed in the midbrain and hindbrain respectively, define the isthmic organiser via mutual repression, which in turn secretes Wnt1 and Fgf8 (Broccoli et al., 1999; Millet et al., 1999). Simultaneous secretion of Shh, initially from the notochord, defines the midbrain along the dorsoventral axis, generating the neurogenic floorplate at high concentrations (Ono et al., 2007; Placzek, 2005). High Shh at the ventral neural tube induces transcription of the key mDA gene forkhead box a2 (Foxa2) (Ang et al., 1993; Sasaki et al., 1997), which together with Otx2, Wnt1 and Fgf8 leads to LIM homeobox transcription factor 1a (Lmx1a), neurogenin 2 (Ngn2), muscle segment homeobox homolog 1 (Msx1) and engrailed 1/2 (En1/2) expression (Andersson et al., 2006a; Andersson et al., 2006b; Omodei et al., 2008; Prakash et al., 2006). Following cell cycle exit, mDA neuroblasts upregulate the orphan nuclear receptor nurr1 and migrate radially toward the mantle zone, with the first mDA neurons appearing by E10.5 (Nelander et al., 2009). This migration is critically dependent on Cxcr4 (C-X-C motif chemokine receptor type 4) signalling from the meninges (Yang et al., 2013). Finally, postmitotic mDA neurons express tyrosine hydroxylase (Th), the rate-limiting enzyme in DA synthesis and paired-like homeodomain transcription factor (Pitx3) (reviewed in Arenas et al., 2015).

The first protocol for the directed differentiation of mDA neurons from hPSCs was reported more than a decade ago, making use of a stromal cell feeder layer (Perrier et al., 2004). While a landmark study at the time, only limited TH⁺ neurons (~40%) were generated, which cri-

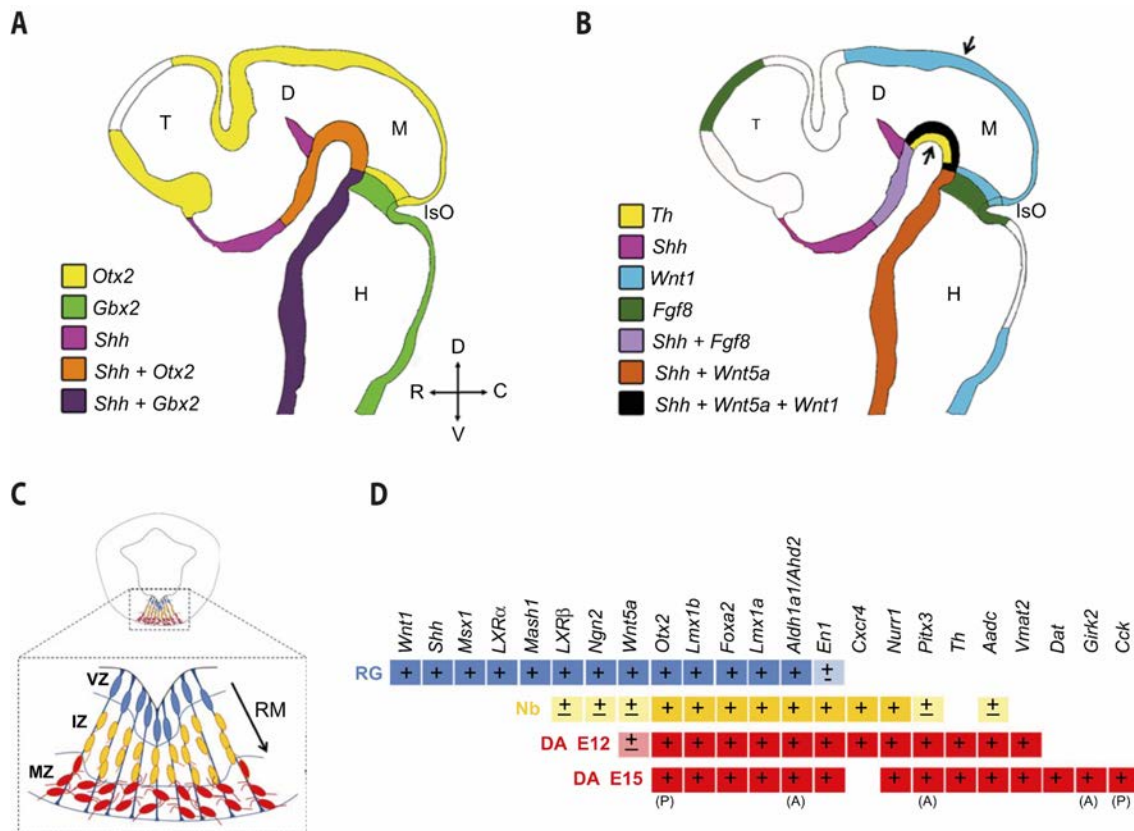


Figure 1.3 – Morphogenetic and transcriptional regulation of mDA development.

(A) Sagittal section at E11.5 of the developing mouse brain. Otx2 and Gbx2 mutual repression delineates where the isthmus organizer (IsO) will form, while Shh is secreted initially by the notochord and subsequently by the floorplate and generates the floorplate at high concentrations. (B) Fgf8, Shh, Wnt1 and Wnt5a together specify the region where Th⁺ mDA neurons are born. (C) Coronal section through the neural tube at E11.5. Immature mDA progenitors (blue) exit the cell cycle and migrate radially as mDA neuroblasts (yellow), finally settling as mature mDA neurons (red) in the mantle zone. (D) Profile of the transcription factors and key proteins in mDA neurogenesis in progenitors (blue), neuroblasts (Nb, yellow) and mature mDA neurons (red). Adapted from Arenas et al. (2015).

tically, did not express the battery of mDA-specific lineage markers we now know delineate mDA neurons (FOXA2/EN1/LMX1A/PITX3/NURR1). It is speculated that the extended neural induction of early protocols favoured default neural programming toward an anterior/forebrain DA fate as evidenced by the presence of rosettes (Chambers et al., 2009; Eiraku et al., 2008; Gaspard et al., 2008). In contrast, recent protocols that mimic *in utero* developmental cascades *in vitro* have obviated the need for stromal cell co-culture and resulted in reproducible differentiation systems, generating *bona fide* mDA neurons (Denham et al., 2012; Kirkeby et al., 2012; Kriks et al., 2011; Nolbrant et al., 2017; Xi et al., 2012). Of note, FGF8, which was thought to be dispensable in the presence of high Wnt signaling, has recently been shown to be crucial in specifying the caudal mDA phenotype responsible for robust motor recovery (Kee et al., 2017; Kirkeby et al., 2017). hPSC-derived mDA neurons are able to functionally integrate and reverse motor deficits in both rodent and non-human primate parkinsonian models, at least partially (Grealish et al., 2014; Hallett et al., 2015; Niclis et al., 2017; Steinbeck et al., 2015).

1.2.4 Strategies to improve DA transplantation

Work from our group as well as others has demonstrated that functional recovery following hPSC-mDA transplantation is directly correlated with DA survival, striatal innervation and release (Kirkeby et al., 2017; Kriks et al., 2011; Niclis et al., 2017). Despite efficient specification of mDA progenitors *in vitro* (>80% FOXA2/OTX2/LMX1A⁺), long-term analysis of the yield of mDA neurons *in vivo* remains low, estimated across multiple studies to be ~5-10% as a proportion of transplanted cells (Kirkeby et al., 2017; Kriks et al., 2011; Niclis et al., 2017) and ~1-50% of total graft-derived cells (Doi et al., 2014; Kirkeby et al., 2012; Niclis et al., 2017; Samata et al., 2016), suggestive of poor survival and/or expansion of poorly specified cells *in situ*. In addition, innervation density from hPSC-derived mDA progenitor grafts is decreased in comparison to human fetal transplants (Grealish et al., 2014). Added to this, proliferative or incorrectly specified cells pose the risk of tumorigenesis or off-target ramifications, as evidenced by 5-HT-induced GID. As a result, significant effort has been directed toward improving mDA transplantation outcomes through targeted sorting strategies or neurotrophic support of grafted DA neurons.

Fluorescent reporter lines have been extensively used for mDA enrichment. In the mouse, Sox1-GFP FACS purification reduces tumorigenicity (Fukuda et al., 2006), while mDA-specific gene isolation strategies improve mDA yield and in some cases motor recovery (Ganat et al., 2012; Hedlund et al., 2008; Jonsson et al., 2009; Nefzger et al., 2012; Thompson et al., 2006). These approaches have, in part, been validated using human embryonic stem cell reporter lines with limited success (Aguila et al., 2014; Watmuff et al., 2015). We have recently shown, utilizing LMX1A and PITX3 reporter lines, that enrichment of mDA neurons *in vivo* is critically related to genetic strategy and differentiation age of the cells at the time of transplantation (de Luzy, et al., unpublished - Appendix 2). FACS isolation of LMX1A⁺ mDA progenitors significantly enriched (>2-fold) the purity of TH⁺ mDA neurons after transplantation contributing to improved behavioural recovery (Figure A2.3). Moreover, LMX1A isolation eliminated 5-HT neurons that may give rise to GIDs (Figure A2.5). In contrast, PITX3-based sorting strategies failed to give rise to viable mDA transplants, likely as a result of PITX3 expression being exclusively limited to postmitotic mDA neurons which die due to mechanical stresses during dissociation, sorting and transplantation. In addition, efforts to adopt more clinically-relevant purification strategies, targeting surface proteins, inclusive of Alcam, Chl1, Gfra1, Igfbp8, CORIN, NCAM/CD29, LRTM1 and IAP have shown evidence for increased DA progenitor purity and yield *in vivo* (Bye et al., 2015; Chung et al., 2011; Doi et al., 2014; Jonsson et al., 2009; Lehen et al., 2017; Ono et al., 2-

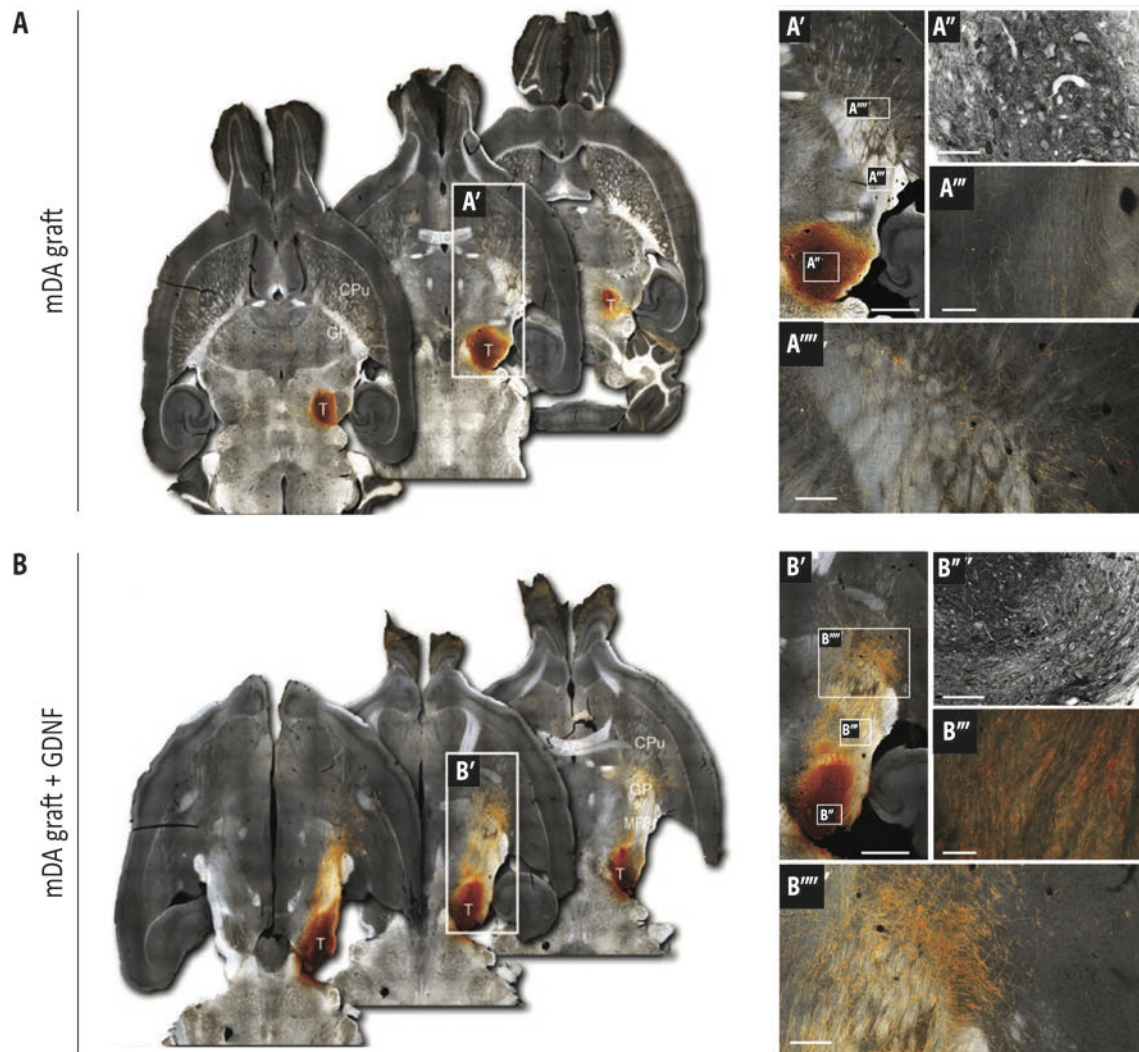


Figure 1.4 – GDNF promotes survival and plasticity of fetal mDA grafts.

(A) Transplantation into the midbrain of mouse fetal mDA, isolated from TH-GFP donor embryos. Staining for GFP through three adjacent sections. mDA neurons project to the host striatum in limited numbers after maturation. (B) In the presence of GDNF (GDNF-AAV induced overexpression within the host striatum), mDA grafts were significantly larger and innervate the host striatum significantly more than mDA grafts alone. Adapted from [Kauhausen et al. \(2013\)](#).

-007; [Pruszek et al., 2007](#); [Samata et al., 2016](#); [Sundberg et al., 2013](#)). Crucially, sorted cells retain the ability to restore host circuitry and provide motor recovery in 6-OHDA rodent models.

Members of the glial cell line-derived neurotrophic factor (GDNF) family, GDNF and the closely related neurturin (NRTN) have been widely studied in the context of mDA development, survival and impact on mDA progenitor transplants targeted for PD. GDNF, first identified as a potent enhancer of mDA survival and differentiation ([Lin et al., 1993](#)), prevents lesion-induced degeneration of mDA neurons and promotes mDA innervation and function in rodent and non-human primate models ([Gash et al., 1996](#); [Hoffer et al., 1994](#); [Kearns and Gash, 1995](#); [Sauer et](#)

al., 1995). Furthermore, GDNF promotes the survival and function of fetal mDA transplants in a dose-dependent manner (Kauhausen et al., 2013; Moriarty et al., 2017; Rosenblad et al., 1996; Thompson et al., 2009; Wang et al., 2016; Winkler et al., 2006; Yurek, 1998), while in hPSC-derived mDA neurons, GDNF blocks MPTP toxicity *in vitro* (Zeng et al., 2006). Strikingly, Kauhausen et al. (2013) demonstrate that GDNF also promotes graft plasticity, boosting striatal reinnervation ~10-fold after homotopic grafting of rodent fetal mDA (Figure 1.4). However, two double-blind clinical trials investigating GDNF infusion failed to show consistent functional outcome, likely due to the low solubility of GDNF in brain parenchyma and the low doses employed (Domanskyi et al., 2015; Gill et al., 2003; Kirik et al., 2017; Kordower et al., 1999). Similarly, NRTN was initially identified as a pro-survival factor for cultured sympathetic neurons and was subsequently shown to exert both neuroprotective and neurorestorative effects in parkinsonian models (Horger et al., 1998; Kotzbauer et al., 1996; Oiwa et al., 2002). However, again, clinical trials in parkinsonian patients failed to improve clinical outcome and significant adverse responses were observed in several patients (Marks et al., 2010). Taken together, combining neurotrophic support of mDA grafts remains an exciting prospect in Parkinson's disease.

1.3 Development of the mammalian neocortex

The evolutionary expansion of the neocortex reaches its peak in primate and hominid lineages. The neocortex, consisting of multiple neuronal subtypes and glia, is characteristically organised in six layers, each with distinct cellular compositions, projections and molecular signatures (Greig et al., 2013). Their precise molecular composition, balance and innervation patterns define the architecture of the cortex and, thus, the computational abilities responsible for the complex cognitive, sensory and motor functions that define human intelligence. Cortical neurons are broadly divided into two classes: (1) glutamatergic excitatory projection neurons, born in the cortical wall of the dorsal telencephalon, which extend both extra- and intra-cortical projections and represent ~85% of cortical neurons and (2) GABAergic inhibitory interneurons, derived predominantly from the ganglionic eminences of the ventral telencephalon, which migrate to the cortex, form local circuits and make up the remaining ~15% of cortical neurons (Sur and Rubenstein, 2005). These two classes can be divided further into hundreds of subtypes defined by their molecular, electrophysiological and functional phenotypes (DeFelipe et al., 2013; Lui et al., 2011; Molyneaux et al., 2007). This review will focus specifically on the

developmental origin of excitatory cortical neurons in mammalian systems, as well as the use of hPSC to model and gain insight into their development.

1.3.1 Early forebrain specification

Experiments in *Xenopus laevis* were the first to describe the default pathway of neuralisation in vertebrate development. In this model, pluripotent cells within the epiblast of the developing embryo default to a neural identity in the absence of exogenous signalling cues (reviewed in [Muñoz-Sanjuán and Brivanlou, 2002](#)) (Figure 1.5A). This work expanded on the pioneering discovery of the vertebrate organiser in neural induction. Transplantation of the newt blastopore lip onto a second embryo induced ectopic nervous system development and the Spemann-Mangold organiser was born ([Spemann and Mangold, 1924](#)). It was initially thought that inductive cues secreted by the organizer led to neuralisation. However, two discoveries altered that perspective. The first showed that loss of Transforming growth factor β (Tgf β) type II receptor signalling induced neuralisation, indicating that Activin/Bone morphogenetic protein (Bmp)-induced Tgf β signalling inhibited neural differentiation ([Hemmati-Brivanlou and Melton, 1992, 1994](#)). In a second set of experiments, it was observed that the Spemann-Mangold organiser secretes multiple inhibitors of Bmp signalling, including noggin, chordin, follistatin and cerberus ([Hemmati-Brivanlou et al., 1994](#); [Piccolo et al., 1999](#); [Piccolo et al., 1996](#); [Sasai et al., 1995](#); [Smith and Harland, 1992](#); [Zimmerman et al., 1996](#)) (Figure 1.5A). Therefore, the inhibition of Bmp signalling (i.e. the absence of morphogenetic cues) by organiser/node secreted antagonists is critical in maintaining the default pathway within the presumptive vertebrate forebrain, a process conserved in mammalian neuralisation ([Levine and Brivanlou, 2007](#); [Valenzuela et al., 1995](#); [Wilson and Houart, 2004](#)). Accordingly, the forebrain fails to develop in mice lacking noggin and chordin ([Bachiller et al., 2000](#)). Fibroblast growth factor and Wnt signalling also play a role in early neural induction, in part by promoting a pre-neural state prior to gastrulation and through inhibition of the intracellular Bmp signal transducer Smad1 ([Pera et al., 2003](#); [Stern, 2005](#)). Indeed, Bmp, FGF, Wnt and Nodal signalling, secreted from the primitive streak, proximal epiblast or extra-embryonic tissues, induce endodermal and mesodermal derivatives.

Following Bmp antagonism and specification of the anterior embryo there is a rapid expansion of the telencephalic anlage in response to Fgf8, secreted from the anterior pole/neural ridge of the primitive telencephalon ([Shimamura and Rubenstein, 1997](#); [Ye et al., 1998](#)). Distinct germinal zones are formed along the dorsoventral axis in response to compleme-

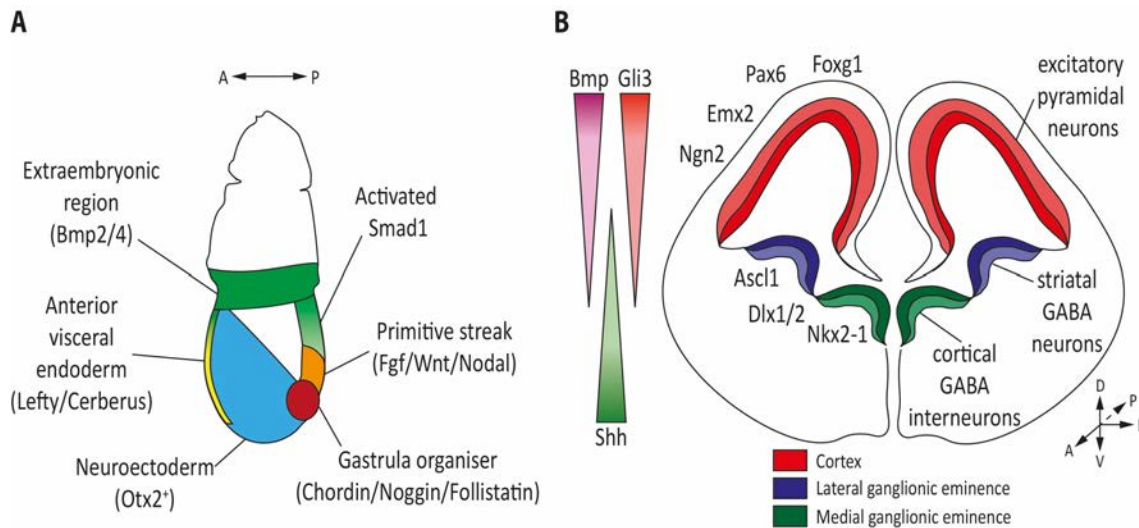


Figure 1.5 – Anterior-posterior and dorsoventral patterning of the forebrain.

(A) Sagittal section through an E7.5 mouse embryo highlighting the major signalling centres. Organiser secreted chordin, noggin and follistatin in conjunction with anterior visceral endoderm secreted lefty and cerberus inhibit Bmp, maintaining the anterior portion of the embryo in its default neural state (blue). (B) Coronal section through an E11.5 mouse telencephalon. Bmp, Shh and Gli3 gradients define dorsoventral patterning and induce the transcriptional determinants of each region. The dorsal forebrain, which goes on to generate all excitatory pyramidal neurons of the cortex expresses Foxg1, Pax6, Emx2 and Ngn2. In contrast, high Shh induces ventral telencephalic fates in the ganglionic eminences defined by Ascl1, Dlx1/2 and Nkx2-1 expression, which give rise to GABAergic striatal neurons or cortical interneurons which migrate tangentially to the cortex during development. (A) adapted from Levine and Brivanlou (2007).

-ntary Bmp, Shh and Gli3 (a repressor of Shh) gradients (Wilson and Rubenstein, 2000) (Figure 1.5B). Gli3 null mice exhibit a dorsal shift of ventral identity, indicating that Shh repression is important in dorsal telencephalic development (Theil et al., 1999). In contrast, Shh is required for ventral forebrain fate and ectopic Shh signalling ventralises putative dorsal tissue (Chiang et al., 1996; Ericson et al., 1995). The ventral telencephalon (subpallium) generates the ganglionic eminences (lateral, medial and caudal) that give rise predominantly to GABAergic projection neurons and interneurons of the striatum and cortex, respectively. In contrast, the dorsal telencephalon gives rise to the excitatory pyramidal neurons of the cerebral cortex (Figure 1.5B). Here, morphogenetic cues induce dorsal transcription factors, including Paired box 6 (Pax6) and Empty spiracles homologue 2 (Emx2), present in opposite rostrocaudal gradients and important in specification and arealisation of the cortex (Götz et al., 1998; Muzio et al., 2002). Pax6^{Sey/Sey} mutants, lacking functional Pax6, display a cell autonomous failure to repress ventral determinants and subsequent expansion into the dorsal telencephalon (Kroll and O'Leary, 2005; Quinn et al., 2007; Stoykova et al., 2000; Yun et al., 2001). The basic helix-loop-helix transcription factor Neurogenin 2 (Ngn2) is also crucial to dorsal identity and its loss leads to the expansion of Ascl1⁺ ventral derivatives dorsally (Fode et al., 2000). Similarly, Forkhead box G1 (Foxg1) and LIM homeobox 2 (Lhx2) repress dorsal midline fates and their loss leads to expansion of the

midline toward dorsal structures (Hanashima et al., 2004; Monuki et al., 2001; Muzio and Mallamaci, 2005). Together, these transcription factors delineate the neuroepithelial stem cell (NESC) population within the dorsolateral cortical wall that gives rise to all excitatory cortical neurons (Figure 1.5B).

1.3.2 Neocortical progenitor diversity

By E10.5 in the mouse and gestational week (GW) 8 in humans, the founder population of NESCs is arranged in a pseudostratified monolayer adjacent to the ventricle, forming the ventricular zone (VZ) (Haubensak et al., 2004) (Figure 1.6A). NESCs undergo interkinetic nuclear migration (IKNM), with mitosis occurring at the ventricular or apical surface, expanding the NESC pool through symmetrical cell divisions, which in turn partly dictates the final size of the cortex (Chenn and McConnell, 1995; Haubensak et al., 2004; Lui et al., 2011; Subramanian et al., 2017). NESCs differentiate directly to form the first cortical neurons or to generate apical radial glia (aRG) that express Pax6, Gfap, Glial, Hes and Vimentin and from which the majority of cortical excitatory neurons are derived (Kriegstein and Alvarez-Buylla, 2009) (Figure 1.6A). Characterised by partial glial identity (Gfap⁺) and long bipolar processes that contact both the ventricular/basal and pial/apical surfaces, aRG were initially thought to provide a migratory scaffold for newborn neurons and subsequently give rise to astrocytes (Levitt and Rakic, 1980; Rakic, 1971; Rakic, 2009). Refuting this, Götz and colleagues were the first to elegantly demonstrate direct neurogenesis from Gfap⁺/Pax6⁺ aRG in the dorsal telencephalon (Heins et al., 2002; Malatesta et al., 2000), a finding quickly confirmed by several others (Miyata et al., 2001; Noctor et al., 2001) and expanded to the entirety of the CNS (Anthony et al., 2004).

Apical RGs typically undergo self-renewing asymmetrical divisions, replenishing the aRG pool and generating a neuron, Tbr2⁺ intermediate progenitor (IPC) or basal RG (bRG) (Englund et al., 2005; Fietz et al., 2010; Hansen et al., 2010; Noctor et al., 2004). bRG, first described in human studies but subsequently discovered in other gyrencephalic species (Reillo et al., 2011), closely resemble aRG but lack an apical process and are thus unipolar (Figure 1.6A). Migration of IPCs and bRG (together basal progenitors, BPs) away from the VZ and their ongoing expansion generate a second, larger germinal zone, adjacent to the VZ, the subventricular zone (SVZ), which appears by E13.5 in the mouse and by GW9-10 in humans. The emergence of BPs and a defined SVZ is specific to mammals and absent or limited in sauropsid (birds and reptiles) corticogenesis (Cheung et al., 2007; Dugas-Ford and Ragsdale, 2015). Indeed, the number of BPs increases progressively in higher order mammals and primates, peaking in human development

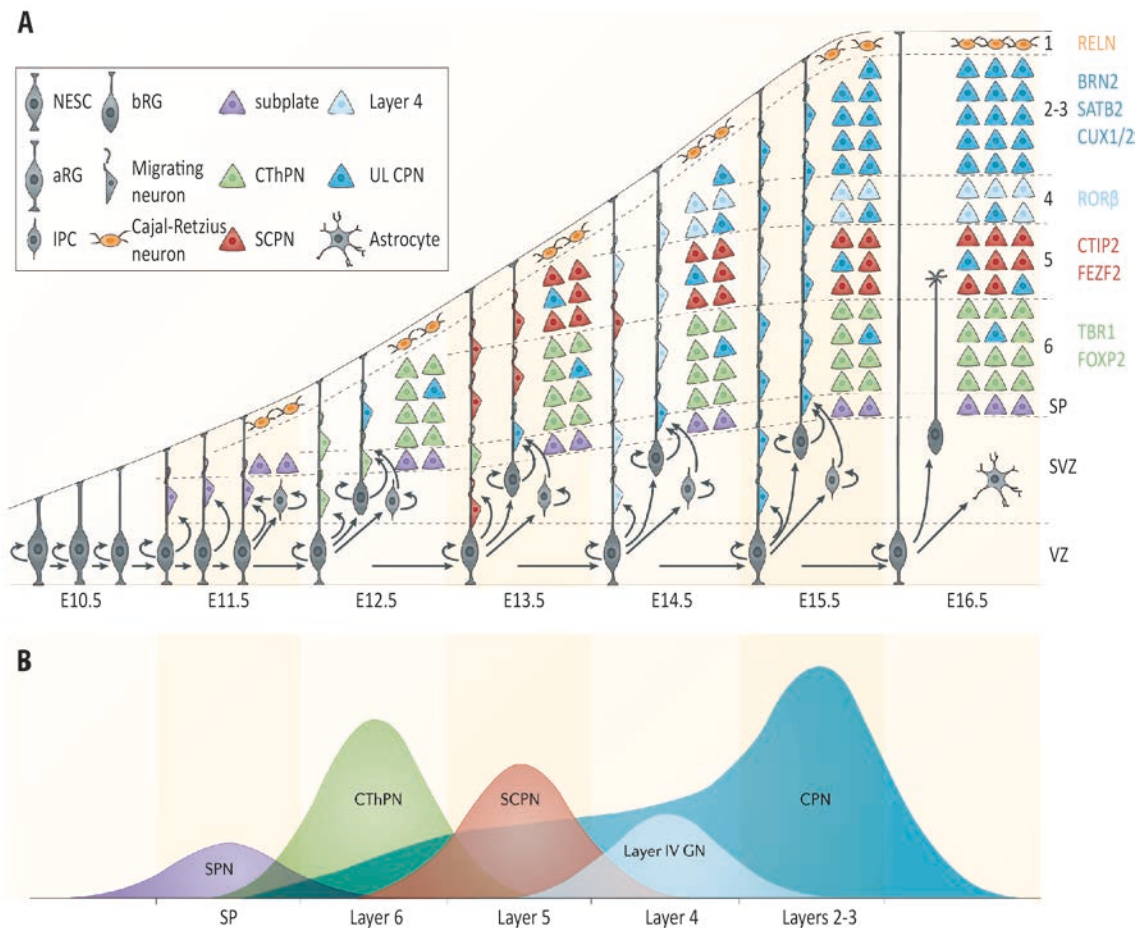


Figure 1.6 – Diverse neocortical progenitors generate sequential cortical layers over time.

(A) Illustrated coronal section through the developing mouse neocortex between E10.5 and E16.5. At E10.5 neuroepithelial stem cells (NESC) divide symmetrically to expand the founding pool of cortical progenitors and become apical radial glia (aRG). aRG in turn generate neurons directly or indirectly through either intermediate progenitor cells (IPC) or basal radial glia (bRG) which migrate basally and form the subventricular zone (SVZ) and outer SVZ (OSVZ) which is heavily expanded in primates. Over time the neurons that are generated shift from a deep layer (DL) fate to an upper layer (UL) fate before finally switching to gliogenesis. (B) Each layer subtype is formed in successive waves, beginning with layer 6 corticothalamic projection neurons (CThPN), and migrates past earlier born neurons, populating the cortical plate in an ‘inside-out’ fashion. Each layer expresses a set of key marker genes (indicated on the right). Of note, the number of UL neurons of layers 2-4 is greatly expanded in primates in concordance with an increase in basal progenitors. Adapted from Greig et al. (2013).

(Kriegstein et al., 2006). In primates the SVZ is further divided into an inner SVZ (ISVZ) or outer SVZ (OSVZ), separated by thin fiber layers (Smart et al., 2002). The primate OSVZ is by far the largest germinal zone, in contrast to the rodent VZ. Strikingly, primate OSVZ expansion between GW11-17 occurs simultaneously with VZ decline, becoming the predominant germinal zone and highlighting its importance in cortical expansion (Dehay et al., 2015; Hansen et al., 2010; Lui et al., 2011).

Concomitant with OSVZ expansion, primate neocortical progenitors display increased diversity when compared to their rodent counterparts. Live imaging studies in the primate have

identified multiple bRG subtypes defined by the presence of apical, basal or dual processes (Betizeau et al., 2013). These bRG readily undergo multiple rounds of division within the OSVZ and can bidirectionally transition between cell states, greatly expanding the neurogenic capacity of primates (Betizeau et al., 2013; Dehay et al., 2015). In addition, Tbr2⁺ IPCs typically undergo only a single amplifying division in rodents (Noctor et al., 2004; Vasistha et al., 2015) but can divide precociously in the primate OSVZ (Lui et al., 2011). Recent work at the single cell level has further expanded our understanding of cortical progenitor diversity (Florio et al., 2015; Nowakowski et al., 2017; Pollen et al., 2015; Thomsen et al., 2016). In particular, extracellular matrix formation and growth factor expression is enriched in bRG, enabling the stability and maintenance of OSVZ stemness in the absence of ventricular contact via STAT3 signalling (Pollen et al., 2015). Evolutionarily recent, human-specific gene duplications have also been implicated in the expansion of the human neocortex, in particular via alteration of RG proliferation/neurogenesis fate decisions and expansion of the progenitor pool (Charrier et al., 2012; Dennis et al., 2012; Fiddes et al., 2018; Florio et al., 2015; Kalebic et al., 2018; Sousa et al., 2017; Suzuki et al., 2018). Hence, the complexity and final organisation of the neocortex is intimately linked to the variety, number, location and fate decisions of the neocortical progenitor pool. In addition, primate neocortical enlargement is a direct result of both exponential progenitor expansion and novel stem cell niches within the developing cortex (reviewed in Dehay et al., 2015; Lui et al., 2011). Such differences in the organisation of the rodent and primate neocortex highlight the importance of species-appropriate models to understand cortical development.

1.3.3 Sequential specification of cortical projection neurons

Cortical projection neurons of the six-layered neocortex are specified in distinctive temporal waves according to the inside-out model of corticogenesis (Gaspard and Vanderhaeghen, 2011) (Figure 1.6). In this model, the earliest born Cajal-Retzius neurons migrate away from the VZ, forming the preplate by E10.5 (Marin-Padilla, 1978). A second wave of migrating neurons infiltrate the preplate, establishing the cortical plate. Over time, increasingly late-born neurons migrate radially, past early-born deep layer neurons (DL: layers 5-6), residing in progressively more upper layers (UL: layers 2-4) within the cortical plate (Molyneaux et al., 2007) (Figure 1.6A). The strict linkage of birthdate and laminar fate indicates that neocortical progenitor competence is gradually restricted over time (Figure 1.6B). In support of this, early RG/IPCs are capable of generating all laminar subtypes after transplantation into a late-stage cortex, while late-stage RG/IPCs only generate late-born neurons when transplanted into a younger donor (Eckler et al.,

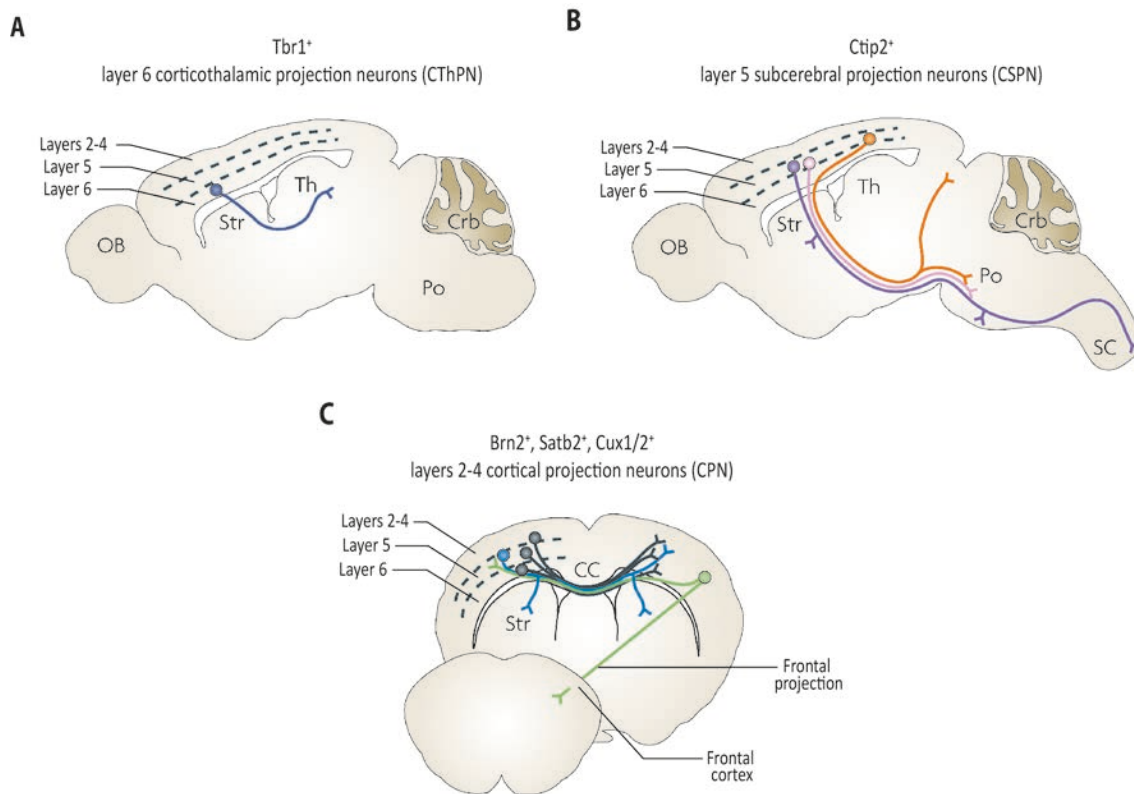


Figure 1.7 – Projection patterns of cortical neurons.

Cortical neurons can be grouped based on their hodology (i.e. projection patterns) into several discrete groups. (A) Tbr1⁺ neurons of layer 6 project to the thalamus and are labelled corticothalamic projection neurons (CThPN). (B) Ctip2⁺ neurons that reside in layer 5 project to multiple subcortical targets, including the tectum, pons and spinal cord and are grouped together as subcerebral projection neurons (SCP). (C) Upper layer (UL) neurons that project either ipsilaterally or contralaterally within the cortex are marked by the expression of Brn2, Satb2 and Cux1/2 and reside predominantly in layers 2-4, although some cortical projection neurons (CPN) are present within layers 5-6. Adapted from Molyneaux et al. (2007).

2015; Frantz and McConnell, 1996; Greig et al., 2013; McConnell, 1988). In addition, *in vivo* lineage tracing experiments reveal diminishing layer competence over time in neocortical progenitors and recapitulation of the developmental sequence *ex vivo* (Luskin et al., 1988; Price and Thurlow, 1988; Shen et al., 2006; Walsh and Cepko, 1988). Several groups have suggested that fate-restricted cortical progenitors reside within the VZ at early stages of neurogenesis. In particular, Cut-like homeobox 2 (Cux2) is expressed in VZ/SVZ progenitors that give rise to neurons of the upper layers (Franco et al., 2012; Zimmer et al., 2004). However, whether specific laminar-competent progenitors contribute significantly to neurogenesis remains controversial (see Eckler et al., 2015; Gil-Sanz et al., 2015; Greig et al., 2013; Guo et al., 2013). Despite this, it is clear that acquisition of laminar fate is intimately linked to neocortical progenitor diversity.

The emergence of large-scale *in situ* hybridisation techniques has enabled thorough characterisation of laminar-specific gene patterns in the mouse in addition to the classical

characterisation based on hodology (i.e. projection pattern) (Molyneaux et al., 2007) (Figure 1.7). Genes with known laminar restriction include: Reelin (Reln) expressed by Cajal-Retzius cells of layer 1, T-box brain 1 (Tbr1) and Forkhead box P2 (Foxp2) expressed in layer 6 corticothalamic projections neurons (CThPN) (Bulfone et al., 1995; Ferland et al., 2003), Coup-TF-interacting protein 2 (Ctip2/Bcl11b) and Fez family zinc finger 2 (Fezf2) expressed in subcerebral projection neurons (SCPN) of layer 5 which innervate the tectum, pons or spinal cord (Arlotta et al., 2005; Leid et al., 2004), Retinoid-related orphan receptor β (Ror β) expressed in cross-callosal corticocortical projection neurons of layer 4 (Schaeren-Wiemers et al., 1997) and Cux1/2, Brain 2 (Brn2) and Special AT-rich sequence binding protein 2 (Satb2) expressed in cortical projection neurons (CPN) of the ULs that innervate the cortex exclusively (Alcamo et al., 2008; Nieto et al., 2004; Sugitani et al., 2002) (Figure 1.7).

In contrast, the molecular determinants of sequential layer competence in neocortical progenitors remain only partially explored (Greig et al., 2013). Laminar restricted genes are often but not always required for subtype specification. For example, Sry box 5 (Sox5) promotes layer 6 CThPN identity, driving Tbr1 expression and repressing Fezf2 via an upstream cis regulatory element (Lai et al., 2008; Shim et al., 2012). Tbr1 is essential to generate these layer 6 CThPNs in early corticogenesis, repressing Fezf2 and Ctip2 determinants of layer 5 SCPN identity (Bedogni et al., 2010; McKenna et al., 2011) (Figure 1.8A). Demonstrating this, Tbr1 null mutants fail to inhibit Fezf2 and Ctip2 in early-born neurons, resulting in a fate switch to a layer 5-like identity (Fezf2⁺/Ctip2⁺), extending axons to subcerebral but not thalamic targets (Han et al., 2011). Further, in Fezf2^{-/-} mice, layer 5 SCPNs fail to develop and Tbr1 expression expands dorsally, while UL (layers 2-4) neurogenesis is unaffected (Chen et al., 2005; Molyneaux et al., 2005). In contrast, forced expression of Fezf2 at E14.5, when UL neurons are being born, reprograms UL neurons to aberrantly project to subcerebral targets, confirming that Fezf2 has a dual role in specifying progenitor competence during DL neurogenesis, as well as in forming correct SCPN identity (Rouaux and Arlotta, 2013). Ctip2 acts downstream of Fezf2, to specify SCPN identity, including pathfinding and axonal sprouting (Arlotta et al., 2005) (Figure 1.8B).

The factors governing the switch from DL to UL neurogenesis are also only beginning to be revealed. Following activation of Fezf2 and Ctip2 and appropriate layer 5 SCPN generation, Satb2 in turn promotes UL neurogenesis by directly repressing Ctip2-driven SCPN fates (Alcamo et al., 2008; Britanova et al., 2008) (Figure 1.8C). SATB2^{-/-} mice fail to generate UL CPNs, displaying aberrant Ctip2 expression in UL neurons (Baranek et al., 2012; Srinivasan et al., 2012).

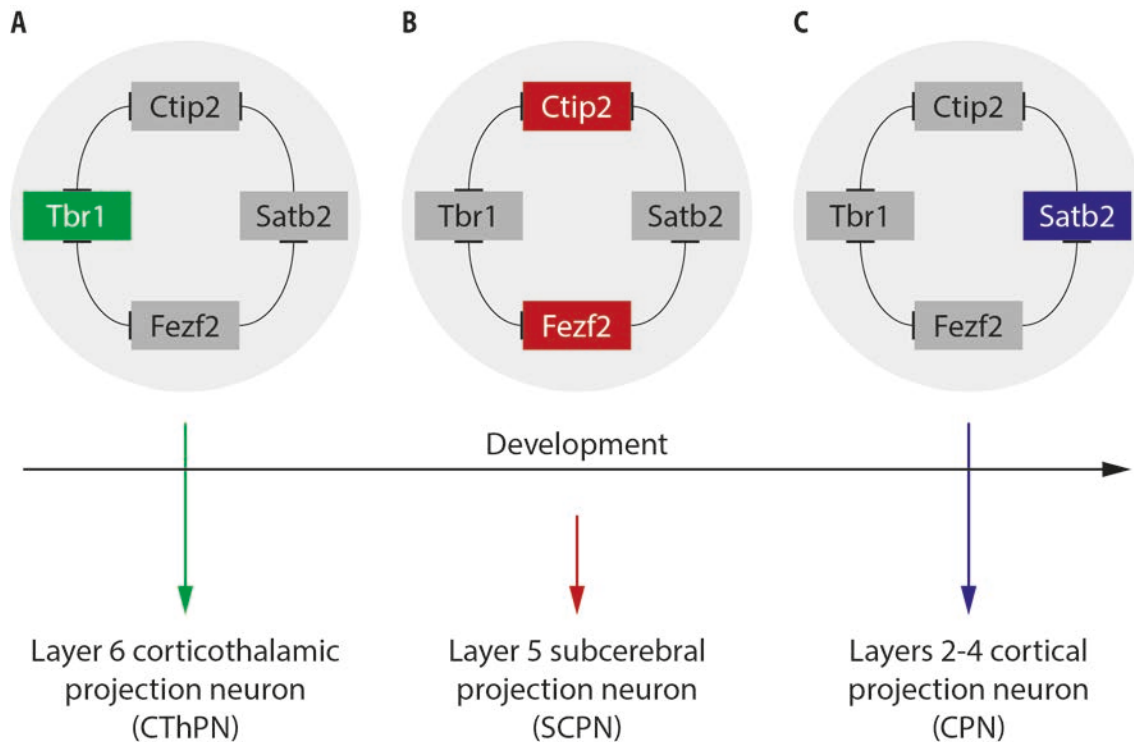


Figure 1.8 – A repression-derepression circuit governs laminar fate specification.

(A) The default excitatory projection neuron phenotype is the layer 6 Tbr1⁺ corticothalamic projection neuron (CThPN). Tbr1 neurogenesis is maintained by repression of Ctip2 and Fezf2. At the onset of layer 5 neurogenesis (~E13.5 in the mouse), expression of Ctip2 and Fezf2 are upregulated and in turn repress Tbr1 expression, switching neurogenesis toward a layer 5 subcerebral projection neuron (SCPN) fate. At the end of layer 5 neurogenesis (~E14.5 in the mouse) Satb2 is upregulated and represses Ctip2 completing the repression-derepression circuit.

Brn2, which begins to be expressed between early- and mid-corticogenesis, is essential for UL neurogenesis (Sugitani et al., 2002). Indeed, Brn2 overexpression in presumptive layer 5-6 progenitors by *in utero* electroporation induces accelerated UL neurogenesis (Dominguez et al., 2013). In summary, following layer 6 Tbr1⁺ CThPN neurogenesis (along the 'default' PN pathway), Fezf2 and Ctip2 repress Tbr1 and switch progenitor identity toward layer 5 Ctip2⁺ SCPNs. Finally, Satb2 represses Ctip2, completing the circuit (Srinivasan et al., 2012; Toma et al., 2014). Taken together, laminar fate specification is determined by a complex repression-derepression circuit (Figure 1.8). This repression-derepression model bears intriguing similarity to temporal progression in *Drosophila melanogaster*, where subtype determinants are expressed sequentially and maintained in postmitotic neurons of each progressive lineage (Bayraktar and Doe, 2013; Isshiki et al., 2001; Rossi et al., 2017). In agreement with this model, Ikaros, a mammalian homologue of *hunchback* that encodes temporal identity in *Drosophila*, promotes DL neurogenesis in the mammalian retina and cortex (Alsiö et al., 2013; Elliott et al., 2008). However, whether temporal progression is cell intrinsic (i.e. a molecular clock) or modulated by extrinsic cues remains opaque, although it is clear that epigenetic and electrical properties also play a role (Vitali et al., 2018; Yoon et al., 2017).

1.3.4 Extrinsic regulation of corticogenesis

The balance between proliferative and neurogenic divisions within the neocortical progenitor pool is crucial for the correct development of the neocortex, specifying both laminar organisation and final cell number. These fate decisions are influenced not only by intrinsic temporal progression but also by extrinsic factors within the neocortical milieu that act to alter gene expression and cell cycle kinetics (Lui et al., 2011). Two pathways that have been studied heavily in cortical development are the Notch and Fgf signalling pathways. The Notch signalling pathway is highly conserved across taxa and controls gene expression through lateral inhibition (Heitzler and Simpson, 1991; Kageyama et al., 2008). In classical lateral inhibition, adjacent cells express differing levels of the Notch ligands delta-like canonical notch ligand (Dll) and Jagged (Jag). High Dll expression stimulates high Notch activation in adjacent cells and transcription of Notch effector genes hairy and enhancer of split 1 and 5 (Hes1/5), which in turn repress Dll, leading to interspersed high- and low-Notch activation states across a population (Lai, 2004). In the neocortex, Hes1/5 expression is restricted to the VZ by E10.5 in the mouse (Sasai et al., 1992) and represses proneural genes, including Ngn2 and Ascl1 (Chen et al., 1997; Ishibashi et al., 1995). Constitutively activated Notch signalling via overexpression of the Notch intracellular domain (NICD) promotes RG proliferation (Gaiano et al., 2000), while loss of Hes1/5 results in depletion of the RG pool (Hatakeyama et al., 2004; Imayoshi et al., 2010). BPs within the SVZ display increased expression of Dll and attenuated expression of the downstream Notch signalling mediator CBF1 indicating that BPs may play a role in maintaining Notch signalling and thus, proliferation within RG (Mizutani et al., 2007; Yoon et al., 2008).

Further, Hes1 directly binds and antagonises itself via a negative autoregulatory loop, leading to an oscillatory pattern of expression (~2-3 hours) within RGs that follows IKNM and cell cycle parameters (Kageyama et al., 2008; Shimojo et al., 2008). Hes1 expression is absent during mitosis and early G1, when RGs are present at the VZ surface, creating a neurogenic period before basal migration and upregulation of Hes1 (Shimojo et al., 2008). Ngn2 and Ascl1 oscillate in a complementary pattern to Hes1. Notably, during mitosis RG basal fibers are inherited by a single daughter cell, which in turn may be able to transduce high SVZ Notch signalling and maintain itself as an RG (Lui et al., 2011; Subramanian et al., 2017). In contrast, low Hes1, coupled with increased Ngn2 and Ascl1 may cause differentiation within the second daughter cell. In addition, Numb, an inhibitor of Notch and Par3 family cell polarity regulator (Pard3), a component of the apical membrane complex, are asymmetrically distributed within

RG and are inherited differentially by RG progeny (Costa et al., 2008; Rasin et al., 2007; Rhyu et al., 1994; Shen et al., 2002). Inheritance of low *Pard3* derepresses *Numb*, inhibiting Notch in one daughter cell, promoting neurogenesis (Bultje et al., 2009). Taken together, asymmetrical inheritance of key Notch signalling factors and the basal fiber in part determine the balance of proliferative/neurogenic divisions within RG (for review see Lui et al., 2011).

The FGF family consists of 22 ligands, transduced by four high affinity Fgf receptors (FgfR), that play pleiotropic roles during neocortical development. FgfRs signal predominantly through mitogen-activated protein kinase (MapK) or protein kinase B/Akt pathways and play a significant role in cell proliferation, differentiation and survival (for review see Mason, 2007). Fgf2 displays maximal expression during early corticogenesis within the VZ (Gonzalez et al., 1995) and is necessary for progenitor expansion (Raballo et al., 2000). In agreement with a role of Fgf2/FgfR1 in RG proliferation, loss of Fgf2 significantly reduces the volume and number of neurons within the cortex (Raballo et al., 2000; Rash et al., 2011; Zheng et al., 2004). In contrast, ectopic expression of Fgf2 results in increased proliferation of RG, delay in neurogenic divisions and expansion of the cortex (Vaccarino et al., 1999). Fgf2-induced Mapk signalling upregulates cyclin D2, resulting in decreased G1-S-phase transition and shortened cell cycle length (Lukaszewicz et al., 2002). Moreover, Fgf signalling inhibits asymmetric divisions and generation of IPCs (Kang et al., 2009). Several studies have demonstrated interplay between Notch and Fgf signalling pathways, which work synergistically to influence cell cycle and proliferation kinetics (Rash et al., 2011; Yoon et al., 2004). While Notch and Fgf signalling have been shown to influence division and differentiation, how they impact cortical lamination is unclear.

1.3.5 Stem cell modelling of neocortical development

The fundamental concepts of cortical development can be recapitulated in *in vitro* PSC models of cortical neurogenesis (Suzuki and Vanderhaeghen, 2015) (Figure 1.9). PSCs cultured in the absence of exogenous cues readily differentiate toward a forebrain neural identity, confirming that the default pathway is conserved across vertebrates (Muñoz-Sanjuán and Brivanlou, 2002; Tropepe et al., 2001). Indeed, directed differentiation approaches incorporating inhibitors of Bmp/Nodal/Tgf β signalling efficiently induce dorsal telencephalic identity in mouse (Eiraku et al., 2008; Gaspard et al., 2008; Pera et al., 2004; Watanabe et al., 2005) and human PSC (Chambers et al., 2009; Espuny-Camacho et al., 2013; Shi et al., 2012) models (commonly referred to as dual-SMAD). Similar to the developing brain, graded morphogenetic cues encode positional information *in vitro* and can be harnessed to generate region- and neurotransmitter-

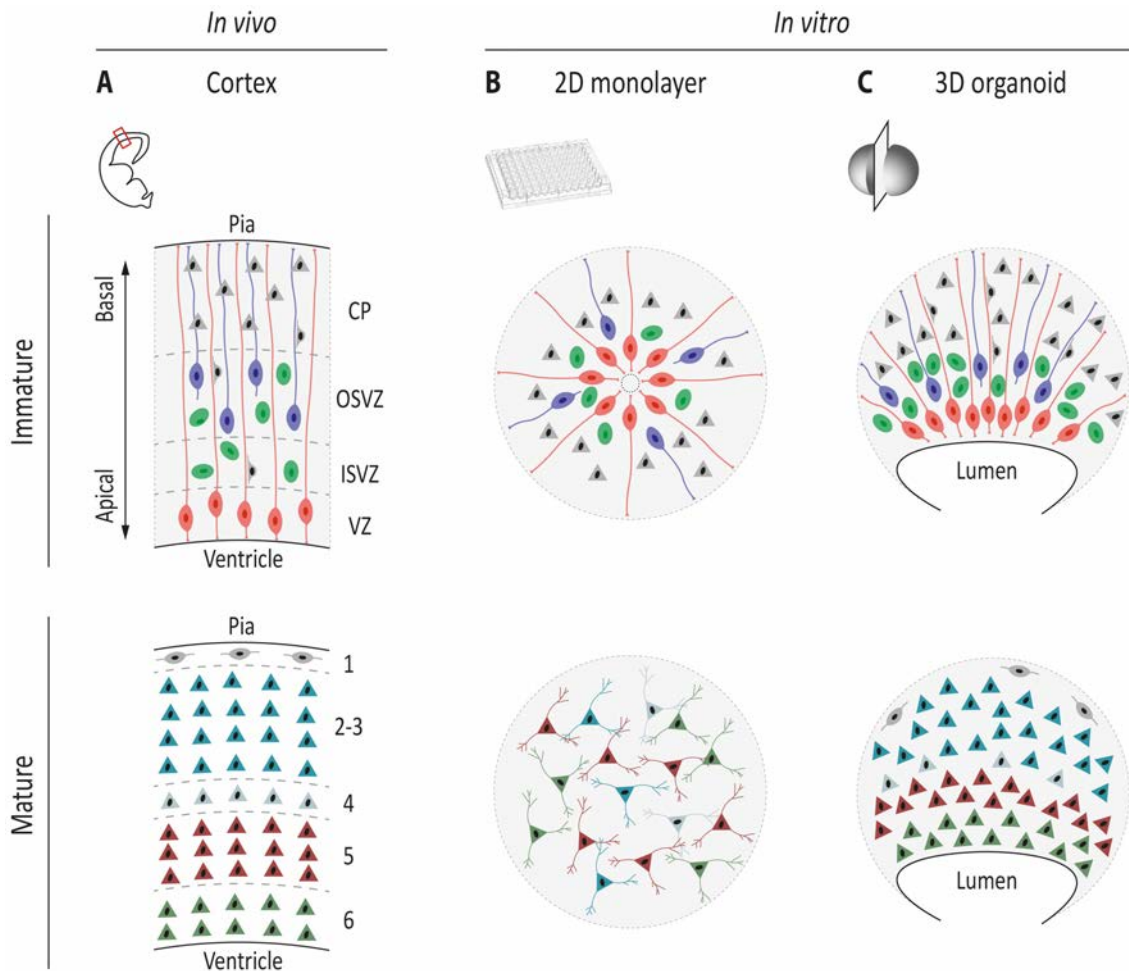


Figure 1.9 - Comparison of *in vitro* stem cell models of corticogenesis.

(A) In the developing telencephalon at E11.5 there is clear apicobasal polarity and delineation between the ventricular zone (VZ), subventricular zone (SVZ) and cortical plate (CP) germinal zones. As development proceeds each subsequent layer of the cortex is born and generates the mature 6-layered structure 'inside-out'. (B) 2D monolayer cultures self-organise to form cortical rosettes, which are analogous to the cortical wall. Rosettes display clear radial orientation with neuroepithelial stem cells (NESC) and apical radial glia (aRG, red) on the inside, intermediate progenitor cells (IPC, green) and basal radial glia (bRG, blue) in the middle and neurons (grey) on the outside. However, 2D monolayer cultures do not mimic the laminar organisation of the cortex. (C) In contrast, *in vitro* organoid cultures show clear progenitor self-organisation akin to the cortical wall and after extended culture also generate clear laminar structure, with early born deep layer (DL) neurons on the inside and later born upper layer (UL) neurons on the outside.

specific populations (for review see Suzuki and Vanderhaeghen, 2015). Shh, which instructs dorsoventral patterning *in utero*, readily generates ventral telencephalic progeny that give rise to GABAergic neurons (Alsanie et al., 2017; Li et al., 2009; Liu et al., 2013; Maroof et al., 2013; Nicholas et al., 2013). Moreover, Wnt and Retinoic acid signalling gradients progressively caudalise telencephalic cultures toward midbrain, hindbrain or spinal cord fates, while distinct FGF signalling gradients, including from the Isthmic organiser or anterior neural ridge act to locally organise several brain regions (Kirkeby et al., 2012; Maury et al., 2014; Shimamura and Rubenstein, 1997; Wichterle et al., 2002). Taken together, the manipulation of developmentally

relevant cues allows for the derivation of individual subtype neurons through post-patterning of the default forebrain identity.

In addition, PSC stem cell models of neocortical development display remarkable self-organisational ability and recapitulate key developmental milestones *in vitro* (Figure 1.9). NESCs first form polarised epithelial structures, termed rosettes, that are analogous to the developing cortical wall and display apicobasal polarity and sequestration of adherence proteins N-Cadherin, Zona occludens 1 (ZO1) and CD133 at the apicoluminal surface (Eiraku et al., 2008; Elkabetz et al., 2008; Zhang et al., 2001) (Figure 1.9B). Moreover, NESCs undergo stereotypical IKNM, undergoing mitosis at the apicoluminal surface *in vitro* (Eiraku et al., 2008; Shi et al., 2012). Increasing evidence also points to species-specific generation of the diverse neocortical progenitor pool (Dehay et al., 2015). Mouse NESCs generate both aRG and IPCs but only limited bRG (Eiraku et al., 2008; Lancaster et al., 2013). In contrast, bRG bearing a single basal process and located within the putative OSVZ are readily generated in both primate and human PSC cultures *in vitro* (Kadoshima et al., 2013; Lancaster et al., 2013; Otani et al., 2016; Shi et al., 2012). The developmental sequence of cortical neurogenesis is also conserved *in vitro*. Sequential waves of Tbr1⁺ and Ctip2⁺ DL and Brn2⁺ and Satb2⁺ UL neurogenesis precede gliogenesis according to intrinsic, species-specific timespans strikingly similar to embryonic development (Suzuki and Vanderhaeghen, 2015). The first mouse neurons *in vitro* are born after only 5-6 days and neurogenesis is complete after 2-3 weeks (Eiraku et al., 2008; Gaspard et al., 2008), while in humans neurons aren't born for 3-4 weeks and neurogenesis proceeds for at least 10 weeks (Espuny-Camacho et al., 2013; Shi et al., 2012).

While 2D protocols do appear to follow the developmental sequence *in vitro* they do not fully capture the embryonic microenvironment, often excluding glia and organised structures (Kelava and Lancaster, 2016). Hence, recent years have seen a burst of interest in 3D models of corticogenesis from PSCs, termed organoids, in order to circumvent the limitations of traditional 2D monolayer cultures (Kadoshima et al., 2013; Lancaster et al., 2013; Pasca et al., 2015) (Figure 1.9C). Regional specificity can be generated by applying the same morphogenetic cues as 2D cultures to generate whole brain (Lancaster et al., 2013), forebrain (Kadoshima et al., 2013), dorsal forebrain (Lancaster et al., 2017; Pasca et al., 2015), ventral forebrain (Bagley et al., 2017; Birey et al., 2017; Xiang et al., 2017), midbrain (Jo et al., 2016) and spinal cord organoids (Ogura et al., 2018). Of particular interest, dorsal forebrain organoids display clear separation between germinal domains, arranging into discrete VZ-, SVZ-, OSVZ- and cortical

plate-like regions analogous to the developing cortex (Figure 1.9C). Indeed, OSVZ expansion is particularly apparent in 3D models in comparison to 2D, likely due to their ability to self-organise and generate a permissive stem cell niche as occurs *in vivo* (Lancaster et al., 2013; Pollen et al., 2015). Moreover, unlike 2D corticogenesis where temporal but not positional developmental patterns are observed, organoid models display clear laminar organisation, with early-born DL neurons residing below late-born UL neurons (Lancaster et al., 2013; Pasca et al., 2015) (Figure 1.9C). However, while heterogeneity of organoids is key to their appeal, it also limits the ability to study specific developmental events or cell types (Kelava and Lancaster, 2016). Thus, there is a need to match particular questions in cortical development with the appropriate *in vitro* model.

1.4 Proposed research

The use of PSCs to derive neuronal populations is a powerful tool for both understanding development and potentially treating neurodevelopmental and neurodegenerative diseases. Critical to both of these approaches is the ability to generate the correct neuronal phenotype in a controlled, developmentally relevant manner. For the treatment of PD, this requires not only increases in the purity of differentiated DA neurons but also a clinically translatable cell product. In addition, hPSC-derived DA neurons do not fully resemble their fetal equivalents. Therefore, it is crucial to employ strategies that boost their survival and plasticity following transplantation in order to improve functional outcome in PD patients. In the context of cortical development, our understanding of the key milestones, cell types and genetic programs that dictate correct laminar organisation and cortical function has increased exponentially over the past two decades. However, how multipotential neocortical progenitors alter their layer-competence over time remains obscure but may be elaborated utilising reductionist, PSC-modelling approaches.

1.4.1 Aims of this thesis

The work of this thesis seeks to develop new stem cell tools for brain repair (aims 1 and 2) and to study development (aim 3) in two distinct regions of the CNS:

1. Generate a defined differentiation system for the derivation of mDA neurons from hPSC that allows for their study *in vitro* and *in vivo* and is readily clinically translatable.

2. Investigate the potential use of the neurotrophic factor GDNF to improve transplantation of hPSC-derived mDA neurons.
3. Understand the role of morphogenetic cues in cortical neurogenesis *in vitro*, specifically whether extracellular signals can alter layer-competence of neocortical progenitors.

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Chapter 2.

MATERIALS AND METHODS

2.1 Pluripotent stem cell maintenance

hESC lines WA09 (WiCell; XX) ([Thomson et al., 1998](#)) and HES3 (WiCell; XX) ([Reubinoff et al., 2000](#)) and iPSC lines 409B2 (CiRA; XX) ([Okita et al., 2011](#)) and RM3.5 (MCRI; XY) ([Kao et al., 2016](#)) were routinely cultured in laminin-521 (BioLamina) coated T25 flasks with mTeSR1 (StemCell Technologies) supplemented with 0.5% penicillin-streptomycin (Life Technologies). Media was changed daily and cells were passaged every 4-5 days or at 70-80% confluence as small clumps using ReLeSR (StemCell Technologies). Mycoplasma infection was routinely tested and all cell lines were negative throughout the study period (MycoAlert, Lonza). The LMX1A- and PITX3-GFP hESC lines were generated in the H9 background as previously described ([Niclis et al., 2017](#); [Watmuff et al., 2015](#)). LMX1A is expressed in the developing midbrain and is an important transcriptional determinant of mDA identity, while PITX3 is specifically expressed in postmitotic mDA neurons. LMX1A- and PITX3-GFP reporter lines were generated to enable purification of mDA progenitors (LMX1A) or visualisation of transplanted mDA neurons (PITX3) in chapters 3 and 4. PAX6-mCherry was generated in the HES3 background as previously described ([Bellmaine et al., 2017](#)) and was used to isolate dorsal telencephalic progenitors in chapter 5. Newly generated lines were karyotypically normal with no aneuploidies detected. For long-term storage of hPSC, cells were dissociated to 5-50 cell clumps with ReLeSR and resuspended in cryopreservation media consisting of 60% mTeSR1, 30% Knockout Serum Replacement (Life Technologies) and 10% dimethyl sulfoxide (Life Technologies) before being placed in a slow rate freezer (EF600M, Grant Instruments) and stored in LN₂. Retrieved cells were rapidly thawed at 37°C, diluted in mTeSR and replated onto laminin-521 coated T25 flasks with the addition of 10µM ROCK inhibitor Y-27632 (ROCKi, Tocris Biosciences). Thawed colonies were passaged twice before commencing experiments.

2.2 Neural differentiation

To initiate differentiation, confluent (70-90%) hPSC cultures were dissociated to single cells with Accutase (StemCell Technologies), counted and plated at $0.375 \pm 0.1 \times 10^6/\text{cm}^2$ in mTeSR1 with

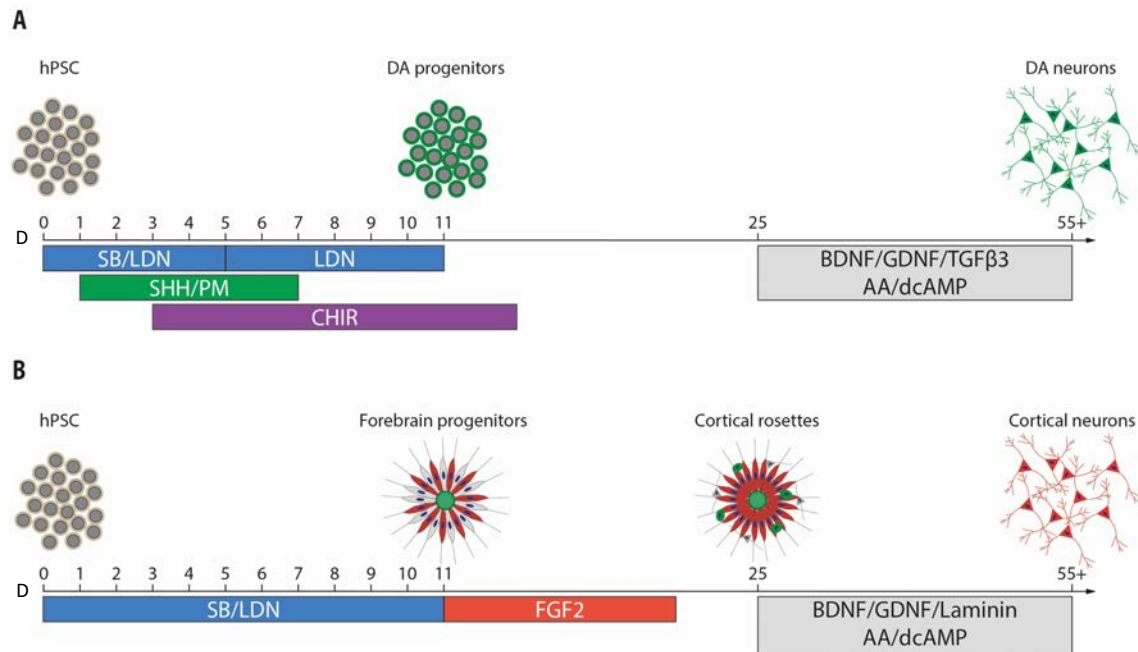


Figure 2.1 - DA and cortical neural differentiation protocols.

(A) DA neuronal differentiation. hPSC undergo dual-SMAD inhibition to generate neuroectoderm cells. SHH and PM ventralise the cultures, generating floorplate progenitors, while the Wnt agonist CHIR caudalises the progenitors. (B) Differentiation protocol to generate excitatory cortical projection neurons. Dual-SMAD inhibition generates neuroectodermal progenitors that are expanded and dorsalised in the presence of FGF2, generating classical rosette structures reminiscent of the developing neural tube. SB: SB43152 (TGF inhibitor). LDN: LDN193189 (BMP inhibitor), SHH: sonic hedgehog, PM: purmorphamine, CHIR: CHIR99021 (Wnt agonist), FGF2: fibroblast growth factor 2, BDNF: brain derived neurotrophic factor, GDNF: glial cell-line derived neurotrophic factor, TGFβ3: transforming growth factor β3, AA: ascorbic acid and dcAMP: dibutyryl cyclic AMP.

Y-27632. Plating density was determined through titration for individual cell lines to ensure 100% confluence the next day. hPSC cultures were washed twice with PBS^{Mg-/Ca-} to remove residual mTeSR and placed into differentiation media one day after replating. Individual differentiation conditions were experiment specific (Figure 2.1). Briefly, all protocols began with dual-SMAD inhibition using LDN193189 (100-200nM, Miltenyi) and SB431542 (10μM, R&D Systems) for 11 days to generate neuroectodermal progenitors which are subsequently patterned towards the desired mDA or cortical neuronal phenotype (Chambers et al., 2009). From days 0-11 mDA neurons were cultured in serum replacement medium (1:1 DMEM/F12:Neurobasal, 15% knockout serum replacement, 1% NEAA and 0.2% β-mercaptoethanol), sequentially replaced with N2 media (DMEM/F12, 1% N2 and 1% ITSA) from day 5. Days 0-11 of cortical differentiation was carried out in 'cortex media' (1:1 DMEM/F12:Neurobasal, 0.5% B27+vitA, 0.5% N2, 0.5% ITSA, 1% GMAX, 0.5% Penicillin Streptomycin and 0.1% β-mercaptoethanol). For mDA specification (Figure 2.1A), neuroectodermal cultures were ventralised via activation of sonic hedgehog signalling between days 1-7 (100ng/ml SHH C2II and 2μM purmorphamine, a smoothened receptor agonist) and caudalised with the canonical Wnt agonist CHIR99021 (3μM,

days 3-13). For cortical excitatory projection neuron specification (Figure 2.1B), neuroectodermal progenitors were further cultured in FGF2 (20ng/ml, days 11-20) before undergoing FACS enrichment of PAX6^{mCherry+} forebrain progenitors. Where possible media was changed exactly 24 hours after the previous change, particularly during early differentiation where differentiating cells are most sensitive to small changes in concentration. For analysis, cultures were fixed for 10 minutes in 4% (w/v) paraformaldehyde at the desired endpoint (in 0.4M Sorensen's buffer, pH 7.3).

2.3 Animals and ethical approval

All animal procedures were conducted in accordance with the Australian National Health and Medical Research Council's Code of Practice for the Use of Animals in Research and were approved by the Florey Institute for Neuroscience and Mental Health Animal Ethics Committee. For all experiments athymic nude mice (BALB/c-Foxn1^{nu}) or rats (CBH-^{nu}) were acquired from the Animal Resource Centre (Canning Vale, Western Australia). Nude rodents display severely impaired adaptive immune responses due to mutations in the Foxn1 gene, allowing for long-term xeno-grafting in the absence of immunosuppression. Experiments were conducted on both male and female rodents, without preference. Animals were group housed (3-5) in individually ventilated cages under a 12:12 hour light/dark cycle with *ad libitum* access to food and water. All surgical and behavioural interventions were conducted in an immunocompromised-specific facility to avoid infection.

2.4 Stereotaxic surgery

Animal procedures were carried out on stereotaxic frames to ensure precise, consistent targeting of toxins, cells or viruses where appropriate (see [Thompson and Parish, 2013](#)). The injection cannula consisted of an extremely fine, heat-pulled glass capillary (Harvard Instruments) attached to a microvolume syringe (Hamilton) via an air-tight, heat-wrapped cuff. Animals were anaesthetised under isoflurane (5% in air) and maintained throughout surgery with persistent 1-2% administration through nosecone inhalation. Paw reflex response was used to indicate sufficient anaesthesia for surgery. Single incisions, exposing the coronal and sagittal sutures were made along the midline and Bregma defined as the intersection of the two. All stereotaxic coordinates are taken relative to this point (table 2.1) ([Paxinos and Franklin, 2004](#);

Table 2.1 - Rodent stereotaxic coordinates.

Target	Species	AP	ML	DV	Use	Chapter
Striatum (central)	Mouse	+0.5	-2.0	-4.0	DA cell transplantation	3, 4
Striatum (dorsolateral)	Mouse	+1.0	-2.0	-2.9	GDNF-AAV transfection	4
Substantia nigra	Mouse	-3.2	-1.4	-4.5	6-OHDA lesions	3, 4
Striatum (central)	Rat	+0.5	-2.5	-4.0	DA cell transplantation	3, 4
Striatum (dorsolateral)	Rat	+0.5	-3.5	-3.5	GDNF-AAV transfection	4
Medial forebrain bundle	Rat	-3.4	-1.3	-6.8	6-OHDA lesions	3, 4

AP: anterior-posterior. ML: mediolateral. DV: dorsoventral axes.

[Paxinos and Watson, 2004](#)). A small burr hole was drilled in the skull and the cannula lowered slowly beneath the dura. A flow rate of 1 μ l/min was used and the cannula left in place for several minutes to ensure diffusion and minimise backflow. The cannula was slowly withdrawn and the skin sutured. Antiseptic was applied and animals received analgesia preoperatively (meloxicam, 3mg/kg). Animals were monitored daily for a week following surgery to ensure recovery and every second day thereafter.

For unilateral 6-OHDA lesioning, the catecholamine-specific neurotoxin 6-OHDA was prepared in a 0.2% ascorbic acid solution and stored in the dark on ice for the duration of the surgeries to prevent oxidation. Mice received 1.5 μ l of 6-OHDA (1.6 μ g/ μ l) directly into the substantia nigra. In rats, 3.5 μ l 6-OHDA was injected into the medial forebrain bundle (3.2 μ g/ μ l). Unilateral loss of DA neurons within the midbrain and concomitant denervation of the striatum following 6-OHDA administration was confirmed by TH immunoreactivity and deficits in motor function (Figure 2.2). For transplantation into parkinsonian rodents, D21 mDA neural progenitors were dissociated with Accutase (10-15 minutes) and triturated through a 1ml pipette to yield 1-5 cell clumps. This solution was rapidly diluted in NBB27 media containing 10 μ M Y-27632 and centrifuged at 380g. Cell pellets were resuspended in NBB27 supplemented with BDNF (20ng/ml), GDNF (20ng/ml), TGF β 3 (1ng/ml), ascorbic acid (200nM), dibutyryl cAMP (0.05mM), DAPT (10 μ M) and Y-27632 (10 μ M), counted, centrifuged and finally resuspended at 100,000/ μ l for transplantation. Cells were maintained on ice throughout surgery. In all studies

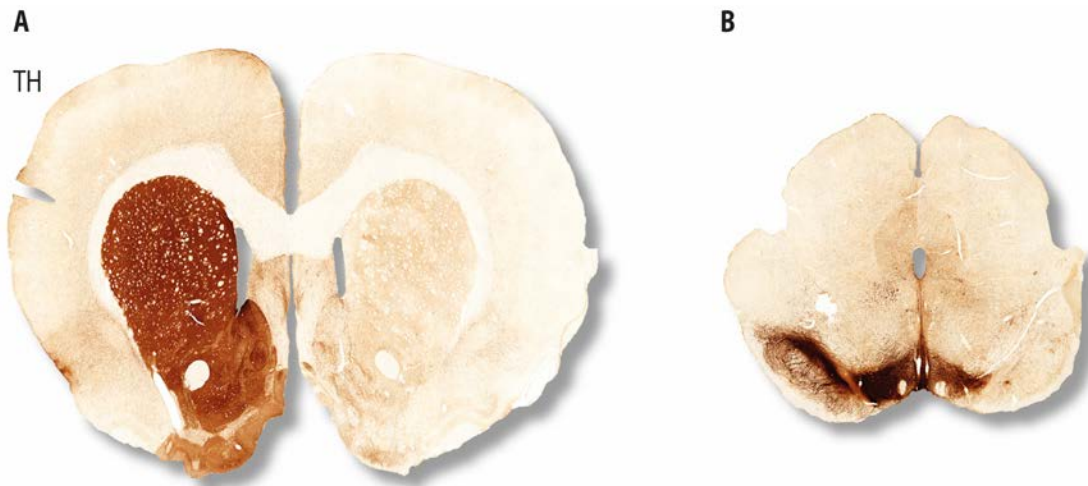


Figure 2.2 – Unilateral 6-OHDA ablation of midbrain DA neurons.

TH immunoreactivity highlights the loss of DA in the rat brain following 6-OHDA lesioning. (A) Coronal section through the striatum showing loss of TH⁺ fibers and innervation of DA-target tissue. (B) Ablation of DA neurons specifically within the substantia nigra pars compacta following 6-OHDA lesion of the medial forebrain bundle.

involving mDA transplantation (chapters 3 and 4) 100,000-150,000 mDA progenitors (100,000/ μ l) were unilaterally transplanted into the denervated striatum in NBB27 media consisting of 1:1 DMEM/F12 and Neurobasal, 1x B27/vitA, 1x N2, 1x ITS-A, 1x NEAA and 0.5x penicillin-streptomycin (All Life Technologies) supplemented with brain-derived neurotrophic factor (BDNF, 20ng/ml, R&D Systems), glial cell-line derived neurotrophic factor (GDNF, 20ng/ml, R&D Systems), recombinant human transforming growth factor type β 3 (TGF β 3, 1ng/ml, Peprotech), ascorbic acid (200nM, Sigma-Aldrich), dibutyryl cAMP (0.05mM, Tocris), DAPT (10 μ M, Sigma-Aldrich) and Y-27632 (10 μ M).

2.5 Behavioural testing of motor function

The unilateral 6-OHDA model of Parkinson's disease recapitulates mDA cell loss and results in stereotyped motor dysfunction contralateral to the lesion (Figure 2.3A). Motor testing was carried out to assess the extent of the lesion as well as recovery following transplantation. Only animals that displayed >300 rotations per hour following amphetamine administration were selected for further experiments and were stratified to reduce variability between experimental groups. All motor testing was conducted blinded in nude rats.

2.5.1 Amphetamine rotation test

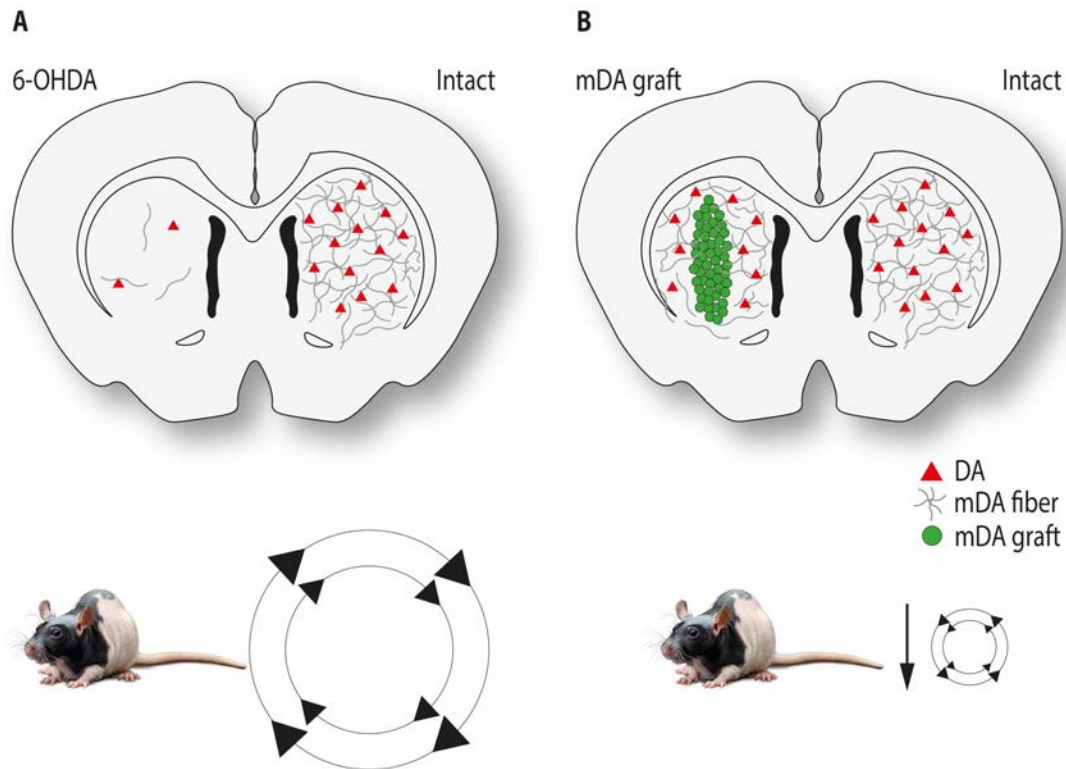


Figure 2.3 – Motor function following 6-OHDA lesioning or mDA transplantation.

(A) Unilateral 6-OHDA lesioning of the substantia nigra (mouse) or medial forebrain bundle (rat) results in a loss of TH+ mDA neurons in the substantia nigra and DA fibers in the striatum (grey). Loss of striatal DA (red) leads to basal ganglia dysfunction and motor symptoms which can be assessed in the amphetamine rotation test. Lesioned animals rotate ipsilateral to the lesion due to DA imbalance. (B) mDA transplantation can restore DA levels and transmission within the striatum, reducing DA imbalance and rotational behaviour after amphetamine administration.

The number of rotations was recorded over 60 minutes and repeated on two independent occasions to confirm deficits prior to transplantation. Following grafting, dopamine release from the transplant acts on sensitised dopamine receptors within the lesioned striatum and can balance DA signalling between lesioned and unlesioned hemispheres, resulting in a decrease in rotational bias. Progressive improvement in motor function (i.e. reduction in rotational turning) was assessed at defined intervals, up to 26 weeks, after grafting.

Animals received a single intraperitoneal injection of D-amphetamine sulfate (5mg/kg; Tocris Bioscience), an indirect DA agonist, which promotes DA release and inhibits reuptake. After 10 minutes, animals were harnessed and placed in a clear circular tube connected to a rotameter (Accuscan Instruments). Lesioned rats rotate ipsilateral to the lesion due to hemispheric DA imbalance (i.e. rats rotate to the right if the lesion is in the right hemisphere, Figure 2.3A).

2.5.2 Cylinder test

Prior to amphetamine rotation testing (to avoid pharmacological interference) animal motor function was probed in the cylinder test, a non-pharmacological assessment of forepaw preference. Rats are placed in a clear cylinder and observed by two investigators, each monitoring a single forepaw. The number of weight bearing touches (defined as clear flexion of the paw) during rearing behaviour is measured. Intact animals have no clear forepaw preference

due to balanced DA release between hemispheres. In contrast, 6-OHDA lesions result in unilateral dysfunction and contralateral forepaw deficits, demonstrating a bias toward use of the ipsilateral paw. A total of 20 touches per animal were recorded and repeated on three consecutive days. Data is displayed as the left paw ratio (left forepaw touches/(left forepaw touches + right forepaw touches))*100. We observed good correlation between rotational and forepaw deficits across all studies.

2.6 Transcardial fixation and tissue processing

Animals were euthanised by intraperitoneal injection of sodium pentobarbitone (100mg/kg, Virbac). Rapidly, an incision along the midline was made and the heart exposed. A needle was placed inside the aorta through a small incision in the left ventricle and the descending vessels clamped. Pre-heated Tyrode solution (37°C) was transcardially perfused through the animal using an external pump, followed by perfusion of 4% paraformaldehyde. Brains were subsequently removed for post-fixing in paraformaldehyde for two hours and transferred to a 20% sucrose solution for cryoprotection overnight. Brains were sectioned at 40µm on a freezing microtome (Leica) in a 1:12 series and stored in a cryoprotectant solution in multi-well plates at -20°C.

2.7 Immunohistochemistry

2.7.1 Chromogenic immunohistochemistry

Free-floating sections were washed (3x) in PBS^{Mg-/Ca-} to remove cryoprotectant and endogenous peroxide activity was quenched in 3% H₂O₂ and 10% methanol diluted in PBS^{Mg-/Ca-} for 20 minutes. Sections were incubated overnight under agitation at room temperature in PBS containing 0.3% triton-X, 10% donkey serum (PADT) and primary antibodies (table 2.2). Sections were washed (3x) in PBS and placed into blocking solution containing 10% donkey serum for an hour before being incubated with appropriate biotinylated secondary antibodies in PADT for two hours. Streptavidin-horseradish peroxidase complex (Vector Laboratories) was added for a further hour. For visualisation, tissue was exposed to di-amino-benzidine (0.5ml/ml; Sigma) for 2-5 minutes before the chromogenic reaction was catalysed by the addition of 1% H₂O₂. Final washes in PBS removed residual di-amino-benzidine before sections were mounted on gel slides

and left to dry. Slides were dehydrated in ascending alcohol series before being cleared in xylene and coverslipped using DePeX mounting media (BDH Chemicals).

2.7.2 Fluorescent immunohistochemistry

Fixed culture wells or free-floating sections were washed (3x) in PBS^{Mg-/Ca-}, permeabilised in 0.3% Triton-X diluted in PBS^{Mg-/Ca-} and incubated overnight in PADT containing primary antibodies (table 2.3). The next day, tissue was blocked in PADT for an hour and incubated for two hours in PADT containing secondary antibodies (table 3). Finally, sections were incubated in 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes, mounted and coverslipped with fluorescent mounting media (DAKO).

2.8 Microscopy and quantification

Fluorescent images were acquired on a Zeiss Axio Observer.Z1 upright, epifluorescence microscope or a Zeiss LSM780 confocal microscope. Brightfield imaging of chromogenic staining was carried out on a Leica DM6000 upright microscope. Light and camera settings were identical across groups and within analyses.

2.8.1 *In vitro* analysis of neural populations

Analysis was carried out on at least three biological replicates (individual differentiations) and three technical replicates (wells within a differentiation) unless otherwise specified within figure legends. For quantification 3-5 fields of view were imaged and quantified first for DAPI to attain the total cell number and subsequently for each protein of interest. The average of the fields of view for a given well and technical replicate wells within a given treatment was taken as a single replicate (n=1). Counting was performed manually with Zen (Carl Zeiss) or ImageJ (NIH) software.

2.8.2 Graft volume, phenotype and innervation analysis

Graft volume: Brightfield images from a 1:12 series (480µm inter-section distance) of NCAM⁺ chromogenic stained grafts were acquired on a Leica DM6000 microscope across the entirety of the graft rostrocaudal distance. The human specific NCAM antibody employed showed immunoreactivity limited to the graft and clearly delineated the borders of the transplant. The

Table 2.2 - List of primary antibodies used in this study.

Gene	Species	Dilution	Source	Catalogue #	Used for
BARHL1	Rabbit	1/300	Novus	NBP1-86513	Non-DA midbrain
BRN2	Goat	1/400	Santa Cruz	Sc-6029	Cortex layers 2-4
CALBINDIN-C28K	Mouse	1/1000	Swant	CB300	DA subtype
CD44	Rat	1/1000	BD Biosciences	550538	Stromal cells
CD9-PE	Mouse	1/100	Gift of M. Pera laboratory		Stem cells
CD90.2-PE-Cy7	Mouse	1/100	Gift of M. Pera laboratory		Stem cells
cFOS	Goat	1/1000	Santa Cruz	sc-52	Immediate early gene
CHOLECYSTOKININ	Rabbit	1/8000	(Frey, 1983)		DA subtype
CTIP2	Rat	1/500	Abcam	AB18465	Cortex layer 5
DAPI		1ug/ml	Sigma Aldrich	D8417	DNA
DARPP32	Rabbit	1/500	Millipore	AB10518	Striatal neurons
FOXA2	Goat	1/200	Santa Cruz	sc-6554	DA progenitors
GCTM2-APC	Mouse	1/100	Gift of M. Pera laboratory		Stem cells
GDNF	Goat	1/1000	R&D Systems	AB-212-NA	Neurotrophic factor
GFAP	Rabbit	1/1000	DAKO	Z0334	Astrocytes
GFP	Chicken	1/1000	Abcam	AB13970	
GFP	Rabbit	1/20000	Abcam	AB290	
GIRK2	Rabbit	1/500	Alomone Labs	APC-006	DA subtype
HUMAN NUCLEI	Mouse	1/300	Millipore	MAB1281	Human nuclei
KI67	Rabbit	1/200	Thermo-Fisher	RM-9106	Proliferative cells
MAP2	Rabbit	1/1000	Millipore	AB5622	Neurons
NURR1	Rabbit	1/200	Santa Cruz	Sc-990	DA progenitors
OCT4	Mouse	1/100	Santa Cruz	sc-5279	Stem cells
OTX2	Goat	1/1000	R&D Systems	AF1979	Forebrain; midbrain
PAX6	Mouse	1/50	DSHB	PAX6-s	Dorsal telencephalon
PH3	Rat	1/1000	Abcam	AB10543	Mitosis
PITX2	Sheep	1/500	R&D Systems	AF7388	non-DA midbrain
PSA-NCAM	Mouse	1/200	Santa Cruz	sc-106	human neural cells
RFP	Rabbit	1/1000	Rockland labs.	600-901-379	mCherry
SATB2	Mouse	1/100	Abcam	AB51502	Cortex layers 2-4
SOX2	Mouse	1/300	R&D Systems	MAB2018	Neural progenitors
SOX2	Goat	1/300	R&D Systems	AF2018	Neural progenitors
SYNAPTOPHYSIN	Mouse	1/1000	Enzo	ADI-905-782	Human synapses
TBR1	Rabbit	1/400	Abcam	AB31940	Cortex layer 6
TBR2	Rabbit	1/500	Abcam	AB23345	Cortex progenitors
TH	Rabbit	1/1000	Pel-freeze	P40101-0	DA neurons
TH	Sheep	1/800	Pel-freeze	P60101-0	DA neurons
TUJ1	Mouse	1/1000	Promega	G712A	Neurons
VIMENTIN	Mouse	1/1000	Chemicon	MAB3400	Cortical progenitors
ZO-1	Mouse	1/200	Invitrogen	339100	Tight junctions

area of each graft section was recorded using Leica Application Suite software and the summed total was corrected according to section number and Cavalieri's principle to attain graft volume.

Table 2.3 - List of secondary antibodies used in this study.

Antibody	Species	Dilution	Source
AlexaFluor-488	Mouse, rabbit, goat, chicken, sheep	1/800	Jackson ImmunoResearch
AlexaFluor-555	Mouse, rabbit, goat, chicken, sheep	1/800	Jackson ImmunoResearch
AlexaFluor-647	Mouse, rabbit, goat, chicken, sheep	1/800	Jackson ImmunoResearch
Biotin	Mouse, rabbit, goat, chicken, sheep	1/1000	Jackson ImmunoResearch

All raised in donkey

GFP and TH quantification: Protein of interest quantification (for GFP and/or TH) was achieved by blinded manual counting of all immunoreactive cells in a single 1:12 series during live imaging. Total cell numbers were subsequently generated by correcting for series.

DA subtype: A9- or A10-like DA subtype was determined by coexpression of TH/GFP with GIRK2 and/or Calbindin. Confocal images of grafts at 1:12 were obtained on a Zeiss LSM780. Every TH⁺ cell was assessed for expression of either GIRK2 or Calbindin. DA cells were assigned as GIRK2⁺, Calbindin⁺ or GIRK2/Calbindin⁺ or GIRK2/Calbindin⁻. Total cell numbers were corrected for series.

GFP and TH fiber density: Three, 40x images brightfield were acquired using a Leica DM6000 within each region of interest (pre-frontal cortex, cingulate cortex, perirhinal cortex, dorsolateral striatum, ventrolateral striatum). Images were colour inverted using Photoshop (Adobe) and the total number of reactive/unreactive pixels quantified using the colour mask tool, resulting in a percentage of immunoreactive GFP or TH pixels.

2.9 Gene expression analysis

Chapters 3-4 (dopamine studies): DA cultures were dissociated with Accutase (5-20 minutes), diluted in PBS^{Mg-/Ca-} and spun at 400g. For *in vivo* analysis, the striatum containing the graft was grossly dissected from the surrounding parenchyma. Briefly, for RNA extraction, cell pellets or brain tissue were lysed in TRIzol (Invitrogen) by trituration with a 1ml pipette, spun at 10,000g and the supernatant collected. Chloroform was added to induce phase separation and the supernatant collected after a further spin. RNA was precipitated with isopropyl alcohol and washed (2x) in 75% ethanol. Extracted RNA was dissolved in RNase-free water and RNA quality was measured using a NanoDrop One spectrophotometer (Thermofisher) before being stored at -80°C. *Chapter 5 (cortex study):* Cell pellets were stored in RNeasy (Qiagen) and total RNA

was extracted using either the RNeasy Mini Kit (Qiagen) or Isolate II Mini Kit (Bioline) according to manufacturer's specifications with on-column DNA digestion.

2.9.1 qRT-PCR

Immediately following RNA quantitation, RNA was converted to cDNA using the SuperScript™ VILO™ Master Mix (Thermofisher) according to manufacturer's specifications. All cDNA reactions were performed under the same conditions at the same time to avoid inefficient reactions using a GeneAmp 9700 (Applied Biosystems) with a thermal profile consisting of 10 minutes at 25°C, 30 minutes at 48°C and 5 minutes at 95°C. Gene primers were designed with PrimerQuest (Integrated DNA Technologies, <https://eu.idtdna.com/Primerquest/home/Index>) or PrimerBank (<https://pga.mgh.harvard.edu/primerbank/index.html>) online platforms. All primers were designed to span exons, generate amplicons between 70-150 bp & generate no nonspecific gene products (see table 2.4 for primer sequences). All primer sequences were validated for efficiency across 3-fold log [cDNA] with amplicon length confirmed through electrophoretic separation. After primer validation, 10ng of sample cDNA was run in triplicate using a two-stage standard cycling protocol with the PowerUp SYBR Green Master kit on the Corbett Rotor-gene (both Thermofisher). Briefly, after UDG inactivation & DNA polymerase activation for 2 m at 50°C and 95°C, respectively, cycles of denaturing at 95°C for 15 s and Annealing/extension 60°C for 1 m were repeated 40 times. Changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method using the housekeeper *HPRT1*.

2.9.2 RNA sequencing

Mixed species samples (i.e containing graft and surrounding host RNA) were used to generate cDNA libraries using the TruSeq stranded mRNA sample preparation kit (Illumina). Samples were adjusted to give approximately 20×10^6 graft-specific reads, utilising qRT-PCR to initially analyse the percentage of graft (human) RNA within the sample. Libraries were then sequenced using paired end, 75bp sequencing on the HiSeq 2000 platform (Illumina). Alignment to the human genome (Hg38) was performed using HISAT2 (2.0.3.3). Paired reads were then cross-checked against the mouse genome (mm10) and aligned reads discarded. Only reads that aligned to human genome and not the mouse genome were analysed. Gene ontology analysis was performed using the DAVID gene ontology browser.

Table 2.4 - List of primers used in this study (continued over page).

Gene	Forward Primer (5'-3')	Reverse Primer (3'-5')
<i>AADC</i>	TGGATTACGTGGCCAACACTACA	AAACGTGTCTGGCTCCTGAG
<i>ADCYAP1</i>	GAGAAGGAGGCAGGGCAC	AGGAGTATTTACATGTCTGGGGT
<i>ALDH1A2</i>	GCTTGCAGACTTGGTGAAC	CCCAGCCTGCGTAATATCGA
<i>AP-1</i>	AACAGGTGGCACAGCTTAAAC	CAACTGCTGCGTTAGCATGAG
<i>BRN2A</i>	CGGCGGTTTGCTCTATTC	ATGGTGTGGCTCATCGTG
<i>C-MYC</i>	GTCAAGAGGCGAACACACAAC	TTGGACGGACAGGATGTATGC
<i>CCK</i>	TAGGCAGCTGAGGGTATCGC	TCCAGGTTCTGCAGGTTCTT
<i>CCND1</i>	CAATGACCCCGCACGATTTTC	CATGGAGGGCGGATTGGAA
<i>CCNE1</i>	GCCAGCCTTGGGACAATAATG	CTTGACGTTGAGTTTGGGT
<i>CTIP2</i>	TCACCCACGAAAGGCATCTGT	TGAAGGGCTGCTTGCATGTTG
<i>CUX1</i>	ACCATCGGCTTCTTCTACAC	ACCATCGGCTTCTTCTACAC
<i>DAT</i>	TTCCTCAACTCCCAGTGTGC	CCGTTCTGCTCCTTGACAAG
<i>DLK1</i>	CTCTCCCGGCTGCCTTCA	GCAGGCCCGAACATCTCTAT
<i>EPHA2</i>	CTCCCTGCTGTGCCATGC	TCCCTGGTCATCTCCTCAGT
<i>FEZF2</i>	AGGGAACACTACAAGAACCACAAG	GGGTGTGCATATGGAAGGTTAG
<i>FGFR1</i>	GAGGGTCAGTTTGAAAAGGAGG	AGCTCCACATCCCAGTTCTG
<i>FOXA2</i>	GCCCCGAGGGCTACTCCTCCG	TCATGTTGCCCCGAGCCGCTG
<i>FOXG1</i>	AGAAGAACGGCAAGTACGAGA	TGTTGAGGGACAGATTGTGGC
<i>GDNF</i>	GGAGAGAGAGAAAACAGCCCT	GCCGTAATCAGTGATGGTG
<i>GFAP</i>	AGATGCTGAAACAGGAGAGAAAAG	GGTATGACACAGCAAGGAAGAG
<i>HES5</i>	AGGCTGGAGAGGCGGCTAAG	TGGAAGGTGACACTGCGTTGG
<i>HPRT1</i>	CTTGCTGCGCTCCGCCT	ATCACTAATCACGACGCCAGGGC
<i>ITGA11</i>	CAGAATGGAACCTAAGCTGGT	GAGGGAGGCCTTGGCATG
<i>LAMC2</i>	ATACAGATCTCAGGGCTCGG	ATCAGGAATGGGGTCAGGTC
<i>LMX1A</i>	CTGCTGGGCAGAGCGGTGAG	TTTGCAGGAGGCGCACTGCA
<i>MANF</i>	GCACGGACCGATTGTAGTC	CCAGGCTTCACATCTGATACAG
<i>MAP2</i>	ACAACATCAAATACCAGCCTAAAG	CTTGTAACAATTTGTACATTTCCG
<i>MASH1</i>	CGCGGCCAACAAAGAAGATG	CGACGAGTAGGATGAGACCG
<i>NCAM</i>	TCCAGGCCAGGCAGAATATT	TCATCGTCTCCTCTTGCTC
<i>NESTIN</i>	AGCGTTGGAACAGAGGTTG	AGGAGGGTCCTGTACGTG
<i>NOTCH1</i>	ATCCTGATCCGGAACCGAG	CGTCGTGCCATCATGCAT
<i>NURR1</i>	TTCTGCCTTCTCCTGCATT	CCCCATTGTTGAAAGTCAC
<i>OCT4</i>	CTGGAGAAGGAGAAGCTGGA	CCTGTGTATATCCAGGGTGA
<i>OSBP2</i>	AGAAATCAGGGTGAAATGGCC	CCCCACTGGTCAGCAAGATA
<i>PAX6</i>	GCGGAAGCTGCAAAGAAATAG	GGGCAAACACATCTGGATAATG
<i>PITX3</i>	CAGCCAACCTTAGTCCGTG	CGGAGGCTGTGAATCGTTG
<i>PSMB4</i>	TGCACTTTACAGAGGTCCAATC	CGTAGGATCCCAGCATGTCT
<i>RBP4</i>	CCCAGAAGCGCAGAAGATTG	TCTTTCTGATCTGCCATCGC
<i>REST</i>	CGGTTGGGGATAACAACCTTTTCA	TCTACGACGCTGAGTTCCAAA
<i>SATB2</i>	CCAAACACACCATCATCAAGTTC	GCAGCTCCTCGTCCTTATATTC
<i>SCN5A</i>	GTTTGAGGAAGGCGAGCAAC	CTCCGTGCCAGGCT
<i>SFRP1</i>	GGCACAGAGCTGCACTATCA	CCTCCTCTCTCGGAGACCAA
<i>SOX2</i>	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG
<i>SOX4</i>	GACCTGCTCGACCTGAACC	CCGGGCTCGAAGTTAAATCC
<i>SOX5</i>	ATAAAGCGTCCAATGAATGCCT	GCGAGATCCCAATATCTTGCTG
<i>SYT12</i>	CTGTCTGATGCCCTCTCCAT	CACTCCTCTGCTGCAATCTG
<i>TBR1</i>	TCCCAGTGCCATGTTCCC	AACCCATTTGCCTCCTTGA
<i>TBR2</i>	AGCTCTCCAAGGAGAAAAGTG	GCCTTCGCTTACAAGCACTG

<i>TH</i>	GAGGCCATCATGGTAAGAGGG	CTGCCTGCGCCCAATGAAC
<i>THBS1</i>	CCCTTGCTGCTCAGAGTGGAT	CACAAGGGATGGGGTAAAACA
<i>VMAT2</i>	ACAGCTAGTTCTCTCAAGTGTAG	GCCAACCAGATAACATGTTAACA

2.10 Flow cytometry

Cells were dissociated using Accutase at the desired timepoint for FACS analysis or transplantation. In general, stem cells and early neural precursors (5-10 minutes) required less enzymatic digestion than maturing neuronal progenitors and neurons (10-20 minutes). Single cell solutions were generated by gentle trituration with a 1ml pipette and then placed into pre-warmed culture medium containing 20% KSR and 10 μ M Y-27632 (ROCKi), spun at 380g and resuspended in FACS wash consisting of 80% culture medium (mTeSR1 for stem cells or NBB27 for neural cells), 20% KSR, 10 μ M Y-27632 and 1 μ g/ml DAPI. Sorting experiments were conducted on a FACS ARIA III (BD) at the Melbourne Brain Centre core facility or a MoFlo XDP (Beckman Coulter) at the Murdoch Children's Research Institute core facility using a 100 μ m nozzle at 20-22PSI. Gating based on forward/side scatter profiles and DAPI exclusion was used to ensure analysis was carried out on live, single cells. Appropriate single stain, secondary only or unstained controls were used where appropriate. Parental H9 or HES-3 hPSC (i.e GFP/RFP) time-matched control differentiations were used to quantify and/or select for GFP/RFP⁺ populations.

2.11 Statistical analysis

Specific details of statistical tests are outlined in figure legends. Data is presented always as mean $\pm$ SEM. Corrections for multiple comparisons were used when appropriate. Analyses were carried out in GraphPad Prism 8 and p-values of <0.05 were considered significant. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

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Efficiently specified ventral midbrain dopamine neurons from human pluripotent stem cells under xeno-free conditions restore motor deficits in parkinsonian rodents. *Stem Cells Translational Medicine* 2017; 6:937-948. Reproduced here in accordance with the Graduate Research Training Policy MPF1321 and with co-author permission.

Chapter 3.

EFFICIENTLY SPECIFIED VENTRAL MIDBRAIN DOPAMINE NEURONS FROM HUMAN PLURIPOTENT STEM CELLS UNDER XENO-FREE CONDITIONS RESTORE MOTOR DEFICITS IN PARKINSONIAN RODENTS

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3.1 Abstract

Recent studies have shown evidence for the functional integration of human pluripotent stem cell (hPSC)-derived ventral midbrain dopamine (vmDA) neurons in animal models of Parkinson's disease. Although these cells present a sustainable alternative to fetal mesencephalic grafts, a number of hurdles require attention prior to clinical translation. These include the persistent use of xenogeneic reagents and challenges associated with scalability and storage of differentiated cells. In this study, we describe the first fully defined feeder- and xenogeneic-free protocol for the generation of vmDA neurons from hPSCs and utilize two novel reporter knock-in lines (LMX1A-eGFP and PITX3-eGFP) for in-depth in vitro and in vivo tracking. Across multiple embryonic and induced hPSC lines, this "next generation" protocol consistently increases both the yield and proportion of vmDA neural progenitors (OTX2/FOXA2/LMX1A) and neurons (FOXA2/TH/PITX3) that display classical vmDA metabolic and electrophysiological properties. We identify the mechanism underlying these improvements and demonstrate clinical applicability with the first report of scalability and cryopreservation of bona fide vmDA

progenitors at a time amenable to transplantation. Finally, transplantation of xeno-free vmDA progenitors from LMX1A- and PITX3-eGFP reporter lines into Parkinsonian rodents demonstrates improved engraftment outcomes and restoration of motor deficits. These findings provide important and necessary advancements for the translation of hPSC-derived neurons into the clinic.

3.2 Introduction

Clinical trials of human fetal tissue grafts in Parkinson's disease patients provide evidence that donor neural cells can reinnervate appropriate targets, increase dopamine neurotransmission and reverse phenotypic symptoms for decades after transplantation ([Lindvall and Bjorklund, 2004](#)). However, immunological rejection as well as temporal and cellular heterogeneity of scarce fetal tissue has resulted in variable symptomatic relief ([Barker et al., 2013](#)). Human pluripotent stem cells (hPSCs) have the potential to circumvent these obstacles, presenting an unrivalled regenerative medicine tool for brain repair. Unlike extensive earlier works, only in recent years have protocols emerged that are capable of generating bona fide ventral midbrain dopamine (vmDA) neurons from hPSCs, with success largely attributed to early floor-plate patterning ([Denham et al., 2012](#); [Kirkeby et al., 2012](#); [Kriks et al., 2011](#); [Xi et al., 2012](#)). Within these studies vmDA neurons were definitively identified using coexpression of key markers. Broadly, ventricular zone equivalent progenitors were confirmed using combinatorial assessment of forebrain/midbrain marker OTX2, basal plate marker FOXA2 and floor plate marker LMX1A expression. Intermediate zone equivalent progenitors were distinguished by FOXA2 coexpression with midbrain identifiers NURR1 or engrailed-1 (EN1). Finally, mantle zone neurons were confirmed by colocalization of FOXA2 and the dopamine (DA) marker tyrosine hydroxylase (TH) and in some cases the cardinal vmDA neuronal identifier PITX3. Recent grafting studies of these hPSC-derived DA precursors demonstrated their capacity to survive, partially innervate the striatum of 6-hydroxydopamine (6-OHDA) lesioned rodents or primates and ameliorate motor function via neuronal integration ([Doi et al., 2014](#); [Grealish et al., 2014](#); [Kirkeby et al., 2012](#); [Kriks et al., 2011](#); [Steinbeck et al., 2015](#)).

Although these new protocols are more sophisticated than early methods that generated non-region-specific DA neurons ([Kawasaki et al., 2000](#); [Perrier et al., 2004](#)), they retain critical components that impede clinical compliance requirements of regulatory authorities. Specifically, all protocols utilize hPSCs adapted to mouse embryonic fibroblast (MEF)

coculture and in several protocols MEF coculture or conditioned media is required immediately preceding or during initiation of neural induction (Kriks et al., 2011; Sundberg et al., 2013; Xi et al., 2012). MEFs impart immunogenic proteins, pluripotency promoting growth factors and extracellular matrix proteins (Hongisto et al., 2012; Martin et al., 2005), as well as exhibiting batch-to-batch stochasticity, which together affect hPSC growth, morphology and differentiation (Villa-Diaz et al., 2009). Although some steps have been taken to move toward a clinically translatable protocol (Doi et al., 2014), all protocols retain poorly defined xenogeneic factors throughout induction and maturation stages, including extracellular matrices such as Engelbreth-Holm-Swarm sarcoma-derived Matrigel or laminin, B27 supplement and knockout serum replacement (KSR) media (Denham et al., 2012; Grealish et al., 2014; Kirkeby et al., 2012; Kriks et al., 2011; Steinbeck et al., 2015; Sundberg et al., 2013; Xi et al., 2012).

Cell replacement therapy requires elimination of xenogeneic components from hPSC expansion and differentiation protocols. To date these changes have not been fully implemented and comparisons have not been made to prior published xenogeneic differentiation protocols. In this study, we describe a fully defined feeder- and xeno-free protocol for the generation of bona fide vmDA neurons. Currently, the dual-SMAD inhibition system reports the highest proportions of correctly patterned vmDA lineage cells, as assessed by colocalization of relevant vmDA proteins (Kriks et al., 2011) and consequently was utilized as a comparative benchmark. Investigation of the newly developed xeno-free system, utilizing novel LMX1A-GFP and PITX3-GFP knock-in reporter lines, demonstrates not only feasibility but also significant improvement in ventral midbrain (VM) patterning, yield and population homogeneity. Generated DA neurons exhibit characteristic vmDA electrophysiological properties and DA metabolism. Moreover, xeno-free vmDA progenitors resulted in improved phenotype specification upon transplantation into Parkinsonian rodents and were shown for the first time to restore functional motor deficits. Finally, we demonstrate that this “next generation” protocol is readily scalable and for the first time report successful cryopreservation of hPSC-derived vmDA progenitors at a time amenable to transplantation. These findings provide critical advancements for the generation and utility of hPSC-derived vmDA neurons for in vitro purposes such as disease modelling and drug development and importantly for clinical transplantation in Parkinson’s disease patients.

3.3 Materials and methods

3.3.1 Maintenance of human pluripotent stem cells

Human embryonic stem cell (hESC) lines HES3 (Somaa et al., 2015), H9 (WA09; WiCell) (Thomson et al., 1998), our newly generated H9 reporter line (H9 LMX1A-GFP knock-in), recently generated H9 PITX3-GFP knock-in (Watmuff et al., 2015) and integration-free human induced pluripotent stem cell (hiPSC) lines RM3.5 (Kao et al., 2016) and 409B2 (Okita et al., 2011), were cultured in xenogeneic or xeno-free conditions. All lines were confirmed to be karyotypically normal and frequently tested for the absence of mycoplasma (MycoAlert detection kit; Lonza). Xenogeneic conditions: hPSCs were cocultured on $0.02 \times 10^6/\text{cm}^2$ γ -irradiated MEFs in Dulbecco's modified Eagle's medium (DMEM)/F12 + GlutaMAX with 20% KSR, 1% NEAA, 0.2% β -mercaptoethanol, 0.4% penicillin-streptomycin (all ThermoFisher Scientific) and 10ng/ml FGF2 (R&D Systems). Xeno-free conditions: hPSCs were cultured on laminin-521 (Biolamina), in xeno-free TeSR2 (StemCell Technologies) supplemented with 0.4% penicillin-streptomycin. hPSC lines were adapted to the TeSR2 xeno-free conditions and maintained for >20 passages. Media were changed daily and EDTA was used to passage the cells every 4–6 days. Cultures were maintained at 37°C with 5% CO₂.

3.3.2 Dopaminergic differentiation

Confluent hPSC cultures were disassociated with EDTA to generate small aggregates. Xenogeneic hPSCs underwent MEF-depletion before differentiation by plating them onto gelatin-coated dishes for 15 minutes in feeder conditioned media (generated by incubating hPSC media with MEFs for 24 hours and 10 ng/ml FGF2 added before use), resulting in preferential attachment of MEFs but not hPSCs. Pluripotent cells were collected in feeder-conditioned media and seeded at either standard ($0.225 \times 10^6/\text{cm}^2$) or high densities ($0.675 \times 10^6/\text{cm}^2$) on tissue culture grade plastic coated with Matrigel. For xeno-free conditions, hPSC suspensions were collected in TeSR2 media and plated at the above densities on plates coated with laminin-521 (10 $\mu\text{g}/\text{ml}$) or vitronectin (10 $\mu\text{g}/\text{ml}$; StemCell Technologies).

Differentiation to vmDA neurons was performed as described previously with modifications (Figure 3.1A) (Kriks et al., 2011). Specifically, differentiation commenced 24 hours after seeding with the addition of serum replacement media (SRM; DMEM/F12 + GlutaMAX with 15% KSR [either xenogeneic or xeno-free variant], 1% NEAA, and 0.2% β -mercaptoethanol) and supplemented with SMAD inhibitors LDN193189 (LDN; 100nM or 200nM; Miltenyi), and SB431542 (SB; 10 μM ; R&D Systems) for neural induction. SB and LDN were removed at day of

differentiation D5 and D11, respectively. SRM media were gradually replaced with N2 media (DMEM/F12 with 1% N2 and 1% ITS-A [ThermoFisher Scientific]) from D5-11. Sonic hedgehog C25II (100ng/ml; R&D Systems) and the smoothened receptor agonist purmorphamine (2 μ M; Stemgent) were added from D1-7 to promote ventralization. GSK3 β inhibitor CHIR99021 (3 μ M; Stemgent) was added from D3-13 to promote caudalization. At D11, media were switched to N2B27 (a 1:1 mix of neurobasal media and DMEM/F12 + GlutaMAX, supplemented with 2% B27 (either xenogeneic or xeno-free), 1% ITS-A, 0.5% GlutaMAX, and 0.4% penicillin-streptomycin) with recombinant human brain-derived neurotrophic factor (20ng/ml; R&D Systems), recombinant human glial cell line-derived neurotrophic factor (20ng/ml; R&D Systems), ascorbic acid (200nM; Sigma-Aldrich), recombinant human transforming growth factor type β 3 (TGF β 3; 1ng/ml; PeproTech), dibutyryl cAMP (0.05mM; Tocris Bioscience), and the notch inhibitor DAPT (10 μ M, Sigma-Aldrich), henceforth known as “maturation media.” At D17, cultures were dissociated with Accutase (StemCell Technologies) and seeded onto surfaces coated with human laminin-521 or poly-L-ornithine (15 μ g/ml; Sigma-Aldrich), mouse laminin (3.5 μ g/ml; ThermoFisher Scientific), and fibronectin (2 μ g/ml; R&D Systems). Maturation media were maintained until desired end point. See supplemental table 3.1 for reagent differences between the two protocols.

3.3.3 Cryopreservation

vmDA neural progenitor cells (NPCs) were collected after 22 days of differentiation (without passage) using EDTA for 5 minutes at 37°C to generate a cell suspension composed of 10 to 200 cell clusters. Cells were resuspended in maturation media and mixed 1:1 with a xeno-free cryopreservation solution (20% dimethyl sulfoxide, 20% TeSR2, 60% xeno-free KSR) and immediately transferred to a slow rate freezer EF600M (Grant Instruments).

3.3.4 Immunocytochemistry and cell quantification

Cells were fixed in 4% paraformaldehyde for 7–10 minutes and antibody staining performed as previously described (Soma et al., 2015). Images were captured using a Zeiss Axio Observer.Z1 or Zeiss Pascal Confocal Microscope. Quantification was carried out on three technical replicates/condition/experiment and repeated on at least three independent culture experiments. Statistical analysis was performed using Graphpad Prism: Student’s t test comparison was performed between all xenogeneic and xeno-free conditions (*p<0.05, **p<0.01, ***p<0.001).

3.3.5 Flow cytometry

Cells were dissociated with Accutase (4 minutes, 37°C) and stained with primary antibodies (Supplemental Table 1) according to previously described methods (Niclís et al., 2013). Appropriate unstained and single antibody controls were used to identify background fluorescence and for compensation respectively, with gating performed according to standard procedures (Supplemental Figure 3.6A–H).

3.3.6 Gene expression analysis

Total RNA was extracted at D0, D11, D25, and D40 using Trizol. RNA was converted to cDNA and subsequently analysed using quantitative real-time polymerase chain reaction (qPCR) for six genes of interest (Supplemental Table 2) using previously described methods (Soma et al., 2015). All qPCR was performed across triplicate technical replicates for each of the four independent biological replicates and normalized against HPRT1.

3.3.7 High-performance liquid chromatography

Dopamine and the metabolite homovanillic acid (HVA) levels were measured in xenogeneic and xeno-free cultures at D40 using reverse phase liquid chromatography with electrochemical detection, as previously described (Parish et al., 2008). Data were expressed as pmol/ml of DA or HVA, and dopamine turnover determined by the ratio of DA to HVA.

3.3.8 Electrophysiology

Whole-cell patch-clamp recordings were performed in vitro on H9 PITX3-GFP hESC-derived DA neurons ($n = 21$) at D55–65 using previously described methods (Parish et al., 2008). Recording pipettes (3.5–5.5 M Ω) were filled with a low Cl⁻ intracellular solution (pH 7.3 and 290 mOsmol). As a consequence, $E_{Cl} = -69$ mV, and inhibitory post synaptic currents (IPSCs) had negligible amplitudes at $V_H = -60$ mV, although more prominent outward current amplitudes were achieved by shifting to $V_H = -40$ mV. All recordings were made using a Multiclamp 700B (Molecular Devices). Signals were sampled at 20 kHz and filtered at 10 kHz (p-Clamp 10.3; Molecular Devices). Voltage clamp measurements: Cells were held at -60 mV and inward spontaneous excitatory postsynaptic currents (EPSCs) were recorded for 3 minutes and blocked by AMPA receptor antagonist NBQX (20 mM; Tocris Bioscience). Cells were held at -40 mV and outward spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded. Membrane

potential measurements: Action potentials (APs) were stimulated in current-clamp mode. Resting membrane potential was recorded in the course of 1 minute. Membrane potential was adjusted to -60mV (current subtraction, ± 8 pA for all cells) and cells depolarized with a series of 400-millisecond current pulses in 10pA increments from -40 to 160pA. Liquid junction potentials were corrected for during data analysis (10.7mV at 32°C) where spontaneous excitatory postsynaptic currents (sEPSCs) were detected by MiniAnalysis (Synaptosoft) and APs counted in Clampfit (version 10.3). Biocytin (0.5%) was added to the recording pipette to fill neurons post-recording and validate DA phenotype (via TH coimmunoreactivity).

3.3.9 Surgical Procedures

All animal procedures were conducted in accordance with the Australian National Health and Medical Research Council's published Code of Practice for the Use of Animals in Research and approved by the Florey Institute for Neuroscience animal ethics committee. Transplantation studies were performed in unilateral 6-hydroxydopamine lesioned athymic mice and rats, as previously described (Bye et al., 2015; Bye et al., 2012; Kauhausen et al., 2013). To assess the survival and maintenance of vmDA fate specification in vivo, H9::LMX1A-GFP hESCs were differentiated for 25 days under xenogeneic or xeno-free conditions. Cultures were subsequently dissociated and resuspended at 100,000 cells/ μ l, with 1 μ l stereotactically implanted into the denervated striatum of mice (n = 6 per group). Mice were killed (100mg/kg pentobarbitone) at 5 weeks. To assess the long-term functional integration of xeno-free vmDA progenitors, grafts were performed into rats because of their greater responsiveness in motor behavioural tests compared with mice. Briefly, 6-OHDA lesioned athymic rats (n = 15) were tested for rotational asymmetry in response to administration of D-amphetamine sulfate (5mg/kg, i.p.) 3 weeks after lesioning and retested for motor improvement at 2, 4, and 6 months after the transplantation of H9 PITX3-GFP derived vmDA progenitors (100,000 cells in 1 μ l injected into the striatum). Rats were killed at 6 months for histological assessment.

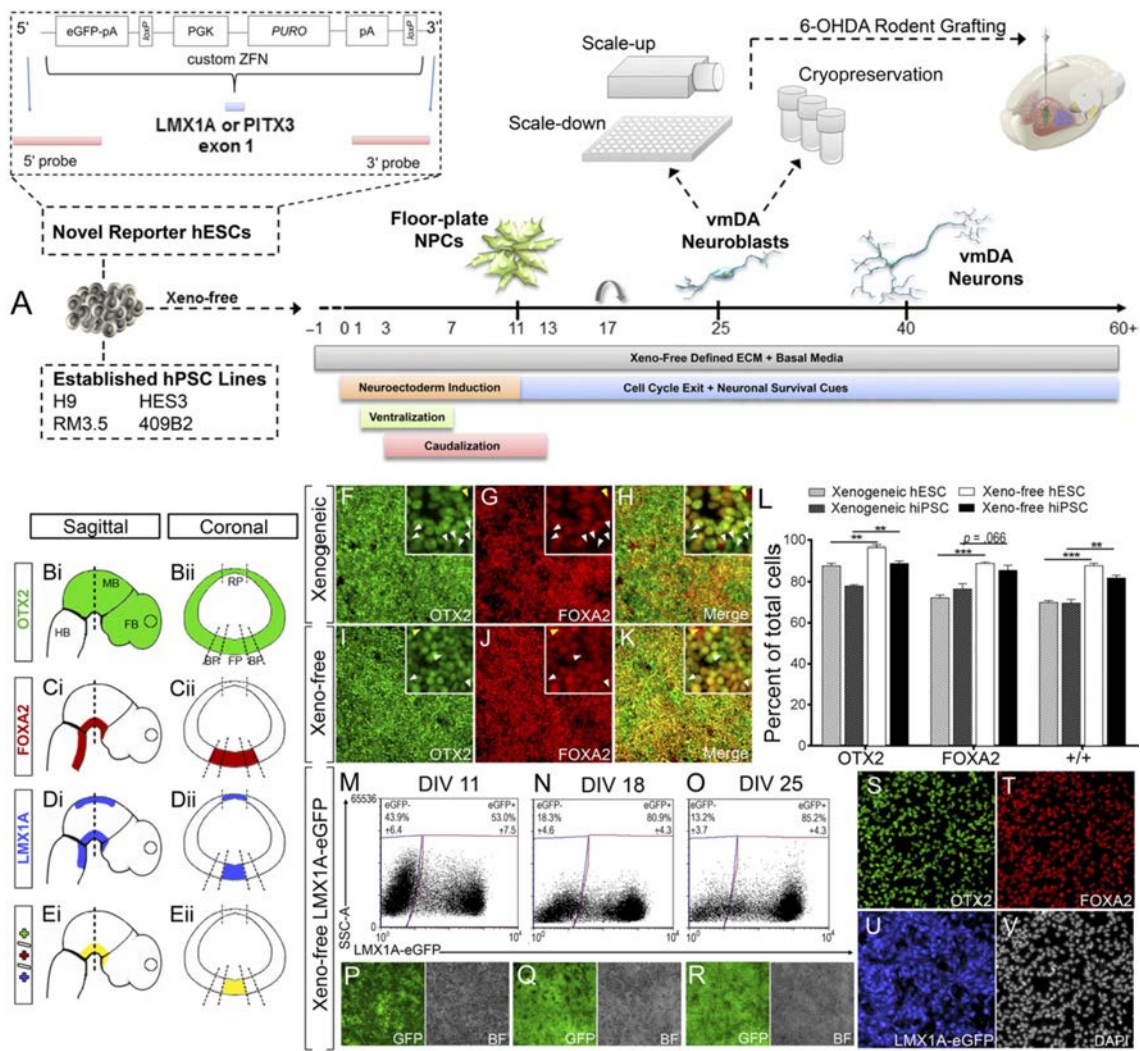
3.4 Results

3.4.1 Establishment of a fully defined feeder-free, xeno-free vmDA differentiation protocol

Differentiation cell-seeding density was optimized at $0.675 \times 10^6/\text{cm}^2$, producing uniform monolayers of tightly compacted pluripotent cells devoid of acellular regions (Supplemental Figure 3.1A–Hii). hPSC lines (Figure 3.1A) were then adapted from xenogeneic KSR media and MEF coculture to TeSR2 xeno-free conditions and differentiated under fully-defined and xeno-free media, which resulted in widespread coexpression of the fore/midbrain marker OTX2 (Figure 3.1B) and basal/floor plate marker FOXA2 (Figure 3.1C) at D11 (Supplemental Figure 3.1Fii, Gii, Hii) (Burbach and Smidt, 2006). Finally, two xeno-free matrix proteins, vitronectin and human laminin-521, were compared for their ability to replace Matrigel (a mouse sarcoma extract). Both matrices facilitated appropriate patterning, however, only laminin-521 supported the long-term attachment of neural precursors (Supplemental Figure 3.1I–P). Next, a detailed assessment of differentiation outcomes in xeno-free conditions was performed using the novel LMX1A- and PITX3-GFP reporter lines, with comparative evaluations to established xenogeneic cultures at stages corresponding to vmDA NPCs (D11–18), neuroblasts/immature neurons (D25), and mature neurons (D40 and D60–75; Figure 3.1A).

3.4.2 Xeno-free conditions generate correctly specified, high-purity vmDA progenitors

Xenogeneic conditions generated OTX2 and FOXA2 expression at levels consistent with previous reports (Grealish et al., 2014; Kirkeby et al., 2012; Kriks et al., 2011; Xi et al., 2012) (Figure 3.1F–H). Xeno-free differentiation significantly increased VMNPC fate acquisition with the proportion of OTX2⁺ cells increasing (from 87.8% to 96.8% for hESC H9, and 77.9%–88.9% for hiPSC RM 3.5), as well as FOXA2⁺ cells increasing (72.2%–89.0% for hESC H9 and 76.5%–85.6% for hiPSC RM 3.5; Figure 3.1I, J, L, Supplemental Figure 3.2H, I). Xeno-free differentiation significantly increased the proportion of OTX2⁺/FOXA2⁺ coexpressing cells, from 70.0% to 88.9% for hESC H9 and from 69.6% to 82.9% for hiPSC RM3.5 (Figure 3.1H, K, L; Supplemental Figure 3.2). Robust OTX2⁺/FOXA2⁺ homogeneity was observed across additional hPSC lines under xeno-free conditions (91.3% of HES3 hESC and 90.3% of 409B2 hiPSCs; Supplemental Figure 3.3A–E, 3.4A–E), indicating reproducibility across multiple ESC and iPSC lines. Developmentally, LMX1A delineates the floor plate along the medial-lateral axis of the neural tube with greater specificity than FOXA2 (Figure 3.1Cii, Dii) (Andersson et al., 2006). Rostrocaudally, LMX1A expression is also more restricted and indicative of the vmDA progenitor zone than FOXA2 (Figure 3.1Ci, Di). Altho-



-ugh LMX1A is also expressed in the dorsal neural tube, this region is FOXA2⁻ and can therefore be excluded using coexpression stains. Assessment of OTX2 coexpression within LMX1A⁺/FOXA2⁺ cells enables further narrowing of the developmental spatial niche along the rostral-caudal axis, with OTX2 excluding the ventral hindbrain portion of the floor plate (Figure

3.1B). To date, studies of hPSC vmDA differentiation have not evaluated expression levels of this highly restricted triple positive neural progenitor (Figure 3.1Ei, Eii). Therefore, to further characterize the specification of the VM progenitors using the xeno-free culture system, a novel LMX1A-GFP knockin reporter hESC line (H9 LMX1A-GFP) was generated to enable tracking of these progenitors in vitro (and subsequently in vivo). This new reporter line was generated and validated using the same methodological approach to our recently derived PITX3-GFP H9 reporter line, whereby a custom zinc finger nuclease pair in combination with a targeting vector delivered to exon 1 of the endogenous LMX1A locus an GFP-puromycin cassette (Figure 3.1A) (Watmuff et al., 2015). Using this novel reporter, live cell imaging and flow cytometric evaluation of xeno-free cultures revealed rapid increases in GFP with $53.0\% \pm 7.5\%$ of progenitors LMX1Ae-GFP⁺ at D11, increasing to $80.9\% \pm 4.3\%$ at D18 and $85.2\% \pm 4.3\%$ by D25 (Figure 3.1M–R, Supplemental Figure 3.7). Cultures stained with anti-GFP and anti-LMX1A/B antibodies showed consistent coexpression, demonstrating the high fidelity of GFP fluorescence in this reporter line (Supplemental Figure 3.7). Furthermore, LMX1A-GFP⁺ quantification at D18 revealed all GFP⁺ cells coexpressed both OTX2 and FOXA2, indicative of bona fide vmDA precursor identity (Figure 3.1S–V; Supplemental Figure 3.7). LMX1A-GFP xenogeneic differentiation outcomes mirrored the decreased efficiencies observed from xenogenic parental H9 and iPSC controls (data not shown).

Ongoing differentiation promoted the expression of the rate-limiting enzyme in dopamine synthesis, TH, within both xenogeneic and xeno-free cultures by D25 (Figure 3.2A–F). Greater uniformity in TH distribution was evident in xeno-free conditions (Figure 3.2B, E; Supplemental Figure 3.5D, I) and the proportion of floor-plate neurons (denoted by TH⁺/FOXA2⁺ coexpression (Bye et al., 2015; Kittappa et al., 2007) was also significantly increased (>60%) (Figure 3.2D–G; Supplemental Figure 3.5E, J). Robust TH and FOXA2 expression were similarly observed across additional hESC (HES3) and hiPSC (409B2) lines in xeno-free conditions (Supplemental Figure 3.3F–J, 3.4F–J). Furthermore, observation of stable FOXA2 expression between D11 and D25 indicates that superior fate specification under newly defined culture conditions is reflective of true gains rather than an accelerated differentiation trajectory (Figure 3.2H).

Definitive vmDA neuronal identity can only be assigned following further narrowing of cell identity with markers such as EN1, nuclear orphan receptor NURR1 or paired-like homeodomain transcription factor PITX3 (Smits et al., 2006). At D25 in vitro, we demonstrate

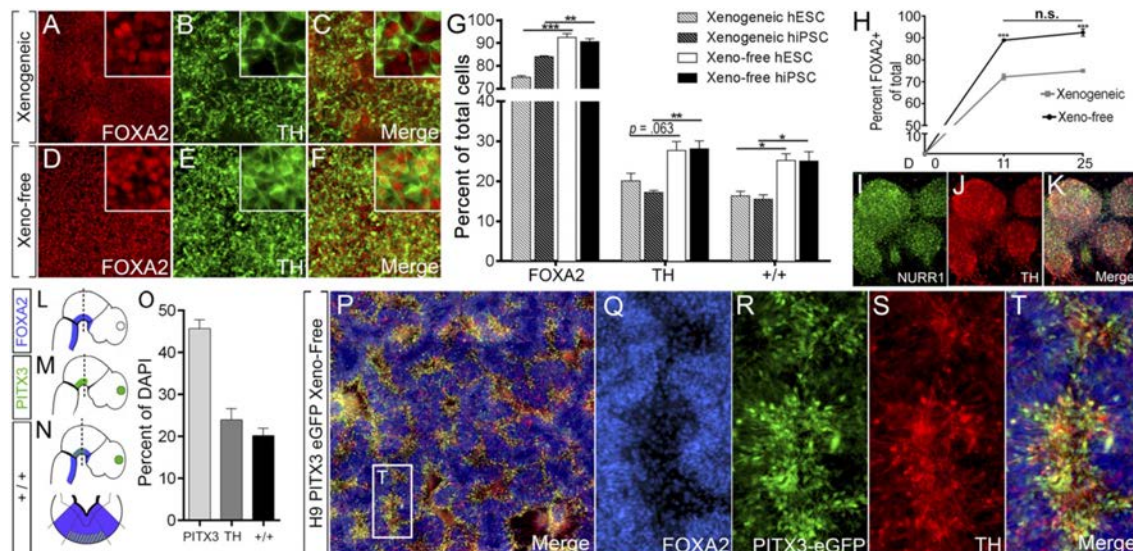


Figure 3.2 – Differentiation of hPSCs under xeno-free conditions increases generation of *bona fide* vmDA neurons at D25.

(A, D) Images of FOXA2, TH (B, E), and FOXA2/TH (C, F) coexpression in H9 cells differentiated under xenogenic (A–C) and xeno-free (D–F) conditions, showing increased FOXA2⁺/TH⁺ colabeled vmDA neurons under xeno-free conditions. (G) Quantification of subpopulations from ESC (H9) and iPSC (RM3.5). (H) Quantification of the temporal expression of FOXA2 reveals maintenance of early fate specification for xeno-free conditions, yet xenogenic cultures fail to increase FOXA2 progenitor yields over time. (I–K) Image of xeno-free differentiated culture expressing NURR1 (I), TH (J), and merge (K). (L–N) Sagittal schematic of the developing embryo illustrating FOXA2 (L), PITX3 (M), and combined FOXA2/PITX3 (N) expression, where vmDA neurons are born. (O–T): Xeno-free differentiation and quantification of the PITX3-GFP reporter line at D25 revealed cultures rich in bona fide vmDA neurons as revealed by FOXA2⁺/TH⁺/PITX3⁺ coexpression. $n = 3-5$ culture replicates, mean $\pm$ SEM. ** $p < .01$, *** $p < .001$. Immunofluorescence images are at x100 magnification. Abbreviations: D, day; DAPI, 4',6-diamidino-2-phenylindole; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; hPSC, human pluripotent stem cell; n.s., not significant; TH, tyrosine hydroxylase; vmDA, ventral midbrain dopamine.

widespread coexpression of NURR1 with TH (Figure 3.2I–K). More definitive, however, is the rostro-caudal and medial-lateral expression of PITX3, which is highly enriched in the VM (Figure 3.2M). Combined PITX3 expression, together with FOXA2 (Figure 3.2L) and TH, unambiguously denotes vmDA neurons (Figure 3.2N) and precludes misclassification with diencephalic or hindbrain TH⁺ populations. To this end we took advantage of our recently generated H9 PITX3-GFP knockin reporter line (Watmuff et al., 2015). Differentiation revealed widespread PITX3 expression throughout xeno-free cultures that robustly correlated with both TH and FOXA2 (Figure 3.2P–T). In all, 45.7% of total cells expressed PITX3 at D25, and strikingly 84.5% of TH⁺ cells were found to coexpress PITX3 (Figure 3.2O).

3.4.3 Identification of mechanism underlying enhanced vmDA specification in xeno-free cultures

In light of the improved differentiation outcomes under xeno-free conditions, we sought to investigate the potential underlining mechanisms. Although it is speculated that fully defined

media improves the homogeneity of cells before differentiation, we observed no differences in the pluripotency of hPSCs cultured in TeSR2 compared with MEF-based xenogeneic conditions, as assessed by OCT4 and SOX2 expression (Figure 3.3A–C, F–H), as well as surface markers CD9 and GCTM2 (Figure 3.3D, I, Supplemental Figure 3.66I, J) - markers that have been shown to discriminate gradients along the pluripotency spectrum (Ho et al., 2011). Instead, using the stromal cell marker CD90.2, a variable fraction of residual feeders was seen to remain within xenogeneic hPSC suspensions at the initiation of differentiation (1.6–11.9% of the total live fraction, Figure 3.3E; mean $5.40\% \pm 3.28\%$) despite undergoing a widely utilized feeder depletion process (Chambers et al., 2009; Delli Carri et al., 2013; Kriks et al., 2011). No feeders were identified in xeno-free hPSC preparations (Figure 3.3J). To determine whether these contaminating cells underlie perturbed differentiation efficiencies xeno-free cultures were “spiked” with 5% of feeders at the initiation of differentiation (Figure 3.3K). CD44⁺ MEF cells could be identified within the culture at D0 (Figure 3.3L–O), however, most inactivated MEFs were lost within 72 hours of differentiation, potentially being overgrown by expanding human NPCs (data not shown). Analysis at D11 revealed a 33.7% decrease in the proportion of mid-forebrain floor plate OTX2⁺/FOXA2⁺ cells following MEF spiking (Figure 3.3P–T), suggesting that the presence of contaminating MEFs elicits a destabilizing effect on early fate specification.

3.4.4 Xeno-free vmDA differentiation is amenable to scalability and cryopreservation of progenitors

Necessary for the clinical applicability of defined differentiation protocols is the requirement for scalability. In this study, we demonstrate the ability to significantly scale-up vmDA differentiation by 450-fold (initiating differentiation in T25-T150 flasks by comparison with previous results produced in 96-well plates). The increased scale had no obvious effect on the survival, morphology or expression of LMX1A-GFP in culture (Figure 3.4A, B), compared with previously observed results (Figures 3.1, 3.2). At D22, the total cell yield averaged $110.4 \pm 9.0 \times 10^6$ per T150 flask (Figure 3.4A), with $80.7\% \pm 1.6\%$ of live cells expressing GFP (Figure 3.4E, F). vmDA NPCs were screened for the presence of pluripotent cells to assess teratoma formation risk following large-scale expansion. No GCTM2⁺/CD9⁺ coexpressing cells were identified (Figure 3.4C–Dii). In addition to the necessity of scalability for clinical translation, we examined the feasibility of cryopreserving vmDA precursors at a stage in the differentiation amenable to cell transplantation. Such approaches could enable scaled standardized differentiation and storage for “on-demand” needs as reported in other hPSC fields. Scaled vmDA NPCs were cryopreserved at day 22 in a xeno-free cryopreservation solution using a controlled rate freezer. Post-thaw vm-

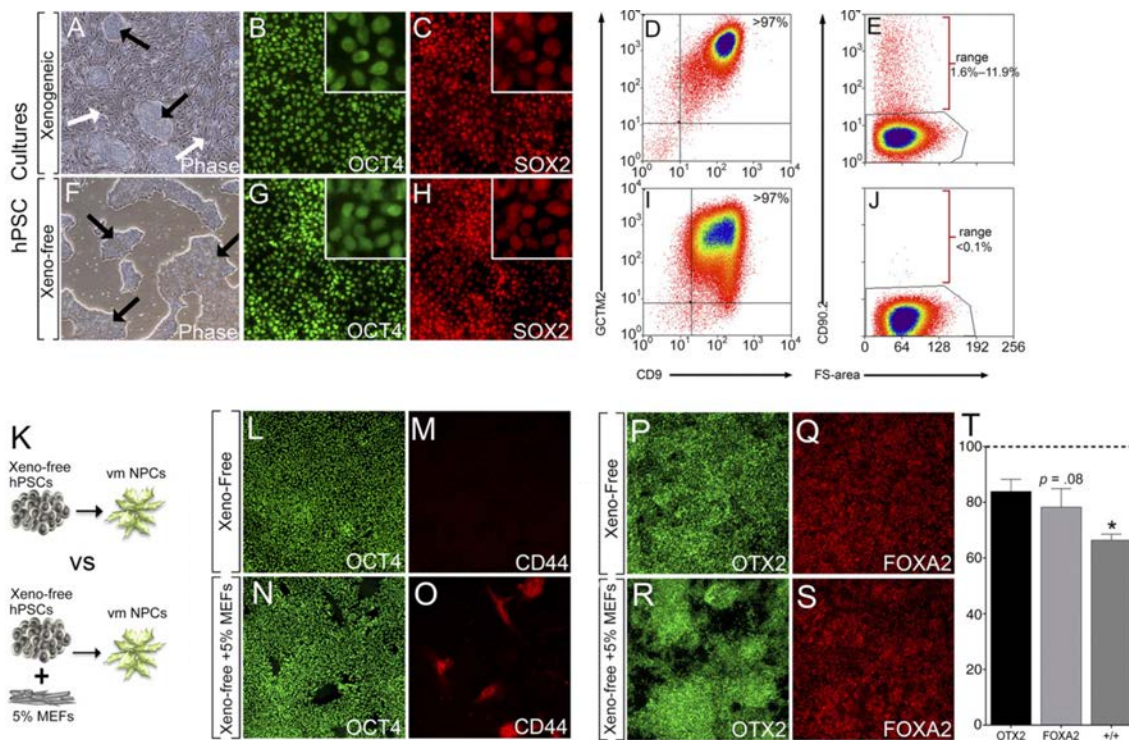


Figure 3.3 – Residual fibroblasts, at the initiation of vmDA differentiation, influence fate specification.

(A, F) Phase contrast image of H9 ESCs cultured in xenogeneic conditions on MEFs (A) or xeno-free conditions on laminin-521 (F). Black arrows: hPSC colonies, white arrows: MEFs. Xenogeneic and xeno-free hPSCs coexpressed pluripotency markers OCT4 (B, G) and SOX2 (C, H). Analysis of pluripotent surface antigens CD9 and GCTM2 by FACS revealed that xenogeneic cultured hPSCs (D) were indistinguishable from xeno-free cultures (I). (E, J) Despite depletion, xenogeneic conditions retained a small fraction of CD90.2⁺ MEFs (E), which were absent in xeno-free conditions (J). (K) Xeno-free vmDA cultures were spiked at initiation with MEFs to elucidate potential effects on differentiation to D11 ventral midbrain NPCs. (L, M) Xeno-free cultures at D0 widely expressed OCT4 and were devoid of CD44⁺ fibroblasts. (N, O): The presence of fibroblasts demonstrated in xeno-free D0 spike cultures confirmed by CD44 reactivity. Confirmation of vmDA precursor specification under xeno-free conditions at D11 by immunolabeling for OTX2 (P) and FOXA2 (Q). Cultures spiked with MEFs demonstrated reduced numbers of OTX2-expressing (R) and FOXA2-expressing (S) cells. (T): Percentage decrease in the proportion of cells in D11 vmDA cultures expressing OTX2, FOXA2, and OTX2/FOXA2 following spiking at D0 with MEFs, compared with standard xeno-free cultures. Note there is a significant reduction in specification of OTX2⁺/FOXA2⁺ DA precursors following MEF spiking of the cultures. $n = 3$ culture replicates, data represents mean $\pm$ SEM. ** $p < .01$, *** $p < .001$. Phase contrast images at $\times 40$, immunofluorescence at $\times 100$ magnification. Abbreviations: DA, dopamine; FACS, fluorescence-activated cell sorting; hPSC, human pluripotent stem cell; NPC, neural progenitor cell; MEF, mouse embryonic fibroblast; vmDA, ventral midbrain dopaminergic.

-DA NPCs, frozen in liquid N₂ for up to 14 days, demonstrated reduced viability (>71%; Figure 3.4H) but retained LMX1A-GFP expression ($76.9 \pm 2.6\%$ post-thaw vs. 80.7% pre-freeze) (Figure 3.4E, G). Furthermore, culture of resurrected vmDA progenitors to day 40 produced correctly patterned vmDA neurons coexpressing LMX1A-GFP, FOXA2 and TH (Figure 3.4I-M). While promising, long term freeze-thaw experiments will be necessary to validate viability over extended time periods (>1 year).

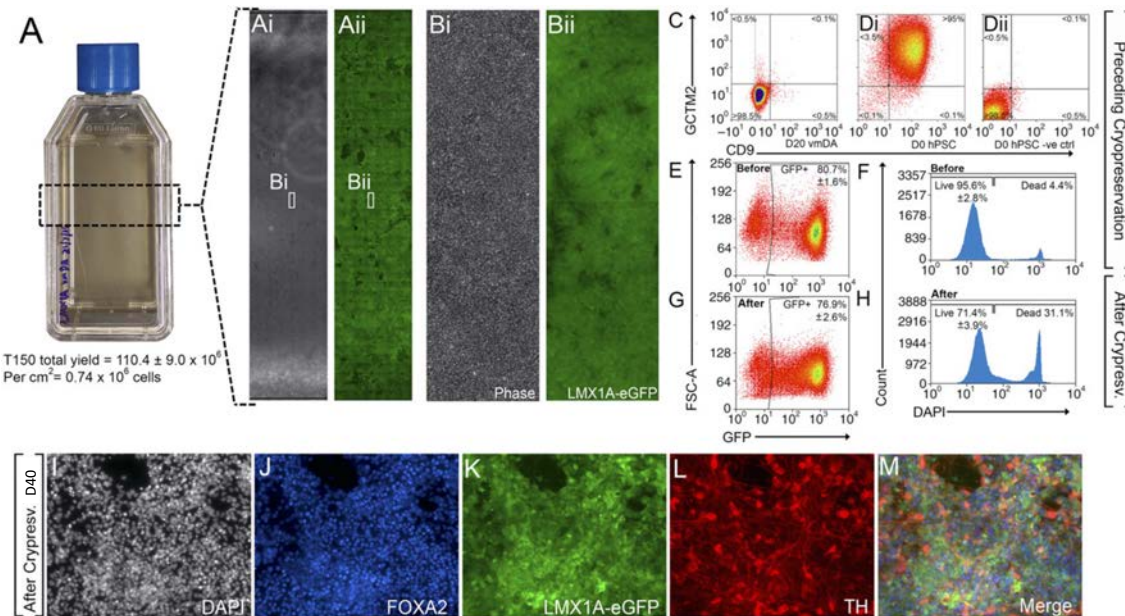
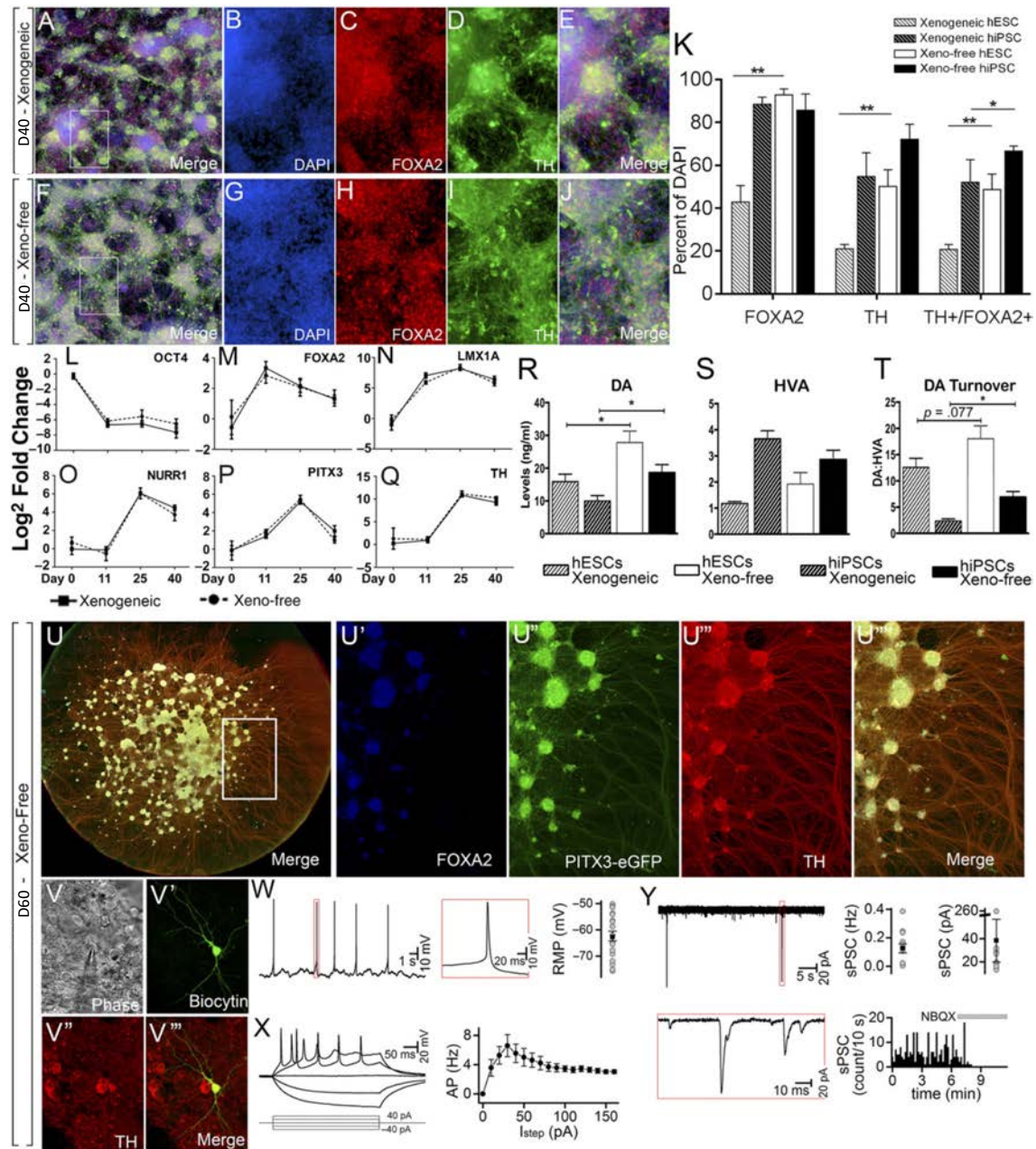


Figure 3.4 – Scaled differentiation and cryopreservation of xeno-free vmDA precursors.

(A, B) Xeno-free differentiation in flasks (T25 pictured) yields high cell quantities (data given for a T150 flask) enriched for LMX1A-GFP at D22. (C, D) Scaled vmDA cultures (C) did not include pluripotent GCTM2/CD9 cells compared with pluripotent controls (Di) and unstained controls (Dii). (E, F) Culture flask expansion had no impact on fate specification as revealed by LMX1A-GFP expression (E) or cell survival (F). (G, H) Cryopreservation and thawing also had no impact on LMX1A-GFP expression (G) and minimal impact on survival (H). (I, J) Cryopreservation and thawing also had no impact on LMX1A-GFP expression (G) and minimal impact on survival (H). After thawing, LMX1A-GFP vmDA progenitors maintained their capacity to differentiate into DA neurons, as revealed by DAPI (I), FOXA2 (J), LMX1A (K), TH (L), and merged (M) expression at D40. $n = 3$ culture replicates, mean $\pm$ SEM. Phase contrast images are at x40, immunofluorescence at x200 magnification. Abbreviations: D, day; DA, dopamine; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; hPSC, human pluripotent stem cell; TH, tyrosine hydroxylase; vmDA, ventral midbrain dopamine.

3.4.5 Xeno-free cultures yield increased proportions of functionally mature DA neurons

Extended in vitro differentiation of vmDA progenitors (until D40) resulted in a significant increase in the proportions of TH⁺ neurons (hESC H9 = 50.1%, HES3 = 55.7%; hiPSC RM3.5 = 76.2%, of total cells) and cells coexpressing FOXA2⁺/TH⁺ (hESC H9 = 48.6%, HES3 = 52.9%; hiPSC RM3.5 = 67.9%, of total cells; Figure 3.5A–K). Complementary to immunocytochemistry findings, temporal gene expression showed profiles indicative of vmDA fate specification for both xenogeneic and xeno-free differentiation cultures, with the early downregulation of the pluripotency gene OCT4, progressive upregulation of vmDA progenitor/neuronal genes FOXA2, LMX1A, and NURR1, and subsequent elevation of mature markers PITX3 and TH (Figure 3.5L–Q). Examination of single gene expression profiles did not reveal the striking differences in fate specification observed between xenogeneic and xeno-free conditions, suggesting that the global measurements of mRNA levels in culture have limited capacity to distinguish differences



in the numbers of individual cells coexpressing the multiple markers required to detect bona fide VM precursor and mature populations.

Critical for the function of DA neurons is their ability to synthesize and utilize dopamine. To this end, we assessed dopamine metabolism in KCl-depolarized neurons using high-performance liquid chromatography (HPLC). Reflective of increases in DA neurons observed at D40, dopamine levels were significantly elevated in both H9 hESC cultures differentiated under xeno-free conditions compared with xenogeneic cultures (27.8 ± 3.5 and 15.9 ± 2.2 pmol/ml, respectively) as well as hiPSC RM3.5 cultures (18.6 ± 2.3 and 9.9 ± 1.7 pmol/ml, Figure 3.5R). Although levels of the dopamine metabolite HVA remained unchanged (Figure 3.5S), dopamine turnover (the ratio of dopamine to HVA, and an indicator of increased DA metabolism) was notably higher for xeno-free differentiated hESC and significantly elevated for xeno-free differentiated iPSC compared with xenogeneic conditions (6.9 ± 1.0 vs. 2.4 ± 0.4 ; Figure 3.5T).

To further validate whether the hPSC-derived vmDA cells generated under xeno-free conditions were functional, we allowed them to mature to 55–65 days and performed whole cell patch clamping. At this stage of maturation, the cells showed morphological features of mature DA neurons - with the majority expressing FOXA2, PITX3-GFP, and TH, in conjunction with extensive axonal growth (Figure 3.5U), which was complemented by electrophysiological features indicative of vmDA neurons. Recorded cells ($n = 21$) exhibited a resting membrane potential of -62mV, with 38% firing spontaneous action potentials of prolonged duration (Figure 3.5W). All neurons generated action potentials upon depolarizing current steps and exhibited inward rectification in response to hyperpolarization steps (Figure 3.5X). These findings are in agreement with previously published data from TH⁺ vmDA neurons (Doi et al., 2014; Parish et al., 2008). Several recorded cells exhibited sEPSCs (0.13 ± 0.03 Hz, 38 ± 18 pA) with fast kinetics that were blocked by administration of the AMPA antagonist NBQX (20 μ M), whereas no cells exhibited outward inhibitory postsynaptic currents (Figure 3.5Y). These findings indicate cells developed excitatory glutamatergic synapses, as previously described for DA neurons. Recorded neurons were confirmed as exhibiting a DA identity by colocalization of TH with biocytin (Figure 3.5V). Combined, these findings suggest that xeno-free cultured cells were capable of differentiating into functional DA neurons possessing morphological, gene, and protein expression and electrophysiological properties of endogenous midbrain DA neurons.

3.4.6 Transplantation of xeno-free DA progenitors results in grafts with improved phenotype composition and restoration of motor deficits in parkinsonian rodents

Next, we examined the capacity of the expanded vmDA progenitors, differentiated under xenogeneic or xeno-free conditions, to survive intrastriatal transplantation in 6-OHDA lesioned mice. The use of the H9 LMX1A-GFP H9 hESC reporter line enabled tracking of the grafted progenitors and assessment of their relative contribution to the graft. In all animals, healthy, viable grafts, confined to the striatum and showing no signs of tumour formation or neural overgrowth were observed. Examination of hPSA-NCAM staining, to identify human specific tissue in situ, highlighted no significant difference in graft size between xenogeneic and xeno-free derived cell grafts, although considerably greater variation in graft size was observed in xenogeneic grafts (Figure 3.6A, G, M: xenogeneic: $0.74 \pm 0.24 \text{ mm}^3$; xeno-free: $0.38 \pm 0.06 \text{ mm}^3$), suggesting that cell survival was similar between both conditions. Examination of GFP staining however revealed that grafts derived from xeno-free differentiation cultures contained notably more LMX1A⁺ progenitors than grafts of xenogeneic-derived cells (Figure 3.6A, G). These findings suggest that improved specification observed under xeno-free conditions in vitro was maintained and influenced the fate of grafted cells in vivo. Further phenotype characterization of grafts at 1 month after implantation revealed high expression of OTX2 (xenogeneic: $75 \pm 4.6\%$; xeno-free: $85 \pm 4.3\%$) and FOXA2 ($41.3 \pm 7.3\%$ vs. $55.0 \pm 5.7\%$), with a nonsignificant trend toward improved composition in xeno-free-derived grafts (Figure 3.6C, D, I, J, N). Significant phenotypic differences were only revealed through the use of the LMX1A-GFP reporter, with a 2.5-fold higher number of LMX1A-GFP⁺ cells in xeno-free compared with xenogeneic derived grafts ($21.7 \pm 9.4\%$ vs. $55.8 \pm 6.6\%$; Figure 3.6E, K, N).

To assess the long-term integration of xeno-free vmDA progenitors we transplanted H9 PITX3-GFP cells into 6-OHDA lesioned athymic rats. Similar to H9 LMX1A-GFP grafts, at 1 month after transplantation, ectopic H9 PITX3-GFP striatal grafts maintained fate restriction with the majority of grafted cells coexpressing FOXA2⁺/TH⁺/PITX3-GFP⁺ (Figure 3.6P–S). At this time grafts showed limited reinnervation of the host tissue (Figure 3.6O). Assessment of motor function revealed a stable motor deficit in 6-OHDA lesioned rats in response to amphetamine induced rotational testing, which was significantly corrected in PITX3-GFP VM progenitor grafted animals by 4 months (Figure 3.6 T). Post-mortem assessment of the grafts at 6 months revealed a dense network of GFP cells within the graft core (Figure 3.6U') and extensive reinnervation of the striatum (Figure 3.6U), most notably in the dorsolateral striatum (Figure 3.6U''). Additional inne-

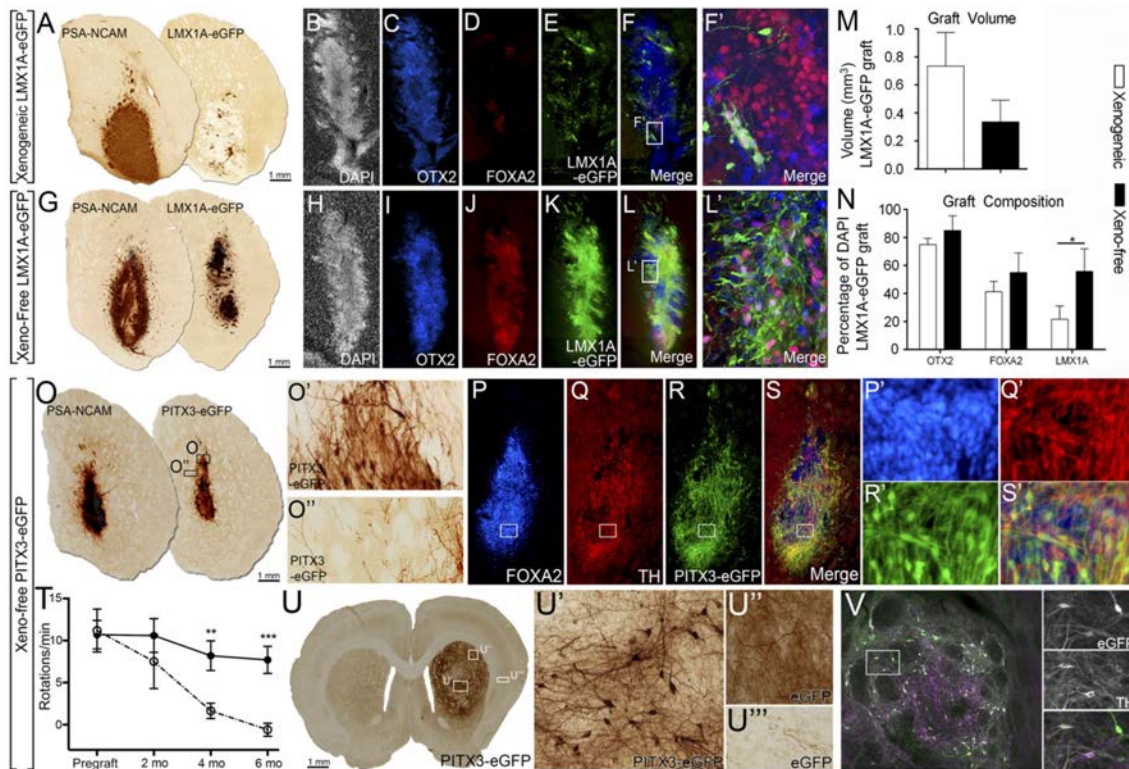


Figure 3.6 – Transplantation of xeno-free vmDA progenitors improved the yield of correctly specified cells within grafts of parkinsonian rodents.

(A, G) Chromogenic PSA-NCAM and GFP staining of transplanted LMX1A-GFP progenitors differentiated for 25 days under xenogenic (A) or xeno-free (G) conditions and analysed at 1 month after implantation. Note similarity in graft size, shown by PSA-NCAM and enrichment of LMX1A-GFP cells under xeno-free conditions. DAPI (B, H), OTX2 (C, I), FOXA2 (D, J), LMX1A-GFP (E, K), and merged images (F, L) show that xeno-free derived grafts were enriched for DA progenitors, as shown by increased OTX2⁺/FOXA2⁺/LMX1A-GFP⁺ coexpression seen in the high- power images: xeno-free (L') compared with xenogenic (F'). (M) Volumetric analysis revealed no difference in graft size between xenogenic or xeno-free conditions. (N) Quantification of progenitors within the graft. (O) Chromogenic PSA-NCAM and GFP staining of transplanted PITX3-GFP progenitors, differentiated for 25 days under xeno-free conditions and analysed at 1 month after implantation. Grafts show high levels of PITX3-GFP staining (O, O') and modest GFP innervation of the host striatum (O''). Immunohistochemistry for FOXA2 (P), TH (Q), PITX3-GFP (R), and merged (S) confirmed that a significant proportion of cells possessed a vmDA phenotype. (P'–S') Higher magnification images of (P–S). Animals grafted with PITX3-GFP progenitors (open circles) showed a significant improvement in amphetamine induced rotational asymmetry compared with 6-OHDA lesioned controls (closed circles). (U) Representative PITX3-GFP graft showing integration into the host striatum at 6 months. (U', U'') Grafts contained a dense network of cells and reinnervation of the dorsolateral striatum (U') as well as other vmDA targets, including the overlying cortex. LMX1A-GFP cell grafts: n = 6 animals per group (xenogenic and xeno-free). PITX3-GFP grafts: n = 3 at 1 month, and n = 6 at 6 months. n = 6 lesion controls. Mean ± SEM. ** p < .01, *** p < .001. Abbreviations: DA, dopamine; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; TH, tyrosine hydroxylase; vmDA, ventral midbrain dopamine.

-rvation was also seen within the overlying cortex (Figure 3.6U'''). Gradual reinnervation of the target striatum is a crucial hallmark of correctly specified vmDA neurons and has been shown to be crucial to behavioural recovery (Barker et al., 2013; Kauhausen et al., 2013). Cells at 6 months maintained a vmDA regional identity with all GFP⁺ graft derived cells coexpressing TH (Figure 3.6V).

3.5 Discussion

In this study, we report a fully defined xeno-free protocol for the generation of vmDA progenitors and mature vmDA neurons from hPSCs. Although the inconsistent nature of feeder coculture is recognized (Villa-Diaz et al., 2009), studies have not previously been performed to directly assess their effects on neural differentiation outcomes. Our findings suggest the carry-forward of a small proportion of MEF cells underlies some of the variability observed during differentiation in a similar manner to MEFs. It would be prudent for future studies to assess whether HEF cells would also reduce differentiation efficiency. Through the removal of feeders and xenogeneic reagents we found a marked improvement in homogeneity and reproducibility of differentiation outcomes across multiple human ESC and iPSC lines. We report a significant increase in the yield of appropriately specified OTX2⁺/FOXA2⁺ progenitors and FOXA2⁺/TH⁺ DA neurons, compared with previous reports (Denham et al., 2012; Kirkeby et al., 2012; Kriks et al., 2011; Sundberg et al., 2013; Xi et al., 2012). Further, we demonstrate elevated DA metabolism and show functional electrophysiological properties reflective of mature vmDA neurons. The use of LMX1A and PITX3 reporter lines enabled further validation of correct vmDA progenitor (LMX1A⁺/FOXA2⁺/ OTX2⁺) and neuronal identity (PITX3⁺/FOXA2⁺/TH⁺) of the resultant differentiated cells, at a level of detail not previously described. Of relevance, widespread use of broad RNA quantification techniques has been depended on to attribute subtype identities to differentiated neural cultures. In this study, we show considerable discrepancy between RNA and protein-based quantification assays, as reported in other systems (Greenbaum et al., 2003; Vogel and Marcotte, 2012). Specifically, qPCR analysis of key identifier genes failed to separate xenogeneic and xeno-free cultures, despite robust separation using immunocytochemistry based counting strategies. As mRNA levels reflect global gene expression, individual cell expression variation may confound RNA assessment methods. Furthermore, qPCR cannot discern colocalization of the multiple markers required to detect bona fide vmDA precursors and mature neurons. These findings highlight the necessity for stringent cytochemical analysis.

Recent studies have highlighted the functional integration of vmDA progenitors but have predominantly relied on human specific antibodies such as human nuclear antigen, PSA-NCAM or constitutively active fluorescent proteins to assess size and integration (Grealish et al., 2014; Kirkeby et al., 2012; Steinbeck et al., 2015; Sundberg et al., 2013), with limited assessments of graft composition. We assessed graft composition and integration using novel LMX1A-GFP and PITX3-GFP reporter lines enabling a specific assessment of the DA contribution to the graft. Both LMX1A-GFP and PITX3-GFP reporter lines differentiated and transplanted with

similar efficiencies as the wildtype H9 parental line. We demonstrated that xeno-free derived grafts contain significantly more correctly specified vmDA progenitors (FOXA2⁺/OTX2⁺/LMX1A-GFP⁺) than xenogeneic- derived cell grafts of a similar size, as well as correctly specified mature DA neurons (FOXA2⁺/PITX3-GFP⁺/TH⁺). Long-term assessment of the grafts showed maintained specification and a dense network of PITX3-GFP vmDA fibers throughout the denervated striatum, thereby providing the first report specifically of the dopaminergic contribution to reinnervate the host, above that previously reported ([Grealish et al., 2014](#); [Kirkeby et al., 2012](#); [Steinbeck et al., 2015](#); [Sundberg et al., 2013](#)). This dense network of vmDA neurons and fibers were derived from a clinically translatable protocol and were capable of correcting motor asymmetry in 6-OHDA lesioned rats. The enhanced homogeneity and progenitor yield achieved within the present study, both in vitro and in vivo are of critical importance in the context of cell-based therapy where undifferentiated cells or incorrectly specified neuronal populations can result in deleterious clinical outcomes.

Finally, we report the capacity for scalability using our xeno-free, defined differentiation protocol, as well as the ability to cryopreserve vmDA progenitors at a stage amenable to transplantation. Scalability from 96-well plates to flasks facilitates utility for a variety of purposes from in vitro disease modelling and drug screening to clinical transplantation trials. Specifically, expansion in T150 flasks resulted in roughly 110 million cells/flask, of which >80% are correctly specified (LMX1A⁺/FOXA2⁺/OTX2⁺) at the transplantation window. The significance of these findings for clinical purposes can be fully realized in the context of preceding clinical trials using fetal tissue where as few as 100,000 surviving DA neurons were sufficient for significant reinnervation of the caudate putamen, F-DOPA uptake and functional improvements ([Brundin et al., 2000](#); [Kordower et al., 1997](#); [Kordower et al., 1996](#)). Furthermore, these cells are amenable to cryopreservation, with no significant impact on viability, progenitor composition or terminal differentiation potential, thereby presenting an “on demand” cell source for clinical application.

Our findings address a number of critical stepping-stones in the advancement of hPSCs for clinical applications. This readily scalable, cryopreservable, and defined xeno-free protocol, primed for clinical translation, provides a highly efficient and standardized system that has been acutely interrogated with novel LMX1A- and PITX3-GFP reporter lines. These findings are highly relevant to a wide variety of downstream applications, from in vitro disease modelling and drug

screening, to the rapidly approaching aspiration of cell replacement therapy for patients with Parkinson's disease.

3.6 Acknowledgements

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3.7 Author contributions

All experiments were performed at the Florey Institute of Neuroscience and Mental Health. Conceptualisation: CG, JN, LT and CP. Investigation: CG, JN, WA, SM and CB. Analysis: CG, JN and CP. Provision of resources: AE, ES, JH, CP, LT and CP. Writing – original draft: CG and CP. Writing – review and editing: CG, JN, CP, LT and CP. Funding: CP.

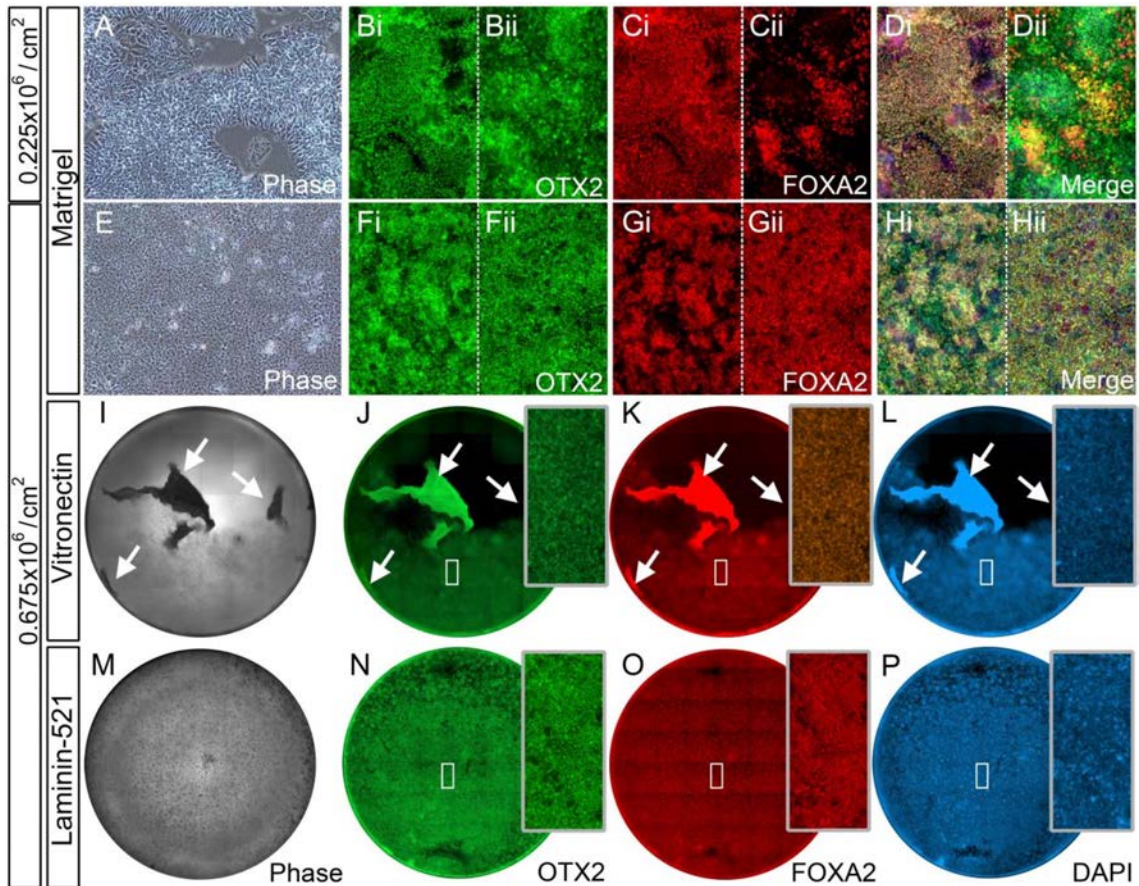
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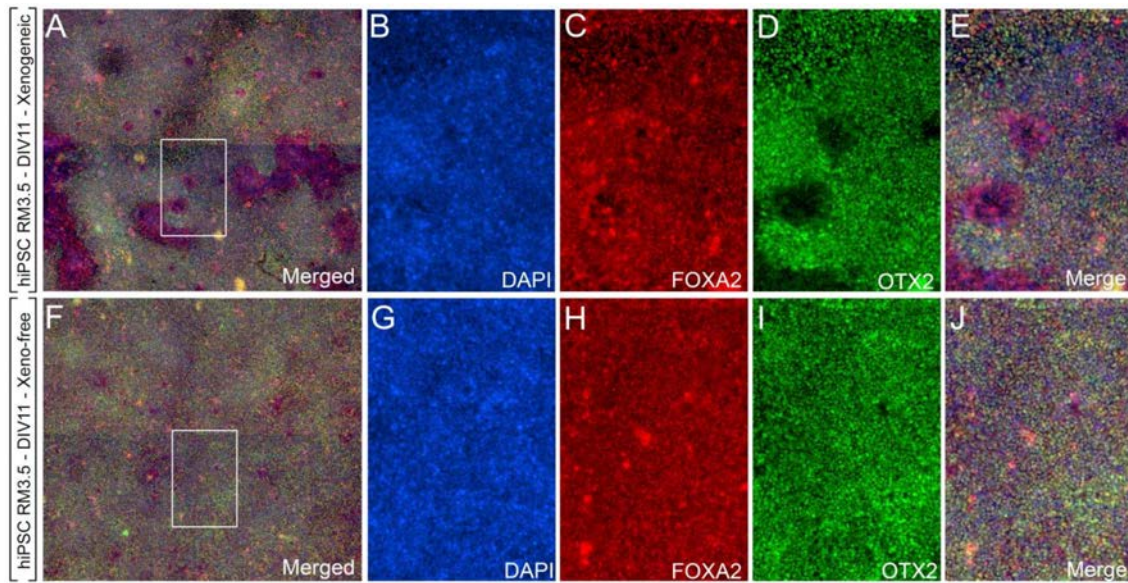
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3.9 Supplementary Figures



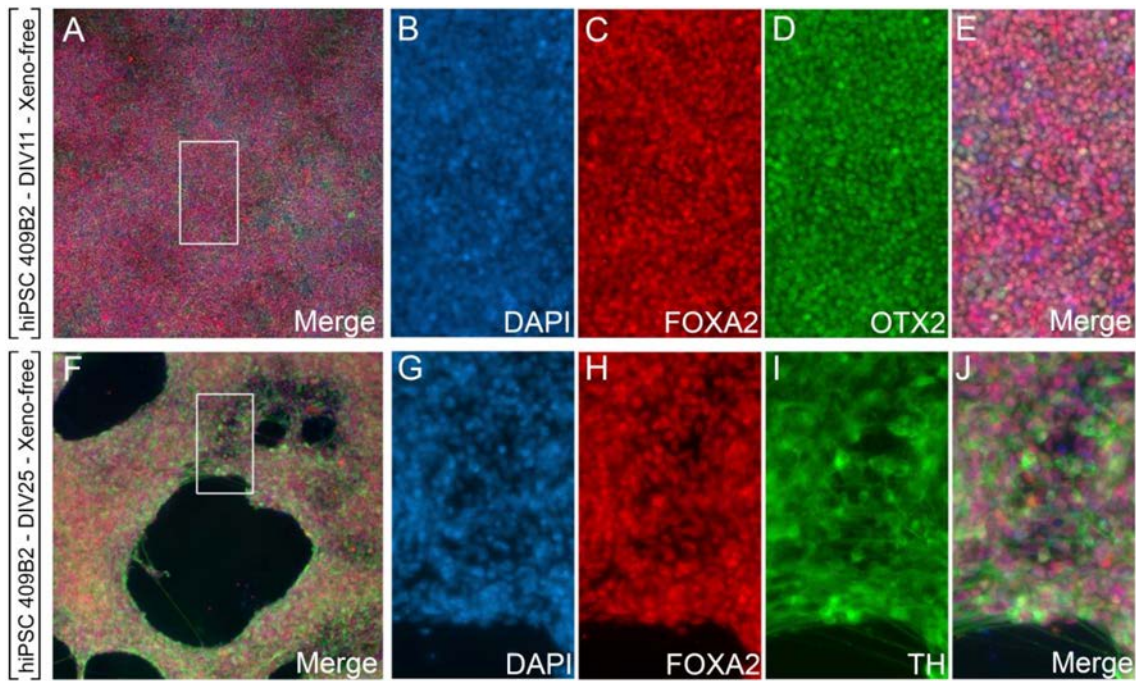
Supplementary Figure 3.1 – Optimisation of vmDA differentiation induction.

(A) Phase contrast of undifferentiated xenogeneic hPSCs prior to initiation of differentiation (day 0), with cells seeded at a standard literature density on MEFs ($0.225 \times 10^6/\text{cm}^2$). Following 11 days of differentiation variable FOXA2 and OTX2 expression patterns were observed across independent cultures; (Bi-Di) robust patterning; (Bii- Dii) variable patterning. Increased xenogeneic hPSC seed densities on Matrigel ($0.675 \times 10^6/\text{cm}^2$, E) improved differentiation reproducibility with robust expression of FOXA2 and OTX2 (Fi-Hi). Widespread OTX2 and FOXA2 expression was also observed from xeno-free hPSCs on Matrigel differentiated in fully defined and xeno-free media (Fii-Hii). Xeno-free hPSCs specification on xeno-free ECMs vitronectin (I-L) and recombinant human Laminin-521 (M-P), both of which exhibited robust OTX2 and FOXA2 expression. Vitronectin proved unreliable for long-term adherence of differentiated cultures (I-L, arrows indicating regions of cultures that have detached) in comparison to rLaminin-521. Immunofluorescence tiled 100x, phase contrast 20x magnification.



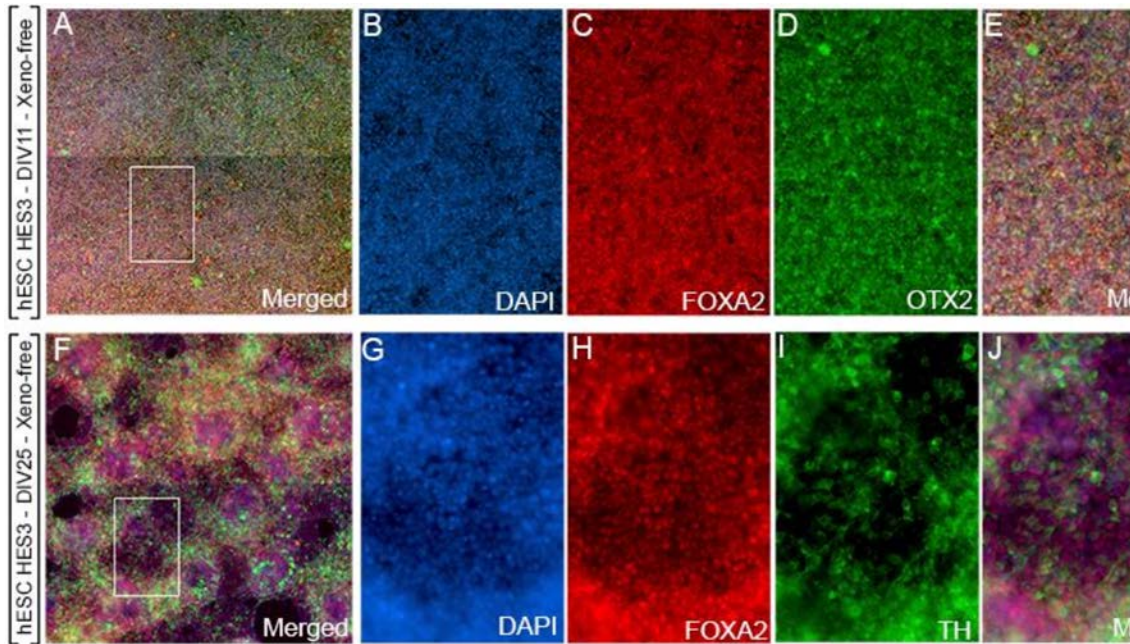
Supplementary Figure 3.2 – Specification of vmDA precursors from hiPSC line RM3.5 under xenogeneic and xeno-free conditions.

Differentiation of hiPSC line, RM3.5, at 11 days in vitro under (A-E) xenogeneic and, (F-J) xeno-free conditions, illustrating DAPI, FOXA2, OTX2 and merged expression. Immunofluorescence tiled 100x magnification



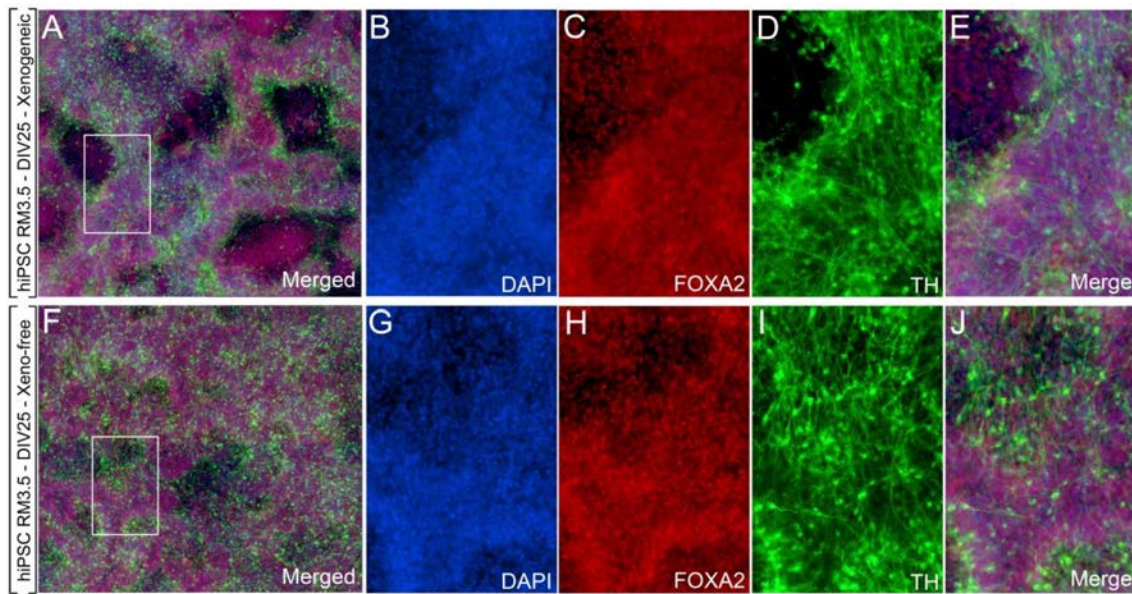
Supplementary Figure 3.3 – Differentiation of hiPSC line 409B2 under xeno-free conditions.

Differentiation of hiPSC line 409B2 under xeno-free conditions resulted in high yields of vmDA precursors at day 11 in culture, as revealed by co-expression of DAPI, FOXA2, OTX2. High power image taken from panel (A) showing (B) DAPI, (C) FOXA2, (D) OTX2 and (E) merged. (F) Maintained differentiation (day 25 in vitro) resulted in specification of vmDA neurons, as confirmed by DAPI, FOXA2 and TH merged expression. High power image taken from panel (F) showing (G) DAPI, (H) FOXA2, (I) TH and (J) merged. Immunofluorescence tiled 100x magnification



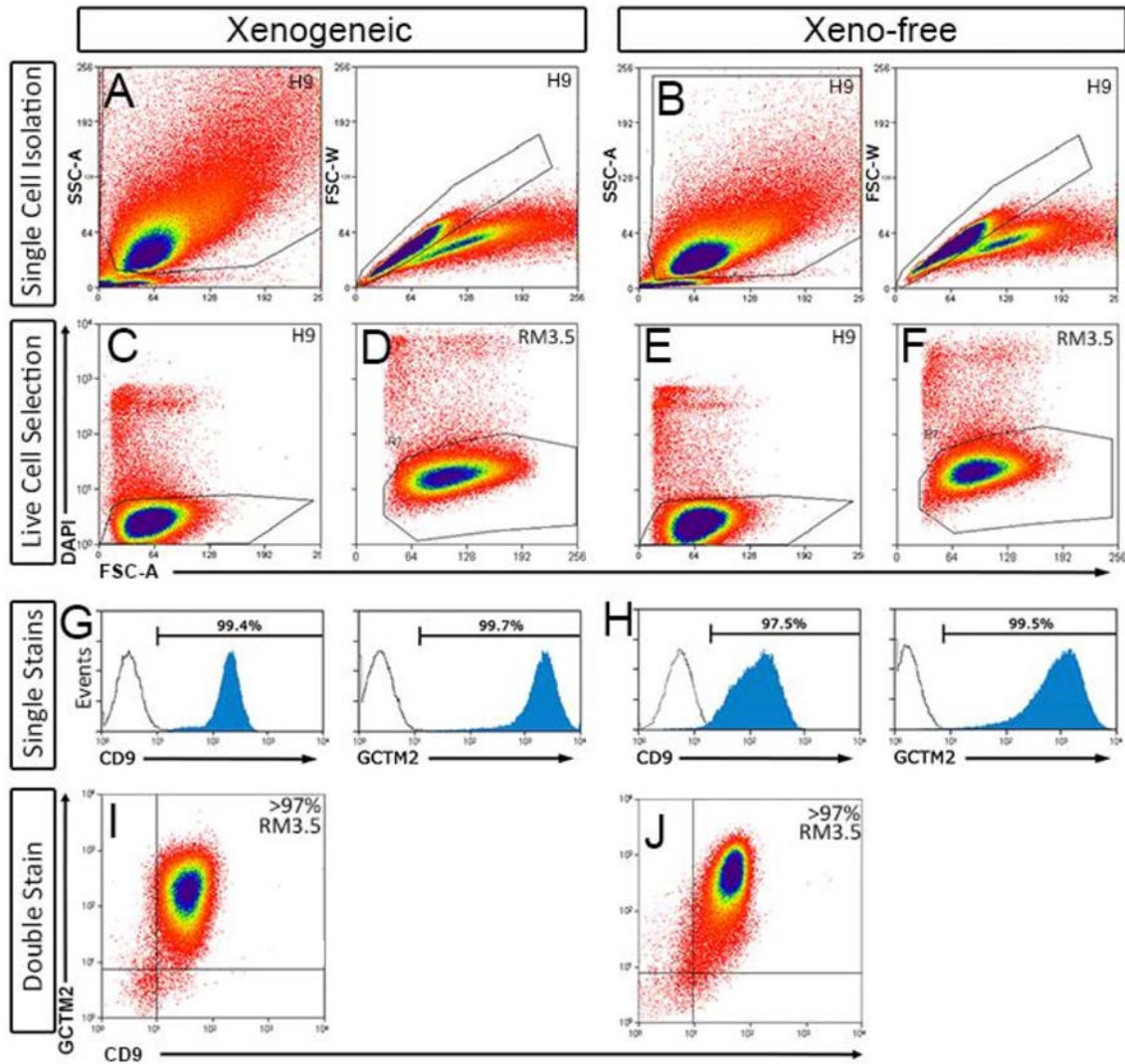
Supplementary Figure 3.4 – Differentiation of hESC line HES3 under xeno-free conditions shows similar properties to the H9 hESC line.

(A) Differentiation of hESC line HES3 under xeno-free conditions resulted in high yields of vmDA precursors at day 11 in vitro, as revealed by co-expression of DAPI, FOXA2 and OTX2. High power image taken from panel (A) showing (B) DAPI, (C) FOXA2, (D) OTX2 and (E) merged. (F) Maintained differentiation (day 25 in vitro) resulted in specification of vmDA neurons, as confirmed by DAPI, FOXA2 and TH merged expression. High power image taken from panel (F) showing (G) DAPI, (H) FOXA2, (I) TH and (J) merged. Immunofluorescence tiled 100x magnification



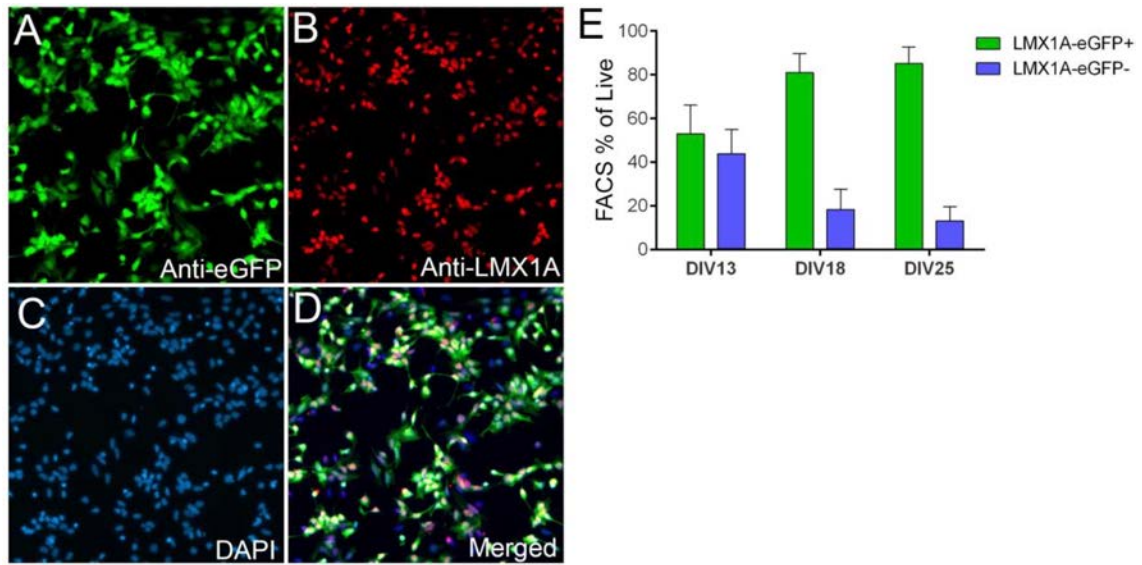
Supplementary Figure 3.5 – Differentiation of RM3.5 hiPSC line for 25 days *in vitro* under xenogeneic or xeno-free conditions.

(A,F) Representative merged images illustrating DAPI, FOXA2 and TH expression. High power image taken from panel (A – xenogeneic and F – xeno-free cultures) showing (B,G) DAPI, (C,H) FOXA2, (D,I) TH and (E,J) merge. Immunofluorescence tiled 100x magnification



Supplementary Figure 3.6 – Flow cytometry gating of pluripotent cells.

Gating strategy for undifferentiated xenogeneic (A,C,D) and xeno-free (B,E,F) hPSC cultures. Single stain controls delineate positive and negative populations (G-H). Representative images of double stained hPSC populations in both xenogeneic (I) and xeno-free conditions (J).



Supplementary Figure 3.7 – LMX1A-GFP reporter line fidelity.

(A) Representative images of vmDA NPCs stained for GFP expression with an anti-GFP antibodies, (B) with anti-LMX1A/B antibodies (C) DAPI and (D) merged showing co-localisation between LMX1A/B expressing cells and GFP expression. (E) Quantification of LMX1A-GFP positive and negative live cell expression levels across a time-course of xeno-free differentiation, n=3 experimental replicates, mean $\pm$ SEM. Immunofluorescence: 200x magnification.

Supplementary table 3.1 – Xeno-free reagents used in study.

Xenogeneic	Catalogue	Xeno-free	Catalogue
Mouse irradiated embryonic fibroblasts (MEF)	Gift from MCRI	TeSR2	StemCell Tech #05860
Mouse laminin	Gibco #23017015	Laminin-521	Biolamina #LN521
KSR	Gibco #10828028	Xeno-free KSR	Gibco #12618013
B27 supplement	Gibco #17504044	Xeno-free B27 supplement	Gibco #A1486701

All other factors were shared between xenogeneic and xeno-free culture conditions. MEFs causally reduce DA neuron differentiation efficiency.

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Chapter 4.

VIRAL DELIVERY OF GDNF IMPROVES THE FUNCTIONAL INTEGRATION OF HUMAN PLURIPOTENT STEM CELL-DERIVED DOPAMINE GRAFTS IN RODENT MODELS OF PARKINSON'S DISEASE

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4.1 Abstract

The derivation of neurotransmitter and region-specific neuronal populations from human pluripotent stem cells (PSC) provides impetus for advancing cell therapies into the clinic. At the forefront is our ability to generate midbrain dopaminergic progenitors, suitable for transplantation in Parkinson's disease (PD). Pre-clinical studies, however, have highlighted the relatively low proportion of DA neurons within these grafts and their inferior plasticity by comparison to human fetal donor transplants. Here we sought to examine whether modification of the host environment, through viral delivery of a developmentally critical molecule, glial cell-line derived neurotrophic factor (GDNF), could improve graft survival, integration and function in Parkinsonian rodents. Utilising LMX1A- and PITX3-GFP hPSC reporter lines, we tracked the response of DA progenitors implanted into either a GDNF-rich environment, or in a second group, after a 3-week delay in onset of exposure. We found that early exposure of the graft to GDNF promoted survival of DA and non-DA cells, leading to enhanced motor recovery in PD rats. Delayed overexpression of intra-striatal GDNF also promoted motor recovery in transplanted rats. Concomitant with behavioural recovery after delayed GDNF we observed enhanced A9/A10 specification, increased DA graft plasticity, greater activation of striatal neurons and

elevated DA metabolism. Lastly, transcriptional profiling of the grafts highlighted novel genes underpinning these changes. Collectively these results demonstrate the potential of targeted neurotrophic gene therapy strategies to improve human PSC graft outcomes.

4.2 Introduction

Clinical trials have provided evidence that human ventral midbrain dopamine progenitors (vmDA), transplanted into the denervated striatum of Parkinson's disease (PD) patients, can structurally integrate, restore DA transmission and alleviate motor symptoms for decades (for review see [Barker et al., 2013](#)). Hindering this therapy has been the reliance on human fetal tissue that, in addition to availability and ethical constraints, is poorly standardized. Consequently, focus has shifted to generating replacement DA progenitors from pluripotent stem cells (PSC). Employing highly standardised procedures, that encompass modelling the developing vm environment and using both tissue-appropriate laminin substrates and extrinsic molecules, recent years have seen rapid advancements in protocols for the generation of *bona fide* vmDA neurons from both human embryonic and induced pluripotent stem cells ([Kirkeby et al., 2012](#); [Kriks et al., 2011](#); [Niclis et al., 2017](#)).

While upon transplantation these human PSC-derived DA progenitors are capable of maturing into DA neurons, adopting appropriate target acquisition and restoring gross motor deficits ([Grealish et al., 2014](#); [Niclis et al., 2017](#)), overall proportions of DA neurons within the grafts remain low. In part, this likely reflects the poor survival of these progenitors/neurons during and immediately following transplantation, as reported for fetal tissue grafts ([Castilho et al., 2000](#); [Olanow et al., 1996](#)). In addition, transplanted hESC-derived DA neurons show inferior axonal growth, compared to human fetal vmDA donor tissue. For example, [Grealish et al., \(2014\)](#), employing a human specific neural cell adhesion molecule and tyrosine hydroxylase co-immunoreactivity to selectively compare graft-derived DA axons from human ESC-derived and human fetal donor grafts, demonstrated reduced innervation of host A9-target tissue (dorsolateral and dorsomedial striatum), from the ESC-derived grafts. Such observations suggest that greater efforts to promote survival and axonal plasticity of human PSC-derived DA neurons following transplantation are required to improve outcomes after grafting and make this therapy competitive.

Unlike the developing brain that contains a cocktail of neurotrophic and morphogenic proteins that influence survival, differentiation and axonal connectivity, the adult brain is a notably less permissive environment for the survival and integration of transplanted progenitors, with many developmental cues downregulated or absent. First identified for its role in the survival and plasticity of embryonic midbrain dopamine neurons in culture (Lin et al., 1993), glial cell line-derived neurotrophic factor (GDNF) has been shown to increase the survival, plasticity and metabolism of DA neurons in pre-clinical and clinical studies for Parkinson's disease – see reviews (Björklund et al., 1997; Kirik et al., 2017; Thompson and Björklund, 2012). Recombinant GDNF protein has also been used to promote the survival of DA progenitors within fetal donor preparations prior to transplantation (Rosenblad et al., 1996), with studies in rodents and non-human primates demonstrating the benefits of prolonged delivery into the host tissue (via infusion (Ahn et al., 2005; Johansson et al., 1995; Sinclair et al., 1996; Yurek, 1998), or injection of viral vectors (Elsworth et al., 2008; Georgievska et al., 2004; Kauhausen et al., 2013; Redmond et al., 2009; Thompson et al., 2009; Wakeman et al., 2014; Winkler et al., 2006) for promoting survival, plasticity and functionally appropriate integration of DA-rich fetal vmDA tissue grafts.

Here we have assessed the impact of long-term GDNF overexpression on human PSC-derived vmDA progenitor grafts. We showed that exposure of the graft to GDNF had a significant impact on survival, plasticity and functional integration and notably that the timing of exposure was an important variable. Using a recombinant AAV vector to overexpress GDNF in the denervated host striatum, we showed that transplanting hESC-derived DA progenitors directly into a GDNF-rich environment promoted survival yet impacted on the capacity of the graft to innervate the host striatum. In contrast, exposure of the DA progenitors to GDNF several weeks after implantation had no effect on survival but positively impacted on DA neuron maturation, innervation of DA-relevant targets and functional outcomes. With human PSC therapies rapidly moving towards the clinic, these findings have important implications for promoting survival, plasticity, integration and thereby function of human PSC derived DA grafts, targeted at restoring DA transmission in PD patients.

4.3 Materials and methods

4.3.1 Human ESC differentiation

The human embryonic stem cell (hESC) reporter lines, H9 PITX3-GFP and H9 LMX1A-GFP, were expanded and differentiated as previously described in detail (Chapter 3) and illustrated in Figure 4.1A. In brief, neural induction was achieved by dual SMAD inhibition using LDN193189 (200 nM, 0–11 days of differentiation, D; Stemgent) and SB431542 (10 μ M, 0–5D; R&D Systems). Ventral midbrain patterning was simultaneously achieved by supplementation of the media with sonic hedgehog (200 ng/mL, 1–7D; R&D Systems), purmorphamine (2 μ M, 1–7D; Stemgent) and CHIR99021 (3 μ M, 3–13D; Miltenyi Biotech). From 11D, cultures were matured in the presence of brain-derived neurotrophic factor (BDNF, 20ng/ml, R&D Systems), glial cell-line derived neurotrophic factor (GDNF, 20ng/ml, R&D Systems), recombinant human transforming growth factor type β 3 (TGF β 3, 1ng/ml, Peprotech), ascorbic acid (200nM, Sigma-Aldrich), dibutyryl cAMP (0.05mM, Tocris) and DAPT (10 μ M, Sigma-Aldrich). At 21D cultures enriched with ventral midbrain progenitors were dissociated using Accutase (StemCell Technologies) in preparation for transplantation.

4.3.2 Surgical procedures

All animal procedures adhered to the Australian National Health and Medical Research Council's (NHMRC) Code of Practice for the Use of Animals in Research and were approved by the Florey Institute for Neuroscience and Mental Health animal ethics committee. Animals were group housed on a 12:12-hour light/dark cycle with *ad libitum* access to food and water. Surgeries were performed on 78 athymic (CBH^{nu}) nude rats and 26 athymic (Foxn1^{nu}) nude mice under 2–5% isoflurane anaesthesia. Animals received unilateral 6-OHDA lesions (rats: 3.5 μ l, 3.2 μ g/ μ l; mice: 1.5 μ l, 1.6 μ g/ μ l) of the medial forebrain bundle (MFB; rat) or substantia nigra (mouse), as previously described (Kauhausen et al., 2013; Niclis et al., 2017).

A subset of animals received an injection of the adeno-associated viral vector carrying GDNF under the chicken β -actin promoter (AAV-GDNF, 0.25 μ l; 7.2×10^{12} gc/ml) or a control virus, expressing mCherry fluorescent protein (AAV-mCherry, 0.25 μ l; 4.1×10^{12} gc/ml) into the dorsolateral striatum (Rats: 0.5mm rostral, 3.5mm lateral to Bregma and 3.6mm below the surface of the brain; Mice: 1mm rostral, 2mm lateral to Bregma and 2.9mm below the surface of the brain). Human PSC-derived DA progenitors were transplanted into the denervated striatum (1 μ l; 100,000 cells/ μ l) (Rats: 0.5mm anterior, 2.5mm lateral to Bregma and 4.0mm below the dural surface; Mice: 0.5mm anterior, 2.5mm lateral, 4.0mm ventral), as previously

described (Kauhausen et al., 2013; Niclis et al., 2017). *Study I - Transplantation of progenitors into a GDNF-overexpressing environment:* AAV-GDNF (or AAV-mCherry as control) was injected into rats 5 weeks after 6OHDA lesioning. After a further 3 weeks, these animals were implanted with vmDA progenitors differentiated from the H9 PITX3-GFP human ESC line (100,000 cells in 1ul). *Study II – Delayed exposure of the transplant to GDNF:* Eight weeks following 6OHDA lesioning, rats and mice received implants of 100,000 FACS isolated GFP-expressing vmDA progenitors, obtained from LMX1A-GFP differentiated hESC cultures. Cell sorting was performed using previously described methods (100µm nozzle at 22PSI, MoFlo XDP (Beckman Coulter)) (Bye et al., 2015). Three weeks later GDNF-AAV was injected into the host striatum.

4.3.3 Behavioural analysis

Four weeks after lesioning, unilateral DA function was assessed using the amphetamine-induced rotation and cylinder tests, as previously described (Niclis et al., 2017; Somaa et al., 2017), with retesting performed at various intervals following transplantation. In brief, *Amphetamine rotations:* net rotations over 60 minutes were analysed 10 minutes after intraperitoneal injection of D-amphetamine sulfate (5mg/kg; Tocris Bioscience). *Cylinder test:* animals were placed within a clear glass cylinder and the first 20 forepaw touches were recorded over three consecutive days. Data is presented as left paw ratio: $(\text{Left}/(\text{Left} + \text{Right})) \times 100$. Upon completion of initial testing 4 weeks post-lesioning, animals displaying a functional deficit (>300 rotations in 60 min) were ranked in order of the percentage rotational asymmetry and evenly distributed across the four treatment groups (Lesion, Lesion + GDNF, Lesion + Graft, Lesion + Graft + GDNF). The behavioural testing and allocation to (4) treatment groups was performed on 2 cohorts of rats, for Study I and Study II, described above.

4.3.4 Immunohistochemistry

Animals received intraperitoneal D-amphetamine sulfate (5mg/kg) one hours prior to receiving an overdose of sodium pentobarbitone (100mg/kg) followed by transcardial perfusion with Trydine solution followed by 4% paraformaldehyde. Brains were coronally or horizontally sectioned (40µm; 12 series) on a freezing microtome (Leica) and immunohistochemistry performed as previously described (Somaa et al., 2017). Primary antibodies and dilutions are shown in table 1. The bound antibody complex was detected either indirectly through enzymatic conjugation of the diaminobenzadine chromagen or using directly conjugated fluorescent secondary antibodies (Jackson ImmunoResearch).

4.3.5 Microscopy and quantification

Fluorescence images were captured on a Zeiss Axio ObserverZ.1 upright epifluorescence or Zeiss LSM 780 confocal microscope. Bright and darkfield images were taken on a Leica DM6000 upright microscope. Grafts were delineated by human PSA-NCAM expression and volume extrapolated using Cavalieri's principle. For GFP, TH and cFOS⁺ quantification, all positive cells were counted from brightfield images and corrected for series number. For GIRK2 and Calbindin quantification, dopamine neurons were first identified using TH immunoreactivity from confocal images. For fiber density assessment, 10 z-stack-sections (1µm per section) were obtained and compressed. TH⁺ fibers were isolated on colour inverted images using the 'colour range' tool on Photoshop (Adobe). Data is expressed as percentage of immunoreactive pixels. All areas were captured in triplicate with conserved settings. Sampling for fiber density and c-FOS labelled neurons was performed in the medial (AP: 0.0, ML: -1.8 and DV: -5.1), dorsolateral (AP: 0.0, ML: -3.8 and DV: -3.9) and ventrolateral (AP: 0.0, ML: -4.0 and DV: -6.9) striatum of rats, (from sections 1 mm anterior to 1.5mm posterior to Bregma).

4.3.6 Gene expression analysis

For transcriptional profiling, the ipsilateral striatal hemisphere containing the LMX1A-GFP transplant was dissected from mice 6 months after transplantation. Total RNA was extracted and RNAseq (Illumina) performed. Alignment against the human genome was conducted to select only human specific sequences for analysis. A number of genes showing significant change in expression (between Graft and Graft + GDNF) were validated using quantitative real time PCR (qPCR), according to previously described methods (Soma et al., 2015). To selectively assess these gene expression changes in the graft and not host tissue, qPCR primers were designed against regions containing nucleotide base-pair differences between the mouse and human gene. Primers were validated for their selectivity on control mouse (no expression) and human (gene expression) tissue, prior to testing on grafted samples. See table 2 for a list of primers.

4.3.7 High-performance liquid chromatography

Using reverse phase liquid chromatography with electrochemical detection, dopamine and its metabolites, 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), levels were measure from mouse striatal tissue 6 months after the transplantation of LMX1A-GFP vmDA

progenitors, in the absence and presence of GDNF, as previously described (Parish et al., 2008). Data was expressed as pmol/ml of DA, DOPAC or HVA and dopamine turnover determined by the ratio of DA to HVA.

4.3.8 Statistical analysis

All data are presented as mean $\pm$ SEM. Statistical tests employed (inclusive of one- and two-way ANOVA and student t-tests) are stated in figure legends. Alpha levels of $p < 0.05$ were considered significant with all statistical analysis performed using GraphPad Prism. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

4.4 Results

4.4.1 Validation of DA differentiation, GDNF expression and GDNF-induced motor recovery

Midbrain dopaminergic neurons were differentiated from PITX3-GFP (PITX3^{WT/GFP}) human embryonic stem cells according to a recent, clinically relevant xenogeneic-free protocol (Figure 4.1A; chapter 3). Enrichment of DA progenitors was confirmed by OTX2⁺/FOXA2⁺ co-expression at 15 days of differentiation (Figure 4.1B), with limited numbers of PITX2, PAX6 or BARHL1⁺ cells observed (indicative of unintended lateral or rostral midbrain contaminants) (Figure 4.1C). While progenitors were isolated at D21 for transplantation, parallel cultures, extended to D25, confirmed the ability of the cells to generate *bona fide* vmDA neurons, displaying high proportions of GFP (PITX3), TH and FOXA2 co-expression (Figure 4.1E). Quantitative PCR analysis of maturing DA progenitors *in vitro* confirmed upregulation of early and late DA determinant genes LMX1A and TH, respectively, and importantly the expression of GDNF receptors, GFR α 1, indicating that the progenitors were capable of responding to GDNF at the time of transplantation (Figure 4.1D).

Long term stable expression of AAV5-GDNF (Figures 4.1F and Supplementary Figure 4.1) and the control AAV5-mCherry (Supplementary Figure 4.2B) were observed at 6 months within the host striatum, as well as along the ipsilateral striatonigral tract, using GDNF and RFP immunohistochemistry, respectively. Rats receiving 6OHDA lesions alone (6OHDA), Lesion + AAV-GDNF (GDNF), Lesion + PITX3-GFP vmDA progenitor transplants (Graft) or Lesion + combin-

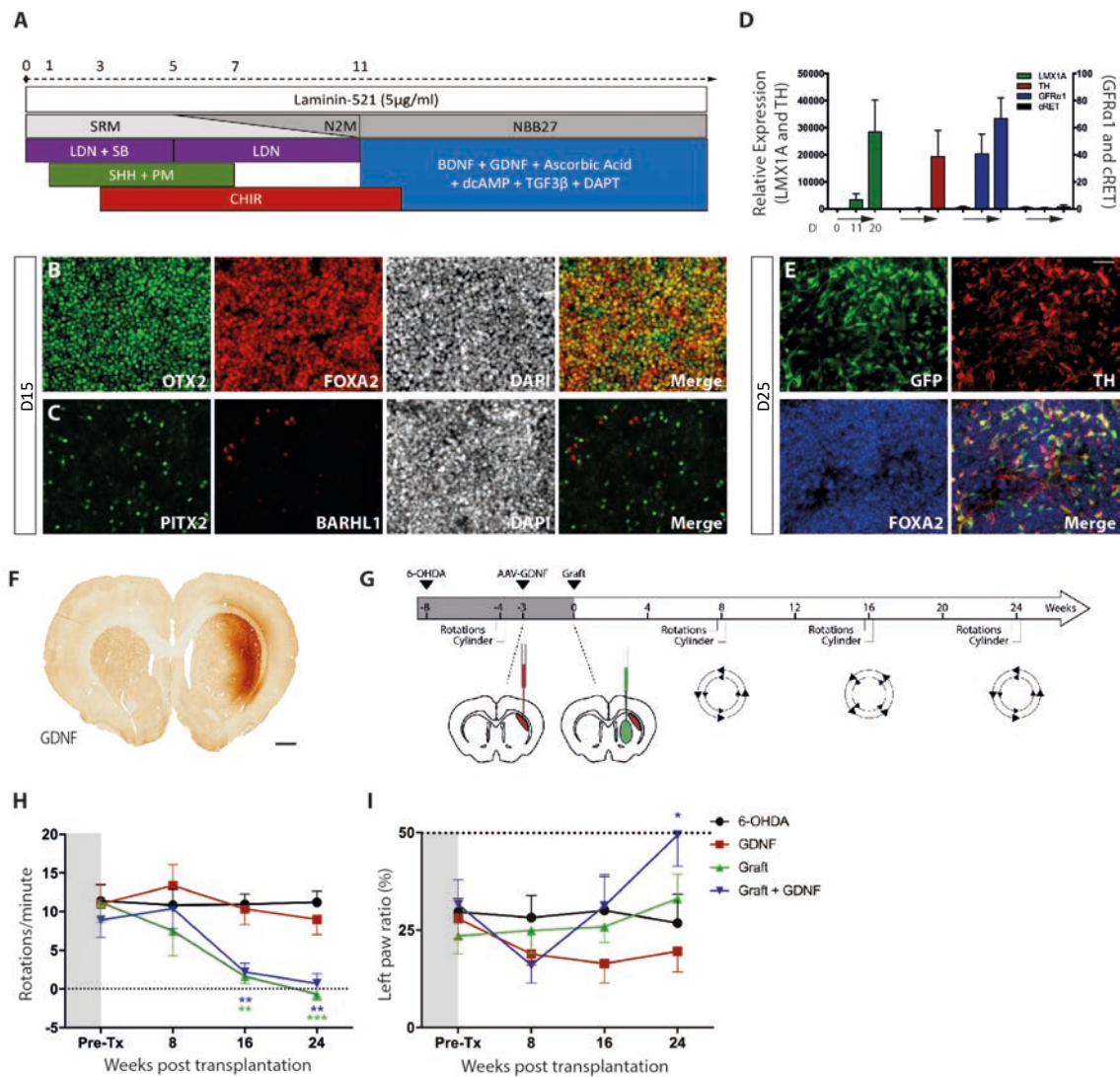


Figure 4.1 – Exposure of hESC-derived mDA transplants to GDNF, from the time of implantation, improved motor deficits in Parkinsonian rats.

(A) Differentiation protocol for hESCs into mDA progenitors, suitable for transplantation. (B) Photomicrographs showing high proportions of OTX2 and FOXA2 co-expression within progenitors at D15. (C) Low numbers of PITX2⁺ and BARHL1⁺ cells, indicative of off-target rostral/forebrain progenitor populations, validated efficiency of the mDA differentiation. (D) Graph showing mRNA levels of mDA progenitor/neuron markers (LMX1A and TH) and GDNF receptors (c-ret and GFRα1) during differentiation, relative to undifferentiated hESCs. All values normalised to HPRT1 expression. n = 3 independent cultures. (E) Validation of mDA differentiation into DA neurons, as confirmed by PITX3-GFP, TH and FOXA2 expression at D25. (F) Coronal section of the rat brain illustrating sustained GDNF expression 27 weeks after intrastriatal injection of AAV-GDNF. (G) Schematic summary of *in vivo* study, inclusive of behavioural testing, GDNF viral delivery and cell transplantation. (H) All grafted animals showed complete reversal of amphetamine-induced rotations at 24 weeks. (I) Correction of forelimb asymmetry in the cylinder test was only observed in rats receiving grafts in the presence of GDNF. (B,C,E) Scale bar, 50µm. (F) Scale bar, 1mm. Lesion, n = 11; GDNF, n = 7; Graft, n = 7 and Graft + GDNF, n = 7. Two-way ANOVA compared to 6-OHDA with Dunnett correction for multiple comparisons.

-ed AAV-GDNF and PITX3-GFP vmDA graft (Graft + GDNF) underwent a battery of motor tests at regular intervals starting at 4 weeks prior to grafting and 8, 16, 24 weeks post-transplantation (Figure 4.1G). Note, all rats included in the study showed an amphetamine induced rotational asymmetry of >5 rotations/min pre-transplantation, that was persistent and stable for 32 weeks

in the 6OHDA lesion control group (Figure 4.1H, blackline). As expected, expression of GDNF from an AAV vector in the absence of a cell transplant did not reverse rotational behaviour in the chronically lesioned animals (Figure 4.1H, red line). In contrast, rats receiving a Graft with or without GDNF showed significant recovery seen at 16 weeks, which progressed to complete recovery by 24 weeks (Figure 4.1H). In the cylinder test (an assessment of spontaneous motor asymmetry), motor dysfunction was reflected in a decrease in weight-bearing contralateral paw touches following lesioning (Figure 4.1I). Strikingly, recovery was only observed in the Graft + GDNF group at 24 weeks (Figure 4.1I, blue line). The time period of recovery (6 months) observed here highlights the necessity of extended observational studies, as previously described for both human fetal (Piccini et al., 2000) and human PSC-derived DA progenitor transplants, (Niclis et al., 2017).

4.4.2 Transplantation of PSC-derived DA precursors into a GDNF-overexpressing environment promotes graft survival but disrupts axonal targeting

At 24 weeks after transplantation, all animals ($\pm$ GDNF) displayed surviving grafts, as confirmed by GFP expression (Figures 4.2A and B). Importantly, we found no gross evidence of aberrant graft growth, i.e. no cell over growth or distortion of host tissue. This was supported by the extremely low proportion of Ki67⁺ cells (<0.2%) within the grafts at 24 weeks, suggesting that the *in vitro* differentiation and subsequent maturation of the pluripotent stem cell-derived progenitors *in situ* was sufficient to deplete proliferative pools. In all animals the GFP⁺ grafts were predominantly confined to the striatum. The validity of PITX3-GFP as an accurate marker of dopaminergic neurons was confirmed by co-expression with the rate-limiting enzyme in dopamine synthesis, tyrosine hydroxylase (TH) using confocal microscopy (Supplementary Figures 4.2C and 4.3D), with > 85% of cells co-expressing TH⁺ and GFP⁺, as previously described for the reporter line (Niclis et al., 2017). GFP⁺ neurons commonly clustered at the periphery of the graft with significant GFP⁺ areas within the graft core (Figures 4.2A-B), as previously observed for both fetal and PSC-derived vmDA grafts (Bye et al., 2015; Kirkeby et al., 2017; Niclis et al., 2017). Volumetric analysis confirmed that GDNF significantly increased graft volume at 6 months (Graft: 7.04 ± 0.96 mm³ and Graft + GDNF: 13.33 ± 1.72 mm³) (Figure 4.2C). Both GFP⁺ (Graft: 5268 ± 654 ; Graft + GDNF: 9476 ± 1462) and TH⁺ (Graft: 4092 ± 602 ; Graft + GDNF: 7986 ± 1375) neuron numbers commensurately increased with graft volume in the presence of GDNF, such that DA cell density was unchanged, indicating that GDNF promoted survival but not differentiation of DA neurons (Figures 4.2D and E). Interestingly, the total GFP⁺ population also increased concomitantly, suggesting that GDNF also influenced

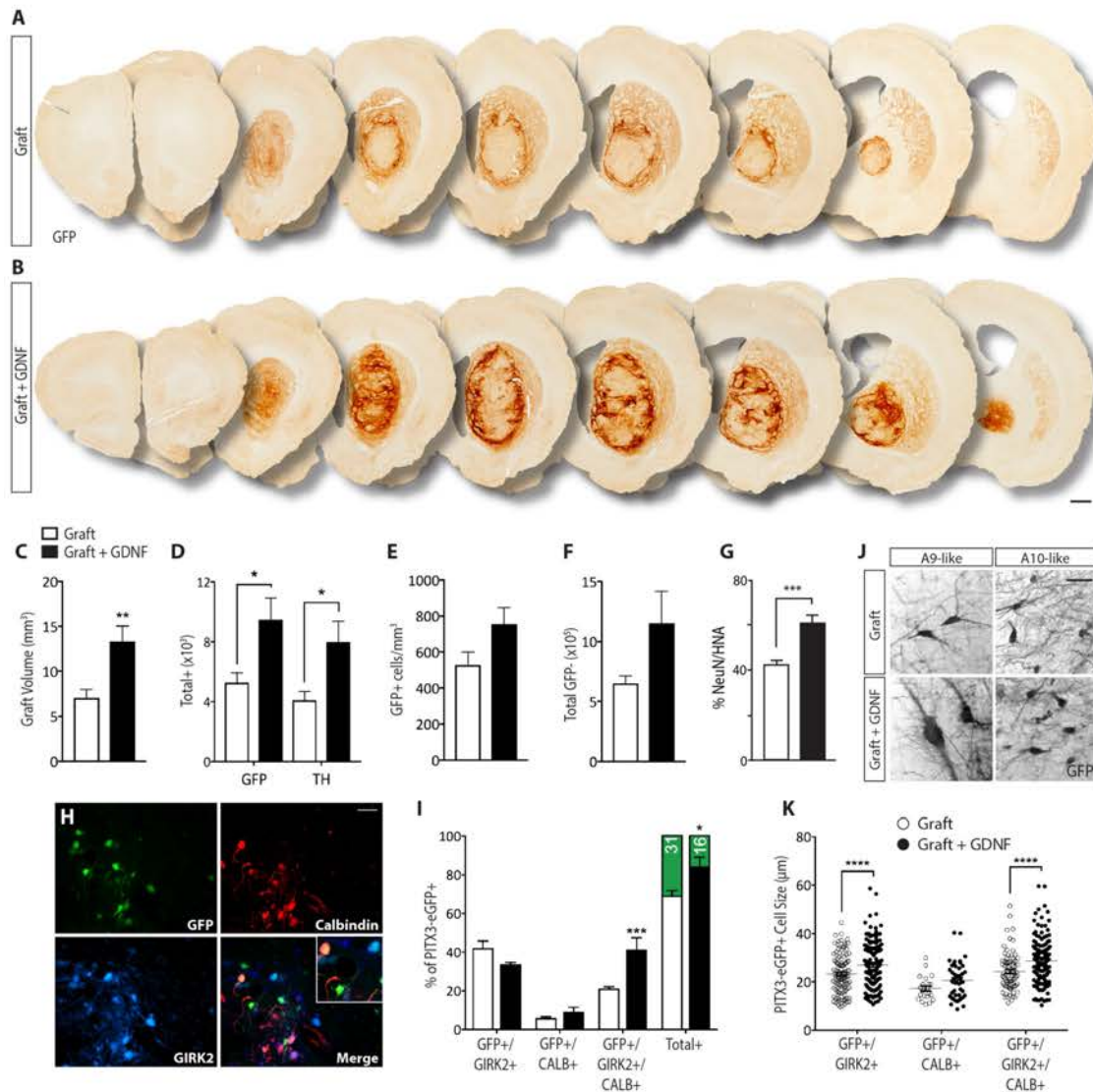


Figure 4.2 – Implantation of hESC-derived mDA progenitors into a GDNF-rich environment promotes graft survival. (A-B) Representative overview of a hPSC-derived mDA progenitor graft in the absence (A) and presence (B) of AAV-GDNF, showing PITX3-GFP expression. Scale bar, 1mm. (C) Assessment of graft volume showed significantly larger grafts in the presence of GDNF. (D-E) GFP⁺ and TH⁺ cell numbers commensurately increases with volume in animals receiving transplants in the presence of GDNF (D), resulting in unchanged GFP⁺ cell density (E). (F) Exposure of the graft to GDNF also increased the number of GFP⁺ cells within the transplants. (G) Cell + GDNF grafts showed enhanced maturation, as revealed by the increased proportion of NeuN⁺ neurons within the graft. Data in C-G expressed as mean $\pm$ SEM, Students t-test ($n = 7-11$). (H-I) Representative image showing the co-expression of GFP (PITX3) with GIRK2 and/or CALBINDIN, indicative of A9- and A10-like mDA neurons (G), and quantification (H). Green bar represents % of GFP⁺ that are neither GIRK or CALBINDIN⁺. Scale bar, 50 μ m. (J) Representative images of A9-like (large, angled soma, commonly found at the graft periphery) or A10-like (small, circular soma, found predominantly in the graft core) $\pm$ GDNF. (K) GFP/GIRK co-expressing neurons were significantly larger in the presence of GDNF suggestive of increased maturation.

non-DA cells within the grafts Figure 4.2F). Consistent with previous reports, TH⁺ DA neurons accounted for $\sim 1\%$ of the total graft (Doi et al., 2014; Niclis et al., 2017; Samata et al., 2016) or $\sim 5\%$ of the 100,000 implanted cells (Kirkeby et al., 2012; Kirkeby et al., 2017; Kriks et al., 2011; Niclis et al., 2017 at 24 weeks and increased to $\sim 9\%$ in the presence of GDNF (Figure 4.2C-D). Note, AAV-mCherry had no impact on graft size (representative graft, Supplementary Figure 4.2-

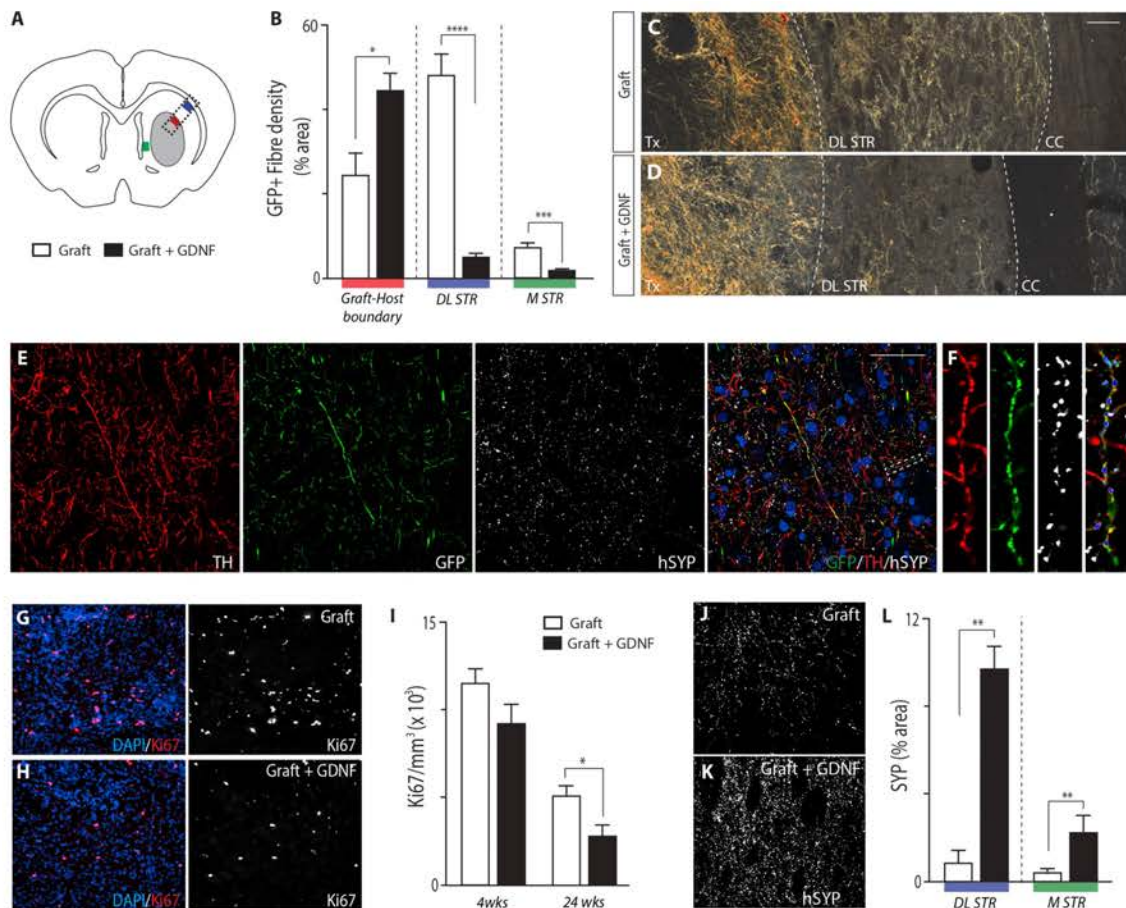


Figure 4.3 – Early exposure of hESC-derived mDA transplants to GDNF impedes axonal sprouting and integration into the host brain.

(A) Schematic showing sampling sites for estimates of graft-derived fiber density, at the graft-host border (red) and within the target dorsolateral (blue) or medial (green) striatum, and region of interest depicted in C-D (dotted line). (B) Transplants in the presence of GDNF showed increased GFP⁺ DA innervation density at the border of the graft, but significant reduction in their capacity to innervate the host dorsolateral and medial striatum ($n = 5-6$). (C-D) Representative photomicrographs illustrating the increase in graft-derived GFP⁺ innervation within the transplant, and decreased innervation in the striatum in animals receiving grafts in the presence (D), compared to absence (C), of GDNF. Tx, transplant; DL STR, dorsolateral striatum; CC, corpus callosum. Scale bar, 100 μ m. (E) Transplants, in the absence or presence of GDNF, were capable of forming synaptic connections within the host striatum, as depicted by the presence of TH⁺, GFP⁺ and human-specific synaptophysin⁺ (hSYP) co-labelling. (G-H) Photomicrographs of Ki67⁺ proliferative cells within a graft, and in the presence of GDNF, at 24 weeks after transplantation. (I) The increase in graft size, observed in the presence of GDNF, was not due to an increase in proliferation as seen by Ki67⁺ analysis within the grafts at either 4- or 24-weeks post-transplantation. (J-K) Representative images, and quantitative assessment of hSYP puncta within the host striatum animals receiving a VM graft in the absence (J) and presence (K) of GDNF. (L) GDNF increased hSYP puncta density within the dorsolateral and medial striatum (DL STR and M STR, respectively), indicative of synaptic maturation and integration of the graft. Scale bar, 50 μ m. (F) Example of synaptophysin immunoreactive puncta along a graft derived, dopaminergic (GFP⁺/TH⁺) neurite within the dorsolateral striatum (magnification of boxed area in E).

-B), composition or motor function compared to grafts of cells alone and were not further analysed (data not shown).

To assess the competence of transplanted DA progenitors to mature into correctly specified A9 or A10 DA subtypes we assessed Calbindin or GIRK2 immunoreactivity in

conjunction with GFP expression (Figures 4.2H-I). Specification of GFP⁺/GIRK2⁺ or GFP⁺/Calbindin⁺ was constant regardless of GDNF expression. In contrast the percentage of GFP⁺/GIRK2⁺/Calbindin⁺ co-expressing neurons significantly increased in Graft + GDNF compared to Graft animals ($21.17 \pm 0.97\%$ and $41.23 \pm 6.24\%$, respectively), such that only 16% of grafted GFP⁺ cells failed to adopt the A9/A10 fate in the presence of GDNF, compared to 31% of GFP⁺ cells in the absence of GDNF (Figure 4.2I, green bar). In addition, GIRK2 expressing cells within the grafts (GFP⁺/GIRK2⁺ and GFP⁺/GIRK⁺/CALB⁺) were significantly hypertrophied in the presence of GDNF (Figures 4.2J-K), findings that collectively suggest GDNF promoted the maturation of DA neurons.

The integration of grafted DA neurons into the host tissue was assessed using GFP innervation density at the graft-host boundary, as well as within the dorsolateral striatum (DL STR), the canonical target region for the A9 DA neurons responsible for motor function (Figure 4.3A). Reflective of the increase in GFP⁺ cells within the grafts, GFP⁺ fiber density at the graft-host boundary was increased in the presence of GDNF (Graft: $24.34 \pm 5.17\%$; Graft + GDNF: $44.05 \pm 10.65\%$ area covered by immunoreactive pixels) (Figures 4.3B-D). Surprisingly, GFP⁺ innervation density within the dorsolateral striatum was significantly diminished in the presence of GDNF (Graft: $47.25 \pm 5.39\%$; Graft + GDNF: $4.8 \pm 0.93\%$). Despite poor host innervation from grafts in the presence of GDNF, those GFP⁺ fibers within the dorsolateral striatum remained capable of forming synapses, as revealed by the presence of human-specific synaptophysin immunoreactivity along GFP⁺ fibers (Figures 4.3E-F).

The surprising reduction in DA innervation of the host striatum by grafted cells exposed to GDNF led us to delve further into understanding the maturation, differentiation and plasticity of these transplants that were capable of improving motor function. Quantitative assessment of KI67⁺ proliferative progenitors within the grafts at 4 and 24 weeks after transplantation revealed a progressive and subsequently significant decrease (Figures 4.3G-I), indicating the pro-maturation (and not proliferative) effect of GDNF on the graft. Reflective of increased neuronal maturation, the density of graft-derived human synaptophysin (hSYP⁺) synapses were also more abundant in the presence of GDNF, within both the dorsolateral (Graft: $1.24 \pm 0.52\%$; Graft + GDNF: $8.52 \pm 1.69\%$ hSYP⁺ area) and medial striatum, (Figures 4.3J-L). Together, these data suggest that whilst GDNF reduced innervation density, it induced graft maturation and synaptogenesis, effects which may underpin behavioural recovery.

4.4.3 Delayed GDNF delivery promotes recovery of motor function without increasing cell survival

Given that DA neurons only accounted for a fraction of the graft, a common observation for human PSC-derived vmDA progenitor grafts (Doi et al., 2014; Kirkeby et al., 2012; Kriks et al., 2011; Niclis et al., 2017; Samata et al., 2016) - and that the delivery of GDNF prior to cell transplantation negatively impacted on the ability of the grafted dopamine neurons to innervate the host dorsolateral striatum, we looked to employ two major refinements to a subsequent study design – the employment of an LMX1a-GFP reporter ESC line, enabling selective isolation of vmDA progenitors prior to transplantation, as well as altering the timing of GDNF delivery to 3 weeks after transplantation. Such modifications would ensure that DA-enriched grafts were exposed to GDNF after a period that would influence survival and rather address the capacity for the neurotrophin to modulate axonal plasticity of the transplanted neurons. As previously demonstrated (Chapter 3), we confirmed the ability of the LMX1A-GFP H9 hESC line to differentiate into vmDA progenitors, with the majority of cells co-expressing GFP, OTX2 and FOXA2⁺ at D15 (Figure 4.4A). On the day of transplantation (D21) the cultures yielded a high proportion of immature GFP⁺(LMX1A)/NURR1⁺/TH⁺ progenitors (Figure 4.4B, white arrows) that could be FACS-purified (>70% GFP⁺, Figure 4.4C) and transplanted directly into the denervated striatum.

Amphetamine induced rotation and cylinder tests were conducted periodically before and after grafting to validate the functionality of LMX1A-GFP derived DA transplants $\pm$ GDNF, (Figure 4.4D). 6-OHDA-induced motor deficits were stable for 34 weeks in 6OHDA lesion control animals in the absence and presence of GDNF (Figure 4.4E, black and red lines, respectively). Complete abolishment of amphetamine-induced rotational asymmetry was observed 26 weeks after grafting in animals receiving transplants ($\pm$ GDNF). However, recovery was notably accelerated in animals with combined DA grafts and GDNF (Figure 4.4E). In the cylinder test, only animals receiving combined graft and GDNF treatment showed significant improvement in contralateral forepaw use (Graft + GDNF: $51.94 \pm 7.66\%$; Graft: $17.72 \pm 7.40\%$; GDNF alone: $7.49 \pm 1.51\%$) (Figure 4.4F).

At 26 weeks post-transplantation, surviving grafts, identified as dense GFP immunoreactive deposits, were again observed in all animals (Figures 4.4G and H). Sustained GDNF expression, restricted to the dorsolateral striatum and overlying cortex, was confirmed only in animals that received AAV-GDNF (Figures 4.4I and J). 6-OHDA lesions consistently ablated

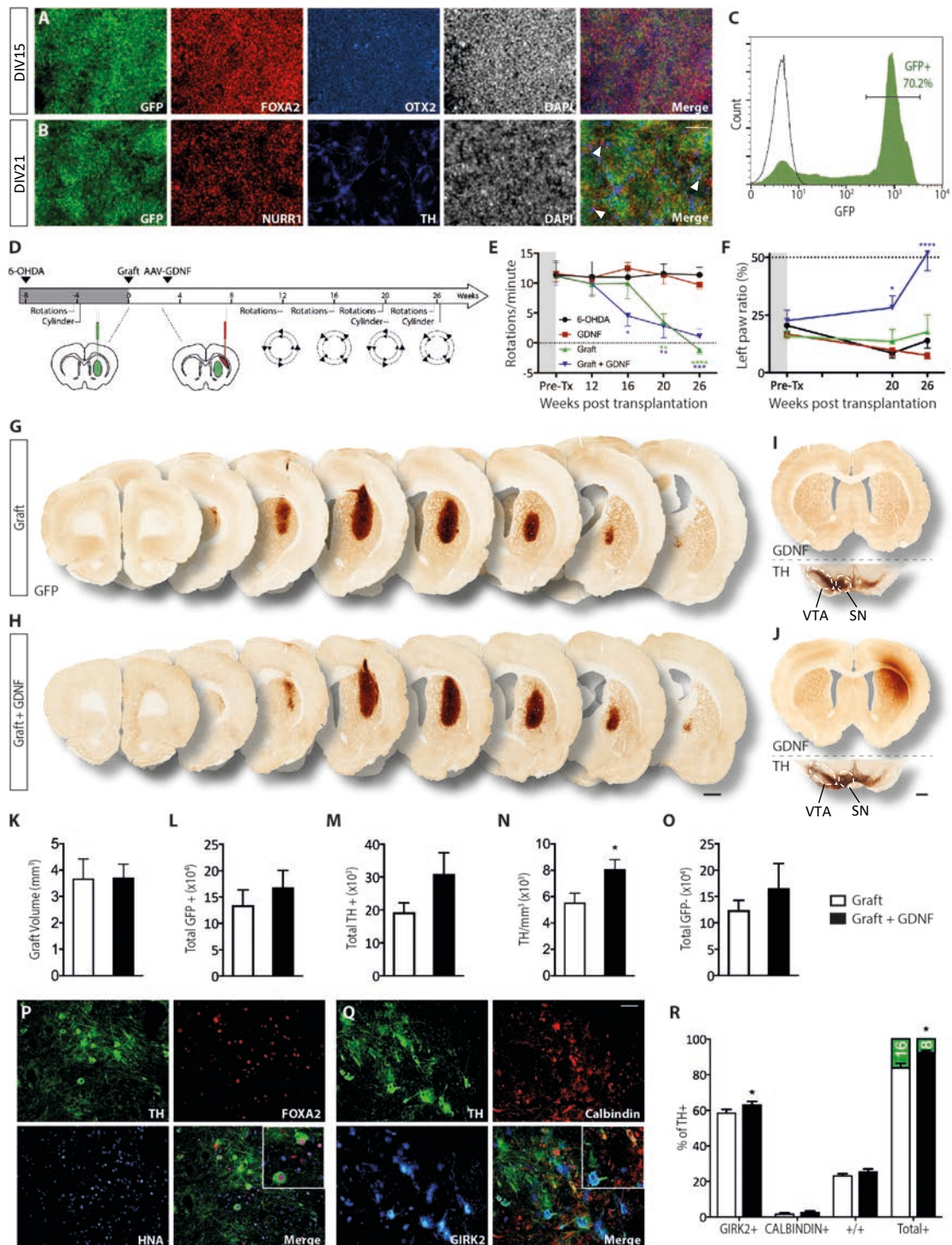


Figure 4.4 – Delayed exposure of hESC-derived mDA grafts to GDNF enhances functional recovery in parkinsonian rats, independent of cell survival.

(A-B) Differentiation of LMX1A-GFP hESC into mDA progenitors was confirmed by the high co-expression of GFP, FOXA2 and OTX2 by D15 (A) as well as GFP, NURR1 and TH at the time of transplantation (D21) (B). Scale bar, 50µm. (C) Fluorescent activated cell sorting plot illustrating the isolation of LMX1A-GFP expressing mDA progenitors from differentiating cultures at D21 for transplantation. (D) Schematic overview of the study design inclusive of behavioural testing, cell transplantation and AAV injections. (E-F) All grafted animals showed restoration of amphetamine-induced rotational asymmetry at 26 weeks after grafting (E), yet only rats receiving transplants in the presence of GDNF showed significant improvements in the use of the impaired (left) paw in the cylinder test (F). Lesion, n = 9; GDNF, n = 8; Graft, n = 7 and Graft + GDNF, n = 7. Two-way ANOVA against 6-OHDA with Dunnett correction for multiple comparisons. (G-H) Representative overview of a hESC-derived vmDA progenitor transplant in the absence (G) and presence (H) of AAV-GDNF. Grafts were immunostained for GFP to specifically identify LMX1A-

Figure 4.4 (continued)

GFP expressing graft deposits. Scale bar, 1mm. (I) Coronal section illustrating the absence of GDNF expression in the adult striatum, and reduction in TH-ir in the lesioned midbrain of the rat brain depicted in (G); white dotted area highlights the position of the VTA and SN. (J) GDNF immunohistochemistry confirmed targeted, long-term delivery of the AAV-GDNF into the dorsolateral striatum, and overlying cortex. TH histochemistry confirmed mDA lesioning. Images are from the same brain depicted in (H). (K-N) Graphs highlighting that delayed GDNF delivery had no significant effect on graft volume (K), the survival of GFP⁺ DA progenitors (L), TH⁺ DA neurons (M) but modestly effect TH⁺ cell density (N). Data presented as mean $\pm$ SEM, Students t-test ($n = 7$). (O) GDNF also had no effect on the total number of non-DA (HNA⁺/TH⁻) cells within the graft. (P) The ability of LMX1A⁺ grafted progenitors within the graft to mature into dopaminergic neurons was confirmed by the co-expression of TH, FOXA2 and HNA. (Q) High proportions of transplanted DA neurons (confirmed by TH-immunoreactivity) expressed CALBINDIN and/or GIRK2, indicative of A10 and A9-like specification. Scale bar (P,Q): 50 μ m. (R) Quantification of TH⁺ neurons that co-express GIRK2 and/or CALBINDIN.

TH⁺ neurons within the substantia nigra, while leaving ventral tegmental area DA neurons relatively spared (Figures 4.4I and J). Isolated LMX1A⁺ grafts were confirmed to be rich in midbrain dopaminergic neurons by 6 months, as indicated by the co-expression of TH, FOXA2 and human nuclear antigen (HNA), Figure 4.4P. In contrast to the first experiment where the grafts were placed into high GDNF containing environments, in this study quantitative assessment revealed no difference in graft volume (Figure 4.4K). Dopaminergic neurons, assessed by TH immunoreactivity showed no significant change between the two groups (Graft: $19,810 \pm 2,828$; Graft + GDNF: $30,381 \pm 6,513$, $P=0.153$) (Figure 4.4M). However, total yields of TH⁺ DA neurons within both Graft and Graft + GDNF were substantially elevated following sorting, accounting for 20-30% of the 100,000 implanted cells, and an estimated 10% of total cells within the transplant (TH⁺/HNA⁺), compared to unsorted vmDA progenitor grafts quantified in Figures 4.2D and F. The high proportion of DA neurons within the graft could be most evidently seen by comparative assessment of PSA-NCAM (total graft), GFP (vmDA progenitors and vmDA neurons) and TH⁺ (DA neurons) (Supplementary Figure 4.3), reflecting the efficacy of the selective isolation and transplantation of appropriately patterned vmDA-specific LMX1A-GFP⁺ progenitors. Graft exposure to GDNF had a modest but significant impact on TH⁺ density ($8095 \pm 730/\text{mm}^3$) compared to grafts of DA progenitors alone ($5555 \pm 699/\text{mm}^3$) (Figure 4.4N). Assessment of the non-dopaminergic fraction of the graft (TH⁻/HNA⁺ cells) revealed that GDNF had no significant effect on their survival and/or proliferation (Figure 4.4O). Co-expression of TH, together with GIRK2 and/or Calbindin again confirmed the ability of DA neurons within the grafts to mature into subtype specific populations with similar proportions in the presence or absence of GDNF (Figures 4.4Q and R). Small but significant increases in the overall proportion of Total GIRK2 and/or Calbindin specified DA neurons were seen in animals receiving GDNF (Graft: $84.44 \pm 1.96\%$; Graft + GDNF: $92.24 \pm 1.41\%$), indicative of improved and/or accelerated maturation of the grafts at 26 weeks (Figure 4.4R).

4.4.4 Delayed delivery of GDNF modulates appropriate innervation of host targets from human stem cell-derived DA neurons

Given the robust number of TH⁺ neurons present within the grafts, we next assessed the modulatory properties of GDNF on graft-derived DA innervation within key target nuclei of the midbrain dopamine system. As GFP was not selectively indicative of DA identity, TH immunoreactivity was used to quantify the striatal innervation density of DAergic fiber terminals. Important for the quantitative assessment of TH⁺ DA fibers emanating from the graft was the need to confirm robust ablation of the host DA system within the ipsilateral striatum and overlying cortex, as observed in 6-OHDA lesioned animals (Figure 4.5A). In contrast, notable TH immunoreactivity was observed along the rostrocaudal axis ipsilateral to the graft in animals that received transplants (+GDNF) (Figures 4.5B and C). Delayed exposure of the graft to GDNF (three weeks post-transplantation) resulted in significant increases in TH⁺ fiber density within multiple intra- and extra-striatal nuclei, including the overlying cingulate cortex (Cing. CTX), perirhinal cortex (Peri. CTX), dorsolateral striatum (DL STR) and ventrolateral striatum (VL STR) (Figures 4.5B-D).

Acute upregulation of the intermediate early response gene, cFOS, in medium spiny neurons (MSN) of the striatum following amphetamine administration has been shown to be modulated by DA release and subsequent receptor activity, thereby providing an indirect measure of DA signalling (Cenci et al., 1992). To facilitate cFOS analysis, all animals were injected with d-amphetamine one hour prior to perfusion. cFOS⁺ activation, was validated in striatal MSNs by CTIP2⁺/DARPP32⁺ co-expression (Figure 4.5E), with quantitative assessments performed within the medial, dorsolateral and ventrolateral striatum (Figures 4.5F-H and Supplementary Figure 4.4). As anticipated, 6-OHDA lesioning (in the absence of a graft) substantially reduced cFOS expression within both the dorsolateral and ventrolateral striatum, in comparison to the contralateral hemisphere and intact control animals (Figure 4.5F and Supplementary Figure 4.4B). Lesioning had no effect on cFOS⁺ cells in the medial striatum (Figure 4.5F). Grafts ($\pm$ GDNF) increased cFOS⁺ density along the rostrocaudal axis of the dorsolateral striatum, an effect particularly pronounced immediately caudal to the site of graft implantation (Figure 4.5G). Within the ventrolateral striatum, grafts in the presence of GDNF also had a significant impact on cFOS expression, with the greatest increase in immunoreactive cells observed rostral, and at the striatal level of implantation (Figure 4.5H). Grafts in the absence of GDNF modestly influence ventrolateral cFOS activation, while GDNF alone had no effect.

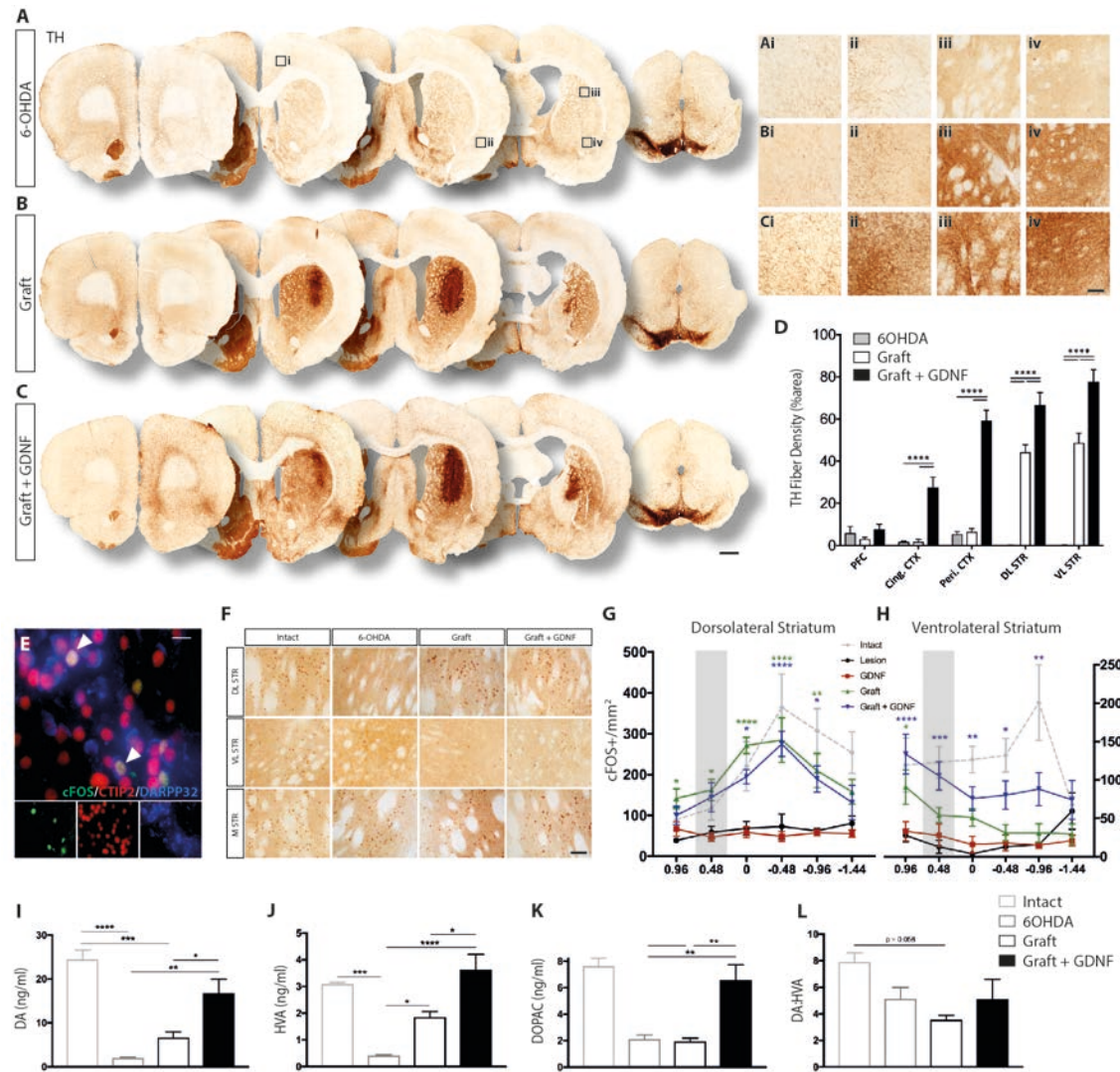


Figure 4.5 – Delayed exposure of the mDA graft to GDNF promoted DA innervation of the host tissue, activation of striatal neurons and upregulated DA biosynthesis.

(A-C) Photomontages illustrating the density of TH⁺ fibers within the rat brain following 6-OHDA lesioning (A) and after cell transplantation in the absence (B) and presence of AAV-GDNF (C). (Ai-Ci) High power images from (A-C) illustrating the density of TH⁺ fibers in the motor cortex, (Aii-Cii) perirhinal cortex, (Aiii-Ciii) dorsolateral striatum and (Aiv-Civ) ventrolateral striatum. Note the increase in TH⁺ fibers in striatal and extrastriatal regions of animals receiving grafts in the presence of GDNF. Scale bar, 1mm (A-C) and 100µm (insets). (D) Quantification of TH fiber density within the host tissue of 6OHDA lesioned rats with and without cell grafts and AAV-GDNF (n = 7-9). Two-way ANOVA, Tukey correction for multiple comparisons. (E) Activation of host striatal neurons was confirmed by co-expression of cFOS, CTIP and DARPP32. Scale bar, 40µm. (F) Photomicrographs of cFOS⁺ cells within the striatum of Intact and 6OHDA lesioned rats ± Grafts ± AAV-GDNF. (G) Quantification of cFOS⁺ cells within the host brain revealed significant increases in activated cells in the dorsolateral striatum of grafted animals, irrespective of GDNF expression, compared to ungrafted rats (Lesion ± GDNF). (H) Rats receiving grafts in the presence of GDNF showed significant increases in cFos activation in the ventrolateral striatum. Two-way ANOVA against 6-OHDA, Dunnett correction for multiple comparisons (n = 6-7). (I-K) High performance liquid chromatography confirmed a significant reduction in dopamine (I) and DA metabolites, HVA (J) and DOPAC (K), in the striatum of lesioned (compared to intact) mice, an effect that could be partially restored by hESC-derived DA grafts, and fully restored when transplants were exposed to GDNF. (L) Reflective of commensurate changes in DA and its metabolites, dopamine turnover (DA:HVA) remained unchanged across lesion and grafted animals. One-way ANOVA with Tukey correction for multiple comparisons (n = 4). Abbreviations: PFC, prefrontal cortex; cing. CTX, cingulate cortex; peri. CTX, perirhinal cortex; DL STR, dorsolateral striatum; VL STR, ventrolateral striatum.

4.4.5 GDNF restores DA levels and modulates graft gene expression

Following the establishment of GDNF's modulatory effect on DA neuronal survival and innervation patterns, we next investigated the effect of GDNF on DA metabolism and gene expression within the graft. A cohort of mice, transplanted with FACS-purified LMX1A⁺ DA progenitors ($\pm$ AAV-GDNF 3 weeks after transplantation), were confirmed to have comparable, viable grafts at 6 months to those observed in rats (Supplementary Figure 4.5). High-performance liquid chromatography (HPLC) was used to assess dopamine turnover within lesioned and grafted animals. A >90% reduction in striatal dopamine levels in 6-OHDA lesioned animals confirmed ablation of the host midbrain dopaminergic system, (6OHDA: 2.0 ± 0.2 pmol/mg of wet tissue; Intact: 24.4 ± 2.2 pmol/mg) (Figure 4.5I). Reflective of the improvement in rotational asymmetry at 26 weeks, grafts in the absence or presence of GDNF showed significant elevations in DA levels (6.7 ± 1.3 pmol/mg and 16.8 ± 3.1 pmol/mg, respectively) (Figure 4.5I), as well as the DA metabolites HVA (1.9 ± 0.2 pmol/mg and 3.6 ± 0.6 pmol/mg) and DOPAC (1.9 ± 0.3 pmol/mg and 6.6 ± 1.1 pmol/mg), compared to 6OHDA lesion alone (HVA: 0.4 ± 0.03 pmol/mg; DOPAC: 2.1 ± 0.3 pmol/mg) (Figures 4.5J and K). However only in the presence of GDNF were DA, DA metabolites and overall DA metabolism (ratio of DA to HVA) restored to levels not significantly different from the intact brain, reflective of restoration of dopamine biosynthesis and function in these animals (Figures 4.5I-L).

To identify transcriptional changes, we performed RNA-seq analysis on host striatal tissue from the same mouse cohort, containing the vmDA grafts $\pm$ GDNF, 6 months after transplantation. Whole genome hierarchical analysis revealed distinct differences in gene expression between the two groups (Graft vs Graft + GDNF), with 489 genes significantly upregulated and 58 genes downregulated in the Graft + GDNF compared to Graft alone (Figure 4.6A); the top 25 up and down regulated genes are depicted in Figure 4.6B. GDNF-upregulated genes were associated with gene ontology biological processes including cell-cell signalling, synaptic signalling, cell communication and secretion (Figure 4.6C). Selective analysis of the top 200 upregulated genes revealed a number of genes with known roles in dopamine development and homeostasis, morphogenesis, axon guidance, cell adhesion and synaptic plasticity (Figure 4.6D).

We subsequently performed qPCR to selectively assess and confirm transcriptionally upregulated genes identified by RNAseq (Figure 4.6E). Human-specific primer sets were designed to enable selective assessment of gene expression changes within the grafts ($\pm$ GDNF),

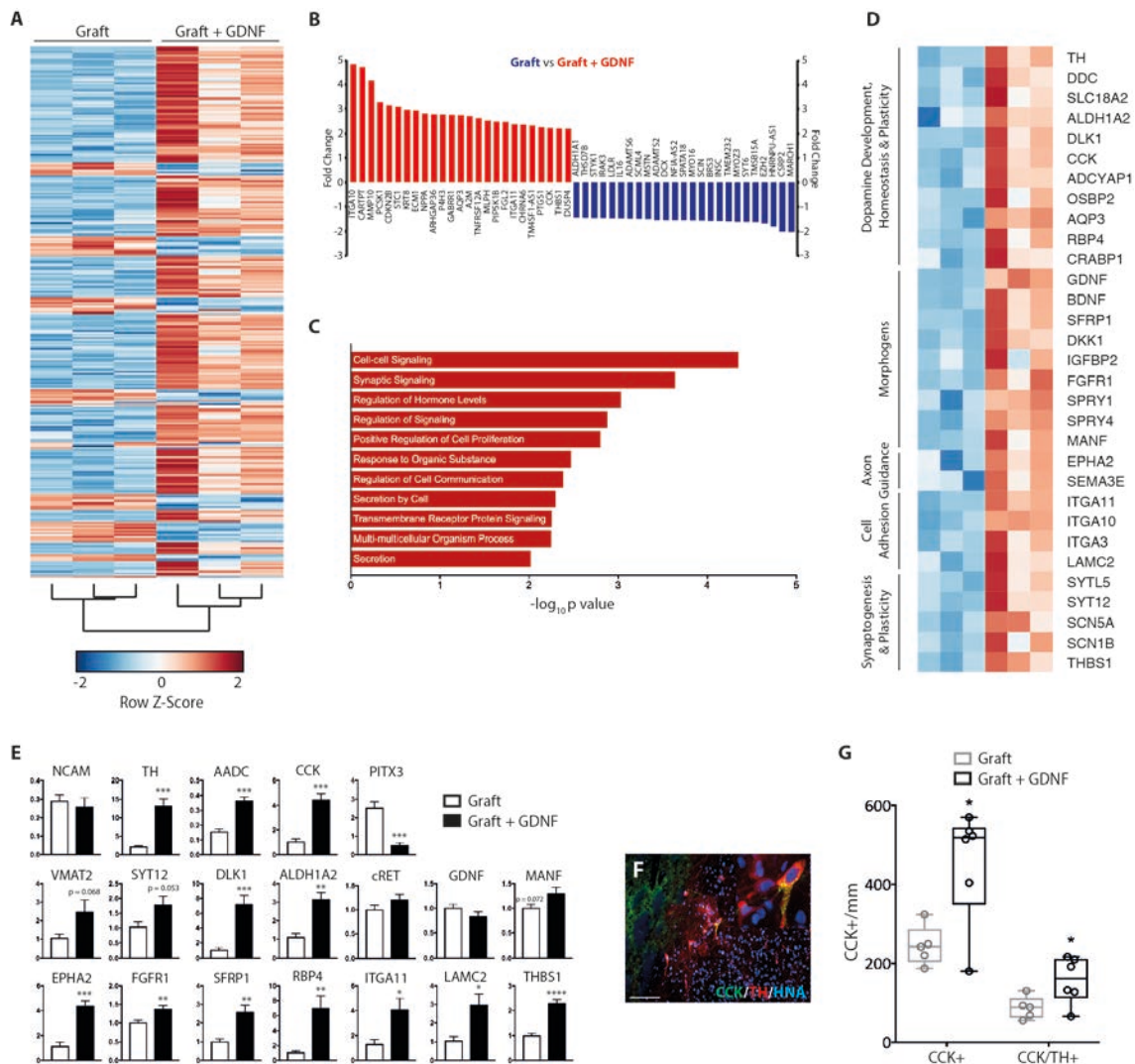


Figure 4.6 – Transcriptional profiling of mDA grafts in the presence and absence of GDNF identified known and novel DA homeostatic, synaptogenesis and axonal-plasticity-related genes.

(A) Unbiased hierarchical clustering analysis of gene expression changes in Graft and Graft + GDNF tissue samples, (n=3/group). (B) Top 25 upregulated (red) and downregulated (blue) genes identified by RNAseq, in Graft+GDNF tissue, compared to mDA Graft alone. (C) Gene ontology analysis clustering of upregulated genes in Grafts exposed to GDNF (<0.01 FDR) ‘Synaptic signalling’ includes related GO terms with equal weighting in ‘Anterograde trans-synaptic signalling’, ‘Trans-synaptic signalling’ and ‘Chemical synaptic transmission’. (D) Heat map of selected genes related to dopamine development & homeostasis as well as gene clusters reflective of morphogenesis and axonal plasticity in all grafts. (E) Quantitative PCR validation of human mRNA expression levels, for transcripts identified by RNAseq, confirmed significant increases in genes related to DA synthesis for grafts in the presence, compared to absence of GDNF. Analysis also verified a number of novel genes, previously ascribed to axonal growth and plasticity in non-DA systems, that may contribute to the observed enhanced integration of Grafts in the presence of GDNF. Students t-test (n = 5). (F) CCK⁺ neurons were readily identified within the graft and often coexpressed TH. Scale bar = 100 μm. (G) Quantification of CCK⁺ and CCK⁺/TH⁺ cells within grafts revealed that both CCK⁺ and CCK⁺/TH⁺ neuronal density was increased in the presence of GDNF.

while excluding detection of host (mouse) gene levels. No change in PSA-NCAM gene expression levels validated previous observations that GDNF had no effect on graft size (Figures 4.4K and 4.6E). Reflective of increase dopamine metabolism (Figures 4.5I-L), genes related to dopamine synthesis (TH and AADC) were significantly elevated in Graft + GDNF tissue. Surprisingly, PITX3,

a transcription factor previously recognised for its role in survival and maintenance of the DA phenotype, was significantly downregulated in grafts exposed to GDNF, an effect that likely reflected the enhanced maturation of these grafts, and in accordance to previous observations of downregulated PITX3 transcript in terminally differentiated human PSC-derived DA neurons (Chapter 3). Reflective of changes in axonal plasticity (i.e. increased TH⁺ fiber density from grafts exposed to GDNF, Figure 4.5), pre-synaptic proteins including synaptotagmin 12 (SYT12) and vesicular monoamine transporter (VMAT2), showed strong trends towards upregulated expression. Both GDNF and the GDNF receptor, c-ret, were present within all grafts, but not modulated by GDNF overexpression. The non-canonical Notch ligand, DLK1, known to influence vmDA neurogenesis and DAT expression in development (Jacobs et al., 2009; Surmacz et al., 2012), be regulated by GDNF (Christophersen et al., 2007), and recently identified as a predictive marker of good human PSC-derived DA graft outcomes (TH⁺ cell number and innervation density) (Kikuchi et al., 2017; Kirkeby et al., 2017), was shown to be highly increased in graft tissue in the presence of GDNF (Figure 4.6E). Also supportive of the increase axonal plasticity of grafts in the presence of GDNF, a number of genes known for their influence on axonal plasticity in development and repair in non-DA systems, were shown to be upregulated, inclusive of Ephrin receptor (EPHA2), fibroblast receptor 1 (FGFR1), soluble frizzled receptor protein 1 (SFRP1), thrombospondin 1 (THSB1), as well as cell adhesion molecules ITGA11 and LAMC2 (Figure 4.6E).

Finally, cholecystokinin (CCK), a neuropeptide known for modulating DA release within midbrain dopamine pathways, including in fetal vmDA transplants, was also shown to be upregulated (Wang et al., 1994). To confirm the observed increase at the transcriptional level, we examined the expression and localisation of CCK protein. In support of upregulated gene expression, we showed vmDA grafts in the presence of GDNF had 1.86-fold more CCK⁺ neurons, with 34.6% co-expressing TH, suggesting that GDNF regulates CCK expression and potentially modulates DA transmission within the grafted brain. Further studies will be required to fully elucidate the role/s of CCK, and other novel GDNF-downstream genes on DA survival, plasticity and function.

4.5 Discussion

This study provides the first evidence of modulating the survival and plasticity of human pluripotent stem cell-derived dopaminergic transplants in the Parkinsonian brain. Employment

of unique GFP-expressing stem cell reporter lines enabled precise tracking of the DA contribution to the grafts (PITX3-GFP), as well as a 10-fold enrichment of DA progenitors (LMX1A-GFP) within the transplants. Utilizing these tools, we were able to demonstrate that the timing of onset for sustained GDNF delivery directly impacted on the mechanisms by which improvements in functional recovery occurred. While all grafted animals showed restoration of amphetamine-induced motor asymmetry by 6 months, reflective of the relatively low number of DA neurons required for correction of this gross motor deficit (estimated to be as few as 1000 human DA neurons ([Grealish et al., 2014](#)), examination of non-pharmacologically-induced sensorimotor tasks revealed distinct benefits of sustained GDNF expression.

We demonstrate that implantation of human PSC-derived DA progenitors into an already GDNF-enriched environment promoted graft survival, yet without selectivity for the DA population. More noteworthy however was the negative impact of early GDNF delivery on the capacity of the graft to innervate the host tissue, such that the majority of graft-derived DA fiber growth was restricted to the graft core. Within these animals we speculate that the functional recovery observed likely reflects increased maturity of the cells and relatively sparse fibers within the dorsolateral striatum and/or their capacity for dopamine turnover. Further studies will be required to fully elucidate such phenomena. In contrast, delayed exposure of the graft to GDNF had no impact on cell survival, yet significantly increased graft-derived reinnervation of the host tissue, elevated striatal DA levels (to levels not different from the intact brain), and consequently, enhanced activation of postsynaptic striatal medium spiny neurons. Interestingly, while increased activation of striatal neurons (the primary output of the transplanted DA neurons) was shown in the dorsolateral striatum of animals receiving grafts in the absence and presence of GDNF, only in the presence of GDNF was cFos activation observed in the ventrolateral striatum, a region of the basal ganglia previously shown to underpin changes in more complex sensorimotor tasks following lesioning and DA transplantation ([Chang et al., 1999](#); [Mandel et al., 1990](#)), and supported by findings in patient fetal grafting studies ([Piccini et al., 2005](#)). Collectively these findings suggest that acute GDNF exposure is required following cell implantation to promote DA survival, but that sustained delivery, initiated prior to cell transplantation, negatively impacts on the capacity of graft-derived DA axons to extend out from the graft core to innervate host tissue. Optimal for promoting DA axonal plasticity and appropriate reinnervation of striatal and extrastriatal tissue, is the requirement for implanted DA progenitors to mature and initiate such axonal growth, prior to sustained GDNF exposure. The importance of these findings is significant in light of former clinical trials where an estimated

15% of patients developed graft-induced dyskinesias, a phenomenon speculated to be, in part, the consequence of incomplete and uneven striatal DA reinnervation (Carlsson et al., 2006; Hagell et al., 2002).

A major contributor to the functional capacity of vmDA grafts is the relative contribution of A9 neurons (Grealish et al., 2010). Previous studies have described differential effects of GDNF on DA subpopulations, such that A10, but not A9 neurons, are protected into adulthood against apoptosis, resulting in increased cortical, but not striatal innervation (Borgal et al., 2007; Kholodilov et al., 2004). By contrast, others have linked GDNF levels with the survival of A9, but not A10, DA neurons (Nossheny et al., 2006). In accordance with our previous work (Niclis et al., 2017), here we report ~70% of TH⁺ cells within the graft adopted an A9- or A10 like identity, with the majority of these fate-specified cells expressing GIRK2 (alone or in combination with Calbindin), while relatively few TH⁺ cells expressed Calbindin alone. Such proportions are reflective of the A9 and A10 contributions within the human ventral midbrain (Reyes et al., 2012) and are supportive of the appropriate differentiation and specification of these cells *in vitro* and subsequently *in vivo*. Surprisingly, in response to GDNF, a significant increase in TH⁺/GIRK2⁺ or TH⁺/GIRK2⁺/CALB⁺ cells was observed, such that fewer than 16% of the TH⁺ cells remained unspecified. This increase in GIRK2-expressing cells in the presence of GDNF was also accompanied by increased soma size, suggestive of a role for GDNF in the maturation of human SN neurons, an effect that may contribute to improvements in complex motor tasks.

In addition to the effects on survival and plasticity of DA neurons, GDNF has been shown to influence dopamine biosynthesis through the regulation of *TH* gene expression, a response dependent on *c-ret* (Xiao et al., 2002). Unlike rodent studies where sustained GDNF induces the long-term downregulation of *TH* mRNA, a compensatory response to increased DA levels from the graft resulting in impaired graft function (Georgievska et al., 2004; Winkler et al., 2006), non-human primate studies have reported maintained increases in TH⁺ cells and fiber density (Elsworth et al., 2008; Eslamboli et al., 2005; Kordower et al., 2000). For the first time, here we report the responsiveness of human dopamine neurons to chronic GDNF exposure. In alignment with non-human primates we observed increases in TH⁺ cells and TH⁺ fibers (dependent on the time of GDNF delivery) that was accompanied by significant upregulation of *TH* mRNA. We also detected elevated levels of aromatic L-amino acid decarboxylase (*AADC*), the enzyme required for the conversion of L-DOPA into dopamine and CCK, a neuropeptide previously reported to modulate striatal dopamine levels, suggestive that GDNF is capable of regulating DA generation

and release at multiple levels. Such observations were also supported by increased DA synthesis from grafts in the presence of GDNF, such that levels were not significantly different to the intact midbrain system. Interestingly, a recent clinical trial of putamenal GDNF infusion also observed an increase in DA via 18F[DOPA] PET scanning but did not show improvement in the OFF state, indicating that exogenous GDNF can promote DA metabolism but that DA release alone is insufficient for behavioural recovery (Whone et al., 2019a; Whone et al., 2019b). Noting previous pre-clinical and clinical studies observations, that developing rather than mature DA neurons are most responsive to GDNF (Elsworth et al., 2008), the maintained elevated enzyme (*TH* and *AADC*) transcript levels observed here likely reflect ongoing graft maturation, an unsurprising observation in light of the duration of human embryonic development as well as the protracted time for integration of human stem cell and fetal DA grafts in pre-clinical and clinical trials to date (Niclis et al., 2017; Piccini et al., 2000). In this regard, extended studies, involving graft assessment at time points when human DA neurons have reached functional maturity and no longer require GDNF, will be needed to determine the effects of sustained GDNF on *TH* and *AADC* transcript levels, resultant DA biosynthesis and motor function.

GDNF expression is recognised to be tightly regulated during embryonic development, with expression levels downregulated in the adult brain, beyond periods of critical cell survival and target acquisition (Kirik et al., 2017). Hence, it is probable and likely that closer attention to the duration of expression may be required when employing gene therapy approaches targeted at promoting neuroprotection and/or plasticity. While the present findings highlight the impact of exposing the grafted cells from the outset of implantation versus delayed expression, the impact of sustained expression remains unknown for human stem cell derived DA neurons. In addition to potential changes in regulatory genes of DA synthesis, further studies will be required to, for example, to determine whether sustained expression may result in increased aberrant innervation of target tissue that results in unregulated synaptic neurotransmission and/or increased innervation of off-targets, as a consequence of suboptimal gene therapy targeting. The dual effects of early versus late GDNF on grafted DA neurons suggest that the desired therapeutic approach may involve biphasic exposure to GDNF – such that acute GDNF delivery at the time of implantation can promote survival, with a subsequent exposure within the target tissue at a defined period (potentially weeks to months) after cell transplantation. Added to this is the requirement for greater control over gene therapy-associated protein dose. Here we report targeted injection of 1.8×10^9 genomic copies of the GDNF-AAV into the striatum of rats. While histochemical analysis confirmed protein expression, resulting in anticipated and

positive impacts on graft survival and/or plasticity, knowledge of absolute protein levels here remained unknown. Previous reports have demonstrated that it is in fact possible to overdose the system and impart effects on other neuronal populations ([Elsworth et al., 2008](#)) as well as negatively regulate DA levels ([Winkler et al., 2006](#)), such that lower chronic doses may be more beneficial. In this regard, several regulatory elements are now being tested to control the level of GDNF expression, as well as fine tune duration, that may be necessary for the optimisation of GDNF gene therapy – see review: [Kirik et al., 2017](#).

In addition to the support of human pluripotent stem cell derived DA grafts, GDNF gene therapy may provide the supplementary advantage of protecting residual host DA neurons within the vm that are undergoing persistent degeneration. Similar to previous reports ([Kauhausen et al., 2013](#); [Thompson et al., 2009](#)), including larger non-human primates ([Elsworth et al., 2008](#)), and evidenced here, viral delivery of GDNF within the target striatal/caudate putamen tissue also resulted in transport and transduction of cells within the midbrain. Although mature DA neurons have been suggested to be less responsive to GDNF than immature DA progenitors/neurons, studies have provided evidence of improved survival in the adult brain, resulting in a number of clinical trials using GDNF (infusion and viral delivery), as well as the closely associated protein Neurturin, targeted at slowing disease progression ([Kirik et al., 2017](#); [Rangasamy et al., 2010](#)).

In summary, here we provide a conclusive body of evidence for the many benefits of GDNF gene therapy to improve the functionality of human pluripotent stem cell-derived DA neural transplants in rodent models of PD. Dependent of timing of gene delivery, we report that GDNF increases graft survival, plasticity/sprouting of DA fibers, DA innervation of host target nuclei, increased activation of striatal neurons and elevated DA metabolism (associated with changes in regulatory synthesis gene expression). These findings suggest that a dual therapeutic approach, involving cell transplantation and targeted gene therapy, to address the shortcoming of poor survival and plasticity of human DA neurons, may have significant implications for the translation of pluripotent stem cell-based therapies into the clinic for the treatment of Parkinson's disease.

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4.7 Author contributions

All experiments were performed at the Florey Institute of Neuroscience and Mental Health. Conceptualisation: CG, LT and CP. Investigation: CG, JK, IDL, JN, VP, CH, CB and CE. Analysis: CG, CB and CP. Provision of resources: CP, DK, LT and CP. Writing – original draft: CG and CP. Writing – review and editing: CG, JK, IDL, JN, VP, CH, CB, CP, DK, LT and CP. Funding: CP.

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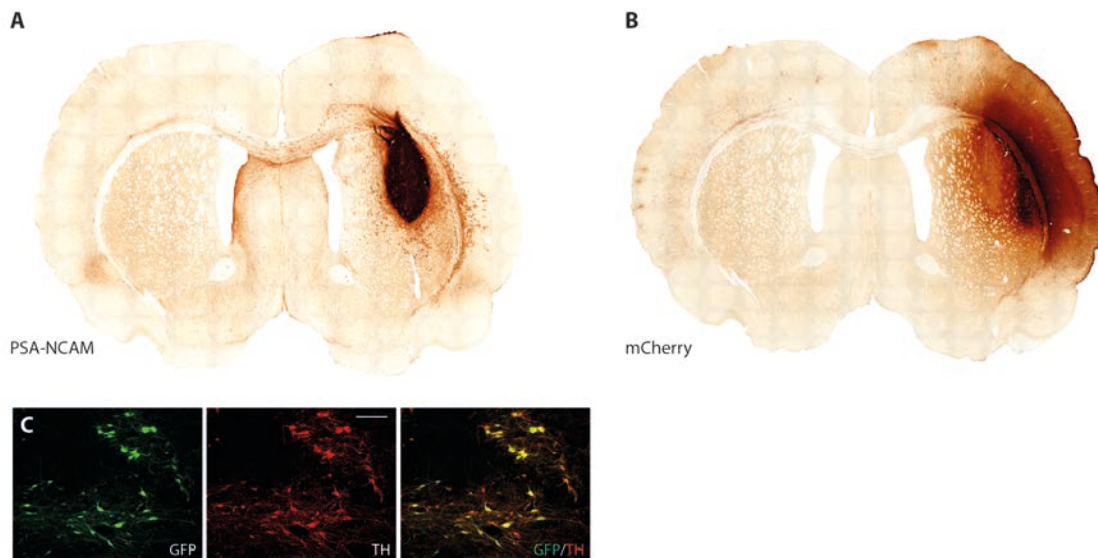
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4.9 Supplementary Figures



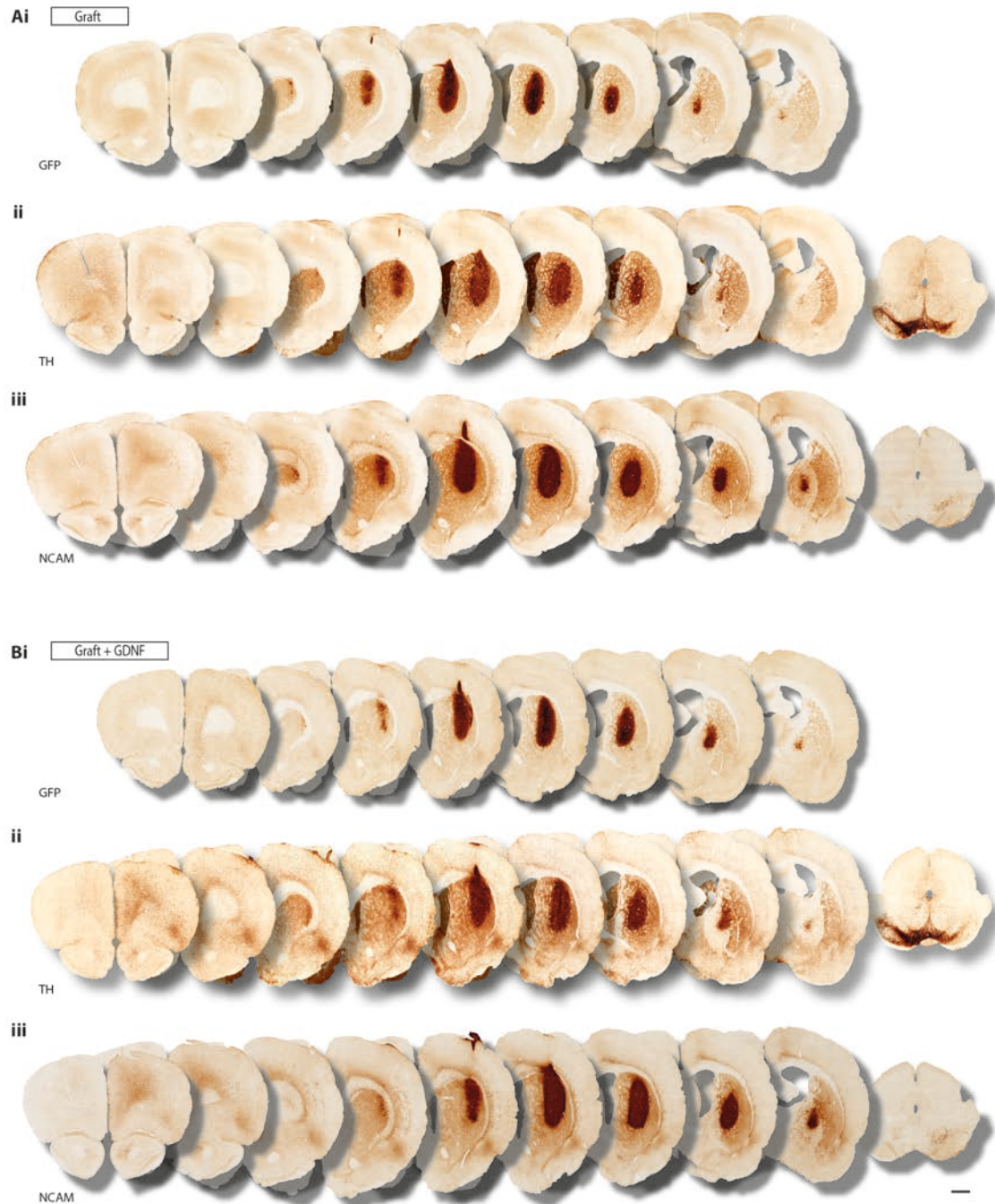
Supplementary Figure 4.1 – Targeted delivery of AAV-GDNF into the host brain.

(A) GDNF immunohistochemistry confirmed sustained expression of the protein within the host tissue 27 weeks after intrastriatal injection. In addition to expression within the host striatum and overlying cortex, GDNF was present within the medial forebrain bundle and ventral midbrain. High power photomicrographs confirmed viral transduction of cells within the (B) striatum, (C) overlying cortex, (D) medial forebrain bundle and (E) ventral midbrain cells. Scale bar, 2mm.



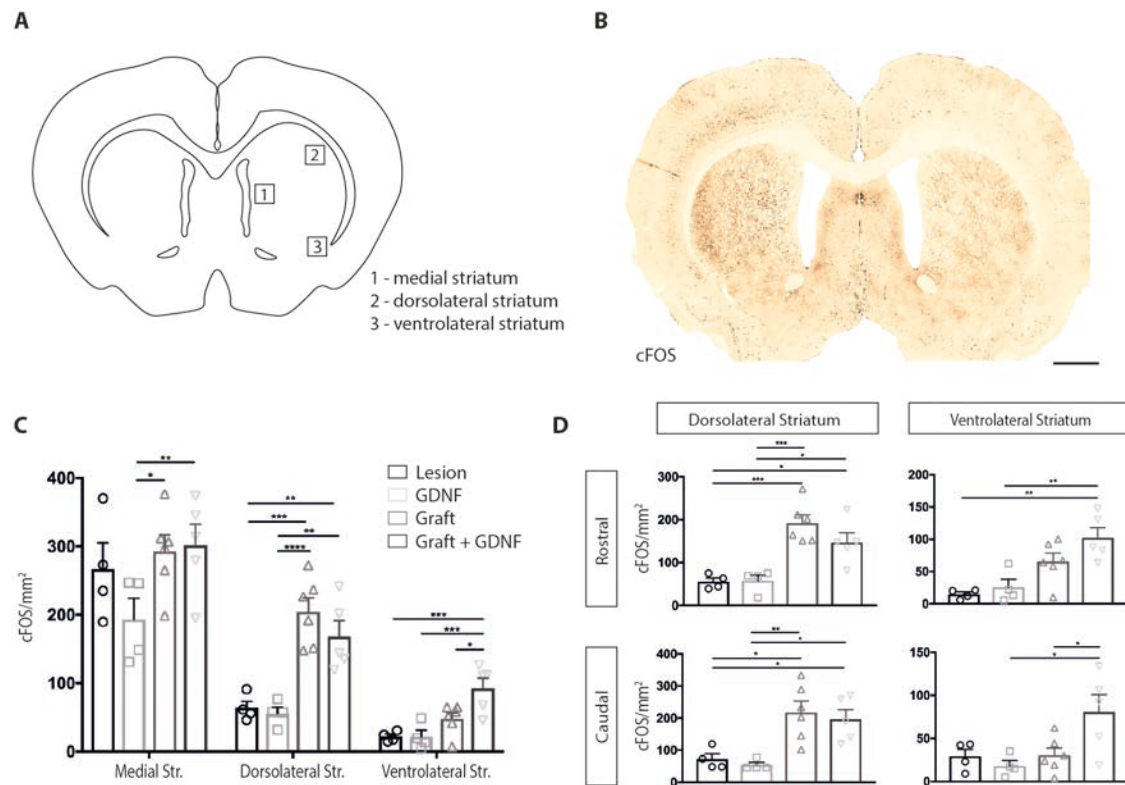
Supplementary Figure 4.2 – Control mCherry-AAV has no effect on the survival or differentiation of hESC-derived mDA progenitor grafts.

(A) Representative image of PSA-NCAM immunoreactivity delineating a hESC-derived PITX3-GFP graft in the presence of the control AAV (AAV-mCherry). (B) Photomicrograph illustrating the targeted localisation of the mCherry virus to the host striatum and overlying cortex. (C) AAV5-mCherry had no effect on the ability of transplanted progenitors to differentiate into dopaminergic neurons as assessed by GFP⁺ (PITX3-GFP) and TH⁺ co-expression.



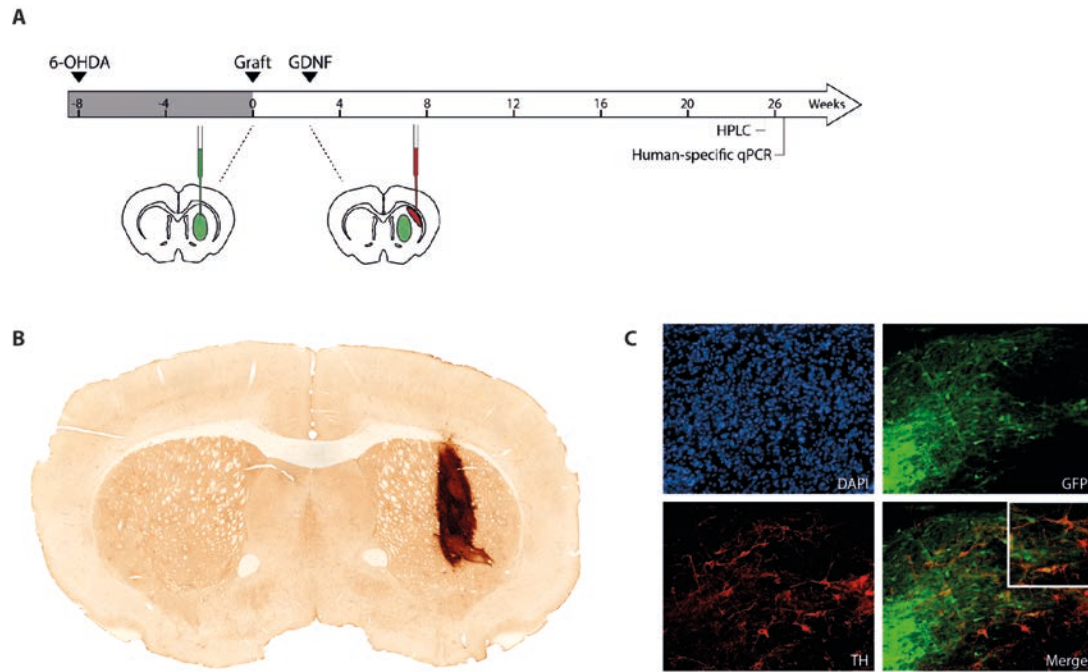
Supplementary Figure 4.3 – Comparative assessment of LMX1A-GFP, TH and NCAM immunoreactivity demonstrates that the vast majority of the transplant, in the absence or presence of GDNF, is dopaminergic at 26 weeks.

(A) Human ESC-derived vmDA progenitor grafts in the absence and (B) presence of GDNF, illustrating maintained GFP expression of the early progenitor marker LMX1A (Ai, Bi), that largely overlaps with the dopaminergic neuronal marker tyrosine hydroxylase (TH; Aii,Bii), as well as the human specific antibody for the neural cell adhesion molecule NCAM (Aiii, Biii). Images shown in A (Ai, Aii and Aiii) are taken from adjacent serial sections of the same brain. Similarly, Images shown in B (Bi, Bii and Biii) are from adjacent serial sections. Collectively these images highlight that a major proportion of the transplant was dopaminergic. Scale bar, 1mm.



Supplementary Figure 4.4 – Quantitative analysis of cFOS⁺ activated striatal cells by hESC-derived mDA transplants.

(A) Schematic coronal view of the rat brain illustrating the three sampling sites for quantification of cFOS-immunoreactive cells. (B) Example photomontage showing diffuse cFos staining in dopaminergic target regions of the rat brain following acute amphetamine administration in a 6-OHDA lesioned animal. Scale bar, 1mm. (C) Quantification of cFOS density within the medial, dorsolateral and ventrolateral striatum of lesion and grafted animals (+ AAV-GDNF). Densities are estimated from 6 serial sections, spanning ~3mm, through the striatum. Two-way ANOVA with Tukey correction for multiple comparisons ($n = 4-6$). (D) Quantification of cFOS⁺ cell density within the rostral and caudal regions of the dorsolateral and ventrolateral striatum. Densities are estimates taken from 3 sections spanning ~1.5mm through the striatum. One-way ANOVA with Tukey correction for multiple comparisons ($n = 4-6$). Abbreviations: Str., Striatum.



Supplementary Figure 4.5 – LMX1A-GFP⁺ hESC-derived mDA progenitor graft in the parkinsonian mouse brain.

(A) Schematic of the experimental design in nude mice, showing the time of lesioning, hESC-derived LMX1A⁺ cell transplantation, AAV delivery and tissue collection (for HPLC and qPCR). (B) Representative image of an LMX1A-GFP⁺ human PSC-derived vmDA progenitor graft in the striatum of a 6OHDA lesioned nude mouse, immunolabeled for GFP. (C) Photomicrographs illustrating graft derived GFP-expressing LMX1A neurons, co-expressing TH⁺ within the graft.

Chapter 5.

MANIPULATION OF DEEP-LAYER NEUROGENESIS IN HUMAN PLURIPOTENT STEM CELL-DERIVED CORTICAL PROGENITORS

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5.1 Abstract

The distinctive six-layered mammalian cortex, heavily expanded in primates, is responsible for higher cognitive functions. During development it is formed in an inside-out fashion whereby deep-layer neurons (Layers 5-6) are generated first, followed by subsequent waves of upper-layer neurons (layers 2-4) and gliogenesis. Despite their heterogeneity, all excitatory neurons are generated from a single founder population of PAX6⁺ neuroepithelial stem cells located in the ventricular zone. However, an understanding of how progenitors alter their layer competence over time is lacking. Here we examine the roles of two major signalling pathways (fibroblast growth factor, FGF, and Notch) that are known to influence cortical cell fate decisions, assessing their impact on progenitor phenotype, cell cycle kinetics and layer-specificity of human pluripotent stem cell derived cortical progenitors. Forced cell cycle exit, via Notch inhibition, led to rapid and exclusive generation of functional TBR1⁺ deep-layer 6 neurons. Continued exposure to FGF2 promoted proliferative divisions, shortened cell cycle length, together maintaining intrinsic progenitor identity and delaying laminar specification. Upon FGF2 withdrawal and Notch inhibition, these progenitors exclusively gave rise to the default TBR1⁺ neuronal phenotype. In contrast, inhibition of the downstream FGF signalling cascade via MAPK/ERK inhibition (MEK inhibition), extends cell cycle length and promotes acquisition of CTIP2⁺, presumptive layer 5, neurons, by repression of both earlier and later laminar fates. Taken together, modulation of Notch and FGF signalling altered the proliferative/neurogenic balance and could accelerate the acquisition of distinct laminar subtypes *in vitro*. These findings provide insight into our understanding of the mechanisms underpinning cortical lamination and present

a novel resource to generate layer specific neurons for the purpose of disease modelling, targeted screening and/or targeted therapeutic approaches.

5.2 Introduction

Neuroepithelial stem cells (NESC) located at the ventricular surface of the developing telencephalon give rise to the diverse populations of neurons and glia within the cortex. These NESCs can divide symmetrically to generate daughter radial glia (RG) as well as asymmetrically to generate early born neurons directly. RG can be further subdivided into at least five distinct subpopulations dependent on their location (within the ventricular or subventricular zone, VZ or SVZ), presence of apical and/or basal processes and gene expression pattern (Betizeau et al., 2013; Nowakowski et al., 2017; Pollen et al., 2015; Thomsen et al., 2016). As corticogenesis proceeds RG divide asymmetrically to generate a neuron and a TBR2⁺ intermediate progenitor cell (IPC). These IPCs migrate basally to populate the superficial germinal zones of the inner and outer subventricular zone (ISVZ and OSVZ) where they act as transit amplifying cells, undergoing further, limited rounds of division before terminal neurogenesis (Lui et al., 2011). The expansion of RG and IPC founder cell diversity is thought to underlie the complexification and enlargement of the primate cortex, particularly the increase in upper layer (UL) neurons. Thus, there is a general progression from PAX6⁺ NESCs toward PAX6⁺ RG and TBR2⁺ IPCs over development that accompanies the generation of neurons (Lui et al., 2011). These spatiotemporal events during corticogenesis are tightly orchestrated resulting in early born neurons becoming deep infragranular layer neurons (layer V/VI), whilst later born precursors become upper supragranular neurons (of layers II-IV). The timing of corticogenesis appears to be dependent on species, with human cortical progenitors demonstrating greater cell-autonomous clonal capacity in comparison to non-human primates and rodents (Otani et al., 2016). In addition to their specific targeted axonal projection, expression of select transcription factors aid in the classification of generated neuronal precursors into each lamina, including TBR1 (Layer 6), CTIP2 (5) as well as CUX1, BRN2 and SATB2 (2-4) (Molyneaux et al., 2007).

Surprisingly similar in the sequence of events has been the *in vitro* specification of human pluripotent stem cell-derived cortical progenitor into layer-specific populations and their organisation into rosette structures, inclusive of a lumen and outer cell layer, reminiscent of apicobasal organisation in the *in vivo* developing mammalian cortex (Eiraku et al., 2008; Espuny-Camacho et al., 2013; Gaspard et al., 2008; Shi et al., 2012). However, despite significant

advances in our understanding of development of the neocortex over the past 2 decades (Greig et al., 2013; Lui et al., 2011) how progenitor diversity and neuronal identity are linked remains elusive and is complicated by the lack of synchronicity across progenitor populations and ability of human cortical progenitors to fluidly transition between NESC and IPC states.

Cell cycle length has been shown to vary over telencephalic development, coinciding with the switch between deep layer (DL) and upper layer (UL) neurogenesis, providing a possible target for investigating laminar specification (Borrell and Calegari, 2014; Dehay and Kennedy, 2007). In the rodent, cell cycle length (T_C) increases over time, corresponding with an increase in consumptive, neurogenic divisions that diminish the initial progenitor pool. In particular, extension of G1 phase length (T_{G1}) alters daughter cell fate, determining the shift from proliferative to neurogenic divisions, while concomitant shortening of the S-phase occurs in gradually more committed neural progenitors (Arai et al., 2011; Turrero García et al., 2016). In contrast, an *ex vivo* non-human primate study has shown two distinct phases of cell cycle behaviour: a gradual extension in T_C and rise in neurogenic divisions corresponding to the generation of UL neurons, followed by a decrease in T_C , that corresponds to a secondary expansion period of OSVZ progenitors and eventual generation of UL neurons (Betizeau et al., 2013). This observation has suggested that the OSVZ provides a specific niche, where signals controlling stemness, proliferation and differentiation are transduced. Additional influences by signals outside the OSVZ, either secreted or via apical and basal processes also play a role. Factors governing the transition between consumptive and proliferative divisions in human cortical progenitors and thus, the relative DL and UL neuronal output remain unexplored.

Fibroblast growth factor (FGF) family members have pleiotropic roles in cortical development (Iwata and Hevner, 2009). In rodents, FGF2 has been shown to shorten T_{G1} and increase proliferative divisions (Lukaszewicz et al., 2002). Moreover, FGF receptor loss in the developing dorsal telencephalon leads to premature exhaustion of the proliferating progenitor pool and acceleration of neurogenesis, partly via loss of downstream Notch signalling. Strikingly, this loss of FGF signalling altered the timing but not the competency of progenitors to form both DL and UL neurons (Rash et al., 2011). Moreover, FGF-ERK signalling is enriched in human cortical development compared to rodents and may play a key role in generation of the OSVZ (Heng et al., 2017). Notch signalling is also well known for regulating self-renewal, in both proliferative and neurogenic modes, whereby the Notch ligand Delta-like 1 (Dll1) induces upregulation of cyclin D1 and suppresses pro-neural genes. Notch activation is mutually active

and oscillatory among aRG, later shifting to a lateral inhibition mode in the neurogenic state, whereby nascent postmitotic neurons expressing notch, prevent neuronal differentiation in adjacent cells. Taken together, FGF and Notch signalling act in concert to maintain RG fate as well as regulate the progression from symmetric to asymmetric divisions and thus, the size and organisation of the cortex via interaction with the cell cycle. However, if and how these extrinsic cues influence the shift in layer competence during human development remains unknown.

Here we assessed the impact of Notch and FGF signalling on laminar fate utilising a PAX6-mCherry human embryonic stem cell reporter line to specifically identify changes within a committed dorsal forebrain progenitor population. We validate that hPSC-derived PAX6⁺ dorsal progenitors give rise to all excitatory layers of the cortex as well as glia. Synchronisation of cell cycle exit through Notch inhibition of early PAX6⁺ progenitors led to the rapid and exclusive generation of functional TBR1⁺ deep-layer neurons. Continued exposure to FGF2 shortened the cell cycle, promoting proliferative divisions and maintain progenitor identity, giving rise exclusively to TBR1⁺ neurons when subsequently exited form the cell cycle. In contrast, and contrary to observations in rodents, inhibition of FGF signalling (via downstream MEK inhibition) biased cell fate toward CTIP2⁺, layer 5 neurons. Together these results shed light on the signalling pathways involved in the sequential generation of excitatory cortical neurons and provide a platform for the derivation of specific cortical layer populations.

5.3 Materials and methods

5.3.1 hPSC maintenance and differentiation

Human embryonic stem cell (hESC) line H9 and reporter line PAX6-mCherry (Bellmaine et al., 2017) were maintained as previously described (Niclis et al., 2017a). Briefly, undifferentiated colonies were maintained on Laminin-521 (BioLamina) in mTeSR1 medium and passaged at 80% confluence every 4-5 days with ReLeSR (both StemCell Technologies).

Cortical induction was achieved adopting a previous protocol with significant adaptations (Shi et al., 2012), Figure 5.1A. To initiate differentiation, hPSCs were dissociated with Accutase (StemCell Technologies) and reseeded onto Laminin-521 coated plates at $0.375 \times 10^6/\text{cm}^2$ in mTeSR1 supplemented with Rock inhibitor Y-27632 (10 μ M, Tocris Bioscience). 24 hours later, cells were washed once in PBS (-Mg²⁺/-Ca²⁺) before undergoing dual-SMAD inhibition for 11 days

in cortex media supplemented with LDN193189 (100nM; Stemgent) and SB431542 (10 μ M; R&D Systems). 'Cortex media' consisted of 1:1 DMEM/F12 and Neurobasal with 0.5x B27, 0.5x N2, 0.5x ITSA, 1x GlutaMAX, 0.5x Penicillin Streptomycin and 50 μ M 2-Mercaptoethanol (All Life Technologies). At 11 days of differentiation (D11) neuroepithelial progenitors were passaged with EDTA (Life Technologies) and seeded 1:2.5 on Laminin-521. The following day cultures were transferred to cortex media containing fibroblast growth factor 2 (FGF2, 20ng/ml, R&D Systems) for 8 days before cell sorting (see below). Following FACS purification cells were plated either at: 4.5 x 10⁶/cm² in cortex media for further maturation, or for cell cycle exit at 0.2 x 10⁶/cm² in cortex media supplemented with the gamma-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, 10 μ M; Sigma-Aldrich), henceforth known as 'exit media'. For ongoing maturation, at 1 day after re-seeding, PAX6⁺ proliferating progenitors were maintained in either basal cortex media, FGF2-supplemented cortex media (20ng/ml) or MEK inhibitor-supplemented cortex media (PD0325901, 1 μ M, Sigma-Aldrich), subsequently referred to as: basal, FGF2 or MEKi conditions, respectively. These cells were passaged 1:2 at D28 before being plated at a final density of 0.2 x 10⁶/cm² in cortex or exit media at D36. One week following final plating neurons were transferred to maturation media containing 1:1 DMEM/F12 and Neurobasal, 1x B27, 1x N2, 1x ITSA, 1x NEAA, 1x GlutaMAX and 0.5x Penicillin Streptomycin, supplemented with brain-derived neurotrophic factor (BDNF, 40ng/ml; R&D Systems), glial cell-line derived neurotrophic factor (GDNF, 40ng/ml), N⁶,2'-O-Dibutyryl adenosine 3',5'-cyclic monophosphate (dcAMP, 0.05mM; Tocris Bioscience), ascorbic acid (200nM; Sigma-Aldrich) and laminin (1 μ g/ml; Sigma-Aldrich).

5.3.2 Flow cytometry

For FACS isolation of PAX6⁺ dorsal telencephalic progenitors D20 cultures were dissociated with Accutase, passed through a 35 μ m cell strainer and resuspended in FACS buffer (80% cortex media, 20% Knockout Serum Replacement (Life Technologies), 10 μ M Y-27632 and 0.5 μ g/ml 4',6-Diamidino-2-Phenylindole (DAPI; Sigma-Aldrich)). Cells were FACS purified using a FACS Aria III (BD Biosciences) or MoFlo XDP (Beckman Coulter) using a 100 μ m nozzle at 20PSI. Threshold PAX6-mCherry positivity was delineated using time-matched H9-derived cortical progenitors. DNA content analysis was carried out using a FxCycle™ kit (Invitrogen), as per the manufacturer's instructions. Cells were pre-fixed by dropwise addition of ice-cold ethanol to a final concentration of 70%. For analysis a univariate cell cycle model was fitted using the cell cycle module within FlowJo.

5.3.3 Cumulative EdU cell cycle analysis

For 5-ethynyl-2'-deoxyuridine (EdU) experiments, D20 FACS-purified PAX6⁺ progenitors were seeded at $0.45 \times 10^6/\text{cm}^2$ in cortex media. 24 hours later cultures were transitioned to cortex media containing FGF2 or 1 μM MEKi. EdU (5 μM) was added to wells sequentially 20, 16, 8, 6, 4, 3, 2, 1 and 0.5 hours prior to simultaneous fixation in 4% PFA. Cultures were permeabilised in 0.3% Triton-X for 20 minutes before visualisation via a click reaction as per manufacturer's instructions (Click-iT EdU kit, Life Technologies). Azide bound Alexa-488 or -555 were used to visualise EdU incorporation followed by secondary immunohistochemistry to delineate specific populations. The growth fraction, defined as the average maximal percentage of Edu⁺/Ki67⁺ of the total Ki67⁺ population (16 and/or 20-hour pulse lengths dependent on total cell cycle length), varied between 85-100% of Ki67⁺ progenitors. For analysis of T_c - T_s and T_s length, linear regression models were plotted and extended during the linear phase of EdU accumulation (0.5-8 hour pulse lengths) (Arai et al., 2011; Nowakowski et al., 1989; Turrero García et al., 2016). T_{G2} was calculated as the half-maximal time of EdU accumulation within mitotic (PH3⁺) progenitors, which represents the average transit duration from S-phase to mitosis (for details of calculation see Arai et al., 2011; Nowakowski et al., 1989; Turrero García et al., 2016).

5.3.4 Gene expression analysis

For quantitative PCR (qPCR), hPSC-derived cortical cultures were isolated using Accutase® (Stem Cell Technologies) & pelleted at 1.6×10^3 rpm for 4 min. Cell pellets were resuspended in 40 μl RNeasy lysis buffer (Qiagen) and transferred to 1.5 ml DNase/RNase-free centrifuge tubes. Samples were then stored at -80°C for subsequent RNA isolation. Briefly, primer-specific mRNA was amplified using in-house designed primers and PowerUp™ SYBR™ Master Mix (ThermoFisher) under the following conditions: 50°C for 2 min, 95°C for 2 min & 40 cycles of; 95°C for 15 s & 60°C for 1 min. All gene expression data was determined using $2^{-\Delta\Delta C_t}$ from at least 3 three independent biological replicates. Table 2.4 contains a list of all primers.

5.3.5 Immunohistochemistry, Microscopy and Quantification

Cells were fixed in 4% (w/v) paraformaldehyde for 10 minutes, washed and incubated in PBS-Azide (0.02% w/v) with 10% (w/v) donkey serum, 0.3% Triton-X and containing primary antibodies (see table 2.2) overnight. Cells were subsequently blocked with 10% donkey serum

before secondary antibodies (AlexaFluor-488, -555 or -647; Jackson ImmunoResearch) were added for 1.5 hours. Nuclei were counterstained with DAPI. Images were captured on a Zeiss Axio ObserverZ.1 upright epifluorescence microscope or a Zeiss LSM780 confocal microscope. For quantification of laminar specification, tiled images across whole 96 wells were generated and at least five fields of view across duplicate wells quantified for TBR1/BRN2/CTIP2 cell number. For cleavage plane analysis a tangential line was drawn at the rosette centre. A second intersecting line was drawn through clearly dividing PH3⁺ cells and the angle between the two lines calculated and binned as vertical (0-30°), i.e. proliferative, or horizontal/oblique (30-90°), reflective of neurogenic division.

5.3.6 Electrophysiology

Whole-cell patch-clamp experiments were performed on PAX6-mCherry derived neurons at D80. Pipettes (3-6M Ω) were filled with low Cl⁻ intracellular solution (pH 7.3 and 290 ± 5 mOsmol). *Voltage-clamp*: following entry into the cell, neurons were maintained in voltage clamp at -60mV and excitatory post synaptic currents were recorded for at least one minute. Cells were then held at -40mV for one minute to elucidate inhibitory synaptic currents. *Current-clamp*: recording was then switched to current clamp (at $I = 0$) and resting membrane potential was recorded for 30 seconds. Next, current was injected into the cell to bring the membrane potential to -60mV and a 10pA stepping protocol ($n=20$, 400ms, first step at -20pA) was applied. Cells were then switched back to voltage clamp and IKA activation and IKA inactivation protocols were applied to validate the quality of recording, according to previously described methods (Niclis et al., 2017b).

5.4 Results

5.4.1 PAX6⁺ cortical progenitors form both deep- and superficial-layer cortical neurons

To assess the role of Notch and FGF signalling pathways in cortical lamination we first validated that both DL- and UL-neurons could be generated in our hands using a wildtype H9 hESC line as well as the HES3 PAX6-mCherry reporter line, that enabled selective purification of human dorsal forebrain progenitors. During primate neurogenesis, PAX6 expression is limited to the VZ, ISVZ and OSVZ and thus is an ideal marker to delineate cortical progenitors *in vitro* (for review

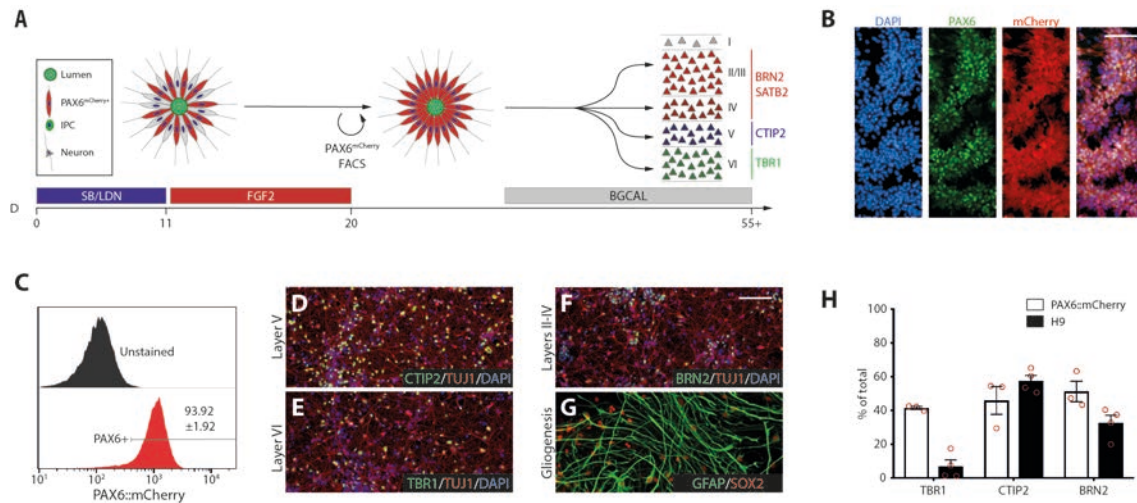


Figure 5.1 - PAX6⁺ cortical progenitors form both DL and UL cortical neurons.

(A) Differentiation schematic. hPSC were exposed to dual-SMAD conditions to generate neuroectoderm before being expanded and pushed to a dorsal telencephalic identity with FGF2. FACS isolation for PAX6-mCherry occurred at D20. Cultures were maintained until at least D55 for analysis of their laminar phenotype. (B) The PAX6-mCherry reporter faithfully mirrored PAX6 protein expression *in vitro*. (C) FACS separation and quantification of PAX6⁺ cortical progenitors at D20 (n = 5). (D-F) Long-term culture (D55) yielded cortical neurons expressing markers of layers 5 (D), 6 (E) and 2-4 (F). (G) Glial differentiation, as seen by GFAP/SOX2 co-expression, was observed after extended culture (>80D). (H) Quantification of TBR1⁺ layer 6 neurons, CTIP2⁺ layer 5 neurons and BRN2⁺ UL 2-4 neurons (n=3-4, data represents mean ± SEM). Scale bars: 50µm (B) and 100µm (D-G).

see Manuel et al., 2015). Immunocytochemical analysis showed mCherry to be a robust indicator of PAX6⁺ dorsal telencephalic progenitors (Figure 5.1B). High efficiency cultures (>90% PAX6⁺) were nevertheless purified to ensure that subsequent experiments were conducted on the selected population only (Figure 5.1C). Replated PAX6⁺ progenitors gave rise to multiple excitatory neuronal subtypes including layer 6 TBR1⁺, layer 5 CTIP2⁺ and layers 2-4 BRN2⁺ neurons by D55, as well as GFAP⁺ astrocytes after further (>80D) culture (Figures 5.1D-G). Long-term neuronal cultures contained a residual population of SOX2⁺ neural progenitors throughout differentiation (Figure 5.1G), highlighting the asynchronicity of cortical differentiation *in vitro*. Similar to previous studies (Espuny-Camacho et al., 2013; Shi et al., 2012), quantification revealed that each layer was formed relatively equally by D55 (Figure 5.1H), however significant line-to-line variability in the generation of TBR1⁺ neurons was observed, likely reflecting intrinsic differences in maturation rate and UL neurogenesis. Despite this, deep-layer TBR1⁺ and/or CTIP2⁺ cortical neurons were generated robustly in our basal culture conditions after PAX6 isolation and therefore provided an amenable system to probe signalling pathways that might alter layer-specificity.

5.4.2 Notch inhibition rapidly generates TBR1⁺, early born cortical neurons

Notch signalling has been shown to regulate proliferation/neurogenic fate decisions in develop-

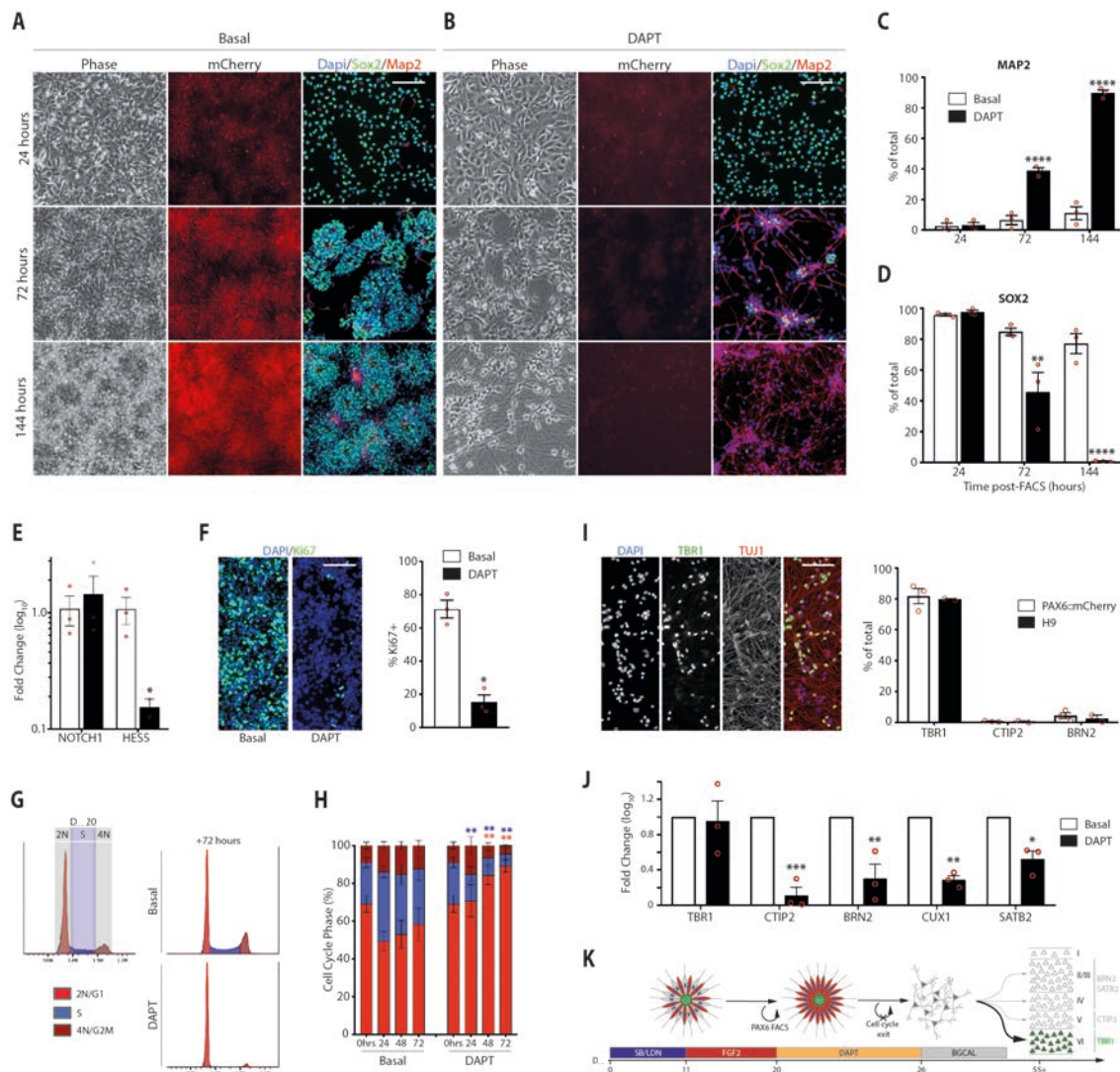


Figure 5.2 - Notch inhibition of PAX6⁺ progenitors rapidly generates TBR1⁺, early born cortical neurons.

(A) Under basal conditions (absence of DAPT), FACS isolated cortical progenitors rapidly reformed PAX6⁺/SOX2⁺ rosettes that were visible within 72 hours, persisted for >7days and showed sparse presence of MAP2⁺ neurons. (B) DAPT treatment of FACS sorted progenitors downregulated PAX6-mCherry expression, rapidly generating neurons and depleting the proliferative Sox2⁺ pool within 72 hours. (C-D) Quantification for MAP2⁺ neurons or SOX2⁺ cortical progenitors after DAPT treatment. (E) DAPT had no effect on the expression of the NOTCH1 receptor but significantly decreased downstream HES5 expression 48 hours after treatment, confirming activity of the inhibitor. (F) Evident of forced cell cycle exit, DAPT rapidly reduced the number of Ki67⁺ cycling progenitors 48 hours after treatment. (G) Example FACS-based PI analysis of DNA content in basal and DAPT-treated cortical progenitors. (H) Quantification revealed that DAPT reduced the number of progenitors entering S-phase within 24 hours. (I) Neurons generated after DAPT-induced cell cycle exit were almost exclusively TBR1⁺ at D55. (J) qPCR confirmed that DAPT-treatment reduced *CTIP2*, *BRN2*, *CUX1* and *SATB2* expression, indicative of other laminar fates. (K) Schematic of DAPT-induced cell cycle exit highlighting that D20 PAX6⁺ progenitors are only competent to generate early born, layer 6 TBR1⁺ neurons. Scale bar is 100µm. Data represents mean ± SEM, Multiple t-tests with Holm-Sidak correction, n=3, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

-ing mammalian cortical progenitors (Fiddes et al., 2018; Mizutani et al., 2007; Rash et al., 2011; Suzuki et al., 2018). To investigate the role of Notch in human cortical lamination *in vitro* we treated cultures immediately following FACS isolation of PAX6⁺ cortical progenitors with the γ-

secretase inhibitor DAPT. DAPT acts to prevent the final cleavage of transmembrane Notch, and thereby decreases levels of the active Notch intracellular domain (NICD). Under basal conditions PAX6-mCherry⁺/SOX2⁺ rosettes rapidly reformed after sorting (Figure 5.2A) and the proportion of neurogenic divisions remained low after a week of further culturing, as confirmed by the low proportion of MAP2⁺ neurons ($11.1 \pm 4.2\%$ MAP2⁺) and persistent presence of SOX2⁺ progenitors (Figures 5.2A-D). In contrast, DAPT treated cultures did not reform rosettes, downregulated PAX6-mCherry expression, lost neural progenitor marker SOX2 and rapidly generated MAP2⁺ neurons ($89.8 \pm 1.9\%$) (Figures 5.2B-D). Repression of the traditional Notch target gene, HES5, after 48 hours confirmed DAPT successfully inhibited Notch signalling downstream of the NOTCH1 receptor (Figure 5.2E).

To further validate the rapid maturation of cortical progenitors after DAPT treatment we assessed their cell cycle state. Assessment of Ki67⁺ cycling progenitors, 48hrs after DAPT treatment, revealed that only $15.3 \pm 4.3\%$ of the cells remained as Ki67⁺ cycling progenitors (compared to $71.2 \pm 5.3\%$ under basal conditions) (Figure 5.2F). In addition, DNA content, analysis using propidium iodide (PI) labelling and FACS analysis (Figure 5.2G), at defined intervals after DAPT treatment (0, 24, 48 and 72 hours), revealed inhibited G1/S-phase transitions as early as 24 hours after treatment, that peaked after 72 hours (Figure 5.2H, blue bars: $6.4 \pm 3.3\%$ DAPT, $29.4 \pm 6.2\%$ basal), while the proportion of cells in G1 increased concomitantly (red bar: $89.3 \pm 3.2\%$ DAPT, 58.4% basal). Strikingly, analysis of the laminar identity of DAPT-induced neurons after ongoing maturation (D55) revealed the almost exclusive expression of TBR1⁺, early-born layer 6 neurons (Figure 5.2I). Importantly, this was consistent across multiple pluripotent stem cell lines (Figure 5.2I), and in contrast to basal conditions where progenitors gave rise to multiple layers of the cortex (Figure 5.1H). The loss of other layer phenotypes, including layers 5 (*CTIP2*) and 2-4 (*BRN2*, *CUX1*, *SATB2*) was confirmed using qPCR (Figure 5.2J). Therefore, D20 PAX6⁺ dorsal forebrain progenitors that exit the cell cycle prematurely in response to Notch inhibition are competent to form layer 6, TBR1⁺ cortical neurons only, consistent with their early maturation state (Figure 5.2K).

5.4.3 Loss of FGF signalling induces layer 5-like CTIP2⁺ neurogenesis

Next, we probed the role of FGF signalling in human cortical progenitor fate either via extending the period of exogenous FGF2 (from D12–20 to D12–35) or by the addition of PD0325901, a potent MAPK/ERK pathway inhibitor (MEKi, from D21–35), downstream of FGFR activity (Figure 5.3A). Following treatment neural progenitors were replated at low density to assess their lami-

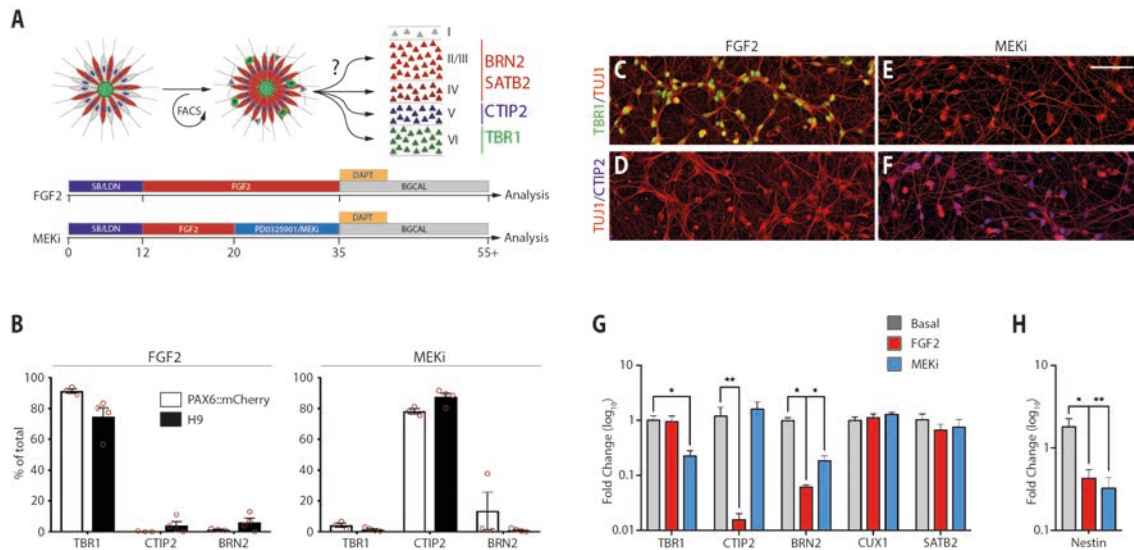


Figure 5.3 – Differential layer phenotype after FGF2 or MEK inhibition.

(A) Sorted PAX6⁺ progenitors were treated with FGF2 or PD0325901, a potent MEK inhibitor (MEKi), between D21-D35 and analysed for their laminar phenotype at D55. (B) Quantification of layer subtype markers at D55 revealed that FGF2 treatment generates TBR1⁺ neurons, while MEK inhibition generates CTIP2⁺ neurons. (C-F) Immunohistochemical staining for TBR1⁺ or CTIP2⁺ neurons at D55. (G) qPCR further confirmed our quantification. FGF2 addition reduced *CTIP2* and *BRN2A* expression, while MEK inhibition decreased *TBR1* and *BRN2* expression, relative to basal conditions, which generates all layer subtypes. (H) FGF2 or MEKi conditions both decrease *Nestin* expression relative to basal, highlighting DAPT-induced maturation and the persistence of immature progenitors within the basal group. Scale bar is 25 μ m. Data represents mean $\pm$ SEM, One-way ANOVA with Dunnet correction, n=3, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

-nar specification after subsequent DAPT-induced cell cycle exit (Figure 5.3A). Neurons born after extended FGF2 treatment almost exclusively became early-born TBR1⁺ cortical neurons by D55 (Figures 5.3B, C), in contrast to basal culture conditions where FGF2 withdrawal at D20 resulted in both DL and UL cortical neurons (Figure 5.1H). To our surprise, cortical neurogenesis following MEK inhibition was restricted to a later-born, layer 5-like CTIP2⁺ phenotype, at the expense of TBR1⁺ neurogenesis (Figures 5.3B, F). Importantly, the shift from TBR1⁺ to CTIP2⁺ neurogenesis under FGF2 or MEKi conditions, respectively, was consistent across two independent ESC lines (Figure 5.3B). Analysis by qPCR at D55, confirmed the specificity of TBR1⁺ neurogenesis after FGF2 addition at the expense of progressively superficial layers expressing CTIP2 or BRN2. MEKi-induced layer 5 CTIP2 selectivity could also be validated by the significant downregulation of layer 6 *TBR1* and UL *BRN2* mRNA expression (Figure 5.3G). FGF2 or MEKi did not alter *CUX1* or *SATB2* expression, likely due to their upregulation in UL-neurons only after extended culture times (Figure 5.3G). *NESTIN*, an intermediate filament protein expressed on neural precursors, was downregulated following DAPT treatment, indicative of neuronal maturation, in contrast to cortical progenitors under basal conditions that showed maintained expression (Figure 5.3H).

Electrophysiological analysis on maturing cultures (D80) revealed that neurons derived after FGF2 and/or MEKi conditions had significantly lower resting membrane potentials compared to basal cultured neurons, indicative of their advanced maturation state (Supplementary Figure 5.1A). Neurons from all groups fired action potentials (AP) in response to depolarising current steps (demonstrating that FGF2/MEKi culture conditions and DAPT-induced exit did not alter neuronal function) and generally displayed a mix of both of mature (AP trains) and immature (AP collapse) AP behaviour (Supplementary Figure 5.1B). Most FGF2-derived TBR1⁺ neurons fired AP trains, in contrast to neurons derived under MEKi or basal conditions which often failed to generate more than a single AP or demonstrated rapid AP collapse (Supplementary Figure 5.1C). Reflective of maturation and glutamatergic neuronal circuitry, spontaneous excitatory post-synaptic potentials (EPSP) were detected in all culture conditions and enriched in the MEKi condition (Supplementary Figure 5.1D).

5.4.4 Aberrant FGF signalling retards normal cortical laminar fate and blocks pro-neural gene expression

Laminar identity has been shown to be directed both cell autonomously during cortical progenitor expansion within the ventricular and subventricular zones (Bayraktar and Doe, 2013; Borrell and Calegari, 2014; Dominguez et al., 2013; Lui et al., 2011), as well as during post-mitotic maturation within the cortical plate (Leone et al., 2015; McKenna et al., 2015; Ozair et al., 2018; Toma et al., 2014). To understand how FGF2 or MEK inhibition generate distinct neuronal phenotypes we, therefore, characterised cultures during their ongoing maturation, prior to cell cycle exit. Cultures were examined acutely (D26) and after extended maturation (D35), corresponding to 5 and 14 days of treatment. Consistent with previous reports (for review see Delaunay et al., 2017), ongoing maturation and consequential asymmetric divisions increasingly generated TUJ1⁺ neurons under basal and MEKi conditions (basal $15.2 \pm 1.9\%$ at D26, $25.8 \pm 3.2\%$ at D35; MEKi $12.5 \pm 1.1\%$ at D26, $35.6 \pm 5.7\%$) (Figures 5.4A-C). However, this effect was blocked after prolonged FGF2 treatment (FGF2 $6.0 \pm 1.3\%$ at D26, $6.2 \pm 1.0\%$ at D35) (Figures 5.4A-C).

The majority of generated neurons were early-born, TBR1⁺ at D26, regardless of culture condition (Figure 5.4D), suggestive of appropriate DL neuronal identity. At this stage CTIP2⁺ neurons were absent (<1%) from all groups at D26, indicating that progenitors were still developmentally immature and competent only to generate TBR1⁺ layer 6 neurons. Under basal conditions TBR1⁺ proportions increased and low numbers of layer 5 CTIP2⁺ neurons could be

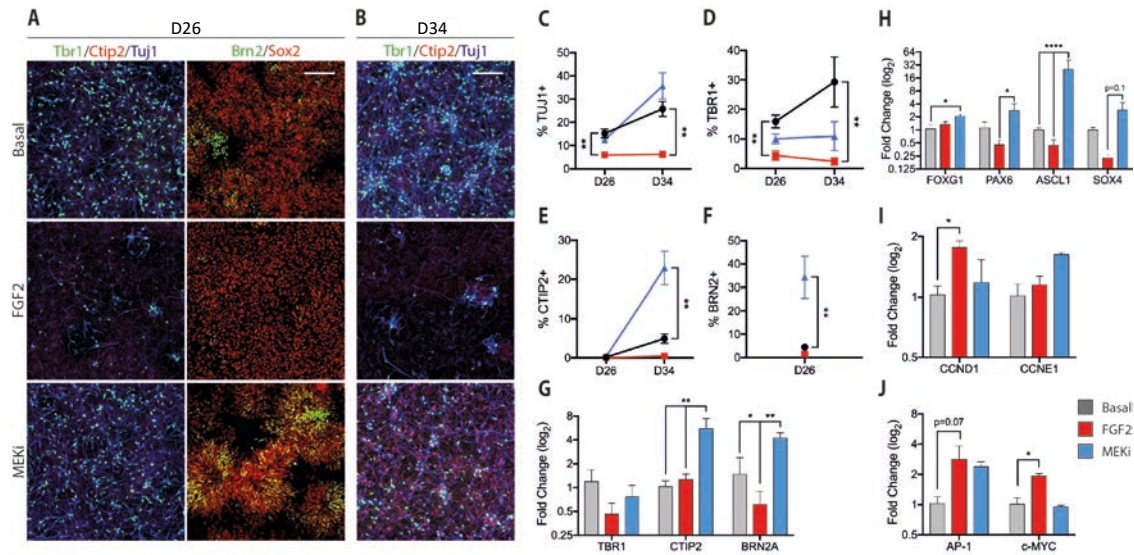


Figure 5.4 – MEK inhibition accelerates the development of CTIP2-competent progenitors through activation of a pro-neural gene network.

(A) Under basal and MEKi conditions TBR1⁺ but not CTIP2⁺ neurons were born at D26. In contrast, FGF2 conditions blocked neurogenesis. BRN2 expression, within SOX2⁺ cortical progenitors, was significantly increased after MEKi. (B) By D35 CTIP2⁺ neurons began to be generated under basal conditions but were absent after FGF2 treatment, while almost all neurons under MEKi became CTIP2⁺. (C-F) Quantification of neurons and layer markers at D26 and D35. BRN2 is quantified only at D26. (G) qPCR at D26 highlights that *CTIP2* and *BRN2A* mRNA expression was increased after acute MEK inhibition. (H) The pro-neural genes *FOXG1*, *PAX6* and *ASCL1* were increased at D26 within MEKi treated cultures, corresponding to an increase in CTIP2⁺ neurogenesis. (I) FGF2 treated cultures had increased levels of *CCND1*, required for G1/S-phase transition, while MEKi did not inhibit cyclin expression. (J) Confirmation that FGF2 induces *AP-1* and *c-MYC*, traditional downstream effectors of FGFR activation. Scale bar is 100μm. Data represents mean ± SEM, One-way ANOVA with Dunnett correction, n=3-5, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

observed within progenitor cultures by D35. CTIP2⁺ cells remained absent following extended FGF2 treatment yet were highly enriched after MEK inhibition (basal $4.9 \pm 1.2\%$ and MEKi $22.9 \pm 4.3\%$) (Figure 5.4E), reflective of an accelerated layer 5 specification. The proportion of BRN2⁺ progenitors (BRN2⁺/SOX2⁺) was increased after MEK inhibition and was absent in progenitors in basal or FGF2 conditions (basal $4.5 \pm 1.0\%$; FGF2 $1.5 \pm 0.9\%$; MEKi $34.4 \pm 8.9\%$) (Figure 5.4A, F). Rare, BRN2⁺/SOX2⁻ cells were observed under basal conditions, suggestive of limited UL neurogenesis (Figure 5.4A).

Supporting these changes in lamination, transcriptional profiling revealed a significant upregulation of the key regulator of layer 5 neurogenesis, *CTIP2*, at D26 in MEKi treated cultures (Figure 5.4G), preceding the appearance of these neurons at D35 (Figure 5.4E). Similarly, *BRN2* mRNA levels reflected increased protein levels at D26. Moreover, chronic regulation of FGF signalling in these cultures resulted in significant changes to the pro-neural transcriptional program after 5 days of treatment (D26) with *FOXG1*, *PAX6*, and *ASCL1* transcripts all increased after MEKi-treatment. *PAX6*-mCherry expression further confirmed its regulation by FGF2 and MEKi (Supplementary Figure 5.2). Critically, these changes in neurogenic competence are not

accompanied by alterations in the expression of cell cycle-related transcripts *CCND1* and *CCNE1*, which are crucial for the G1/S-phase transition, indicating that in early progenitors, upregulation of pro-neural genes does not force cell cycle exit (Figure 5.4I). Confirming the responsiveness of cortical progenitors to exogenous FGF2, we revealed an upregulation of downstream transcriptional genes *AP-1* and *c-MYC* (Figure 5.4J). Responsiveness to MEKi treatment was validated by downregulation of the MAPK/ERK pathway gene *c-MYC*, while expression of *AP-1*, a JNK pathway effector, was unchanged (Figure 5.4J).

5.4.5 Altered FGF signalling impacts cortical progenitor dynamics and cleavage plane orientation

Under basal cortical differentiation conditions, rosettes recapitulate key features of the developing human cortex, as previously described (Eiraku et al., 2008; Espuny-Camacho et al., 2013; Gaspard et al., 2008; Shi et al., 2012). Notably, apicobasal polarity of PAX6-mCherry⁺/SOX2⁺/Vimentin⁺ radial glia cells (Figures 5.5Ai-ii), radial orientation around a ZO-1⁺ lumen (Figure 5.5Aiii), apical mitoses (Figure 5.5Aiii) and emergence of peripheral TBR2⁺ intermediate progenitor cells at a distance from the lumen (Figure 5.5Aiv), together mimicking developing mammalian cortical progenitor behaviour *in vivo*. Under basal conditions at D26, $88.2 \pm 1.7\%$ of cells were SOX2⁺ neural progenitors (data not shown) and $67.2 \pm 3.3\%$ were proliferating Ki67⁺ progenitors (Figure 5.5C). By D35 only $35.1 \pm 1.9\%$ remained Ki67⁺ while the proportion of TBR2⁺ IPCs increased from $18.1 \pm 1.7\%$ to $39.8 \pm 4.6\%$ (Figure 5.5D), in line with a progressive maturation of cortical cultures. FGF2 treatment notably reduced rosette formation and apicobasal polarity, resulting in a decrease in ZO-1⁺ lumen size (Figures 5.5Aiii, E). Strikingly, despite similar proportions of proliferative cells (Ki67⁺) to basal conditions, <5% of progenitors progressed to TBR2⁺ IPCs at D35 following extended FGF2 treatment (Figure 5.5D). In contrast, acute MEK inhibition resulted in larger rosettes with larger lumens (ZO1⁺ diameter: MEKi, $13.8 \pm 0.2\mu\text{m}$ and basal, $9.3 \pm 0.8\mu\text{m}$ at D26; Figure 5.5E), that otherwise resembled basal conditions in cytoarchitectural organisation and capacity for generating TBR2⁺ IPCs (MEKi $37.6 \pm 3.1\%$ and basal $39.8 \pm 4.6\%$ at D35; Figure 5.5D). Importantly, caspase-3 analysis confirmed that FGF2 or MEKi did not alter progenitor dynamics via selective apoptosis or death above those observed under basal conditions (Supplementary Figure 5.3).

Symmetric divisions generating two proliferating daughter cells are associated with a perpendicular, vertical cleavage plane relative to the ventricular surface, while parallel, oblique

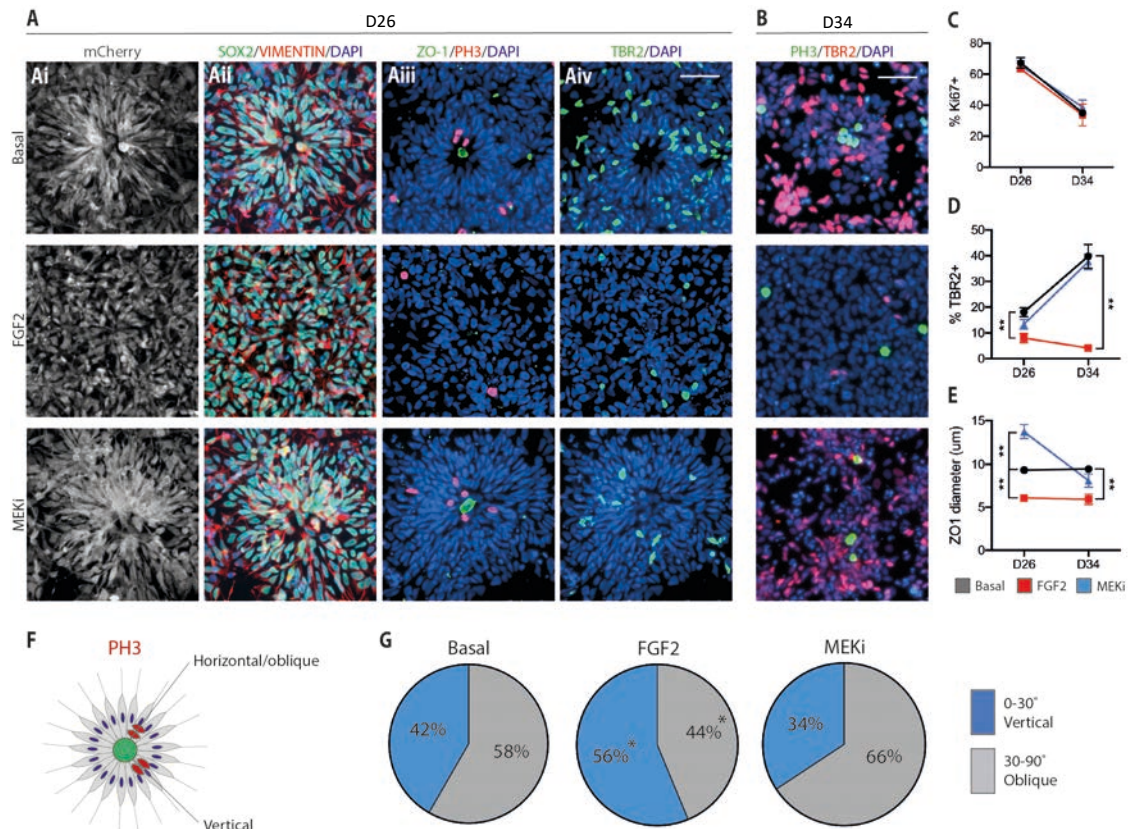


Figure 5.5 – Progenitor organisation and cleavage plane highlight immaturity of FGF2-treated progenitors.

(A) Cortical rosettes under basal conditions recapitulated *in vivo* cortical development, generating apicobasal polarity, ZO1⁺ lumens, PH3⁺ apical mitoses and TBR2⁺ intermediate progenitor cells (IPC) located basally to the lumen. Rosette organisation and TBR2⁺ IPC were severely diminished after FGF2 treatment and unaffected by MEK inhibition. (B) At D35, increased IPCs were seen in basal and MEKi conditions but were extremely rare after FGF2 treatment. (C) No difference in the total number of Ki67⁺ cycling progenitors was observed. (D) Quantification for TBR2⁺ IPCs at D26 and D35 highlighted that FGF2 treated cultures do not generate IPCs. (E) MEK inhibition generated larger rosettes as demonstrated by increased ZO-1 lumen diameter. (F) Diagram of quantification method for determining the cleavage angle of dividing, PH3⁺ cortical progenitors. (G) FGF2-treated progenitors preferentially underwent symmetrical, proliferative divisions as shown by the increase in vertical cleavage planes. Scale bar is 50µm. Data represents mean ± SEM, One-way ANOVA with Dunnett correction, n=3-5, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

cleavage plane angles are associated with asymmetric division and neurogenesis (Kosodo et al., 2004; Noctor et al., 2004; Subramanian et al., 2017), likely due to differential inheritance of key cellular and molecular machinery (Chenn and McConnell, 1995; Fish et al., 2006). In order to assess cleavage plane within our *in vitro* culture system we labelled fixed cultures at D26 with ZO-1 and PH3 and assessed the cleavage plane of recently divided cells at the ventricular surface, relative to the radial orientation of the rosette (Figure 5.5F). Under basal conditions both horizontal/oblique (58%) and vertical (42%) cleavage planes were observed, reflecting the proliferative and neurogenic mix of divisions within heterogeneous cortical cultures (Figure 5.5G). After FGF2 treatment, significantly more (56%) divisions had a proliferative-associated vertical cleavage plane, while MEKi treated cultures were unchanged from basal conditions (Figure 5.5G). Taken together, FGF2 inhibits the normal development of cortical progenitors, restricting

Table 5.1 Cell cycle analysis of total cortical progenitor pool at D26 and D35 under basal, FGF2 or MEKi conditions.

D	Group	T _C T _S	T _S	T _S (% of T _C)	T _C	T _{G2}	T _M	T _{G1}	T _{G1} (% of T _C)
26	Basal	16.5	15.5	(48.4)	32.1	4.7	0.7	11.1	(34.7)
	FGF2	15.6	12.7	(44.7)	28.3	5.4	0.4	9.9	(34.9)
	MEKi	16.8	17.1	(50.3)	33.9	4.9	0.8	11.1	(32.9)
35	Basal	18.3	21.1	(53.5)	39.4	5.8	0.4	12.1	(30.7)
	FGF2	16.8	15.5	(47.9)	32.3	5.9	0.2	10.8	(33.4)
	MEKi	20.3	16.6	(44.9)	36.9	6.4	0.7	13.2	(35.9)

Cumulative EdU labelling at D26 and D35 were used to quantify the average cell cycle phase duration within cultures under basal, FGF2 or MEKi conditions. FGF2 treated cultures demonstrated a consistently lower total cell cycle length (T_C) further reinforcing their early phenotype.

them to a proliferative, early VZ-like phenotype. In contrast, MEKi appears to accelerate acquisition of layer V-like CTIP2⁺ neurons without precocious commitment to a fully differentiated phenotype.

5.4.6 Cell cycle analysis highlights early progenitor characteristics of FGF2 treated cultures

The observation that FGF2 treated cultures upregulated CCND1 and preferentially divided with a vertical cleavage plane led us to further investigate how FGF2 and MEKi influence cell cycle kinetics. Utilising a cumulative EdU labelling protocol (Nowakowski et al., 1989), at D26 and D35 we observed consistent EdU labelling restricted to the Ki67⁺ cycling progenitor pool at all timepoints and across all three conditions (Figure 5.6A, representative image). Unfortunately, due to the nature of the EdU staining protocol we could not readily distinguish progenitor subpopulations (e.g RG, IPC, etc.). Therefore, we restricted our analysis to the total pool of proliferating progenitors. No significant difference in the proportion of total cells (DAPI⁺Ki67⁺) entering the cell cycle was observed. Over time, an increasing proportion of cycling progenitors entered S-phase and incorporated EdU (Figure 5.6B). T_C-T_S ((total cycle length)-(S-phase length)) is indicated by the plateau in EdU reactivity, where EdU⁺ cells have completed a full cycle and re-enter the S-phase (Figure 5.6B – arrows). As previously shown in both rodents and primates (Arai et al., 2011; Betizeau et al., 2013; Subramanian et al., 2017; Turrero García et al., 2016), under basal conditions the total length of the cell cycle increased over development from 32.1 hours at D26 to 39.4 hours at D35, predominantly via extension of the S-phase (+5.6 hours) (Figures 5.6B, D; Table 5.1). Extension of total cell-cycle length over time was common across all groups (Figure 5.6D; Table 5.1). We found that FGF2 cultures consistently had shorter cell-cycle lengths across the two timepoints analysed, while MEKi had only a small, acute effect on

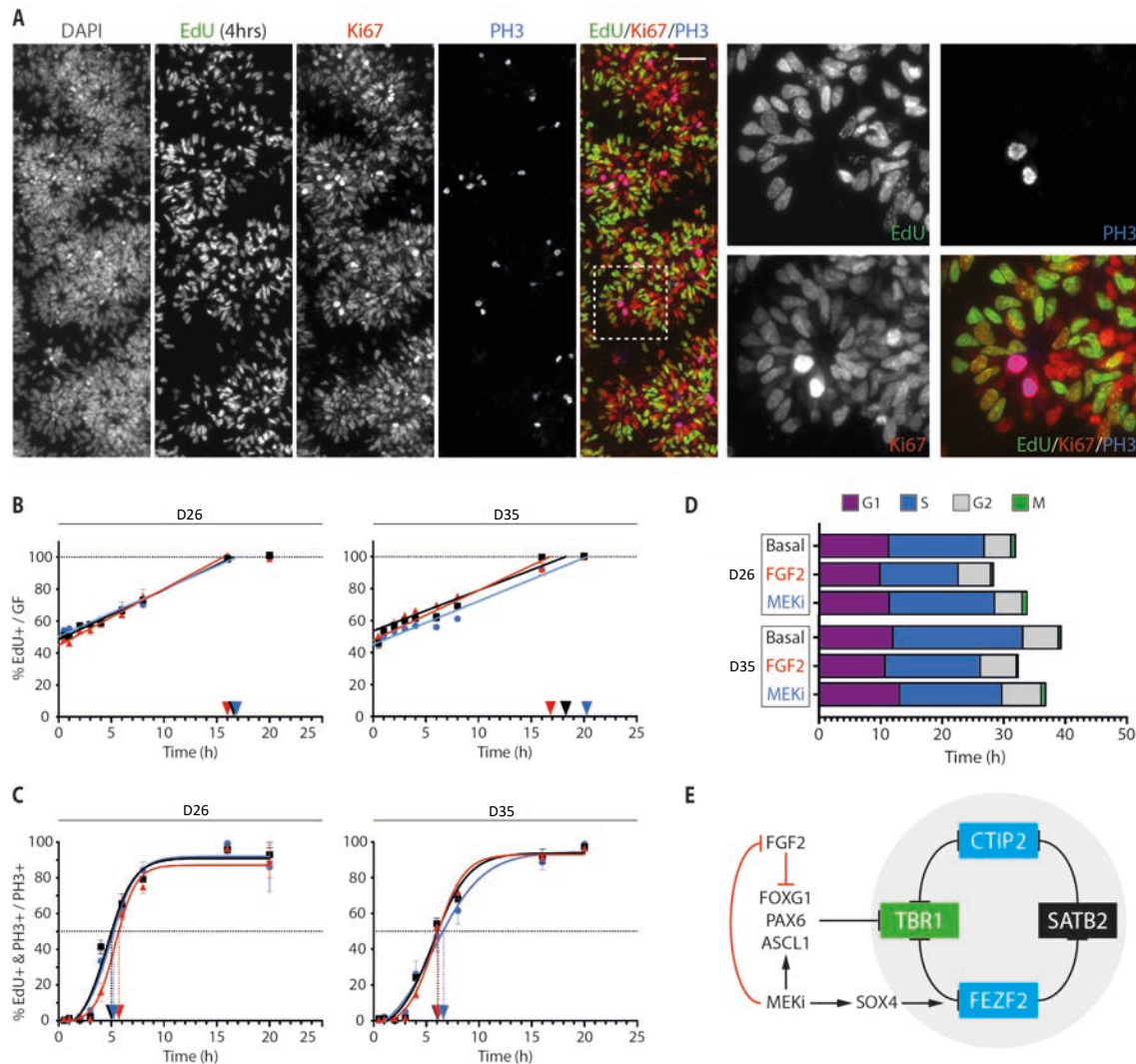


Figure 5.6 – Cell cycle analysis highlights early progenitor characteristics of FGF2 treated cultures

(A) Cumulative EdU labelling was used to probe the cell cycle kinetics of cortical progenitors in basal, FGF2 or MEKi conditions. EdU is incorporated in the S-phase and the total cycling pool was delineated by Ki67 immunoreactivity. Inset shows high magnification of the dotted area. Note that the two PH3⁺ mitotic progenitors are EdU⁻ at this time point (4 hours), indicating that they had exited S-phase prior to EdU addition. (B) Plots of EdU⁺ cycling progenitors normalised to the growth fraction (GF) at D26 and D35. GF is defined as the maximal percentage of EdU⁺/Ki67⁺ of total Ki67⁺ proliferating cells and ranged between (85.5-96.1%). Arrow heads represent the average time taken to reach EdU saturation and represent the T_C - T_S interval. The x-intercept is T_S . Data is from 3 independent experiments in duplicate. (C) Plots of EdU⁺/PH3⁺ of total PH3⁺ and fitted sigmoidal curves at D26 and D35. The time taken to 50% is the average time from S-phase incorporation of EdU to mitosis and hence represents average G2. Data from 3 independent experiments in duplicate. (D) Representation of total cell cycle and individual G1, S, G2 and M-phase length under basal, FGF2 or MEKi conditions highlights that FGF2-treated cultures have shorter cell cycle lengths. (E) Simplified diagram highlighting the possible roles of FGF2 and MEK inhibition in laminar competence within cortical progenitors. FGF2 represses the expression of pro-neural genes and in conjunction with alterations in cell cycle kinetics maintains progenitors in an early TBR1-competent state. In contrast, MEKi induces the pro-neural network which represses TBR1, in turn derepressing the layer 5 CTIP2⁺ transcriptional cascade and may additionally exert a layer 5 fate through cis-activation of FEZF2.

lengthening the cell cycle (Figures 5.6B, D; Table 5.1). Analysis of the average G2 duration revealed only minor differences between the groups across both timepoints (Figures 5.6C, D; Table 5.1). Finally, analysis of the relative proportion of each major cell-cycle phase revealed that FGF2-treated progenitors consistently exhibit a shortened S-phase (Figure 5.6D). These

findings are in line with *in vivo* reports where FGF2 serves to expand the progenitor pool in RG (Rash et al., 2011) and further confirms that FGF2 maintains hPSC-derived cortical progenitors as immature by promoting proliferative divisions and limiting development of multiple neurogenic and intermediate characteristics. However, the small differences observed between groups at each timepoint indicates that total cell cycle length may only play a small role in laminar progression.

5.5 Discussion

In this study we show, in a reductionist model system, that FGF2-MAPK signalling can influence the laminar fate of human PSC-derived DL neurons *in vitro*. Utilising a PAX6-mCherry reporter line to selectively isolate dorsal telencephalic progenitors, we confirmed that PAX6⁺ cortical progenitors sequentially generate progressively superficial neurons *in vitro*. DAPT-induced repression of Notch, a technique widely used to force cell cycle exit and promote maturation *in vitro*, was used to ‘crystallise’ the temporal identity of PAX6⁺ progenitors at D20 and demonstrated that these progenitors are competent only to generate TBR1⁺ neurons. Together, these experiments highlight the temporal window of DL neurogenesis and validity of the PAX6⁺ sorting strategy to assess DL neurogenesis within a defined population. Manipulation of FGF-MAPK signalling, by treatment with FGF2 or MEKi, can halt or accelerate the development of progressive layer competence within the human cortical progenitor pool. Exogenous FGF2 signalling resulted in the maintenance of an early RG phenotype, reflected in gene expression patterns, cell cycle kinetics, cell fate decisions and laminar competence. In contrast, MEKi, a downstream effector of FGFR signalling, accelerates the acquisition of layer 5 competence at the expense of TBR1⁺ layer 6 neurons, such that DAPT-induced cell cycle exit at D35 yielded CTIP2⁺ neurons almost exclusively. Furthermore, we demonstrate that this effect is, in part, mediated through upregulation of a pro-neural gene network including *ASCL1*, *PAX6* and *FOXG1* that together repress the default TBR1⁺ layer 6 identity and promote CTIP2⁺ neurogenesis (Figure 5.6E).

In the rodent FGFR1 and FGFR3 signalling have been shown to be critical for controlling proliferation and size of the cortex, with their loss leading to premature depletion of the progenitor pool and decreased cortical volume (Maric et al., 2007; Raballo et al., 2000; Rash et al., 2011). In contrast, FGFR2 signalling controls the differentiation and maturation of RG from NESCs (Sahara and O’Leary, 2009; Yoon et al., 2004). FGF2, which binds FGFR1 and FGFR3 with

high affinity, exerts a mitogenic effect on cortical progenitors by altering cell cycle and fate decisions, consistent with their role *in vivo* (Lukaszewicz et al., 2002; Maric et al., 2007). Moreover, FGF2 is routinely used as a mitogenic agent in hPSC culture and differentiation protocols. However, the role of FGF2 signalling on human cortical progenitors has not previously been explored. The findings reported here suggest that FGF2 exerts a similar effect on human cortical progenitors as that previously shown in the rodent. In particular, FGF2 blocks the developmental progression of RG, such that, D35 cultures remain competent only to generate TBR1⁺ neurons. In our hands, FGF2 treated cultures failed to undergo differentiative/neurogenic divisions that generate TBR2⁺ IPCs and TUJ1⁺ neurons and instead cells preferentially underwent proliferative, symmetrical divisions as observed in the mouse (Kang et al., 2009). Consequently, we conclude that FGF2 is a crucial signal mediating the expansion and subsequent differentiation of human cortical progenitors but not laminar competence. Of particular interest, this result confirms, in human cortical progenitors, that laminar competence is not linked to the number of divisions, as has been demonstrated in the mouse (Ohtsuka and Kageyama, 2019; Okamoto et al., 2016). Instead, FGF2 shortens cell cycle length and diminishes laminar progression, supporting cell cycle length as a critical mediator in facilitating the build-up of neurogenic determinants (Calegari et al., 2005; Dehay and Kennedy, 2007; Ohtsuka and Kageyama, 2019; Pilaz et al., 2009; Watanabe et al., 2015).

While the repression-derepression model within neocortical neurons is widely accepted, whereby transcription factors driving each layer repress other layer identities (Figure 5.6E, circle), the factors that govern temporal progression in RG and thus, layer-competence, have remained relatively unknown. *Acsl1* and *Neurog2* are required for progression from default *Tbr1* to *Ctip2* neurogenesis in rodents by modulating *Foxg1* (Dennis et al., 2017), which itself determines the sequence of layer generation by repressing *Tbr1* (Kumamoto et al., 2013; Toma et al., 2014). SOX factors mediate progressive DL specification postmitotically by *cis* activation/repression of a *FEZF2* upstream promotor sequence (Kwan et al., 2008; Lai et al., 2008; Shim et al., 2012). In addition, RAS/ERK signalling has been linked to pro-neural lineage decisions during development and disease (Dee et al., 2016; Li et al., 2014), generation of bRG and is increased in human VZ progenitors (Heng et al., 2017). Consistent with a role of FGF2 in delaying progenitor maturation, we observed a downregulation of the pro-neural genes *ASCL1*, *PAX6* and *SOX4* after FGF2 treatment. In the mouse, *Ascl1* is sufficient to specify *Ctip2*⁺ neurogenesis in DL progenitors while loss of *Ascl1* results in decreased *Ctip2*⁺ neurogenesis and expansion of *Tbr1*⁺ neurons into presumptive layer 5 (Dennis et al., 2017). Here, MEK inhibition

rapidly led to increases in *FOXP1* and *ASCL1*, exhaustion of TBR1⁺ neurogenesis and acceleration toward CTIP2⁺ neurogenesis. Together our results suggest that ERK-mediated genetic programs may provide a context-dependent switch during laminar specification, whereby during DL neurogenesis inhibition of FGF-ERK signalling may act to break default TBR1 repression of CTIP2 and thus, drive layer 5 neurogenesis. Further studies will be necessary to confirm the role of FGF-ERK regulation *in vivo* and whether the effect is FGF-specific (i.e. do other factors that influence MAPK-ERK signalling also slow temporal progression of cortical progenitors?).

The unique presence of an OSVZ in primates contributes disproportionately to layers 2-4 and is accompanied by an expanded repertoire of cortical progenitors, including multiple RG subtypes and IPCs (Betizeau et al., 2013; Dehay et al., 2015; Lui et al., 2011; Reillo et al., 2011). In contrast, 2D hPSC-models struggle to generate large numbers of basal progenitors and generate disproportionately low numbers of UL neurons. For these technical reasons, here we have focused on the role of FGF2-MAPK signalling in DL neurogenesis as these layers are generated in a robust temporal sequence *in vitro*. In the mouse, FGF2 expands the initial NESC progenitor pool (Vaccarino et al., 1999), blocks RG/IPC transitions (Kang et al., 2009) and is downregulated by the mid-stage of corticogenesis (Raballo et al., 2000), allowing appropriate time for DL neurogenesis. Our findings suggest that FGF2 may similarly act to block NESC-to-RG transitions and maintain early layer fate exclusively *in vitro*. It will be interesting to explore whether longer-term FGF2 treated cortical progenitors subsequently reproduce the developmental sequence after FGF2 withdrawal. However, maintaining cells under these conditions presents challenges associated with long-term survival and neurogenesis in our hands. FGF-ERK signalling is crucial for aRG-to-bRG transitions during OSVZ expansion (Heng et al., 2017) and therefore MEK inhibition may simultaneously promote CTIP2 identity, while blocking bRG generation and UL neurogenesis. This raises the possibility that FGF-ERK signalling may have a biphasic role in cortical development, first promoting NESC/RG expansion and then later being required for OSVZ expansion and UL neurogenesis. This model fits with observed biphasic changes in cell cycle length during primate corticogenesis (Betizeau et al., 2013). It is tempting to speculate that the prolonged period of human corticogenesis in comparison to primate and rodent may be, in part, due to cell-autonomous increases in FGF-MAPK signalling, which act to simultaneously maintain DL neurogenesis and expansion of the progenitor pool (Otani et al., 2016). Studies utilising organoid culture systems, which more fully give rise to appropriate OSVZ and laminar organisation may illuminate the role of FGF-ERK signalling in human UL neurogenesis.

Taken together these findings provide new insights into the function of FGF/ERK signalling in deep layer specification of the human cortex, roles that are both unique to human development but also display similarities to the rodent brain. Finally, while traditional human PSC-derived cortical differentiation systems produce asynchronous, heterogenous populations of pyramidal neurons and glia, we demonstrate rapid and homogenous differentiation of deep layer specific cortical projection neurons (layer 6 or 5) that may prove valuable for the study, modelling and/or therapeutic development for laminar-specific disorders ([Espuny-Camacho et al., 2013](#); [Shi et al., 2012](#)).

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5.7 Author contributions

All experiments were performed at the Florey Institute of Neuroscience and Mental Health. Conceptualisation: CG, JN, CH and CP. Investigation: CG, CH, VP. Analysis: CG and CH. Provision of resources: LT and CP. Writing – original draft: CG. Writing – review and editing: CG, CH and CP. Funding: CP.

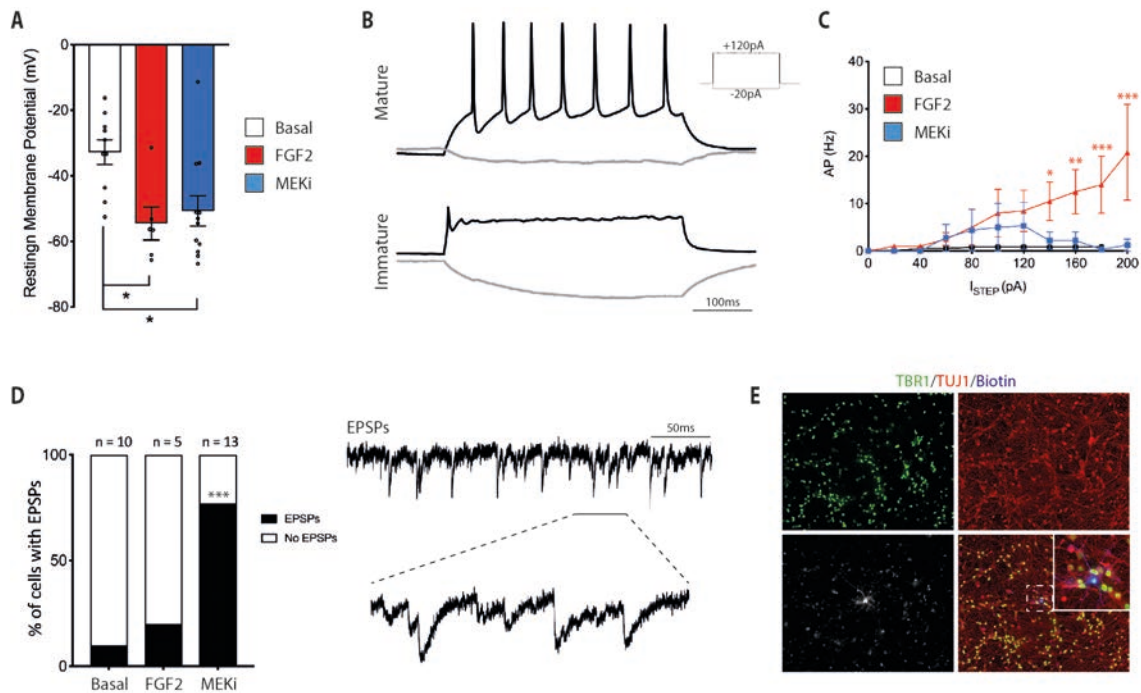
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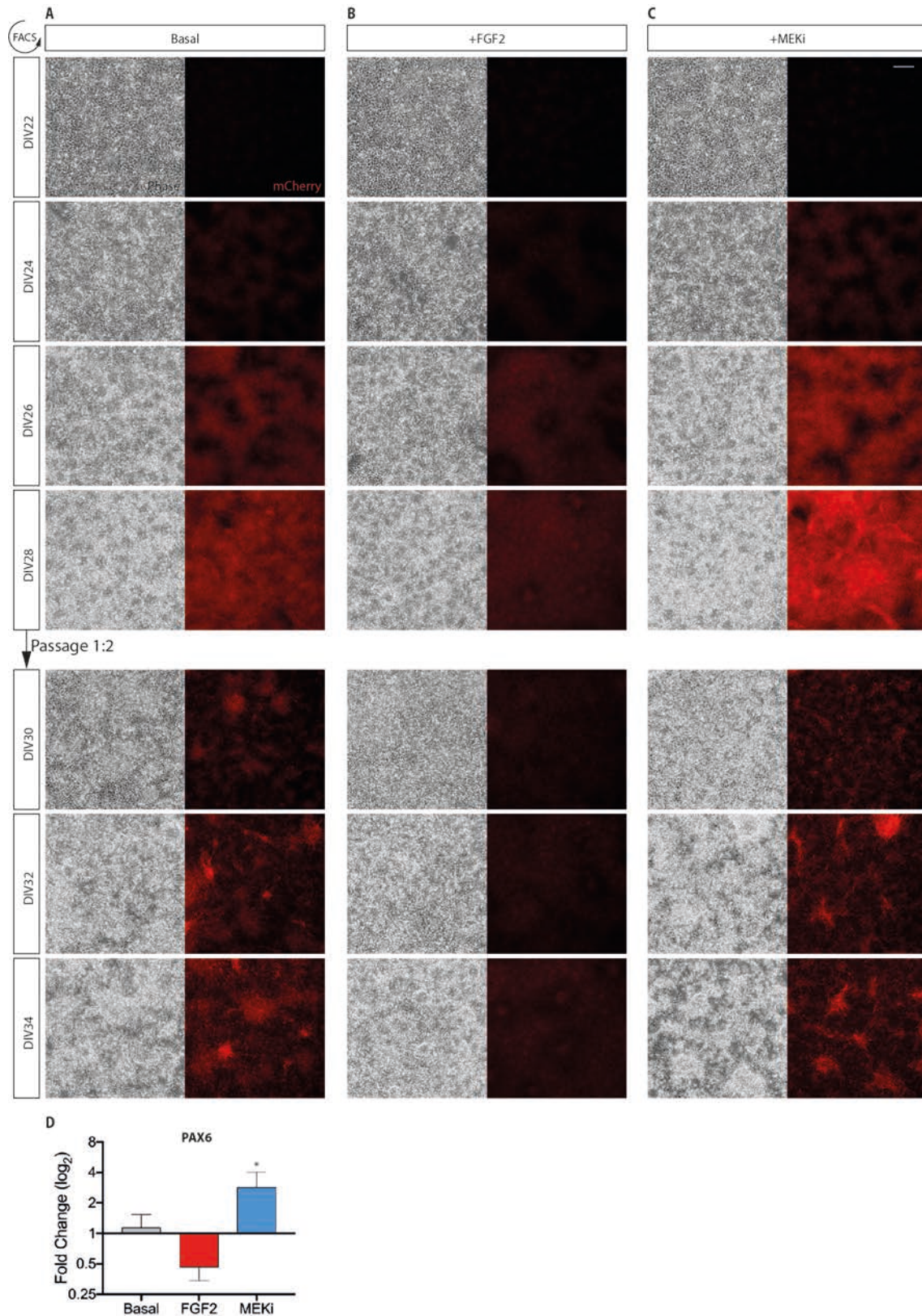
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5.9 Supplementary Figures



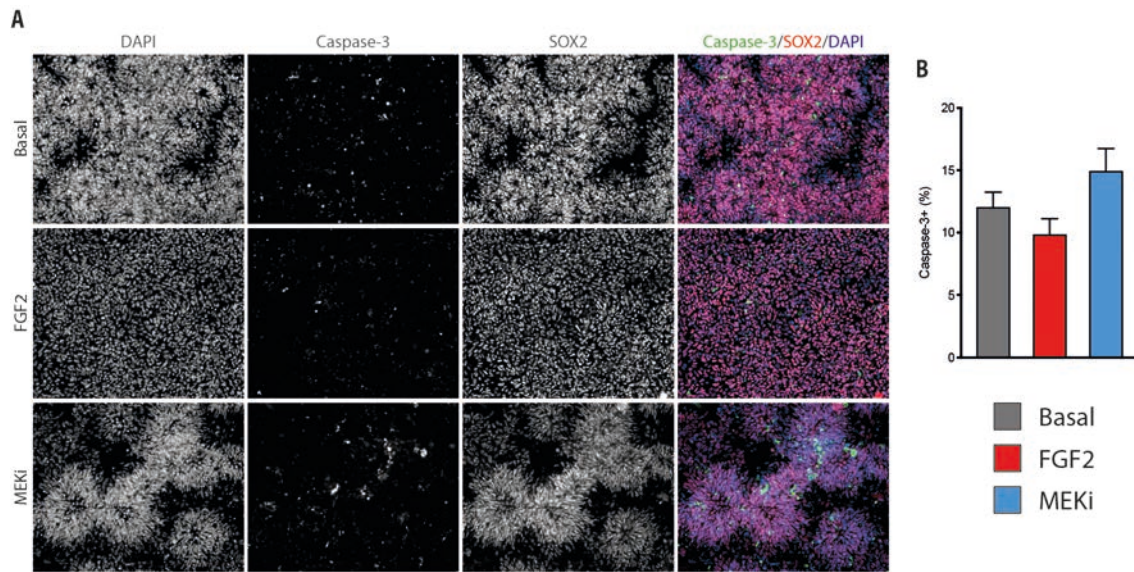
Supplementary Figure 5.1 – Electrophysiological analysis of basal, FGF2 or MEKi treated cultures at D80.

(A) FGF2 and MEKi treated cultures, which underwent DAPT-induced cell cycle exit, displayed more mature resting membrane potentials (Basal $n = 10$, FGF2 $n = 5$, MEKi $n = 13$; from two separate experiments). (B) Neurons from all groups were capable of firing action potentials (AP) that existed along a gradient of mature and immature firing patterns depending on the number induced and AP collapse. (C) FGF2 treated neurons fired significantly more APs in response to current injections. (D) In contrast, neurons derived from MEKi treated progenitors received a greater number of excitatory postsynaptic potentials (EPSP). Example traces of EPSP events within a MEKi treated neuron. (E) Representative biocytin filled, TBR1⁺ cortical neuron.



Supplementary Figure 5.2 – PAX6-mCherry expression in cortical progenitors after FGF2 or MEKi addition.

(A) After FACS, PAX6⁺ progenitors decrease mCherry expression due to dissociation-induced stress. After replating, mCherry expression gradually increases as rosettes reform. Ongoing neurogenesis results in heterogeneous cultures of PAX6⁻ cells with neuronal morphology and PAX6⁺ progenitors. (B) PAX6-mCherry expression is notably reduced after FGF2 treatment but remains detectable by FACS. (C) In contrast, MEKi treated cortical progenitors increase PAX6-mCherry reporter expression. Following a further passage, cultures contain PAX6⁻ neurons and progenitors as well as PAX6⁺ cortical progenitors. (D) qPCR analysis of PAX6 expression confirms mCherry observations.



Supplementary Figure 5.3 – Extended FGF2 or MEKi treatments do not induce apoptosis.

(A) D26 cultures treated with FGF2 or MEKi displayed no changes in toxicity relative to basal conditions as assessed by staining for cleaved caspase-3. A basal level of apoptosis (10-15%) was present within all conditions. (B) Quantification of caspase-3⁺ cells within cortical progenitor cultures.

Chapter 6.

CONCLUSIONS AND FUTURE DIRECTIONS

The discovery of human pluripotent stem cells in the late 1990s led to massive anticipation about their use in understanding human development and regenerative medicine. Since then concerted efforts have been made to recapitulate development by fate restricting these stem cells into specialised, terminally differentiated neuronal subpopulations. Both mDA neurons and cortical pyramidal neurons have been extensively studied and can now be derived routinely *in vitro*.

6.1 Generating xenogeneic-free cultures for clinical translation

Underpinning the attention and progress in generating mDA neurons has been the recognition that this discrete population of cells is capable of restoring motor function in PD patients. However, necessary for the translation of these hPSC-derived cells into the clinic is a fully defined differentiation system free from all animal products. Within this thesis we have shown that not only are the xeno-free mDA neurons functional, both *in vitro* and *in vivo*, but that they are generated at increased efficiency compared to traditional differentiation systems. Mechanistically, this phenomenon was linked, in part, to the use of mouse embryonic fibroblasts (MEF) as feeder cells for the culture of expanding, undifferentiated hPSC (i.e. prior to commencement of directed differentiation). MEF co-culture systems have historically been used to maintain hPSC in their undifferentiated state (Thomson et al., 1998). However, as with many biological or cell products, they remain, essentially, black boxes. As our understanding of pluripotency has increased so too has our ability to use defined media to culture hPSC. In this study, we have shown that feeder-free culture of hPSC (in mTeSR throughout this thesis) reduces the variability of differentiation by eliminating MEF contamination. MEFs secrete TGF β and FGF ligands, as well as BMP antagonists (Villa-Diaz et al., 2009), which, when carried over into the crucial first days of differentiation (even in seemingly small numbers), can act to inappropriately pattern hPSC and/or inhibit dual-SMAD approaches for neuroectodermal differentiation. In contrast, mTeSR media is a fully defined media containing FGFb, TGF β , GABA, pipecolic acid and insulin, factors which individually and in concert promote clonal proliferation of hPSC and

repress differentiation (Ludwig et al., 2006). The ability to directly remove these reagents by replacing mTeSR with differentiation medium allows for tight synchronisation of differentiation and improves differentiation efficiency. Additionally, the removal of xenogeneic components from the differentiation, including serum, geltrex/matrigel matrices and recombinant mouse proteins, was demonstrated to have no negative effect on the ability of hPSC to differentiate to mDA progenitors and neurons. This factor is crucial, as a clinical product for PD must be good manufacturing practice (GMP) compliant. The newly established xeno-free protocol described here provided a critical foundation for the subsequent studies in Chapter 4 and Appendices 1, 2 and 3.

6.2 Employment of reporter cell lines for aiding cell characterisation, isolation and tracking

Reporter cell lines provide valuable tools for stem cell and developmental biology, aiding in the identification, isolation and/or tracking of defined populations. The use and validation of LMX1A- and PITX3-GFP reporter lines, employed in the present studies, enabled characterisation of mDA progenitors and neurons *in vitro* and following transplantation in unprecedented detail. In particular, LMX1A-GFP⁺/FOXA2⁺/OTX2⁺ coexpression eliminates the potential for basal plate contamination of mDA cultures that would be undetectable with traditional FOXA2⁺/OTX2⁺ antibody staining alone (see Figure 3.1 B-E). While this combination allows for greater precision, it does not give rise exclusively to mature mDA neurons and should be viewed as a refinement not a definitive identification (Kee et al., 2017). However, LMX1A-GFP did delineate between successful and unsuccessful graft outcomes above and beyond classification with FOXA2⁺/OTX2⁺ alone (see Figure 3.6). Moreover, sorting strategies utilising LMX1A-GFP to purify mDA progenitors improved mDA graft outcome and provided a platform for the analysis of neurotrophic interventions (Chapter 4 and Appendix 3). While GFP reporters are an invaluable tool in preclinical research, their use in a clinical setting remains difficult. Several groups have reported GFP-immunogenic-induced toxicity *in vivo* in both rodent and primate models (Ansari et al., 2016; Morris et al., 2004). Thus, the benefit of sorting strategies that remove unwanted cell types must be balanced with the potential for cytotoxicity and immune modulation in any clinical setting. However, the preclinical use of lineage-restricted reporter lines may also aid in identifying cell surface antigens that are applicable for clinical purification strategies.

PITX3-GFP labels with high specificity postmitotic mDA neurons and has been invaluable in understanding how hPSC-derived grafts integrate within the host following transplantation. Unlike traditional methods, which utilise whole-graft staining (such as human specific NCAM) or TH staining (which fails to discriminate graft from host neurons), PITX3-GFP selectively marks the mDA component of the graft within the host brain. We first validated that PITX3-GFP hPSC differentiate appropriately *in vitro* and that PITX3-GFP is a faithful marker of DA identity (TH⁺/GFP⁺; Chapter 3). This work allowed an analysis of the relative DA/non-DA contribution within hPSC grafts, a crucial step in understanding their behaviour *in vivo* (Appendix 1) (Niclís et al., 2017). Crucially, targeted tracing experiments show that the two major subclasses of mDA neurons, the A9 and A10, project to developmentally appropriate host regions. However, this study also provided a warning. mDA neurons remained the significant minority within the graft (5% of transplanted cells) and non-DA neurons projected widely across the host CNS. Non-DA innervation was observed across the rostrocaudal and mediolateral axes of the brain. However, we could not, using the PITX3-GFP reporter, discern distinct projection patterns (e.g. GABAergic, glutamatergic, etc.) that may also project in a target-specific fashion. Of note, GABAergic precursors have been linked to behavioural recovery in the 6-OHDA, indicating that other non-DA populations may play a role in recovery (Martínez-Cerdeño et al., 2010). Indeed, while the effect of this non-DA innervation remains to be explored, it highlights the need for continuous improvement of mDA differentiation approaches as well as methods to increase survival and plasticity *in vivo*.

6.3 GDNF improves DA graft function

Our ability to derive a clinically relevant mDA population *in vitro*, coupled with their limited purity and innervation following transplantation, led us to next seek to improve transplantation outcome through modulation of the host environment. Work from our group has demonstrated that GDNF, a potent neurotrophic protein, can improve the survival, innervation and function of rodent fetal mDA transplants (Kauhausen et al., 2013; Thompson et al., 2009). Therefore, we investigated whether GDNF could also improve hPSC-derived mDA grafts. Crucially, no study has investigated the effect of GDNF on hPSC-derived mDA transplants *in vivo*, despite its widespread use in *in vitro* differentiation systems. Here, we show that GDNF is able to improve motor function in animals receiving hPSC-derived mDA transplants. Importantly, the effect of GDNF is critically dependent on the timing of GDNF exposure.

Exposure of the graft to GDNF at the time of transplantation (i.e. early GDNF) increased the survival of transplanted progenitors. Notably, this effect was not specific to the mDA component as evidenced by the concomitant increase in PITX3-GFP⁺ cells in addition to PITX3-GFP⁺/TH⁺ mDA neurons. The expression of GFR α 1 and the immediate survival effect together demonstrate that mDA progenitors are capable of transducing GDNF-mediated pro-survival signalling at the time of transplantation (see Figures 4.1 and 4.2). The effect of GDNF on hPSC-derived mDA neurons mimicked rodent mDA and fetal mDA grafts in several important aspects. First, behavioural recovery in a non-pharmacological test was improved and recovery was positively correlated to PITX3-GFP⁺/TH⁺ number. Second, a greater proportion of transplanted TH⁺ mDA neurons went on to express GIRK2 or Calbindin, markers of mature A9- or A10-like mDA subtypes. Lastly, mDA neurons displayed significant hypertrophy in the presence of GDNF. However, hPSC-derived mDA neurons less efficiently targeted the dorsolateral striatum in the presence of GDNF and displayed premature, aberrant sprouting of mDA fibers within the graft core. A similar axonal entrapment and sprouting phenotype has been observed in both dopaminergic and motor neurons following GDNF therapy (Blits et al., 2004; Georgievska et al., 2002; Love et al., 2005; Tannemaat et al., 2008). Intriguingly, behavioural recovery did not correlate to striatal reinnervation, implying that other mechanisms may be responsible for motor recovery.

In a refinement of the work we sought to split the survival and innervation effects of GDNF. By transplanting mDA progenitors, prior to GDNF exposure (i.e. late GDNF), we allowed the graft to undergo the normal apoptotic process after grafting, which predominantly occurs within the first week and consequently no differences in survival were observed (see Figure 4.4). In addition, we utilised our recent LMX1A sorting approach to enrich for mDA neurons (Appendix 2). Three weeks later the grafts were exposed to GDNF-AAV. Behavioural recovery was strikingly similar to that of the early GDNF study in that only animals receiving combined GDNF and mDA grafts recovered in the cylinder test. Again, we observed that grafts increasingly matured into subtype specific mDA neurons in the presence of GDNF. However, in contrast to the early GDNF cohort, mDA neurons were able to appropriately innervate outside of the graft core after delayed exposure to GDNF, indicating that 3-4 weeks is sufficient for maturing mDA neurons to target their projections appropriately. Indeed, there was a significant increase in axonal sprouting and fiber density within both intrastriatal (dorsolateral and ventrolateral) and extrastriatal (perirhinal and cingulate cortex) mDA target regions (see Figure 4.5). Of functional significance, increased innervation improved DA-mediated cFOS expression within the

ventrolateral striatum, a region known to play a role in complex sensorimotor tasks. Additionally, the metabolic parameters of mDA grafts in the presence of GDNF were indistinguishable from intact hosts and significantly boosted in comparison to mDA grafts alone. Our transcriptional analysis further confirmed this phenotype, revealing large increases in *TH*, *AADC*, *DLK1* and *CCK* mRNA, which likely underpin increased DA metabolism. In the rodent, long-term exposure to GDNF reduces *TH* mRNA levels through autoregulatory feedback on multiple levels (Georgievska et al., 2002). It is intriguing that this effect was not observed in our study. However, it's crucial to recognise that the intrinsic developmental program is lengthened in the human and that there are differential requirements of GDNF in developing and adult mDA neurons. Together, these data demonstrate that GDNF has significant effects on DA metabolism, innervation and maturation that result in augmented behavioural recovery. Although no direct comparison between the early GDNF and later GDNF studies can be made, it is likely that these same changes in DA metabolism and maturation, in conjunction with its pro-survival effect, mediate the GDNF-induced recovery observed in the early GDNF study.

6.4 Future directions of hPSC-derived mDA research

Almost 30 years after the first clinical transplantation of fetal mDA in Sweden, the first patient has been transplanted with hPSC-derived mDA progenitors (Takahashi group, Kyoto, 2018 – for press release in Nature news, see <https://www.nature.com/articles/d41586-018-07407-9>) and several other groups are poised to begin trials. While genuinely exciting, extensive improvements remain to be made in generating mDA neurons, ensuring their safety and improving their function *in vivo*.

Here, we have focused on improving mDA differentiation to improve cell replacement therapy in the context of PD (Chapter 3). However, improved, standardised protocols have relevance for several other areas of research. In particular, the ability to derive reproducible populations of mDA neurons allows for targeted *in vitro* disease modelling and drug screening approaches, which have already increased our understanding of familial PD aetiology (Chung et al., 2016; Cooper et al., 2012; Hsieh et al., 2016; Woodard et al., 2014). Patient-derived monogenic iPSC along with isogenic controls provide a platform for the study of familial PD. However, over 90% of PD cases are idiopathic. The creation of large-scale iPSC banks alongside sophisticated barcoding and analysis techniques will allow for mixed, automated assessment of

polygenic and/or idiopathic PD iPSC-derived mDA neurons, greatly increasing the rate of discovery and understanding of the disease. One caveat of iPSC disease modelling for age-associated disorders is the difficulty and cost of deriving truly mature cultures *in vitro*, which can often take months. Two promising strategies have emerged to generate aged cultures *in vitro*: (1) genetic or pharmacological induction of aging and (2) transdifferentiation of adult somatic cells to the desired cell type. Progerin, which is a truncated form of *LMNA* (a gene involved in the positive regulation of cell ageing), induces premature ageing in Hutchinson-Gilford progeria syndrome (HGPS). iPSC-derived mDA neurons, which typically lose their aged phenotype during reprogramming, rapidly resemble aged controls after treatment with progerin (Miller et al., 2013). These aged iPSC-derived mDA neurons were selectively vulnerable in PD models, validating this approach. Telomerase inhibition has also been shown to accelerate age-related characteristics *in vitro* (Vera et al., 2016). In contrast to iPSC-derived mDA, transdifferentiation approaches open the possibility of generating the desired cell type without reversion to the pluripotent state and loss of the aged signature. This approach has been used to generate DA (Caiazzo et al., 2011; Pfisterer et al., 2011; Rivetti di Val Cervo et al., 2017), glutamatergic (Pang et al., 2011) and GABAergic (Yang et al., 2017) neurons. Exactly how similar induced neurons are to their *in vivo* counterparts remains to be fully explored. Together, the generation of iPSC-derived or adult-derived neurons bearing an aged signature will be crucial in investigating neurodegenerative diseases, which often take decades to develop in patients.

While the transplantation of mDA progenitors is sufficient to induce behavioural recovery in parkinsonian rodents, it also provides an opportunity to study human mDA neurons in a chimeric host. Indeed, transplantation approaches may allow for the study of mature, human mDA neurons above and beyond that which can be achieved *in vitro*. Transplantation into embryonic animals, when the appropriate growth and guidance factors are expressed, may accelerate the development of these models. Additionally, the use of blastocyst complementation approaches, which allow for selective development of specific regions with altered genetic material, may be used to generate humanised rodent models of the mDA system (Chang et al., 2018). It will be fascinating to know whether human cells are able to develop selectively into mDA neurons after interspecies blastocyst complementation, although this remains controversial (Wu et al., 2017). These approaches may be particularly amenable to understanding the prion-like transmission of α -synuclein pathology in PD and other neurodegenerative diseases (Brundin et al., 2010; Olanow and Brundin, 2013). Indeed, the transplantation of mutated α -synuclein iPSC-derived human mDA into humanised rodent

models will facilitate our understanding of α -synuclein pathology and may also allow for the development of targeted therapies (Thakur et al., 2017).

In Chapter 4, the effect of GDNF was observed to be time-dependent. One future approach may be to generate inducible systems that allow for control of the time and dosage of GDNF and/or other neurotrophic factors. Inducible GDNF viral constructs are now available and have been shown to be responsive after transplantation (Akhtar et al., 2018; Eggers et al., 2019). Following on from the findings observed in this thesis, it is clear that a biphasic strategy may prove beneficial. For example, GDNF may be acutely induced prior to transplantation to maximise graft survival and subsequently shut off to allow developing mDA neurons to project appropriately. A subsequent, second pulse of GDNF may be used to increase the innervation, sprouting and biosynthesis of mDA neurons. Here, we suggest that three weeks is sufficient to increase innervation density; however, further study is necessary to illuminate the ideal duration and dose of GDNF. We have also focused exclusively on ectopic transplantation of mDA neurons. Homotopic transplantation approaches, which typically innervate the striatum poorly, may also benefit from GDNF overexpression. We have previously shown that GDNF improves innervation in a rodent mDA context (Kauhausen et al., 2013; Thompson et al., 2009) and it is worth noting that GDNF is expressed along the striatonigral tract after striatal targeting of GDNF-AAV, potentially providing a GDNF-enriched scaffold for graft-derived mDA projections. The use of restrictive promoters to aid in targeting GDNF overexpression appropriately may also be beneficial. Placing GDNF under the control of the DARPP32 promoter may limit GDNF expression to medium spiny neurons, the traditional target of mDA neurons, encouraging directed growth of mDA fibers towards GDNF. We and others are also exploring the use of biomaterials as reservoirs for neurotrophic factors and have demonstrated that GDNF can be delivered within a supportive scaffold (Moriarty et al., 2017; Wang et al., 2016).

Several other axon growth and guidance cues have been implicated in mDA pathfinding and sprouting during development. These include the classical guidance ligands and their cognate receptor families Ephrin/EphR, Netrin/DCC, Slit/ROBO and Semaphorin/SemaR, WNT, FGF and SHH morphogens, as well as additional pro-survival/plasticity cues such as neurturin and Rho Kinase inhibitors (ROCKi) (Van den Heuvel and Pasterkamp, 2008). While the expression of these guidance cues generally decreases during development, receptor expression is maintained in both adult and immature hPSC-derived mDA neurons (Giger et al., 2010). Therefore, their ability to interact with and attract/repel developing hPSC-derived mDA neurons

after transplantation is an attractive area of research. One intriguing example is ephrinA5, which is expressed in a high ventrorostral-low dorsocaudal gradient within the striatum (Cooper et al., 2009; Deschamps et al., 2009). This pattern suggests that ephrinA5 may act to divide responsive and unresponsive mDA neurons within the striatum and thus potentially determine A9/A10 patterning. Indeed, single cell transcriptomic analysis reveals that different clusters of developing mDA progenitors express unique subsets of guidance cues that may determine their pathfinding and phenotype postmitotically (La Manno et al., 2016; Poulin et al., 2014). Further functional analysis of these subtype-specific guidance cues may facilitate targeted differentiation systems *in vitro*, as well as directed projection of developing hPSC-derived mDA neurons *in vivo* (Brignani and Pasterkamp, 2017).

6.5 Stem cell modelling of cortical neurogenesis

Unlike in PD, where the loss of a single neuronal population results in stereotypical motor dysfunction, neurodevelopmental disorders of the cortex often effect multiple neuronal and glial subtypes and result in diverse pathologies (Birnbaum and Weinberger, 2017; Bourgeron, 2015; Marín, 2012). Our understanding of these complex disorders is invariably limited by our inability to monitor human development *in utero*. Compounding this, the human cortex is arguably the most altered structure during hominid evolution, making the use of traditional model animals a challenge. As a consequence, our understanding of the unique developmental roadmap of human corticogenesis remains opaque. In this light, our ability to harness the remarkable developmental potential of hPSCs *in vitro* provides a powerful tool to understand both development and disease (Ardhanareeswaran et al., 2017). However, as with DA cell replacement therapy, a thorough understanding of development is crucial to our ability to derive cell types of interest. Thus, research into the development of the cortex utilising hPSC may in turn be able to, in a positive feedback loop, improve our ability to derive specific populations *in vitro*.

Neurogenesis takes 17 weeks in the human and less than 1 in the mouse. However, increases in developmental time alone do not predict correct species-specific neuronal numbers, indicating that other amplifying modifications occur during human corticogenesis (Dehay et al., 2015; Lui et al., 2011). A major contribution is the expansion of the NESC founder pool and emergence of a second major germinal zone – the OSVZ. The proportion of neurogenic/proliferative divisions within this progenitor pool directly dictates the size and final

number of neocortical neurons and thus cognitive and processing abilities. Despite its importance, our understanding of how cortical fate decisions and temporal identity are encoded in the developing human brain remains limited and several outstanding questions remain. Namely, what are the determinants of proliferative vs. neurogenic fate decisions? How does the diversity of hominid neocortical progenitors give rise to the six-layered neocortex? And finally, how is temporal progression mediated during development to give rise to the distinctive waves of progressively superficial neurogenesis?

In Chapter 5 we investigated the role of the Notch and FGF pathways during *in vitro* human corticogenesis. These two pathways were chosen for multiple reasons. Firstly, both have been heavily implicated in *Drosophila* and mouse corticogenesis (Iwata and Hevner, 2009; Koch et al., 2013; Ohtsuka and Kageyama, 2019). Secondly, Notch and/or Notch inhibitors, as well as FGF ligands are regularly utilised in hPSC models of neural development to bias differentiation toward specific neuronal lineages and to promote proliferation, survival or maturation. In addition, while Notch and FGF modulation is readily used and permissive to cortical differentiation *in vitro*, their effects on key developmental criteria during corticogenesis have not been robustly investigated. Finally, the PAX6-mCherry reporter line enabled us to isolate, with high specificity, a homogenous population of telencephalic progenitors amenable to modulation with these key extrinsic factors. We first confirmed that isolated mCherry-PAX6 progenitors were capable of differentiating toward the major cell types of interest, including neuroepithelial stem cells, DL and UL neurons (defined by expression of laminar-restricted transcription factors) and glia. Of note, the generation of UL neurons requires protracted protocols, reflective of their status as late-born neurons during *in vivo* corticogenesis and they are often produced less robustly than preceding DL neurons during 2D monolayer culture (Espuny-Camacho et al., 2013; Otani et al., 2016; Shi et al., 2012). Therefore, we limited our investigation to the critical period for DL neurogenesis, between DIV20 PAX6⁺ progenitors capable of forming TBR1⁺, layer 6 neurons to multipotent DIV35 progenitors which generate both TBR1⁺ and CTIP2⁺, layer 5 neurons (Figure 5.1A).

In the mouse, Notch signalling represses pro-neural gene expression and promotes maintenance of stem cell identity within NESCs and RG. In its absence, progenitors rapidly differentiate to neurons and the stem cell pool is diminished, reducing the overall size of the cortex (Imayoshi et al., 2010). We found a similar, essential role for Notch signalling during human NESC expansion (Figure 5.2). PAX6⁺ NESCs exposed to DAPT, a potent γ -secretase/Notch

inhibitor, rapidly exited the cell cycle and generated homogenous populations of TBR1⁺, layer 6 neurons. As a result of the rapid cell cycle exit and neurogenesis, we concluded that the competency of the exited progenitor is crystallised at, or close to, the time of DAPT addition. Therefore, DIV20 PAX6⁺ progenitors are competent only to form TBR1⁺, layer 6 cortical neurons at this time. The loss of any cycling progenitors in the absence of Notch indicates that it is essential during human cortical progenitor expansion. Several studies suggest that Notch activation promotes RG fate in the mouse (Gaiano et al., 2000) and hPSC-derived cultures (Duan et al., 2015) but does not influence laminar competence (Okamoto et al., 2016). In this regard, further investigation into the potential role of activating Notch signalling (via addition of DLL/JAG ligands) is ongoing within our laboratory.

We next investigated the laminar outcome of extending or blocking FGF2 treatment. In most cortical differentiation protocols, FGF2 is used to enhance proliferation and telencephalic identity, promoting rosette formation within neuroepithelial cultures (Espuny-Camacho et al., 2013; Shi et al., 2012). We extended the time period of FGF2 addition from DIV12-20 to DIV12-35 (Figures 5.3-5.6). Surprisingly, the neurons generated by DIV55 (DAPT treatment at DIV35) almost exclusively became TBR1⁺, layer 6 neurons, indicating that progenitor maturation had stalled during FGF2 addition (Figure 5.1B). In agreement with this hypothesis, FGF2 treated cultures increasingly underwent proliferative/symmetrical divisions, displayed a faster cell cycle length and failed to appropriately localise apical-specific proteins, including ZO-1. As a result, FGF2-treated cultures displayed a concomitant decrease in neurogenesis, failed to generate CTIP2⁺ or BRN2⁺ neurons and decreased expression of pro-neural genes *PAX6*, *ASCL1* and *SOX4*. Together, these results confirm that FGF2 blocks the temporal development of cortical progenitors in a manner that is strikingly similar to the mouse (Kang et al., 2009; Lukaszewicz et al., 2002; Rash et al., 2011), where FGF signalling has a crucial role in slowing neurogenesis and ensuring the timely generation of cortical neurons. We hypothesise that FGFR signalling, in part, controls the relative extension of human corticogenesis by ensuring appropriate time for DL neurogenesis. In agreement with this model, FGF-ERK signalling is upregulated in primate neocortical VZ/OSVZ NESC and RG (Heng et al., 2017). Unfortunately, in the absence of other survival and proliferation cues *in vitro*, long-term FGF2 treated cultures eventually become differentiated/non-neural (>45 days in FGF2), precluding investigation of the full potency of FGF2-treated progenitors to subsequently recapitulate the layer sequence *in vitro*.

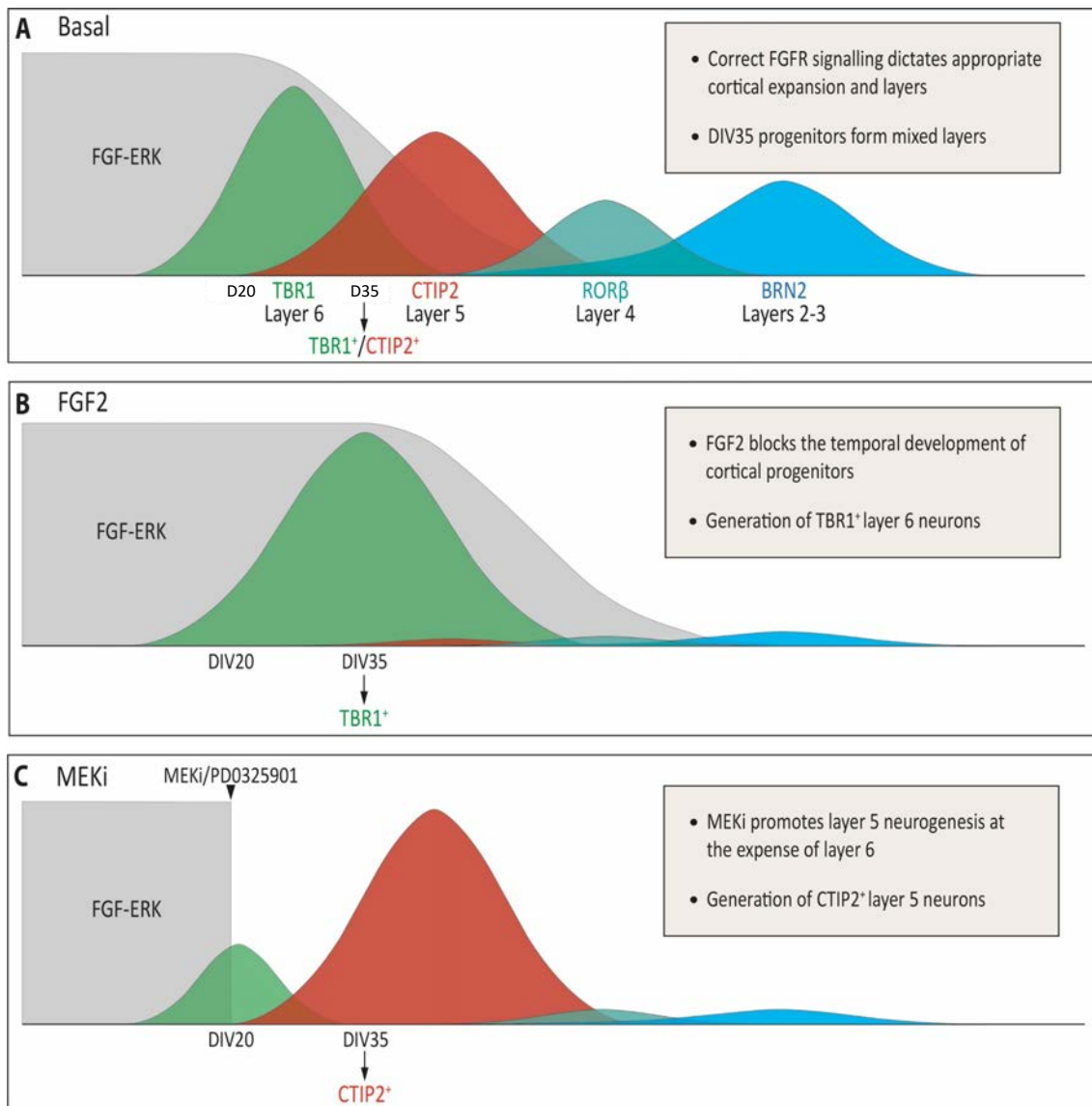


Figure 6.1 – Laminar consequences of FGF2 or MEK inhibition on human cortical progenitors *in vitro*.

(A) Under basal conditions hPSC-derived cortical progenitors recapitulate the developmental sequence, generating progressive layers over time, albeit with low efficiency for layers 2-4 under 2D conditions. At D35, progenitors are competent to generate both layer 6 TBR1⁺ and layer 5 CTIP2⁺ cortical neurons. (B) When exogenous FGF2 treatment is extended the temporal maturation of cortical progenitors is blocked and they remain competent only to generate TBR1⁺ neurons at DIV35. (C) In contrast, MEKi-treated cultures lose FGF-ERK signalling and repress the TBR1⁺ fate and instead accelerate CTIP2⁺ laminar fate.

In attempting to understand the role of FGF-ERK signalling in cortical lamination and development we utilised an inhibitor of MEK1/2 (Mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK)), PD0325901 (MEKi). The ERK pathway is a major downstream effector of FGFR activation heavily implicated in proliferation and differentiation (Mason, 2007). Strikingly, MEKi-treatment accelerated the acquisition of CTIP2-competent cortical progenitors *in vitro* (Figures 5.3, 5.4 and 6.1). It is notable that MEKi did not block TBR1⁺ neurogenesis or TBR2⁺ IPC generation acutely (at DIV26, 5 days of MEKi) but instead promoted CTIP2⁺ neurogenesis over extended culture times (at DIV35, 14 days of MEKi). The exclusive

generation of CTIP2⁺ neurons after DAPT treatment at DIV35 likely reflects the loss of early-born TBR1⁺ neurons during the passaging step, indicating that the remaining progenitors are competent after 15 days of MEKi to generate only CTIP2⁺ neurons. PD0325901 has been recently utilised to rapidly mature hPSC-derived cortical neurons in concert with Notch and pan-FGF inhibition (Qi et al., 2017). However, in our hands, MEKi drives a temporal fate shift but not maturation *per se*. We observed an acute increase in the length of the cell cycle, as occurs over development in the mouse, but no increase in TBR2⁺ IPC or TUJ1⁺ neurogenesis in MEKi-treated cultures compared to basal conditions. Instead, we demonstrate alterations in expression of the pro-neural genes *FOXG1*, *PAX6*, *ASCL1* and *SOX4*. *FOXG1* has been shown to directly repress TBR1 (Kumamoto et al., 2013), while *ASCL1* is necessary for appropriate CTIP2⁺/FEZF2⁺ layer 5 neurogenesis (Dennis et al., 2017). Finally, *SOX4* binds to the E4 cis-activation element of FEZF2 (Shim et al., 2012). Together, our data indicate that MEKi, downstream of FGFR signalling, may be crucial in initiating the repression of the default TBR1⁺ transcriptional program in glutamatergic cortical progenitors and thus promote the rapid acquisition of CTIP2, layer 5 competence. It is interesting to note that FGFR expression is downregulated at the midpoint of rodent neurogenesis, possibly supporting our interpretation (Raballo et al., 2000). This study is an important first step in understanding how FGFR activation and repression in human cortical development controls the timely generation of the cortical laminae.

6.6 Future directions in cortical modelling

We have established a general role for FGF-ERK signalling in proliferative/neurogenic fate decisions and temporal progression in human cortical progenitors, demonstrating that it is key in correctly timing DL neurogenesis. However, several additional experiments may expand this finding. In particular, analysing the role of FGF-ERK signalling in models that more fully recapitulate both DL and UL neurogenesis may shed light on what role FGF-ERK plays in UL neurogenesis. Cortical organoids closely mimic the timing and structural organisation of the developing cortical wall (Kadoshima et al., 2013; Lancaster et al., 2013; Pasca et al., 2015) and may provide an ideal humanised model to probe FGF-ERK modulation further. Their size and structure also make them amenable to targeted genetic interventions akin to *in utero* electroporation. For example, targeted plasmids can be injected into the ventricle/lumen and infect NESCs and RG that display ventricular contact as is routinely done during mouse development (Cardenas et al., 2018; Pacary and Guillemot, 2014; Shimogori and Ogawa, 2008). Moreover, single cell analysis of cortical development is providing a wealth of new cell type-

specific markers that can be used to successfully target specific progenitor sub populations in human corticogenesis. Indeed, HOPX and FAM107A expression is limited to bRG within the OSVZ ([Pollen et al., 2015](#)), while CRYAB is expressed exclusively within vRG ([Thomsen et al., 2016](#)). Manipulation within these specific populations may start to tease apart context-dependent regulation of FGF-ERK. On the other hand, postmitotic DL neurons are known to influence the fate of later-born UL neurons via negative feedback ([Ozair et al., 2018](#); [Toma et al., 2014](#)). Therefore, genetic knockdown or activation of FGF-ERK signalling in the maturing neuronal population may also modulate temporal progression within the progenitor pool.

While hPSC models of corticogenesis are invaluable tools, rodent models may still provide key insights into how FGF-ERK signalling behaves during cortical development. Critically, Chapter 5 addressed the role of FGF-ERK signalling within a generalised PAX6⁺ telencephalic progenitor pool. However, it is well established that progenitor behaviour, response to extrinsic factors and genetic programs differ between specific areas of the cortex (for review see [Greig et al., 2013](#); [Lukaszewicz et al., 2005](#)). PAX6 is expressed in a rostralateral-high, caudomedial-low gradient, while EMX2 is expressed in a complementary pattern. In addition, commissural plate expression of FGF8, which signals preferentially through FGFR3, acts in a classic morphogen gradient to progressively specify rostromedial fates at increasing concentrations ([Shimamura and Rubenstein, 1997](#); [Shimogori and Grove, 2001](#)). Therefore, it cannot be ruled out that the cell cycle changes, progenitor behaviour and laminar specificity induced by exogenous FGF2 addition within Chapter 5 are partially a result of differential arealisation. Studies in the mouse may allow for manipulation of FGF-ERK signalling within specific regions of the cortex to investigate this phenomenon further. Transcriptomic analysis is also providing regional markers that allow the refinement of *in vitro* differentiation protocols toward area-specific subtypes. In addition, several other extrinsic factors contribute to the regulation of corticogenesis, including WNT, SHH and BMP family members ([Grove et al., 1998](#); [Shimogori et al., 2004](#)). Further investigation is necessary to elucidate their respective role and interaction with FGF-ERK signalling during human corticogenesis.

Finally, while the utility of hPSC models of cortical development is well established, the derivation of layer-specific cortical subtypes also has implications for disease modelling and drug development. Here we report a relatively rapid protocol for the generation of specific TBR1⁺ or CTIP2⁺ cortical neurons. One tangible possibility is the use of these layer neurons to study the relative susceptibility of SCPN subtypes in *in vitro* models of motor neuron diseases (MND).

MNDs are a diverse range of diseases that affect the upper and/or lower motor neurons and result in muscle wastage. Amyotrophic lateral sclerosis (ALS), the most common MND, is characterised, in part, by the loss of both layer 5 SCPNs of the motor cortex in addition to lower motor neurons within the spinal cord (Hardiman et al., 2017). In contrast, Primary lateral sclerosis and Hereditary spastic paraplegia affect specific subpopulations of SCPN upper motor neurons based on spinal projection pattern (Salinas et al., 2008; Tartaglia et al., 2007). The modelling of these disorders is hindered by an inability to robustly generate specific cell types of interest (Sances et al., 2016). Most notably, current protocols generate mixed layer identities, making targeted screening approaches difficult and laborious. Combining our new protocol with ALS, PLS and/or HSP patient-derived iPSC may prove to be an invaluable platform for large scale drug-screening approaches (for review see Chen, 2018).

6.7 Concluding remarks

The promise of hPSCs is rapidly giving way to concrete therapeutic and developmental discoveries – a remarkable feat given the nascent stage of the field. Researchers can now readily combine a toolbox of control and patient hPSC-derived cell types and genetic manipulation with traditional animal models to validate and explore disease and development. In combination, this approach may lead to improved translation of therapeutics from bench to bedside. However, variability and limited reproducibility within and between laboratories continue to hinder progress. The work outlined in this thesis seeks to develop new hPSC-based tools, providing insight into cell replacement therapy strategies in PD and furthering our understanding of human cortical development. It is hoped that this thesis will contribute to the development of meaningful interventions for patients suffering from neurodevelopmental or neurodegenerative diseases.

6.8 References

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Appendix 1.

A PITX3-EGFP REPORTER LINE REVEALS CONNECTIVITY OF DOPAMINE AND NON-DOPAMINE NEURONAL SUBTYPES IN GRAFTS GENERATED FROM HUMAN EMBRYONIC STEM CELLS

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A1.1 Abstract

Development of safe and effective stem cell-based therapies for brain repair requires an in-depth understanding of the in vivo properties of neural grafts generated from human stem cells. Replacing dopamine neurons in Parkinson's disease remains one of the most anticipated applications. Here, we have used a human PITX3-EGFP embryonic stem cell line to characterize the connectivity of stem cell-derived midbrain dopamine neurons in the dopamine-depleted host brain with an unprecedented level of specificity. The results show that the major A9 and A10 subclasses of implanted dopamine neurons innervate multiple, developmentally appropriate host targets but also that the majority of graft-derived connectivity is non-dopaminergic. These findings highlight the promise of stem cell-based procedures for anatomically correct reconstruction of specific neuronal pathways but also emphasize the scope for further refinement in order to limit the inclusion of uncharacterized and potentially unwanted cell types.

A1.2 Introduction

Human pluripotent stem cells (PSCs) are a promising source of transplantable neural progenitors for cell-replacement therapies to treat neurological conditions. One of the most anticipated applications is in Parkinson's disease (PD), where proof of principle for efficacy has already been established in clinical trials using fetal midbrain grafts ([Hagell and Brundin, 2001](#); [Lindvall and Hagell, 2000](#)). Although these trials have been problematic due to variability in clinical outcome, more than 30 years of basic and clinical research has led to refinement of the approach, and identification of important criteria for establishment of stem cells as an alternative and potentially superior donor source ([Barker et al., 2015](#); [Thompson and Bjorklund, 2012](#)), for example the requirement that the implanted dopamine (DA) neurons establish a functional terminal network with appropriate targets in the host brain.

Detailed characterizations of fetal graft composition and structural integration have been fundamentally important for understanding graft-host connectivity and the relationship to functional outcome. Early xenografting studies using species-specific antibodies to identify fiber outgrowth from human or porcine fetal midbrain grafts placed homotopically into rat midbrain provided some of the first evidence that grafted neurons retained an intrinsic capacity for target-directed outgrowth, even in the adult host ([Isacson et al., 1995](#); [Wictorin et al., 1992](#)). The development of transgenic reporter mice led to more precise characterization of fiber outgrowth, including the first demonstrations of connectivity on a neuronal-subtype-specific basis (for review see [Thompson and Bjorklund, 2009](#)). Grafting of fetal midbrain from TH-GFP ([Sawamoto et al., 2001](#)) or Pitx3-EGFP ([Zhao et al., 2004](#)) mice allowed for precise identification of graft-derived (GFP⁺) DA fiber patterns, even in the presence of residual host DA fibers, and showed that implanted DA neurons not only innervated the host striatum but also innervated appropriate extra-striatal targets, including the cortex, amygdala, and septum ([Thompson et al., 2005](#)). These studies also showed that the major A9 and A10 DA neuronal subclasses maintained developmentally appropriate target preference, innervating striatum and frontal cortex, respectively, and importantly that A9-specific innervation of dorsolateral striatum was required for restoration of motor function ([Grealish et al., 2010](#)).

The first histological characterizations of neural grafts generated from human stem cells were limited to identification of the graft core using antibodies specific for a human nuclear antigen ([Brederlau et al., 2006](#); [Cho et al., 2008](#); [Park et al., 2005](#); [Sonntag et al., 2007](#); [Yang et al., 2008](#)). Recently however, more sophisticated approaches using human stem cell lines

ubiquitously expressing fluorescent reporters (Denham et al., 2012b; Doerr et al., 2017; Espuny-Camacho et al., 2013; Lu et al., 2014; Niclis et al., 2017a; Qi et al., 2017; Steinbeck et al., 2012) and human-specific antibodies against cell-surface proteins, such as polysialated nuclear cell adhesion molecule (NCAM) (Grealish et al., 2014; Kirkeby et al., 2017; Kriks et al., 2011; Sundberg et al., 2013), have dramatically improved our understanding of the capacity of stem cell-derived grafts to integrate within the host CNS. These studies have shown that grafted neurons, generated from either embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), are capable of extensive axonal growth over long distances, particularly via host white matter tracts (Grealish et al., 2014; Kriks et al., 2011; Sundberg et al., 2013). However, it has thus far not been possible to characterize the specificity of these outgrowth patterns on the basis of neuronal phenotype.

Here we report that not only are grafted neurons generated from stem cells capable of long-distance innervation of host nuclei, but also that they have the capacity to do so with neuronal subtype specificity. Using a human ESC reporter line in which EGFP expression is driven by the endogenous PITX3 promoter (PITX3-EGFP) allowed us to selectively identify DA-specific patterns of growth and connectivity. The results reveal the extent and specificity of dopaminergic innervation from ventral midbrain grafts generated from human PSCs. The DA neurons innervate developmentally appropriate A9 and A10 target structures with remarkable specificity, but represent only a minority of total graft outgrowth, which is largely non-dopaminergic and extends widely throughout the host brain.

A1.3 Materials and methods

A1.3.1 Animals and ethics

All procedures were conducted in accordance with the Australian National Health and Medical Research Council's published Code of Practice for the Use of Animals in Research, and experiments were approved by the Florey Institute of Neuroscience and Mental Health Animal Ethics Committee.

A total of 23 male athymic (CBH^{nu}) rats at 8–9 weeks of age (~150 g) were used at the beginning of this study. The animals were group housed in individually ventilated cages with

Alpha-dri paper bedding material (Abel Scientific, Perth) to reduce skin and eye irritation and housed on a 12:12-hr light/dark cycle with ad libitum access to food and water.

A1.3.2 Human pluripotent stem cell culture

The human embryonic stem cell PITX3-EGFP reporter line was generated on an H9 background as originally described ([Watmuff et al., 2015](#)) and expanded and differentiated under xeno-free conditions also as previously described in detail ([Niclis et al., 2017b](#)). In brief, neural induction was achieved via dual SMAD inhibition using LDN193189 (200 nM, days 0–11; Stemgent) and SB431542 (10 mM, days 0–5; R&D Systems). Ventral midbrain patterning was achieved by addition of sonic hedgehog (C25II, 100 ng/mL; R&D Systems), purmorphamine (2 mM, days 1–7; Stemgent), and CHIR99021 (3 mM, days 3–13; Miltenyi). At day 19, cultures rich in ventral midbrain progenitors were dissociated with Accutase (Innovative Cell Technologies) and prepared for transplantation as a cell suspension in day-19 culture medium supplemented with 0.05% DNase (Sigma).

A1.3.3 Surgical procedures and rotational behaviour

Anesthesia for all surgical procedures was induced and maintained by inhaled isoflurane (2%–5% in air). All 23 athymic rats received stereotaxic injection of 3.5 mL 6-hydroxydopamine (6-OHDA) (3.2 mg/mL free base) unilaterally into the medial fore-brain bundle (3.4 mm caudal and 1.3 mm lateral to bregma and 6.8 mm below the dural surface) as previously described ([Thompson et al., 2013](#)). Three weeks later, all animals were tested for rotational asymmetry after administration of D-amphetamine sulfate (2.5 mg/kg, intraperitoneally) as described by [Thompson et al. \(2013\)](#). A cohort of 13 out of 23 of the animals satisfied our criteria of >7 full turns/min for designation of stable rotational asymmetry. For longitudinal assessment of rotational behavior, these animals were divided into a control group (n = 7) and a transplantation group (n = 6). The remaining 10 animals received grafts of ventral midbrain progenitors for additional histological analysis only.

One week after pre-graft rotational assessment (4 weeks after 6-OHDA) a total of 16 6-OHDA-lesioned animals received single 1-mL injections of pre-differentiated human PITX3-EGFP donor cells (1×10^5 cells/mL) into the rostral head of the striatum as previously described in detail ([Thompson et al., 2013](#)). Assessment of amphetamine-induced rotational behavior was performed at 8, 16, and 28 weeks after transplantation.

For retrograde labeling of grafted neurons, a Neuros syringe (Hamilton) was used to microinject 0.1 mL of a 2% solution of Fluorogold (Fluorochrome) into either the dorsolateral striatum (n = 5; 1.2 mm rostral, 3.4 mm lateral to bregma; 3.6 mm below dura) or frontal cortex (n = 5; 2.7 mm rostral, 1 mm lateral to bregma; 2 mm below dura). Tracing was performed 1 week prior to the experimental endpoint at 28 weeks.

A1.3.4 Immunohistochemistry

For histological assessment, animals were perfusion fixed (4% paraformaldehyde) and the brains were cut on the coronal plane using a freezing microtome as previously described ([Thompson et al., 2005](#)). Four animals were taken for histological assessment 4 weeks after transplantation to confirm successful engraftment, with the remainder taken at 28 weeks (n = 10). Additionally, 2 animals were perfused at 10 and 18 weeks due to spontaneous development of skin irritations that are not uncommon with long-term housing of athymic rats. This facilitated useful, albeit qualitative information on the progressive development of DA innervation of the host striatum over time (Figure A1.1B).

Immunohistochemical procedures on free-floating tissue sections were as described previously in detail ([Thompson et al., 2005](#)). Primary antibodies and dilutions included: 5HT (mouse, 1:10,000, Immunostar: 20080); CALBINDIN (mouse, 1:1,000, Swant Biotech: CB300); ChAT (rabbit, 1:1,000, Millipore: AB143); DAT (rat, 1:1,000, Millipore: MAB369) FoxA2 (rabbit, 1:300, SCBT: sc-6554); GFP (chicken, 1:5,000, Abcam: AB13970); GABA (rabbit, 1:5,000, Sigma: A2052); GIRK2 (rabbit, 1:500, Alomone Labs: APC-006); NeuN (mouse, 1:200, Millipore: MAB377); dopamine- β -hydroxylase (mouse, 1:10,000, Millipore: MAB308); NCAM (mouse, 1:500, clone eric-1, SCBT: sc-106); TH (rabbit or sheep, 1:800, PeIFreeze: P40101-0 or P60101-0); VMAT2 (rabbit, 1:1,000, Millipore: AB1598P). The bound primary antibody was detected either through enzymatic conjugation of the diaminobenzidine chromagen or fluorescent secondary antibodies (Jackson ImmunoResearch).

A1.3.5 Imaging and graft reconstruction

A Leica microscope (DM6000) with a motorized x-y stage was used to capture photomontages of whole coronal sections with chromogen-labeled EGFP or NCAM. Images were acquired either

in bright field or dark field to visualize graft-derived fiber outgrowth. Photomicrographs of immunofluorescence were acquired using a Zeiss (Z780) microscope.

For analysis of cells labeled by retrograde FG uptake, cell size was recorded as the longest linear diameter for each FG⁺ cell. The relative position of each FG⁺ cell within the graft was calculated by determining the radial size of the graft on the dorsoventral and mediolateral axes and determining the distance from the edge of the graft along the nearest axis. To normalize for different graft sizes, the results are reported as a fractional distance along the axis where the graft periphery and center are assigned as 0 and 1, respectively.

High-resolution photomontages of whole coronal sections immunolabeled for EGFP and NCAM were used to schematically illustrate the extent and pattern of DA (EGFP) and total (NCAM) outgrowth. The “select>color-range” tool (Photoshop 13.0.6, Adobe) was initially used to define and select the bulk of the EGFP or NCAM signal. Conservative threshold levels were used to avoid selection of background signal. Each section was subsequently annotated manually by carefully tracing over the remaining fibers using a tablet-stylus interface (Intuos Pro, Wacom) and Canvas Draw software (3.0.5, ACD Systems).

A1.3.6 Quantification and statistics

Rotational response to amphetamine was quantified using an automated system as described by [Bye et al. \(2015\)](#) and group difference in mean assessed separately at each time point by Student's un-paired t test (control, n = 7; graft, n = 8 at each time point).

The mean number of EGFP cells at 4 weeks (n = 4) and 28 weeks (n = 10) was determined by counting all cells in 6 series for each animal. Double-counting error was corrected by the method of Abercrombie according to section thickness and average cell diameter ([Abercrombie, 1946](#)). The size of 200 EGFP⁺ cells was measured as the largest linear diameter and was used to compare cell size in the periphery of the graft - defined as within 300 mm of the host-graft interface - with those located in the center. Graft volumes were calculated in the same animals by extrapolation of the cumulative area measured in 6 series using Cavalieri's principle ([Cavalieri, 1966](#)). Difference in mean volume between the 4-week (n = 4) and 28-week (n = 10) time point was assessed using Student's t test.

The overlap between TH and EGFP was determined through careful inspection of 267 EGFP⁺ cells in confocal datasets including separate channel information on the x, y, and z axes. Similarly, the GIRK2⁺/CALBINDIN⁺ profile of grafted DA neurons was assessed through careful inspection of all EGFP⁺ cells in grafts (n = 5) in single sections captured via confocal microscopy. The relative density of graft-derived DA (EGFP⁺/NCAM⁺) or non-DA (EGFP/NCAM⁺) fiber networks was assessed in specific host nuclei by measurement of EGFP⁺ (n = 7) and NCAM⁺ (n = 3) immunolabeled fibers as the fractional pixel contribution to single photomicrographs acquired using a 403 objective. Difference in the mean density at each location was assessed using Student's t test.

A1.4 Results

A1.4.1 Ventral midbrain differentiation of a human PITX3-EGFP ESC line

Here we used a reporter line with monoallelic insertion of EGFP into exon 1 of the PITX3 gene so that both EGFP and PITX3 were driven by PITX3 regulatory elements (Figure A1.1A). To generate ventral midbrain progenitors, we used the now widely favored approach ([Denham et al., 2012a](#); [Kirkeby et al., 2012](#); [Kriks et al., 2011](#); [Niclís et al., 2017b](#)) that employs early signaling to instruct regional identity in parallel with neural induction (Figure A1.1B). As we have recently reported in detail under fully defined, xeno-free conditions ([Niclís et al., 2017b](#)), this results in rapid and efficient neural (NESTIN⁺) specification of ventral midbrain (FOXA2⁺/OTX2⁺) progenitors as shown after 11 days in vitro (DIV) and acquisition of midbrain DA identity evident at the time of transplantation (19 DIV; Figure A1.1C). Prior to transplantation we affirmed that differentiated DA progenitors reached criterion for transplantation (>80% FOXA2⁺/OTX2⁺).

A1.4.2 Function and composition of human PITX3-EGFP grafts

The experimental design is illustrated in Figure A1.2A, showing the timeline of rotational testing and the small subsets of animals taken for histology over the course of 7 months. The first group for histology at 4 weeks was to confirm successful engraftment, and single animals were taken at 10 and 18 weeks as a result of unrelated skin and respiratory complications. Rotational response to D-amphetamine showed that the grafts significantly reversed asymmetry previously induced by unilateral removal of the host midbrain DA neurons (Figure A1.2B). This was evident

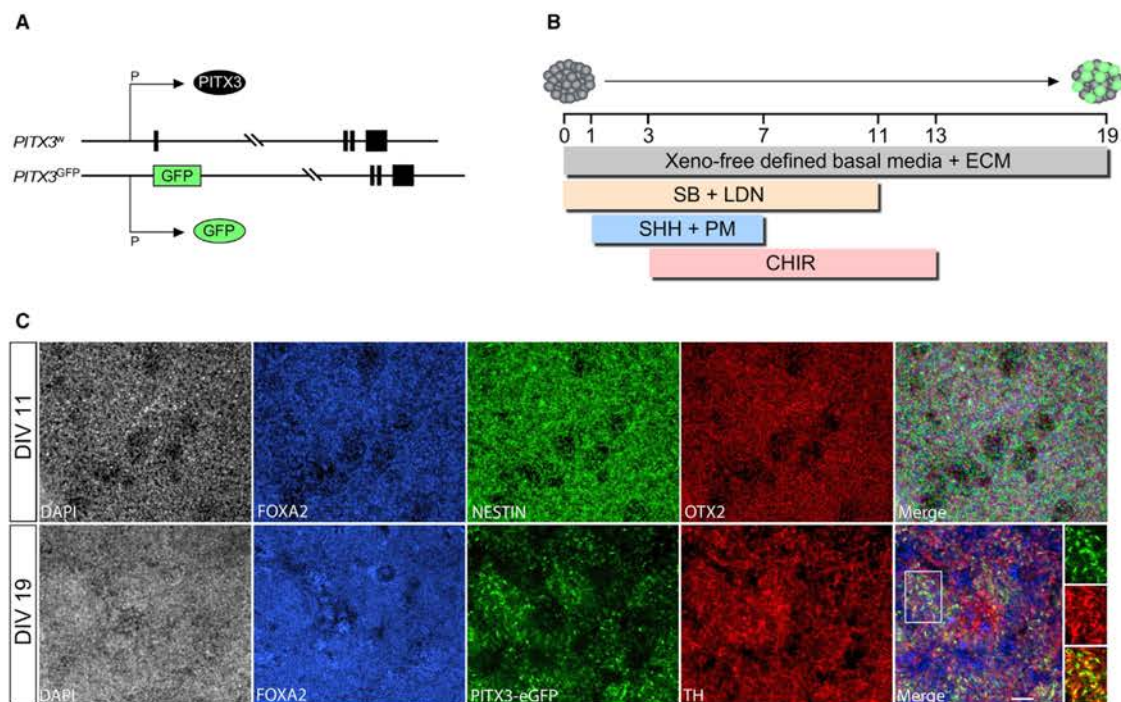


Figure A1.1 – A human PITX3-eGFP ES reporter line for identification of midbrain DA neurons.

(A) Monoallelic insertion of EGFP into exon 1 of the PITX3 gene in the H9 human embryonic stem cell line to act as a reporter for PITX3 protein expression. (B) Schematic illustration of the differentiation procedure for specification of neural progenitors with ventral midbrain identity under defined, xeno-free conditions. (C) Differentiating cells show robust expression of markers consistent with neural (NESTIN⁺) and ventral midbrain/forebrain (FOXA2⁺/OTX2⁺) identity by 11 DIV and begin to show expression of PITX3-driven EGFP and TH at the time of transplantation (19 DIV). Scale bar, 50 μ m. CHIR, CHIR99021; DIV, days in vitro; ECM, extracellular matrix; LDN, LDN193189; PM, purmorphamine; SB, SB431542; SHH, sonic hedgehog; TH, tyrosine hydroxylase.

by 16 weeks and sustained up until the experimental endpoint at 28 weeks. Histological analysis showed a progressive, graft-specific, EGFP⁺ innervation of the host striatum by human DA neurons (Figure A1.2C). At 4 and 10 weeks, prior to significant change in rotational scores, few EGFP⁺ fibers were detected outside the immediate vicinity of the graft. At 18 weeks, dense areas of terminally ramified EGFP⁺ fiber networks could be observed, and this expanded to cover larger areas of the host striatum by 28 weeks. The grafts grew significantly in size from $0.8 \pm 0.28 \text{ mm}^3$ at 4 weeks ($n=4$) to $7.04 \pm 0.91 \text{ mm}^3$ by 28 weeks ($n = 10$; Student's *t* test, $p < 0.01$). The number of GFP⁺ DA neurons, however, did not expand over this time frame: $6,081 \pm 659$ at 4 weeks compared with $5,268 \pm 654$ at 28 weeks (Student's *t* test, $p = 0.49$).

Immunohistological analysis showed EGFP⁺ to be a robust indicator of DA neuronal identity in vivo with the vast majority (86.5%) of EGFP⁺ cells also identified as tyrosine hydroxylase positive (TH⁺) (Figure A1.3A). Grafted DA neurons also expressed mature markers indicative of the capacity to store and release dopamine, including both the vesicular monoamine transporter (VMAT2) and the dopamine transporter (DAT; Figure A1.3B). The EGFP⁺

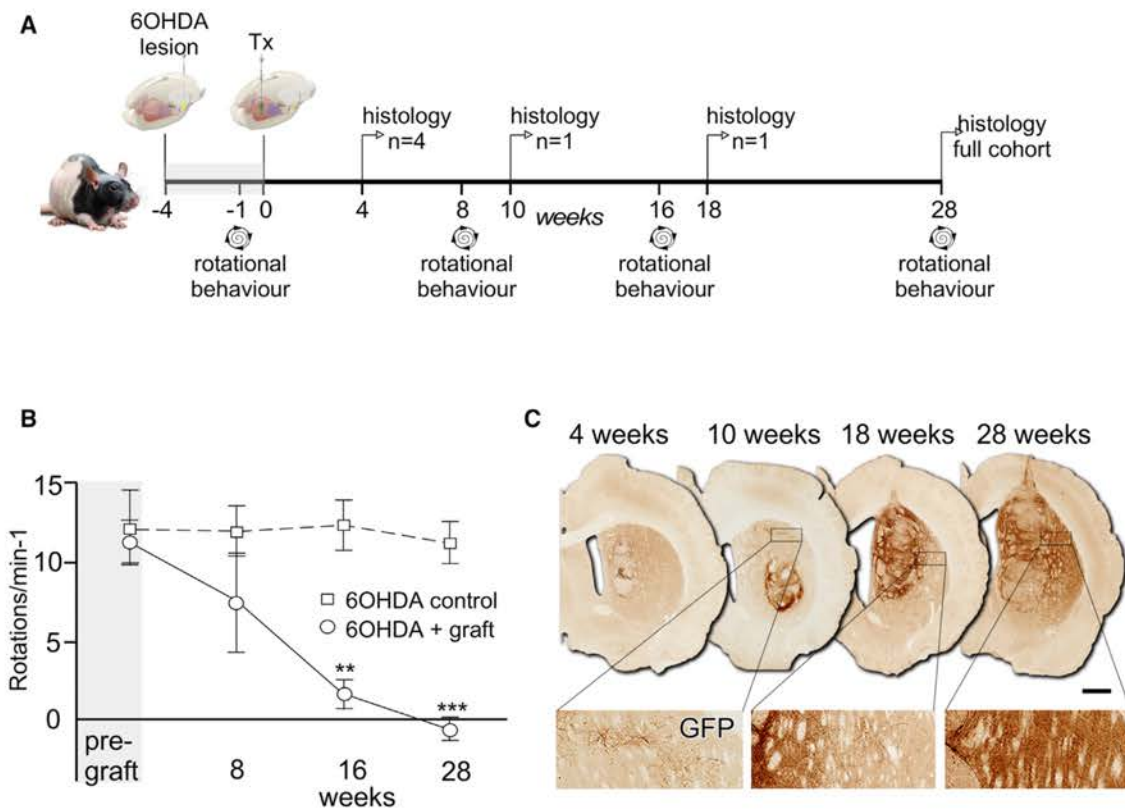


Figure A1.2 – PITX3-eGFP grafts progressively innervate the host and correct motor function.

(A) Schematic illustrating experimental design and timeline. Tx, transplantation. (B) Animals with unilateral 6-OHDA lesions showed strong rotational asymmetry in response to 2.5 mg/kg (intraperitoneal) D-amphetamine that was significantly reduced in the group (mean $\pm$ SEM) that received transplants by 16 weeks and maintained at 28 weeks (Student's t test; $n = 7$ for control, $n = 6$ for grafted; ** $p < 0.01$, *** $p < 0.005$). (C) Immunohistochemistry for EGFP in representative examples of grafted animals at 4, 10, 18, and 28 weeks showed the progressive innervation of host striatum over 6 months. Scale bar, 1 mm.

neurons were clearly heterogeneous in nature and could be distinguished based on features representative of both the major A9 and A10 classes of midbrain DA neurons. This included smaller diameter (~ 15 – 20 μ m), spherical neurons consistent with A10 morphology (Figures A1.3C and A1.3H) and also larger (~ 20 – 50 μ m), pyramidal neurons typical of A9 phenotype (Figures A1.3D and A1.3H). Labeling for GIRK2 and CALBINDIN also showed a mix of DA subtypes (Figures A1.3E–3H). Large, GIRK2⁺ DA neurons that did not express CALBINDIN were often found in clusters near the edge of the grafts, while CALBINDIN⁺ DA subtypes were found throughout the grafts. Cell counting showed that the fractional contribution of distinct EGFP⁺ DA phenotypes based on expression of GIRK2 or CALBINDIN was: $46.8\% \pm 8.1\%$ GIRK2⁺/CALBINDIN⁻, $20.8\% \pm 1.1\%$ GIRK2⁺/CALBINDIN⁺, and $5.0\% \pm 1.6\%$ GIRK2⁻/CALBINDIN⁺ (Figure A1.3I). A remaining $27.4\% \pm 5.7\%$ of EGFP⁺ cells did not express either GIRK2 or CALBINDIN.

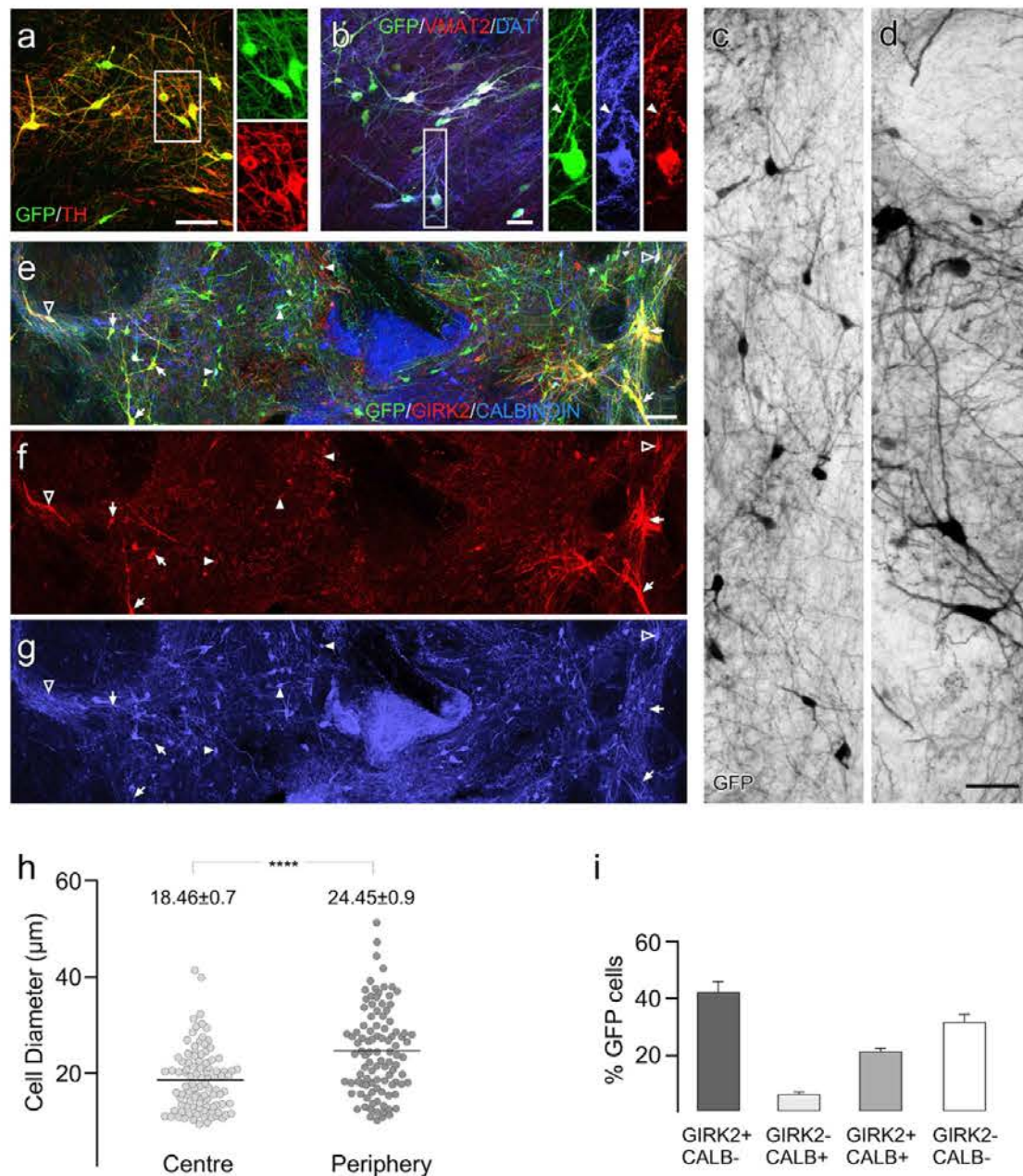


Figure A1.3 – Immunohistochemical identification of DA neuronal subtypes in grafts at 28 weeks.

(A) The vast majority of EGFP⁺ cells were TH⁺ with typical midbrain dopamine neuron morphology. (B–G) Most EGFP⁺ DA neurons also expressed VMAT and DAT with a punctate pattern typical for these proteins (closed arrowheads). Cytoplasmic distribution of EGFP showed a mix of neuronal morphologies including smaller spherical neurons typical for A10 identity (C), as well as large, angular cell soma typical for A9 neurons (D). The GFP⁺ neurons were also mixed based on GIRK2 and CALBINDIN expression and included EGFP⁺/GIRK2⁺/CALBINDIN⁻ (E–G; arrows, particularly at the periphery of the grafts), EGFP⁺/GIRK2⁺/CALBINDIN⁺ (E–G; open arrowheads), and a smaller contribution of EGFP⁺/GIRK2⁻/CALBINDIN⁺ neurons (E–G; closed arrowheads). (H) Comparison of the mean diameter (horizontal lines) of EGFP⁺ cells in the periphery of the graft (n = 100; sampled across 3 grafts) showed these cells were significantly larger than those located more centrally (n = 100; sampled across 3 grafts, Student's t test: ****p < 0.001). (I) Fractional contribution of GIRK2/ CALBINDIN-expressing cell subtypes as a proportion of EGFP⁺ cells (mean ± SEM; n = 5 grafts). CALB, CALBINDIN. Scale bars, 100 µm (A, E–G) and 50 µm (B–D).

A1.4.3 Human stem cell-derived DA neurons specifically innervate appropriate host targets

Dark-field imaging of chromogen-labeled immunohistochemistry for EGFP revealed a remarkably specific pattern of axonal outgrowth by the grafted DA neurons (Figure A1.4). Within the host striatum, EGFP⁺ fibers formed a dense fiber network covering large areas of the head of the striatum (Figures A1.4A and A1.4D). The EGFP⁺ fibers also extended long distances to innervate extra-striatal areas. A notable example included prominent growth along white matter tracts, coursing anterior to the graft through forceps minor to provide a robust innervation of the frontal cortex in a layer-specific pattern, and also the cingulate cortex directly overlying the graft (Figures A1.4A and A1.4B). A smaller innervation of the frontal cortex in the contralateral hemisphere via fibers projecting through the corpus callosum was also observed in some animals (Figure A1.4C). Other structures with distinct EGFP⁺ innervation included the perirhinal cortex (Figure A1.4E) and the islands of Calleja through the ventral pallidum and septum (Figures A1.4F and A1.4G).

A1.4.4 Retrograde tracing reveals neuronal subtype specificity for axonal outgrowth patterns

To characterize the neuronal identity of graft-derived innervation of distinct host target areas, we microinjected Fluorogold (FG) into either the dorsolateral striatum (Figures A1.5A and A1.5B) or frontal cortex (Figures A1.5C and A1.5D) in separate groups of animals. The deposits of FG were verified as being discretely contained within the intended target sites and clearly separated from the graft itself (Figures A1.5A and A1.5C). Across 4 animals where FG was injected into the dorsolateral striatum, 79 FG⁺ cells were identified within the grafts and the majority of these (54; ~68%) were EGFP⁺ DA neurons. These neurons appeared as predominately A9 in phenotype with 45 of 54 (~83%) presenting as GIRK2⁺/CALBINDIN⁻ and a relatively large average diameter of 35.49 μ m, while a smaller fraction of cells (9 of 54) presented as either GIRK2⁻/CALBINDIN⁺ or GIRK2⁺/CALBINDIN⁺ with a smaller average diameter of 20 μ m (Figures A1.5B and A1.5E).

Retrograde uptake of FG from the frontal cortex identified a markedly different pattern of FG⁺ cells, with the vast majority across 3 animals having a non-DA identity (93/105; ~89%). The 12 FG⁺/EGFP⁺ DA neurons were mixed in phenotype with respect to typical A9/A10 attributes. A total of 5 were GIRK2⁺/CALBINDIN⁻ and variable in size with an average diameter of 32.8 μ m; while the remaining 7 expressed CALBINDIN—either alone or with GIRK2—and were

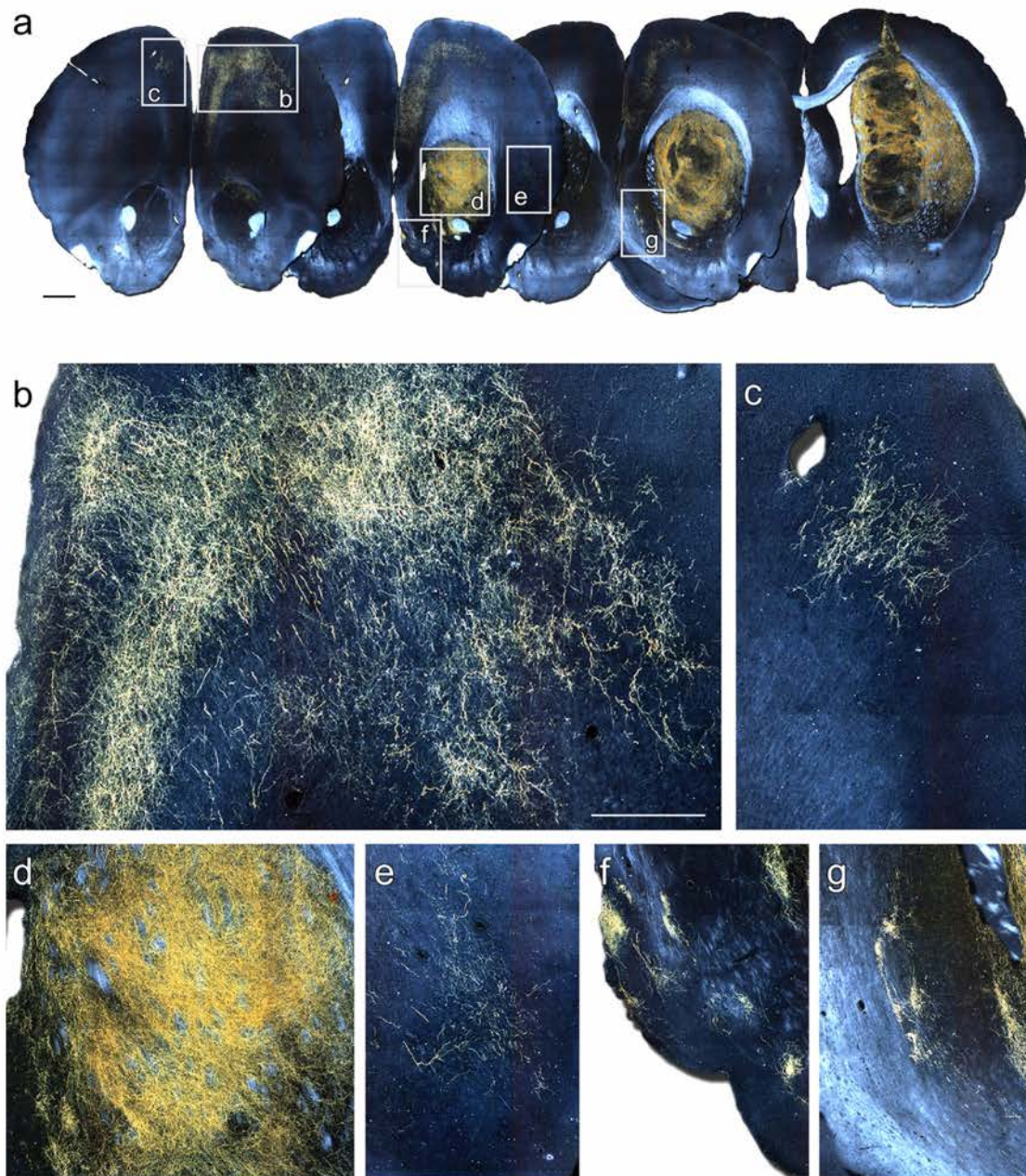


Figure A1.4 – PITX3-eGFP reveals specific patterns of graft-derived DA neuron connectivity.

(A) Representative dark-field photomontages of chromogen-labeled GFP 28 weeks after grafting. (B–G) Boxed areas from (A) are shown at higher magnification to illustrate EGFP⁺ innervation of various host territories, including: frontal cortex ipsilateral (B) and contralateral (C) to the graft; the head of the striatum immediately rostral to the graft core (D); perirhinal cortex (E); and the islands of Calleja through the ventral palladium (F) and septum (G). Scale bars, 1 mm (A) and 500 mm (B–G).

notably smaller, with an average diameter of 21.86 mm (Figures A1.5D and A1.5E). Regardless of the FG injection site, larger cells with A9 features tended to reside toward the periphery of the graft, while the smaller, CALBINDIN-expressing fraction was distributed closer to the center (Figure A1.5E). A number of the non-DA neurons innervating the cortex co-expressed γ -aminobutyric acid (GABA) (Figure A1.5F), although the dense GABA immunoreactivity of the sur-

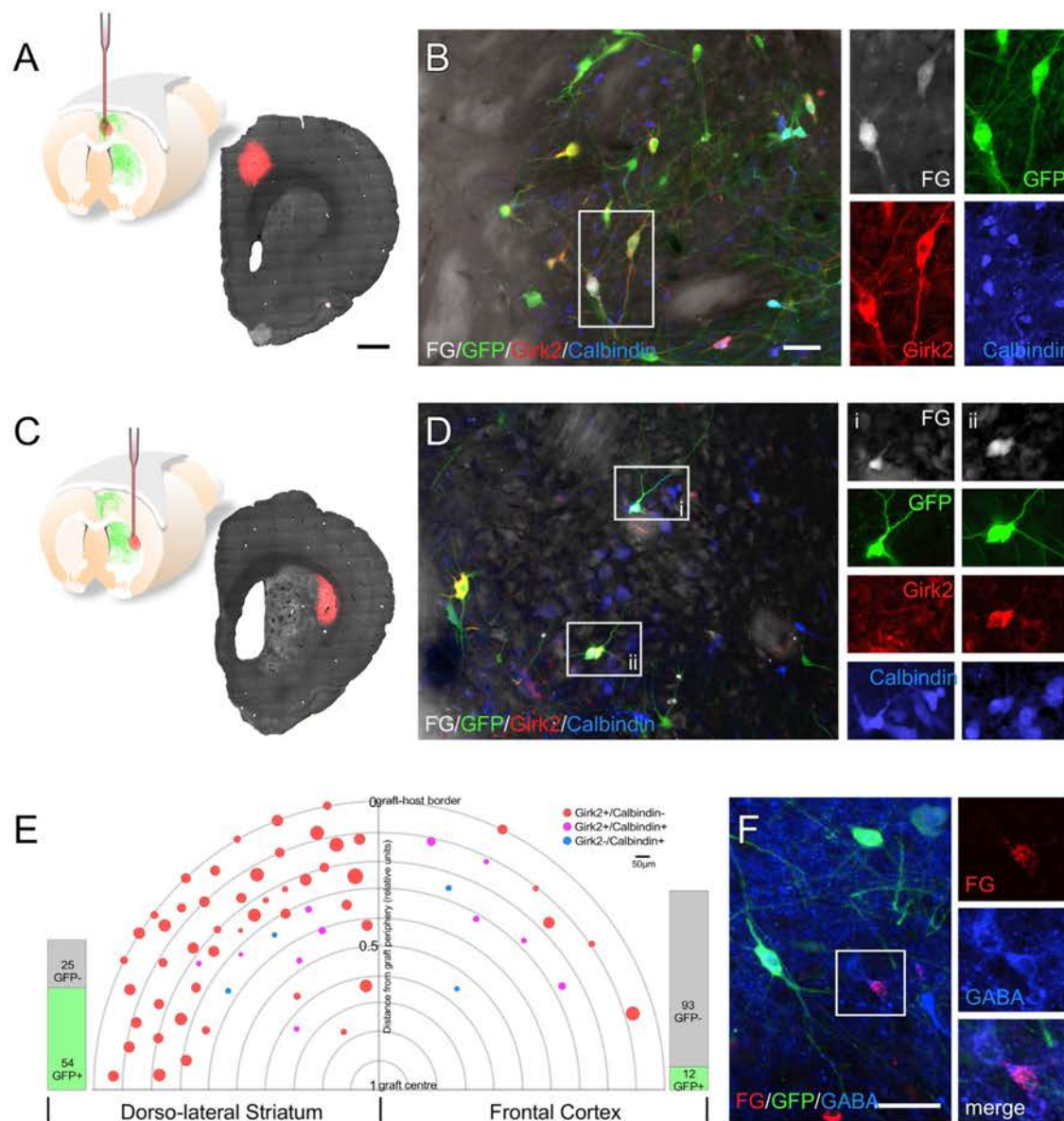


Figure A1.5 – DA neuronal-subtype-specific innervation of host targets.

Small deposits of Fluorogold (FG) were injected into the dorsolateral striatum (A) or frontal cortex (C) 27 weeks after grafting. The deposits were verified as being located remotely to the graft core for each animal as shown in photomontages of fluorescent detection of EGFP (grayscale; note EGFP⁺ fiber network can be seen in the striatum) and FG (red) in representative coronal sections (A and B). Detection of FG (grayscale), EGFP (green), GIRK2 (red), and CALBINDIN (blue) in animals receiving FG in either the dorsolateral striatum (B) or frontal cortex (D) highlight representative examples of DA neuronal phenotypes projecting to these regions. A total of 79 FG⁺ grafted cells were detected in animals injected with FG in the dorsolateral striatum, of which 54 were EGFP⁺ DA neurons and a total of 105 were detected in animals injected in the frontal cortex, of which only 12 were EGFP⁺ DA neurons (E). For each FG⁺ cell the relative location on the axis between the center of the graft and the graft-host border is indicated as well as the soma size (maximum diameter) and the GIRK2/CALBINDIN expression profile. Dopamine neurons (EGFP⁺) retrogradely labeled (FG⁺) from the dorsolateral striatum are plotted on the left of the graph and EGFP⁺/FG⁺ neurons retrogradely labeled from frontal cortex were also observed in the grafts (F). Scale bars, 1 mm (A and C); 50 μ m (B, D, and F). The schematic in (A) is modified from an original created by Bengt Mattsson (Lund University, Sweden).

-rounding neuropil made it difficult to unambiguously quantify the proportion of FG⁺ cells.

A1.4.5 Non-dopaminergic neurons in stem cell-derived ventral midbrain grafts show extensive patterns of connectivity in the host brain

Immunohistochemistry for EGFP and NeuN showed that the majority of differentiated neurons in the grafts were non-dopaminergic (EGFP⁺/NeuN⁺; Figure A1.6A). Many of these had a ventral identity, based on labeling for FOXA2, with examples also of NeuN⁺/FOXA2⁺ cells, particularly in central parts of the grafts, which may represent relatively immature ventralized progenitors. In terms of neurotransmitter phenotype, the grafts were highly immunoreactive for GABA (Figure A1.6B). The labeling pattern for GABA made it difficult to reliably identify GABA⁺ cell bodies, precluding quantification; however, some cases of clearly GABA⁺ neuronal profiles could be seen intermingled with grafted DA neurons (Figure A1.6C). We were not able to identify serotonin-, acetylcholine-, or noradrenaline-containing neurons.

Detection of human-specific NCAM allowed for identification of the entirety of graft-derived fiber outgrowth (Figure A1.6D), which was substantially more extensive than the EGFP⁺ fiber patterns. The NCAM⁺ labeling was particularly prominent in frontal cortical areas (Figures A1.6E and A1.6F) and, again, notably more extensive than the EGFP⁺ fiber innervation. NCAM⁺ fibers extended widely throughout the rostrocaudal axis of both hemispheres, predominately coursing along host white matter tracts, to innervate many structures not targeted by EGFP⁺ DA innervation. This included discrete areas of concentrated fiber innervation indicative of target-directed growth, including the lateral septal nuclei (Figures A1.6D and A1.6G), perirhinal and entorhinal cortex (Figures A1.6D and A1.6I), basolateral amygdala (Figures A1.6D and A1.6J), periaqueductal gray, and caudal aspects of the ventral hippocampus (Figures A1.6D and A1.6L). Another notable feature was the presence of NCAM glia, which migrated extensively throughout the corpus callosum and adjacent cortical parenchyma and along the internal capsule as far caudally as the entopeduncular nucleus and cerebral peduncles (Figures A1.6D, A1.6H, and A1.6K). An overlay of camera lucida-style reconstructions of immunohistochemistry for NCAM and EGFP illustrates the topographical distribution and relative density of dopaminergic and total (largely non-dopaminergic) patterns of graft-derived fiber outgrowth in the host brain (Figure A1.7A). Photomicrographs of the corresponding sections showed that the majority of TH⁺ fibers in the striatum were graft-derived EGFP⁺ origin, while in the cortex the EGFP⁺/TH⁺ fibers were clearly intermingled with a residual host derived EGFP⁻/TH⁺ fiber network (Figure A1.7B). Comparison of EGFP⁺ and NCAM⁺ fiber densities in specific host nuclei illustrated the relative contribution of DA and non-DA innervation of these areas (Figure A1.7C). There was no significant difference in EGFP⁺ and NCAM⁺ density in the dorsolateral striatum, indicating a

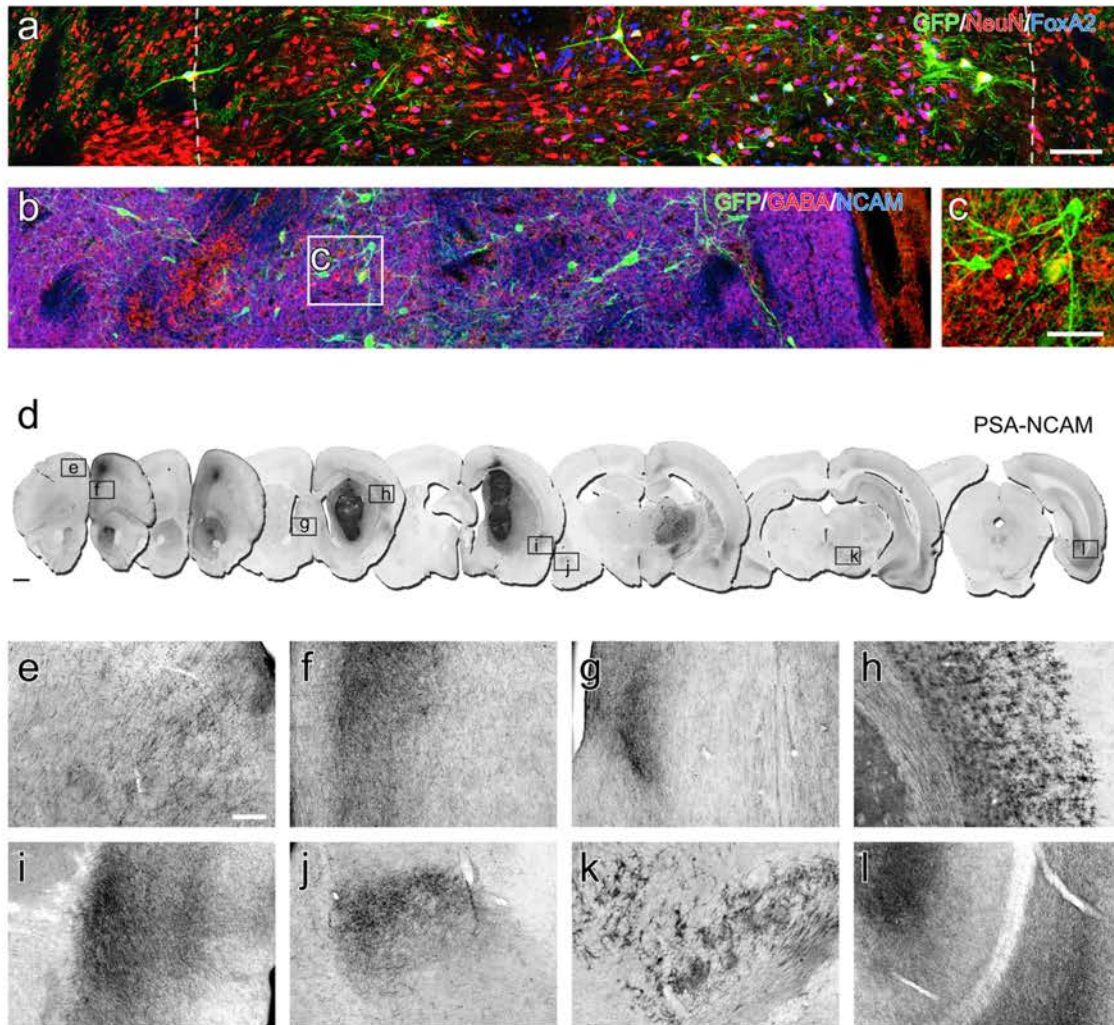


Figure A1.6 – Grafts are heterogeneous in composition and contain predominantly non-DA neuronal subtypes.

(A) Immunohistochemistry for EGFP, NeuN, and FOXA2 at 28 weeks illustrates the relative contribution of DA neurons (EGFP⁺/NeuN⁺/ FOXA2⁺) and non-DA neurons (NeuN⁺/GFP⁺) as well as ventralized (FOXA2⁺) cell types which included both neurons (NeuN⁺) and NeuN⁻, presumably immature, cell types (dashed lines approximate the graft-host border). (B and C) The graft was highly immunoreactive for GABA (red), where human NCAM defines the graft (blue) and EGFP (green) is included to show DA neurons (B). Individual GABA neurons are difficult to resolve but can be delineated in the enlarged boxed area (C). (D–L) Human-specific detection of NCAM in representative coronal sections (D) illustrates the overall extent of graft-derived fiber outgrowth, with prominent innervation of various host territories including, but not limited to: the frontal cortex contralateral (E) and ipsilateral (F) to the graft; lateral septal nuclei (G); perirhinal cortex (I); basolateral amygdala (J, contralateral hemisphere shown); and caudal aspects of the ventral hippocampus (L). Cells with glial morphology migrated extensively through host white matter tracts, particularly adjacent to the corpus callosum (H), and migrated long distances as far caudally as the midbrain (K). Scale bars, 100 mm (A and B), 50 mm (C), 1 mm (D), 200 mm (E; applies to E–L).

largely dopaminergic innervation, while all other areas showed significantly greater NCAM⁺ density and, thus, substantial contribution from non-DA neuronal subtypes.

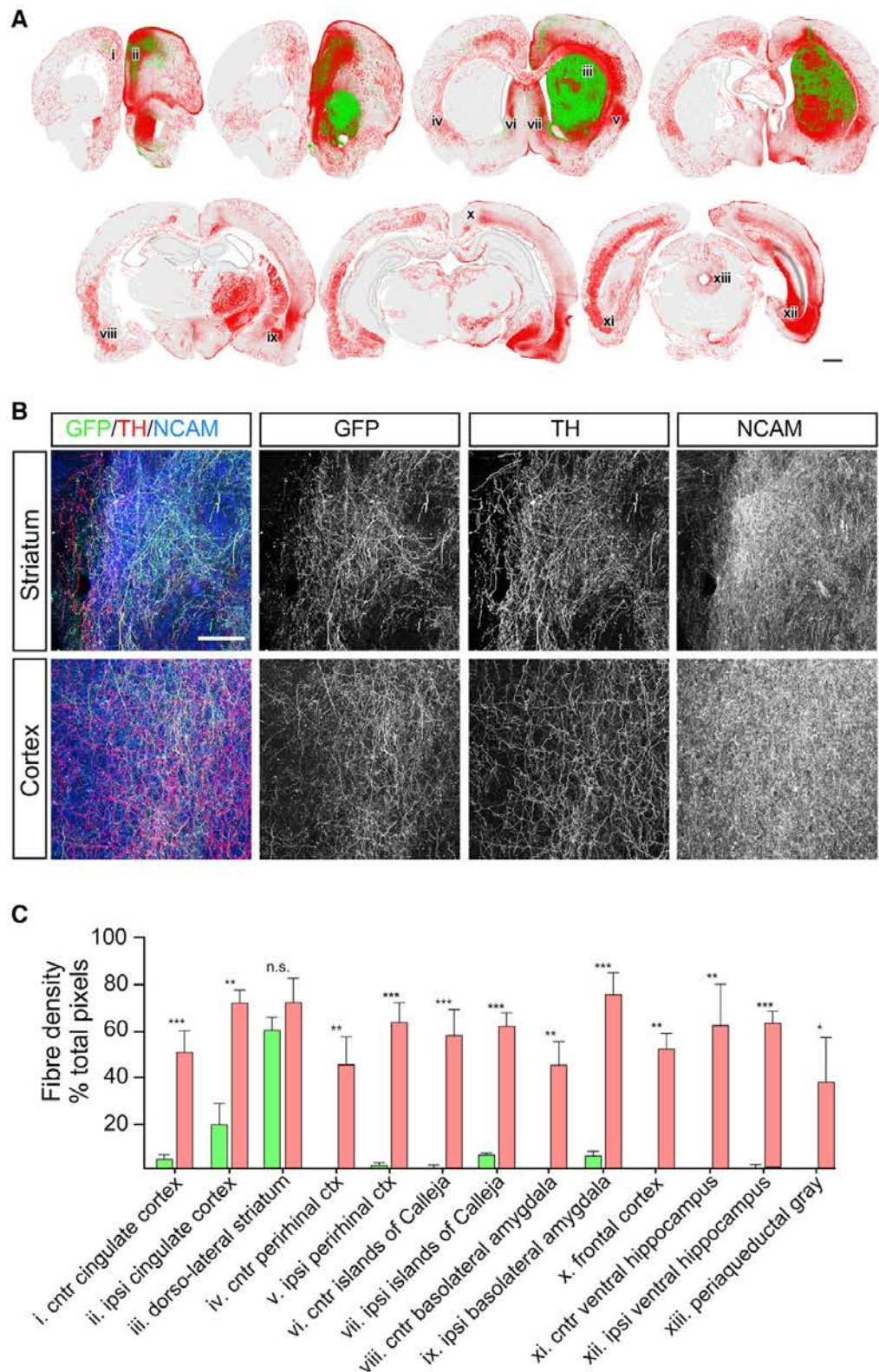


Figure A1.7 – The non-DA component in ES-derived grafts provides extensive innervation of the host.

Schematic reconstruction of immunohistochemistry for NCAM and EGFP illustrates the extent and pattern of DA-specific innervation of the host (EGFP⁺/NCAM⁺, green) relative to the total non-DA innervation (EGFP⁺/NCAM⁺, red) in a representative example 28 weeks after grafting (A). Photomicrographs from the striatum and cortex show dense fiber EGFP⁺, TH⁺, and NCAM⁺ fiber networks and illustrate the relative contribution of NCAM⁺ fibers as substantially greater than the graft-derived DA innervation in both areas (B). Quantification of NCAM⁺ and EGFP⁺ fiber density (mean ± SEM; n = 5 grafts) in specific host nuclei illustrates the relative contribution of DA and non-DA innervation in each region (C; *p < 0.05, **p < 0.01, ***p < 0.005; n.s., not significant). Scale bars, 1 mm (A) and 100 mm (B).

A1.5 Discussion

These results demonstrate the extent and specificity of host innervation from grafted DA neurons generated from human ESCs using contemporary differentiation and transplantation procedures. Under these conditions, PITX3-driven EGFP expression was highly specific for bona fide midbrain DA identity and thus allowed for precise characterization of DA-specific patterns of connectivity. The findings are similar to those from studies using fetal donor tissue from TH-GFP or Pitx3-GFP reporter mice ([Grealish et al., 2010](#); [Thompson et al., 2005](#)), showing that the grafts contain a mix of the major A9 and A10 DA neuronal subtypes and retain an intrinsic capacity to innervate multiple, developmentally appropriate host structures on a neuronal-subtype-specific basis. There were differences, however, that likely reflect species-specific aspects of midbrain cytoarchitecture and projection patterns.

The relative contribution of cell subtypes on the basis of GIRK2/CALBINDIN expression showed the predominant phenotype as GIRK2⁺/CALBINDIN⁻ (~47%) and only a minority as GIRK2⁻/CALBINDIN⁺ (~5%). This differs from grafts of fetal mouse midbrain, where there is a more marked distinction between GIRK2⁺ and CALBINDIN⁺ DA subtypes ([Grealish et al., 2010](#); [Thompson et al., 2005](#)) and may represent species-specific differences in GIRK2 expression - which is broader in the human midbrain, incorporating A10 areas ([Reyes et al., 2012](#)) - but also the relatively greater ratio of A9 to A10 DA neurons in the primate brain compared to rodents (for review see [Bjorklund and Dunnett, 2007](#)). Innervation of extra-striatal territories was notably more extensive than has been reported in studies using donor tissue from reporter mice ([Thompson et al., 2005](#)), including a particularly robust innervation of frontal and cingulate cortices. Compared with rodents, the A9 DA neurons in the primate brain are known to provide a substantially greater innervation of the cortex ([Bjorklund and Dunnett, 2007](#); [Fallon and Loughlin, 1995](#); [Lewis and Sesack, 1997](#)). In line with this, the retrograde tracing experiments showed that while innervation of the dorsolateral striatum was overwhelmingly provided by grafted DA neurons with A9 properties, the cortex, which is predominately innervated by A10 subtypes in rodent grafting studies ([Thompson et al., 2005](#)), received innervation from both A9 and A10 subtypes. Thus overall, grafted DA neurons generated from human ESCs retain an intrinsic capacity for neuronal-subtype-specific patterns of target-directed outgrowth. These observations indicate that GIRK2 and/or CALBINDIN are accurate markers of A9/A10 innervation identity after ~6 months *in vivo*. This is in contrast to early development where the expression of GIRK2/CALBINDIN is less restricted. These results also highlight the requirement for differentiation procedures resulting in appropriately patterned A9 neurons for establishment of

terminal networks in territories relevant for restoration of motor function, including the dorsolateral striatum.

Both the time course for impact on motor function and the development of a graft-derived DA terminal network in the striatum were notably protracted. The first significant reduction in amphetamine-induced rotational asymmetry was apparent from 16 weeks and was complemented by the progressive development in density of striatal innervation between 10 and 28 weeks. This is consistent with other recent grafting studies using human embryonic stem (Kirkeby et al., 2017; Kriks et al., 2011; Niclis et al., 2017b) or induced pluripotent stem (Doi et al., 2014; Kikuchi et al., 2017) donor cells, where functional impact required 3–5 months, and also observations in PD patients receiving fetal tissue grafts, in whom clinical improvement occurs gradually between 6 and 24 months (Lindvall and Hagell, 2000). It supports the view that, irrespective of the fetal or stem cell origin of the donor material, therapeutic benefit requires that grafted midbrain DA neurons establish a functional terminal network and is unlikely to be related to indirect mechanisms, such as neuroprotection or host neuroplasticity, as has been suggested in recent studies using parthenogenetic stem cells (Gonzalez et al., 2016). Such conclusions are also supported by recent studies using light-activated opsins (Steinbeck et al., 2015) or synthetic ligand-receptor systems (Chen et al., 2016) to illustrate the relationship between correction of motor function and functional integration of implanted DA neurons.

Use of a PITX3-EGFP reporter line provided the unique opportunity to assess the relative contribution of host innervation from midbrain DA neurons compared with the total extent of graft-derived host connectivity. The results showed that the overwhelming majority of graft-derived outgrowth was non-dopaminergic, even in DA-specific target areas including the striatum and cortex. Non-dopaminergic axonal growth extended predominately via host white matter tracts to provide extensive innervation of remote nuclei throughout both hemispheres. In some areas, including amygdaloid nuclei, the pattern of connectivity appeared highly targeted, suggesting innervation by phenotypically specified neuronal subtypes. The overall pattern of fiber outgrowth was similar to those reported in fetal ventral mesencephalic xenografting studies (Isacson and Deacon, 1996; Thompson et al., 2008), which also include a predominately non-DA component. To some degree this may reflect developmentally appropriate outgrowth of non-DA ventral midbrain neuronal subtypes, for example GABAergic projection neurons of the substantia nigra pars reticulata, ventral tegmental area, and mammillary region that are known to project extensively to thalamus and cortex (Beckstead et

al., 1979; Cornwall and Phillipson, 1988; Shibata, 1992). We identified GABA-containing cells in the stem cell-derived grafts, including those that innervated the host cortex; however, both here and in other recent studies (Doi et al., 2014; Grealish et al., 2014; Hallett et al., 2015; Kirkeby et al., 2017; Kriks et al., 2011; Samata et al., 2016), the majority of grafted neurons were not readily identified based on neurochemical features. This may indicate that many of the immature neural progenitors in the donor cell preparations are insufficiently specified to acquire a terminally differentiated phenotype after transplantation. In this case it is possible that the axonal growth trajectories will be through permissive growth corridors determined by the host environment, rather than intrinsically specified patterns of target-directed connectivity. Consistent with such an explanation, very similar growth patterns characterized by extensive elongation along host white matter tracts has been observed also after transplantation of other donor cell identities, including cortical cell types (Denham et al., 2012b; Niclis et al., 2017a; Somaa et al., 2017). The functional consequences of non-DA connectivity remain largely unknown. While this is an area for ongoing work, it is worth noting that the similarly large contribution of non-DA neuronal outgrowth from fetal grafts has not been identified as problematic or offsetting the overall therapeutic impact provided by the DA neuronal replacement. One notable exception is the inclusion of serotonin neurons, which have been reported as a risk for graft-induced dyskinesia (Carlsson et al., 2007, 2009; Politis et al., 2010).

The average yield of DA neurons in the grafts at 28 weeks was $5,268 \pm 654$ per 100,000 cells implanted. This figure is consistent across a number of recent grafting studies using similar experimental designs, including reports of $\sim 3,716$ (Kirkeby et al., 2017), $\sim 6,000$ (Kriks et al., 2011), and $\sim 8,338$ (Kirkeby et al., 2012) for ESCs and $\sim 3,436$ (Doi et al., 2014) and $\sim 8,849$ (Samata et al., 2016) for iPSCs. Thus the final yield of DA neurons using currently available differentiation and transplantation techniques is in the order of 3.4%–8.8% of cells implanted. This fractional contribution may become considerably smaller with continued growth of the non-DA component over time. Here we observed that the number of DA neurons did not expand between 4 ($6,081 \pm 659$) and 28 weeks ($5,268 \pm 654$), while there was a significant increase in graft volume from $0.8 \pm 0.28 \text{ mm}^3$ to $7.04 \pm 0.91 \text{ mm}^3$ over the same time frame. We have previously reported that neural grafts derived from human PSCs exhibit ongoing cell proliferation in vivo as the basis for expansion of the graft volume (Denham et al., 2012b; Niclis et al., 2017a). Human fetal grafts are also known to expand in vivo (Labandeira-Garcia et al., 1991) and, thus, expansion of stem cell-derived grafts may represent normal developmental growth properties of human neural tissue. On current evidence, however, one cannot

unambiguously exclude the possibility of slow but potentially pathological growth over clinically relevant post-transplantation time frames. This is an area that warrants further attention and various strategies are actively being pursued to safeguard against this risk, including the use of cell-sorting techniques to remove unwanted cell types (Doi et al., 2014; Gennet et al., 2016; Samata et al., 2016) or ligand-activated suicide genes to eliminate proliferating cells in vivo (Itakura et al., 2017; Tieng et al., 2016). A recent study by Kirkeby et al. (2017) highlights that there is also scope for ongoing refinement of differentiation procedures to optimize graft composition, with increased efficiency in specification of midbrain DA identity in vitro resulting in a significant increase in DA neuron density in the resulting grafts.

In summary, we report here that midbrain DA neurons generated from human ESCs establish highly specific patterns of growth in vivo, including innervation of developmentally appropriate targets on a DA neuronal-subtype-specific basis. The delayed onset of functional recovery and protracted course of striatal innervation by midbrain DA neurons with predominately A9 features reinforces the notion that therapeutic benefit from stem cell-based treatments for PD requires functional integration of correctly specified DA neuronal subtypes. Furthermore, the relatively small contribution of DA neurons to the over-all pattern of neuronal integration highlights the scope for further refinement of current differentiation and transplantation protocols. This should include efforts to limit the inclusion of unwanted or uncharacterized cell types. The inability to standardize and characterize fetal tissue preparations prior to transplantation has been recognized as one of the key factors underlying highly variable clinical outcomes to date (Barker et al., 2015; Thompson and Bjorklund, 2012). It is therefore imperative that the same issues are avoided as enthusiasm builds for advancement of human PSCs toward clinical trials in patients with PD. Recent advances in differentiation (Kirkeby et al., 2017) and cell sorting (Doi et al., 2014; Gennet et al., 2016; Samata et al., 2016) procedures give cause for optimism.

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A1.7 Author contributions

Conceptualization, J.C.N., C.L.P., and L.H.T.; Methodology, J.C.N., J.A.K., C.L.P., and L.H.T.; Investigation, J.C.N., C.G., C.H., J.A.K., J.D., C.L.P., and L.H.T.; Provision of Resources, J.M.H. and C.W.P.; Writing – Original Draft, J.C.N., C.L.P., and L.H.T.; Writing – Review and Editing, J.C.N., C.G., C.W.P., C.L.P., and L.H.T.; Funding Acquisition, C.L.P. and L.H.T.

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Appendix 2.

ISOLATION OF LMX1A VENTRAL MIDBRAIN PROGENITORS IMPROVES THE SAFETY AND PREDICTABILITY OF HUMAN PLURIPOTENT STEM CELL-DERIVED NEURAL TRANSPLANTS IN PARKINSONSIAN DISEASE

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A2.1 Abstract

Human pluripotent stem cells (hPSC) are a promising resource for the replacement of degenerated ventral midbrain dopaminergic (vmDA) neurons in Parkinson's disease. Despite recent advances in protocols for the *in vitro* generation of vmDA neurons, the asynchronous and heterogeneous nature of the differentiations results in transplants of surprisingly low vmDA neuron purity. As the field advances towards the clinic it will be optimal, if not essential, to remove poorly specified and potentially proliferative cells from donor preparations to ensure safety and predictable efficacy. Here, we utilize two novel hPSC knock-in reporter lines expressing GFP under the LMX1A and PITX3 promoters, to selectively isolate vm progenitors and DA precursors, respectively. For each cell line, Unsorted, GFP+ and GFP- cells were transplanted into male or female Parkinsonian rodents. Only rats receiving Unsorted cells, LMX1A-eGFP+ or PITX3-eGFP- cell grafts showed improved motor function over 6 months. Post-mortem analysis revealed small grafts from PITX3-eGFP+ cells, suggesting these DA precursors were not compatible with cell survival and integration. In contrast, LMX1A-eGFP+ grafts were highly enriched for vmDA neurons, and importantly excluded expansive proliferative populations and

serotonergic neurons. These LMX1A-eGFP+ progenitor grafts accelerated behavioural recovery and innervated developmentally appropriate forebrain targets whilst LMX1A-eGFP- cell grafts failed to restore motor deficits, supported by increased fibre growth into non-dopaminergic target nuclei. This is the first study to employ a hPSC-derived reporter line to purify vm progenitors, resulting in improved safety, predictability of the graft composition, and enhanced motor function.

A2.2 Introduction

Clinical studies, employing human fetal tissue enriched with dopaminergic (DA) progenitors, have provided the necessary evidence that new DA neurons transplanted into the brains of Parkinson's disease (PD) patients can functionally integrate and alleviate motor symptoms for decades ([Barker et al., 2013](#)). Poor standardisation procedures, limited tissue availability and ethical consideration associated with the use of fetal tissue has resulted in a shift towards the use of human pluripotent stem cells (PSC) to generate donor cells. Despite significant advancements in ventral mesencephalic differentiation protocols in recent years ([Denham et al., 2012](#); [Kirkeby et al., 2012](#); [Kriks et al., 2011](#); [Niclis et al., 2017a](#)), cultures remain asynchronous and heterogeneous, with even the most advanced current protocols reporting ~10-15% of cells fail to adopt a vm progenitor fate at times amenable to transplantation ([Kirkeby et al., 2017](#); [Niclis et al., 2017a](#)). While this appears to represent a relatively high specification efficiency, upon transplantation studies report low yields of tyrosine hydroxylase-expressing DA neurons within the grafts ([Doi et al., 2014](#); [Kirkeby et al., 2012](#); [Kirkeby et al., 2017](#); [Kriks et al., 2011](#); [Niclis et al., 2017b](#); [Samata et al., 2016](#)). These observations indicate significant expansion of other vm progenitors not destined to become DA neurons as well as proliferation of incorrectly specified non-dopaminergic progenitors *in vivo* that may also pose a risk of neural overgrowths/tumours. One also recognises the risk of incorrectly specified neuronal populations, such as serotonergic neurons within grafts, that may induce dyskinetic behaviours ([Carlsson et al., 2007](#); [Politis et al., 2011](#)).

A key strategy to overcome such conundrums and ensure the reproducible generation of safe and predictable cell products for clinical translation is to selectively enrich for appropriately specified vm progenitors prior to transplantation. While a number of rodent

studies have successfully isolated vm progenitors, using reporter mice/cell lines and antibodies targeted against extracellular proteins - employing both fluorescent activated cell sorting (FACS) as well as magnetic beads (MACS) (Bye et al., 2015; Fukuda et al., 2006; Ganat et al., 2012; Hedlund et al., 2008; Jonsson et al., 2009; Nefzger et al., 2012; Thompson et al., 2006), isolation from human PSC cultures has been met with variable success. In part, this has been due to breadth of expression of the transgene/protein, timing of expression of the gene/protein and hence progenitor isolation occurring weeks prior to transplantation, and/or suboptimal specificity (or availability) of antibodies for human cells (Aguila et al., 2014; Doi et al., 2014; Lehnen et al., 2017; Samata et al., 2016)

With the field rapidly advancing to the clinic (Barker et al., 2017), there is a persistent and inherent need to identify a reliable candidate marker for the enrichment of DA progenitors from human PSC-derived vm cultures. Here we assessed the capacity to isolate vm progenitors and DA precursors based upon two cardinal genes involved in vmDA development – LMX1A, an early vm determinant (Andersson et al., 2006; Yan et al., 2011) and PITX3, a gene required for the post mitotic maturation of DA progenitors (Smidt et al., 2004). Both genes have been used to isolate vm progenitors/precursors from mouse ESC cultures (Hedlund et al., 2008; Nefzger et al., 2012). We demonstrate that following FACS isolation and transplantation LMX1A-eGFP+ progenitors, but not PITX3-eGFP+ DA precursor cells, resulted in a higher density of TH+ DA neurons within grafts, appropriate target innervation and consequential improved motor function, whilst critically eliminating proliferative and serotonergic populations from the grafts.

A2.3 Materials and methods

A2.3.1 Human ESC culture and differentiation

Human ESC H9 reporter lines, LMX1A-eGFP and PITX3-eGFP, were cultured and differentiated under xeno-free conditions as previously described (Niclis et al., 2017a). In brief, cells were cultured on Laminin-521 (10µg/ml; BioLamina) and exposed to dual SMAD inhibition (SB431542, 10µM, 0-5 days in vitro, DIV, R&D systems; and LDN193189, 200nM, 0-11DIV, Stemgent) to promote neuralisation. Regionalisation to a ventral midbrain floor plate identity was achieved by exposure to Sonic hedgehog C25II (100ng/ml; R&D systems) and Purmorphamine (2µM; Stemgent) from day 1-7DIV, in addition to Wnt agonist, CHIR 99021 (3µM; Stemgent, 3-13DIV). From 11DIV, cells were cultured in a 'maturation media' consisting of NBB27 supplemented with

GDNF (20ng/ml; R&D systems), BDNF (20ng/ml, R&D system), TGFβ3 (1 ng/ml; PeproTech), DAPT (10 μM, Sigma-Aldrich), ascorbic acid (200nM; Sigma-Aldrich) and dibutyryl cAMP (0.05mM; Tocris Bioscience).

A2.3.2 Fluorescence-activated cell sorting

At 21 DIV, cultures rich in vm progenitor/precursors were dissociated in Accutase (Innovative Cell Technologies) to a single-cell suspension and resuspended into a sorting buffer containing 90% maturation media, 10% knockout serum replacement, and ROCK inhibitor Y-27632 (10μM, Tocris Bioscience) to a final density of $5-8 \times 10^6$ cells/ml. Cells were filtered through cell strainer caps (35μM mesh, Falcon) to remove the presence of cell doublets and clusters. Sorting was performed on a MoFlo cell sorter (Beckman Coulter, MoFlo) or FACS Aria III using a 100-μM nozzle, sheath pressure of 17-22 psi. A fraction of cells were passed through the cell sorter, without selection, forming the Unsorted group, whilst eGFP+ and eGFP- cells were isolated using a highly restrictive gating strategy to exclude doublets and debris based on forward and side-scatter parameters, as well as dead cells using the viability marker 4'-6-Diamidino-2-Phenylindole (DAPI). Background auto-fluorescence was compensated for by an empty auto fluorescence channel (FL2-580/30), and a H9 parental cell line at the same stage of differentiation was used as a GFP- control (data not shown). FACS-isolated cell fractions were either (i) replated at 300,000 cells/cm² in 96-well plates, (ii) passed back through the FACS machine, (both targeted at confirming efficacy of the FACS isolation), or (iii) resuspended in maturation media containing ROCK inhibitor (Y27632, 10μM, Sigma) at 100,000 cells/μL and stored on ice until the time of transplantation. Flow cytometric analysis was conducted using FlowJo software.

A2.3.3 6-hydroxydopamine lesioning and cell transplantation

All animal procedures were conducted in agreement with the Australian National Health and Medical Research Council's published Code of Practice for the Use of Animals in Research, and experiments approved by The Florey Institute of Neuroscience and Mental Health Animal Ethics committee. Animals (of either sex) were group housed on a 12:12-hour light/dark cycle with ad libitum access to food and water. Surgeries were performed on athymic nude rats and nude mice under 2% isoflurane anaesthesia. Unilateral 6-hydroxydopamine (6-OHDA) lesions of the rat medial forebrain bundle (3.5μl, 3.2μg/μl) or mouse substantia nigra pars compacta (1.5μl,

1.6 µg/µl) were performed to ablate the host midbrain dopamine pathways, as previously described (Kauhausen et al., 2013; Niclis et al., 2017a).

Six weeks after lesioning, animals received a unilateral injection of either human PSC-derived LMX1a-eGFP vm progenitors or PITX3-eGFP DA precursors into the denervated striatum. For each cell line, grafts were of Unsorted, GFP+, or GFP- progenitors (1µl; 100,000 cells/µl). Stereotaxic co-ordinates were as follows in rats: 0.5mm anterior, 2.5mm lateral to Bregma and 4.0mm below the dura surface, and in mice: 0.5mm anterior, 2.5mm lateral, 4.0mm ventral), as previously described (Kauhausen et al., 2013; Niclis et al., 2017b). See Figure 1 for study design overview.

A2.3.4 Behavioural testing

Rotational asymmetry, following intraperitoneal administration of D-amphetamine sulphate (3.5mg/kg, Tocris Bioscience), was assessed 4 weeks after 6OHDA lesioning. Only animals capable of >300 rotations in 60 minutes, confirmed on 2 independent tests/rat, were included in the study. Behavioural testing was repeated at 16, 20 and 24 weeks after cell grafting to assess the functional integration of the transplants.

A2.3.5 Immunocytochemistry

In vitro cultures were fixed with 4% paraformaldehyde at day 21, or at 6 hours after FACS and *in vitro* re-plating. Rats and mice were killed by an overdose of sodium pentobarbitone (100mg/kg), and transcardially perfused with Tyrode solution followed by 4% paraformaldehyde. Brains were coronally sectioned (40µm; 12 series) on a freezing microtome (Leica). Immunohistochemistry was performed in accordance to previously described methods (Soma et al., 2017). Primary antibodies and dilutions were as follows: 4',6-diamidino-2-phenylindole (DAPI, 1:5000; Sigma Aldrich), rabbit anti-OTX2 (1:4000; Millipore), goat anti-FOXA2 (1:200; Santa Cruz), chicken anti-GFP (1:1,000; Abcam), rabbit anti-GFP (1:20,000; Abcam), sheep anti-tyrosine hydroxylase (TH, 1:800, Pelfreeze), rabbit anti-TH (1:1,000; Pelfreeze), rabbit anti-BARHL1 (1:300, Novus Biologics), sheep anti-PITX2 (1:500, R&D Systems), mouse anti-neural cell adhesion molecule (NCAM, 1:500; Santa Cruz), rabbit anti-NeuN (1:100; R&D Systems), mouse anti-GAD67 (1:100, Millipore), goat anti-choline acetyltransferase (ChAT, 1:100, Millipore), mouse anti-dopamine-β-hydroxylase (DBH, 1:5000,

Millipore, MAB308), rabbit anti-glial fibrillary acidic protein (GFAP, 1:500, DAKO), mouse anti-adenomatous polyposis coli, clone CC1 (APC, 1:100, Abcam), mouse anti-rat endothelial cell antigen-1 (RECA1, 1:500, Serotec), rabbit anti-Claudin V (1:200, Abcam) rabbit anti-GIRK2 (1:500; Alomone Labs), mouse anti-calbindin (1:1,000; Swant Biotech), rabbit anti-KI67 (1:1,000; ThermoFisher), goat anti-doublecortin (DCX, 1:1,000; Santa Cruz), mouse anti-human nuclear antigen (HNA, 1:300; Millipore) and rabbit anti-5HT (1:1000; Immunostar). Secondary antibodies for direct detection were used at a dilution of 1:200—DyLight 488, 549 or 649 conjugated donkey anti-mouse, anti-chicken, anti-rabbit, anti-goat or anti-sheep (Jackson ImmunoResearch).

A2.3.6 Microscopy and quantification

Fluorescence images were captured using a Zeiss Axio Observer Z.1 epifluorescence or Zeiss LSM 780 Confocal Microscope, while bright and dark field images were obtained using a Leica DM6000 microscope. Grafts were delineated based upon PSA-NCAM expression and volume estimated using the Cavalieri's principle. Total number of human nuclear antigen (HNA+), green fluorescent protein (GFP+), tyrosine hydroxylase (TH+), serotonergic (5HT+) and proliferative (KI67+) cells were counted from images captured at 20X magnification. To quantify GIRK2+ and CALBINDIN+ cells within the grafts, dopamine neurons were first identified based upon TH immunoreactivity from acquired confocal images.

The density of TH and NCAM labelled fibres were assessed at predetermined sites within known dopaminergic (dorsolateral striatum and cortex) and non-dopaminergic (periventricular thalamic nucleus and fimbria of the contralateral hippocampus) target nuclei, from compressed z-stacked images. Images were analysed using ImageJ software.

A2.3.7 Experimental design and statistical analysis

All behaviour testing and histological assessments were performed with the researcher blinded to the experimental group. A total of 48 female athymic nude rats (CBHrnu) and 13 nude mice (BALB/c-*Foxn1*^{nu}/Arc) were transplanted from two independent differentiations (into rats and subsequently mice, with n=3-8 animals per group. All data are presented as mean + SEM. Statistical tests employed (inclusive of one-way ANOVA, two-way ANOVA and student t-tests), as well as number of animals/group for each analysis are stated in figure legends. Alpha levels

of $p < 0.05$ were considered significant with all statistical analysis performed using GraphPad Prism. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

A2.4 Results

A2.4.1 Isolation of vm progenitor/precursors from LMX1A-eGFP and PITX3-eGFP human ESC-lines

Enhanced GFP reporter human ESC lines for the early vm progenitor gene LMX1A and later DA precursor gene PITX3 (Figure A2.1A-C), were differentiated according to the recently developed, and clinically relevant, xenogeneic-free protocol (Niclis et al., 2017a). At the time corresponding to the FACS isolation of progenitors for transplantation, 21 days in vitro (DIV), the efficiency of the differentiating cultures could be confirmed in both stem cell lines by the co-expression of the forebrain-midbrain marker OTX2 and basal plate marker, FOXA2 (Figure A2.1D,E,J,K), accounting for >85% of all cells. At this time, the LMX1A-eGFP reporter line revealed that a significant proportion of the cultures also co-expressed LMX1A (i.e. GFP+, >70%), as previously described (Niclis et al., 2017a), with few maturing TH+ cells emerging (Figure A2.1F-I). In contrast, PITX3 expression, visible by GFP immunocytochemistry in the differentiated PITX3-ESC cultures, showed sparse expression (<2%) that tightly overlapped with TH (Figure A2.1L-O). Importantly, we showed low proportions of BARHL1 and PITX2 immunoreactive cells, indicative of off-target, developmentally adjacent subthalamic progenitors (Kee et al., 2017), within differentiating LMX1A-GFP (Figure A2.1P) and PITX3-GFP (data not shown) cell lines.

FACS analysis of the LMX1A-GFP and PITX3-GFP cells from differentiating cultures revealed 70.2% and 1.2% of cells, respectively, expressed GFP (Figure A2.1R,V). Efficiency of the sorting was confirmed by re-analysis of the isolated fractions by flow cytometry, as well as replating of the sorted fractions, and cytochemistry at 6 hours after seeding (Figure A2.1Q,S-U,W-Y). Within the LMX1A-eGFP positive (LMX1A-GFP+) fraction, >99.2% of cells expressed GFP, with all GFP cells confirmed to co-express cardinal vm progenitor markers OTX2 and FOXA2 (data not shown). Within the re-plated LMX1A-eGFP negative fraction (LMX1A-GFP-), 10% of cells were GFP+, a likely reflection of ongoing, asynchronous maturation of the cultures during the period of both sorting and replating (Figure A2.1U). Assessment of the sorted PITX3-eGFP cell line

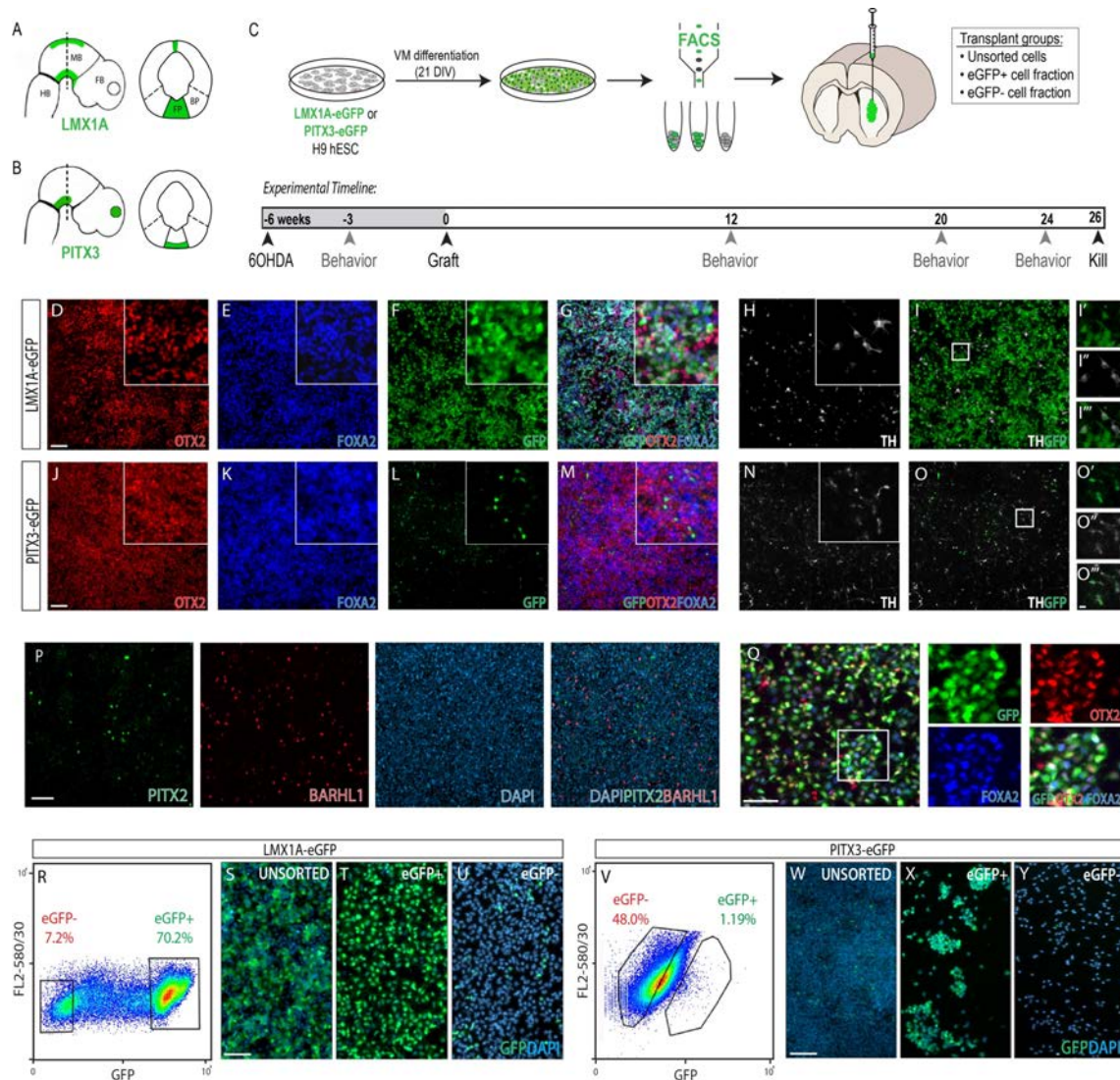


Figure A2.1 – In vitro differentiation and purification of vm progenitors/precursors using LMX1A-eGFP and PITX3-eGFP human embryonic stem cell reporter lines.

(A-B) Schematic coronal and sagittal views of the developing embryo depicting expression patterns of two key vmDA determinant transcription factors, (A) LMX1A and, (B) PITX3. eGFP was expressed under the promoter for these two transgenes within H9 hESC lines for the purpose of cell sorting. (C) Schematic detailing the differentiation, isolation and transplantation of cells derived from LMX1A-eGFP and PITX3-eGFP hESC reporter lines, and the experimental timeline for *in vivo* procedures. (D-G, J-M) Validation of efficient VM differentiation by co-expression of OTX2, FOXA2 and eGFP, demonstrated robust specification of vm progenitors by 21DIV. (I,O) Maturing dopamine neurons present within the culture at 21DIV tightly overlapped with eGFP expression. (P) Low proportion of PITX2+ and BARHL1+ cells confirmed cultures were not of unintended subthalamic identity. (Q) Immunohistochemical labelling of LMX1A-GFP+ progenitors, 6 hours after sorting and replating, showing co-expression of cardinal vmDA progenitor markers OTX2, FOXA2 and LMX1A-GFP. (R, V) Flow cytometric plots indicating the gating for enrichment of eGFP+ and eGFP- populations within differentiating cultures at 21DIV. Successful isolation of specified populations (Unsorted, GFP+ and GFP-) from the (S-U) LMX1A-eGFP, and (W-Y) PITX3-eGFP hPSC line was confirmed by replating of cells and immunohistochemical staining for GFP and DAPI. Abbreviations: DIV, days in vitro; eGFP, enhanced green fluorescent protein; hESC, human embryonic stem cells. Scale bar: (D-O, S-U, W-Y) 200µm, (I'-I''', O'-O''') 20µm.

confirmed >95% of the replated cells from the positive fraction expressed GFP (Figure A2.1V), whilst GFP+ cells within the negative fraction were extremely rare (Figure A2.1Y).

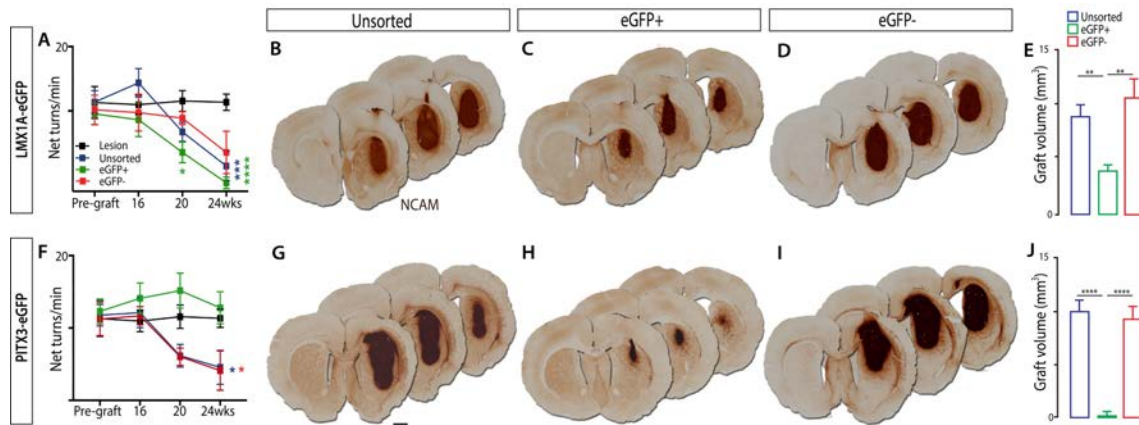


Figure A2.2 - LMX1a-eGFP⁺ progenitors, but not PITX3-eGFP⁺ precursors, show engraftment and amelioration of behavioural deficits in 6-OHDA lesioned rats.

(A) Transplantation of LMX1A-eGFP⁺ progenitors (green) resulted in an accelerated correction of rotational asymmetry in 6OHDA athymic rats, compared to grafts of Unsorted (blue) and eGFP- vm progenitors (red), Two-way ANOVA with Dunnett correction for multiple comparisons relative to 6-OHDA, Group, $F_{(3,88)} = 9.131$, $p < 0.0001$, $n=6-8/\text{group}$). (B-D) Human specific neural cell adhesion protein (NCAM) enables visualisation of the LMX1A progenitor grafts within the host striatum. (E) Quantitative assessment of LMX1A-eGFP progenitor grafts highlighting the smaller size of grafts derived from eGFP⁺ progenitors, (One-way ANOVA with Tukey's post-hoc testing, $F_{(2,14)} = 9.8$, $p = 0.0022$, $n=6-8/\text{group}$). (F) Grafting of PITX3-eGFP⁻ and Unsorted, but not PITX3-eGFP⁺ cells, corrected gross motor deficits in 6OHDA rats, Two-way ANOVA, $F_{(3,120)} = 3.188$, $p = 0.0263$, $n=6-8/\text{group}$). (G-I) Coronal sections of the rat brain showing large viable grafts 6months after transplantation of Unsorted and PITX-eGFP⁻ cells. PITX3-eGFP⁺ DA precursors showed poor survival and engraftment following transplantation, (J) confirmed by significantly reduced graft volume, (One-way ANOVA, $F_{(2,16)} = 30.61$, $p = <0.0001$, $n=6-8/\text{group}$). All data represents Mean $\pm$ SEM. Scale bar: (B-D, G-I) 1mm.

A2.4.2 Transplanted LMX1A-EGFP⁺, but not PITX3-GFP⁺ DA precursors, restore functional deficits in Parkinsonian rats

All rats included in the study showed an amphetamine-induced rotational asymmetry >5 rotations/min, prior to transplantation, that persisted over 26 weeks as a stable motor deficit in ungrafted animals (Figure A2.2A, F). As anticipated, and in alignment with human PSC-derived vm progenitor grafts by us and others (Doi et al., 2014; Kirkeby et al., 2012; Kirkeby et al., 2017; Kriks et al., 2011; Niclis et al., 2017a; Niclis et al., 2017b; Samata et al., 2016), transplants of unsorted cells, from both LMX1a-GFP and PITX3-GFP cell lines, resulted in near complete recovery of rotational asymmetry by 24 weeks. Grafts of LMX1A-GFP⁺ progenitors also showed accelerated, complete recovery, evident at 20weeks. Interestingly, PITX3-GFP⁻ but not PITX3-GFP⁺ cell grafts showed improvements in motor asymmetry at 6 months.

Post-mortem analysis showed viable grafts, identified by human PSA-NCAM, in all rats 6 months after implantation, (Figure A2.2B-D,G-I). Volumetric analysis revealed that LMX1A-GFP⁺ grafts were significantly smaller than both Unsorted and LMX1A-GFP⁻ cell grafts (3.9mm^3

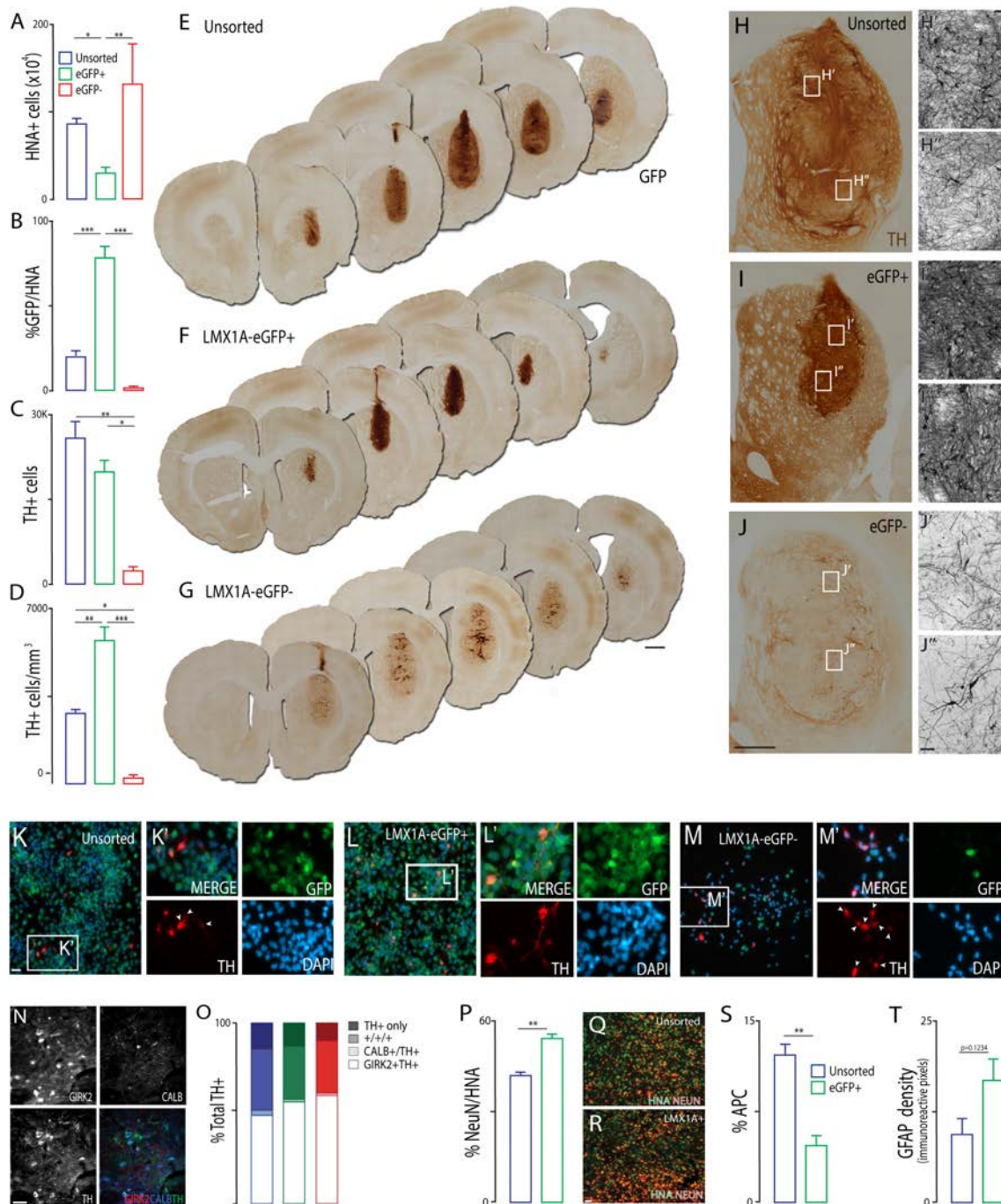


Figure A2.3 – Transplants of LMX1A-eGFP+ progenitors results in discrete, homogeneous grafts with enriched density of dopaminergic neurons of maintained A9/A10 subtype specification.

(A) Quantitative assessments of transplants revealed significantly fewer HNA+ cells (One-way ANOVA, $F_{(2,14)} = 9.445$, $p = 0.0025$, $n=6-8$ /group), and (B) enrichment of GFP expressing cells within grafts of LMX1A-GFP+ progenitors, compared to both Unsorted and LMX1A-GFP-, (One-way ANOVA, $F_{(2,13)} = 42.15$, $p < 0.0001$, $n=6-8$ /group). (C) While TH+ cell number did not differ from Unsorted progenitor grafts, (One-way ANOVA, $F_{(2,12)} = 8.445$, $p = 0.0051$, $n=6-8$ /group), (D) TH density was significantly elevated within LMX1A-GFP+ progenitor grafts, in comparison to transplants of Unsorted and LMX1A-GFP- cells, (One-way ANOVA, $F_{(2,12)} = 19.68$, $p = 0.0002$, $n=6-8$ /group). (E) Representative overview of Unsorted, (F) LMX1A-GFP+, and (G) LMX1A-GFP- grafts showing GFP expression. (H-J) Photomicrograph showing the distribution and density of TH+ cells within grafts derived from Unsorted, LMX1A-GFP+ and LMX1A-GFP- progenitors. (**H'**, **H''**) Note the heterogeneity in TH cell density seen in Unsorted grafts, (**I'**, **I''**) by comparison to the dense homogeneous distribution observed in LMX1A-GFP+ grafts. (J) Transplants of LMX1A-GFP- progenitors showed few TH+ cells scattered throughout the transplant. (K-M) Representative images of Unsorted (K), LMX1A-GFP+ (L) and LMX1A-GFP- (M) progenitors 6 hours after sorting and replating showing the co-expression of GFP with TH. Note that while TH+GFP+ cells numbers were low in all replated cultures, reflective of the stage of differentiation (21DIV), the

LMX1A-GFP- cultures showed a high proportion of TH+GFP- cells (white arrow heads), indicative of an earlier born, non-vm, dopaminergic or adrenergic/noradrenergic cell population. (N) Representative images showing the co-expression of TH together with GIRK2 and/or CALBINDIN, indicative of A9- and A10-like specification. (O) Quantification of TH+ neurons that showed co-expression of GIRK2 and/or CALBINDIN, confirmed that transplanted LMX1A-GFP+ progenitors retained their propensity to form mature vm DA neurons of both A9- and A10-phenotype. (P) Isolation of LMX1A-GFP+ cells resulted in grafts significantly enriched with NeuN+ neurons, compared to unsorted VM progenitor grafts, (Unpaired t test, $t = 7.76$ $df = 4$, $p = 0.0015$, $n=3-4$ /group). (Q-R) Representative photomicrographs of NeuN immunolabelled neurons within an Unsorted and GFP+ graft. (S) The proportion of APC+ mature oligodendrocytes was significantly reduced in LMX1A-GFP+ grafts (Unpaired t test, $t = 5.32$, $df = 4$, $p = 0.006$, $n=3-4$ /group), while the relative contribution of GFAP+ astrocytes remained unchanged (Unpaired t test, $n=3-4$ /group). All data represents Mean $\pm$ SEM, Scale bars: (E-G, H-J) 1mm, (H'-J'', N) 50 μ m.

± 1.0 , $8.9\text{mm}^3 \pm 0.9$, $10.7\text{mm}^3 \pm 1.7$, respectively). Grafts of Unsorted PITX3-GFP cells were, not surprisingly, comparable to the Unsorted LMX1a-GFP cells ($9.9\text{mm}^3 \pm 1.1$), with PITX3-GFP- also of similar size ($9.3\text{mm}^3 \pm 1.5$), Figure A2.2E,J. Most evident was the reduced size of the PITX3-GFP+ cell grafts ($0.3 \pm 0.1 \text{mm}^3$). Recognising that midbrain DA progenitors exit the cell cycle and become post-mitotic at a similar time to the onset of PITX3 expression during vm development, combined with knowledge that post-mitotic neuroblasts poorly survive transplantation, we speculated that these factors contributed to the small graft size observed. GFP labelling of these grafts confirmed low numbers of PITX3-GFP+ cells, and whilst showing a higher density of GFP+ cells (data not shown), TH+ DA neurons were notably sparse (591 ± 84 , $n=8$), highlighting the lack of suitability of this reporter to isolate and enrich DA progenitor/precursors for the purpose of transplantation.

A2.4.3 Transplantation of LMX1A-GFP⁺ vm progenitors enriches for DA neurons that are capable of innervating developmentally appropriate targets in the host brain

Reflective of the smaller size of LMX1A+ transplants (Figure A2.2E), these grafts contained significantly fewer (HNA+) cells compared to both Unsorted or LMX1A-GFP- cell grafts (Figure A2.3A). Assessment of GFP staining confirmed the efficiency of the sorting, with LMX1A-GFP+ progenitor grafts showing enrichment of GFP+ cells; both total GFP+ cells (Unsorted: $162,159 \pm 14,703$; LMX1A-GFP+: $225,700 \pm 48,832$; LMX1A-GFP-: $19,523 \pm 5,937$) and proportion, Figure A2.3B,E-G. Similar to *in vitro* observations, transplants of LMX1A-GFP- progenitors resulted in grafts containing pockets of GFP+ cells (Figure A2.3G), likely reflective of the asynchronous maturation of younger progenitors adopting an LMX1a+ fate after the time of sorting and implantation.

Unsurprisingly, the total number of TH+ DA neurons quantified within LMX1A-GFP+ and Unsorted grafts was not significantly different ($19,310 \pm 2,828$ and $23,318 \pm 3,697$), Figure A2.3C,H,I. Most striking, however, was the density and distribution of these neurons (Figure A2.3D,H-J). In comparison to Unsorted grafts, that contained a heterogeneous distribution of TH neuron including areas of high TH+ cell numbers (Figure A2.3H') and areas of low TH+ (Figure A2.3H''), transplants of LMX1A-GFP+ progenitors resulted in a dense, homogenous network of TH+ cells (with >2-fold increase in density), that minimally displaced host striatal tissue (Figure A2.3I, I',I''). Reflective of the selection for non-VM progenitors, LMX1A-GFP- grafts showed sparse TH+ DA neurons (Figure A2.3J). Interestingly, upon analysis of the replated LMX1a-GFP- fraction in vitro, a number of GFP-/TH+ cells were observed, indicative of a non-vm DA cell population (Figure A2.3M, arrowhead) that was not seen in the replated LMX1A-GFP+ cell fraction (Figure A2.3L). Within LMX1A+ grafts, $79.2\% \pm 8.2\%$ of cells were of appropriate vm lineage (LMX1A+FOXA2+OTX2+) of which ~7% became mature (TH+) DA neurons, 2-fold higher than unsorted grafts.

To confirm that selection of LMX1A-GFP+ progenitors did not affect the propensity (or proportion) of DA neurons to adopt an A9 substantia nigra pars compacta-like or A10 ventral tegmental area-like fate, we assessed the phenotype of TH+ cells within the grafts. As previously reported ([Grealish et al., 2014](#); [Niclis et al., 2017b](#)), >85% TH+ cells in all grafts showed expression of the A9 and/or A10 proteins, GIRK2 and Calbindin, respectively, with no significant difference between any of the transplant groups (Figure A2.3K,L). In alignment with post-mortem assessment of the human midbrain ([Reyes et al., 2012](#)), the majority of TH+ cells co-expressed GIRK2, alone or together with Calbindin, whilst few TH+ neurons expressed only Calbindin+.

Necessary for the functional efficiency of vm DA grafts is their capacity to send afferent projections to the host striatum. In accordance with the similar numbers of TH neurons, LMX1A+ grafts showed comparable density of innervation of the dorsal striatum and cortex to grafts of Unsorted progenitors (Figure A2.4A-D). In contrast, LMX1A-GFP- progenitor grafts poorly innervated these motor-function associated nuclei (Figure A2.4A,B,E), rather showing increased connectivity with non-dopaminergic target nuclei, inclusive of the periventricular thalamic nucleus (PVN) and hippocampus (Figure A2.4F-L).

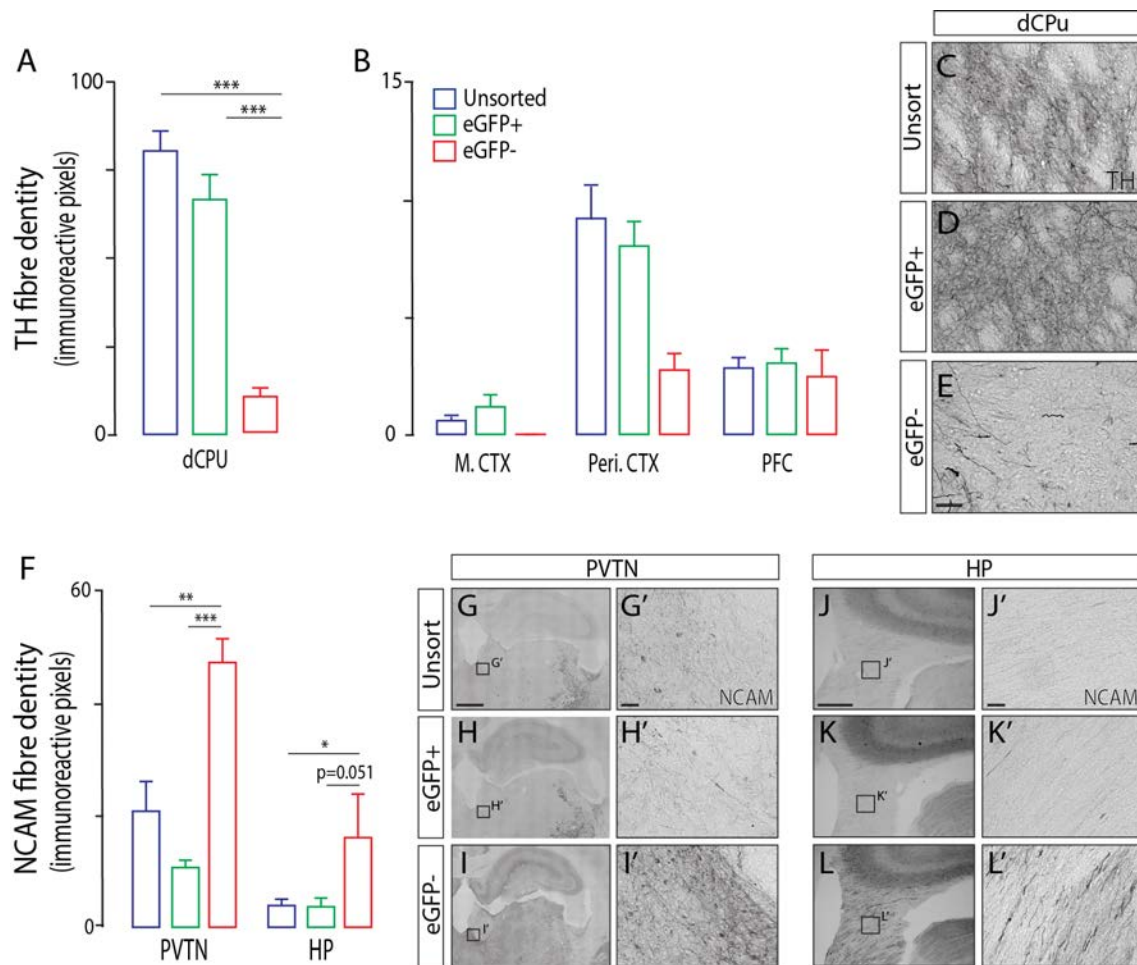


Figure A2.4 - Grafts containing LMX1A- cells preferentially innervate non-dopaminergic targets.

(A) Quantitative assessment of TH+ fibre density in the dorsal caudate putamen (dCPU, One-way ANOVA, $F_{(2,10)} = 32.77$, $p = <0.0001$, $n=4-6/\text{group}$), (B) and cortex, inclusive of the motor cortex (M.CTX), perirhinal cortex (Peri.CTX) and pre-frontal cortex (PFC). (C-E) High power images illustrating the increased density of TH+ fibres in the striatum of Unsorted and LMX1A-GFP+ cell grafts compared to grafts derived from LMX1A-GFP- progenitors. (F) Quantification of human NCAM+ fiber density in the periventricular thalamic nucleus (PVTN, One-way ANOVA, $F_{(2,13)} = 16.75$, $p = 0.0003$, $n=4-6/\text{group}$) and fimbria of the contralateral hippocampus (HP, One-way ANOVA, $F_{(2,13)} = 4.308$, $p = 0.0367$, $n=4-6/\text{group}$) from grafted animals. (G-I) Representative photomicrographs showing increased density of NCAM+ fibers within the PVTN and, (J-L) HP in animals transplanted with LMX1A-GFP- progenitors, compared to grafts of Unsorted or LMX1A-GFP+ cells, demonstrative of off-target innervation. Data presented as mean $\pm$ SEM. Scale bars: (C-E) 50 μ m, (G-I, J-L) 200 μ m, (G'-I', J'-L') 20 μ m.

Whilst we confirmed a low proportion of STN progenitors within our cultures prior to transplantation (a rostral vm population known to express LMX1A (Kee et al., 2017)), residing within the appropriately specified, more caudal, domain of the developing vm are other non-dopaminergic LMX1A-expressing progenitors. With <9% of the GFP+ cells expressing TH within GFP+ progenitor grafts (or 19%, as a proportion of total implanted cells), we sort to perform a more detailed assessment of the composition of these grafts – focusing on neural specification. All neural lineages were present within the transplants with >55% of HNA+ cells co-expressing the postmitotic neuronal marker NeuN within the GFP+ progenitor grafts, significantly greater

than unsorted cell grafts (Figure A2.3P-R). In contrast, sorting reduced the contribution of APC+ mature oligodendrocytes (Figure A2.3S), and no change was observed in the density of GFAP+ astrocytes (Figure A2.3T). More detailed assessment of neuronal subpopulations revealed that DBH+, ChAT+ and GAD67+ cells were noticeably rare within the GFP+ grafts (data not shown), suggesting that the many neurons present appeared to adopt a mature neurotransmitter identity. Staining against vascular proteins targeted to the host (RECA1) versus broader/non-species specific (Claudin V), revealed that the sparse density of vessels within the grafts were of host, and not graft, origin (data not shown). Whilst quantitative assessment of GFAP+ astrocytes was not feasible (due to the dense network of staining and inability to resolve graft from host cells with certainty), there was at least some evidence of HNA+ cells failing to adopt any of the three neural lineages. Such observations reflect our incomplete knowledge of the lineage trajectories of the LMX1A+/FOXA2+/OTX2+ progenitor, and whilst recently recognised as having the capacity to give rise to mature astrocytes *in vitro* (Holmqvist et al., 2015), other populations may remain to be identified.

A2.4.4 Isolation of LMX1A-GFP⁺ progenitors reduces proliferating cell populations and eliminates serotonergic neurons from human PSC-derived vm grafts

Reflective of the general efficiency of the *in vitro* differentiation of the human PSC LMX1A-GFP cell line, and the *in vivo* maturation of the implanted progenitors, no gross neural overgrowths or tumors were observed in any grafted animals after 26 weeks. The increase in total cells, from 100,000 implanted cells to a total of $836,883 \pm 54,083$ cells within the Unsorted grafts at 6 months reflect the proliferative capacity of the progenitors at the time of implantation. Whilst the majority of cells adopted a post-mitotic fate, a small dividing population remained present at 26 weeks, identified by doublecortin and KI67 staining within Unsorted and LMX1A-GFP-progenitor grafts. Supportive of the smaller graft volumes observed, transplants of LMX1A-GFP+ progenitors contained significantly fewer dividing cells, ($0.18\% \pm 0.01\%$ KI67+/HNA+), Figure A2.5A-D.

Finally, we assessed grafts for the presence of 5HT+ serotonergic neurons, a population known to contribute to the development of graft-induced dyskinesias (GID) in preclinical and clinical studies (Carlsson et al., 2007; Politis et al., 2011). Transplants of LMX1A-GFP+ progenitors eliminated serotonergic neurons, with just 2 grafts containing a single 5HT immunoreactive neuron, compared to the significantly higher numbers observed in both Unsorted ($7,346 \pm$

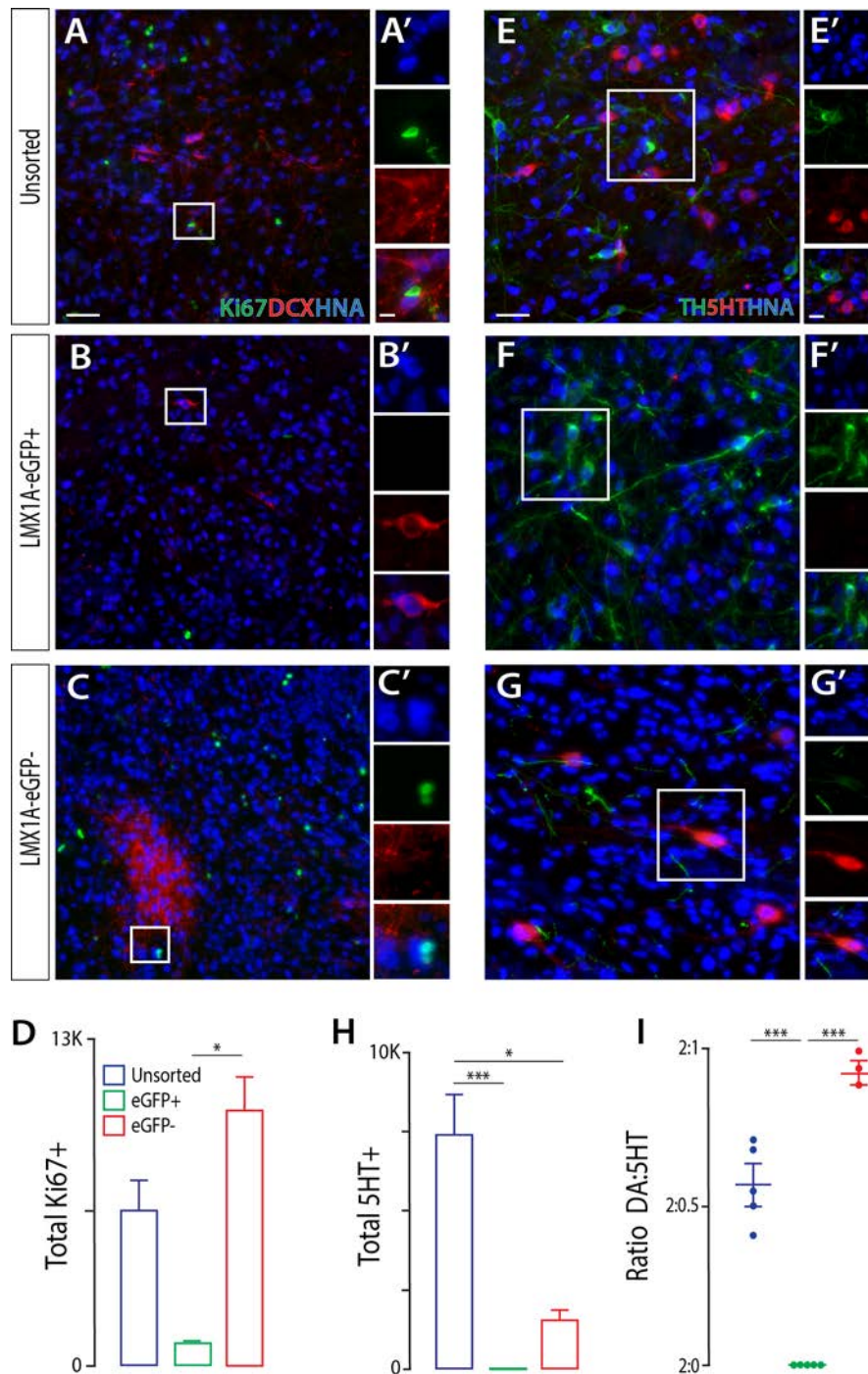


Figure A2.5 - Transplantation of LMX1A-GFP+ progenitors reduces the presence of proliferating cells, and eliminates incorrectly specified serotonergic neurons within grafts.

(A-C) Immunohistochemical labelling of transplants with human nuclear antigen (HNA, blue), doublecortin (DCX, red), Ki67 (green) illustrating the reduction in proliferative progenitors at 6 months within transplants derived from LMX1A-GFP+. (D) Quantification of Ki67+ cells within the LMX1A-eGFP cell grafts, One-way ANOVA, $F_{(2,6)} = 8.248$, $p = 0.019$, $n=4-6$ /group. (E-G) Representative photomicrographs showing dopaminergic (TH+, green), serotonergic (5HT+, red) and HNA immunolabeling within transplants of Unsorted GFP+ and GFP- progenitor grafts. (H) Quantification of 5HT+ cells (One-way ANOVA, $F_{(2,13)} = 17.26$, $p = 0.0003$, $n=4-6$ /group), and, (I) ratio of dopaminergic to serotonergic (DA:5HT) neurons within the transplants (One-way ANOVA, $F_{(2,11)} = 57.19$, $p = <0.0001$, $n=4-6$ /group), confirmed the near complete elimination of the dyskinetic-contributing cell population from grafts derived from LMX1A-GFP+ progenitors. Data represents Mean + SEM. Scale bars: (A-C, E-G) 50um, (A'-C', E'-G') 20um.

1,382) and LMX1A-GFP- ($2,168 \pm 359$) grafts, and consequently also the proportion of serotonergic to dopaminergic neurons, (DA:5HT, Figure A2.5E-I), a recognised contributing factor in the incidence of induced dyskinesias (Garcia et al., 2011; Politis et al., 2011).

Demonstrating the utility and reproducibility of sorting for LMX1A-expressing progenitors, we repeated FACS isolation and transplantation into 6OHDA lesioned athymic mice. Again, we showed smaller grafts with enrichment for both GFP and TH neurons (Figure A2.6A-I). TH+ DA neuronal density within the grafts was surprisingly similar to the rats; with significant increases in density (and homogeneity) observed in the LMX1A-GFP+, compared to Unsorted and LMX1A-GFP- grafts (Figure A2.6F, I). No 5HT neurons were observed in grafts of LMX1A-GFP+ progenitors (Figure A2.6J-L).

A2.5 Discussion

Heterogeneity in graft composition following the implantation of hPSC-derived vm progenitors presents ongoing concerns for the safety and functional predictability of these cells for future clinical application in Parkinson's Disease. For these reasons, efforts to improve the homogeneity of the donor preparation, and thereby graft compositions are of significant importance. In this study we developed two GFP-expressing reporter lines, LMX1A-GFP and PITX3-GFP, to enable the selective isolation of correctly specified vm progenitors/precursors from differentiating human pluripotent stem cell cultures, at differing developmental stages. We demonstrate that transplants of LMX1A-GFP expressing progenitors result in discrete grafts with increased density of DA neurons and greater, target-appropriate innervation, resulting in accelerated functional recovery of motor symptoms, compared to Unsorted progenitor grafts. In contrast, transplants of PITX3-GFP+ cells resulted in notably small grafts with low TH+ DA neuron yields. Whilst PITX3-GFP populations have been successfully isolated from mouse ESC cultures (Ganat et al., 2012; Hedlund et al., 2008), the present findings are in accordance with fetal tissue grafting studies using PITX3-GFP embryos; recognising the vulnerability of this post-mitotic population to shear trauma during periods of culture detachment/dissociation, FACS and implantation (Jonsson et al., 2009). Such findings highlight the need to identify a developmentally relevant differentiation stage of hPSC-derived vm progenitors that are sufficiently specified and also amenable for grafting, as has been achieved for rodent and human

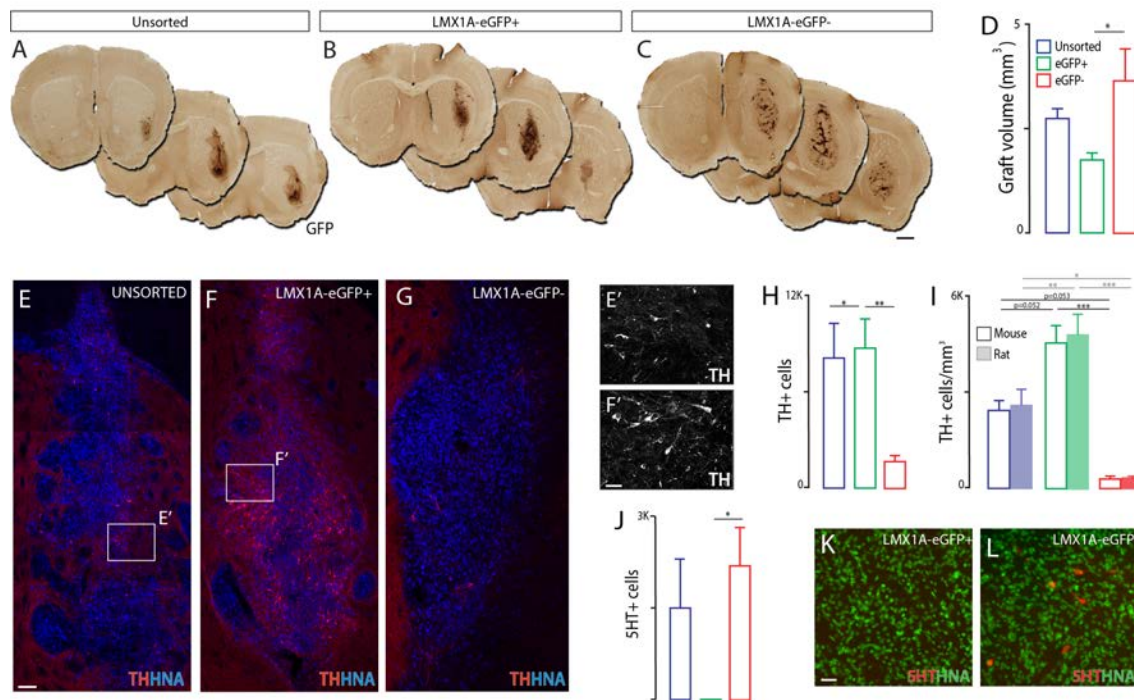


Figure A2.6 – Transplantation of LMX1A-GFP+ progenitors shows high reproducibility across repeated rodent transplantation studies.

Photomicrographs showing GFP immunohistochemical labelling of representative grafts in the mouse brain following transplantation of (A) Unsorted, (B) LMX1A-GFP+ and (C) LMX1A-GFP- progenitors. (D) Volumetric assessment revealed that LMX1A-GFP+ cells form discrete grafts that were significantly smaller than LMX1A-GFP- grafts, One-way ANOVA, $F_{(2,10)} = 4.544$, $p = 0.0395$, $n = 4-5/\text{group}$. (E-G) Photomontages of representative grafts in mice illustrating TH+ (red) and HNA+ (blue) labelling, and (F') demonstrating the enrichment of dopaminergic neurons within LMX1A-GFP+ transplants. (H) Quantification of total TH+ cells (Rats: One-way ANOVA, $F_{(2,12)} = 8.445$, $p = 0.0051$, $n = 4-6/\text{group}$; Mice: One-way ANOVA, $F_{(2,15)} = 7.566$, $p = 0.0053$, $n = 4-5/\text{group}$), and (I) TH cell density (Rats: One-way ANOVA, $F_{(2,12)} = 19.68$, $p = 0.0002$, $n = 4-6/\text{group}$; Mice: One-way ANOVA, $F_{(2,9)} = 18.91$, $p = 0.0006$, $n = 4-5/\text{group}$) within grafts derived from Unsorted, LMX1A-GFP+ and LMX1A-GFP- progenitors and transplanted into mice (open bars) and rats (closed bars). (J) Quantification of 5HT+ cells within LMX1A progenitor grafts in mice (One-way ANOVA, $F_{(2,9)} = 9.816$, $p = 0.0055$, $n = 4-5/\text{group}$), showing reproducible elimination of serotonergic neurons (as seen also in transplants in rats). (K-L) High power images highlighting the elimination of 5HT neurons in LMX1A-GFP+ grafts. Data presented as Mean $\pm$ SEM. Scale bars (A-C) 1mm, (E-G) 500um, (E'-F') 20um, (K-L) 50um.

fetal tissue (Bye et al., 2012; Freeman et al., 1995; Torres et al., 2007) as well as mouse PSCs (Ganat et al., 2012).

In addition to the significance of enriching for DA neurons within grafts is the concomitant capacity to eliminate poorly specified and high proliferative populations that may lead to overgrowth or graft-induced dyskinesias. Whilst incorrectly specified cells are in the minority *in vitro*, due to advances in hPSC neuronal differentiation protocols (Kriks et al., 2011; Niclis et al., 2017a), these populations have a propensity for expansion following implantation and/or can impart negative effects on transplant function. On average, studies have reported a yield of 5-6% TH+ DA cells in the grafts per 100,000 cells implanted (Doi et al., 2014; Kirkeby et al., 2012; Kirkeby et al., 2017; Kriks et al., 2011; Niclis et al., 2017b; Samata et al., 2016). This

yield however is notably lower when expressed as a proportion of the total number of cells within the graft, given the proliferation of the vm progenitors following implantation (Niclis et al., 2017b; Samata et al., 2016). The impact of the incorrectly specified cells has been most evidently demonstrated by Niclis et al., where despite >85% correctly specified vm DA progenitors present in culture (confirmed by FOXA2/OTX2/LMX1A co-expression) and maintained expression in the majority of cells observed 4weeks post-implantation, by 28weeks graft volume increased 20-fold with no increase in TH+ cell numbers (Niclis et al., 2017a; Niclis et al., 2017b). Also recognising this challenge, Kirkeby et al., describe the lack of predictability of grafting outcomes across many (>30) human PSC vm differentiations, despite consistent (>80% FOXA2/OTX2+) *in vitro* specification of the progenitors prior to transplantation (Kirkeby et al., 2017). In the present study, the discrete size of LMX1A-GFP+ grafts, lack of proliferating cells, and increased proportion of TH+ DA neurons observed at 6months, is a significant improvement and a stark contrast to grafts of the LMX1A-GFP- populations. It is important to note, however, that multiple lineages develop from the midbrain LMX1A+ population, including GABAergic, Glutamatergic and glial derivatives observed in grafts of LMX1A-GFP+ grafts. Together these results highlight the dramatic impact of eliminating poorly specified and/or proliferating cells and underline the necessity for vm progenitor enrichment prior to grafting.

The benefit of eliminating poorly specified neural progenitors can be further appreciated in the context of recent work highlighting the level of off-target innervation made by human PSC-derived vm progenitor grafts (Niclis et al., 2017b). While earlier studies were unable to specifically track the relative contribution of dopaminergic and non-dopaminergic innervation from the graft into the host brain, Niclis et al., made use of the PITX3-eGFP reporter line to demonstrate that the dopaminergic neurons within the grafts innervated developmentally appropriate host targets, inclusive of the striatum and cortex. However, the vast majority of graft-derived fibre outgrowth was non-dopaminergic, resulting in extensive axonal growth through permissive white matter tracts and significant innervation of off-target nuclei (Niclis et al., 2017b). Supporting these findings, here we demonstrate that the dopamine-enriched LMX1A-GFP+ vm progenitors grafts maintain preferential innervation of appropriate dopaminergic target nuclei with low level innervation of other nuclei. In contrast, LMX1A-GFP- grafts show enhanced innervation of target nuclei that typically have low midbrain dopaminergic innervation, such as the thalamus and hippocampus which carry unknown consequences, as well as low level dopaminergic innervation of the appropriate forebrain targets.

Elimination of serotonergic neurons, a population of cells that have been shown to contribute to graft-induced dyskinesias in both pre-clinical and clinical studies is an additional requisite in the refinement of human PSC grafting targeted for Parkinson's disease therapy (Carlsson et al., 2007; Politis et al., 2011). These undesirable neurons, generated in the ventral basal plate of the rostral hindbrain, reside outside the region of expression of LMX1A (see figure A2.1B, (Kirkeby et al., 2012; Mishima et al., 2009)), in contrast to other target genes, such as *FolR1* and *Corin* that have been employed to enrich for DA progenitors (Doi et al., 2014; Gennet et al., 2016). Here we demonstrate the capacity to eliminate 5HT+ serotonin cells from within LMX1A-GFP+ progenitor grafts, in both rat and mouse grafting studies. Of note, the significant reduction in 5HT+ neurons observed in grafts of LMX1A-GFP- progenitors, in comparison to the Unsorted cell grafts, reflects the stringency of the FACS gating employed within the study (Figure A2.1R), with an estimated 23% of cells excluded from both the GFP+ or GFP- pool.

The importance of cell sorting approaches for clinical applications is by no means a new concept. Most evidently demonstrated, and clinically translated, has been the purification of haematopoietic stem cells from blood for the treatment of, for example, some blood-related cancers and autoimmune diseases. Preclinical studies focused on the isolation of vm progenitors from differentiating human PSC cultures is also not novel in the context of improving the safety and predictability of transplants for PD. While a number of rodent studies successfully isolated vm progenitors, using fluorescent activated cell sorting (FACS) or magnetic beads, targeting intracellular and extracellular proteins, respectively (Bye et al., 2015; Fukuda et al., 2006; Ganat et al., 2012; Hedlund et al., 2008; Jonsson et al., 2009; Nefzger et al., 2012; Thompson et al., 2006), isolation from human PSC cultures has been met with variable success.

In a number of studies, candidate markers employed for selection purposes have been recognised as too broad in their expression to selectively isolate VM progenitors - such as the pan-neuronal marker CD56/NCAM (Pruszek et al., 2007), ventral floorplate marker FOXA2 (Aguila et al., 2014), ventral midbrain floor and basal plate markers Integrin Associated Protein and LMRT1 (Aguila et al., 2014; Lehnert et al., 2017; Samata et al., 2016), as well as ventral midbrain/hindbrain marker CORIN (Doi et al., 2014). Studies also conducted sorting early in the differentiation (due to timing of expression of the selectable protein) and required extended periods of replating prior to transplantation (Doi et al., 2014; Lehnert et al., 2017; Samata et al., 2016). Such replating steps undermine initial purification and may introduce downstream variability in cell composition at the time of grafting, and have been shown to result in poor TH+

survival (Doi et al., 2014). Alternatively, cultures were sorted late in the vm differentiation yet were not grafted (Xia et al., 2017).

With the rapid advancement of human PSC-derived DA progenitor grafts towards the clinic, cell sorting studies have increasingly focused on the identification of cell surface proteins, to avoid reporter cell lines. A key challenge here has been the poor translation of findings from rodent to human – with several studies employing rodent tissue to identify clinically-applicable cell surface antigens on DA progenitor populations, yet translation of these approaches showed limited success as antibodies failed to show the same restrictive isolation or immunoreactivity in human cells (Bye et al., 2015; Gennet et al., 2016; Samata et al., 2016) and our unpublished observations. The narrowing attention to cell surface markers for selection has to some degree distracted from the primary goals of demonstrating the full potential of vm DA progenitor enrichment, and the safety goals.

Focused on these tasks of safety, predictability and reproducibility, here we present the first evidence of using a fluorescent transgene to isolate and engraft hPSC-derived vm progenitors with superior outcomes to cell-surface based purification strategies. Whilst we do not claim LMX1A as the optimal target gene (recognising expression within other non-dopaminergic vm progenitors), this work provides the important impetus for sorting using a human targeted gene. Moving forward, the recent work by Kirkeby et al, (2017) may provide new insight into additional and/or superior sorting candidates – having recently identified genes (including ETV5, EN1, SPRY1, WNT1 and CNPY1) expressed during vm differentiation that are predictive of positive DA graft outcomes. Interestingly, several genes commonly used to monitor DA differentiation (that have been employed in sorting studies e.g. FOXA2 and CORIN), have shown poor correlates with graft outcomes (Kirkeby et al., 2017). While our selected transgene, LMX1A, was shown to correlate positively with DA density in grafts (Kirkeby et al., 2017), the emphasis remains that additional markers are also likely needed. At this point the field is now left to ponder the risk-to-benefit ratio of taking GFP cell lines into the clinic, noting that GFP transgenes have previously been employed in tracing and targeted gene therapy clinical trials (*clinicaltrials.gov*) or the necessity of identifying a cell surface target.

In summary, improved pre-clinical sorting strategies may enable the identification of novel protein, epigenetic or transcriptomic identifiers that underpin positive DA transplantation outcomes and therefore, inform further clinically relevant interventions.

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Appendix 3.

TRANSCRIPTIONAL PROFILING OF XENOGENEIC TRANSPLANTS: EXAMINING HUMAN PLURIPOTENT STEM CELL-DERIVED GRAFTS IN THE RODENT BRAIN

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A3.1 Abstract

Human pluripotent stem cells are a valuable resource for transplantation, yet our ability to profile xenografts is largely limited to low throughput immunohistochemical analysis or restricted by the ability to readily isolate grafts for transcriptomic and/or proteomic profiling. Here we present a methodology, utilising differences in the RNA sequence between species, to discriminate xenograft from host gene expression (using qPCR or RNA sequencing). To demonstrate the approach, grafts of undifferentiated human stem cells and neural progenitors in the rodent brain were assessed. Xenograft-specific qPCR provided sensitive detection of proliferative cells, identified germ layer markers and appropriate neural maturation genes across the graft types. Xenograft-specific RNAseq profiling of the complete transcriptome enabled identification of total gene expression and an unbiased characterisation of graft composition. Such specific profiling will be crucial for pre-clinical characterisation of grafts and batch-testing of therapeutic cell preparations to ensure safety and functional predictability prior to translation.

A3.2 Introduction

Since the derivation of human pluripotent stem cell lines there has been great hope and anticipation for the use of these cells and their derivatives in regenerative medicine. One of the most anticipated applications is their restricted fate specification to defined cellular populations for the purpose of transplantation, aimed at treating an acute and chronic disorders. Some of the most advanced examples are human PSC-derived islet cells for the treatment of diabetes, retinal pigment epithelia for application in visual impairment conditions, and dopamine neurons for the replacement of those cells lost to Parkinson's disease, PD ([Trounson and McDonald, 2015](#)).

Prior to clinical translation of human PSC-derived cells it will be imperative that graft composition is well defined – to ensure safety and functional predictability. The significance of understanding graft composition is perhaps most evidently demonstrated in hPSC-derived dopamine (DA) progenitor transplantation studies in PD models. Despite significant advancements in differentiation protocols resulting in high yields of correctly specified progenitors for grafting ([Kriks et al., 2011](#); [Kirkeby et al., 2012](#); [Niclis et al., 2017b](#)), following protracted graft analysis (>6months) only a fraction of the transplant is comprised of mature DA neurons ([Kriks et al., 2011](#); [Kirkeby et al., 2012](#); [Doi et al., 2014](#); [Samata et al., 2016](#); [Kirkeby et al., 2017](#); [Niclis et al., 2017a](#)). Such outcomes suggest that the small proportion of poorly specified cells present in the cultures subsequently expand following transplantation to dominate the graft. Histochemical assessment has been able to confirm that these grafts are predominantly 'neural' in origin and of an appropriate regional identity ([Kriks et al., 2011](#); [Samata et al., 2016](#)), however due to limited antibody availability to selectively assess the human cells, as well as not knowing what to specifically look for, the identity of much of these grafts remains unknown. This raises the concern of what impact these cells may have graft function, as well as host tissue.

Transcriptionally profiling grafts has been hindered by the inability to selectively isolate the graft, which is inter-dispersed with the host tissue. When the graft can be clearly identified, (e.g. grafts of reporter stem cell lines), careful laser capture microdissection can reduce the level of contaminating host cells. However, laser capture-based approaches are labour intensive, require meticulous tissue processing to maintain RNA quality, and are associated with low RNA yields. An alternative approach is isolating the grafted cells by tissue dissociation and applying fluorescent or magnetic cell sorting strategies. This approach relies on the dissociation of post-

mitotic cells and can result in poor survival. Single cell genomic profiling of cells within the graft has also increasingly been employed to assess molecular and cellular diversity within defined populations (Tang et al., 2010; Etzrodt et al., 2014; La Manno et al., 2016). While providing an accurate assessment of the cell identity within the graft, such approaches are also onerous and sample just a fraction of the graft.

Circumventing these challenges, here we develop a method that employs a selective detection process to discriminate the graft genome within a mixed graft-host tissue pool. This simple approach relies on identifying differences in RNA sequences between the host and graft species for a specific gene when designing primers for the purpose of real-time qPCR (qPCR), and similarly recognising species-specific reads within a gene during RNA sequencing. The utility and rigor of these techniques are demonstrated by comparing different transplant populations of human cells in the rodent brain.

A3.3 Materials and methods

A3.3.1 Cell culture, differentiation and sorting

Mouse RNA, containing mixed cell populations, inclusive of naïve pluripotent stem cells through to maturing cells from all germ line lineages (ecto-, meso- and endoderm), was obtained by pooling together undifferentiated mouse pluripotent stem cells (E14TG2a obtained from ATCC, USA), whole mouse embryos (embryonic day (E) 9.5 and E12.5) and maturing ventral midbrain (VM) progenitors isolated from E10.5, E12.5, E14.5 and postnatal day 1 pups.

Human RNA, containing mixed cell populations, was generated by pooling undifferentiated human pluripotent stem cell together with embryoid bodies (consisting of all germ layers), and varying maturation stages of VM neural progenitors and mature dopamine neurons (derived from differentiated human ESC, as described below).

The H9 human embryonic stem cell line, expressing a green fluorescent reporter under the LMX1A promoter (H9 LMX1A-GFP) was cultured as previously described (Niclis et al., 2017b). Maintenance of pluripotency, prior to transplantation, was confirmed by morphology and the

co-expression of OCT4 and SOX2, Supp figure A3.1A-D. Undifferentiated hPSCs for transplantation were isolated by Accutase treatment and resuspended at 10,000 cells/ μ L.

To obtain a pool of purified neural progenitors of known origin, PSCs were differentiated to a VM fate as previously described (Niclis et al., 2017b), with correctly specified cells isolated by fluorescent activated cell sorting (FACS) for the GFP reporter gene, LMX1A. At 21 days in vitro (DIV), differentiating cultures were dissociated in Accutase to a single-cell suspension and fluorescent activated cell sorting (FACS) performed on a MoFlo cell sorter (Beckman Coulter). The generation of neural progenitors (of VM identity) was verified by co-expression of OTX2, FOXA2 and LMX1A (LMX1A-GFP+), Supp figure A3.1E-I. As previously described, >95% of GFP+ cells co-expressed the ventral floorplate marker FOXA2 and forebrain-midbrain marker OTX2 (Niclis et al., 2017b), indicating that isolation of this GFP fraction (>80% of cells, Supp Figure A3.1J) could enrich for correctly specified VM progenitors from the heterogenous differentiated cultures by fluorescent activated cell sorting. The FACS-isolated cell fraction was resuspended at 100,000 cells/ μ L in maturation media and stored on ice until the time of implantation.

A3.3.2 Cell transplantation

All animal procedures were approved by The Florey Institute of Neuroscience and Mental Health Animal Ethics committee. Surgeries were performed on 30 athymic (Foxn1nu) nude mice under 2% isoflurane anaesthesia. One microliter of cells was stereotactically injected into the brains at the following co-ordinates, relative to bregma: 0.5mm anterior, 2.1mm lateral, and 3.2mm below the surface of the dura), as previously described (Kauhausen et al., 2013). Transplant groups (n=10/group) included: (1) undifferentiated hPSC implanted for a period of 1month; (2) VM progenitors, implanted for 1month; and (3) VM progenitors, implanted for 6 months.

A3.3.3 Tissue processing and immunohistochemistry

After the prescribed period of graft survival (1 or 6 months), a subset of animals (n=5/group) were killed by an overdose of sodium pentobarbitone (100mg/kg) and transcardially perfused with 4% paraformaldehyde and cryosectioned. Immunohistochemistry was performed on fixed cell cultures or brain sections as previously described (Soma et al., 2017). Primary antibodies and dilutions were as follows: 4',6-diamidino-2-phenylindole (DAPI, 1 μ g/ml; Sigma Aldrich), Goat anti-FOXA2 (1:200; Santa Cruz), chicken anti-GFP (1:1,000; Abcam), rabbit anti GFAP (1:800, DAKO), mouse anti-human nuclear antigen (HNA, 1:300; Millipore), rabbit anti-KI67

(1:1,000; ThermoFisher), mouse anti-NESTIN (1:200; Millipore), mouse anti Neuroligin 3 (Nlgn3, 1: 100, Synaptic systems), mouse anti-OCT4 (1:100, Santa Cruz), rabbit anti Olig1 (1:200, Millipore), rabbit anti-OTX2 (1:4000; Millipore), mouse anti PSA-NCAM (1:200, Santa Cruz), goat anti-SOX2 (1:200; R&D), mouse anti synaptophysin (hSYP, 1:1000, Enzo Life Sciences), sheep anti-tyrosine hydroxylase (TH, 1:800, Pelfreeze), rabbit anti-TH (1:1,000, Pelfreeze). For quantification of HNA+, KI67+, GFP+ and TH+ cells, images were captured 20X magnification using a Zeiss Axio Observer Z.1 epifluorescence. The density of Nestin-labeling (%immunoreactive pixels) was assessed from captured images and analysed using ImageJ software.

A3.3.4 RNA isolation and real-time quantitative PCR

The remaining animals (n=5/group) were killed, the brains removed and the striatum (containing the transplant) dissected from surrounding tissues and snap frozen. Tissue was homogenised using a TissueLyser LT (Qiagen) and total RNA extracted using the RNeasy Mini Kit (Qiagen). RNA yield and integrity were assessed using a Nanodrop One Spectrophotometer (ThermoFisher Scientific) and confirmed using a Qubit (ThermoFisher Scientific) and TapeStation (Agilent). The RNA was analysed by qPCR or RNAseq, Figure A3.1A.

Species-specific primers were designed using Primer3 ([Untergasser et al., 2012](#)), aimed at containing a minimum of 5 bp mismatches between graft and host, or 2 mismatches in the 5 bp at the 3' end between species. Primers were targeted manually to regions of dis-similarity (identified by blasting paralogue genes) or using Primer-BLAST and selecting both the xenograft and host species (Homo sapiens and Mus musculus, respectively in the present context) under organism in the “Primer Pair Specificity Checking Parameters.” This identified primers specific only to the xenograft species (i.e. human). All xenograft-specific primers used in this study are listed in Figure A3.1B. Lineage specific genes were selected from established markers used in the characterisation of embryoid bodies ([Tsankov et al., 2015](#); [D'Antonio et al., 2017](#)), and genes associated with vascular/endothelial cell differentiation identified from the MGI Gene Ontology Browser and Park et al ([Park et al., 2013](#)). Genes were selected for confirmation with xenograft specific qPCR based on showing highly significant upregulation in undifferentiated/teratoma grafts compared to neural progenitor grafts.

First-strand reverse transcription of 500 ng of RNA into cDNA as conducted using the SuperScript® VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer's recommendations. Real-time qPCR was carried out on 25ng of cDNA using the SYBR GreenER™ qPCR SuperMix Universal (Invitrogen) and run on a Rotor-Gene 6000 (Qiagen). To control for the size of the xenograft, a xenograft-specific reference (housekeeping) gene was identified. PSMB4, MTHFD1, CHAMP2A and HPRT1 were tested and PSMB4 selected as the optimal housekeeping gene due to its high specificity and stable expression across the 3 graft types (Supp Figure A3.1K). All qPCR data was analysed using the $\Delta\Delta CT$ method (Pfaffl, 2001), using the xenograft-specific reference gene and expressed relative to the Undifferentiated grafts. Five independent biological replicates were analysed per group.

A3.3.5 RNA Sequencing

cDNA libraries (containing graft and host RNA) were prepared using the TruSeq stranded mRNA sample preparation kit (Illumina). For Sequencing the final cDNA concentration of each sample adjusted to generate a target of 20 million xenograft-specific reads using the percentage xenograft RNA calculated from the qPCR data. Note, due to the low percentage of xenograft RNA in the immature grafts, 5.5 million reads were targeted. Libraries were subject to paired-end, 75 bp sequencing on an Illumina HiSeq 2000 platform (Illumina) with 3 independent biological replicates analysed per group.

Analysis was conducted on the Galaxy web platform (Afgan et al., 2018) using the Galaxy Australia server, and using Bioconductor (Huber et al., 2015) in the statistical analysis environment R (<https://www.R-project.org/>). Alignment to the human genome (Hg38) was performed using HISAT2 (2.0.3.3). Paired concordant reads were then mapped against the mouse reference genome (mm10). Under our high stringency approach, all reads that aligned to both the human and mouse genome were discarded, accounting for an average of 5% of human reads.

Read counts for each gene were generated using HTSeq-count (0.6.1) on union mode (Anders et al., 2015). Differential expression analysis and Principal Component Analysis was conducted with DeSeq2 (2.11.38) (Love et al., 2014). Expression heat maps and unsupervised clustering were performed on log transformed row scaled expression values using the gplots

package in R (Pfaffl, 2001). Gene ontology enrichment analysis on biological process was performed using DAVID gene ontology browser (Huang da et al., 2009). Lists of human transcription factors and axon guidance cues and receptors were sourced from the HumanTFDB (Hu et al., 2019) and the KEGG PATHWAY Database (Kanehisa et al., 2017) (hsa04360, Homo sapiens Axon guidance), respectively. The RNA-seq data generated from this study has been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE126804.

A3.3.6 Statistical analysis

All data are presented as mean $\pm$ SEM. Statistical tests employed (inclusive of one-way ANOVA and student t-tests) are stated in figure legends. Alpha levels of $p < 0.05$ were considered significant with all statistical analysis performed using GraphPad Prism, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

A3.4 Results

A3.4.1 Design and validation of xenograft-specific primers for real-time qPCR

To identify xenograft-specific sequences using qPCR, primers were designed targeting regions of dissimilarity between the 2 species. Specifically, primers targeted an RNA sequence containing a minimum of 5 bp mismatches between xenograft and host, or 2 mismatches within the 5 bp at the 3' end of the primer (e.g. Ki67 and LMX1A forward primers), Figure A3.1B. To confirm the specificity of the primers for xenograft transcript, primers were tested with *in vitro* pools of mouse or human cells known to express the target genes. A total of 30 primers were designed and tested (Figure A3.1B). Primer specificity for xenograft transcripts (over mouse) ranged from 500 to 1.0×10^7 times greater, with a median specificity of 174,000 (Figure A3.1C). Using an arbitrary cut-off of 1000 times (1000x) greater specificity for the human pool compared to mouse, primers for 97% of genes (29/30) were deemed as specific.

With success at designing species-specific primers, as validated *in vitro*, next the feasibility of this approach *in vivo* was determined – targeted at confirming the ability to discrim-

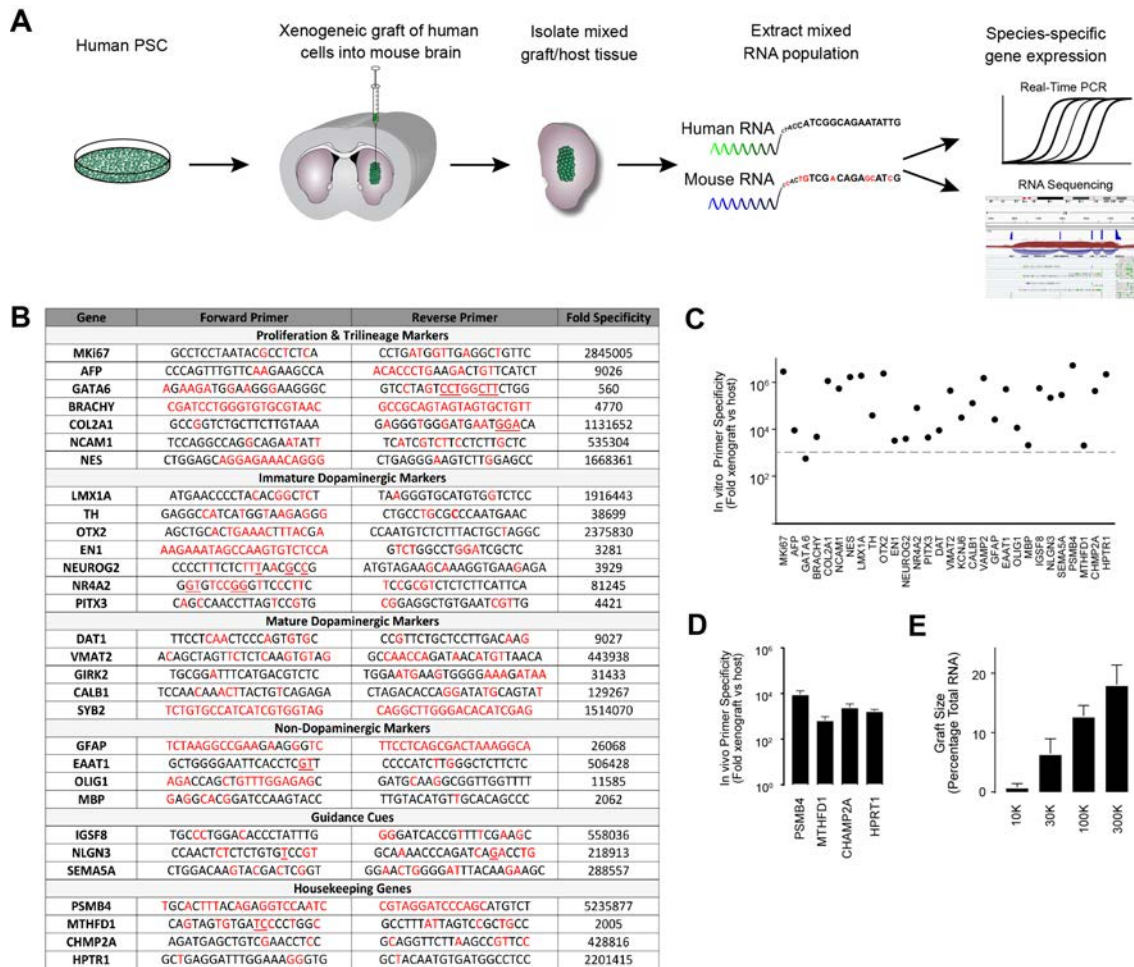


Figure A3.1 - Design and validation of xenograft-specific primers for real-time qPCR.

(A) Schematic of the experiment paradigm. Human PSC-derived cells were transplanted into the rodent brain. Tissue containing both transplanted cells and host tissue was dissected, and the RNA isolated to produce a mixed species RNA pool. Xenograft gene expression was discriminated from the host using species-specific primers for qPCR, or by RNA sequencing to profile the entire genome. (B) Table of human xenograft-specific primers designed for the present study. Nucleotide bases shown in red correspond to mismatches between the human and mouse RNA sequence, and underlined bases represent the presence of insertions or deletions. (C) Graph of the specificity of xenograft-specific primers for human transcript relative to rodent host transcript *in vitro* showing an average specificity of 5,000 times that of the host (also represented numerically 'fold specificity' in panel B). An arbitrary cut-off of 1000-fold (grey line), represents an ideal specificity threshold, with 96% of primers designed in this study exceeding this threshold. (D) *In vivo* specificity of xenograft-specific primers for 4 constitutively expressed transcripts, showing an average specificity of ~4,000 times greater in the transplanted, compared to untransplanted host. (E) Estimation of xenograft size using a xenograft-specific primer, PSMB4, showed a significant correlation ($r^2 = 0.78$) with actual number of cells implanted into the host. (D-E) Data represents mean $\pm$ SEM, $n = 4$ grafts/group.

-inate between xenograft and host transcripts. To achieve this, transplants of human stem cells in the striatum of immune-compromised athymic mice were analysed using qPCR. The specificity of the primers for xenograft RNA were confirmed *in vivo* by measuring the ability to detect the expression of four constitutively expressed genes in grafted tissue compared to un-grafted tissue (i.e. mouse striatal tissue containing no xenograft), Figure A3.1D. The 4 primers tested specifically detected xenograft transcripts *in vivo*, with expression in the grafted host greater

than the host only tissue for PSMB4 (10,349x), MTHFD (769x), CHAMP2A (2,775x) and HPRT1 (1,726x).

To access the capacity of species-specific primers to provide a readout of graft size, we implanted a known number of neural progenitors (10,000, 30,000, 100,000 or 300,000 cells) into the rodent brain and assessed expression levels of the housekeeping gene after 2 weeks. A xenograft-specific primer for a housekeeping gene was used to assess graft size, whilst a host-specific primer enabled assessment of the amount of host tissue present within the isolated tissue. Expressed as a proportion of total RNA, xenograft RNA constituted $0.8\% \pm 0.60$ (10,000 cells), $6.5\% \pm 2.48$ (30,000 cells), $12.8\% \pm 1.78$ (100,000 cells) or $18.1\% \pm 3.31$ (300,000 cells) of the RNA population (Figure A3.1E). The estimate of xenograft RNA showed a tight and significant correlation with the number of cells transplanted ($r^2 = 0.78$), demonstrating the utility of the method to rapidly estimate graft size.

A3.4.2 Characterisation of xenografts using species-specific real-time qPCR

To demonstrate and validate the utility of species-specific transcriptional profiling of xenografts, RNA from 3 distinct xenografts was compared: (i) Grafts derived from undifferentiated PSCs, anticipated to proliferative populations and cells from all 3 germ layers after 1 month *in situ* (subsequently referred to as the 'Undifferentiated' grafts), (ii) Transplants of VM neural progenitors, analysed 1 month after implantation and anticipated to show characteristic signatures of immature neuronal progenitor neurons (subsequently referred to as 'Immature neuronal' grafts) and, (iii) Grafts of VM neural progenitors, allowed to mature for 5 months *in situ* into neuronal populations inclusive of dopamine neurons (denoted 'Mature Neuronal' grafts). In parallel, tissue was collected from separate animals for immunohistochemistry to provide verification of the gene expression results.

Using an antibody specific for human cells (human nuclear antigen, HNA) that enabled delineation of the graft, size and cell number were determined. Grafts of Undifferentiated cells were large and expansive ($7.0 \pm 3.5\text{mm}^3$ containing $2.03 \times 10^6 \pm 0.43 \times 10^6$ cells), while Immature Neuronal grafts were small ($0.43 \pm 0.07\text{mm}^3$ with $0.49 \times 10^5 \pm 0.11 \times 10^5$ cells), and of moderate size following ongoing maturation (Mature Neuronal grafts: $2.4 \pm 0.25\text{mm}^3$ containing 1.51×10^5

$\pm 0.31 \times 10^5$ cells), Figure A3.2A-D. Transcriptional estimation of graft size, by xenograft-specific qPCR, measured the proportion of xenograft RNA at $33.0 \pm 8.9\%$ in the Undifferentiated grafts, $1.8 \pm 0.4\%$ in the Immature Neuronal grafts and $9.2 \pm 0.9\%$ in the Mature Neuronal grafts (Figure A3.2E), reflective of graft sizes determined histologically.

Necessary for the safe clinical translation of cell transplantation is the removal of proliferative cells prior to implantation, or their reduction to low levels shortly thereafter. Hence in an effort to characterise graft composition the expression of the proliferative gene *Ki67*, was assessed. A large population of *Ki67*+/HNA+ dividing cells were identified in the Undifferentiated grafts ($9.5\% \pm 1.0$), that was significantly reduced in Immature Neuronal grafts ($0.74\% \pm 0.16$), and reduced further in the Mature Neuronal grafts ($0.28\% \pm 0.06$), Figure A3.2A-D,F. Xenograft-specific gene expression (expressed relative to the Undifferentiated grafts), showed *Ki67* expression in the Immature grafts was reduced to 0.11 ± 0.02 -fold, and to 0.05 ± 0.01 -fold in Mature grafts (Figure A3.2G), corresponding closely to the cell counts, and highlighting the capacity for xenograft-specific qPCR to detect rare cell populations.

The capacity of the technique to detect different cellular populations within a graft was demonstrated using primers targeted against genes restricted to defined germ layers (endoderm, mesoderm and ectoderm) (Tsankov et al., 2015; D'Antonio et al., 2017). Unsurprisingly, endoderm (AFP and GATA6) and mesoderm (Brachyury and Collagen2A type 1, COL2A1) genes were lowly expressed in the Immature and Mature Neuronal grafts, Figure A3.2H-K. In contrast, expression of the ectoderm marker neural cell adhesion marker (NCAM), was elevated in the Immature and Mature Neuronal grafts (2.25 ± 0.39 and 1.58 ± 0.38 , respectively), Figure A3.2L. The neuroectodermal stem cell marker, Nestin, expressed in the Immature neuronal grafts, was reduced in the mature graft, reflective of downregulation of the gene during neuronal maturation as observed in development (Wiese et al., 2004), Figure A3.2M, and confirmed by Nestin immunohistochemistry, Figure A3.2N-Q. Greater differences in neuroectodermal gene expression between the Undifferentiated and neuronal fate restricted grafts (Immature Neuronal and Mature Neuronal) were not observed largely due to the preferential default neural fate acquisition of PSCs (Munoz-Sanjuan and Brivanlou, 2002).

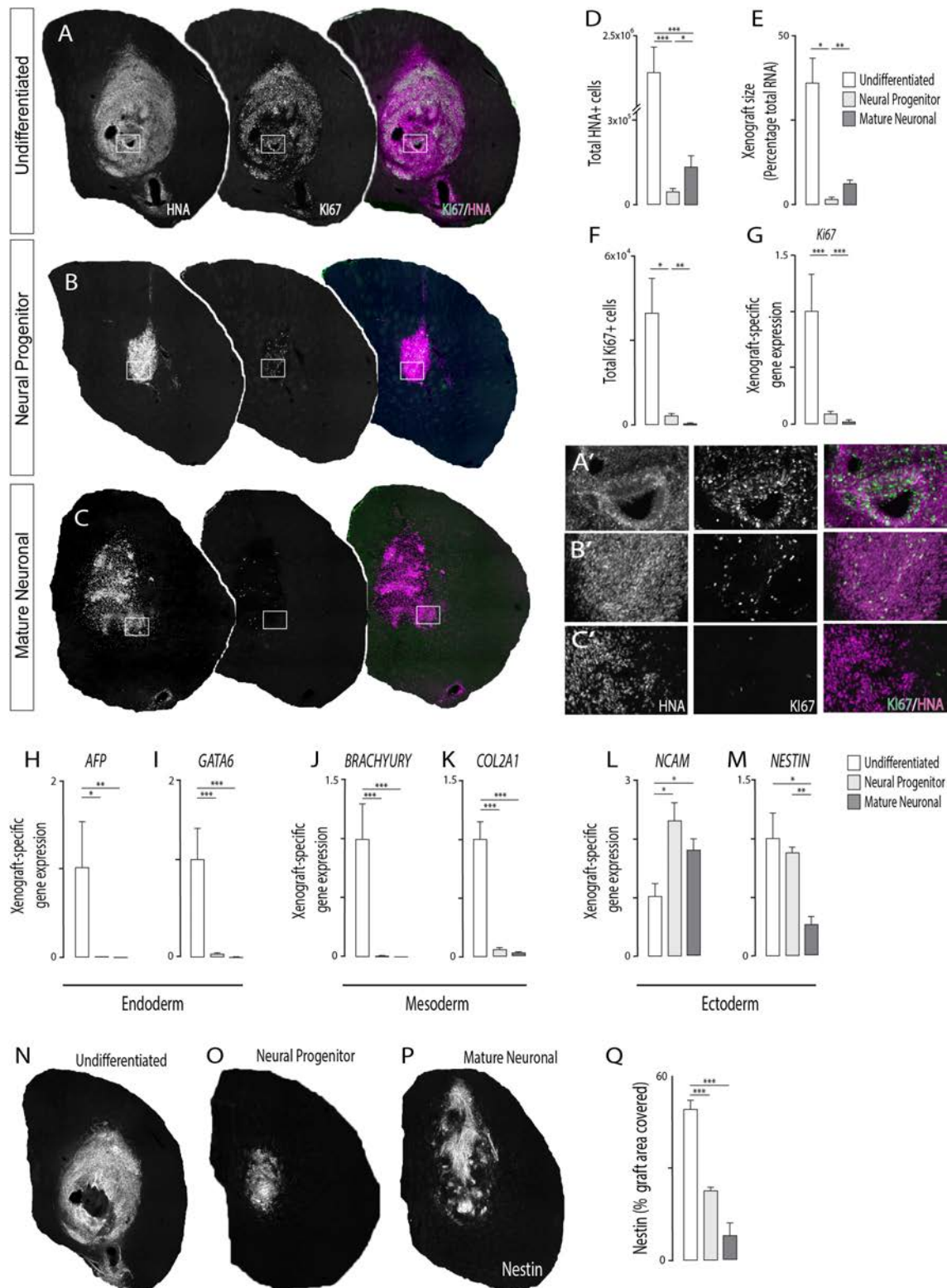


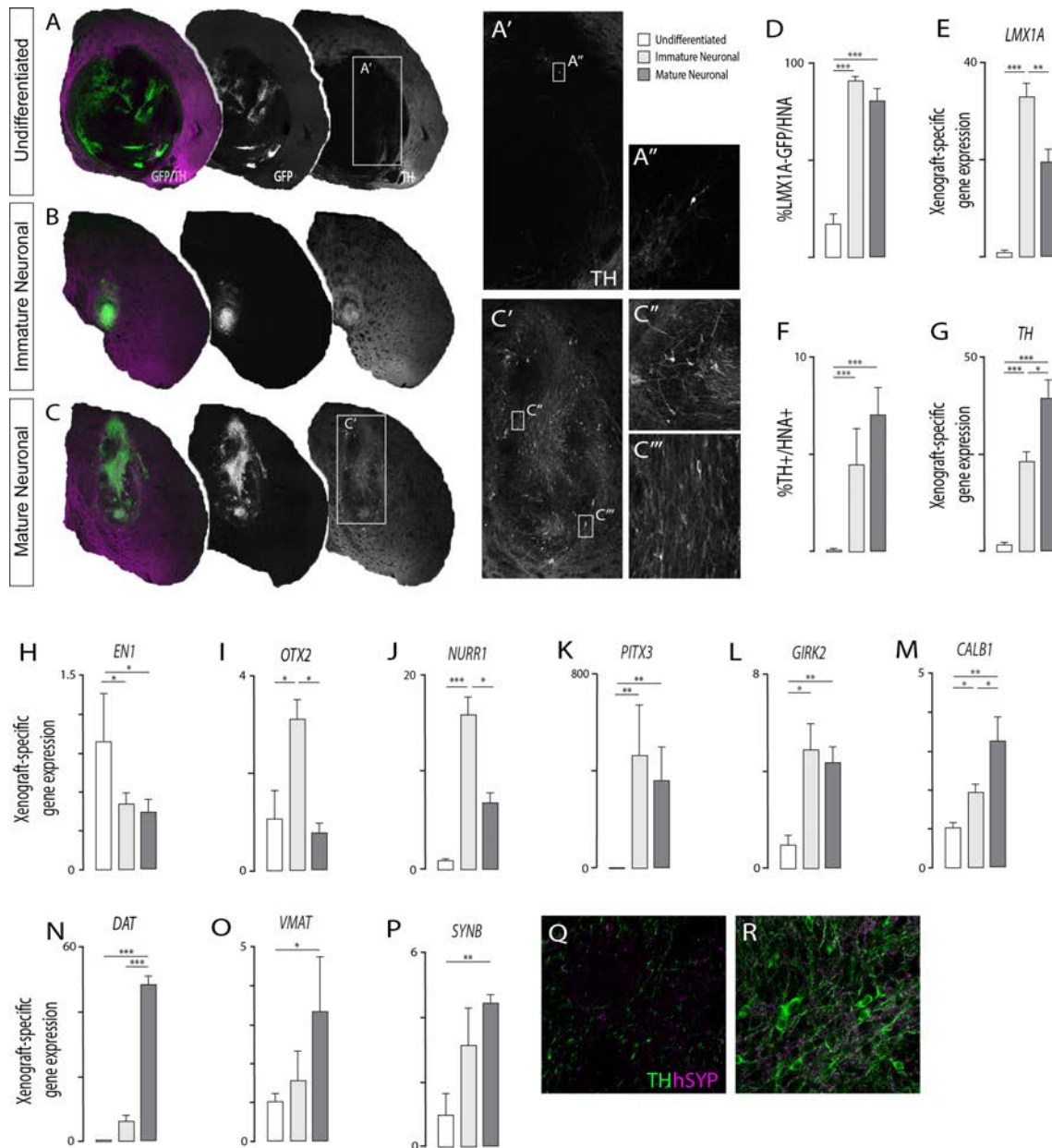
Figure A3.2 - In vivo validation of a xenograft profile using species-specific qPCR.

(A) Representative micrograph depicting a graft of undifferentiated human pluripotent stem cells (hPSC) 1 month after implantation, (B) a neural progenitor graft at 1 month and (C) neural progenitor graft at 5 months. Human nuclear antigen (HNA) labelled all human donor cells within the host, while KI67 labelled proliferative cells. (D) Quantification of HNA+ cells within the grafts (E) closely mirrored the proportion of human-specific transcript (as a percentage of total transcript). (F) Quantification of KI67+ proliferative cells, significantly elevated in grafts of undifferentiated hPSC, (G) reflected RNA transcript levels of the gene. (H-M) Trilineage specification of cells within grafts of undifferentiated hPSCs was demonstrated by high transcript expression of Endoderm (AFP, GATA6), Mesoderm (BRACHYURY, COL2A1)

and ectoderm (NCAM, NESTIN) genes. Appropriately, neural progenitor grafts (immature and mature neuronal grafts) contained only neuro-ectodermal gene expression. (N-P) Representative images of NESTIN-immunoreactive cells. (Q) Comparative levels Nestin transcript was validated by the proportion of the graft showing NESTIN-immunoreactivity. (D-G, H-M, Q) Data represents mean $\pm$ SEM, One-way ANOVA, $n=5$ grafts/group. Scale bar (A-C, N-P) = 500um, (A'-C') = 100um.

Next the sensitivity of the approach to detect more subtle changes present between Immature Neuronal and Mature Neuronal grafts was assessed. Immunohistochemistry against GFP (to identify the LMX1A-GFP+ cells) and TH (Tyrosine Hydroxylase) identified immature VM neural progenitors and mature dopaminergic neurons respectively, Figure A3.3A-C. Counts revealed $21 \pm 4\%$ of cells in the Undifferentiated grafts expressed GFP, a proportion that was enriched in the Immature Neuronal grafts ($82 \pm 1\%$) and maintained in the Mature Neuronal grafts ($76 \pm 4\%$), Figure A3.3A-D. Xenograft-specific gene expression showed LMX1A was increased 32.7 ± 1.7 -fold in the Immature Neuronal grafts, and reduced to 17.2 ± 2.3 -fold in the Mature Neuronal grafts (Figure A3.3E), commensurate with the cell counts and in accordance to the previously described downregulation of the gene in maturing midbrain dopamine neurons during development. Counts of the mature dopaminergic marker TH identified $0.3\% \pm 0.04$ cells in the Undifferentiated grafts (TH+/HNA+), significantly increased in the Immature Neuronal grafts ($4.4\% \pm 1.5$) grafts, and further in the Mature Neuronal grafts ($7.2\% \pm 0.9$), Figure A3.3F. Xenograft-specific gene expression analysis supported the increase in TH with expression 8- and 12-fold greater in the Immature and Mature Neuronal grafts, respectively, compared to those of Undifferentiated cells, Figure A3.3G.

To demonstrate the utility of the approach to rapidly and comprehensively profile graft composition, 9 additional genes anticipated to be expressed predominantly in neural specified grafts were assessed. Four genes expressed within VM neural progenitors included the midbrain-hindbrain restrictive gene *Engrailed-1* (EN1), forebrain-midbrain gene *OTX2*, dopaminergic precursor, *Nurr1* (NR4A2), and pro-survival gene *PITX3*, Figure A3.3H-K. As expected, in comparison to undifferentiated grafts, upregulation of *OTX2* (3-fold) and *NR4A2* (16-fold) were observed in the immature neuronal grafts (rich in dopaminergic progenitors), but not the more mature (dopaminergic neuronal) grafts, reflective of the transient expression of these genes in embryonic development (Niclis et al., 2017b). *PITX3* was upregulated in both the immature and mature neuronal grafts, compared to the undifferentiated grafts. Surprisingly, *EN1* was downregulated in the neuronal grafts, a result that likely reflects its many roles in early



development inclusive of embryonic segmentation and limb formation. The five mature neuronal genes examined included: G-protein-regulated inward-rectifier potassium channel 2 (GIRK2/KCNJ6) and Calbinin-1 (CALB1), markers of mature midbrain dopamine neuron subpopulations; dopamine transporter (DAT/SLC6A3) and vesicular monoamine transporter 2 (VMAT2/SLC18A2), both responsible for dopamine recycling; and vesicle-associated membrane protein Synaptobrevin 2 (SYNB/VAMP2), a mature presynaptic protein (Figure A3.3L-P). Both GIRK2 and CALB1 were significantly increased in the Immature and Mature Neuronal grafts. Not surprisingly, synaptic protein and transmitter associated genes DAT (49-fold), VMAT and SYNB showed significantly elevated expression in Mature Neuronal grafts, compared to Undifferentiated and Immature neuronal grafts. The increased synaptic integration of the Mature Neuronal grafts was supported by increased histochemical labelling for human Synaptophysin in Mature (Figure A3.3R) compared to Immature Neuronal grafts (Figure A3.3Q).

A3.4.3 Unbiased characterisation of xenograft composition and identification of transcriptional changes using RNAseq

To validate use of the approach for characterising xenograft composition, we profiled the RNA of the 3 distinct xenografts: (i) Grafts generated from the implantation of undifferentiated pluripotent stem cells, anticipated to yield highly proliferative populations and consist of cells from all 3 germ layers 1 month after implantation (subsequently referred to as the 'Undifferentiated' group, (ii) Transplants of partially differentiated ventral midbrain progenitors, analysed 1 month after implantation and anticipated to show characteristic signatures of immature neurons (referred to as 'Neural Progenitor' grafts) and, (iii) Grafts derived from the transplantation of ventral midbrain progenitors and allowed to mature for 6 months *in situ* into mature neuronal populations inclusive of dopamine neurons (denoted 'Mature Neuronal' grafts). Tissue was collected in parallel for immunohistochemistry to provide verification of the gene expression results.

Quantification of xenograft size was conducted by immunohistochemistry using an antibody specific for human cells (human nuclear antigen, HNA) to enable delineation of the graft core and total cell counts. HNA immunostaining showed the presence of large expansive grafts in the Undifferentiated grafts, small discrete grafts in the Neuronal Progenitor grafts and moderate grafts in the Mature neuronal group (Figure A3.2A-D). Transcriptional characterisation of graft composition required the identification of a stable xenograft-specific housekeeping gene across the 3 graft types to control for the size of the xenograft (generating

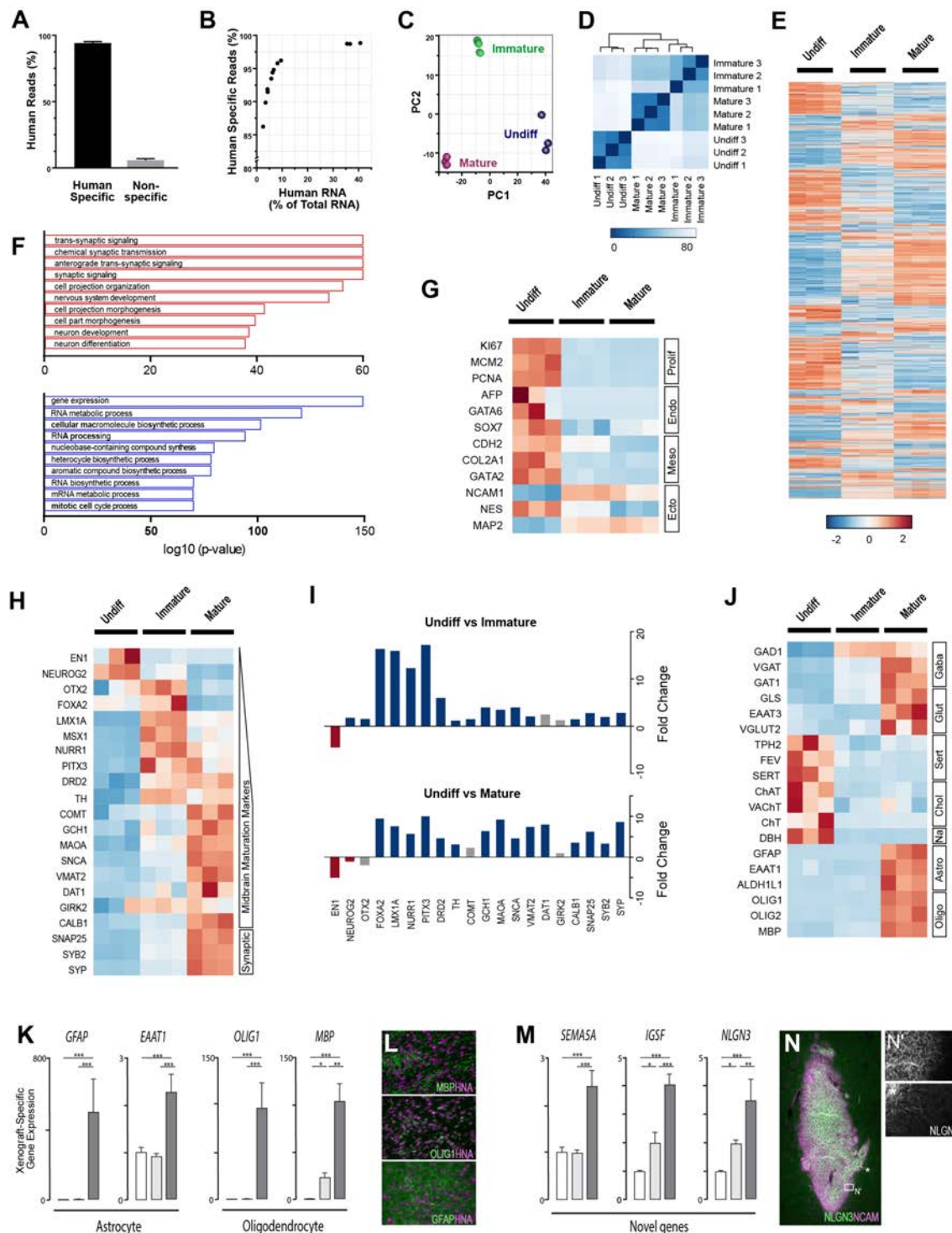


Figure A3.4 – Unbiased Characterisation of xenograft composition and identification of transcriptional changes using RNAseq.

(A) Graph showing 94.4% of the RNAseq reads were uniquely mapped to the human genome (xenograft-specific) compared to 5.6% mapping to both human and host (ambiguous origin). (B) Graph of the percentage of xenograft-specific reads compared to the size of the xenograft showing that xenografts contributing $\geq 2.5\%$ of the total RNA provide greater than 86% uniquely identified xenograft reads. (C) Principal component analysis comparing the global gene expression profile of each xenograft revealed the samples clustered into 3 distinct groups corresponding to the 3 graft types. (D) A dissimilarity matrix of the global RNA profile correlation and unsupervised clustering showed the highest similarity between the replicates of each group. (E) Heatmap of xenograft-specific gene expression. (F) Graph of enriched gene ontology categories between undifferentiated and immature neuronal grafts. (G) Heatmap of markers of germ layer lineages across the different graft types. (H) Heatmap and (I) fold change in gene expression for early and late VM dopaminergic neuron markers across the 3 graft types. (J) Heatmap of non-dopaminergic neural

cell type markers across the 3 graft types. (K) Real-time qPCR verification of the expression of astrocyte (GFAP, EAAT1) and oligodendrocyte (OLIG1, MBP) genes. (L) Representative immunohistochemistry confirmed the presence of astrocytes and oligodendrocytes in the mature grafts. RNAseq identified a number of previously unidentified genes expressed in the mature neuronal grafts that could be verified by (M) qPCR (SEMA5A, IGSF8, NLGN3). (N) Immunohistochemical staining against NLGN3 (green) and human specific NCAM confirmed the presence of this plasticity-associated gene within the mature neuronal grafts. Data represents mean $\pm$ SEM, One-way ANOVA. (A-J) n=3 grafts/group, (K,M) n=5 grafts/group. Scale bar (L,N') = 50um, (N) = 250um.

a graft specific expression value). PSMB4, MTHFD1, CHAMP2A and HPRT1 were tested). The housekeeping gene PSMB4 was selected due to its high specificity and stable expression across the 3 graft types. Estimation of graft size using qPCR measured the proportion of xenograft RNA at $33.0 \pm 8.93\%$ in the Undifferentiated grafts, $1.8 \pm 0.40\%$ in the immature grafts and $9.2 \pm 0.91\%$ in the mature grafts (Figure A3.2E), reflective of graft sizes determined histologically.

Necessary to ensure the safety of cell transplantation therapies is the removal of proliferative cells prior to implantation, or relatively low contribution shortly thereafter. Hence in an effort to validate the technique and characterise graft composition we accessed the expression of Ki67, a gene/protein expressed during all active phases of cell cycle division. Cell counts of Ki67-immunoreactive cells in the grafts (Ki67⁺/HNA⁺) identified a large population of dividing cells in the Undifferentiated grafts, that was significantly reduced in Immature Progenitor grafts, and reduced further in the Mature Neuronal grafts (Figure A3.2A-D,F). Xenograft-specific gene expression (expressed relative to the Undifferentiated grafts), showed expression in the immature grafts was reduced to 0.12 ± 0.02 -fold, and to 0.05 ± 0.01 -fold in mature grafts (Figure A3.2G), corresponding closely to the cell counts. These findings suggest that xenograft-specific qPCR is sensitive enough to detect rare cell populations within a tissue.

The capacity of the technique to detect different cellular populations was demonstrated using primers against genes restricted to defined germ layers (endoderm, mesoderm and ectoderm) and expressed in the Undifferentiated grafts (resulting from the transplantation of pluripotent cells), compared to the immature and mature grafts which were restricted to an ectodermal lineage prior to transplantation (Figure A3.2H-M). Not surprisingly, in comparison to the Undifferentiated grafts, endoderm (AFP and GATA6) and mesoderm (Brachyury and Collgen2A type 1, COL2A1) genes were lowly expression in the Neural Progenitor and Mature Neuronal grafts (Figure A3.2H-K). In contrast, expression of the ectoderm marker neural cell adhesion marker (NCAM), was elevated in the Neural Progenitor and Mature Neuronal grafts (2.25 ± 0.39 and 1.58 ± 0.38 , respectively). The neuroectodermal stem cell marker, Nestin, was also expressed in the Neural progenitor grafts, yet significantly reduced in the mature graft

reflective of downregulation of the gene during neuronal maturation as observed in development. Greater differences in neuroectodermal gene expression between the Undifferentiated and neuronal fate restricted grafts (Neural Progenitor and Mature Neuronal) were not observed largely due to the preferential default fate acquisition for pluripotent stem cells to acquire a neural phenotype. This was confirmed by the extensive expression of Nestin immunoreactivity within Undifferentiated grafts (Figure A3.2N, compared to Mature Neuronal grafts that showed an absence of Nestin labelling at the graft periphery, where the more mature cells reside (Figure A3.2N-Q). The xenograft-specific gene expression accurately identified changes in markers across the 3 germ layers, and closely reflected the observed immunohistochemical labelling and quantification.

We next assessed the sensitivity of the approach to detect more subtle changes present between neural progenitor and mature neuronal grafts. Immunohistochemistry against GFP (to identify the LMX1A-GFP expressing cells) and TH (Tyrosine Hydroxylase) identified immature ventral midbrain progenitors and mature dopaminergic neurons respectively (Figure A3.3A-C). Cell counts revealed 21% (GFP⁺/HNA⁺) of Undifferentiated grafts expressed the ventral midbrain progenitor marker, a proportion that was significantly increased in the Neural Progenitor grafts (82%), and largely maintained in the Mature Neuronal grafts (76%), (Figure A3.2A-D). Xenograft-specific gene expression expressed relative to the Undifferentiated grafts, showed the expression of LMX1A was increased 32.7 ± 1.7 -fold in the Neural Progenitor grafts and reduced to 17.2 ± 2.3 -fold in the Mature Neuronal grafts (Figure A3.3E), commensurate with the cell counts and in accordance to the previously described downregulation of the gene in maturing midbrain dopamine neurons during embryonic development ([Laguna et al., 2015](#)). Cell counts of the mature dopaminergic marker TH identified immunoreactive cells in the Undifferentiated grafts (TH⁺/HNA⁺), which were significantly increased in the Neural Progenitor grafts, and maximal in the Mature Neuronal grafts, accounting for $7.2\% \pm 0.9$ of all cells (Figure A3.3F). Xenograft-specific gene expression analysis supported the increase in TH with expression 8.0 ± 0.7 -fold greater in the Neural Progenitor grafts, and of 12.9 ± 1.9 in the Mature Neuronal grafts, compared to the Undifferentiated grafts (Figure A3.3G).

To demonstrate the utility of the approach to rapidly and comprehensively profile the composition of a graft, 9 additional genes expressed predominantly in immature or mature grafts were then assessed. Four primer pairs were designed, capable of identifying key transcription factors expressed within ventral midbrain neural progenitor inclusive of the

forebrain-midbrain gene OTX2, midbrain-hindbrain restrictive gene Engrailed-1 (EN1), dopaminergic precursor Nurr1 (NR4A2), and pro-survival gene PITX3. OTX2 expression was significantly increased only in the Neural Progenitor graft (3.2 ± 0.7), reflective of its transient expression in development. Whilst Engrailed was unexpectedly downregulated in the Mature neuronal group, PITX3 was significantly increased in the both Neural Progenitor and Mature Neuronal grafts (476.2-fold $\pm$ 239.2 and 381-fold $\pm$ 185.1 respectively, compared to Undifferentiated grafts). The five mature neuronal genes examined included: the dopamine transporter (DAT) and vesicular monoamine transporter (VMAT), both responsible for the recycling of dopamine; G-protein-regulated inward-rectifier potassium channel 2 (GIRK2) and Calbinin-1 (CALB1), markers of mature midbrain dopamine neuron subpopulations; and vesicle-associated membrane protein (VAMP)/Synaptobrevin, a presynaptic protein involved in the docking of synaptic vesicles (Figure A3.3M-Q). Not surprisingly, synaptic protein and transmitter associated genes DAT, VMAT and VAMP2 showed significantly elevated expression in Mature Neuronal grafts, compared to Undifferentiated and Neural Progenitor grafts. Both GIRK2 and CALB1 (labelling the A9 and A10 midbrain dopamine neuron subpopulations, respectively) were significantly increased in the Neural Progenitor and Mature Neuronal grafts (GIRK2: Progenitor graft 5.1 ± 1.4 , Mature 4.1 ± 0.9 ; CALB1: Progenitor graft 1.9 ± 0.2 , Mature graft 3.0 ± 0.7 -fold change relative to Undifferentiated grafts).

A3.4.4 Unbiased characterisation of xenograft composition and identification of novel transcriptional changes using RNAseq

Expanding on our species-specific transcriptional profiling approach, we sought to demonstrate the capacity for whole genome profiling using RNA sequencing. Unlike selected qPCR, RNAseq enables unbiased characterisation of graft composition, confirming both known/anticipated gene expression and the presence of previously unidentifiable cell populations. RNAseq can also provide new insight into gene expression, including recognition of mechanistic pathways contributing to graft function. Mixed species cDNA libraries were prepared from the 3 graft types and subject to paired-end, 75 bp sequencing to a target depth of approximately 20 million human reads (using the estimation of percentage xenograft RNA described in Figure A3.2E). A rapid and computationally efficient analysis pipeline was then established and implemented on the freely available Galaxy platform to make it accessible without specialist bioinformatics infrastructure or expertise. Reads were first mapped against the human genome, with only concordant (successfully aligned) reads subsequently mapped to the mouse genome. To

minimise the risk of incorrectly specified reads, a high stringency approach was adopted where all human reads found to also map to the mouse genome were discarded. The resulting reads were designated as unique to the human genome, and therefore originated from the xenograft. An average of $94.4 \pm 1.5\%$ of reads were identified as unique to the human genome under these criteria across the 3 graft groups (Figure A3.4A). An average of $5.6 \pm 1.5\%$ of reads aligned to both human and mouse genome and were therefore discarded from analysis. These discarded reads mapped to 327 genes (defined as having an average of >10 reads) and showed no enrichment for any gene ontology category or pathway. Visual analysis of the 10 most highly expressed genes from this list (Supp Figure A3.2A-H) showed the reads did not provide broad coverage across genes, aligning to only to isolated fragments in both exonic and intergenic regions. The reads did not map preferentially to highly conserved regions or repeat elements, and displayed profiles consistent with originating from mouse RNA (significant mismatches to human genome, not overlapping with human specific reads).

Taking advantage of the variation in graft size across the experimental groups, an estimate of the percentage of xenograft RNA required in the host tissue to provide a sufficient level of unique reads for analysis was assessed. Samples in which human RNA constituted just 2.5% of the total RNA (human RNA + mouse RNA) provided 86% reads unique to the human transcript using our high stringency approach. This specificity increased exponentially such that with 6.7% human RNA, greater than 95% of the reads were classified as uniquely human, while grafts containing 35% human RNA and yielded 99% unique reads (Figure A3.4B). Overall these results demonstrate the analysis pipeline provided a highly efficient yield of species-specific reads from within a graft-host pool of tissue/RNA, including when the graft size was limited.

To determine if the identified xenograft-specific reads were consistent with xenograft gene expression, transcripts were assembled for analysis. Examination of the global gene expression profile by principal component analysis showed that the grafts clustered tightly as 3 distinct populations corresponding to the 3 grafted cell types (Figure A3.4C). A dissimilarity matrix and unsupervised clustering also showed the highest similarity between the replicates of each group, with Immature Neuronal and Mature Neuronal grafts the most closely related graft types (Figure A3.4D). Combined, these findings show that at a global expression level the xenograft-specific RNAseq provided a highly specific expression profile consistent with the expected graft relationships.

To demonstrate the ability to use RNAseq data to investigate known and unknown genes, xenograft-specific differential expression analysis was conducted (Figure A3.4E). A total of 11,010 differentially expressed genes were identified between Undifferentiated and the Immature Neuronal grafts (Supp Table 2) and 12,449 genes between Undifferentiated and Mature Neuronal grafts (Supp Table 3). Enriched gene ontology categories between Undifferentiated and Immature Neuronal grafts identified upregulation of genes associated with synaptic signalling, axonal plasticity and neural development, while RNA expression/processing and regulation of cell cycle genes were downregulated (Figure A3.4F). Analysis of genes at this comparison identified 233 transcription factors potentially involved in the development of mature dopaminergic populations (Supp Table 4), and 751 genes coding for axon guidance cues and receptors (Supp Table 5), including several associated with the classical axon guidance superfamilies (Supp Figure A3.3A). Many of the identified guidance cues have previously been implicated in dopamine axonal plasticity in rodents, but not yet human. These results provide an expansive list of targets for the potential modulation of cell populations or axon growth and plasticity in the maturation and integration of neuronal grafts.

The utility of the RNAseq data to provide an unbiased characterisation of the xenograft composition was first confirmed by assessment of known genes. Reflective of qPCR observations, Ki67 was highly expressed in the Undifferentiated grafts, (Figure A3.2G). Two additional pro-proliferation genes, DNA replication licensing factor MCM2 and proliferative cell nuclear antigen (PCNA), were similarly upregulated (Figure A3.4G). Expression of three markers of an endodermal, mesodermal and ectodermal lineage were all shown to be highly expressed in the Undifferentiated cell group. In accordance with qPCR findings, endodermal and mesodermal genes were downregulated in the Immature and Mature Neuronal grafts, while ectodermal marker remained expressed, or were downregulated with ongoing graft maturation, reflective of neural developmental. Further validating the approach, numerous vascular-associated genes, such as essential hematopoietic transcription factor TAL1 and vasculogenesis gene, FLT1 were upregulated in the undifferentiated PSC grafts (i.e. teratomas), but not neural progenitor grafts, and could be validated by qPCR, Supp Figure A3.4.

Genes of known association with dopamine development and function showed increased expression in Immature and Mature Neuronal grafts compared to the Undifferentiated. Expression levels were represented as a heatmap (Figure A3.4H) and graphed as fold change for each of the comparisons between the graft types (Figure A3.4I). Markers of dopamine progenitors NEUROG2, FOXA2, OTX2 and LMX1A were most highly expressed in the Immature Neuronal grafts. NR4A2, PITX3, TH and DRD2 were maintained at high levels in the both Immature and Mature Neuronal grafts, whilst the mature genes GCH1, MAOA, SNCA (α -synuclein), VMAT2/SLC18A2 and DAT1/SLC6A3 were expressed most highly in the Mature Neuronal grafts. Confirming graft maturation, synaptic markers SNAP25 (Synaptosome Associated Protein 25), Synaptobrevin2 (SYNB/VAMP2) and SYP (Synaptophysin) were all significantly upregulated in the Immature Neuronal grafts, and even greater fold changes in the Mature Neuronal grafts (Figure A3.4I).

With less than 5% of VM neural progenitor grafts consisting of dopamine neurons, yet little knowledge of the identity of the remaining >95% of cells, the data set was screened for markers of other neural (non-dopaminergic) cell populations in the 3 grafted cell groups (Figure A3.4J). GABAergic (GAD1, VGAT/SLC32A1, GAT-1/SLC6A1) and glutamatergic (GLS, EAAT2/SLC1A2, VGLUT2/SLC17A6) related genes were found to be expressed in the Mature Neuronal grafts, suggestive of the presence of these neuronal populations, while low expression of serotonergic (TPH2, FEV, SERT/SLC6A4), cholinergic (CHAT, VACHT/SLC18A3, Ch/TSLC5A7) noradrenergic (DBH) relative to Undifferentiated grafts inferred few of these neuronal populations were present within midbrain-differentiated grafts. Strikingly, non-neuronal cell types showed unexpectedly high expression within Mature grafts, including astrocyte-associated genes GFAP, GLAST/SCL1A3, and ALDH1L1 as well as oligodendrocyte genes OLIG1, OLIG2 and MBP (Figure A3.4J). Xenograft-specific qPCR profiling and immunohistochemistry for the astrocyte markers GFAP and EAAT1/SLC1A3 as well as oligodendrocyte markers OLIG1 and MBP confirmed the presence of these cell populations in the mature grafts (Figure A3.4K,L). Retrospective assessment of matured VM progenitor grafts from four previous grafting studies performed in the laboratory validated and confirmed the significant proportions of these glial populations within grafts (data not shown). The identification of non-target cell types in the mature grafts demonstrates the utility of using xenograft-specific profiling to screen a broad range of phenotypic markers and cell populations. In addition, the presence of non-DA cells within grafts of LMX1A-GFP+ sorted progenitors highlight the broad lineage potential of these cells, above and beyond the DA lineage alone.

Finally, the data set was utilised to detect unique candidate genes that may participate in the maturation and plasticity of human PSC-derived VM progenitor grafts. We identified and examined three genes that show significantly elevated expression within the Mature Neuronal graft – SEMA5a (Semaphorin 5A), IGSF8 (Immunoglobulin superfamily member 8) and NGLN3 (Neurologin 3). Each of these genes were confirmed by qPCR (Figure A3.4M) and NGLN3 further validated by immunohistochemistry, showing punctate expression throughout the graft and evident along graft-derived axonal fibres (Figure A3.4N,N'). A lack of reliable commercially available antibodies against Semaphorin A5 and IGSF8 prevented confirmation of expression at the protein level. These findings provide the first efforts and evidence for identifying the true composition of VM neural progenitor grafts and highlight the utility for this transcriptional profiling methodology to understand graft composition. In the present context the identification of new genes involved in graft maturation may present candidate proteins for manipulation in the future, targeted at promoting graft plasticity, integration and function.

A3.5 Discussion

Here we describe an approach to transcriptionally profile xenografts by discriminating the graft species RNA from host. The technique is designed to allow standardisation and characterisation of graft composition. The method is simple to implement, requires minimal experience and is highly reproducible. The rapid design of primers and large quantity of RNA generated allows a greatly enhanced number of genes to be measured in a relatively short timeframe, enabling a more thorough characterisation of grafts inclusive of proteins/genes that are currently difficult to discriminate. When combined with RNAseq, the technique provides an unbiased means to screen graft composition, investigate key questions pertaining to their function, and provide insight into strategies to enhance grafting outcomes.

Within this study we demonstrated the utility of this technique by analysing 3 distinct human grafts within the rodent brain - grafts derived from undifferentiated human PSCs, grafts of immature neural progenitors, and grafts of neural progenitors left to mature *in situ* over several months. Xenograft-specific qPCR and RNAseq were both demonstrated to accurately and efficiently discriminate xenograft gene expression from host. In addition, to cross validate these

qPCR and RNAseq outcomes, findings were confirmed by immunohistochemistry, where antibodies were available, with quantification across the 3 methodologies generating highly correlated results.

Validated both *in vitro* and *in vivo*, the specificity of xenograft-specific (human) qPCR primers designed for the 30 genes examined in the present study showed 1000 to >1,000,000-fold increased expression over the mouse host. As such, the technique allowed the presence of a xenograft to be confirmed and provided an estimate of the graft size. The proportion of xenograft RNA in individual pools of both graft and host tissue correlated with predetermined cell numbers, whilst cell counts in experimental groups quantified in parallel using immunohistochemistry also closely matched these estimates, as did the proportion of xenograft RNA estimated by RNAseq. The technique was also able to accurately detect both large- and small-scale changes in graft cell populations, such as the expression of germ layer lineage specific markers in Undifferentiated cell grafts, compared to the more subtle changes between immature and mature neuronal grafts.

A key advantage of this transcriptional profiling approach is the ease of tissue and RNA isolation, allowing standardisation across laboratories and by separate operators. Here, grafts were placed into the striatum as it provided an easily identifiable structure for gross tissue dissection, yet can be transplanted in any location. Following gross tissue dissection, RNA was readily isolated using a standard column-based protocol. The large amount of tissue ensured the RNA extraction was robust against the effects of RNA loss or degradation and allowed the RNA quality and quantity to be analysed using the commonly available nanodrop spectrophotometer. The mixed species RNA population could then be directly interrogated with PCR or RNAseq, making the process easily achievable for most laboratories without necessary expertise in microdissection, single-cell sorting or RNA handling. To make the RNAseq analysis methods employed here accessible without specialist bioinformatic experience or infrastructure, we implemented the analysis pipeline in the freely available Galaxy web platform (www.usegalaxy.org/), which provides point-and-click access to all the required bioinformatic tools (Afgan et al., 2018).

Analysis of graft composition is often hindered by the availability of reliable antibodies, or the presence of a protein of interest in both the graft and host tissue, thereby preventing the

ability to discriminate the graft. This later challenge is especially relevant were cells migrate and intersperse within host tissues or, in the context of neural transplants, where axons innervating regions distal to the site of implantation cannot be accurately separated. Xenograft-specific qPCR can readily detect any gene of interest in a graft, irrespective of the expression in the host or localisation of the cell within surrounding tissues. As evidence, here we successfully designed primers against NURR1/NR4A2, EN1 and PITX3, examples where antibodies are unavailable or unreliable. Primers were also designed against genes expressed in both the graft and host tissue for which human specific antibodies are not available, including broadly expressed neural (Nestin and GFAP), dopamine specific (TH, DAT1/SLC6A3 and VMAT2/SLC18A2), and synaptic genes (SYB2/VAMP2). Added to this was the ability to detect rare events within transplants, such as proliferative Ki67 cells. Importantly, validity of the species-specific qPCR technique was demonstrated by similarities in gene expression levels and cell counts within the grafts.

The inability to easily apply high throughput gene or protein analysis to xenografts has been a major impediment to understanding graft integration, and to identify targets to enhance survival, maturation and plasticity that may impact overall graft function. Xenograft-specific RNAseq provides an easily accessible tool for the interrogation of the entire transcriptome of the transplant, allowing the investigation of previously unknown genes and pathways active in the graft. Importantly, the RNAseq data also contains host specific reads, thereby providing the means to examine interactions between the host tissue and the xenograft. Here we have described the ability of xenograft-specific profiling to characterise graft composition. The ability to measure all the expressed genes in the graft allows an unbiased assessment and the ability to rapidly screen a range of cell phenotypes. No prior assumptions are required (as with antibody selection or qPCR primer design). The capture of all genes allows an expanded examination of markers, providing a thorough analysis of the target cell populations. The advantage of an unbiased and through coverage of phenotypic markers was exemplified here by the surprising expression of astrocyte and oligodendrocyte transcripts within grafts derived from FACS sorted VM neural progenitors. The presence of these cell types predominantly in mature grafts highlights the importance of long-term transplantation to accurately assess the efficiency of differentiation protocols. The presence of small pools of poorly specified cells can expand to become a significant component of the graft at therapeutically relevant timeframes yet are not easily detected *in vitro*. These cell types are difficult to detect using conventional approaches as host astrocyte and oligodendrocyte cells can migrate to infiltrate the graft and can only be directly detected using transgenic reporters. As the use of transgenic reporters is not feasible in

a clinical setting, xenograft-specific profiling may present the only approach for pre-clinical testing of cell differentiation protocols and cell batch verification prior to transplantation in patients. This is especially relevant in light of the 2 recently commenced clinical trials (www.nature.com/articles/d41586-018-07407-9), (Barker et al., 2017), that adopt similar differentiation protocols to the one used here for the generation of midbrain dopamine neurons in the treatment of Parkinson's disease.

Finally, we demonstrated the utility of the species-specific RNAseq approach to identify previously unidentified genes within grafts. Here we identified the expression of *Nlgn3*, *Sema5A* and *IGSF8* within mature neuronal grafts (compared to immature). These proteins have previously been implicated in axonal and synaptic plasticity (Kantor et al., 2004; Bariselli et al., 2018), or shown to be expressed on dopaminergic progenitors (Bye et al., 2015), but their roles in dopaminergic graft maturation remains to be explored. With previous work showing that human pluripotent stem cell-derived dopamine neurons show inferior plasticity to grafts derived from fetal tissue, identification of such genes may present new targets for modulating axonal growth and graft integration.

While we highlight the many benefits of whole tissue xenograft-specific transcriptomics, we also acknowledge the limitations. Here the transcript levels of individual cells are not captured individually, as in single cell approaches. This restricts the classification of complex cell populations in the graft, with the xenograft-specific expression profile reflecting expression over the entire graft and unable to identify how many cells express a given gene or the level of expression within a cell. The approaches described here are thus highly complementary. One also recognises that whilst RNAseq profiling of the grafts is highly sensitive and informative, it is also expensive – especially when attempting to detect low numbers of grafted cells as small grafts require much higher sequencing depth to achieve sufficient reads for accurate expression profiling.

In summary, we describe an alternative approach to profile xenografts. While demonstrated in the context of pluripotent stem cell-derived dopamine grafts for cell therapy in Parkinson's disease, this technique holds promise for profiling all xenografts, both within and outside the brain.

A3.6 Acknowledgements

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A3.8 Supplementary Figures

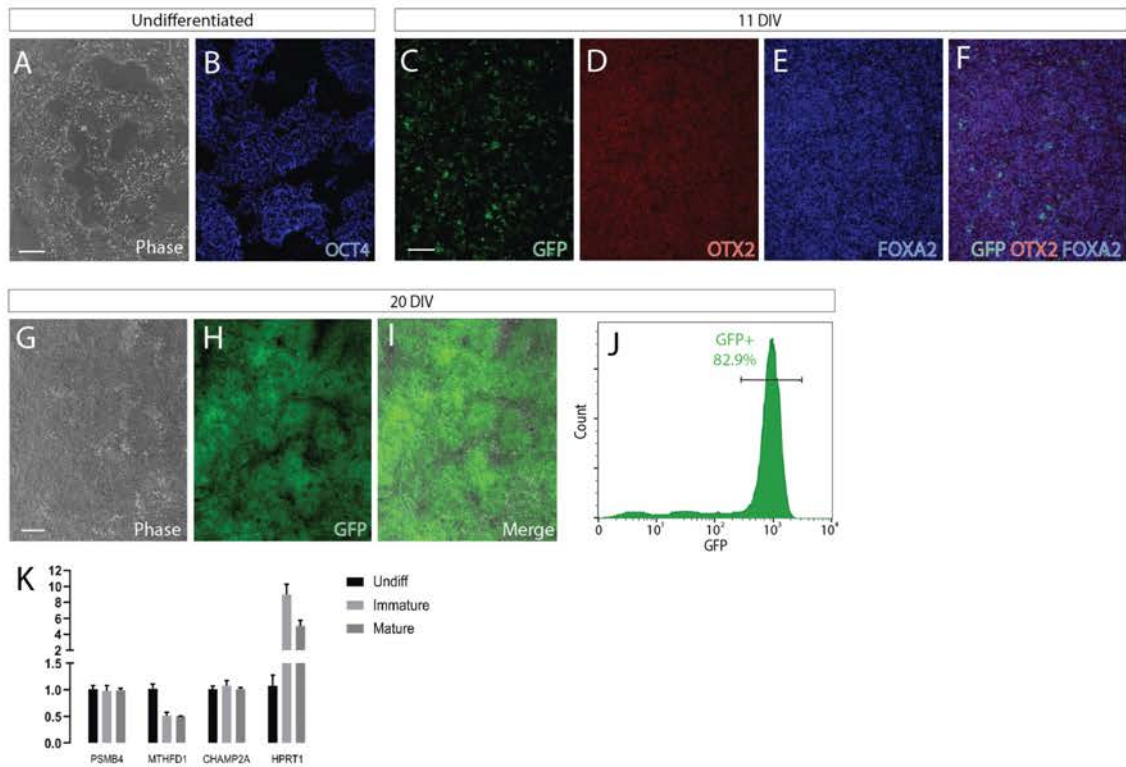


Figure A3.S1 – Characterization of undifferentiated and neural specified progenitors in vitro.

(A) Phase contrast illustrating the morphology of undifferentiated human embryonic stem cells (H9 LMX1A-GFP) (B) and their expression of pluripotent gene OCT4. (C) Ventral midbrain specification of human ESCs was confirmed at 11 days in vitro (11DIV) by the co-expression of the LMX1A-GFP reporter (GFP), (D) midbrain-forebrain gene OTX2 and (E) ventral identity transgene FOXA2. (F) Merged image of LMX1A-GFP, OTX2 and FOXA2 illustrating that the vast majority of cells showed co-labeling, indicative of ventral midbrain patterning, inclusive of dopamine progenitors. (G) Phase contrast and (H) GFP labeling (I) and merge, of day 20 ventral midbrain progenitor cultures used for isolation of LMX1A-expressing cells for transplantation. (J) FACS plot showing >80% of cells in culture expressed GFP (LMX1A-GFP) at 20DIV. (K) Screening of xenograft-specific reference (housekeeping) genes identified PSMB4 as having high specificity and stable expression across the 3 graft types assessed. Data represents Mean + SEM, n=4 grafts/group. Scale bars (A-B) 100um, (C-F) 200um, (G-I) 200um.

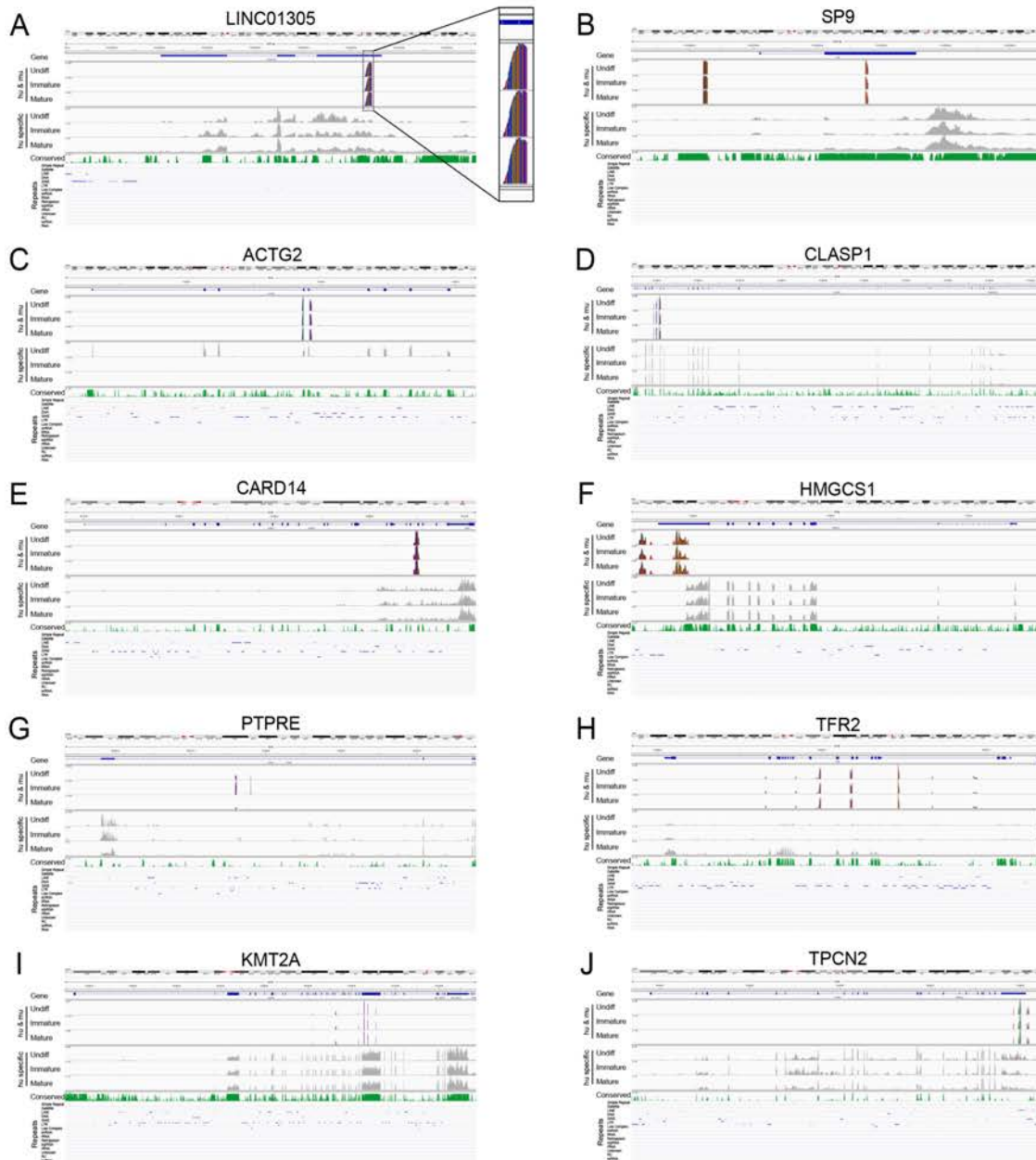


Figure A3.S2 – Alignment of non-specific human reads.

RNAseq coverage for human aligned reads that also aligned to the mouse genome were designated as non-specific. (A-J) shows coverage for the 10 genes with the largest average number of reads across samples mapped to the human genome and displayed with Integrated Genome Viewer (IGV). Coverage for 1 sample per group shown for non-specific (human & mouse) and specific (human only) reads with perfect matches represented in grey and mismatched represented in colors (see enlarged insert in A) corresponding to the percentage of alternative bases. Annotation tracks showing conserved genomic regions from Phastcons (x20) represented in green, and repeat elements from Repeatmasker represented beneath in blue. Non-specific reads aligned to small fragments in exonic and intergenic regions and did not provide consistent coverage across genes. The reads showed a high percentage of variation relative to the human genome, with little variation seen in the human specific reads. Non-specific reads in exonic regions corresponded with highly conserved regions for a majority (LINC01305, SP9, ACTG2, SCTG2, CARD14, PTPRE, TFR2, KMT2A), but not all genes (CLASP1, HMGCS1, TPCN2), with a majority of conserved regions in the genome showing no non-specific reads mapped. No overlap of non-specific reads with repeats was observed (n=3 grafts/group).

A

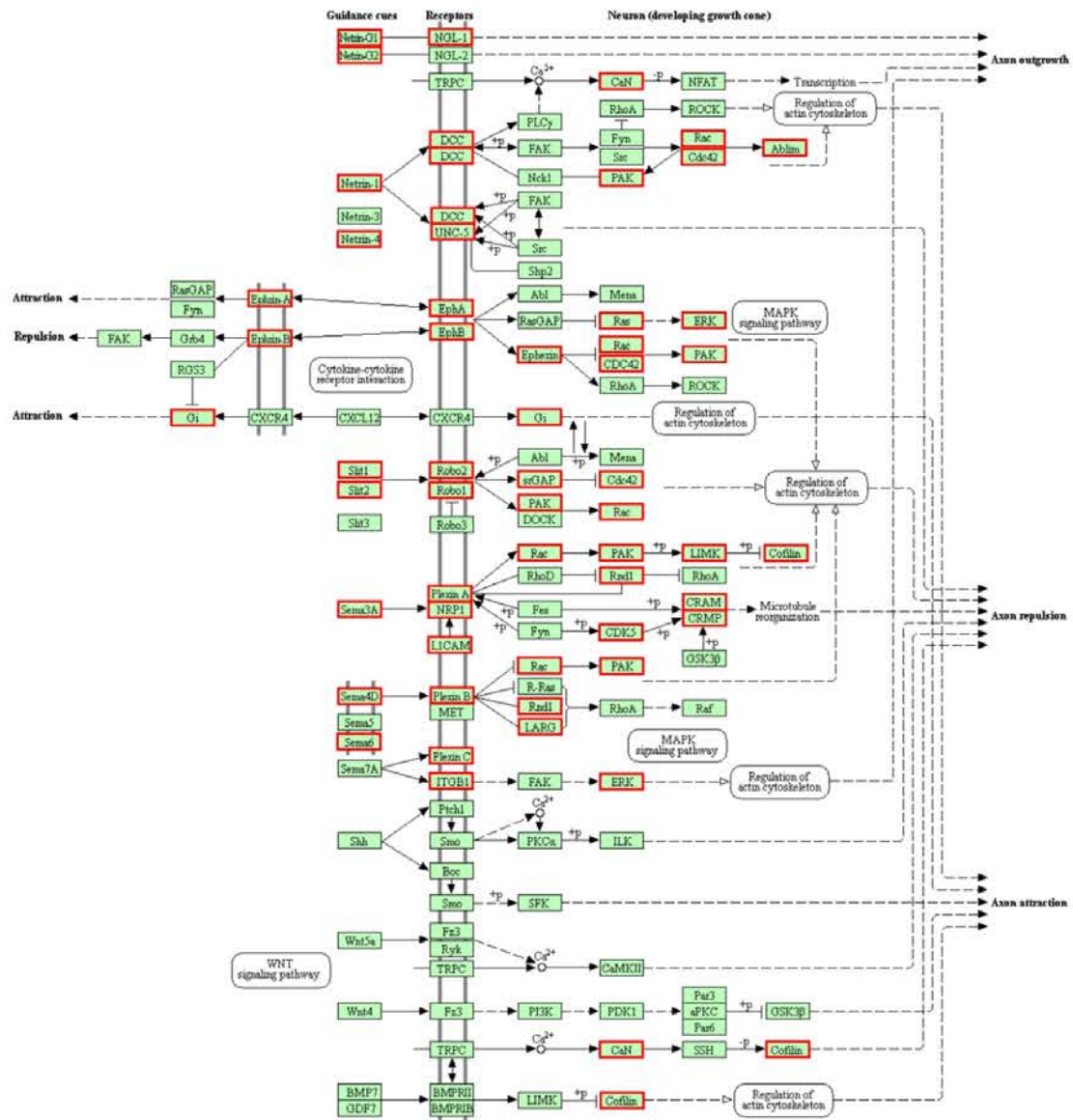


Figure A3.S3 – Axon guidance genes upregulated in the Neuronal grafts.

(A) KEGG Axon guidance pathway showing genes upregulates in Immature Neuronal grafts (highlighted with a red outline).

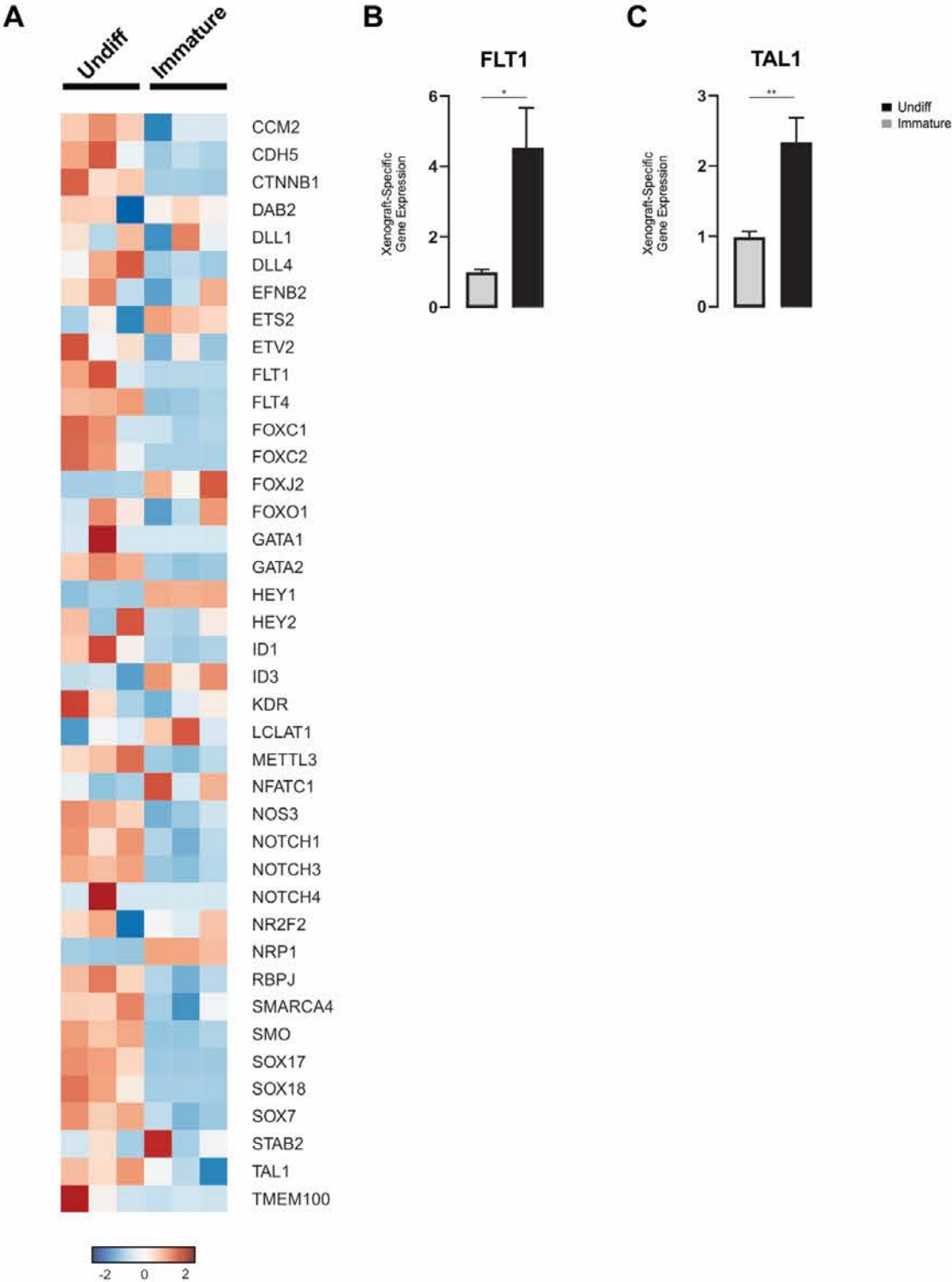


Figure A3.S4 – Transcriptional profiling of vascular-associated genes.
Heatmap of gene expression changes for undifferentiated and immature neural progenitor grafts for vascular-associated genes expressed in development, highlighting the increased expression of angiogenesis/vasculogenesis transcription factors within the undifferentiated/teratoma-like grafts, n=3 grafts/group. (B) Real-time qPCR verification for the expression of FLT1 and, (C) TAL1, (B-C) n=5 grafts/group. Data represents Mean + SEM, * p<0.05, ** p<0.01.