INVESTIGATING THE DEVELOPMENT OF MIDBRAIN DOPAMINERGIC NEURONS USING MOUSE EMBRYONIC STEM CELL REPORTER LINES

A thesis submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

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Chapters 3 and 4, "BMP, Shh and Wnt signalling pathways on dopaminergic neuron development" and "Enrichment and characterisation of Lmx1a positive cells" are parts of a paper published in *Stem Cells (2012)*.

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In the case of Chapters 3 and 4 my contribution to the work involved the following:

Name	Nature of contribution
Colin Su	Execution of experimental work, composition of manuscript (Est. 35% of total work)
Christian Nefzger	Planning and execution of experimental work, composition of manuscript (Est. 35% of total work)
Stewart Fabb	Generation of reporter cell lines (Est. 10% of total work)
Brigham Hartley	Execution of experimental work (Est. 7.5% of total work)
Siew Beh	Execution of experimental work (Est. 7.5% of total work)
Wendy Zeng	Execution of experimental work (Est. 5% of total work)
John Haynes	Consultative role in planning and execution of experimental work, reviewing of manuscript drafts
Colin Pouton	Consultative role in planning and execution of experimental work, reviewing of manuscript drafts

Declaration by co-authors

The undersigned hereby certify that:

1. They meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
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COMMUNICATIONS

The following communications, resulting from studies conducted in this thesis were presented as conference abstracts and posters:

1. **Colin T. Su**, Warren S. Raye, John M. Haynes and Colin W. Pouton (2009). Differentiation of mouse embryonic stem cell-derived neural rosettes into neurons. *Proceedings of the 7th Annual Meeting for the International Society for Stem Cell Research.*

2. **Colin T. Su**, Warren S. Raye, John M. Haynes and Colin W. Pouton (2009). Differentiation of mouse embryonic stem cell-derived neural rosettes into neurons. *Proceedings of the 2nd Annual Meeting for the Australasian Society for Stem Cell Research.*

3. **Colin T. Su**, Christian M. Nefzger, Stewart R. Fabb, Colin W. Pouton and John M. Haynes (2011). The role of Lmx1a during *in vitro* monolayer differentiation. *Proceedings of the 4th Annual Meeting for the Australasian Society for Stem Cell Research.*

4. **Colin T. Su**, Christian M. Nefzger, Stewart R. Fabb, Colin W. Pouton and John M. Haynes (2012). The role of Lmx1a during *in vitro* monolayer differentiation. *Proceedings of the 32nd Annual Meeting for the Australian Neuroscience Society.*

PUBLICATIONS

The following papers, resulting from studies conducted in this thesis were published:

1. Christian M. Nefzger[*], **Colin T. Su**[*], Stewart R. Fabb, Brigham J. Hartley, Siew J. Beh, Wendy R. Zeng, John M. Haynes and Colin W. Pouton.
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   Stem Cells (2012)

   * These authors contributed equally

SUMMARY

Embryonic stem (ES) cells possess the capability to self-renew indefinitely and are capable of generating any cell of the three primary germ layers, making them an attractive source of material to investigate both basic physiological properties and neurodegenerative processes. Although ES cells can be directed into specific cell lineages, the differentiation of ES cells results in heterogenous cultures. To date, there are few differentiation protocols that produce homogenous populations of any desired cell type. Many methods have been used in an effort to obtain homogenous populations of cells; from forced expression of genes involved in developmental pathways, to FACS isolation of cells expressing markers of interest. There has been a considerable focus on generating homogenous populations of midbrain dopaminergic progenitors (or neurons) for Parkinson's disease which involves the degeneration of a specific population of midbrain dopaminergic neurons. In this thesis, I investigate the development of mouse embryonic stem cells into midbrain dopaminergic neurons using reporter cell lines.

In the first experimental chapter (Chapter 3), I investigate the expression of Lmx1a and Msx1; two key transcription factors implicated in dopaminergic neuronal development. I also examine the impact of the BMP, Shh and Wnt signalling pathways on dopaminergic neural differentiation. Activation of the BMP and Wnt pathways resulted in inhibition of neural induction and the expression of both Lmx1a and Msx1. In contrast, antagonising these signalling pathways increased the yield of tyrosine hydroxylase (TH) expressing neurons. Activating or inhibiting the Shh pathway did not affect Lmx1a, Msx1 or TH

expression. These experiments show that early Lmx1a expression is not indicative of the number of dopaminergic neurons produced. Furthermore, many of the TH positive neurons derived from monolayer cultures were not of midbrain origin.

In the following experimental chapter (Chapter 4), I used immunocytochemistry and qPCR to characterise the population of cells expressing Lmx1a. The downstream targets of Lmx1a, Msx1 and Wnt1, and midbrain dopaminergic neuron markers, Lmx1b and En1, were significantly upregulated in Lmx1a positive cells. The Lmx1a positive fraction was enriched with neural progenitors, and give rise to highly neural cultures. However, the majority of neurons in the terminally differentiated cultures derived from Lmx1a positive cells were GABAergic. Immunocytochemistry identified these cells as forebrain GABAergic neurons with upper-layer identity. Furthermore, the isolated Lmx1a positive cells were not responsive to patterning cues, indicating that they were already committed towards a GABAergic neuron fate. To show that these Lmx1a+ progenitors could generate dopaminergic neurons I used an alternative differentiation paradigm, the PA6 co-culture method. Expression of Lmx1a in PA6 co-cultures was different from monolayer cultures; the percentage of Lmx1a positive cells increased throughout the differentiation period. In addition, PA6 co-culture derived TH positive cells were found to co-express Lmx1a, an occurrence that was uncommon in monolayer cultures.

The ionotropic glutamate receptors on neurons derived on adherent monolayer and PA6 co-cultures were functionally characterised in Chapter 5. Previously, antagonism of ionotropic glutamate receptors has been reported to improve behavioural assay scores in

Parkinsonian animal models (Johnson *et al.*, 2009). Terminally differentiated monolayer cultures and PA6 co-cultures responded differently to stimulation with glutamate, AMPA kainate and NMDA. The ionotropic glutamate receptors of midbrain dopaminergic and GABAergic neurons derived from both culture systems were further investigated. An initial characterisation indicates distinct differences between the glutamate receptor populations in monolayer and PA6 co-cultures. It appears that monolayer differentiation generates AMPA expressing midbrain dopaminergic neurons in comparison to the NMDA receptors evident following PA6 differentiation. Interestingly, these differences in receptor expression appear restricted by culture method, rather than neuronal subtype, i.e. monolayer neurons expressed AMPA receptors, regardless of whether they were TH+ or GAD67+. Similarly both TH+ and GAD67+ neurons appeared to express NMDA receptors following PA6 differentiation. At present the significance of these findings is unknown. In addition, the effect of Wnt5a on cell responses to glutamate agonists was examined. Wnt5a was able to potentiate cell responses to sub-maximal concentrations of certain glutamate agonists depending on the differentiation paradigm performed.

ABBREVIATIONS

$[Ca^{2+}]_i$	intracellular free calcium ion concentration
°C	degrees Celsius
5-HT	5-hydroxytryptamine/serotonin
AADC	L-aromatic amino acid decarboxylase
Aldh	aldehyde dehydrogenase
ALS	amyotrophic lateral sclerosis
AMP	ampicillin resistance gene
AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APC	Adenomatous polyposis coli
BDNF	brain-derived neurotrophic factor
BF	brain factor
bHLH	basic-helix-loop-helix
BMP	bone morphogenic protein
BMPR	bone morphogenic protein receptor
bp	base pairs
BSA	bovine serum albumin
CamKII	calcium/calmodulin-dependent kinase II
cDNA	complementary DNA
CGP55845	(2S)-3-[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl)phosphinic acid

CHIR99021	6-(2-(4-(2,4-dichlorophenyl)-5-(4-methyl-1H-imidazol-2yl)pyrimidin-2-ylamino)ethylamino)nicotinonitrile
Chrd	chordin
CM	conditioned medium
CNS	central nervous system
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
cRET	RET receptor tyrosine kinase
Cyclopamine	(2'*R*,3*S*,3'*R*,3'a*S*,6'*S*,6a*S*,6b*S*,7'a*R*,11a*S*,11b*R*)1,2,3,3'a,4,4',5',6,6',6a,6b,7,7',7'a,8,11,11a,11b-Octadecahydro-3',6',10,11b-tetramethyl spiro[9*H*-benzo[*a*]fluorene-9,2'(3'*H*)-furo[3,2-*b*]pyridin]-3-ol
DAPI	4',6-diamidino-2-phenylindole
DAT	dopamine transporter
DDC	dopa decarboxylase
Dkk	dickkopf
Dll	delta-like ligand
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DVL	Dishevelled
E	embryonic day
EB	embryoid bodies
EC cells	embryonal carcinoma cells
EDTA	ethylenediaminetetraacetic acid

EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
En	Engrailed
ES cells	embryonic stem cells
FACS	fluorescent activated cell sorting
FCS	fetal calf serum
FGF	fibroblast growth factor
FLUO-4AM	FLUO acetoxymethyl ester
Foxa2	forkhead box a2
Frt	flippase recognition target
FRzB	Frizzled-related protein
Fz	Frizzled
g	grams
xg	g-force
GABA	gamma-amino butyric acid
GAD	glutamic acid decarboxylase
GDF	growth and differentiation factor
GFAP	glial fibrillary acid protein
GIRK	G-protein-gated inwardly rectifying K^+ channel
GLAST	glutamate-aspartate transporter
GMEM	Glasgow's modified essential medium
gp	glycoprotein
GSK	glycogen synthase kinase

HD	Huntington's disease
HEPES	N-2-[hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
hES cells	human embryonic stem cells
HNF-3 β	Hepatocyte nuclear factor 3 β
ICM	inner cell mass
IGF2	insulin-like growth factor 2
IGFBP4	Insulin-like growth factor binding protein 4
IL	interleukin
iPS cells	induced pluripotent stem cells
IRES	internal ribosome entry site
Jag	Jagged
JNK	Jun N-terminal kinase
KSR	knockout serum replacement
L	litre
L-DOPA	L-dihydroxyphenylalanine
LEF	lymphoid enhancer-binding factor
LIF	leukemia inhibitory factor
LIFR	leukemia inhibitory factor receptor
Lmx	LIM homeobox transcription factor
LY235959	[3S-(3a,4aa,6b,8aa)]-Decahydro-6-(phosphonomethyl)-3-isoquinolinecarboxylic acid)
M	molar
MAO	monoamine oxidase

MEM	minimum essential medium
MEFs	mouse embryonic fibroblasts
mES cells	mouse embryonic stem cells
Msx	msh homeobox
n	number of experiments
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
Neo	neomycin
Ngn	neurogenin
NMDA	N-methyl-D-aspartate
Nog	noggin
NS cells	neural stem cells
nt	nuclear transfer
Otx2	orthodenticle homologue 2
PBS	phosphate buffered saline
PBST	PBS with 0.1% Tween 20
PD	Parkinson's disease
Pitx3	Pituitary homeobox 3
Pen-Strep	penicillin-streptomycin
PKC	protein kinase C
PSD-95	post-synaptic density protein-95
Ptc	Patched
PTN	pleiotrophin
qPCR	quantitative polymerase chain reaction

RA	retinoic acid
RAR	retinoic acid receptor
RNA	ribonucleic acid
R-NSC	rosette neural stem cell
RRF	retrorubal field
SANT-1	N-[3,5-dimethyl-1-phenyl-1H-pyrazol-4yl)methylene-4-(phenylmethyl)-1-piperazinamine
SEM	standard error of the mean
SDF	stromal cell-derived factor
sFRP	secreted Frizzled-related protein
Shh	Sonic hedgehog
Smo	Smoothened
SMA	spinal muscular atrophy
SNpc	substantia nigra pars compacta
SVZ	subventricular zone
TAE	tris-acetate-EDTA
TBR-1	T-box brain 1
TGFβ	transforming growth factor β
TH	tyrosine hydroxylase
TOPRO-3	Quinolinium, 4-[3-(3-methyl-2(3H)-benzothiazolylidene)-1-propenyl]-1-[3-(trimethylammonio)propyl]-, diiodide
TTX	tetrodotoxin
TUJ-1	neuronal class III β-tubulin

VMAT	vesicular monoamine transporter
VTA	ventral tegmental area
v/v	volume per unit volume
Wnt	Wingless-type Mouse Mammary Tumor Virus integration site family member
w/v	weight per unit volume
ZO	Zonula Occludens

CHAPTER 1

Introduction

1.1 Embryonic stem cells

The stem cell field has advanced rapidly since the derivation of embryonic stem (ES) cells more than 20 years ago. Prior to the discovery of ES cells, pluripotent cells were first identified in the 1950s, when Stevens and Little (1954) observed spontaneous testicular teratomas in 1% of mice. Following that, Stevens discovered a high occurrence of teratomas in testes grafted with genital ridges (Stevens, 1964). Also in 1964, Kleinsmith and Pierce isolated a single cell from a tumour which produced all somatic cell types of a differentiated carcinoma (Kleinsmith and Pierce, 1964). In 1970, two studies showed that teratocarcinomas were produced after mouse embryos were grafted into extra-uterine sites of adult mice (Solter *et al.*, 1970; Stevens, 1970). Embryonal carcinoma (EC) cells, found in teratocarcinomas, have the capability to self-renew, which means these cells can be isolated and propagated in culture using feeder cells, leading to the generation of multiple pluripotent teratocarcinoma cell lines *in vitro* (Martin and Evans, 1974). These EC cell lines retain pluripotency after expansion and give rise to cells of the three primary germ layers: ectoderm, mesoderm and endoderm (Kleinsmith and Pierce, 1964; Martin and Evans, 1975). A single EC cell transplanted into a host resulted in the formation of a new tumour (Kleinsmith and Pierce, 1964). However, there was distinctive variability between EC cell lines. Many cell lines were reported to differentiate poorly *in vitro* and *in vivo*, hardly produced germline chimaeras and developed embryonic tumours in host animals (Smith, 2001; Solter, 2006). EC cells typically have an abnormal karyotype; they are aneuploid, therefore unable to proceed to meiosis to produce mature reproductive cells (Smith, 2001). Despite all the drawbacks, studies done on EC cells provided a basis for understanding, isolating and maintaining ES cells.

ES cells were first derived from the inner cell mass (ICM) of a mouse blastocyst in 1981 (Evans and Kaufman, 1981; Martin, 1981). The two main characteristics of ES cells are similar to EC cells; they are pluripotent, meaning they are capable of generating derivatives of all primary germ layers (Figure 1), and they have the ability to self-renew without losing their differentiation potential. The core transcription factors Oct4, Nanog and Sox2 form a transcriptional network regulating ES cell pluripotency (Boiani and Scholer, 2005; Boyer *et al.*, 2005; Loh *et al.*, 2006). However unlike EC cells, ES cells are able to differentiate extensively *in vitro* and *in vivo* and are also capable of generating germline chimaeras (Solter, 2006). A single ES cell is capable of giving rise to clonal populations under appropriate culture conditions (Smith, 2001). Several pathways that regulate ES cell self-renewal have been identified, although our understanding of the mechanisms involved is still limited (Niwa, 2007). The advantage of using ES cells over EC cells is that they can be genetically modified, which allows for the creation of cell lines that bear specific reporters, and also allows for the knockout, mis- and overexpression of certain genes of interest (Keller, 2005). Because ES cells have the ability to differentiate into all cell lineages, they are important as an indefinite source for regenerative medicine and pharmacological studies as well as drug screening programs (Pouton and Haynes, 2007).

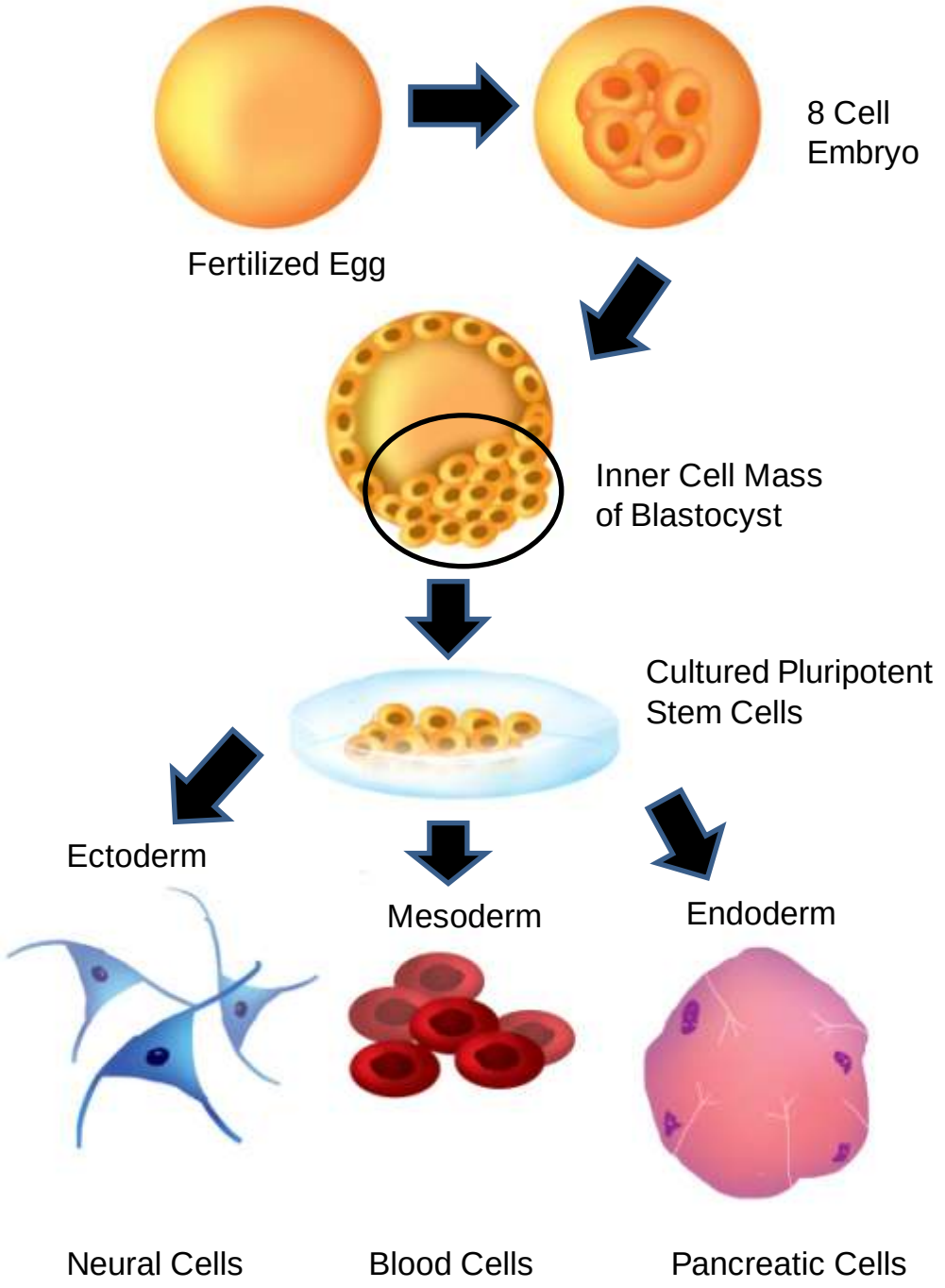

Figure 1: Isolation and differentiation of embryonic stem cells into a variety of cell types.

1.1.1 Mouse embryonic stem cells

ES cells maintain pluripotency by preventing differentiation and promoting propagation (Niwa, 2007). Mouse embryonic stem (mES) cells were first isolated and cultured on feeder layers of mitotically inactivated mouse embryonic fibroblasts (MEFs) (Evans and Kaufman, 1981; Martin, 1981). mES cells divide symmetrically every 12 hours in culture (Niwa, 2007). The essential component secreted by MEFs that supported ES cell pluripotency was later found to be the cytokine leukemia inhibitory factor (LIF) (Smith *et al.*, 1988; Williams *et al.*, 1988). Since then, mES cells have been cultured in the absence of MEFs, but in the presence of LIF and serum (Smith *et al.* 1988; Williams *et al.* 1988). After the withdrawal of LIF mES cells continue to proliferate, but also start to differentiate into the three germ layers (Niwa *et al.*, 1998).

The most clearly established pathway that maintains mES cells self-renewal is the LIF/STAT3 signalling pathway. LIF activates the STAT3 pathway which is sufficient to maintain mES cells self-renewal in the presence of serum (Niwa *et al.*, 1998; Matsuda *et al.*, 1999). Serum contains various growth factors, most of which are stimulatory to growth (Mannello and Tonti, 2007). In the absence of serum, LIF is unable to block differentiation and maintain pluripotency (Ying *et al.*, 2003a). The essential factor in serum was later identified as Bone Morphogenic Protein 4 (BMP4), which in combination with LIF, is able to maintain ES cells self-renewal and offers a chemically defined method for mES cells maintenance (Ying *et al.*, 2003a). Bone Morphogenic Protein 2 (BMP2) and growth and differentiation factor 6 (GDF6) are other alternatives for mES cell expansion in serum-free culture (Ying *et al.*, 2003a).

LIF was first identified as a molecule inducing macrophage differentiation in M1 myeloid leukemic cells and belongs to the cytokine interleukin-6 (IL-6) family, which is involved in survival, apoptosis, proliferation, differentiation and suppression of differentiation of cells (Williams *et al.*, 1988; Starr *et al.*, 1997; Kristensen *et al.*, 2005). LIF binds a low-affinity LIF receptor (LIFR) which works as a heterodimer with the signal transducer glycoprotein 130 (gp130) (Matsuda *et al.*, 1999). It has been shown that gp130 is essential in mediating signals for self-renewal of mES cells and LIFR alone is insufficient (Starr *et al.*, 1997; Niwa *et al.*, 1998). The heterodimer activates the JAK-STAT pathway and the MAP-ERK pathway, both implicated in ES cell self-renewal (Boulton *et al.*, 1994).

1.1.2 Human embryonic stem cells

Studies on primate ES cells eventually led to the derivation of human embryonic stem (hES) cells (Thomson *et al.*, 1998). hES cells were first maintained on feeder cells in the presence of serum (Thomson *et al.*, 1998). hES cells grow at a much slower rate than do mouse cells, they have been reported to divide symmetrically every 36 hours in contrast with 12 hours for mouse ES cells (Odorico *et al.*, 2001; Amit and Itskovitz-Eldor, 2002). Unlike mES cells, the fibroblast growth factor (FGF) and the transforming growth factor β (TGFβ)/Activin/Nodal signalling pathways have been identified as the key signalling pathways that maintain hES cell pluripotency (Beattie *et al.*, 2005; James *et al.*, 2005; Vallier *et al.*, 2005; Xu *et al.*, 2005a; Xu *et al.*, 2005b). Identification of these signalling pathways has permitted the development of feeder/animal product free systems for the maintenance of hES cells.

1.1.2.1 FGF2 and TGFβ/Activin/Nodal

FGFs are a family of 22 polypeptide growth factors involved in a variety of processes within the body. The first identified FGF, Fibroblast Growth Factor 2 (FGF2) or basic Fibroblast growth factor (bFGF), is the key mitogen that maintains self-renewal and pluripotency of hES cells (Amit *et al.*, 2000; Levenstein *et al.*, 2006; Diecke *et al.*, 2008). It is essential in cultures with or without the presence of serum and/or feeder cells (Liu *et al.*, 2006). FGF2 principally activates the MAP-ERK pathway to maintain self-renewal of hES cells, although there are suggestions of MAP-ERK independent FGF2 signalling (Dvorak *et al.*, 2005; Li *et al.*, 2007; Diecke *et al.*, 2008). The FGF signalling pathway works in association with the Wnt, TGFβ, Hedgehog and Notch pathways but antagonises the BMP signalling pathway, which promotes differentiation of hES cells into trophoblast (Xu *et al.*, 2002; Delaune *et al.*, 2005; Greber *et al.*, 2007; Dhara and Stice, 2008). Indirectly, FGF2 modulates TGFβ1 signalling and co-operates with activin A to support self-renewal (Beattie *et al.*, 2005; Greber *et al.*, 2007).

TGFβs are proteins that have many functions including controlling cell proliferation, differentiation, regulating cell cycle, apoptosis, migration, extracellular matrix deposition and responding to injuries or diseases. The TGFβ superfamily consists of more than 40 members, including activin, nodal and BMPs (Valdimarsdottir and Mummery, 2005). TGFβ1, via TGFβ1 receptors (Alk5, TβRII) and activin (and Nodal) share via the Alk4, ActRIIB receptors (Vallier *et al.*, 2005) activate the Smad 2/3 pathway (James *et al.*, 2005). Activin A with FGF2 was found to maintain hES cells undifferentiated without feeder cells, conditioned medium (CM) or STAT3 activation (Beattie *et al.*, 2005). Although

Activin/Nodal signalling is essential for maintenance of hES cells pluripotency and self-renewal, Activin or Nodal on their own are insufficient to maintain hES cells pluripotency (Vallier *et al.*, 2005). The Activin/Nodal pathway co-operates with the FGF pathway to maintain long term hES cell pluripotency without feeder cells, conditioned medium or serum replacement (Vallier *et al.*, 2005).

1.1.3 induced Pluripotent stem cells and transdifferentiation

ES cell research has been controversial ever since the derivation of hES cells. Many ethical issues have been raised as the generation of pluripotent human ES cell lines involves the destruction of human embryos (Robertson, 2010). However, a breakthrough discovery was made in 2006 by Takahashi and Yamanaka where mouse somatic cells were successfully reprogrammed back into a pluripotent state using retroviral transduction. These cells are known as induced Pluripotent Stem (iPS) cells. From twenty-four genes associated with pluripotency, four (Oct4, Sox2, c-Myc and Klf4) were identified as factors necessary for the generation of iPS cells. These cells displayed ES cell like morphology and characteristics i.e. self-renewal, differentiation into all germ layers, upregulation of pluripotency genes and teratoma formation (Takahashi and Yamanaka, 2006). Although the first generation iPS cells had DNA methylation errors and were unable to produce adult chimaeras (Takahashi and Yamanaka, 2006), they generated widespread interest within the stem cell community and research on iPS cells has been rapidly progressing since. A year later, mouse iPS cells with germline transmission were generated (Okita *et al.*, 2007; Wernig *et al.*, 2007). In the same year, another landmark discovery was made; iPS cells were reprogrammed from human somatic cells by two separate groups.

Yamanaka's group used a retroviral method with the same four genes (Oct4, Sox2, c-Myc and Klf4) which they previously identified were crucial for reprogramming mouse somatic cells (Takahashi *et al.*, 2007); Thomson's group identified four genes (Oct4, Sox2, Nanog and Lin28) which were sufficient to reprogram human somatic cells using a lentiviral system (Yu *et al.*, 2007). Several groups then generated iPS cells from patients with neurodegenerative diseases, including; Parkinson's disease (PD) (Soldner *et al.*, 2009), Huntington's disease (HD) (Park *et al.*, 2008), amyotrophic lateral sclerosis (ALS) (Dimos *et al.*, 2008) and spinal muscular atrophy (SMA) (Ebert *et al.*, 2009). Although Yamanaka established a standard approach for reprogramming iPS cells, there remain disadvantages with this method i.e. (i) c-Myc and Klf4 are oncogenic factors which may cause tumours, (ii) possible insertional mutagenesis from viral integration, (iii) low reprogramming efficiency (0.01% - 1.0%), and (iv) incomplete nuclear reprogramming (Takahashi and Yamanaka, 2006). To overcome those problems, new methods have been developed to produce iPS cells. These new methodologies include replacing retroviruses with plasmids (Okita *et al.*, 2008), omitting the oncogenic factors (Huangfu *et al.*, 2008b; Wernig *et al.*, 2008a; Kim *et al.*, 2009b), using small molecule compounds (Huangfu *et al.*, 2008a; Shi *et al.*, 2008), recombinant proteins (Kim *et al.*, 2009a; Zhou *et al.*, 2009) and microRNAs (Judson *et al.*, 2009; Anokye-Danso *et al.*, 2011; Miyoshi *et al.*, 2011).

The recent success with iPS cells has renewed interest in transdifferentiation, which was attempted over two decades ago but not capitalised on. Transdifferentiation refers to the regression of differentiated cells from one lineage to give rise to other cell types without going through a pluripotent state (Thowfeequ *et al.*, 2007; Zhou and Melton, 2008). A

study done by Davis and group (1987) showed overexpression of a single factor (myoD) converts fibroblasts into myoblasts. However, the same gene overexpressed in hepatocytes did not convert the liver cells into muscle cells (Schafer *et al.*, 1990). Subsequent studies have shown conversion of one cell type to another of related cell lineages, such as pancreatic into liver cells (Shen *et al.*, 2000), or from B cells into macrophages (Xie *et al.*, 2004). In 2008, Zhou and colleagues made a key discovery where they transdifferentiated pancreatic exocrine cells to insulin secreting β-cells *in vivo*. From a screen of more than 1100 genes, nine transcription factor candidates exhibiting β-cell developmental phenotypes when mutated were selected. Adenoviral infection with the combination of three factors (Ngn3, Pdx1 and Mafa) converted up to 20% of exocrine cells into β-cells with the first insulin positive cells appearing by day 3 (Zhou *et al.*, 2008b). In 2010, Wernig and group adopted a similar strategy to convert mouse fibroblasts into functional neurons. Among the 19 genes screened, three genes (Ascl1, Brn2 and Myt1l) were sufficient to reprogram almost 20 percent of fibroblasts into neurons with the capability of forming synapses (Vierbuchen *et al.*, 2010). Recently, Wernig and group reprogrammed human fibroblasts into neuronal cells using the same techniques (Pang *et al.*, 2011) while Pfisterer and colleagues successfully converted human fibroblasts into dopaminergic neurons with additional expression of Lmx1a and Foxa2, two genes involved in dopaminergic neuron development, with Ascl1, Brn2 and Myt1l (Pfisterer *et al.*, 2011). These findings represent a huge discovery within the transdifferentiation field, as the study proved that transdifferentiation can be accomplished between germ layers without going through an embryonic state. The recent advances with transdifferentiation may shift focus towards transdifferentiated cells as the risk of tumour formation is much

lower and the procedure of generating transdifferentiated cells is faster and easier compared to iPS cell reprogramming. However more studies are needed to determine if large scale production is possible and whether transdifferentiation is applicable for all cell types (Nicholas and Kriegstein, 2010).

1.2 Neural development *in vivo*

Neural induction is the earliest phase of neural development and was first discovered in 1924 when Hilde Mangold and Hans Spemann grafted the dorsal lip of a gastrulating salamander embryo to the ventral side of a host salamander embryo, resulting in the formation of a second embryonic axis which consisted of almost the whole CNS derived from the host ectoderm rather than the graft (Spemann and Mangold, 1924). For many decades it was believed that an 'organiser' provided instructive signals to promote cells towards a neural identity. However, it is now understood that the default embryonic ectoderm pathway is neural. During neural induction, BMP4 inhibits neuralisation and promotes an epidermal fate (Sasai *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995); Noggin, follistatin and chordin, which antagonise BMP signalling, induce neural progenitor cells within the ectoderm to form the neuroepithelium (Lamb *et al.*, 1993; Hemmati-Brivanlou *et al.*, 1994; Sasai *et al.*, 1994; Sasai *et al.*, 1995; Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996; Fainsod *et al.*, 1997). Neurulation occurs after neural induction where the neuroepithelium, also termed the neuroectoderm, thickens to form the neural plate. The neural plate then folds up and forms the neural tube. The neural tube further differentiates, forming the brain at the anterior end and the spinal cord at the posterior end (Altmann and Brivanlou, 2001; Wurst and Bally-Cuif, 2001; Wallingford, 2005; Stiles

and Jernigan, 2010). Before the neural tube closes, the anterior end of the tube expands and is subdivided into three vesicles that will form the adult brain: prosencephalon, mesencephalon and rhomboncephalon. Prosencephalon, which is the most anterior of the vesicles, becomes the forebrain; the middle vesicle, mesencephalon, becomes the midbrain; the most posterior vesicle, rhomboncephalon, becomes the hindbrain. These vesicles further develop to form five secondary vesicles: The prosencephalon divides into the telencephalon, which becomes the paleocortex, corpus striatum, and neocortex, and the diencephalon which becomes the epithalamus, thalamus, hypothalamus, and infundibulum. The rhomboncephalon gives rise to the metencephalon, which becomes the cerebellum and pons, and myelencephalon which becomes the medulla oblongata. The mesencephalon does not divide into secondary vesicles but forms the tectum, tegmentum, and cerebral peduncles. The five brain vesicles align at the anterior-posterior axis of the embryo and together with the spinal cord form the CNS (Altmann and Brivanlou, 2001; Stiles and Jernigan, 2010). The development of the nervous system is illustrated in Figure 2.

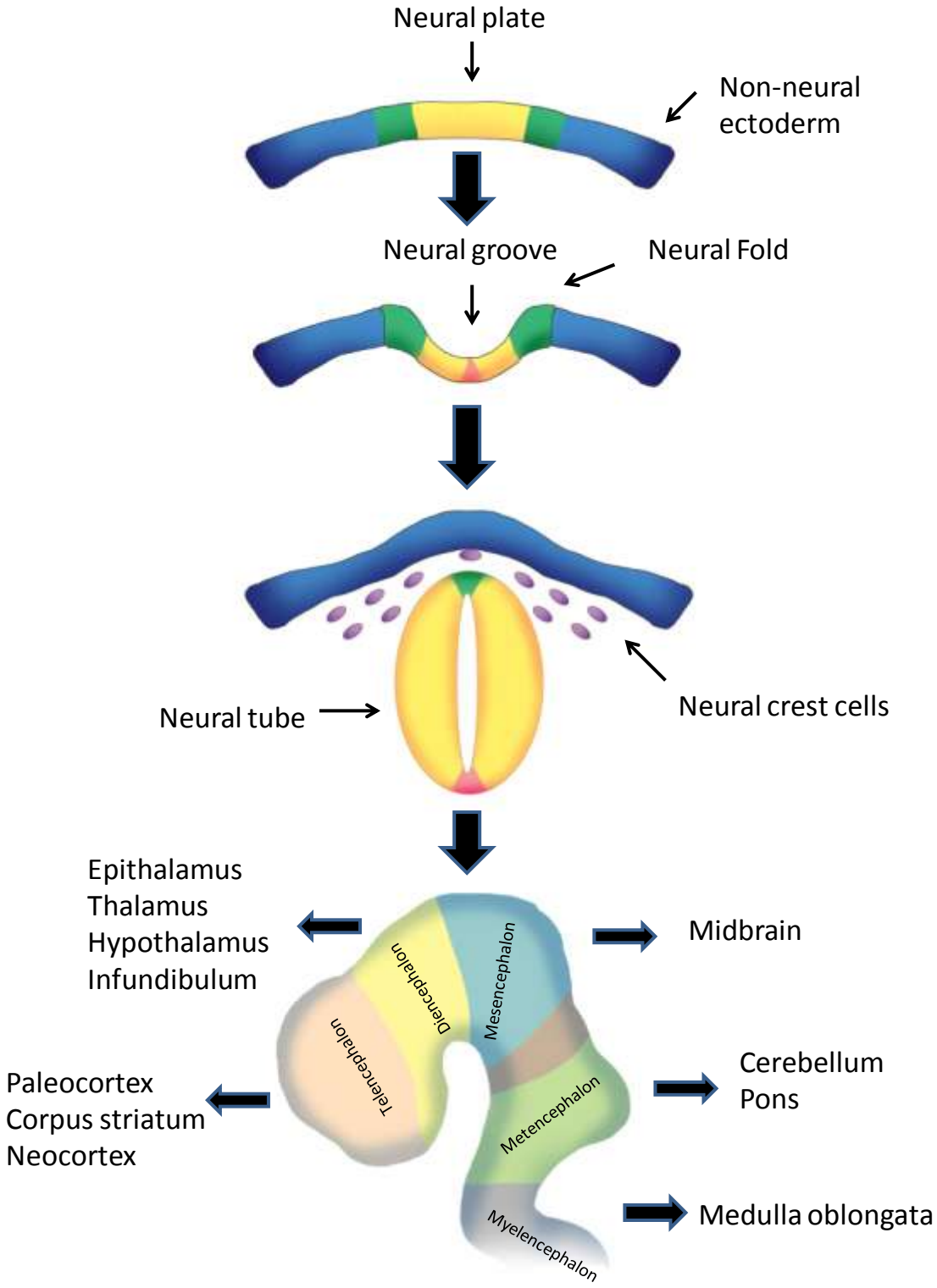

Figure 2: Development of the nervous system.

1.2.1 Midbrain dopaminergic neurons

Dopaminergic neurons are mainly found in the mesencephalon (midbrain) but also in other CNS regions; the forebrain, olfactory bulb, and retina. Midbrain dopaminergic neurons are located in three distinct nuclei: retrorubal field (RRF, A8), the substantia nigra pars compacta (SNpc, A9) and ventral tegmental area (VTA, A10). Dopaminergic neurons of the SNpc project to the dorsolateral striatum (caudate putamen) and form the nigro-striatal pathway involved in control of motor functions. Axons of VTA dopaminergic neurons project to the ventromedial striatum, cortical areas and the limbic system forming the mesocorticolimbic pathway involved in emotional behaviour and mechanisms of natural motivation and reward. The neurons of the RRF on the other hand project to the SNpc, VTA and dorsal striatum (Simon *et al.*, 2003; Wallén and Perlmann, 2003; Ang, 2006; Prakash and Wurst, 2006a; Smidt and Burbach, 2007; Alavian *et al.*, 2008; Gale and Li, 2008). Dopaminergic neurons are generally identified by expression of tyrosine hydroxylase (TH), the rate limiting enzyme responsible for converting tyrosine to L-dihydroxyphenylalanine (L-DOPA) in catecholamine biosynthesis (Nagatsu *et al.*, 1964; Kawano *et al.*, 1995; Dunkley *et al.*, 2004).

The development of midbrain dopaminergic neurons from neural progenitors can be divided into three distinct steps: (1) an early stage induction of neural progenitors that can potentially generate midbrain dopaminergic progenitors, (2) an intermediate stage specification of midbrain dopaminergic neuronal fate of progenitors and (3) late stage maturation and terminal differentiation of dopaminergic progenitors (Prakash and Wurst, 2006b). The two key morphogens, essential and sufficient for the induction of ventral

midbrain dopaminergic neurons during neurogenesis are Shh, from the floor plate, and FGF8, from the mid-hindbrain organiser (isthmus) (Hynes *et al.*, 1995a; Hynes *et al.*, 1995b; Ye *et al.*, 1998). Wnt1 and TGFβs have also been identified as crucial factors for the generation midbrain dopaminergic neurons (Prakash *et al.*, 2006). The stages of dopaminergic neuron development can be characterised by the expression of molecular markers. An early marker identified in midbrain dopaminergic progenitors aldehyde dehydrogenase 1 (Aldh1 also known as Aldh2), capable of oxidising retinaldehyde into retinoic acid (Lindahl and Evces, 1984). It is expressed in the ventral midbrain by proliferating progenitors at E9.5 and also in post mitotic neurons (Wallén *et al.*, 1999; Wallén *et al.*, 2001). More transcription factors have been identified in the past few years: Lmx1a/b, Msx1, Ngn2, Otx2, Foxa2, Engrailed 1/2 (En1/2) (Simon *et al.*, 2001; Puelles *et al.*, 2003; Puelles *et al.*, 2004; Vernay *et al.*, 2005; Andersson *et al.*, 2006a; Andersson *et al.*, 2006b; Kele *et al.*, 2006; Thompson *et al.*, 2006; Ferri *et al.*, 2007; Kittappa *et al.*, 2007; Bonilla *et al.*, 2008). As dopaminergic progenitors differentiate and become post mitotic, Nurr1 and Pitx3 are expressed in immature post-mitotic and terminally differentiated midbrain dopaminergic neurons (Saucedo-Cardenas *et al.*, 1998; Wallén *et al.*, 1999; Wallén *et al.*, 2001; Hwang *et al.*, 2003; Nunes *et al.*, 2003; Smidt *et al.*, 2004; Maxwell *et al.*, 2005). A simplified schematic representation of the genetic network regulating the development of midbrain dopaminergic neurons can be seen in Figure 3.

The homeodomain transcription factor orthodenticle homologue 2 (Otx2) is expressed in the epiblast and the anterio-visceral endoderm before its expression is progressively

restricted to the anterior neuroectoderm that defines the fore- and midbrain (Broccoli *et al.*, 1999; Simeone *et al.*, 2002). Initial studies reported that Otx2 plays a critical role in specification and differentiation of midbrain dopaminergic neuron, including activation of Ngn2 (Puelles *et al.*, 2003; Puelles *et al.*, 2004; Vernay *et al.*, 2005). However, a study by Prakash *et al.* (2006) suggested that the role of Otx2 is to repress Nkx2.2, a Shh responsive gene encoding a type II transcriptional regulator necessary for specification of ventral cell types in both the hindbrain and spinal cord. The LIM homeodomain transcription factors Lmx1a and Lmx1b are expressed in the ventral midbrain, playing different roles in the development of midbrain dopaminergic neurons (Smidt *et al.*, 1997; Andersson *et al.*, 2006b). Lmx1b was first reported to be required for maintenance of TH+ midbrain dopaminergic neurons and regulation of the Pitx3 gene (Smidt *et al.*, 2000). Lmx1b initiates expression of FGF8 and maintains expression of Wnt1 as well as En1/2 in the isthmic organiser (Smidt *et al.*, 1997; Adams *et al.*, 2000; Matsunaga *et al.*, 2002; Guo *et al.*, 2007; Chung *et al.*, 2009). Wnt1 is involved in the activation and maintenance of Otx2 expression and is suggested to regulate Pitx3 expression through a yet to be identified mechanism (Prakash *et al.*, 2006; Chung *et al.*, 2009). Unlike its orthologue, Lmx1a is a midbrain dopaminergic fate specifier, initially thought to be induced by Shh (Andersson *et al.*, 2006b). However, a more recent study revealed that Wnt1 is responsible for inducing Lmx1a expression via the β-catenin pathway forming an autoregulatory loop (Chung *et al.*, 2009). Lmx1a activates msh homeobox homolog 1 (Msx1) which acts synergistically with Lmx1a to activate expression of Ngn2, a proneural gene, while repressing Nkx6.1, a homeodomain protein broadly expressed in ventral progenitor cells (Andersson *et al.*, 2006b). Ngn2 is part of a family of basic-helix-loop-helix

(bHLH) transcriptional regulators involved in neurogenesis but not specification of midbrain dopaminergic fates. Expression of Ngn2 is required for the generation of Nurr1+ post-mitotic immature neurons which further differentiate into TH+ mature neurons (Andersson *et al.*, 2006a; Kele *et al.*, 2006). The Engrailed homeobox genes (En1/2) are crucial in early development for the generation and later for the survival of midbrain dopaminergic neurons (Simon *et al.*, 2001). The Engrailed genes, also regulated by Wnt1, regulate α-synuclein, a gene genetically linked to PD (Danielian and McMahon, 1996; Simon *et al.*, 2001). In recent years, hepatocyte nuclear factor-3 β (HNF-3 β) (or forkhead box a2, Foxa2), a member of the *fork head/HNF-3* family of DNA binding transcription factors expressed in the ventral midline of the neural tube, has emerged as another marker as it is now known that midbrain dopaminergic neurons originate from the midbrain floor plate (Joksimovic *et al.*, 2009a). Shh from the notochord induce Foxa2 expression through Gli binding sites which in turn, directly induces Shh expression in floor plate cells (Sasaki *et al.*, 1997; Jeong and Epstein, 2003). Foxa2 regulates midbrain dopaminergic development by repressing Nkx2.2, regulating neurogenesis through activation of Ngn2 and subsequently inducing mid to late dopaminergic genes (Ferri *et al.*, 2007). Furthermore, Foxa2 has been implicated in the survival and maintenance of dopaminergic neurons (Kittappa *et al.*, 2007; Arenas, 2008). One of the earliest markers identified for proper midbrain dopaminergic neuron development is the orphan nuclear receptor Nr4a2 (Nurr1). Nurr1 is important for the differentiation and maintenance of midbrain dopaminergic neurons (Zetterström *et al.*, 1997; Saucedo-Cardenas *et al.*, 1998). It is also required for the expression of several proteins involved in dopamine synthesis and regulation such as TH, vesicular monoamine transporter 2 (VMAT2), dopamine

transporter (DAT) and RET receptor tyrosine kinase (cRET) (Zetterström *et al.*, 1997; Saucedo-Cardenas *et al.*, 1998; Wallén *et al.*, 1999; Wallén *et al.*, 2001; Smits *et al.*, 2003). The paired homeodomain transcription factor, Pituitary homeobox 3 (Pitx3) is uniquely expressed by all midbrain dopaminergic neurons in the central nervous system (Smidt *et al.*, 1997). It is required for proper differentiation and regulates TH expression of a subset of neurons (Maxwell *et al.*, 2005). Pitx3 is essential for survival of dopaminergic neurons in the adult brain, especially in the SNpc and also in the VTA (Hwang *et al.*, 2003; Munckhof *et al.*, 2003; Nunes *et al.*, 2003; Smidt *et al.*, 2004).

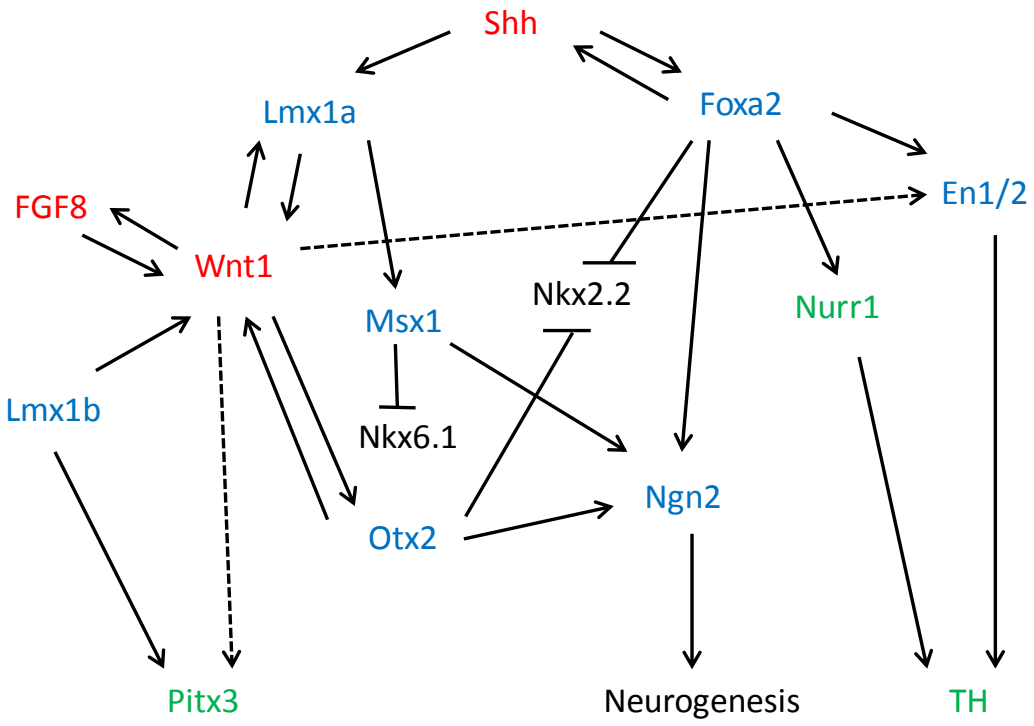

Figure 3: A simplified schematic diagram representing the genetic network regulating the development of midbrain dopaminergic neurons. Red indicates patterning factors. Blue indicates genes expressed by dopaminergic progenitors. Green indicates markers for post-mitotic dopaminergic neurons. Broken arrows indicate interactions that have not been elucidated.

1.2.2 Neural patterning

During neural induction to the development of the brain and spinal cord, multiple signalling factors are involved in dictating regionalisation. Independent patterning of two axes, anterior-posterior and dorsal-ventral, establish the structure of the CNS (Simon *et al.*, 1995). As previously mentioned, the neural tube is subdivided along the anterior-posterior axis into the forebrain, midbrain, hindbrain and spinal cord (Altmann and Brivanlou, 2001; Stiles and Jernigan, 2010). In the developing neural tube, the roof plate is located at the dorsal end and the floor plate at the ventral pole with neuroblasts and motor neurons between the two plates. Signals from the notochord and the mesoderm lateral to the neural tube are major influences in shaping the dorsoventral structure of the neural tube (Wilson and Maden, 2005). Here, several key signalling factors involved in patterning the neural tube are described below.

1.2.3 BMP signalling

The establishment of neural induction as the default differentiation was based on studies reporting BMP4 inhibiting neural differentiation while promoting epidermal differentiation (Hawley *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995). An early study on the TGFβ pathway using misexpression of a dominant-negative activin receptor reported inhibition of mesodermal induction and surprisingly generated neural tissue which suggested secretion of an neural suppressing factor which was a member of the TGFβ superfamily (Hemmati-Brivanlou and Melton, 1992). However in an experiment to test the epidermalisation of neural cap cells using a purified recombinant activin protein, cells adopted a mesodermal instead of an epidermal fate (Wilson and Hemmati-

Brivanlou, 1995). Subsequently, BMP4 emerged as a candidate involved in repressing neural differentiation. In the early ectoderm there is widespread expression of BMP4 but is extinguished during neural induction (Fainsod *et al.*, 1994; Hemmati-Brivanlou and Thomsen, 1995; Schmidt *et al.*, 1995). Further assays supplied evidence that BMP4 inhibited neural differentiation and promoted animal cap cells into a mesodermal lineage (Wilson and Hemmati-Brivanlou, 1995). These findings led to the proposal that the inhibition of BMP activity by an organiser-induced signal promotes a neural fate (Tanabe and Jessell, 1996). Three secreted factors expressed in the organiser were identified to induce neural activity – noggin, follistatin and chordin. Noggin was first screened from a functional library screen (Smith and Harland, 1992) and promoted neural induction without first inducing mesodermal differentiation (Lamb *et al.*, 1993). The second proposed factor, follistatin, was initially reported as an activin antagonist (Nakamura *et al.*, 1990) with the capability of promoting neural activity (Hemmati-Brivanlou *et al.*, 1994). Chordin was identified by its expression in the organiser region (Sasai *et al.*, 1994) and direct neural inducing activity (Sasai *et al.*, 1995). All three inducing factors have been reported as binding partners of BMP that antagonise the BMP signalling pathway (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996; Fainsod *et al.*, 1997).

BMPs also regulate dorsal-ventral patterning of the neural tube. The epidermal ectoderm releases BMP signals that induce a dorsal cell fate for neural plate cells; neural crest cells and roof plate formation are mediated by BMP signalling. The roof plate secretes BMP proteins to induce neural markers such as Pax3 and Msx and gives rise to dorsal interneurons. Moreover, the epidermal ectoderm and roof plate are unable to generate

dorsal interneurons when BMP signalling is inhibited (Liem *et al.*, 1995; Liem *et al.*, 1997; Lee and Jessell, 1999). In addition, ablation of the roof plate in mice revealed that dorsal interneuron specification is heavily dependent on BMP signals from the roof plate (Lee *et al.*, 2000a). In zebrafish mutants with compromised BMP signalling, different neuronal phenotypes were detected along the dorsal-ventral axis depending on the severity of the mutation providing evidence that there is concentration-dependent gradient of BMP action along the neural tube (Barth *et al.*, 1999). An assay using a constitutively active BMP receptor in chicks, to manipulate BMP expression levels, resulted in an upregulation of Cath1, the most dorsal bHLH protein, and repression of Cash1 and neurogenins (Ngns) in dorsal neural cells and specification of dI1 and dI3 interneuron subtypes in the neural tube (Timmer *et al.*, 2002). In BMPR1a and BMPR1b double knockout mice, Math1 expressing dI1 interneurons are lost and there is a significant reduction in DI2 neurons (Wine-Lee *et al.*, 2004). Together these findings demonstrate that a gradient of BMP signalling influences the neuronal phenotype along the neural tube dorsal-ventral axis (Figure 4).

Besides the roof plate, several BMP genes (BMP2, BMP4, BMP5, BMP6, BMP7) are expressed in the early developing brain (Furuta *et al.*, 1997). BMP2 and BMP4 induce the expression of Msx1 and inhibit BF-1 in the dorsal forebrain (Furuta *et al.*, 1997). Msx1 is a target of BMPs in the spinal cord and hindbrain in chick embryos (Graham *et al.*, 1994; Liem *et al.*, 1995) while BF-1 is expressed in the developing telencephalon and eye (Tao and Lai, 1992). Chordin and noggin double null ($Chrd^{-/-}$; $Nog^{-/-}$) mutants resulted in defects in each three primary embryonic axis, whereas $Chrd^{-/-}$; $Nog^{+/-}$ displayed multiple head

defects from loss of rostral structures to anterior neural and craniofacial defects. The absence of Sonic hedgehog (Shh) in the rostral mesendoderm and rostral ventral midline, as well as reduced FGF8 signalling in the anterior neural ridge, contribute to the defects observed in the mutant embryos (Anderson *et al.*, 2002). The effects of BMP antagonists during brain development provide evidence of the importance of confining BMP signalling to the dorsal region of the forebrain.

1.2.4 Wnt signalling

Wnt signalling is involved in a vast array of cellular processes including development of the CNS (Moon *et al.*, 2004; Ciani and Salinas, 2005). To date, three different Wnt-signalling pathways, involving different proteins and downstream targets, have been identified. The canonical Wnt-signalling pathway is activated when a Wnt molecule binds to Frizzled (Fz) receptors, leading to the formation of a complex with LRP5/6, Dishevelled (DVL) and Adenomatous polyposis coli (APC). This complex blocks the activity of glycogen synthase kinase 3β (GSK3β), allowing β-catenin to enter the nucleus and interact with lymphoid enhancer-binding factor 1 (LEF1) to activate Wnt target genes. The other two non-canonical Wnt signalling pathways are the Wnt-Jun N-terminal Kinase (JNK) pathway and the Wnt-Ca^{2+} pathways; both are independent of β-catenin (Kohn and Moon, 2005; Inestrosa and Arenas, 2010).

An early study on the effect of Wnt on neural induction by Baker *et al.* (1999) revealed that Wnt signalling promotes neural induction. In Xenopus neuroectodermal explants, ectopic activation of Wnt signalling inhibits BMP4 signalling leading to neural

differentiation in the animal cap tissue (Baker *et al.*, 1999). This observation was supported by a later study which showed that activation of β-catenin induces noggin and chordin expression in animal caps, promoting neural induction (Wessely *et al.*, 2001). However, a study done by Heeg-Truesdell and LaBonne (2006) reported that inhibition of Wnt-β-catenin signalling is required for neural induction. According to them, neural fate is inhibited by Wnt/β-catenin in a cell autonomous fashion which is consistent with an earlier study reporting neural induction in animal caps by stabilised β-catenin was always non-cell autonomous (Domingos *et al.*, 2001).

Wnt signalling is involved in patterning of both anterior-posterior and dorsal-ventral neuraxes of the neural tube. The earliest evidence of a role for Wnt in anterior-posterior patterning was observed in animal cap explants where Xwnt-3a increased expression of posterior genes while suppressing anterior markers (McGrew *et al.*, 1995). Other studies conducted using the ectopic expression of Wnts (McGrew *et al.*, 1997) or modulations of the Wnt signalling pathway (Darken and Wilson, 2001; Hamilton *et al.*, 2001; Kiecker and Niehrs, 2001) provided further evidence that Wnts act as a posteriorising signal. On the contrary, inhibition of Wnt signalling through loss of Wnt8 function (Glinka *et al.*, 1997; Erter *et al.*, 2001; Lekven *et al.*, 2001) and Wnt antagonists (McGrew *et al.*, 1997; Glinka, 1998; Hsieh *et al.*, 1999) both led to an expansion of forebrain markers and a loss of posterior markers. Thus an endogenous Wnt/β-catenin signalling gradient regulates the specification of cells during patterning of anterior-posterior axis (Kiecker and Niehrs, 2001; Nordstrom *et al.*, 2002). Multiple Wnt antagonists such as Dickkopf1 (Dkk1) (Glinka *et al.*, 1998; Kazanskaya *et al.*, 2000; Mukhopadhyay *et al.*, 2001), secreted Frizzled-

related proteins (sFRPs) (Pera and De Robertis, 2000), Frizzled-related proteins (FRzBs) (Leyns *et al.*, 1997; Bradley *et al.*, 2000; Pera and De Robertis, 2000) and Cerberus (Bouwmeester *et al.*, 1996; Piccolo *et al.*, 1999), which are expressed in the anterior ectoderm and mesendoderm, play crucial roles in the head region. By suppressing Wnt signalling activity, they enable specification of the anterior neural tube. The posteriorising effect of Wnts, coupled with anterior specification of Wnt inhibitors contribute to the establishment of a Wnt signalling gradient along the neuraxis (Figure 4).

Among the 19 Wnt genes that have been identified, Wnt1 and Wnt3a are expressed in the roof plate (Parr *et al.*, 1993; Hollyday *et al.*, 1995; Cauthen *et al.*, 2001). Wnt3 and Wnt8b are also expressed dorsally (Parr *et al.*, 1993; Hollyday *et al.*, 1995; Cauthen *et al.*, 2001). Wnt5a, Wnt7b and Wnt7b expression are restricted at the ventral spinal cord (Parr *et al.*, 1993). Wnt4 is found at both the dorsal neural tube and the floor plate (McGrew *et al.*, 1992; Parr *et al.*, 1993; Hollyday *et al.*, 1995; Ungar *et al.*, 1995). The first evidence of Wnt signalling regionalisation along the dorsal-ventral axis was through a study by Muroyama and group (2002) using Wnt1/Wnt3a double mutant mice to disrupt the canonical Wnt pathway, leading to reduced dorsal-most interneurons and expansion of ventral cell types. These results indicated that Wnts regulate specification of dorsal interneurons. In ventral chick explants, an increasing gradient of Wnt3a addition led to an increase of cells expressing dorsal markers Pax6 and Ngn2 (Gunhaga *et al.*, 2003). In addition, ectopic activation of the Wnt/β-catenin pathway in chicks expanded expression of dorsal markers including Pax6 and Pax7, and inhibited ventral genes such as Nkx6.1/6.2, Nkx2.2, Olig2 and Foxa2 (Alvarez-Medina *et al.*, 2008). Other studies have

suggested that Wnts act as mitogens to regulate expansion of dorsal cells, including neural crest cells (Dickinson *et al.*, 1994; Ikeya *et al.*, 1997) and that the neural progenitor cell cycle is regulated by the canonical Wnt pathway (Megason and McMahon, 2002; Zechner *et al.*, 2003; Chesnutt *et al.*, 2004; Bonner *et al.*, 2008). BMPs have also been suggested to regulate Wnts in the neural tube (Marcelle *et al.*, 1997; Panchision *et al.*, 2001; Chesnutt *et al.*, 2004). However, the exact interaction between the BMP and Wnt signalling pathways to induce dorsal cell fates during neural development is not fully understood.

1.2.5 Shh signalling

Sonic Hedgehog (Shh) plays a crucial role during the development of the brain and spinal cord. The notochord, which is ventral to the neural tube, secretes Shh thereby inducing formation of the floor plate, and subsequent ventral cell types including motor neurons, ventral interneurons and spinal cord oligodendrocytes (Echelard *et al.*, 1993; Yamada *et al.*, 1993; Hynes *et al.*, 1995a; Marti *et al.*, 1995; Roelink *et al.*, 1995; Ericson *et al.*, 1996; Pringle *et al.*, 1996; Tanabe and Jessell, 1996; Orentas *et al.*, 1999; Jessell, 2000). Floor plate cells in turn express Shh to direct neural progenitors into a ventral fate (Yamada *et al.*, 1993; Hynes *et al.*, 1995b).

Shh which is first secreted from the notochord then the floor plate affects patterning of the neural tube, forming a concentration gradient along the dorsal-ventral axis. Ericson and group (1997) observed different classes of ventral cell types based on Shh concentration suggesting Shh acts as a morphogen in the neural tube. In chick neural

explants, increasing Shh concentrations specify more ventral fates in a graded manner (Briscoe *et al.*, 2000). In a study using Shh-GFP protein by Chamberlain *et al.* (2008), expression of Shh was visualised in the developing neural tube. The highest concentration of Shh-GFP ligands were detected at the most ventral progenitor cells with decreasing levels in the more dorsal domains of the neural tube. These ventral progenitor cells were also exposed to Shh signalling for a longer duration of time compared to dorsal cells (Chamberlain *et al.*, 2008). This was consistent with the progressive emergence of ventral progenitor cells which require increasing concentrations of durations of Shh exposure at the ventral midline (Jeong and McMahon, 2005). Thus, it is now established that sequential induction of ventral genes along the Shh concentration gradient is dependent on concentration and duration to Shh exposure, leading to patterning of the ventral neural tube (Figure 4).

Developments of midbrain dopaminergic and hindbrain serotonergic neurons are induced by Shh signalling from the floor plate (Yamada *et al.*, 1993; Hynes *et al.*, 1995b; Wang *et al.*, 1995; Ye *et al.*, 1998). An early study carried out by grafting an additional notochord or floor plate to ectopic positions or eliminating both cell groups resulted in distorted neuronal differentiation, with the identity and location of the distinctive cell types changed along the dorsal-ventral axis of the neural tube (Yamada *et al.*, 1991). The study suggested that the notochord and floor plate cells influence the development of various neuronal classes along the dorsal-ventral axis of the neural tube. Multiple studies have shown that different ventral neural cell types can be generated when neural explants are cultured with a Shh signalling source (Patten and Placzek, 2000). Yamada and group

(1991) demonstrated that grafted notochords placed next to neural tubes lead to an increase in motor neuron marker expression even in the absence of a floor plate. In addition, the group also proved that by placing grafted notochords next to the neural tube at the hindbrain level, an ectopic floor plate was induced which in turn generated serotonergic neurons (Yamada *et al.*, 1991). A further study by Yamada and group (1993) validated that a factor secreted by the notochord and floor plate specifies generation of motor neurons. In a study conducted by Hynes *et al.* (1995b), E9 embryos, devoid of the endogenous presumptive floor plate region, were cultured with E12 spinal cord floor plate explants resulted in a high number of midbrain dopaminergic neurons produced around the exogenous floor plate. Ericson *et al.* (1997) showed that a distinctive classes of ventral interneurons were induced depending on the concentration of Shh exposure to neural explants. Shh also influences the induction of spinal cord oligodendrocyte precursors (Orentas *et al.*, 1999). Inhibition of Shh signalling using blocking antibodies prevented induction of the floor plate and ventral neural cell types (Marti *et al.*, 1995; Ericson *et al.*, 1996; Orentas *et al.*, 1999).

The importance of Shh in patterning the neural tube is further supported by genetic manipulation studies. As previously mentioned, Foxa2 expression induced by Shh from the notochord, directly induces Shh expression in floor plate cells (Sasaki *et al.*, 1997; Jeong and Epstein, 2003). In embryos with ectopic expression of Foxa2, floor plate associated genes were expressed in the dorsal ectopic region of Foxa2 and endogenous Foxa2 expression was autoactivated (Sasaki and Hogan, 1994). Similar results were obtained in transgenic mice ectopically expressing the zinc-finger transcription factor

Gli1; ventral markers were induced and dorsal genes were suppressed in the mid- and hindbrain (Hynes *et al.*, 1997). In mutant mice with disrupted Shh signalling activity, the notochord degenerated and the floor plate failed to develop. Furthermore, the ventral neural tube of the mice expressed high levels of Pax3 and reduced levels of Pax6, similar to normal roof plate characteristics (Chiang *et al.*, 1996).

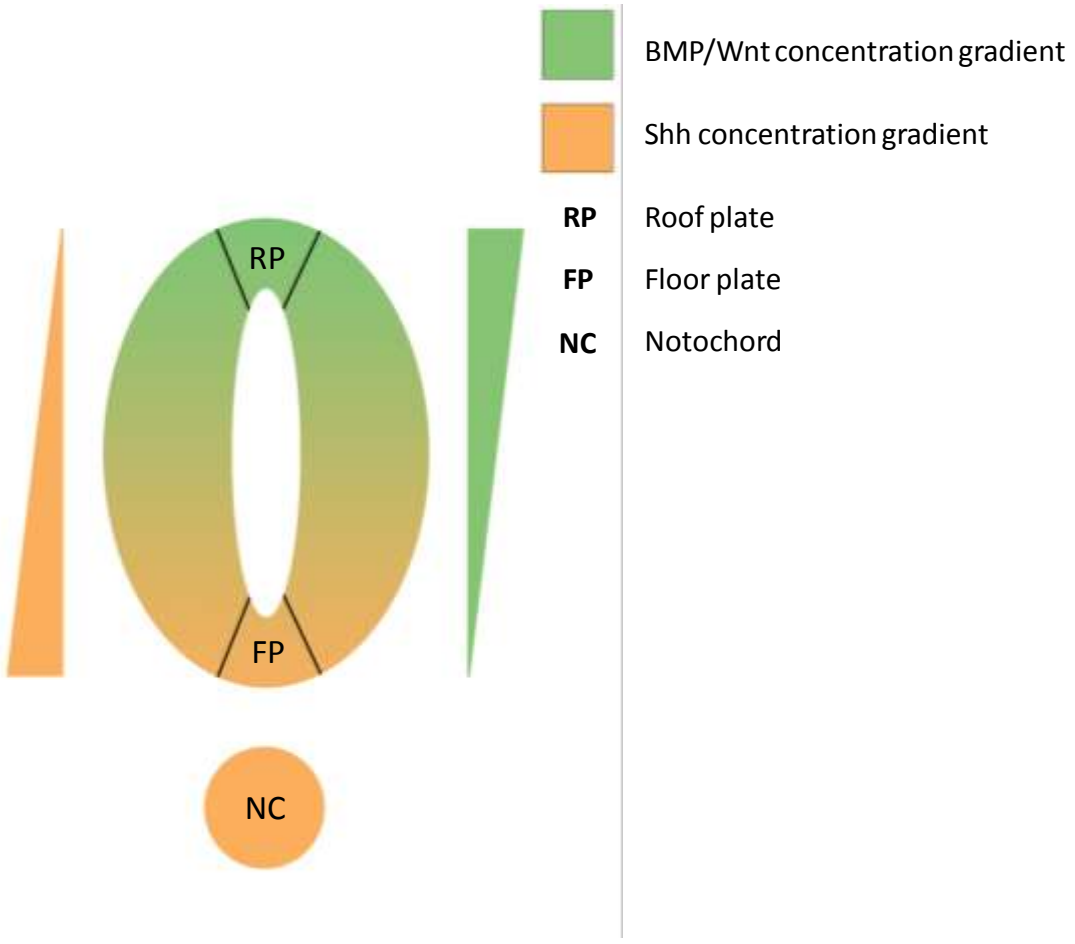

Figure 4: Concentration gradients of BMP, Wnt and Shh in the neural tube. The roof plate secretes BMPs and Wnts and the floor plate secretes Shh.

1.3 Neural development *in vitro*

Similar to neural development *in vivo*, the differentiation of ES cells into neurons *in vitro* involves intrinsic factors and extrinsic cytokines patterning of ES cells into specific neuronal phenotypes (Barberi *et al.*, 2003). As adult neurogenesis is limited, only occurring in the subventricular zone (SVZ), hippocampus, substantia nigra and the cortex (Baizabal *et al.*, 2003), deriving specific neuronal phenotypes from ES cells *in vitro* is important as potential therapeutic use for neurodegenerative disorders such as PD and Alzheimer's disease. To date, many protocols effective in generating neurons from ES cells have been established.

1.3.1 Current methods of *in vitro* differentiation

The default differentiation pathway of ES cells *in vitro* is towards a neural lineage (Tropepe *et al.*, 2001; Munoz-Sanjuan and Brivanlou, 2002). The long-established method of neuronal differentiation is by formation of embryoid bodies (EBs); where LIF is withdrawn from ES cells left in suspension, cells differentiate spontaneously and aggregate to form EBs, which contain cells from all three germ layers (Bishop *et al.*, 2002). Although cell-cell interactions within EBs are necessary for differentiation, the presence of endodermal and mesodermal cells in the aggregates complicates specification of neural cells and confounds the identification of the growth factors involved in neural lineage commitment (Keller, 2005). In addition, extracting neural cells from EBs is difficult due to their three-dimensional structures. Addition of retinoic acid (RA) in EB formation protocols has been reported to improve the yields of neural cells including neurons and glia (Fraichard *et al.*, 1995; Strübing *et al.*, 1995; Bain *et al.*, 1996). A variant of the EB

formation protocol known as the five-stage protocol was developed by Lee *et al.* (2000b); first, the expansion of ES cells (stage 1), then formation of EBs (stage 2) followed by selection of neuronal precursors from plated EBs (stage 3), expansion of selected neuronal precursors with FGF2 in chemically defined media (stage 4) and lastly, patterning the cells and allowing them to terminally differentiate (stage 5). Through this method Lee and group reported the generation of a high number of dopaminergic (>30%) and serotonergic (>11%) neurons.

An alternative approach used to derive neurons from ES cells is by co-culturing ES cells on a monolayer of bone marrow derived stromal cells. Kawasaki and group (2000) differentiated ES cells on PA6 stromal cells in serum replacement media, resulting in a highly neuronal cultures consisting predominantly dopaminergic neurons (30%), comparable to the five-stage protocol. The fact that neither Shh nor FGF8, both critical for midbrain dopaminergic neuron specification, were added to the cultures during the differentiation process implies that PA6 cells secrete intrinsic signals to promote ES cells. Vazin *et al.* (2009) identified a combination of four factors, stromal cell-derived factor 1 (SDF-1), pleiotrophin (PTN), insulin-like growth factor 2 (IGF2) and insulin-like growth factor binding protein 4 (IGFBP4), that were able to reproduce the effects of stromal cells. Selective derivation of other neural cell types is possible using this protocol by exposing cells to different combinations of cytokines. Thus, Barberi *et al.* (2003) used a MS5 stromal line and demonstrated that, depending on culture conditions, dopaminergic, GABAergic, serotonergic, cholinergic and motor neurons as well as NS cells, astrocytes and oligodendrocytes could be selectively generated. This protocol offers a rapid and

efficient, but ill-defined method of generating various central nervous system cell types. Another disadvantage with all stromal cell protocols is the difficulty in separating ES cell derived cells from the stromal cells (Keller, 2005).

In a work designed to eliminate the confounding influence of stromal cell co-culture, Ying *et al.* (2003b) developed a monolayer differentiation protocols using a chemically defined media, N2B27. In this method, ES cells can be grown in a monolayer on extra-cellular matrices such as gelatin or laminin, or tissue culture plastic. As a serum free media is used in this method, the effects of individual morphogens on self-renewal or differentiation of ES cells can be examined. The development of a single cell or colony can also be tracked throughout the whole differentiation process. Furthermore, cells are uniformly exposed to exogenous factors that are added to the cultures (Shin *et al.*, 2006). As this monolayer protocol utilises a serum free, chemically defined media, it offers the most attractive option for ES cell neural differentiation and is the preferred method of choice for the majority of this work.

During a typical ES cell monolayer differentiation, cells are seeded at low density and commence differentiation upon removal of LIF. A neural fate is induced in the absence of any extrinsic factors (Munoz-Sanjuan and Brivanlou, 2002). By day 4 of differentiation, more than 60% of cells are positive for Sox1, a specific neuroectodermal marker. Sox1 expression reaches a maximum between days 5 to 7 (Ying *et al.*, 2003b). Neuroepithelial cells organise radially into rosette-like structures known as neural rosettes or rosette neural stem cells (R-NSCs) (Ying *et al.*, 2003b; Elkabetz *et al.*, 2008). R-NSCs are early NS

cells that are able to give rise to a wide range of neuronal phenotypes (Elkabetz *et al.*, 2008). During neural differentiation, FGFs play an important role in maintaining survival (Ying *et al.*, 2003b; Smukler *et al.*, 2006). FGFs have been used in many neural differentiation protocols to maintain cell survival and enable the expansion of neural progenitors (Barberi *et al.*, 2003; Stavridis and Smith, 2003; Ying *et al.*, 2003b; Conti *et al.*, 2005). It has been shown that FGF2, in combination with epidermal growth factor (EGF), enables the propagation of neurosphere derived NS cells *in vitro* for extended periods (Conti *et al.*, 2005). Withdrawal of FGF2 leads to terminal differentiation of neural progenitors into glial cells and functional neurons (Fraichard *et al.*, 1995). Unlike stromal cell co-cultures, neurons derived from adherent monolayer cultures are biased towards an anterior fate and are mainly GABAergic (Jain *et al.*, 2003; Ying *et al.*, 2003b; Gaspard *et al.*, 2008). It has even been suggested that GABAergic neurons are the default neuronal phenotype of differentiated ES cells (Westmoreland *et al.*, 2001). Upon replating the neural precursors and exposing them to Shh and FGF8, there is a significant increase in dopaminergic neurons indicating neural precursors from monolayer cultures are responsive to external cues (Ying *et al.*, 2003b).

The main drawback with attempts to derive specific neuronal subtypes *in vitro* is the heterogeneity within stem cells cultures. To date, there has not yet been a successful report of a method of generating cultures consisting of pure neuronal precursors or neurons. To enrich for cells of interest, other options have been explored. One strategy is to use fluorescent activated cell sorting (FACS) extraction of knock-in fluorescent reporter cell lines (Chung *et al.*, 2006) or cell surface antigens (Carpenter *et al.*, 2001; Pruszak *et*

al., 2007). Another approach is to overexpress a gene involved in the development of a cell type of interest (Friling *et al.*, 2009).

1.3.2 Derivation of dopaminergic neurons from stem cells

Dopaminergic neurons have been generated from multiple stem cell sources - ES cells, iPS cells and neural stem (NS) cells using different patterning protocols and genetic manipulation. Stem cell cultures contain multiple cell types and there has yet been a method of generating a homogenous population of midbrain dopaminergic progenitors or neurons. Typically, extrinsic factors such as Shh and FGF8 are added after neural induction of ES cells to improve yields of TH+ neurons (Lee *et al.*, 2000b; Barberi *et al.*, 2003; Ying *et al.*, 2003b; Perrier *et al.*, 2004). The highest amounts of dopaminergic neurons allegedly through differentiation of ES cells were reported by Perrier *et al.* (2004) and Cho *et al.* (2008) with 79% and 86% of cultured neurons positive for TH respectively. As differentiation protocols have not reproducibly generated large numbers of dopaminergic neurons, research groups have turned to forced or overexpression of genes to increase midbrain dopaminergic neuron numbers. In a study by Andersson *et al.* (2006b), a Lmx1a cDNA was inserted into expression vector driven by a Nestin enhancer (NesE). Differentiation of mES cells transfected with the NesE-Lmx1a vector resulted in *bona fide* dopaminergic neurons, as defined by the co-expression of TH with Nurr1, Pitx3, En1/2, Lmx1a, Lmx1b and DAT (Andersson *et al.*, 2006b). A further study by the same group reported that differentiation of the mouse NesE-Lmx1a cell line generated 75% - 95% neurons expressing TH, with 90% - 98% of those TH+ neurons co-expressing additional midbrain dopaminergic neuron markers such as Lmx1a, Lmx1b, Pitx3, En1/2,

Nurr1, Foxa2, DAT and VMAT2 (Friling *et al.*, 2009). Furthermore, the Lmx1a-induced neurons expressed physiological properties characteristic of *bona fide* midbrain dopaminergic neurons (Friling *et al.*, 2009). FACS sorting reporter cell lines or cell surface antigens has been used to enrich for dopaminergic progenitors and/or neurons. Hedlund and group (2008) obtained an enriched Pitx3+ population by FACS sorting a Pitx3-eGFP cell line. Sorted Pitx3+ cells co-cultured with primary astrocytes showed co-localisation of Pitx3, Nurr1, En and L-aromatic amino acid decarboxylase (AADC) and the majority of the cells also expressed Lmx1a and TH (Hedlund *et al.*, 2008). In a recent report, Otx2+ Corin+ cells sorted from an Otx2-GFP cell line co-expressed Lmx1b, Foxa2 and GLAST and terminally differentiated into midbrain dopaminergic neurons expressing midbrain neuronal markers such as Pitx3, Lmx1b, Nurr1, DAT and dopa decarboxylase (DDC) (Chung *et al.*, 2011).

1.4 Parkinson's disease

Parkinson's disease (PD) was named after James Parkinson, who first described this condition in his monograph "An essay of the shaking palsy" in 1817. It is a neurodegenerative disorder of the central nervous system (CNS) which affects 0.3% of the total population in developed countries and approximately 1% of the population over the age of 60 (Samii *et al.*, 2004). PD is characterised by the loss of dopaminergic neurons from the substantia nigra especially in ventral component of the pars compacta. The pathological hallmark of PD is the accumulation of α-synuclein-immunoreactive inclusions known as Lewy bodies present in the remaining neurons. The exact cause of PD is still unknown but there is evidence that ageing, genetic mutations and environmental factors

contribute to the onset of the disorder (Samii *et al.*, 2004; Davie, 2008). The cardinal signs of PD are tremor, rigidity and bradykinesia. Postural instability is sometimes included as a cardinal sign in certain reports although it is not common early in the disease. Non-motor complications include dementia and psychosis, depression, autonomic dysfunction, oculomotor abnormalities and sleep disorders (Gelb *et al.*, 1999; Samii *et al.*, 2004; Poewe, 2006; Davie, 2008; Jankovic, 2008). Currently there are no methods of slowing disease progression, although symptomatic therapies for PD have been developed. The main drugs used for motor symptom treatments during early stages of PD are levodopa, which is the most potent anti-Parkinsonian drug, dopamine agonists and monoamine oxidase-B (MAO-B) inhibitors (Samii *et al.*, 2004; Davie, 2008). As the duration of PD increases, drug effectiveness at treating the symptoms diminishes, eventually producing dyskinesias. In severe cases where drugs are ineffective, surgery has been performed on PD patients using deep brain stimulation of the thalamus, internal globus pallidus or the subthalamic nucleus (Samii *et al.*, 2004; Davie, 2008).

1.5 Cell replacement therapy

The prospect of grafting dopaminergic neurons derived from stem cell cultures to replace degenerating and lost neurons in PD patients has received widespread attention. The first replacement therapy for PD was tried 20 years ago, using fetal mesencephalic tissue (Lindvall and Kokaia, 2009). Although occasionally successful, ethical issues, poor graft viability, increased effects of dyskinesias, aggregation of Lewy bodies and possible placebo effect from open label clinical trials hamper the widespread use of this tissue (Lindvall and Kokaia, 2009; Sonntag *et al.*, 2009; Arenas, 2010; Vidaltamayo *et al.*, 2010).

Due to the limitations of fetal mesenphalic tissues, stem cells have arisen as an alternative source for dopaminergic neurons for grafting in PD. As stem cells are able to self-renew indefinitely, they could provide an unlimited numbers of dopamine-secreting neurons suitable for clinical trials and animal studies. Furthermore, transplantation of dopaminergic neurons generated from iPS cells would not involve any ethical or immunoreactivity issues. For induction of clinical improvement after transplantation, stem-cell-derived dopaminergic neurons (i) require the ability to synthesise and release dopamine (in addition to exhibiting morphological, molecular and electrophysiological properties of mature midbrain dopaminergic neurons), (ii) are able to abolish motor symptoms in animal models of PD, (iii) show long term survival (at least 100,000 dopaminergic neurons) in each human putamen, (iv) have the capability to re-establish a dense dopamine releasing network in the striatum and (v) functionally integrate into existing neural circuitries (Lindvall and Kokaia, 2009). One of the earliest stem cell transplantation studies by Ben-Hur and co-workers (2004) showed partial functional recovery in rats using transplanted hES-cell-derived neural progenitors (Ben-Hur *et al.*, 2004). Since then several studies have reported survival and successful integration of dopaminergic neurons derived from different sources – human ES cells (Roy *et al.*, 2006; Yang *et al.*, 2008), nuclear transfer (nt) ES cells (Tabar *et al.*, 2008), iPS cells (Wernig *et al.*, 2008b; Swistowski *et al.*, 2010; Rhee *et al.*, 2011) and NS cells (Tønnesen *et al.*, 2011) into host animals, resulting in behavioural improvements. The successes of transplantation studies, especially with iPS cell derived dopaminergic neurons, have enhanced interest in application of stem cells in PD cell replacement therapy. However, applications for iPS cells in clinical studies have not yet been realised. Several issues have to be resolved

before iPS cell-derived dopaminergic neurons can be clinically used. These include (i) teratoma formation/neural outgrowth, (ii) possible mutations, polymorphisms or epigenetic marks leading to increased susceptibility to neurodegeneration in PD, (iii) heterogeneity of iPS cells i.e. whether every iPS cell clone is completely reprogrammed and (iv) determining whether iPS cell derived midbrain dopaminergic neurons from different lineages are functionally similar (Lindvall and Kokaia, 2009; Yamanaka, 2009; Arenas, 2010).

1.6 Aims

The genetic network underlying midbrain dopaminergic neuron development has been extensively studied *in vivo*. This project focuses on identifying and deriving *bona fide* midbrain dopaminergic neurons from mES cells based on markers that have been previously reported in literature. The signalling pathways that pattern midbrain dopaminergic neurons *in vivo* are also examined to determine whether these pathways affect the development of midbrain dopaminergic neurons *in vitro*. Selection of only the *bona fide* midbrain dopaminergic neurons, while eliminating other neuronal phenotypes, is essential for successful cell replacement therapies in Parkinson's Disease. In this study, I utilise reporter cell lines to investigate the development of ES cells into dopaminergic neurons *in vitro* using the adherent monolayer and stromal cell co-culture differentiation protocols. Furthermore the functional properties of neurons derived via different protocols are explored.

The main aims of this project are:

1. To investigate the effects of BMP, Shh and Wnt signalling on *in vitro* dopaminergic neuron differentiation.
2. To enrich for midbrain dopaminergic precursors.

CHAPTER 2

General Methods

2.1 Cell lines

Several mES cell lines were used in this study: E14-tg2a, Sox1-GFP, Lmx1a-AMP-IRES-eGFP, Msx1-AMP-IRES-eGFP and Pt4-1. E14-tg2a is a subclone of the E14 cell line and was derived by Hooper *et al.* in 1987 from the mouse strain 129/Ola (Downing and Battey, 2004). Sox1-GFP, which was engineered by Smith and colleagues in 2003, has one copy of the Sox1 gene replaced by a reporter cassette containing the open reading frames of GFP (Green Fluorescent Protein) and the puromycin resistance gene. Both cell lines were received as a courtesy of Stem Cell Sciences. The Lmx1a-AMP-IRES-eGFP and Msx1-AMP-IRES-eGFP were targeted using Lmx1a-AMP-IRES-eGFP-FneoF and Msx1-AMP-IRES-eGFP-FneoF targeting vectors respectively. The vectors were designed to replace exon 1 of the respective genes with the gene for AMP (β-lactamase), enhanced green fluorescent protein (eGFP), separated by an internal ribosomal entry site (IRES), and a neomycin (neo) gene cassette flanked by flippase recognition target (Frt) sites. The Pt4-1, Pitx3-eGFP cell line was given as a gift from Dr. C. O'Brien, details of the this line are described by Zhao *et al.* (2004).

2.2 Cell culture

2.2.1 Maintenance of mES cell cultures

E14-tg2a, Lmx1a-AMP-IRES-eGFP, Msx1-AMP-IRES-eGFP and Pitx3-eGFP ES cells were seeded at 3 - 4 x 10^4 cells/cm^2 on 0.1% (w/v) gelatin coated plates and routinely cultured in DMEM (Dulbecco's Modified Essential Medium; Invitrogen, Australia) supplemented with 10% ES cell qualified fetal calf serum (FCS; Invitrogen, Australia), 0.1 mM β-mercaptoethanol (Sigma, Australia), 50 units/mL penicillin-50 µg/mL streptomycin (Pen-

Strep; Invitrogen, Australia) and 10^3 units/mL LIF (Millipore, Australia). For the Sox1-GFP line, GMEM (Glasgow Modified Essential Medium; Sigma, Australia) supplemented with 10% FCS, 1 mM sodium pyruvate (Sigma, Australia), 0.1 mM MEM non-essential amino acids (Sigma, Australia), 0.1 mM β-mercaptoethanol (Sigma, Australia), 2.75 g/L sodium bicarbonate (Sigma, Australia) and 50 units/mL-50 µg/mL Pen-Strep (Invitrogen, Australia) with 10^3 units/mL LIF (Millipore, Australia) was used as culture medium. Media was changed every 48 hours until cells were confluent.

To passage the cultures, media was aspirated and cells washed with Phosphate Buffered Saline (PBS; see Appendix I) and incubated with accutase (Sigma, Australia) for 5 minutes at 37°C. mES media was added to stop digestion of cells which were then counted with a haemocytometer. Cells were transferred to a Falcon conical tube and centrifuged at 200*xg* for 5 minutes at room temperature. Media was removed and cells were resuspended with fresh culture medium. The cell suspension was then transferred to gelatin coated plates and incubated at 37°C with 5% CO_2 until confluent. All flasks and wells were gelatin coated for at least 15 minutes.

2.2.2 Maintenance of PA6 stromal cells

PA6 stromal cells (Riken, Japan) were maintained in α-MEM (Invitrogen, Australia) supplemented with 10% FCS and 50 units/mL-50 µg/mL Pen-Strep. These cells were passaged at a 1:3 ratio whenever cultures reached 80% confluency, see 2.2.1. Media was renewed every other day.

2.2.3 Cryopreservation of cells

For long term storage, cells were first accutased and centrifuged as described in 2.2.1. 5 x 10^5 cells were resuspended in 1 mL cryo-mix consisting 40% culture medium, 50% FCS and 10% DMSO (Sigma, Australia) then subsequently transferred into a cryo-vial. Cryo-vials were frozen down at -1°C/minute in a 'Mr. Frosty' (Nalgene, Australia) for at least 2 hours and left overnight at -70°C before being transferred to a liquid nitrogen dewar for storage.

To thaw cells, frozen cryo-vials were removed from the liquid nitrogen dewar and placed in a water bath at 37°C. Once thawed, cells were resuspended in culture media and centrifuged at 200*xg* for 5 minutes at room temperature. Media was then aspirated from the pelleted cells and replaced with fresh culture media supplemented with LIF. Cells were resuspended and transferred onto gelatin coated flasks or wells.

2.2.4 Neural differentiation

2.2.4.1 Adherent monolayer

Monolayer neural differentiation was undertaken using a modified Ying *et al.* (2003) protocol. Briefly, ES cells were dissociated with accutase and replated onto gelatin coated plates at 4.5 x 10^3 cells/cm^2 and cultured in ES maintenance media with LIF for 24 hours. The next day, cells were washed once with PBS and supplemented with N2B27. N2B27 consists of a 1:1 mix of DMEM/F12 medium (Invitrogen, Australia), Neurobasal medium (Invitrogen, Australia) supplemented with 5 mL/L N2 (Invitrogen, Australia), 10 mL/L B27

(Invitrogen, Australia), 10 mg/mL insulin (Sigma, Australia) and 25 mg/L bovine serum albumin (BSA) fraction V (Invitrogen, Australia). Media was renewed every other day.

2.2.4.2 PA6 co-culture

Prior to ES cell differentiation, PA6 cells were seeded at 5 x 10^4 cells/cm^2 in maintenance media and then switched to GMEM supplemented with 15% Knockout Serum Replacement (KSR), 2 mM Glutamax, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids and 0.1 mM β-mercaptoethanol. ES cells were seeded at 100 cells/cm^2 onto PA6 containing wells. After 8 days, media was switched to N2B27. From day 3 of differentiation onwards media was renewed at least every other day.

2.3 Immunocytochemistry

All procedures were performed at room temperature unless stated otherwise. Cells were washed once with PBS then fixed with 4% (w/v) paraformaldehyde (Sigma, Australia) in PBS for 20 minutes. After fixation cells were washed twice with PBST (see Appendix I) and permeabilised with 0.5% (v/v) Triton-X (Sigma, Australia) in PBST for 30 minutes. Cells were then washed twice with PBST and blocked with 5% (v/v) donkey serum (Sigma, Australia) in PBST for 30 minutes. Afterwards cells were washed 3 times with PBST for 5 minutes each and exposed to primary antibodies in PBST overnight on a shaker at 4°C. The list of primary antibodies used can be found in Table 1. Anti-GFP primary antibody was not used in any experiments as GFP expression was detectable after the previous steps.

Table 1: Dilutions and suppliers of primary antibodies used in immunocytochemistry experiments.

Primary Antibody	Dilution	Supplier
Rabbit anti-tyrosine hydroxylase (TH)	1:200	Millipore (Australia)
Mouse anti-Foxa2	1:4000	Developmental Studies Hybridoma Bank (DSHB; USA)
Mouse anti-class III β-tubulin (TUJ-1)	1:200	Covance (Australia)
Mouse anti-Nestin	1:200	Millipore (Australia)
Mouse anti-glial fibrillary acid protein (GFAP)	1:200	Millipore (Australia)
Mouse anti-GAD67	1:1000	Millipore (Australia)
Mouse anti-SatB2	1:40	Abcam (United Kingdom)
Rabbit anti- Zonula Occludens-1 (ZO-1)	1:100	Invitrogen (Australia)
Rabbit anti-TBR-1	1:400	Abcam (United Kingdom)
Rabbit anti-5-HT	1:2000	Sigma (Australia)

Rabbit anti-GABA	1:1000	Sigma (Australia)
Rabbit anti-Musashi-1	1:300	Abcam (United Kingdom)

The following day, cells were again washed 3 times with PBST for 5 minutes each then incubated for 1 hour with fluorophore conjugated secondary antibodies in 1% (v/v) donkey serum in PBST. Secondary antibodies used can be found in Table 2. Negative controls were only exposed to secondary antibodies. After incubation cells were washed 3 times with PBS and exposed to nuclear counter stains TOPRO-3 (1 µM; Invitrogen, Australia) or DAPI (0.5 µg/mL; Sigma, Australia) for 15 minutes. Then, cells were washed 3 times with PBS and covered with 0.1% (v/v) sodium azide in PBS for fluorescent imaging.

Table 2: Dilutions and suppliers of secondary antibodies used in immunocytochemistry experiments.

Secondary Antibodies	**Dilution**	**Supplier**
Alexa Fluor 488 donkey anti-mouse	1:500	Invitrogen (Australia)
Alexa Fluor 567 donkey anti-mouse	1:500	Invitrogen (Australia)

Alexa Fluor 567 donkey anti-rabbit	1:500	Invitrogen (Australia)
Alexa Fluor 594 donkey anti-rabbit	1:500	Invitrogen (Australia)
Alexa Fluor 637 donkey anti-mouse	1:200	Invitrogen (Australia)
Alexa Fluor 637 donkey anti-rabbit	1:200	Invitrogen (Australia)

2.4 Imaging and quantification

Fluorescent images were taken with a Nikon A1R confocal microscope (Nikon, Japan). For quantification, cells positive for markers were counted manually in 9 random fields of view (6x magnifications) or with the Metamorph® v6.1ro imaging software (Universal Imaging Co., USA) taken from 3 separate experiments.

In order to assess cell proliferation cultured cells stained with TOPRO-3 were scanned with the Odyssey Infrared Imaging System (Li-cor Biosciences, USA). Emission values of each well were standardised to control wells with subtraction of background values.

2.5 Flow cytometry analysis

Cultures were dissociated into single cells with accutase and resuspended in culture media containing FCS. Once resuspended, cells were centrifuged at 200*xg* for 5 minutes

and media was then removed. For analysis of GFP expression, cells were resuspended in 5% (v/v) FCS in PBS. Wild type cells were subjected to comparable differentiation conditions and used as negative controls. To analyse AMP expression, cells were loaded with the LiveBLAzer™-FRET B/G Loading Kit (Invitrogen, Australia) according to manufacturer's specifications for 2-3 hours. After incubation, cells were centrifuged again at 200*xg* for 5 minutes. Media was removed and cells were resuspended in 5% (v/v) FCS in PBS. Wild type cells submitted to the staining procedure were used as negative controls. Prior to flow cytometry analysis, cell cultures were run through a 40 µm strainer (BD Biosciences, Australia) to remove clumps and all samples were incubated with SYTOX® Red stain (5 nM; Invitrogen, Australia) for at least 15 minutes at room temperature to select for live cells. For all experiments, gates were set so that 99.0 – 99.5% of the wild type population registered as negative. Flow cytometry was performed with a FACS Canto II analyser (BD Biosciences, Australia).

2.6 Statistical analysis

For all experiments, results are presented as the mean and standard error of the mean (SEM) of $n \geq 3$. Statistical analyses were performed using the PRISM v5.00 (Graphpad Software, USA). All raw data were analysed accordingly by Student's t-test, one-way analysis of variance (ANOVA) with either post-hoc Bonferroni's test or Dunnett's test. In all cases, $p < 0.05$ were considered to be statistically significant.

CHAPTER 3

BMP, Shh and Wnt signalling pathways on dopaminergic neuron development

3.1 Introduction

Knock-in ES reporter cell lines provide a means of identifying and quantifying cells at any time during the differentiation. Critically, these cells can be enriched through FACS sorting, eliminating the issue of heterogeneity. Although there have been attempts to direct ES cells into specific neuronal phenotypes (Barberi *et al.*, 2003), the generation of homogenous populations of neurons has not yet been achieved. From previous studies using a Sox1-GFP line, the majority of ES cells on adherent monolayer differentiate into Sox1+ neuroectodermal cells by default (Ying *et al.*, 2003b). Around 75% of ES cell cultures commit to a neural fate, however, the number of those cells that differentiate into dopaminergic neurons has yet to be reported. The recent identification of transcription factors involved in midbrain dopaminergic neuron development has increased the selection of markers that can be used to identify dopaminergic progenitors/neurons. Andersson *et al.* (2006b) identified two key transcription factors, Lmx1a and Msx1, as determinants of midbrain dopaminergic neurons. Lmx1a is expressed in the ventral midline at E9 and expands dorsally (Andersson *et al.*, 2006b). It was previously only implicated in development of the dorsal neural tube (Millonig *et al.*, 2000; Chizhikov and Millen, 2004). In chick embryos, overexpression of Lmx1a using a retroviral vector resulted in ectopic expression of dopaminergic neurons in the ventral midbrain, while silencing of Lmx1a RNA in the ventral midbrain resulted in a drastic loss of Nurr1+ cells suggesting Lmx1a is sufficient and required for generation of dopaminergic neurons (Andersson *et al.*, 2006b). The expression of Msx1 in ventral midline cells was detected later than Lmx1a. It is also broadly expressed in the developing dorsal neural tube as well as in the ventricular/subventricular (VZ/SVZ) of the ventral midline (Ramos and Robert,

2005; Andersson *et al.*, 2006b). However, unlike Lmx1a, overexpression of Msx1 was insufficient to induce dopaminergic neurons in the midbrain. As previously mentioned (see 1.4), Msx1 represses Nkx6.1 expression and induces Ngn2 and neuronal differentiation in the ventral midbrain (Andersson *et al.*, 2006b).

3.1.1 Aims

In this chapter, I focus on monolayer neural differentiation using knock-in reporter cell lines: Sox1-GFP, Lmx1a-AMP-IRES-eGFP and Msx1-AMP-IRES-eGFP. Lmx1a and Msx1 expression patterns *in vitro* have not been previously explored by other groups. Therefore, the aims of this study are to investigate the expression of both genes from ES cells to neurons in culture and to explore the effects of three major signalling pathways involved in neural patterning (BMP, Shh and Wnt signalling) on dopaminergic neuron differentiation.

3.2 Methods

Unless detailed below, the experimental methods used in this chapter have been described in Chapter 2.

3.2.1 Neural differentiation

Initiation of neural differentiation is as described in 2.2.4.1. BMP4 (10 ng/mL), Noggin (500 ng/mL), Shh (200 ng/mL), SANT-1 (1 µM; Tocris Bioscience, United Kingdom), Cyclopamine (1 µM; Sigma, Australia), Wnt1 (50 ng/mL), Wnt3a (50 ng/mL), CHIR99021 (3 µM; Axon Medchem, The Netherlands) or Dkk1 (100 ng/mL) were added on days 0 or 4 as

indicated. RA (Sigma, Australia) was only added from day 4. No cytokines were added to the control wells.

Terminal differentiation was performed on E14-tg2a wild type cells. N2B27 medium was supplemented with L-ascorbic acid (200 µM; Merck, Australia) from day 12 onwards. All cytokines were purchased from Peprotech (Israel) unless stated otherwise.

3.2.2 Quantification of TH+ Foxa2+ cells

Stacks (50 µm, 2.5 µm/step) were taken at 10x magnifications from random fields of view with the Nikon A1R confocal microscope (Nikon, Japan) to confirm co-labelling for both markers. Only cells expressing both TH and Foxa2 on the same plane of a stack were counted.

3.3 Results

3.3.1 Sox1, Lmx1a and Msx1 expression during neural differentiation

The Sox1-GFP cell line was used to evaluate the degree of neural induction achieved in our differentiation protocol (Ying *et al.*, 2003b). The percentage of Sox1 positive cells indicates the proportion of cells in culture that have committed to a neural fate. FACS analysis was performed at selected time points with gates set to exclude 99% to 99.5% of wild type cells (Figure 5A). In our hands, Sox1-GFP expression during ES cell differentiation was similar to previously reported (Figure 5B). Sox1 expression steadily increased between days 0 to 8 of differentiation. 65% of cells expressed Sox1-GFP by day 4, indicating that the first stages of commitment to neural lineages had taken place. The

maximum percentage of Sox1+ cells were observed at day 8 (75%) but this was not significantly higher than the estimate on day 4. The number of Sox1+ cells declined after day 8.

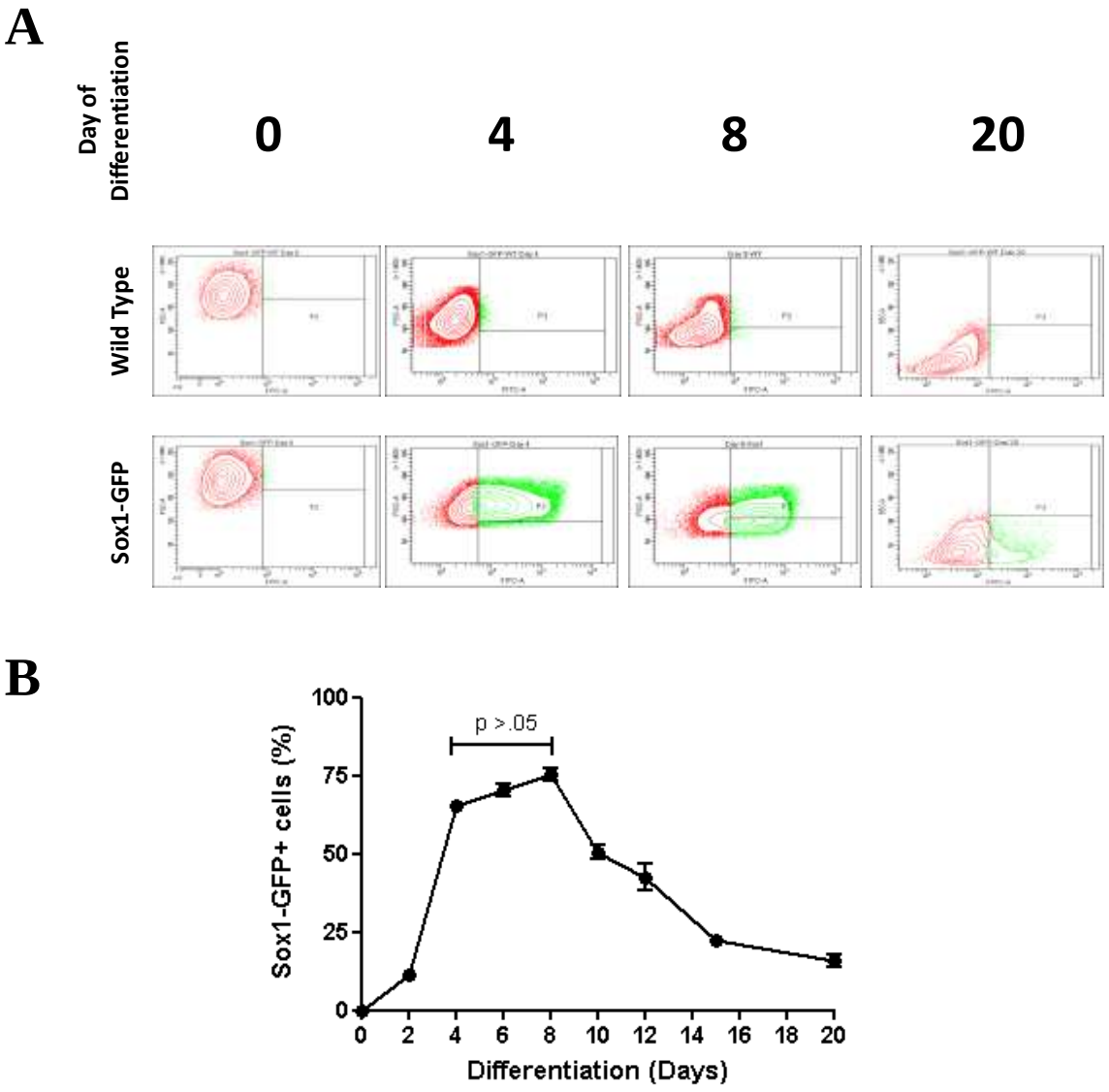

Figure 5: Sox1-GFP expression during ES cell differentiation in N2B27. (A) Flow cytometry plots of forward scatter or side scatter vs. FITC (GFP) for wild type and Sox1-GFP cells. (B) Time course for Sox1-GFP expression during differentiation. Results are presented as the mean ± SEM of at least 3 independent experiments.

Having shown a robust differentiation into a neural phenotype, the expression of both Lmx1a and Msx1 during were assessed. FACS analysis was performed at the same points as the previous experiment. The Lmx1a-AMP-IRES-eGFP and Msx1-AMP-IRES-eGFP knock-in cell lines were differentiated similarly to the Sox1-GFP cell line to determine the proportion AMP expressing cells in N2B27. The AMP expression profiles of both cell lines were indistinguishable throughout the 20 day differentiation period. Lmx1a and Msx1 both increased up to day 8 and steadily declined thereafter (Figure 6A + 6B).

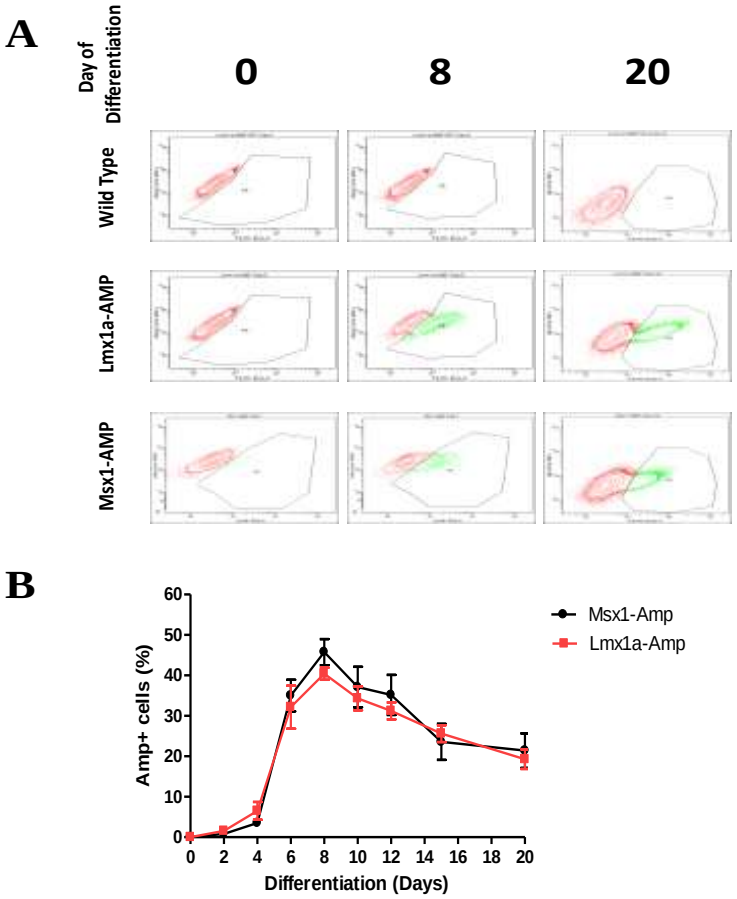

Figure 6: Expression of Lmx1a-AMP and Msx1-AMP during ES cell differentiation in N2B27. (A) Flow cytometry plots of Alexa Fluor 488 vs. Pacific Blue for Lmx1a-AMP-IRES-eGFP cells and Msx1-AMP-IRES-eGFP cells. (B) Time courses for Lmx1a-AMP and Msx1-AMP during differentiation. Results are presented as the mean ± SEM of at least 3 independent experiments.

As the Lmx1a-AMP-IRES-eGFP cell line contained the sensitive catalytic reporter β-lactamase (e.g. the ampicillin resistance gene, AMP) and enhanced green fluorescent protein (eGFP) co-expressed under control of the endogenous Lmx1a promoter, I investigated if GFP expression was comparable to AMP expression. The expression pattern of GFP was similar to AMP, although with a 2 day delay before the apparent onset of GFP expression (Figure 7). The difference in apparent onset may be explained by the higher sensitivity of the AMP assay. Peak expression of GFP was detected at day 10, with 35% of cells positive for the reporter at a time when the percentage of AMP-positive cells was already in decline, reflecting the considerably shorter half life of AMP (~3.5 hours) (Zlokarnik *et al.*, 1998) compared to GFP (~26 hours) (Corish and Tyler-Smith, 1999).

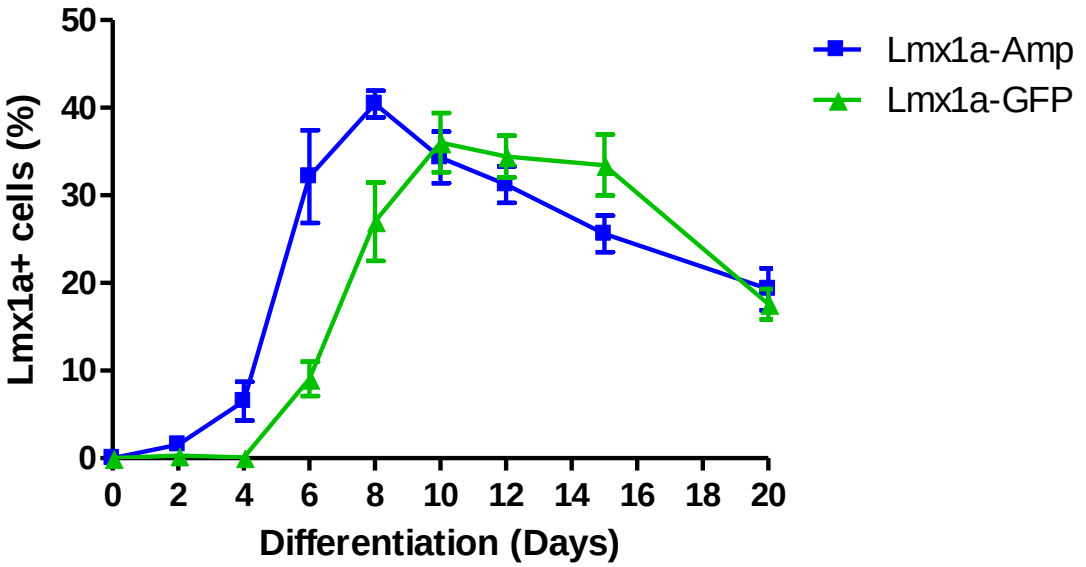

Figure 7: Time courses for AMP and eGFP expression under control of the Lmx1a promoter. Results are presented as the mean ± SEM of at least 3 independent experiments.

3.3.2 BMP, Shh and Wnt signalling on early stages of differentiation

To investigate BMP, Shh and Wnt signalling on neural differentiation, the effects of agonists and antagonists of these signalling pathways on Sox1, Msx1 and, in particular, Lmx1a expression were examined. Day 8 of differentiation was chosen as the time point to evaluate the expression of all three reporter cell lines as peak expression of Lmx1a-AMP was detected at that particular day. Factors modulating pathways that have been associated with the induction of Lmx1a expression *in vivo*, namely the canonical Wnt-signalling, Shh-signalling, and BMP signalling pathways were chosen for examination (Millonig *et al.*, 2000; Chizhikov and Millen, 2004; Arenas, 2008; Chung *et al.*, 2009; Joksimovic *et al.*, 2009a). Pathway agonists and antagonists were added to cultures either at the start of differentiation process (day 0) or after neural conversion (day 4).

Table 3: Agonists and antagonists used to activate/inhibit the BMP, Shh and Wnt signalling pathways.

Factor	Signalling Pathway	Agonist/Antagonist
BMP4	BMP signalling	Agonist
Noggin	BMP signalling	Antagonist
Shh	Shh signalling	Agonist
Cyclopamine	Shh signalling	Antagonist
SANT-1	Shh signalling	Antagonist
Wnt1	Wnt signalling	Agonist
Wnt3a	Wnt signalling	Agonist
CHIR99021	Wnt signalling	Agonist
Dkk1	Wnt signalling	Antagonist

The impacts of the factors were first investigated on the Sox1-GFP cell line to determine whether neural differentiation was influenced by these exogenous factors. Among the factors added from day 0, BMP4, Wnt3a and CHIR99021 (one-way ANOVA post-hoc Dunnett's test, $p < 0.01$, $n=3$) and SANT-1 (one-way ANOVA post-hoc Dunnett's test, $p < 0.05$, $n=3$) significantly decreased percentage of Sox1-GFP expressing cells as compared to control cells. None of the factors significantly increased the Sox1-GFP+ cells in the cultures (Figure 8A).

When added from day 4 onwards, only BMP4 (one-way ANOVA post-hoc Dunnett's test, $p < 0.01$) and CHIR99021 (one-way ANOVA post-hoc Dunnett's test, $p < 0.05$, $n=3$) still significantly decreased the number of Sox1-GFP expressing cells. Similarly, none of the cultures exhibited a significant increase in Sox1-GFP+ populations when treated from day 4 (Figure 8B).

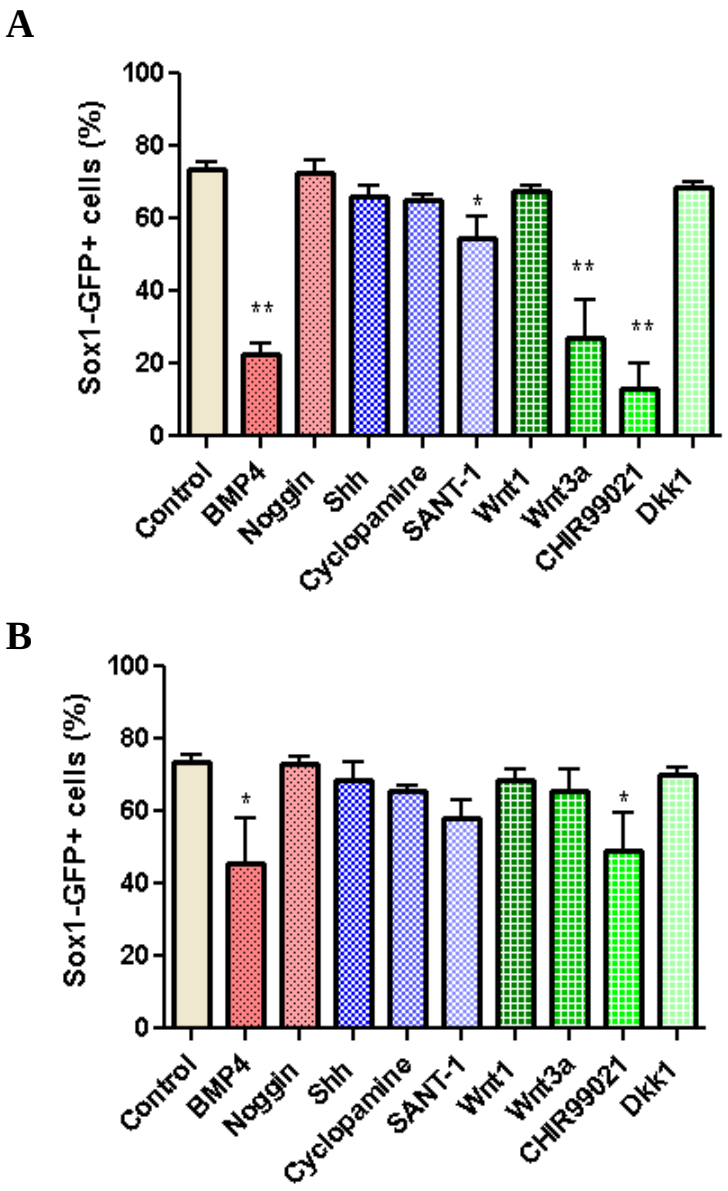

Figure 8: Percentages of Sox1-GFP positive cells on day 8 in response to factor treatments. (A) Factors added continuously from start of differentiation (day 0 to day 8). (B) Factor treatments after neural conversion (day 4 to day 8). Results are presented as the mean ± SEM of 3 independent experiments. *, ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared to control with one-way ANOVA plus post-hoc Dunnett's test.

As neural induction was affected by four of the factors when they were added to the culture medium from the start of differentiation, I next investigated the effect of all factors on Lmx1a-AMP expression. BMP4, Wnt3a and CHIR99021 significantly reduced the Lmx1a-AMP+ cells in cultures (one-way ANOVA post-hoc Dunnett's test, $p < 0.01$, $n=3$). However, Lmx1a-AMP expression was not affected by the addition of SANT-1. None of the other factors induced a significant increase in the percentage of Lmx1a-AMP+ cells (Figure 9A).

The only factors that affected the percentage Lmx1a-AMP+ cells when treated from day 4 were BMP4 (one-way ANOVA post-hoc Dunnett's test, $p < 0.01$, $n=3$) and CHIR99021 (one-way ANOVA post-hoc Dunnett's test, $p < 0.05$, $n=3$) as both significantly decreased the number of Lmx1a-AMP expressing cells. None of the other factor treatments significantly increased Lmx1a-AMP+ cells in cultures (Figure 9B).

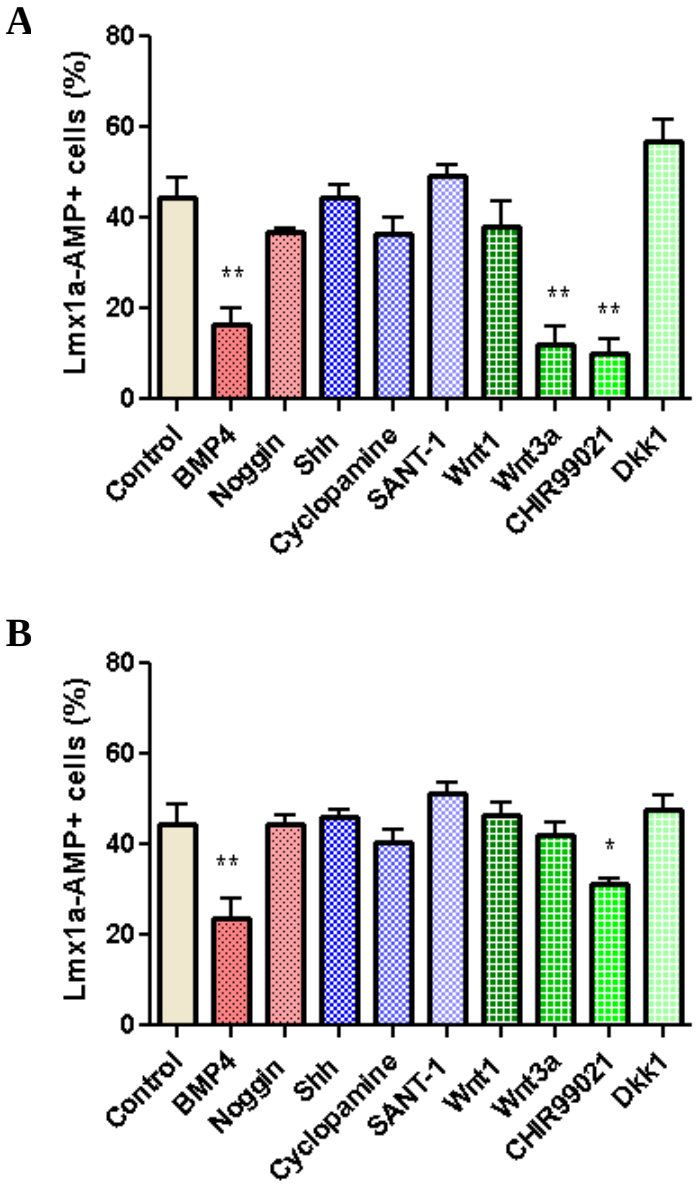

Figure 9: Percentages of Lmx1a-AMP positive cells on day 8 in response factor treatments. (A) Factors added continuously from start of differentiation (day 0 to day 8). (B) Factor treatments after neural conversion (day 4 to day 8). Results are presented as the mean ± SEM of 3 independent experiments. *, ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared to control with one-way ANOVA plus post-hoc Dunnett's test.

As Msx1 is a direct downstream target of Lmx1a and its expression mirrors that of Lmx1a in our monolayer cultures, we expected the Msx1-AMP-IRES-eGFP line to be similarly affected. In Msx1-AMP-IRES-eGFP cultures treated with the factors either continuously from day 0 or from day 4, only treatment with CHIR99021 from day 0 caused a significant reduction in Msx1-AMP+ cell population (one-way ANOVA post-hoc Dunnett's test, $p < 0.01$, $n=3$) (Figure 10A + 10B).

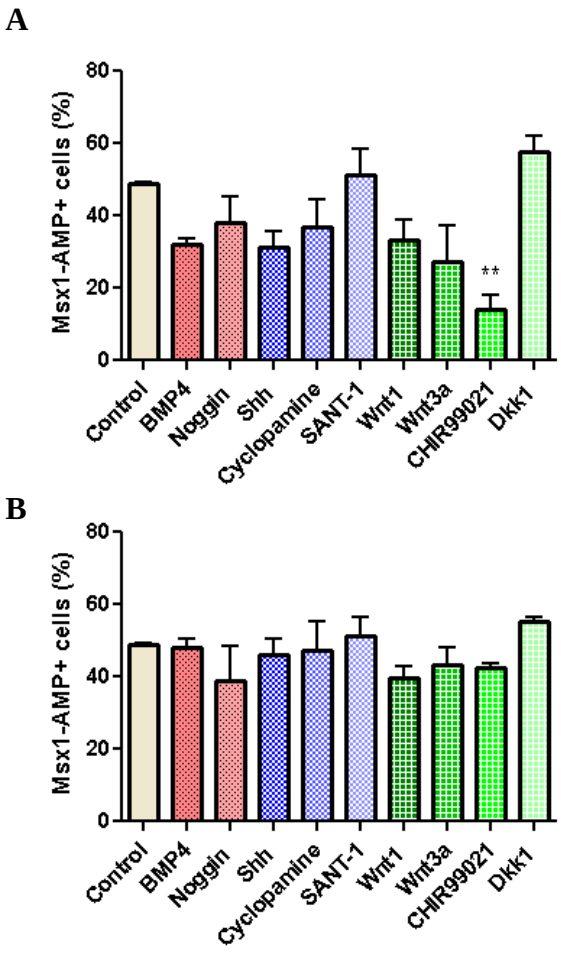

Figure 10: Percentages of Msx1-AMP positive cells on day 8 in response to factor treatments. (A) Factors added continuously from start of differentiation (day 0 to day 8). (B) Factor treatments after neural conversion (day 4 to day 8). Results are presented as the mean ± SEM of 3 independent experiments. ** indicates $p < 0.01$ compared to control with one-way ANOVA plus post-hoc Dunnett's test.

3.3.3 BMP, Shh and Wnt signalling on late stages of differentiation

To investigate the effect of the exogenous factors on late stages of differentiation, cultures were allowed to progress towards terminal differentiation then immunolabelled with antibody against TH, a marker used to identify mature catecholaminergic neurons (Nagatsu *et al.*, 1964; Kawano *et al.*, 1995; Dunkley *et al.*, 2004) (Figure 11).

While none of the treatments increased Lmx1a or Msx1 expression at day 8, exposure to noggin or dkk1 from day 0 to day 12 significantly increased numbers of TH+ cells on day 20 (one-way ANOVA post-hoc Dunnett's test, $p < 0.05$, $n=3$). Exposure of the other factors did not significantly affect TH+ counts (Figure 12A). The numbers of TH+ cells obtained from day 4 to day 12 treated cultures were not significantly different from the control wells (Figure 12B).

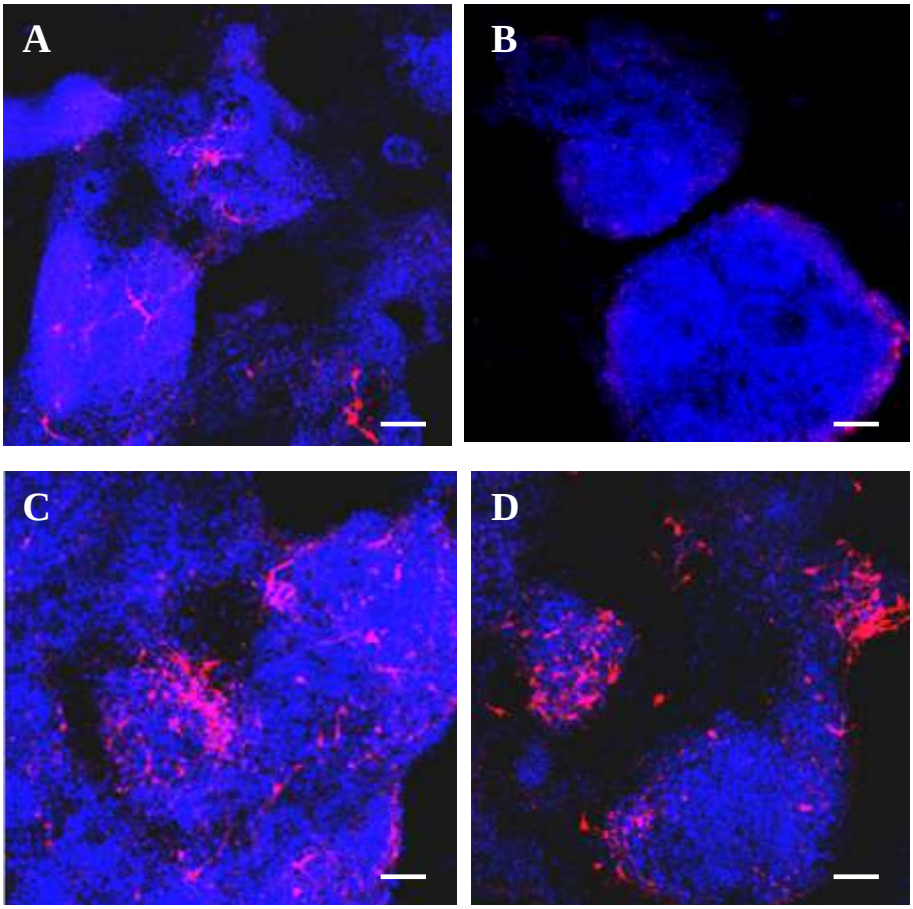

Figure 11: Terminally differentiated cultures 20 days after the induction of differentiation. Immunolabelling of day 20 cultures exposed to (A) no factors, (B) BMP4, (C) noggin and (D) dkk1 from day 0 to day 12 for TH (red) and nuclear dye TOPRO-3 (blue). Scale bar = 100 µm.

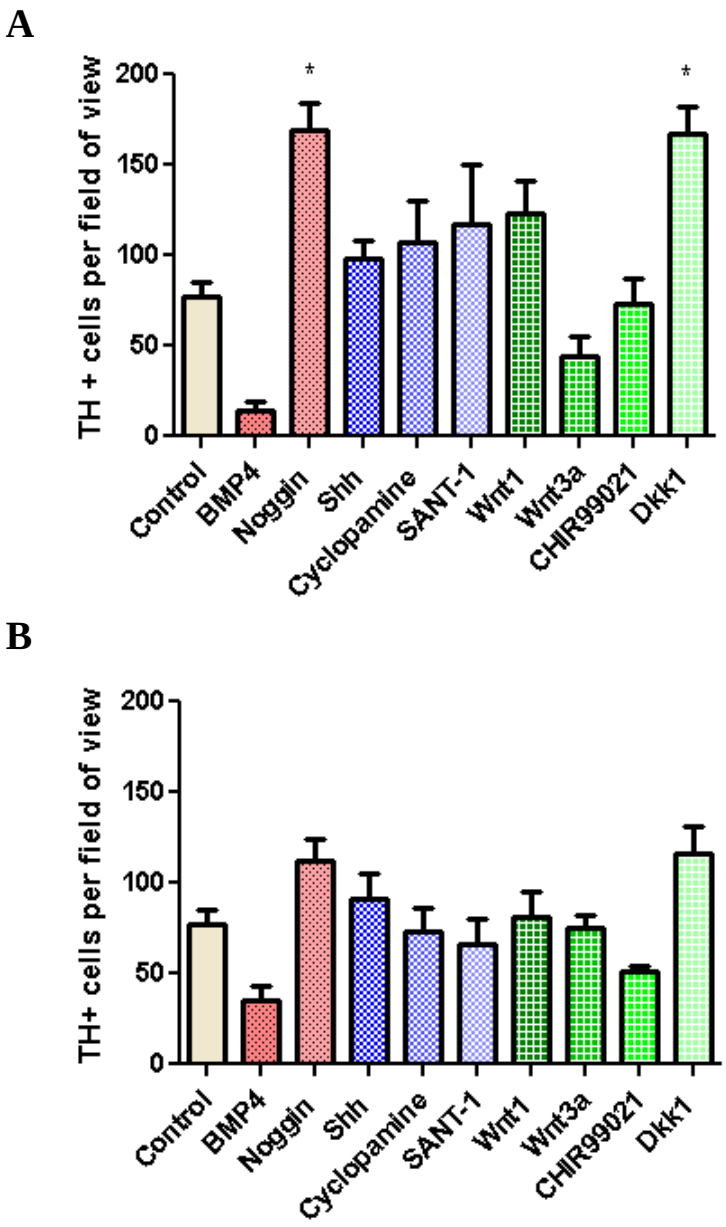

Figure 12: Number of TH positive cells on day 20 of differentiation. (A) Factors added continuously from start of differentiation (day 0 to day 12). (B) Factor treatments after neural conversion (day 4 to day 12). Results are presented as the mean ± SEM of 3 independent experiments. * indicates $p < 0.05$ compared to control with one-way ANOVA plus post-hoc Dunnett's test.

To determine if the increase in TH+ cell numbers were due to increased cell proliferation, cultures were labelled with nuclear dye TOPRO-3 (Figure 13A). Emission values of cultures exposed to CHIR99021 from days 0 and 4 to day 12 were significantly higher than the control wells (one-way ANOVA post-hoc Dunnett's test, $p < 0.001$, $n=3$). There was no significant difference in TOPRO-3 emission values for other factor treatments from either day 0 or day 4 (Figure 13B + 13C).

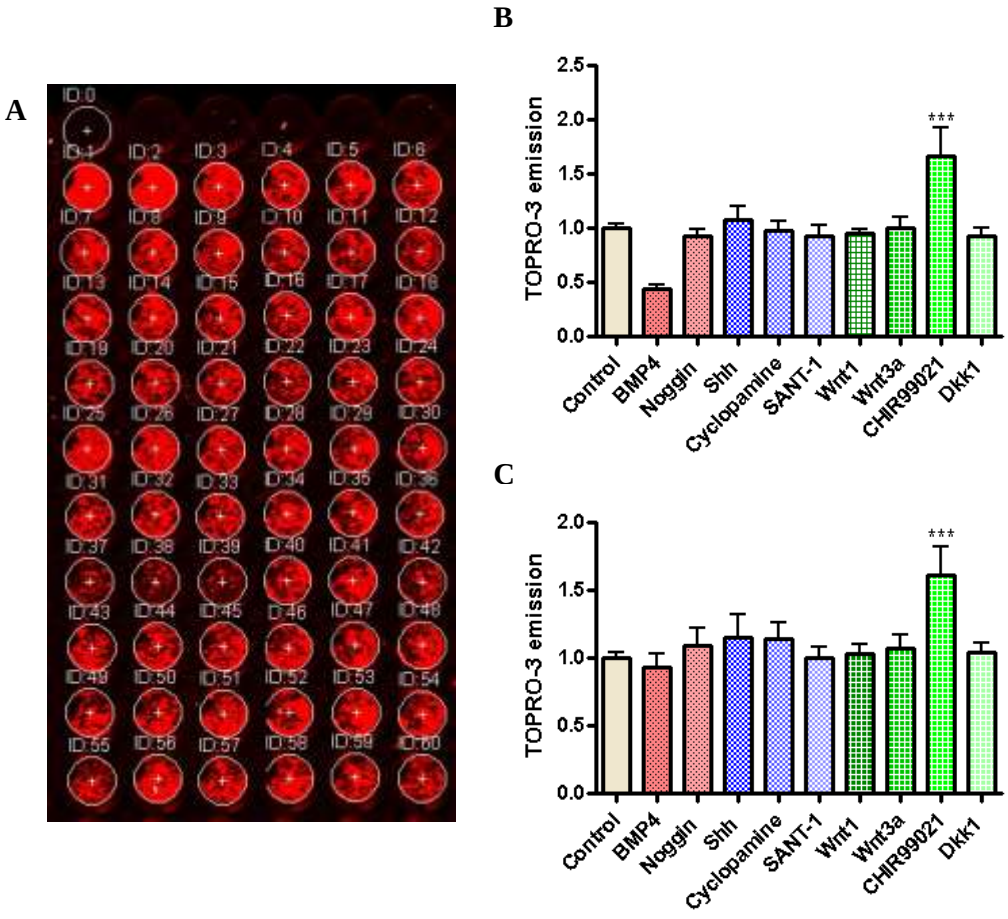

Figure 13: Assessment of cell proliferation on day 20 of differentiation. (A) Cultures in a 96 well plate labelled with TOPRO-3 and analysed with the Odyssey. Emission values for TOPRO-3 of cultures treated from (B) day 0 to day 12 and (C) day 4 to 12. Results are presented as the mean ± SEM of 3 independent experiments. *** indicates $p < 0.001$ compared to control with one-way ANOVA plus post-hoc Dunnett's test.

To further examine whether the TH+ cells in culture were midbrain dopaminergic neurons, cultures were immunolabelled for TH and Foxa2 (Figure 14). Only cells that co-labelled for both markers were considered to be dopaminergic neurons of midbrain origin (Figure 15). No significant differences were detected between the number of cells co-expressing TH and Foxa2 in cultures exposed to factors from day 0 with control wells (Figure 16A). TH+ cells expressing Foxa2 as a percentage of total TH+ cells were also not significant (Figure 16B). Results from day 4 treated cells were similar, with no significant changes in number of TH+ or Foxa2+ cells, or percentage of TH+ cells expressing Foxa2 (Figure 17A + 17B).

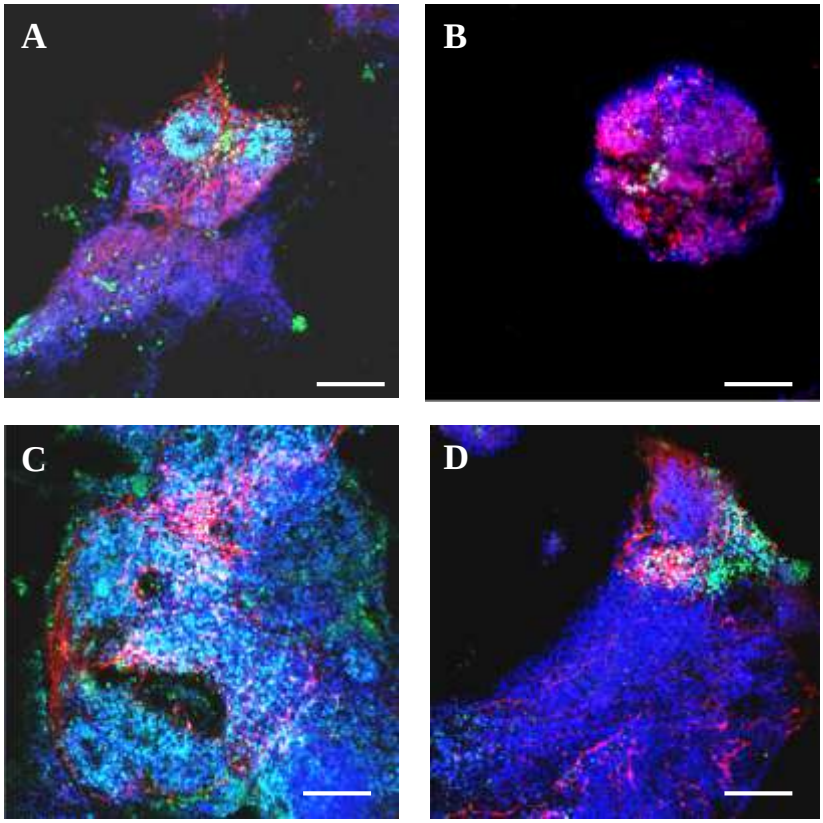

Figure 14: Terminally differentiated cultures 20 days after the induction of differentiation. Immunolabelling of cultures exposed to (A) no factors, (B) BMP4, (C) noggin and (D) dkk1 from day 0 to day 12 for TH (red), Foxa2 (green) and TOPRO-3 (blue). Cell that co-labelled for TH and TOPRO-3 are in purple. Scale bar = 100 µm.

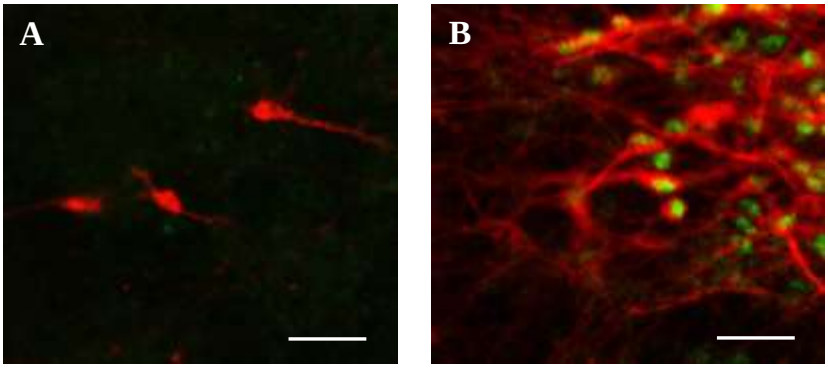

Figure 15: Immunolabelling of day 20 cultures for TH (red) and Foxa2 (green). (A) Cells labelled for TH but not Foxa2. (B) Cells labelled for both TH and Foxa2. Scale bar = 50 µm.

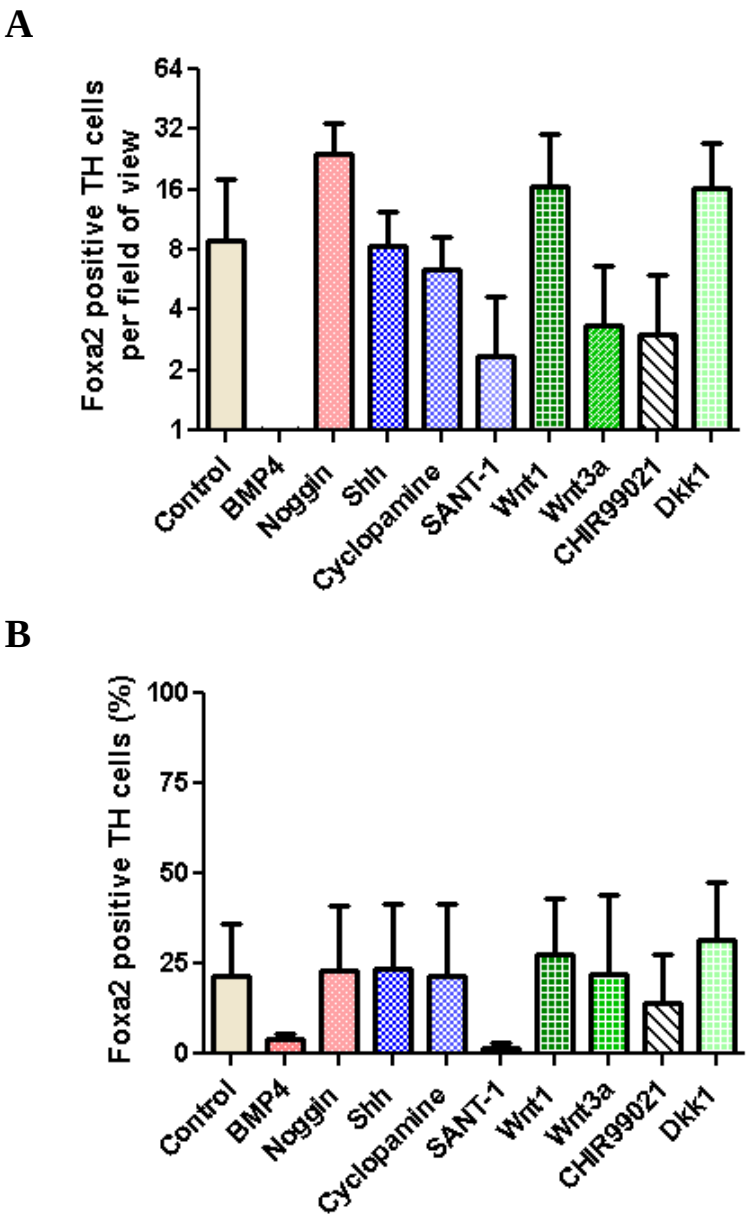

Figure 16: Analysis of TH+ Foxa2+ cells in day 20 cultures treated with factors from day 0 to day 12. (A) Number of cells co-labelled for TH and Foxa2. (B) Percentages of TH+ cells that labelled for Foxa2. Results are presented as the mean ± SEM of 3 independent experiments.

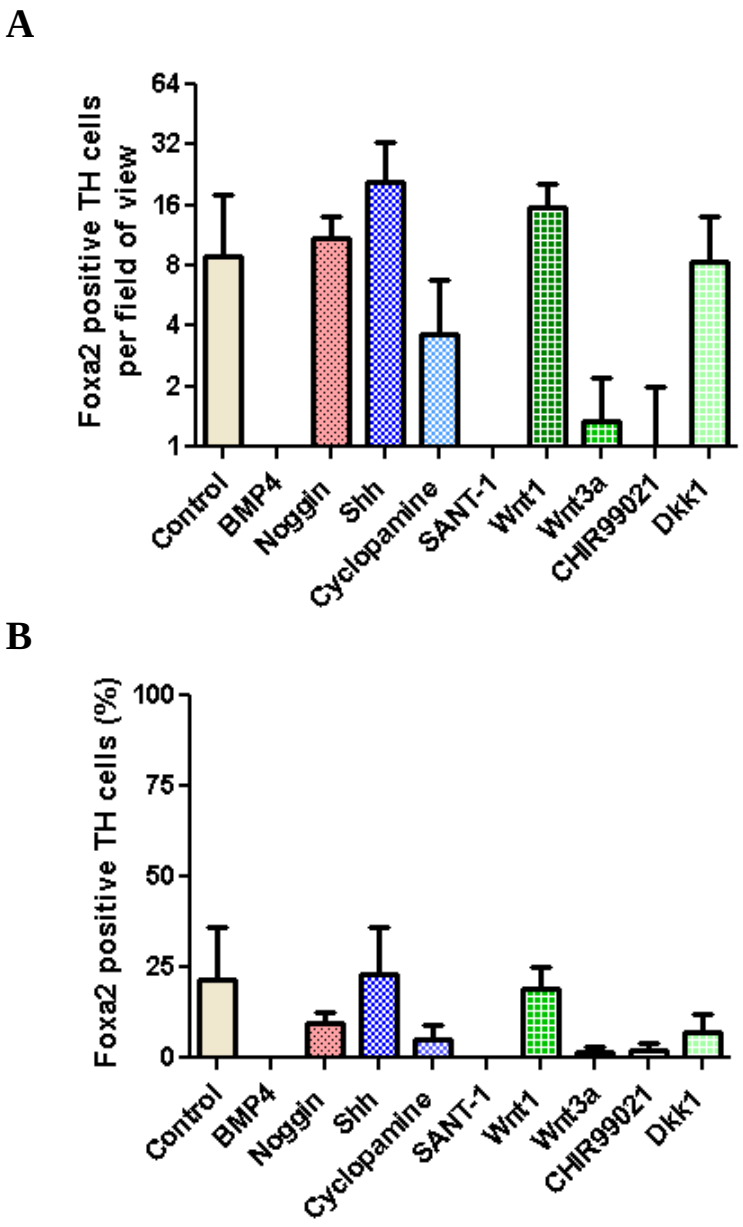

Figure 17: Analysis of TH+ Foxa2+ cells in day 20 cultures treated with factors from day 4 to day 12. (A) Number of cells co-labelled for TH and Foxa2. (B) Percentages of TH+ cells that labelled for Foxa2. Results are presented as the mean ± SEM of 3 independent experiments.

3.3.4 Effects of Retinoic Acid on neural differentiation

RA has been implicated as a factor involved in neurulation and neural patterning of the CNS (Maden, 2002) and has been reported to improve neural differentiation of ES cells (Fraichard *et al.*, 1995; Strübing *et al.*, 1995; Bain *et al.*, 1996). To investigate the effects of RA on Sox1, Lmx1a and Msx1 expression, the same differentiation protocol was applied, with exposure of RA on day 0 and day 4. A range of concentrations (10^{-10} M to 10^{-6} M) was used in all RA studies. However, all cultures incubated with RA from day 0 did not survive up to day 8 (data not shown).

The addition of RA to the Sox1-GFP and Msx1-AMP-IRES-eGFP cell lines did affect Sox1-GFP+ and Msx1-AMP+ expression (Figure 18A + 18C). However, a significant increase in AMP+ cells was detected in Lmx1a-AMP-IRES-eGFP cultures treated with 10^{-8} M RA (one-way ANOVA post-hoc Dunnett's test, $p < 0.05$, $n=3$) while exposure to 10^{-6} M RA resulted in a significant decrease in Lmx1a-AMP+ cells (one-way ANOVA post-hoc Dunnett's test, $p < 0.05$, $n=3$) (Figure 18B).

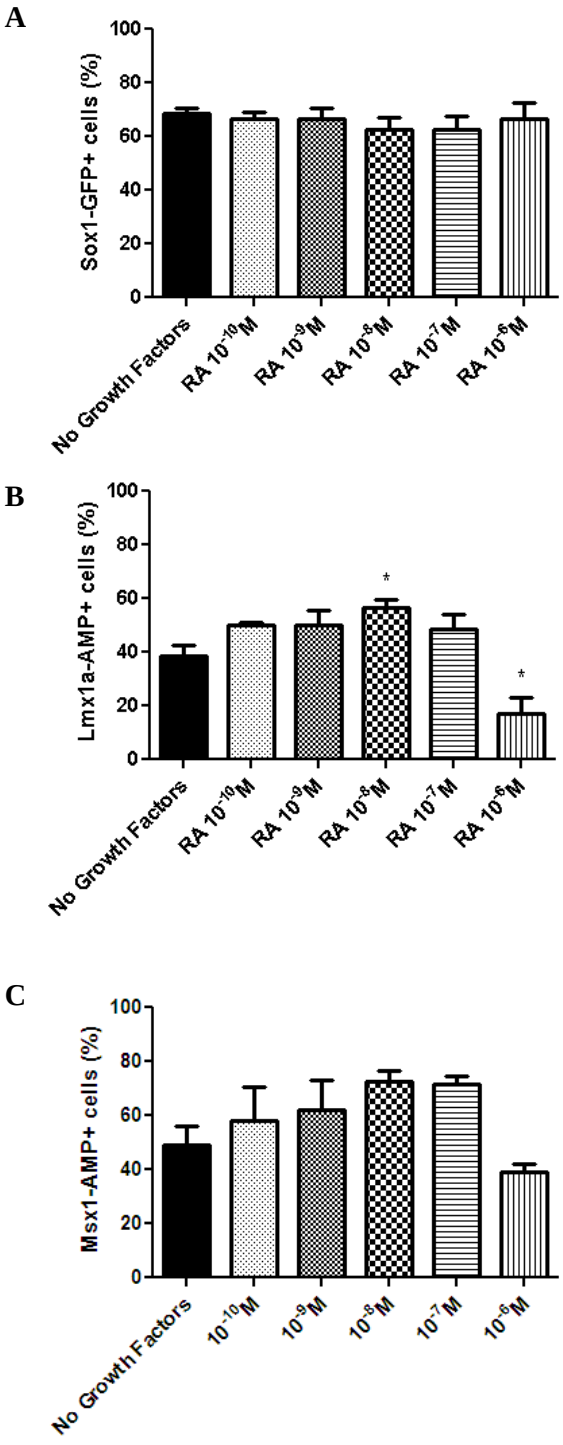

Figure 18: Analysis of reporter positive cells in response to RA treatments from day 4. Percentages of (A) Sox1-GFP+ cells, (B) Lmx1a-AMP+ cells and (C) Msx1-AMP+ cells on day 8. Results are presented as the mean ± SEM of 3 independent experiments. * indicates $p < 0.05$ compared to control with one-way ANOVA plus post-hoc Dunnett's test.

Next, the effect of RA on TH expression and cell proliferation was examined. Similar to previous experiments, exposure of RA from day 4 was prolonged to day 12 before addition of AA for terminal differentiation then cultures were immunolabelled for TH and TOPRO-3 (Figure 19). Only RA at 10^{-6} M significantly influenced TH+ numbers, causing a reduction in number of TH+ cells in day 20 cultures (one-way ANOVA post-hoc Dunnett's test, $p < 0.05$, n=3) (Figure 20A). TOPRO-3 emission values of cultures exposed to 10^{-7} M and 10^{-6} M RA were significantly lower than the controls (one-way ANOVA post-hoc Dunnett's test, $p < 0.01$, n=3) (Figure 20B).

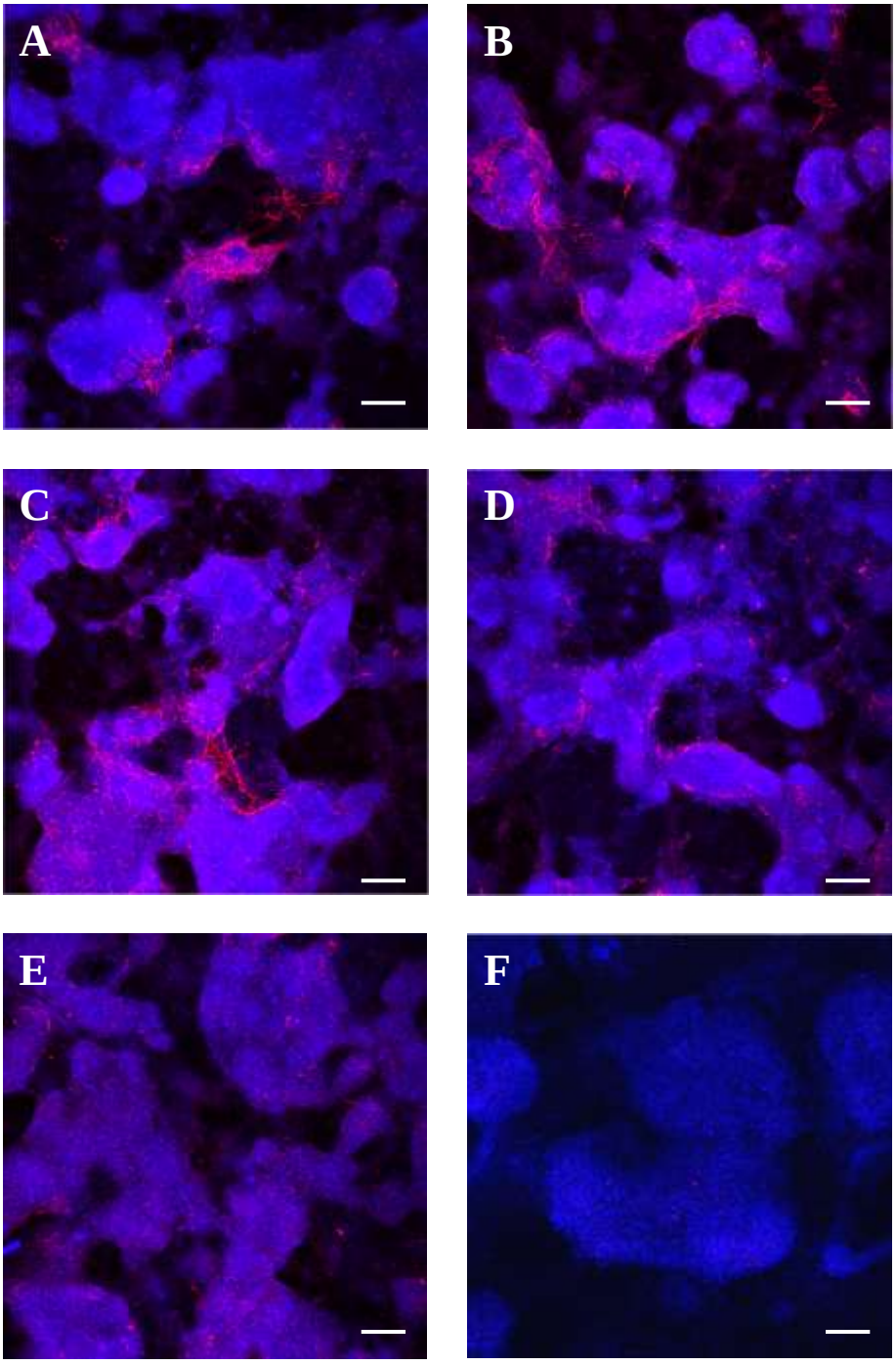

Figure 19: Terminally differentiated cultures 20 days after the induction of differentiation. Immunolabelling of cultures exposed to (A) no factors, (B) 10^{-10} M, (C) 10^{-9} M, (D) 10^{-8} M, (E) 10^{-7} M and (F) 10^{-6} M RA from day 4 to day 12 for TH (red) and TOPRO-3 (blue). Cell that co-labelled for TH and TOPRO-3 are in purple. Scale bar = 100 µm.

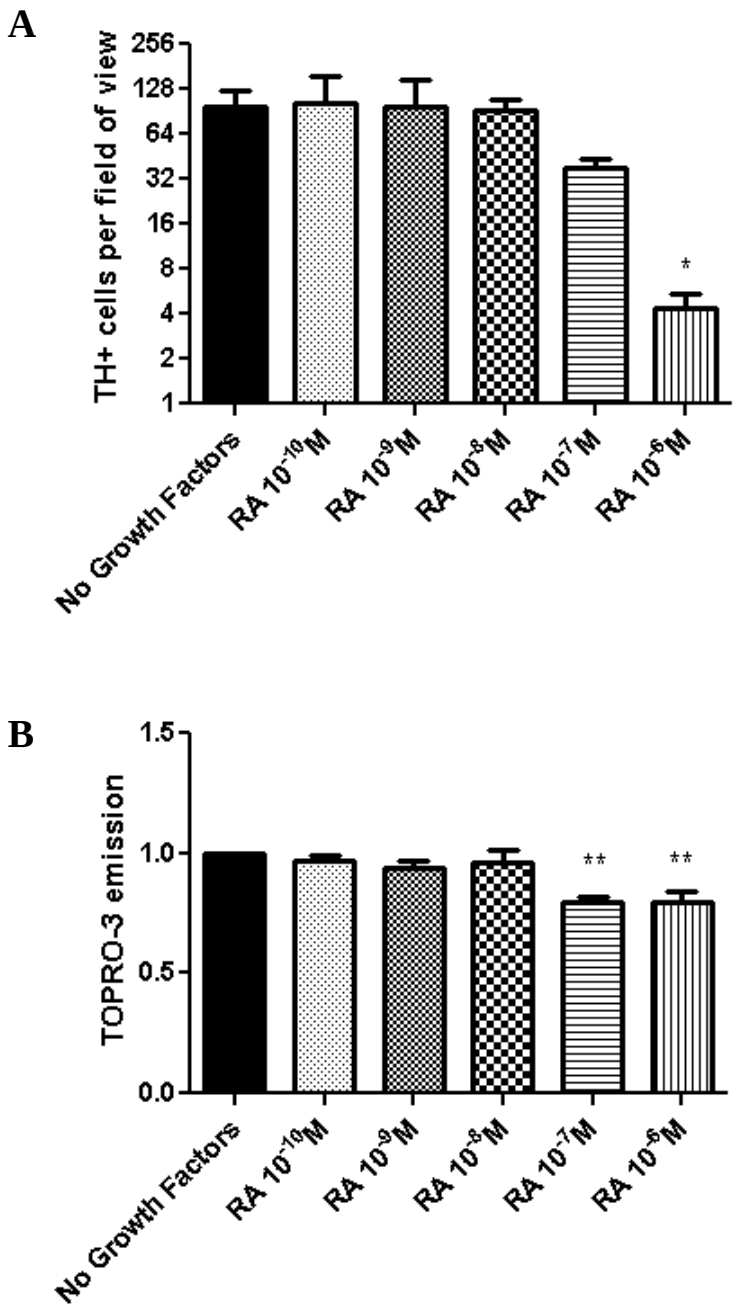

Figure 20: Effects of RA on dopaminergic neurons and cell proliferation on day 20 cultures. (A) Number of TH+ cells after RA treatment. (B) TOPRO-3 emission values of RA treated cultures. Results are presented as the mean ± SEM of 3 independent experiments. *, ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared to control with one-way ANOVA plus post-hoc Dunnett's test.

3.4 Discussion

Genetic reporters offer a powerful means to track differentiation of ES cells, provided that the expression of the reporter faithfully reproduces endogenous patterns of expression. In our laboratory, homologous recombinant ES cell lines are used to ensure high-fidelity expression. In this chapter, I showed the expression of Sox1, Lmx1a, and Msx1 during ES cell differentiation using adherent monoculture as well as the impact of activators and inhibitors of the BMP, Wnt and Shh signalling pathways on gene expression as well as TH. In addition, I have also shown the effects of different concentrations of RA on neural differentiation.

Lmx1a, and its downstream target Msx1, have emerged as key transcription factors in midbrain dopaminergic neuron differentiation (Andersson *et al.*, 2006b). In our laboratory, two reporter cell lines were generated by targeting a dual-reporter (AMP-IRES-eGFP) under the control of the endogenous Lmx1a and Msx1 promoters. A dual reporter was used so cells could be visualised using GFP if expression was strong enough, but could also introduce a substrate for β-lactamase (i.e. using the AMP reporter gene) to give a very sensitive assay or FACS sorting method. The Sox1-GFP line was given to our laboratory as a courtesy of Stem Cell Sciences.

3.4.1 Lmx1a and Msx1 expression in adherent monolayer cultures

The monolayer differentiation method was chosen to study ES cell differentiation in culture as this approach would ultimately give the best control over differentiation, as opposed to using EBs or the SDIA method. A previous study utilising the Sox1-GFP line

had already established Sox1 expression during the initial ten days ES cell monolayer differentiation (Ying *et al.*, 2003b), therefore differentiation of the same knock-in cell line using the same method allowed comparison of whether neural differentiation in our hands was indeed as efficient.

The percentage of Sox1+ cells detected during the early stages of differentiation was comparable to that previously (Ying *et al.*, 2003b). In our cultures, a large percentage of cells expressed GFP by day 4 with peak Sox1 expression detected on day 8, but as there was no significant difference between the percentages of positive cells at the two time points, we conclude that the majority ES cells converted into neuroectodermal cells by day 4 of differentiation. The decline of Sox1 expression on day 10 indicates that terminal differentiation in ES cell cultures commences at that time point, as Sox1 is expressed by mitotically active neural progenitors but is downregulated when neural progenitors exit mitosis (Pevny *et al.*, 1998; Ying *et al.*, 2003b).

In chemically defined conditions on adherent monolayer, the expression profile of Msx1 reporter line was indistinguishable from that obtained with the Lmx1a reporter line, implying that the endogenous Lmx1a transcript translates into functional protein at levels sufficient for activation of downstream gene expression. Interestingly, expression of both Lmx1a and Msx1 were widespread in the differentiating ES cell cultures with peak expression of around 40% positive cells for each reporter on day 8. Considering that the monolayer differentiation protocol is notorious for producing limited numbers of dopaminergic neurons without addition of patterning factors (Ying *et al.*, 2003b),

widespread expression of both genes seen in our cultures was unexpected. As the detection of GFP by flow cytometry requires 3 orders of magnitude more molecules of GFP per cell than the catalytic reporter AMP (Zlokarnik *et al.*, 1998), AMP expression was detected at earlier time points than GFP, resulting in an apparent lag in the proportion of cells expressing GFP. Therefore, it is possible that Sox1 is expressed two days before GFP detection however this can only be confirmed with a Sox1-AMP reporter.

3.4.2 Impact of exogenous factors on Sox1 expression

Factors that were tested in this study were chosen based on the signalling pathways previously reported to regulate Lmx1a expression *in vivo.* BMP signalling, which plays a critical role induces dorsal cells in the neural tube including the roof plate, is sufficient and necessary to induce Lmx1a expression (Millonig *et al.*, 2000; Chizhikov and Millen, 2004). Shh was first reported by Andersson *et al.* (2006b) to induce Lmx1a expression in the ventral midbrain. Contrary to that finding, Chung *et al.* (2009) reported that Wnt and Lmx1a form an autoregulatory loop that cooperates with a Shh-Foxa2 loop to regulate midbrain dopaminergic neuron development. At least one agonist and antagonist selected for stimulation or inhibition of each pathway. Stimulation of the BMP (through BMP4) and canonical-Wnt signalling pathways (through Wnt3a and CHIR99021) and inhibition of the Shh signalling pathway (through SANT-1) antagonised neural commitment of ES cells. These findings were consistent with previous reports that activation of the BMP and canonical Wnt pathways block neural differentiation (Finley *et al.*, 1999; Tropepe *et al.*, 2001; Aubert *et al.*, 2002; Xu *et al.*, 2002; Ying *et al.*, 2003a; Watanabe *et al.*, 2005; Ying *et al.*, 2008). In this study, noggin does not affect neural

induction of mES cells. This is also consistent with previous work (Finley *et al.*, 1999; Ying *et al.*, 2003b; Watanabe *et al.*, 2005). Results from dkk1 addition were similar to a previous study which showed that addition of dkk1 does not significantly increase Sox1-GFP+ cells unless added in combination with the Nodal antagonist, Lefty (Watanabe *et al.*, 2005). There have been no previous reports detailing the effect of Shh signalling on Sox1 expression although Cai *et al.* (2008) confirmed that Shh is critical for neuronal generation *in vitro* and Shh is exclusively expressed by Sox1-GFP+ cells. In the same study, the group showed that a Hh antagonist, Cur199691, decreases total cell number but does not in fact affect percentage of Sox1-GFP+ cells in culture (Cai *et al.*, 2008). Through the addition of Shh, we found that Shh does not affect neural induction. However, one of the two Shh antagonists that were examined, SANT-1, decreased the percentage Sox1-GFP+ cells. Cylopamine and SANT-1 are both small molecule antagonists that inhibit Smoothened (Smo), a 7-pass transmembrane receptor that is activated by the binding of Shh ligand to Patched 1 (Ptc1) in the Shh signalling pathway. Although both antagonists inhibit Smo, the mechanisms of action may differ (Chen *et al.*, 2002), which may explain why SANT-1 but not Cyclopamine decreased expression of Sox1. Decreases in Sox1 expression were more pronounced when cultures were exposed to the factors from day 0 onwards indicating that additions of factors early on inhibit neural induction.

3.4.3 Impact of exogenous factors on Lmx1a and Msx1 expression

Lmx1a expression was similarly affected by the same factors which decreased Sox1 expression. This suggested inhibition of neural differentiation through agonism of the BMP and canonical-Wnt pathways indirectly reduced the occurrence of Lmx1a+ cells. That

none of the factors increased the expression of both genes further support this hypothesis. As Msx1 is a direct downstream target of Lmx1a and its expression during differentiation of ES cells was determined from this study to be comparable to that of Lmx1a, similar effects were expected. However in the case of Msx1, only the addition CHIR99021 from day 0 onwards significantly decreased Msx1 expression. Unlike Sox1 and Lmx1a expression, additions of BMP4 from day 0 or 4 did not cause a decline in Msx1 expression. Although Lmx1a and Msx1 are downstream of BMPs and Wnt1 (Suzuki *et al.*, 1997; Millonig *et al.*, 2000; Chizhikov and Millen, 2004; Chung *et al.*, 2009), exogenous additions of these factors do not stimulate increases in reporter expression. As decreases in Lmx1a and Msx1 expression were observed when Sox1 expression was decreased, these findings suggest that Lmx1a and Msx1 expression are highly dependent on the efficiency of neural induction of ES cells. Further work can be carried out to identify if both transcription factor expression are restricted to Sox1+ neural progenitors or within entire cultures.

3.4.5 Impact of exogenous factors on midbrain dopaminergic neuron numbers

Despite none of the tested factors significantly increasing Lmx1a or Msx1 expression in early cultures, interestingly, exposure of noggin and dkk1 from day 0 onwards significantly increased numbers of dopaminergic cells in day 20 cultures; more than a doubling of the number of TH+ neurons was observed. Exposure to Shh from either day 0 or 4 onwards, did not increase TH+ numbers on day 20. In addition, inhibition of Shh signalling (either by SANT-1 or Cyclopamine) did not alter the number of TH^+ as well. These findings were surprising because of the obligatory role of Shh during the *in vivo*

differentiation of midbrain dopaminergic neurons. A possible explanation is that the activity Shh alone is insufficient to promote specification of ES cells into a dopaminergic neuronal fate and FGF8 and/or Wnt1 exposure is needed in combination to induce differentiation of TH+ neurons. Another possible reason is that unlike Ying *et al.* (2003b), our cultures were not replated onto extra-cellular matrices, such as fibronectin or laminin, that promote neuronal differentiation (Ma *et al.*, 2008). Cultures are generally replated when cultures contain high percentages of Sox1+ cells onto combinations of extra-cellular matrices for neural progenitor expansion and subsequent neuronal differentiation (Ying *et al.*, 2003b). I chose not to replate the cultures as the effects of extra-cellular matrices would obscure the results. Noggin improves neuronal differentiation and the yield of dopaminergic neurons obtained from mES cells cultured on PA6 stromal cells and hES cells in general (Pera *et al.*, 2004; Sonntag *et al.*, 2007; Chiba *et al.*, 2008; Zhou *et al.*, 2008a). No studies have so far reported an increase in TH+ neurons in adherent monocultures with exposure to noggin, thus the result obtained in this study was somewhat unexpected. Conversely, the significant increase in TH+ cells from dkk1 exposure is consistent with the study by Cajanek *et al.* (2009), where the group obtained enhanced yields of dopaminergic neurons from mES cells in feeder free protocols when dkk1 was added during early stages of differentiation. However treatments of noggin and dkk1 after neural induction (day 4) failed to significantly improve TH+ numbers in day 20 cultures. It is notable that Wnt1, reported to be crucial for midbrain dopaminergic neuron generation and regulation of Lmx1a expression did not affect TH+ or Lmx1a+ cells within these cultures. These findings demonstrate that timing of factor exposure affects dopaminergic differentiation of ES cells and early treatments

with noggin or dkk1 in adherent monolayer boost the generation of TH+ cells. In addition, these data show that under chemically defined monolayer conditions, early Lmx1a expression is not indicative of TH expression. Further investigations should be carried out on the combinatorial effects of both noggin and dkk1 on neural induction and generation of dopaminergic neurons from mES cells. There has been a suggestion via communication with other research groups that the recombinant Wnt1 used in these experiments is actually inactive although no assays were carried out in this study to test its activity. As there were significant increases in TH+ cells in noggin and dkk1 treated cultures, it was unclear whether the observed increases were a result of induction activities from the exogenous factors or due to increased cell proliferation. This was determined by analysis of TOPRO-3 emission values of all culture wells standardised to the values obtained from controls wells. Treatments with CHIR99021, a glycogen synthase kinase-3 (GSK-3) inhibitor, either from the start of differentiation or after neural induction resulted in a significantly larger number of cells. CHIR99021 is able to enhance ES cell growth capacity and viability, but it also induces non-neural differentiation (Ying *et al.*, 2008). It is likely that the addition of CHIR99021 promoted the expansion of ES cells and also non-neural differentiation in our cultures, as evidenced by the decrease in Sox1 expression as well as increased TOPRO-3 emission values.

The majority of dopaminergic neurons (75%) are located in the midbrain (Parmar and Li, 2007). The non-midbrain dopaminergic neurons express TH but not markers specific to the midbrain. Since all midbrain dopaminergic neurons are derived from the floor plate (Joksimovic *et al.*, 2009a), day 20 cultures were immunolabelled with antibodies against

Foxa2 and TH. TH+ cells were generally observed within mounds consisting of cells within terminally differentiated cultures. Stacks, which are multiple Z-planes at various depths, of mounds were taken to identify TH+ cells co-expressing Foxa2 on the same plane of a field of view. Approximately 22% of TH+ neurons derived via default differentiation of ES cells were of midbrain origin. Although noggin and dkk1 are able to increase TH+ cells, the percentages of TH+ cells expressing Foxa2 from each treatment was similar to the control. Both noggin, in the context of 'dual Smad' inhibition in the absence of caudalising factors, as well as dkk1, have been reported to promote or enhance the generation of anterior neural fates from ES cells (Watanabe *et al.*, 2005; Chambers *et al.*, 2009; Fasano *et al.*, 2010). It is possible that the majority of TH+ cells generated from monolayer cultures are of forebrain phenotype since predominantly forebrain neurons were derived from adherent monolayer cultures in previous studies (Gaspard *et al.*, 2008) and quantification of cells co-labelled with TH with a forebrain marker will have to be performed. These results reflect previous reports that monolayer differentiation in Neurobasal medium, with or without patterning factors, is a poor source of midbrain dopaminergic neurons (Gaspard *et al.*, 2008; Konstantoulas *et al.*, 2010). As mainly GABAergic neurons are derived from monolayer cultures in the absence of patterning factors (Jain *et al.*, 2003; Ying *et al.*, 2003b), it is likely that ES cells default into a GABAergic neuronal pathway under monolayer conditions.

3.4.6 Caudalising effects of RA

As none of the factors previously used increased the number of midbrain dopaminergic neurons, I speculated that terminally differentiated monolayer cultures consist of anterior populations of neurons. Therefore, I investigated whether RA could induce caudalised fates to our cultures. RA is a caudalising factor which induces the expression of multiple posterior genes (Durston *et al.*, 1989; Sive *et al.*, 1990; Conlon, 1995; Simeone *et al.*, 1995; Blumberg *et al.*, 1997; Maden, 2002). *In vitro* studies have shown that RA promotes neural differentiation of ES cells (Fraichard *et al.*, 1995; Strübing *et al.*, 1995; Bain *et al.*, 1996) and specifies a caudalised fate to ES-cell derived neural precursors (Okada *et al.*, 2004; Irioka *et al.*, 2005). RA signalling causes a stronger level of caudalisation compared to other caudalising signals such as Wnt and FGF (Irioka *et al.*, 2005). It is mainly used to generate motor neurons, which are located in the posterior regions of the CNS (hindbrain and spinal cord) (Wichterle *et al.*, 2002; Barberi *et al.*, 2003). Additions of RA, regardless of concentration, from day 0 of neural differentiation, but not from day 4, resulted in cell death indicating that RA suppresses ES cell survival early on. Findings from this study revealed that treatments of RA after neural induction do not impact on Sox1+ progenitors but do, however, affect the percentage of Lmx1a+ cells in monolayer cultures. These results imply that RA signalling is suppressing Lmx1a expression at a high concentration (10^{-6} M) but induces Lmx1a expression at a lower concentration (10^{-8} M). Although a similar expression pattern was observed with Msx1, effects of RA were not significant. Previous studies have shown that RA signalling promotes caudalisation in a dose-dependent manner. At higher concentrations RA induces a hindbrain phenotype while at lower concentrations a forebrain phenotype was induced (Okada *et al.*, 2004; Irioka *et al.*,

2005). In the same study, the group proved that later additions of Wnt3a on its own were insufficient for caudalisation but in the presence of RA, Wnt3a exerts a caudalising effect (Irioka *et al.*, 2005). Although Lmx1a expression in our cultures were not affected when Wnt3a was added from day 4 onwards, it is possible that the addition of both RA and Wnt3a could reduce Lmx1a-AMP+ cells. The fact that Lmx1a expression is downregulated by the caudalising effects of RA leads me to propose that the Lmx1a expressing cells in adherent monoculture are primarily of an anterior population with only a minute fraction of those cells deriving from the floor plate. Consistent with this proposal, labelling of day 20 cultures showed that there were less TH+ cells in cultures treated with higher concentrations of RA. This finding further strengthens my proposal that Lmxa+ cells derived from monolayer cultures are an anterior population and addition of high dose RA promotes differentiation of ES cell cultures into posterior neurons while suppressing anterior fates (Okada *et al.*, 2004; Irioka *et al.*, 2005). In day 20 cultures, lower concentrations of RA did not significantly elevate the numbers of TH+ cells. It may be that additional factor signals are required in combination to generate more dopaminergic neurons. The decrease in TH+ cells can be partly attributed to reduced total cell numbers in culture as significant reductions were detected in cultures treated with higher concentrations of RA (10^{-7} M and 10^{-6} M). In addition to promoting differentiation, transcription activation by RA receptors (RARs) has been reported to trigger cell cycle arrest (Donato *et al.*, 2007) and apoptosis (Altucci *et al.*, 2001; Kitareewan *et al.*, 2002; Donato and Noy, 2005) in other types of cells. It is possible that RA acts through same mechanism in ES cell cultures, thereby reducing cell numbers.

3.5 Conclusion

Through these experiments, the expressions of both Lmx1a and Msx1 *in vitro* have been shown to be widespread in culture using a chemically defined monolayer differentiation method. However, early expression of Lmx1a was not indicative of the number of TH+ neurons that were generated, indicating that Lmx1a is not a specific marker for dopaminergic neurons in monolayer cultures. Furthermore, the majority of TH+ neurons generated were not derived from the midbrain and were most likely to be forebrain dopaminergic neurons as the lack of Foxa2 expression suggests. These findings indicate that early Lmx1a+ cells are anterior neural progenitors which give rise to cultures containing forebrain but not midbrain dopaminergic neurons and the identity of these cells could be further explored.

CHAPTER 4

Enrichment and characterisation of Lmx1a positive cells

4.1 Introduction

A major obstacle to the use of stem cells for cell transplantation or screening studies is the common observation that neuronal differentiation protocols produce heterogeneous populations (Kawasaki *et al.*, 2000; Ying *et al.*, 2003b). These cultures may contain the desired cell type, but also contain other neuronal subtypes, non-neural derivatives, undifferentiated cells and unwanted types cells. Cell grafts contaminated with undifferentiated cells are unacceptable due to their predisposition to form tumours (Björklund *et al.*, 2002; Nishimura *et al.*, 2003), while for *in vitro* applications, in particular the screening of novel compounds, the presence of high numbers of unwanted cell types is likely to obscure results. The notion of extracting committed mitotic progenitors is attractive since their proliferative potential makes subsequent expansion for *in vitro* and *in vivo* applications a possibility. Although Lmx1a is prominent in midbrain dopaminergic neuron differentiation, it is also expressed in three unrelated neural tissues during development: (i) the cortical hem, a forebrain structure that gives rise to cortical and hippocampal neurons (Chizhikov *et al.*, 2010); (ii) the roof plate of the developing cerebellum (Mishima *et al.*, 2009; Chizhikov *et al.*, 2010) and (iii) the non-neurogenic roof plate of the neural tube (Millonig *et al.*, 2000; Chizhikov and Millen, 2004). In addition to upregulating Msx1 and Wnt1 (Andersson *et al.*, 2006b; Chung *et al.*, 2009), Lmx1a cooperates with other transcription factors such as Lmx1b and Foxa2 to regulate the development of midbrain dopaminergic neurons (Lin *et al.*, 2009; Nakatani *et al.*, 2010; Yan *et al.*, 2011). Chung *et al.* (2009) suggested that two autoregulatory loops synergistically control midbrain dopaminergic differentiation. These are the Wnt-Lmx1a and Shh-Foxa2 loops. Lmx1a cooperates with Lmx1b to regulate specification,

proliferation and differentiation of midbrain dopaminergic progenitors (Yan *et al.*, 2011). However, while Lmx1a is essential for neurogenesis, Lmx1b is dispensable for the generation of midbrain dopaminergic neurons from its progenitors (Guo *et al.*, 2007; Yan *et al.*, 2011). Lmx1a indirectly induces floor plate differentiation through activation of Msx1. Suppression of Nkx6.1 via Msx1 expression is necessary for floor plate marker expression in midbrain dopaminergic progenitors (Andersson *et al.*, 2006b; Nakatani *et al.*, 2010; Yan *et al.*, 2011). The key transcription factor involved in the other loop, Foxa2, functions upstream of Lmx1a and positively regulates Lmx1a and Lmx1b to induce a dopaminergic fate as well as control floor plate differentiation (Lin *et al.*, 2009; Nakatani *et al.*, 2010). Lmx1a specification of midbrain dopaminergic fate is restricted to Foxa2 expressing progenitors (Nakatani *et al.*, 2010). Both Foxa2 and Lmx1a cooperate to promote common targets in the midbrain dopaminergic neuron development network, Ngn2 and Nurr1. Foxa2 alone can directly inhibit Nkx2.2, and regulate TH expression (Ferri *et al.*, 2007; Lin *et al.*, 2009).

4.1.1 Aims

Based on the results from the previous chapter, I explored the identities of Lmx1a positive and negative populations derived from monolayer neural differentiation. The aim of this chapter is to determine whether genes associated with midbrain dopaminergic neuron development including Lmx1b, Msx1 and Foxa2 are expressed by the Lmx1a positive cells derived from our ES cell cultures. Furthermore, the differentiation potential of FACS sorted Lmx1a positive cells is examined to establish whether enrichment of cells

expressing Lmx1a generates a 'purer' population of midbrain dopaminergic neurons, than that obtained using normal differentiation paradigms.

4.2 Methods

Unless detailed below, the experimental methods used in this chapter have been described in Chapter 2.

4.2.1 FACS separation

Day 8 cultures were dissociated with accutase, resuspended in PBS with 5% FCS (v/v) and run through a 40 µm strainer (BD Biosciences, Australia) to remove clumps. Flow cytometry was performed with a FACS Aria I (BD Biosciences, Australia) for cell sorting experiments. For all experiments, the highest and lowest 25% of cells expressing AMP on day 8 were collected avoid the inclusion of cells from the other fraction into the respective sorted populations (Figure 21). After FACS extraction, Lmx1a positive and negative fractions were either used for RNA extraction, neurosphere formation or directly replated onto laminin/fibronectin/poly-d-lysine (1 µg/cm^2 each)-coated wells for terminal differentiation.

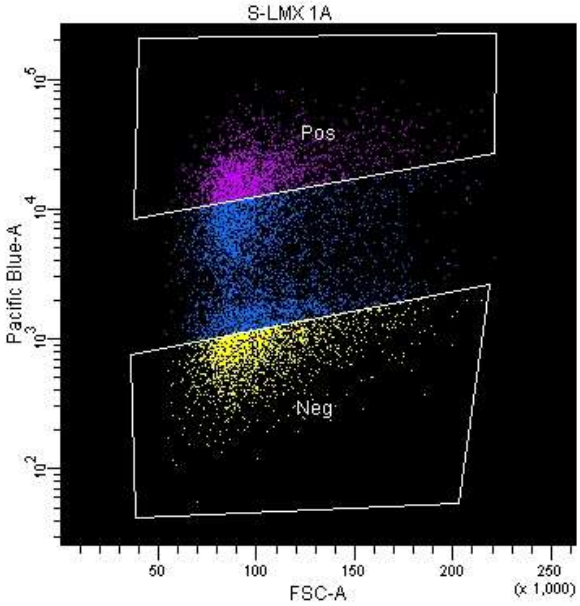

Figure 21: Typical flow cytometry blot of Pacific Blue vs. forward scatter for extraction of Amp positive (pos) and negative (neg) cells on day 8 of differentiation.

4.2.2 Quantitative Polymerase Chain Reaction

4.2.2.1 RNA extraction and cDNA synthesis

Total RNA was extracted from >1 million cells using the RNeasy Mini Kit (Qiagen, Australia) according to manufacturer's specifications. After cells were harvested, Buffer RLT with β-mercaptoethanol was added to lyse the cells and lysate was either stored at -80°C or used immediately. RNA samples were quantified using the Nanodrop® ND-1000 (Thermo Scientific, USA) spectrophotometer. For the generation of cDNA, 100 ng of RNA were used in 10 µl reaction mix together with oligodTs from the SuperScript III First-Strand Synthesis kit (Invitrogen, Australia) according to manufacturer's specifications. Synthesised cDNA were either stored at -80°C or used immediately.

4.2.2.2 Primer sequences and qPCR conditions

All quantitative Polymerase Chain Reactions (qPCR) were performed on at least 3 independent samples in triplicate using SYBR Green I Master Mix (Roche, Australia) and the Light Cycler 480 System (Roche, Australia). 1 µl of cDNA was added to a PCR Mix containing 7 µl PCR-grade water, 2 µl PCR primer pairs and 10 µl 2x Master Mix. General PCR conditions were 95°C for 10 minutes followed by 50 cycles (95°C for 10s, 60°C for 30s). For the En1 primers, conditions for the 50 cycles were 95°C for 10s, 60°C for 20s and 72°C for 30s. After PCR amplification, samples were held at 4°C for 10 seconds. For optimisation of primers, 9 µl of each sample mixed with 1 µl orange-G (Sigma, Australia) were loaded into a 1.5% (w/v) agarose gel (Progen Biosciences, Australia) containing GelRed^{TM} (1:10 000; Biotium, USA) in TAE buffer (see Appendix I). Gels were run at 70V for 50 minutes then visualised under ultraviolet (UV) light using a Gene Flash Syngene Bio Imaging and Video Graphic Printer UP-895MD system. Specificity of the used primer pairs was verified by DNA sequencing of products (Micromon, Australia). Lmx1a and Foxa2 primer sequences were previously described by Čajánek *et al.* (2009) and the En1 primer sequences by Kim *et al.* (2002). The β-actin and TATA box binding protein primers were provided by Ebba Lagerqvist from our laboratory at Monash. All other primer pairs were designed using the program "Primer Premier 3.0". Primer sequences can be found in Table 3. See Figure 22 for real time melt curve and Figure 23 for real time trace.

Data analysis was performed using the $2^{-\Delta\Delta CT}$ method (Livak *et al.*, 2001). Relative quantification values were obtained by standardising C_T values of each target gene to averaged C_T values of two housekeeping genes (β-actin and TATA box binding protein).

Table 4: Primer sequences and conditions used for qPCR.

Primer	Sequence	Size (bp)	Annealing Temperature (°C)
β-actin	5’-CTAAGGCCAACCGTGAAAAG-3’ 5’-ACCAGAGGCATACAGGGACA-3’	104	60
TATA box binding protein	5’-CTGCTGTTGGTGATTGTTGG-3’ 5’-AACTGGCTTGTGTGGGAAAG-3’	100	60
Lmx1a	5’-GAGACCACCTGCTTCTACCG-3’ 5’-CCTCCTTCAGGACAAACTCG-3’	211	60
Lmx1b	5’-GCCAAGAGGTTCTGTCAAGC-3’ 5’-GCTACTTCCGTAGGGGCTCT-3’	110	60
Msx1	5’- AAGTTCCGCCAGAAGCAGTA-3’ 5’-GAGGAAAAGAGAGGCCGAAG-3’	202	60
Wnt1	5’-CTACTGGCACTGACCGCTCT-3’ 5’-GAATCCGTCAACAGGTTCGT-3’	104	60
Wnt3a	5’-GGGACCCCAGTACTCCTCTC-3’ 5’-GGGCATGATCTCCACGTAGT-3’	109	60
Wnt5a	5’-AGACAGGCATCAAGGAATGC-3’ 5’-GTCTCTCGGCTGCCTATTTG-3’	112	60
Foxa2	5’–CATCCGACTGGAGCAGCTA-3’ 5’–CATAGGATGACATGTTCATGGAG-3’	171	60

BF-1	5’-CTGACGCTCAATGGCATCTA-3’ 5’-TCACGAAGCACTTGTTGAGG-3’	118	60
Sox1	5’-AGACAGCGTGCCTTTGATTT-3’ 5’-TGGGATAAGACCTGGGTGAG-3’	124	60
Sox2	5’-GAACGCCTTCATGGTATGGT-3’ 5’-TCTCGGTCTCGGACAAAAGT-3’	125	60
En1	5’-TCAAGACTGACTCACAGCAACCCC-3’ 5’-CTTTGTCCTGAACCGTGGTGGTAG-3’	381	60
Nestin	5’-CTCGAGCAGGAAGTGGTAGG-3’ 5’-GCCTCTTTTGGTTCCTTTCC-3’	140	60
BMP2	5’-GAACCCAGGTGTCTCCAAGA-3’ 5’-TGACGCTTTTCTCGTTTGTG-3’	143	60
Hoxb4	5’-CAGGTCCTGGAGTTGGAGAA-3’ 5’-GTTGGGCAACTTGTGGTCTT-3’	159	62
Aldh1a1	5’-GGGCTGACAAGATTCATGGT-3’ 5’-GGAAAATTCCAGGGGATGAT-3’	109	60
Notch1	5’-TGTTGTGCTCCTGAAGAACG-3’ 5’-GCAACACTTTGGCAGTCTCA-3’	110	60
Jag1	5’-AAAGACCACTGCCGTACCAC-3’ 5’-GGGGACCACAGACGTTAGAA-3’	115	60
Dll4	5’-TCAGCCAAATCATCATCCAA-3’ 5’-ACTGCAGATGACCCGGTAAG-3’	110	60

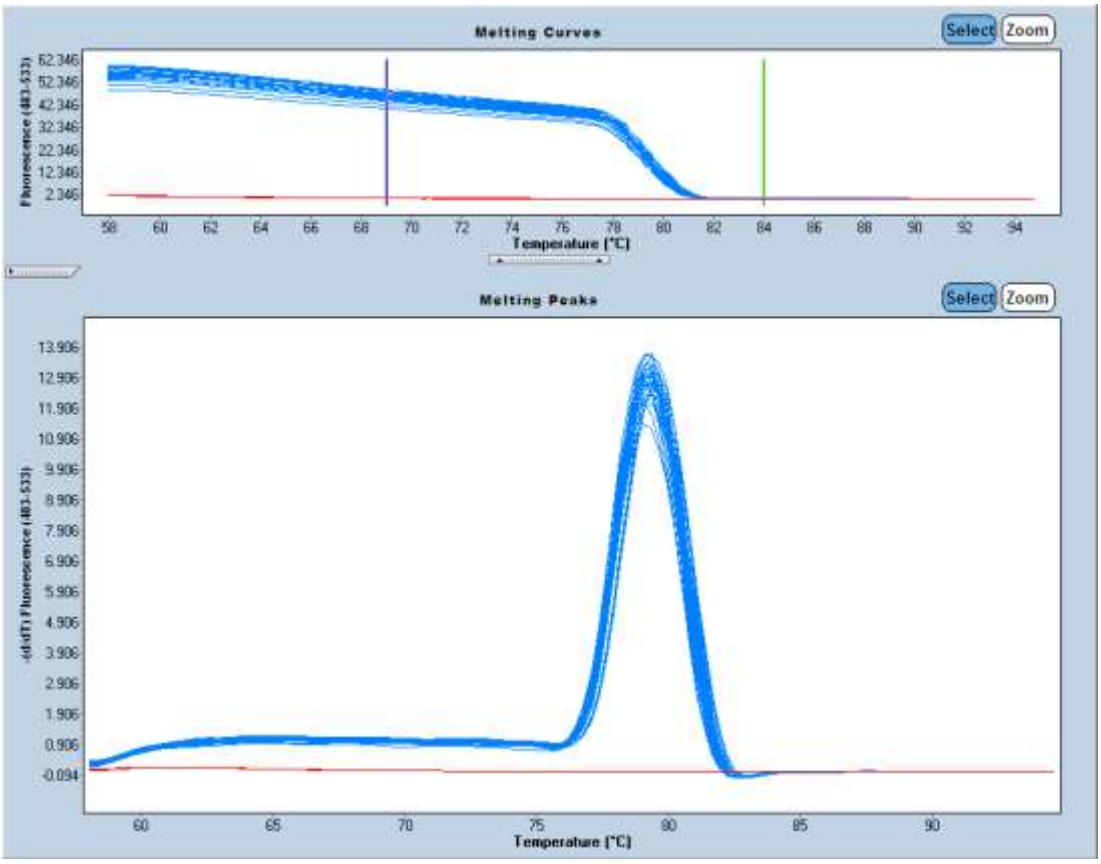

Figure 22: Real time melt curves of PCR samples. Graph representing fluorescence vs. temperature during a typical qPCR experiment. Samples melted at the same temperature, indicating they contain products of similar size.

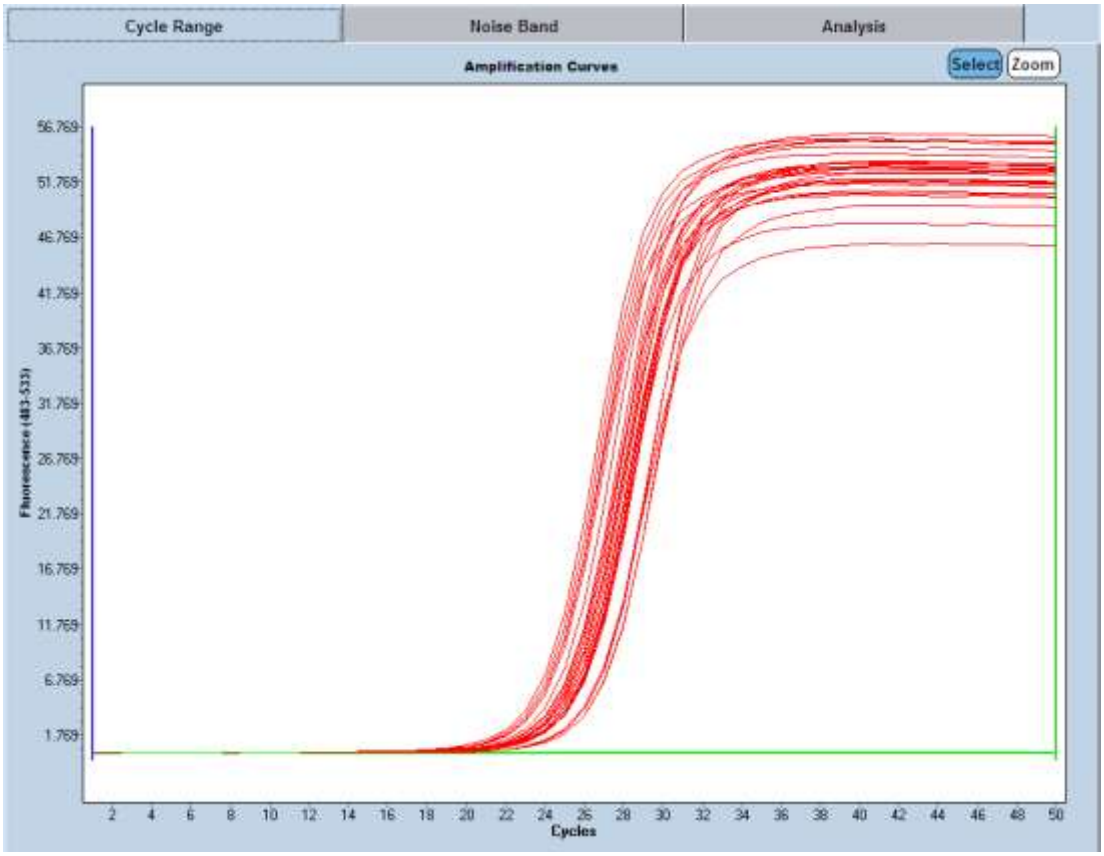

Figure 23: Graph representing fluorescence vs. number of cycles, showing amplification of PCR products in real time. C_T values for samples are determined from the qPCR curves.

4.2.3 Formation and terminal differentiation of neurospheres

Both fractions of sorted cells were seeded at 5 x 10^4 cells/well in ultra low-adherent 96-well plates in StemPro® NS cell culture medium (Invitrogen, Australia) supplemented with FGF2 (10 ng/mL; Peprotech, Israel) and EGF (10 ng/mL; Invitrogen, Australia). After 5 days, 10 - 20 neurospheres were directly plated onto laminin/fibronectin/poly-d-lysine (1 µg/cm^2 each)-coated 96-well plates in N2B27 medium supplemented with FGF2 (20 ng/mL; Peprotech, Israel) for 3 days. The media was then replaced with N2B27 containing BDNF (20 ng/mL; Peprotech, Israel) and L-ascorbic acid (200 µM; Merck, Australia) for a further 10 days

4.2.4 Quantification of viable cells in neurospheres

Neurospheres were centrifuged at 200*xg* for 2 minutes. Media was aspirated and the neurospheres incubated with accutase (Sigma, Australia) for 15 minutes at 37°C. Neurospheres were vigorously resuspended by pipetting up and down to ensure neurospheres were broken down into single cells, before the addition of mES media to stop the digestion of cells. 10 µl of the single cell suspension was then mixed with 10 µl of trypan blue and the live cells immediately counted with a haemocytometer. Only cells that did not take up trypan blue were deemed to be viable.

4.2.5 Direct differentiation of FACS sorted cells

Sorted positive and negative cells were replated at either 3 x10^5 cells/cm^2 (low-density) or 1.5 x 10^6 cells/cm^2 (high-density). Extracted cells were plated in N2B27 media supplemented with FGF2 (20 ng/mL; Peprotech, Israel) alone or in combination with Shh

(200 ng/mL; Peprotech, Israel) and FGF8 (100 ng/mL; Peprotech, Israel) or Shh (500 ng/mL; Peprotech, Israel) and RA (10^{-6} M; Sigma, Australia). After 5 days the medium was replaced with N2B27 supplemented with BDNF (20 ng/mL; Peprotech, Israel) and L-ascorbic acid (200 µM; Merck, Australia) for a further 7 days.

4.2.6 Rosette formation and patterning

Cells that had been differentiated for 7 days on adherent monolayer, as described in 2.2.4, were replated at 4 x 10^5 cells/cm^2 onto laminin/fibronectin/poly-d-lysine (1 µg/ cm^2 each) wells in N2B27 supplemented with Shh (500 ng/ml; Peprotech, Israel), Jagged1 (Jag1; 500 ng/mL; R&D Systems, USA) and Delta-like ligand 4 (Dll4; 500 ng/ml; R&D Systems, USA) for an additional 6 days. For neural patterning experiments, day 13 cultures were replated at 5 x 10^4 cells/cm^2 onto laminin (1 µg/cm^2) coated wells supplemented with FGF2 (20 ng/mL; Peprotech, Israel), Shh (200 ng/mL; Peprotech, Israel) and FGF8 (100 ng/mL; Peprotech, Israel) (dopaminergic neurons) or FGF4 (100 ng/mL; Peprotech, Israel) (serotonergic neurons) for 3 days followed by withdrawal of FGF2 for a further 3 days. After 6 days, the medium was replaced with N2B27 supplemented with BDNF (20 ng/mL; Peprotech, Israel) and L-ascorbic acid (200 µM; Merck, Australia) for further 7 days.

4.3 Results

4.3.1 Analysis of sorted day 8 Lmx1a+ and Lmx1a- cells

The presence of mRNA for markers involved in brain development was examined using qPCR. Markers of midbrain dopaminergic progenitors (Lmx1a, Msx1, Aldh1a1, Lmx1b, En1), forebrain (BF-1), hindbrain (Hoxb4), ventral floor plate (Foxa2), dorsal roof plate (BMP2), Wnt signalling (Wnt1, Wnt3a, Wnt5a), Notch signalling (Notch1, Jag1, Dll4) and NS cells (Sox1, Sox2, Nestin) were selected for investigation. Primers were first optimised on unsorted day 8 monolayer cultures for reverse transcriptase PCR prior to qPCR runs. Bands obtained from gel electrophoresis confirmed the presence of the selected markers in day 8 cultures (Figure 24).

Figure 24: PCR bands for unsorted day 8 samples run on agarose gel and visualised under UV light using a Gene Flash Syngene Bio Imaging and Video Graphic Printer UP-895MD system. Primers were first optimised before used in actual qPCR runs.

To determine the efficiency of FACS separation, the fold changes in Lmx1a expression was determined by comparing of C_T values of FACS separated Lmx1a+ and Lmx1a- cells against unsorted day 1 cells. qPCR analysis of the Lmx1a positive and negative fractions showed that transcript levels of Lmx1a over two orders of magnitude higher in the positive fraction compared to the negative fraction (Student's t-test, $p < 0.001$, $n=3$) (Figure 25).

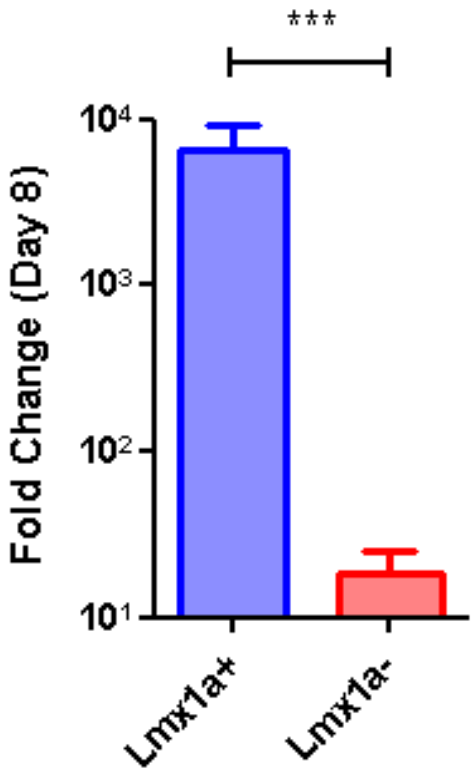

Figure 25: Fold up-regulation of Lmx1a transcript levels in day 8 Lmx1a positive and negative fractions compared to unsorted day 1 cells. *** indicates $p < 0.001$ between Lmx1a+ and Lmx1a- cells with Student's t-test. Results are presented as the mean ± SEM of at least 3 independent experiments.

After confirming the efficiency of FACS separation, qPCR runs were carried with the rest of the primers that have been optimised (see Figure 24). The fold changes of every gene in Lmx1a+ and Lmx1a- cells compared to day 1 unsorted cells are detailed in Figure 26.

Gene	Lmx1a+ cells	Lmx1a- cells
Sox2	1.1 ± 1.2	- 1.7 ± 0.54
Aldha1a	1.9 ± 1.2	4.7 ± 2.2
Jag1	2.7 ± 1.3	3.7 ± 0.9
Wnt5a	2.9 ± 1.3	1.7 ± 0.4
Foxg1	3.4 ± 1.5	21.3 ± 13.7
Dll4	3.4 ± 1.5	10.6 ± 3.1
Notch1	4.3 ± 1.5	6.0 ± 3.4
Bmp2	6.5 ± 4.9	21.1 ± 9.1
En1	9.7 ± 4.0	5.4 ± 2.9
Hoxb4	10.1 ± 8.7	33.9 ± 21.2
Foxa2	16.3 ± 2.5	263.8 ± 150.3
Sox1	21.6 ± 8.0	40.2 ± 6.9
Nestin	57.5 ± 25.7	31.5 ± 16.7
Lmx1b	321.0 ± 186.4	53.2 ± 35.2
Wnt3a	903.7 ± 286.5	177.8 ± 105.1
Msx1	1428.0 ± 1182.7	123.0 ± 52.7
Lmx1a	6612.52 ± 4680.69	18.41 ± 11.65
Wnt1	101303.3 ± 68276.0	7482.7 ± 7192.3

Figure 26: Fold differences in expression levels for selected genes of day 8 Lmx1a+ and Lmx1a- cells compared to unsorted day 1 cells. Results are presented as the mean ± SEM of at least 3 independent experiments.

The fold differences in expression levels for the genes in the positive fraction were compared to the negative fraction (Figure 27). Nestin, En1 and Lmx1b expression were significantly higher in the positive fraction (Student's t-test, $p < 0.05$, $n \geq 3$). Interestingly, the differences in expression of Wnt3a and the downstream targets of Lmx1a, Msx1 (Student's t-test, $p < 0.01$, $n \geq 3$) and Wnt1 (Student's t-test, $p < 0.001$, $n \geq 3$) between both fractions were even more pronounced. In the negative fraction, expression of Aldh1a1, Dll4, BF-1, BMP2 and intriguingly, Foxa2 were significantly upregulated (Student's t-test, $p < 0.05$, $n \geq 3$) compared to the positive fraction.

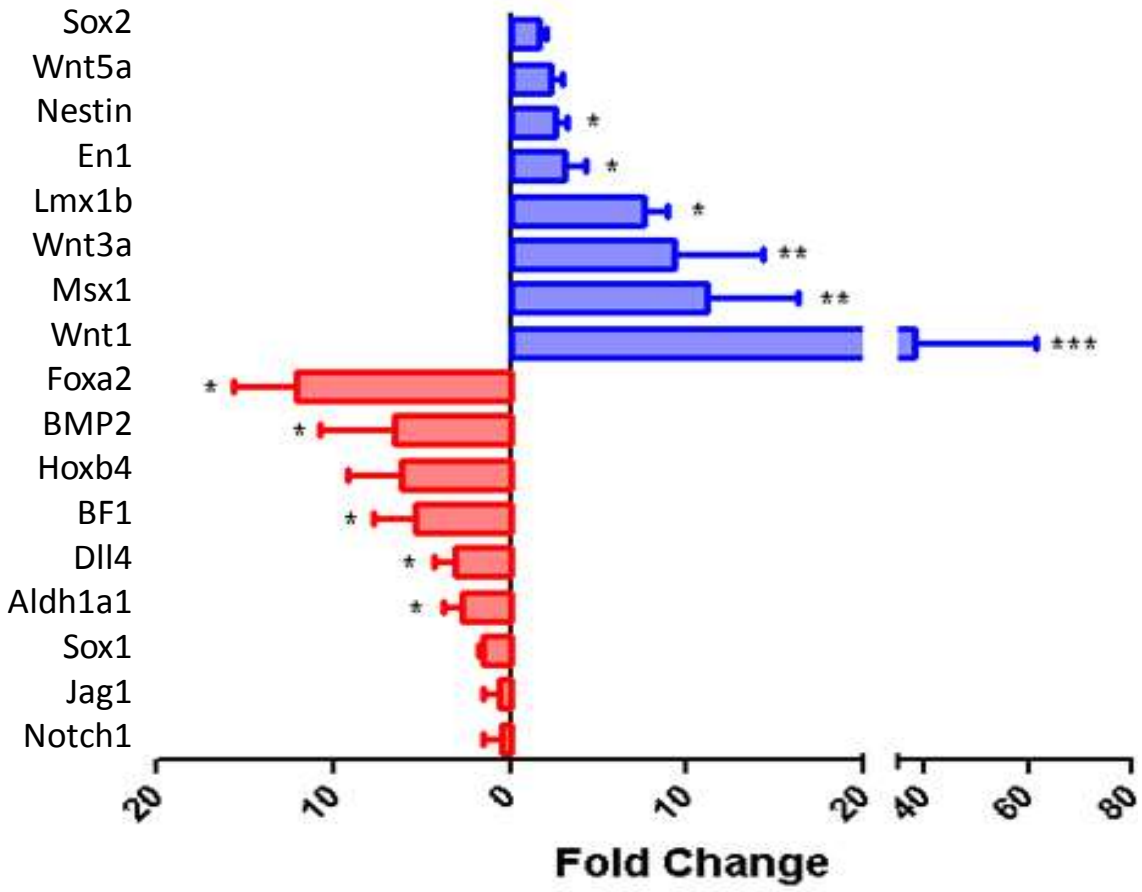

Figure 27: Fold differences in expression levels for selected genes of the positive fraction compared to the negative fraction. Blue bars represent upregulation in the positive fraction. Red bars represent upregulation in the negative fraction. *, **, *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, between Lmx1a+ and Lmx1a- cells with Student's t-test. Results are presented as the mean ± SEM of at least 3 independent experiments.

4.3.2 Formation of neurosphere from FACS sorted cells

Neurospheres are spherical clusters, formed by committed neural progenitors in suspension, which can be propagated *in vitro*. The neurosphere assay has been predominantly used to isolate NS cells and progenitors and to generate neurons as well as glia (Bez *et al.*, 2003; Conti *et al.*, 2005; Reynolds and Rietze, 2005; Dottori and Pera, 2008; Zeng *et al.*, 2011). As there was a significant difference in Nestin, an intermediate filament protein used as a marker for neural progenitors in both the central and peripheral nervous system (Lendahl *et al.*, 1990), expression between Lmx1a+ and Lmx1a- cells, I speculated that the majority of the neural progenitors were in the positive fraction. Therefore, the capability of these separated cells to form neurospheres was investigated. When seeded into ultra low-attachment plates, both fractions of sorted cells were able to form spheres in suspension within 5 days (Figure 28).

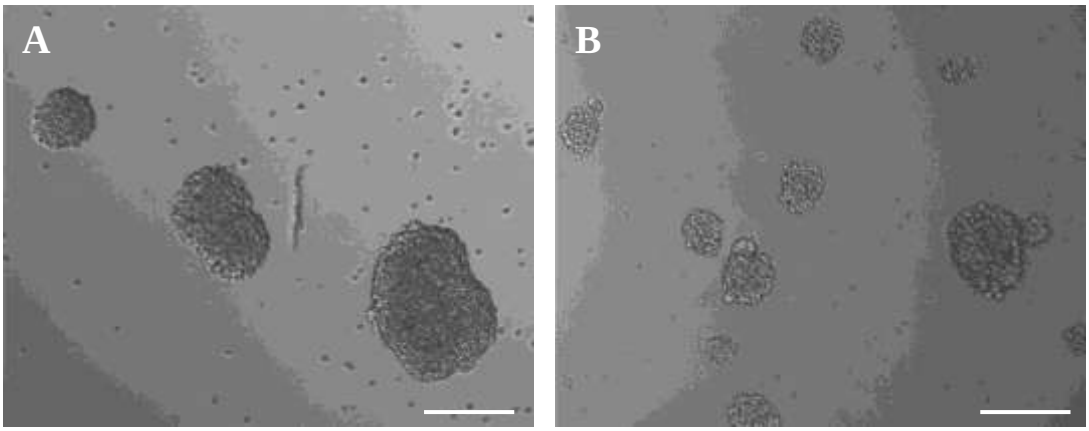

Figure 28: Spheres formed by separated Lmx1a cell fractions after 5 days in suspension. White light images of spheres formed by (A) Lmx1a+ and (B) Lmx1a- cells. Scale bar = 100 µm.

GFP expression was analysed 5 days after cells were seeded in suspension. GFP expression, instead of AMP expression, was chosen for analysis as GFP detection did not require incubation with any substrates. After 5 days, neurospheres formed by Lmx1a+ cells still retained GFP expression in parts of the spheres while no GFP expression was observed in the Lmx1a- neurospheres (Figure 29).

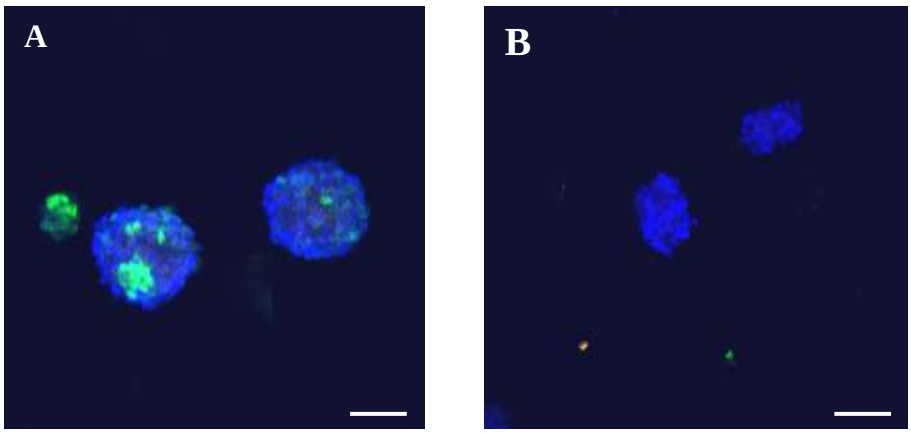

Figure 29: Spheres formed by separated Lmx1a cell fractions after 5 days in suspension Nuclear staining of spheres formed by (A) Lmx1a+ and (B) Lmx1a- cells with TOPRO-3 (blue). Lmx1a-eGFP+ cells are in green. Scale bar = 100 µm.

The total number of spheres per well formed by Lmx1a+ and Lmx1a- cells were similar, with no significant difference in total number of spheres (Figure 30A). However, there was a significant difference in the total viable cells counted per 96 well, with more than twice the number of live cells from Lmx1a+ neurospheres compared to Lmx1a- neurospheres (Student's t-test, $p < 0.05$, $n=3$) (Figure 30B). Furthermore after 5 days in suspension, 45% of the Lmx1a+ cells expressed GFP, a significantly higher amount than

the percentage of Lmx1a- cells expressing GFP (2.5%) (Student's t-test, p <0.001, n=3) (Figure 30C). The neurospheres formed by Lmx1a+ cells were also significantly larger than Lmx1a- neurospheres, with an average diameter 120 µm compared to 65 µm (Student's t-test, p <0.01, n=3) (Figure 30D).

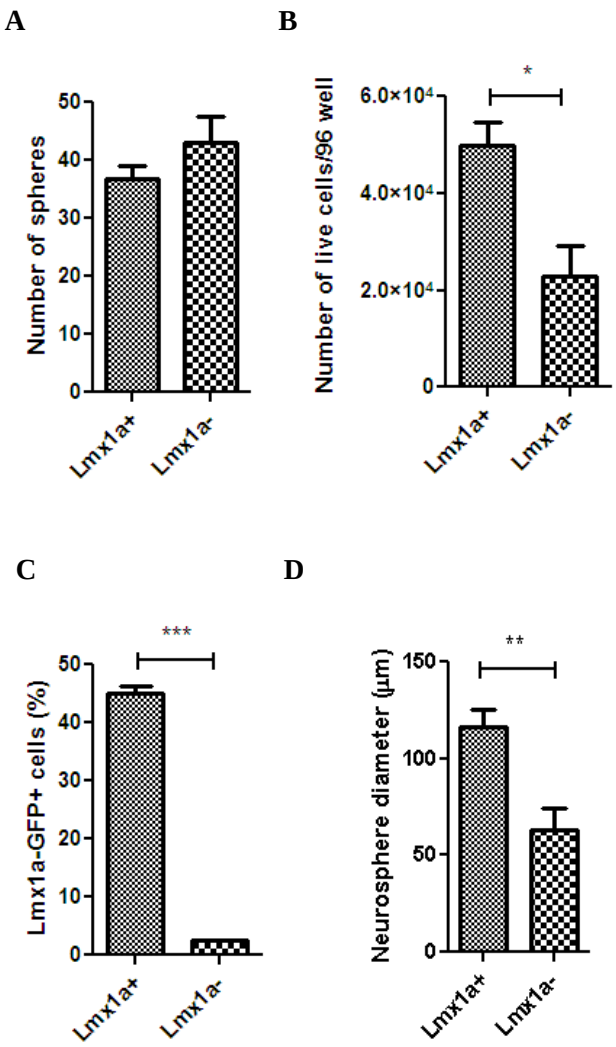

Figure 30: Comparison of neurospheres formed by separated Lmx1a cells. Differences in (A) total spheres, (B) number of live cells within spheres, (C) percentage of Lmx1a-eGFP+ cells and (D) diameter of neurospheres of Lmx1a positive and negative cells. *, **, *** indicate p <0.05, p <0.01 and p <0.001, respectively, between Lmx1a+ and Lmx1a- cells with Student's t-test. Results are presented as the mean ± SEM of 3 independent experiments.

To determine the neurogenic potential of the neurospheres to generate neurons, neurospheres were directly plated and terminally differentiated in the absence of any patterning factors. Immunocytochemistry against TUJ-1 was then performed. TUJ-1 is a pan-neuronal marker expressed in the central and peripheral nervous systems (Alexander *et al.*, 1991; Katsetos *et al.*, 1998) and is used to identify neurons in ES cell cultures (Shim *et al.*, 2004; Erceg *et al.*, 2008). Terminally differentiated cultures from Lmx1a+ neurospheres were highly immunoreactive towards TUJ-1 (Figure 31A). In contrast, only a small number of cells were TUJ-1+ in Lmx1a- neurosphere cultures (Figure 31B). This indicates that neurospheres formed by Lmx1a+ cells are capable of producing highly neuronal cultures, while the Lmx1a- spheres presumably give rise to non-neuronal cell types as the lack of TUJ-1 expression suggests.

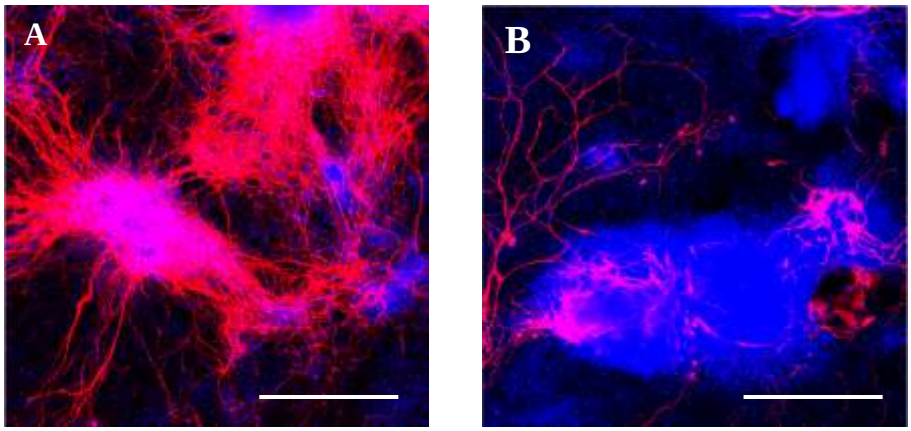

Figure 31: Terminally differentiated cultures 13 days after plating of neurospheres. Immunolabelling of cultures arising from (A) Lmx1a+ and (B) Lmx1a- neurospheres for TUJ-1 (red) and TOPRO-3 (blue). Scale bar = 100 µm.

4.3.3 Terminal differentiation of Lmx1a+ cells

As Lmx1a+ cells were able of form neurospheres capable of generating highly neuronal cultures, the differentiation potential of these cells was further examined. Lmx1a+ cells were directly plated and allowed to terminally differentiate. Immunolabelling for various neural markers was performed on the terminally differentiated cultures. The terminally differentiated cultures contained a large number of cells immunoreactive for GABA, Nestin, TUJ-1 and glial fibrillary acidic protein (GFAP) (Figure 32A+ 32B + 32C + 32E). Gamma-amino butyric acid (GABA) is a major inhibitory neurotransmitter in the CNS and GABA producing neurons are known as GABAergic neurons (Farrant, 2002; Watanabe *et al.*, 2002). GFAP is the major protein constituent of glial intermediate filaments in astrocytes of the CNS (Eng, 1985). In contrast to the proportion of GABA+ cells, the number of TH immunoreactive cells observed was low (Figure 32A + 32D). GFP expression was detected in only a small number of cells (Figure 32B + 32D + 32E). Among the TH+ cells observed, only a few of them co-localised with either Lmx1a or Foxa2 expression (Figure 32D). Moreover, no TH+ cells co-localising with both Lmx1a and Foxa2 expression were detected in any of the cultures. In addition, cells that labelled for GABA and/or TUJ-1 were also mostly Lmx1a- cells (Figure 32E). The presence of cells expressing Islet1, which is required for the generation of motor neurons (Pfaff *et al.*, 1996), was detected in the cultures (Figure 32F).

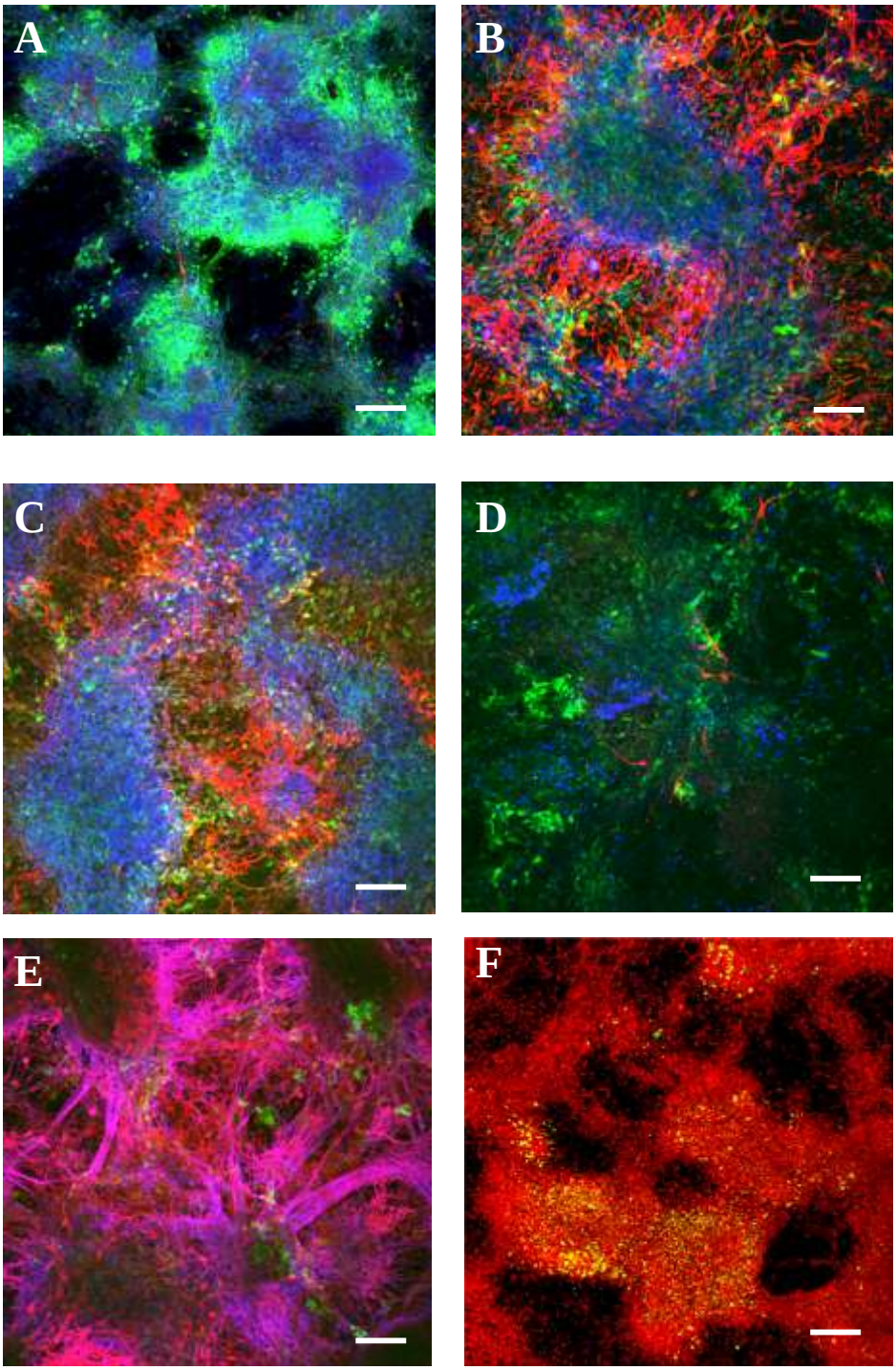

Figure 32: Terminally differentiated cultures 12 days after plating of Lmx1a+ cells. (A, B) Cultures labelled for (A) TH (red) and GABA (green) and (B) Nestin (red) and TUJ-1 (green). DAPI (blue) was used as a nuclear counter stain. (C, D, E) Cultures labelled for (C) GFAP (red) and DAPI (blue), (D) TH (red) and Foxa2 (blue) and (E) GABA (red), and TUJ-1 (blue). Lmx1a-eGFP+ cells are in green. (F) Culture labelled for Islet1 (green) and TOPRO-3 (red). Scale bar = 100 µm.

Quantification of GABA+, TH+ and Islet1+ cells within the terminally differentiated cultures revealed that the vast majority of the cells expressed GABA (Figure 33). The number of GABA+ cells was significantly higher than TH+ and Islet1+ cells (one-way ANOVA post-hoc Bonferroni's test, $p < 0.001$, $n=3$). The difference in number of TH+ and Islet1+ cells was also significant (one-way ANOVA post-hoc Bonferroni's test, $p < 0.001$, $n=3$). Upon closer examination, the GABA+ cells frequently labelled for GAD67 (Figure 34A) and SatB2 (Figure 34B). Glutamic acid decarboxylase (GAD) is the rate limiting enzyme in GABA synthesis and is a specific marker for GABAergic neurons within the CNS (Farrant, 2002; Watanabe *et al.*, 2002). SatB2 is a specifier and marker of upper-layer neurons of the neocortex (Britanova *et al.*, 2008). Co-expression of GABA with both markers indicates that these cells are forebrain GABAergic neurons with upper-layer identity. Immunolabelling for TBR-1, a regulator and marker for deep-layer cortical neurons (Hevner *et al.*, 2001), showed that TBR-1+ cells were rare in the cultures (Figure 35), further confirming the upper-layer identity of the GABA+ cells.

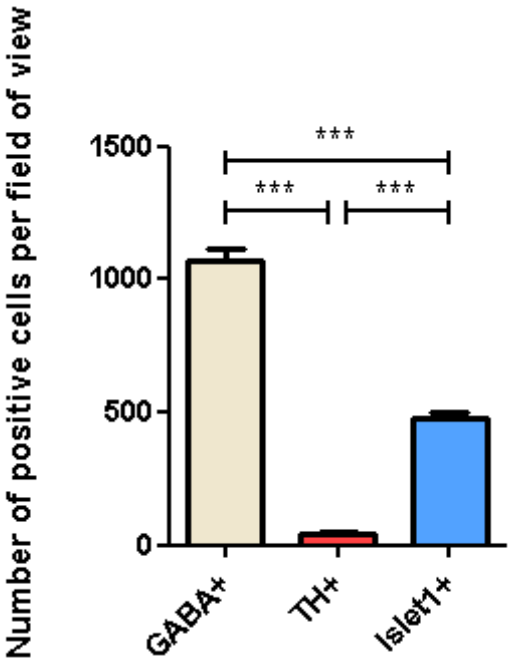

Figure 33: Number of cells labelling for GABA, TH and Islet1 in a single field of view of approximately 1.6 x 10^5 µm^2. *** indicates p <0.001, respectively, between GABA+,TH+ and Islet1+ cells with one-way ANOVA post-hoc Bonferroni's test, p <0.001, n=3. Results are presented as the mean ± SEM of 3 independent experiments.

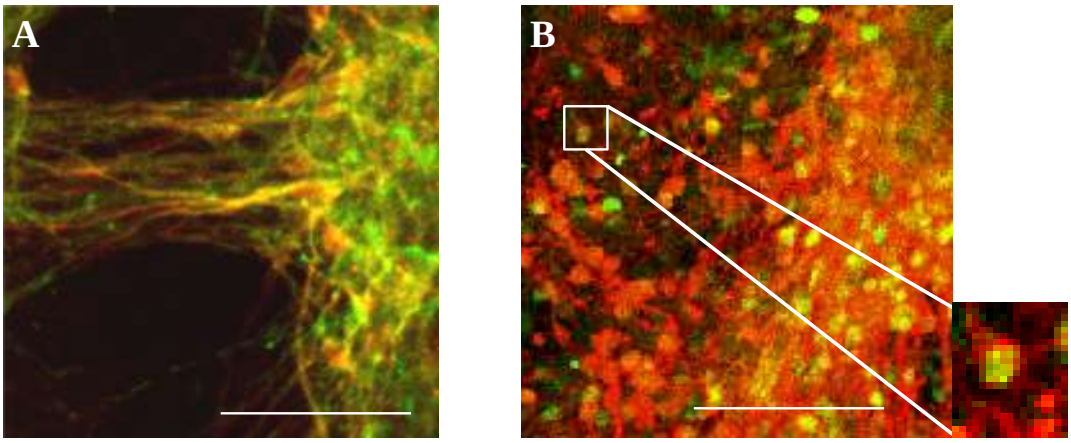

Figure 34: Terminally differentiated cultures 12 days after plating of Lmx1a+ cells. Immunolabelling of cultures for (A) GABA (red) and GAD67 (green) and (B) GABA (red) and SatB2 (green). Scale bar = 100 µm.

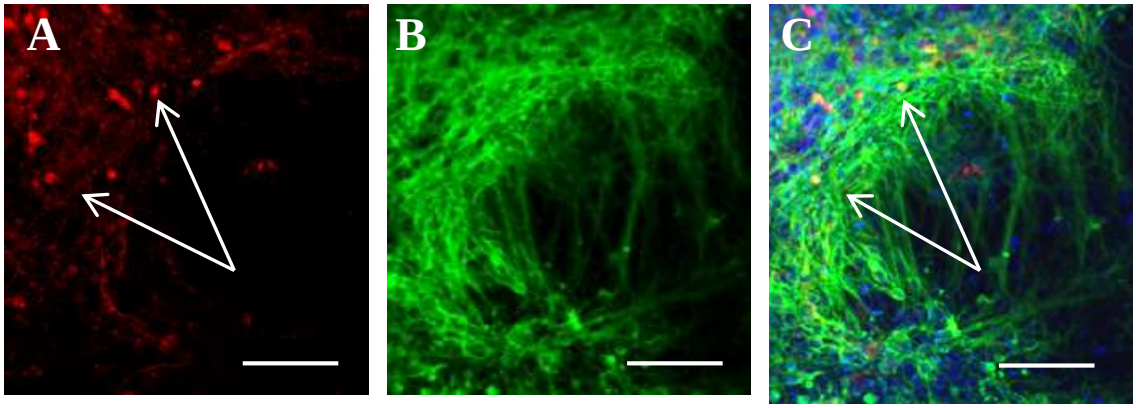

Figure 35: Terminally differentiated cultures 12 days after plating of Lmx1a+ cells. Immunolabelling of cultures for (A) TBR-1 (red) and (B) TUJ-1 (green). (C) Overlay of TBR-1 and TUJ-1 with DAPI (blue). Arrows indicate TBR-1+ cells co-localised with TUJ-1 and DAPI. Scale bar = 100 µm.

As terminally differentiated Lmx1a+ cells gave rise to highly GABA+ cultures in the absence of patterning factors, the effects of Shh, FGF8 and RA combinations on the development of distinct neuronal phenotypes were investigated. The combination of Shh and FGF8 is commonly used to generate dopaminergic neurons from ES cell cultures while additions of Shh together with RA are used to obtain motor neurons (Barberi *et al.*, 2003). The majority of GABA+, TH+ and Islet1+ cells in all cultures co-localised with TUJ-1 immunolabelling, indicating GABAergic, dopaminergic and motor neurons were present (Figure 36A + 37A + 38A). The addition of patterning factors did not significantly increase the number of GABA+, TH+ or Islet1+ neurons in all terminally differentiated cultures (Figure 36B + 37B + 38B).

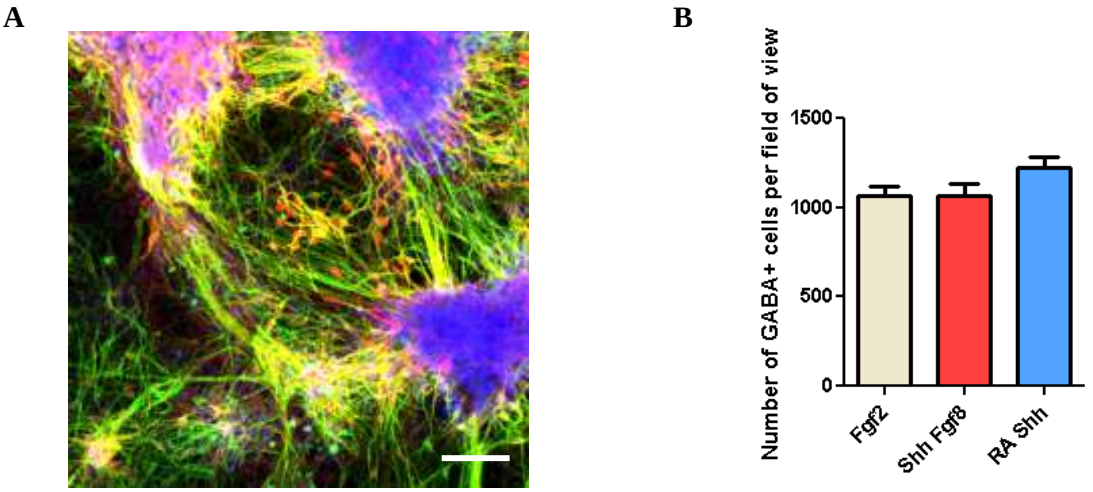

Figure 36: Quantification of GABA+ cells in terminally differentiated cultures from Lmx1a+ cells. (A) Cultures immunolabelled for GABA (red), TUJ-1 (green) and DAPI (blue). (B) Number of GABA+ cells in cultures exposed to different combinations of growth factors. Cell that co-labelled for GABA and TOPRO-3 are in purple. Scale bar = 100 µm. Results are presented as the mean ± SEM of 3 independent experiments.

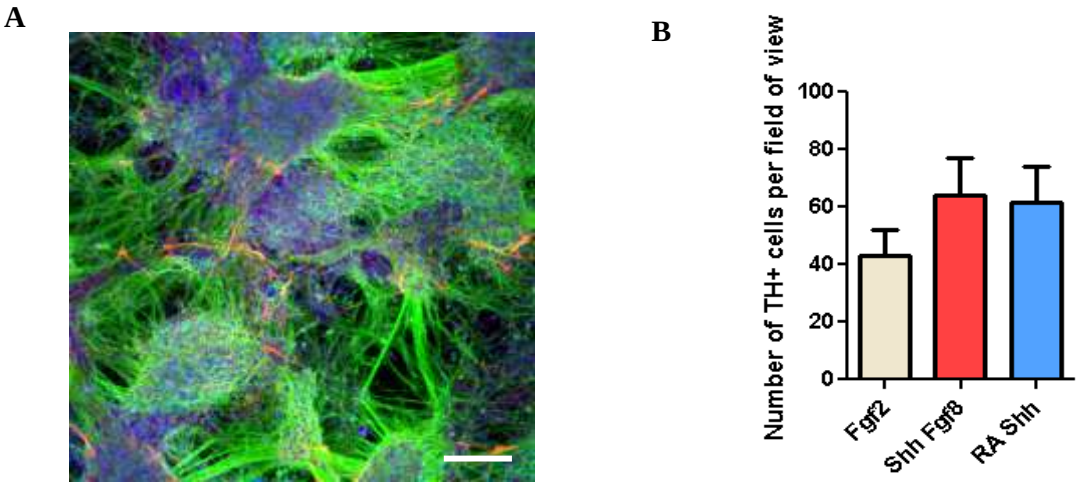

Figure 37: Quantification of TH+ cells in terminally differentiated cultures from Lmx1a+ cells. (A) Cultures immunolabelled for TH (red), TUJ-1 (green) and DAPI (blue). (B) Number of TH+ cells in cultures exposed to different combinations of growth factors. Cell that co-labelled for TH and TOPRO-3 are in purple. Scale bar = 100 µm. Results are presented as the mean ± SEM of 3 independent experiments.

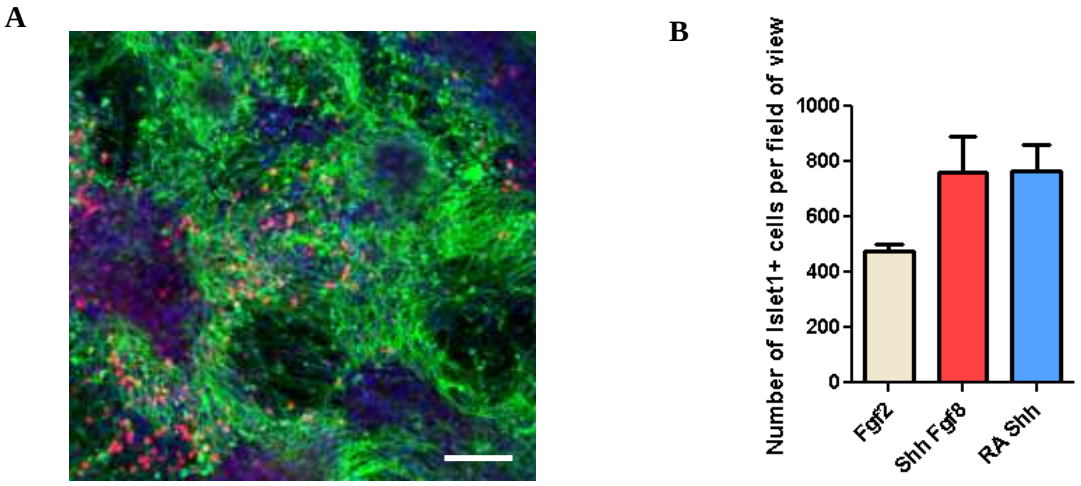

Figure 38: Quantification of Islet1+ cells in terminally differentiated cultures from Lmx1a+ cells. (A) Cultures immunolabelled for Islet1 (red), TUJ-1 (green) and DAPI (blue). (B) Number of Islet1+ cells in cultures exposed to different combinations of growth factors. Cell that co-labelled for Islet and TOPRO-3 are in purple. Scale bar = 100 µm. Results are presented as the mean ± SEM of 3 independent experiments.

Although the number of cells immunoreactive to GABA, TH and Islet1 were unaffected, I examined whether the patterning factors affected cell proliferation using the nuclear dye TOPRO-3. No significant differences in TOPRO-3 emission values were detected between the different culture conditions (Figure 39).

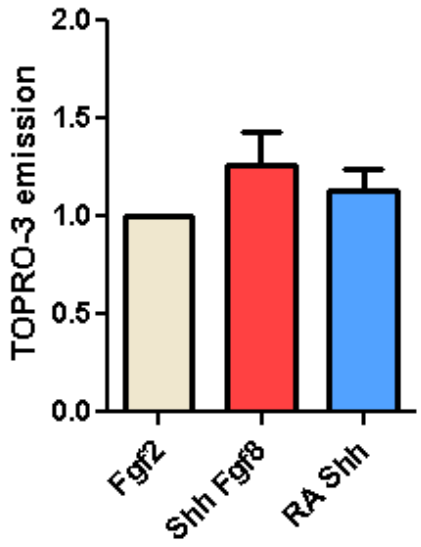

Figure 39: Assessment of cell proliferation 12 days after plating of Lmx1a+ cells. TOPRO-3 emission values from the 700 nm channel of the Odyssey of cultures exposed to different patterning factors, normalised to FGF2 treated cultures. Results are presented as the mean ± SEM of 3 independent experiments.

4.3.4 Formation of neural rosettes from isolated cells

A remarkable feature of the Lmx1a+ fraction, when seeded at high density, was the formation of neural rosettes (Figure 40A). These rosettes were characterised by ZO-1+ foci surrounded by Nestin+ cells. At low density, fewer rosettes were detected in Lmx1a+ cultures (data not shown). Formation of neural rosettes was rarely observed in cultures of the Lmx1a- fraction (Figure 40B).

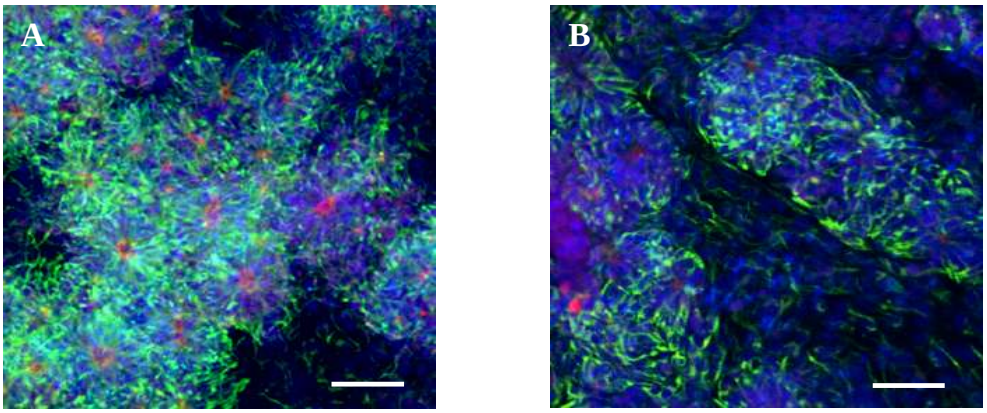

Figure 40: Cultures of separated Lmx1a cells 5 days after replating at high density. Immunolabelling of cultures arising from (A) Lmx1a+ and (B) Lmx1a- cells for ZO-1 (red), Nestin (green) and DAPI (blue). Scale bar = 100 µm.

4.3.5 Neural rosettes in monolayer cultures

In monolayer cultures, neural rosettes could be observed by day 7 of differentiation (Figure 41A). Besides Nestin (Figure 41B + 41C), cells surrounding the ZO-1+ lumens were also immunoreactive to Musashi-1 (Figure 41D), a marker for NS cells (Sakakibara *et al.*, 1996; Kanemura *et al.*, 2001). At the edges of neural rosettes, TUJ-1 immunoreactivity was detected (Figure 41E). Although neural rosettes were detected in cultures, the presence of cells with rosette morphology was not always consistently obtained.

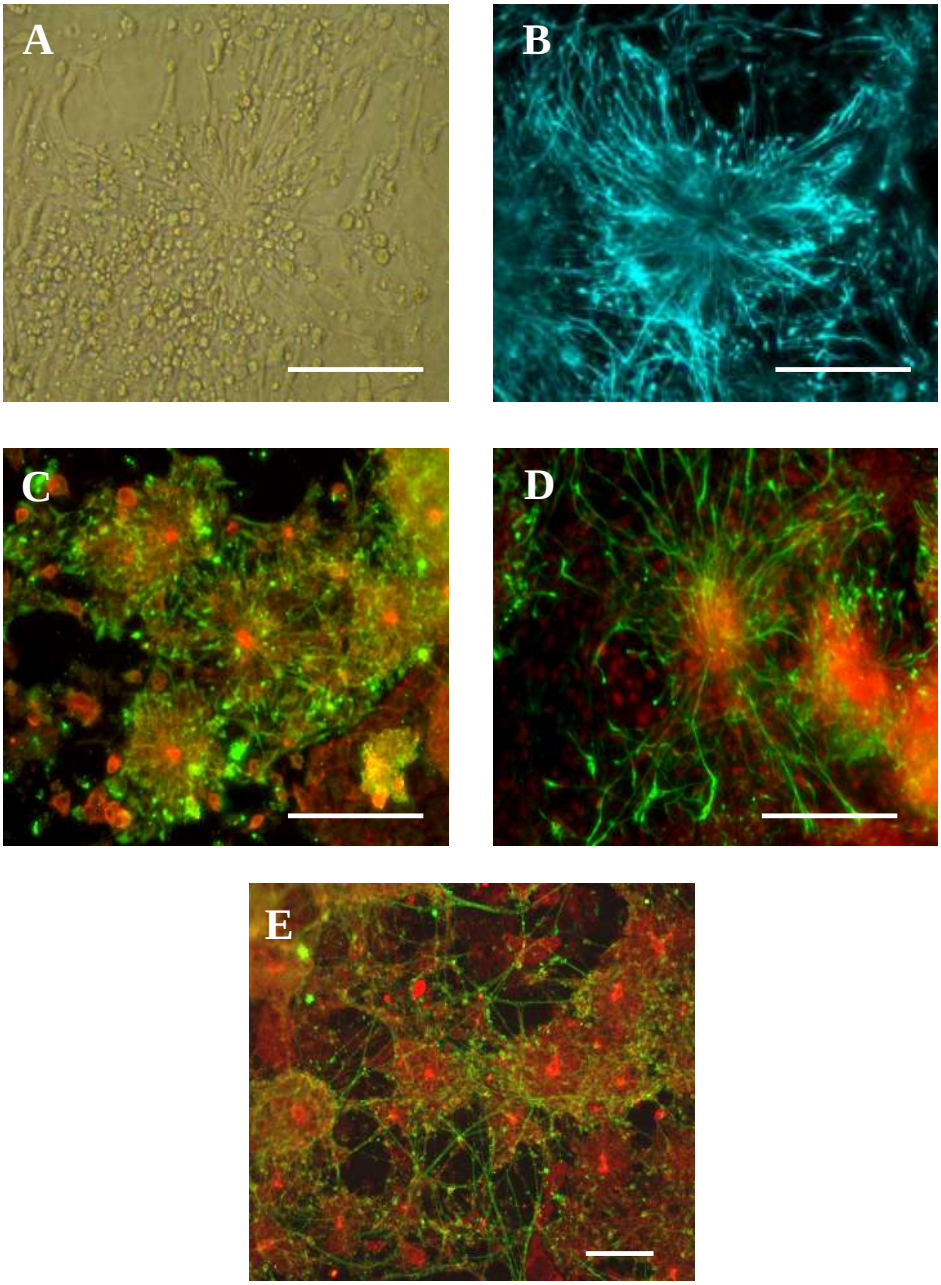

Figure 41: Neural rosettes in monolayer cultures during neural differentiation. (A) White Light image of a neural rosette. Cultures labelled for (B) Nestin, (C) ZO-1 (red) and Nestin (green), (D) Musashi-1 (red) and Nestin (green), and (E) ZO-1 (red) and TUJ-1 (green). Scale bar = 100 µm.

When cultures containing neural rosettes were replated and exposed to patterning factors such as Shh, FGF8 and FGF4, 5-hydroxytryptamine (5-HT/serotonin) and TH positive cells were detected in the cultures 13 days after replating (Figure 42). The 5-HT+ and TH+ cells co-localised with TUJ-1 labelling indicating that serotonergic and dopaminergic neurons could be obtained from differentiated neural rosettes.

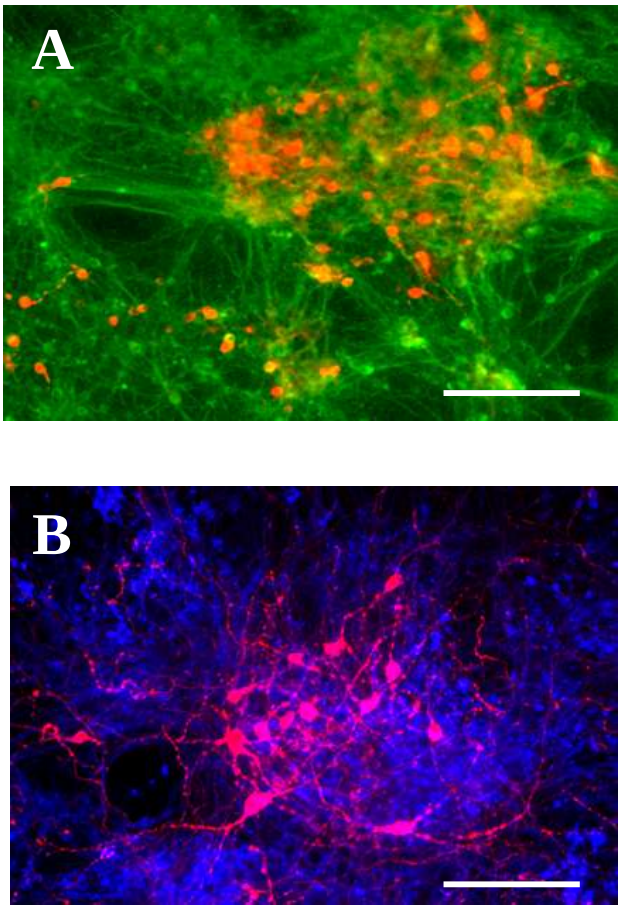

Figure 42: Terminally differentiated cultures arising from neural rosettes. Immunolabelling of cultures for (A) TH (red) and TUJ-1(green) and (B) 5-HT (red) and TUJ-1 (blue). Scale bar = 100 µm.

4.3.5 PA6 co-culture

As monolayer cultures under chemically defined conditions did not yield increased numbers of dopaminergic neurons, I turned to the PA6 co-culture protocol, a method that has been shown to generate high numbers of midbrain dopaminergic neurons (Kawasaki *et al.*, 2000; Barberi *et al.*, 2003). Analysis of Lmx1a expression under this culture condition showed that the percentage of Lmx1a+ cells did not peak on day 8, as observed in monolayer cultures, but gradually increased to >20% positive cells by the end of the differentiation period (day 15) (Figure 43).

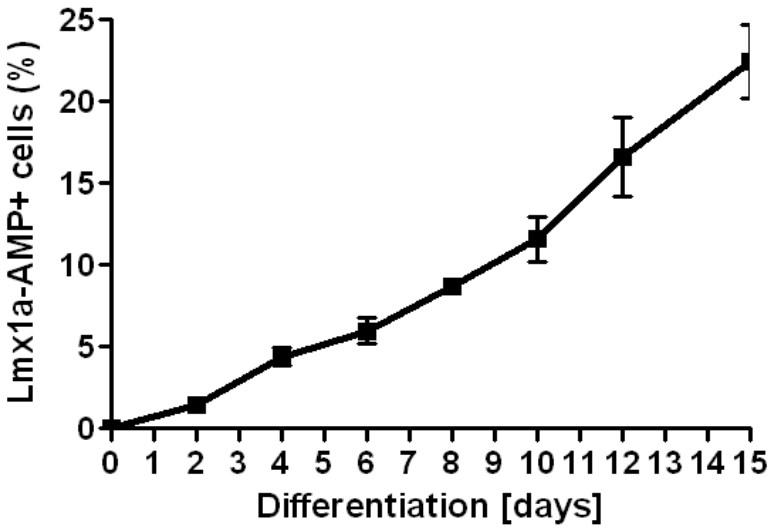

Figure 43: Time course of Lmx1a-AMP+ cells during PA6 co-culture differentiation. Results are presented as the mean ± SEM of at least 3 independent experiments.

Immunocytochemistry at day 8 showed that Lmx1a-eGFP+ cells were TUJ-1- and Nestin+ (Figure 44A + 44B). By day 15 of a typical PA6 co-culture differentiation all colonies were TUJ-1 positive (data not shown), and 70% ± 5% of the colonies contained patches of Lmx1a-eGFP+ cells. There was a strong correlation between the presence of Lmx1a and the occurrence of TH+ neurons in these cultures, and co-expression of the two markers was observed frequently at the edges of colonies (Figure 44C). Furthermore, the presence of Lmx1a+ cells was significantly higher in colonies that contained ≥10 TH+ neurons compared to colonies with <10 TH+ neurons (Student's t-test, $p < 0.01$, $n=3$) (Figure 44E). However, the number of cells co-expressing Lmx1a and TH could not be quantified as single Lmx1a+ cells could not be distinguished in the colonies.

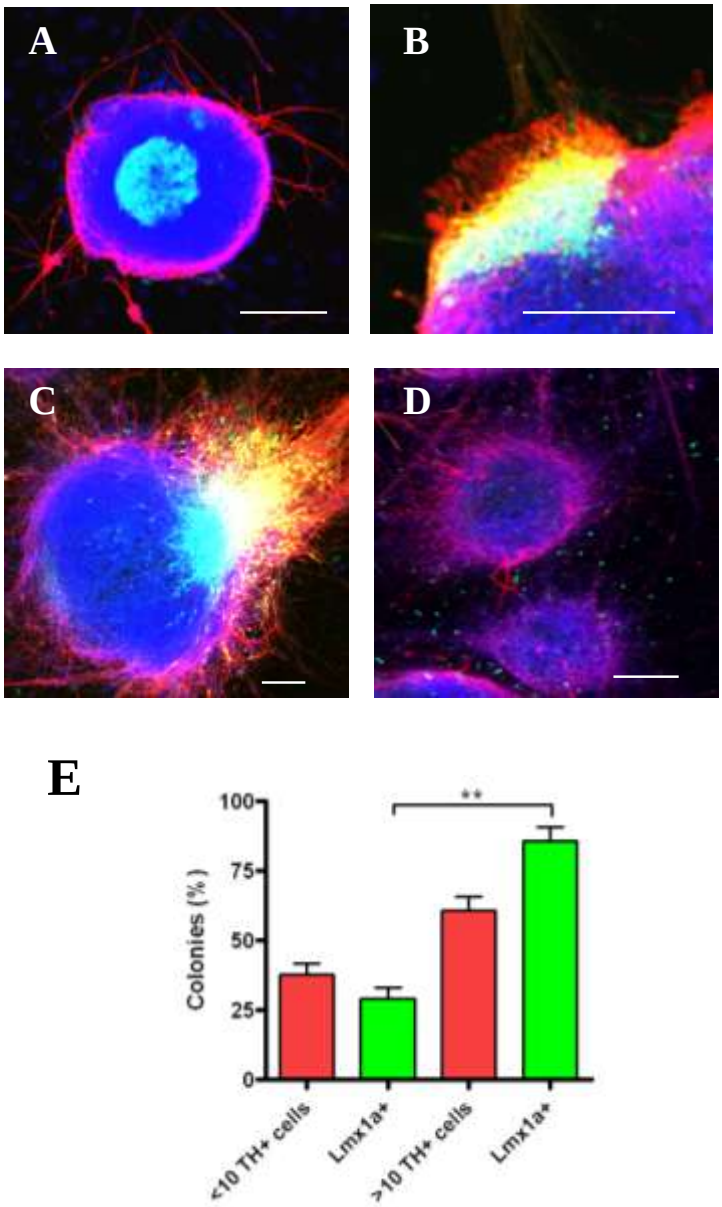

Figure 44: Lmx1a expression during PA6 co-culture. (A, B) Labelling of day 8 cultures for (A) TUJ-1 (red) and (B) Nestin (red); Lmx1a-eGFP+ cells are in green and DAPI (blue) was used as nuclear counter stain. (C, D) Labelling of day 15 cultures for TH; Lmx1a-eGFP+ cells are in green and DAPI (blue) was used as nuclear counter stain. (E) Percentage of colonies (day 15) that contain more or less than 10 TH+ cells (red bars); neighbouring bars (green) indicate the percentage of these colonies that are positive for Lmx1a reporter GFP. ** indicates $p < 0.01$ between colonies containing less than and more than 10 TH+ cells expressing Lmx1a with Student's t-test. Results are presented as the mean ± SEM of 3 independent experiments.

4.4 Discussion

In this chapter, I attempted to enrich Lmx1a+ cells derived from adherent monolayer cultures via FACS sorting and further characterise the isolated cells. I examined the expression of selected genes involved in neural development in the fractions of day 8 separated Lmx1a-AMP-IRES-eGFP cultures. Furthermore, the differentiation potential of FACS sorted Lmx1a+ cells was also investigated.

4.4.1 Expression profiling of isolated day 8 Lmx1a positive and negative cells

Based on results from the previous chapter, when peak Lmx1a expression was observed on day 8 of monolayer differentiation, it was intriguing to examine whether Lmx1a+ cells express other genes associated with midbrain dopaminergic neuron development. Although 40% cells expressed Lmx1a by day 8, only the top 25% AMP expressing cells were isolated to minimise the chances of Lmx1a- cells mixed into the positive population. qPCR analysis showed that transcript levels of Lmx1a in the positive fraction was 360 times higher than the negative fraction. This demonstrates that our reporter line allowed robust separation of Lmx1a expressing cells from the rest of the population. The fold increase detected in the negative fraction was possibly due to the inclusion of Lmx1a expressing cells which had failed to take up the fluorescent substrate from the loading kit.

Expression of NS cell markers (Sox1, Sox2, Nestin) were examined as our previous results showed that Sox1 expression was also peaked on day 8 in our cultures. Sox2 is marker for neural progenitors/stem cells (Graham *et al.*, 2003) as well as a key pluripotency marker (Boiani and Scholer, 2005; Boyer *et al.*, 2005; Loh *et al.*, 2006). There were no significant

differences in Sox1 and Sox2 expression between the positive and negative fractions. However, the expression of Nestin was >2 fold higher in the positive fraction compared to the negative fraction, indicating that the Lmx1a+ cells are enriched for neural progenitors/stem cells. Data from the neurosphere formation studies further reflected this result; Lmx1a+ cells were capable of forming larger spheres consisting of higher numbers of viable cells compared to Lmx1a- cells.

The importance of Wnt signalling during neural patterning and midbrain dopaminergic neuron development has already been detailed in Chapter 1. The expressions of three Wnt genes (Wnt1, Wnt3a and Wnt5a) in the Lmx1a positive and negative fractions were examined. Wnt1 forms an autoregulatory loop with Lmx1a to control midbrain dopaminergic neuron development (Chung *et al.*, 2009). Wnt3a and Wnt5a have also been implicated in dopaminergic neuron proliferation and differentiation. Wnt3a does not affect the number of TH+ cells but instead regulates proliferation of Nurr1+ cells and Wnt5a promotes maturation of Nurr1+ cells into dopaminergic neurons (Castelo-Branco *et al.*, 2003). Among the three Wnt genes examined, Wnt5a had the lowest fold changes with no significant difference in both separated fractions. Conversely, Wnt1 and Wnt3a were considerably upregulated in both fractions. Expression levels of Wnt1 and Wnt3a were significantly higher in the positive fraction, with a more pronounced increase observed for Wnt1. As Wnt1 is a reported downstream target of Lmx1a, it was not surprising that the transcript levels of Wnt1 in the positive fraction was >13 times higher compared to the negative fraction.

BMP2 and Foxa2 were selected as the markers of roof plate and floor plate respectively to be investigated. BMPs, like Lmx1a, are expressed in the dorsal roof plate (Furuta *et al.*, 1997); however BMP2 expression was primarily upregulated in the negative fraction with only modest increases in the Lmx1a+ fraction compared to day 1 transcript level. Similarly, the fold increase of Foxa2, which is known to be expressed in the ventral midline well before Lmx1a (Joksimovic *et al.*, 2009b), was also significantly upregulated in the negative fraction. The results from qPCR analysis of Wnt1 and Foxa2 expression was consistent with the report of Chung *et al.* (2009), with Wnt1 being regulated by Lmx1a+ cells and Foxa2 expression primarily in the Lmx1a- population. As Lmx1a and Foxa2 expression are upregulated in distinct cell populations, it is possibly the major reason for the lack of Lmx1a+ Foxa2+ cells observed in our cultures.

Notch signalling is essential for the maintenance of NS cells and is required for R-NSC self-renewal (Artavanis-Tsakonas *et al.*, 1999; Elkabetz *et al.*, 2008; Imayoshi *et al.*, 2010). In the study by Elkabetz *et al.* (2008), additions of the Notch ligands, Jag1 and Dll4, in combination with Shh were able to maintain proliferation of R-NSCs without losing rosette morphology. Although there were no significant differences in Jag1 and Notch1 expression between the positive and negative fractions, a significant upregulation of Dll4 expression was detected in the negative fraction. The winged-helix gene BF-1 is a forebrain marker also expressed by R-NSCs (Hatini *et al.*, 1994; Elkabetz *et al.*, 2008) while the homeobox protein Hoxb4 is expressed in the hindbrain and is induced by a retinoid pathway (Gould *et al.*, 1998). There was significant upregulation of BF-1 in the negative fraction. As upregulation of genes associated with R-NSCs were mainly in the negative

fraction, this indicates that neural rosettes are present in day 8 Lmx1a- cells during monolayer differentiation. No significant difference in Hoxb4 transcript levels between separated fractions was detected. qPCR analysis of midbrain dopaminergic progenitor markers revealed that expression of Msx1, Lmx1b and En1 were largely within the positive fraction whereas Aldh1a1 was significantly upregulated in the negative fraction. Msx1, Lmx1b and En1 have been associated with Lmx1a. As previously mentioned, Msx1 is a direct downstream target of Lmx1a (Andersson *et al.*, 2006b) while Lmx1b has been implicated in regulation of midbrain dopaminergic neurons in co-operation with Lmx1a (Yan *et al.*, 2011). En1 is an indirect target of Lmx1a as it is downstream target of the Wnt1 signalling pathway (Danielian and McMahon, 1996). No connections between Lmx1a and Aldh1a1 have been previously reported. In addition, Aldh1a1 is expressed in multiple cancer stem cell types (Li *et al.*, 2009; Penumatsa *et al.*, 2010; Raz *et al.*, 2012). It is probable that Aldh1a1 expressing non-neural cells were present in the Lmx1a- fraction. Taken together, qPCR results suggested that the Lmx1a+ fraction consisted of neural progenitors, presumably, progressing towards a dopaminergic fate while roof plate and floor plate cells of forebrain identity were present in the negative fraction.

4.4.2 Neurogenic potential of Lmx1a+ cells

To determine the neurogenic potential of the FACS separated cells, neurosphere formation assays were performed. Neurospheres were then plated as monolayer cultures. As only neural progenitors are competent in forming neurospheres, non-neural cells are eliminated in the serum-free selective media. In 96-well culture plates, the number of spheres observed was similar, however, the number of viable cells from the dissociated spheres was significantly different, with positive spheres containing >2 times the number of live cells compared to the negative spheres, indicating that only a proportion of spheres formed by Lmx1a- cells were neurospheres and the remaining spheres were aggregates formed by dead cells. I have already shown that Nestin is expressed by both fractions of cells, with significant upregulation in the positive fraction, and these results further support the qPCR data. In addition, the majority of spheres formed by positive cells were considerably larger and displayed a more spherical morphology compared to the Lmx1a- spheres. After 5 days of neurosphere culture, GFP expression of Lmx1a+ cells decreased considerably, with around 45% GFP positive cells detected. In the negative spheres around 2.5% cells expressed GFP. These results implied that GFP expression cannot be maintained through neurosphere formation and negative cells begin to express Lmx1a although it remains to be determined if there would be an increase in GFP+ cells with prolonged culture. When plated and terminally differentiated, Lmx1a+ neurospheres gave rise to predominantly TUJ-1+ cells. On the other hand, noticeably less TUJ-1+ cells were identified in the Lmx1a- cultures. This indicates that neurosphere formation of Lmx1a+ cells and subsequent differentiation provides a method of deriving highly neuronal cultures.

As Lmx1a+ cells displayed high neurogenic potential via neurosphere formation, the ability these cells to directly differentiate into neurons was investigated. In the absence of any patterning factors, cultures enriched with neurons and glia were obtained. GFP expression was downregulated in a large amount cells and the majority of remaining GFP+ cells did not co-localise with other neural markers. It was surprising that the vast majority of neurons were GAD67+ GABAergic neurons as Lmx1a expression has not been previously associated with GABAergic neuron differentiation *in vitro*. The number of GABAergic neurons outnumbered dopaminergic neurons by 20:1 and motor neurons by >2:1. Considering day 8 Lmx1a+ cells also expressed Msx1 and En1, I expected to obtain a larger proportion of TH+ cells. Furthermore when patterning factors were added, the number of TH+ and Islet1+ cells remained constant, indicating that day 8 Lmx1a+ cells were primarily programmed towards a GABAergic fate irrespective of exogenous factors. In another study, FACS extracted monolayer derived Sox1-GFP+ cells were subjected to patterning factors (FGF2, FGF8 and Shh) and similarly failed to yield increased TH+ neurons (Parmar and Li, 2007). Considering results from both these studies, it appears that midbrain dopaminergic neuron specification occurs very early on during *in vitro* monolayer differentiation of mES cells.

It is worth noting that Shh signalling was able to offset RA inhibition of cell proliferation. Shh is a mediator of R-NSC and NS cell proliferation (Ahn and Joyner, 2005; Elkabetz *et al.*, 2008). In the previous chapter, exposure of neural progenitors to 10^{-6} M RA resulted in decreased cell proliferation. However, total cell numbers in cultures exposed to RA in combination with Shh were not influenced.

The GABAergic neurons were mainly SatB2+ but did not express TBR-1, indicating Lmx1a+ cells default into GABAergic neurons with upper-layer identity. The cerebral cortex is grouped into six layers; SatB2 is expressed by upper-layer neurons (Britanova *et al.*, 2008) and TBR-1 by deep-layer neurons (Hevner *et al.*, 2001). Results from qPCR support this hypothesis; Wnt3a, which was primarily upregulated in the Lmx1a+ fraction, is also a marker for the cortical hem (Yoshida *et al.*, 2006). BF-1 is expressed throughout the forebrain but by embryonic day 10.5 BF-1 expression is excluded from the dorsomedial telencephalon which includes the cortical hem (Furuta *et al.*, 1997). Wnt1 has been implicated as a cortical hem marker in chick (Ligon *et al.*, 2003), but not in the mouse system (Grove *et al.*, 1998). This assumption is complicated by reports indicating that the cortical hem is also a site of BMP2 expression (Grove and Tole, 1999). In this study, during monolayer differentiation, BMP2 expression was primarily upregulated in the negative fraction with only modest increases in the Lmx1a+ fraction compared to day 1. Most importantly, *in vivo*, the cortical hem is primarily a source of Cajal-Retzius cells, a type of marginal zone glutamatergic neuron (Yoshida *et al.*, 2006), while our monolayer derived Lmx1a+ cells predominantly give rise to GAD67+ GABA+ neurons with upper layer identity. As the cortical hem is the primary site of Lmx1a expression in the developing forebrain (Chizhikov *et al.*, 2010), it is tempting to speculate that monolayer derived Lmx1a+ cells are the *in vitro* equivalent of this structure. As these cells were resistant to dopaminergic patterning cues they were in a committed state predisposed to the generation of layer specific GABAergic neurons.

4.4.3 Formation of R-NSCs in monolayer cultures

When replated at high density, extracted Lmx1a+ cells organised to form rosettes structures. Conversely, R-NSCs were rarely observed in Lmx1a- cultures. In general, fewer Nestin+ cells were detected in plated Lmx1a- cultures indicating the presence of other lineages. Neural rosettes have previously been detected during monolayer neural differentiation of mES cells (Ying *et al.*, 2003b). Elkabetz and group (2008) then managed to derive and characterise R-NSCs from hES cells. According to the group, R-NSCs are early NS cells of forebrain identity that can be maintained for several passages without losing differentiation potential in the presence of Shh, Jag1 and Dll4. Furthermore, R-NSCs can be patterned into region specific neuronal phenotypes, which had not been previously proven with NS cells derived from ES cells (Elkabetz *et al.*, 2008). Observations from cultures derived from extracted cells were intriguing as results from qPCR showed that genes associated with R-NSCs and their maintenance were primarily upregulated in the negative fraction. A possible explanation for this is that in the absence of Lmx1a- cells, replated Lmx1a+ cells begin to secrete endogenous Notch ligands to 'compensate' for the loss of inductive Notch signalling from Lmx1a- cells. The Notch ligands would in turn promote Lmx1a+ cells to form the radially organised R-NSCs.

As enriched Lmx1a+ cells failed to increase the number of TH+ cells, I attempted to generate a R-NSC rich culture in chemically defined conditions without the need for FACs extraction to increase numbers of dopaminergic neurons. Using a modified protocol of Elkabetz *et al.* (2008), ES cell cultures were subjected to exposure of Shh, Jag1 and Dll4 after neural induction. The appearance of neural rosettes, defined by Nestin, ZO-1 and

Musashi-1 immunolabelling could be detected by day 7 of monolayer differentiation. Detection of TUJ-1 immunoreactivity around but not within the rosette structures implied that terminal differentiation occurs in cells surrounding the neural rosettes while R-NSCs still retain the potential to further differentiate. When cultures containing R-NSCs were exposed to various patterning factors during terminal differentiation, these cells were able to generate TH and 5-HT positive cells, confirming their multipotent potential. Although R-NSCs were detected in cultures, differentiation of ES cells did not consistently yield large numbers of R-NSCS. The derivation and subsequent expansion of neural rosettes has been applied in many hES cell neural differentiation protocols. However, there have been no studies reporting R-NSC rich cultures derived from mES cells, except by Elkabetz *et al.* (2008). The growth rate of hES cells is slower than mES cells; The population-doubling time of hES cells is 36 hours compared to ~12 hours for mES cells (Odorico *et al.*, 2001; Amit and Itskovitz-Eldor, 2002; Niwa, 2007). While mature neurons can be derived from mES cells after 21 days, based on results from our laboratory (Watmuff *et al.*, 2012), neural differentiation of hES cells require at least 4 weeks of culture to generate neurons (Lee *et al.*, 2000b; Perrier *et al.*, 2004). Because of a higher growth ratio, it is possible that neural rosettes in mES cell cultures only emerge during a short period of time before further differentiating. Therefore unlike hES cultures, it would be difficult to mechanically harvest neural rosettes from mES cell cultures.

4.4.4 Expression of Lmx1a in PA6 co-cultures

Since monolayer differentiation, a culture method that is now strongly associated with the occurrence of forebrain phenotypes (Gaspard *et al.*, 2008), produced Lmx1a+ cells predisposed to become GABAergic neurons, I wanted to explore the fate of Lmx1a+ cells produced during PA6 co-culture, a method that efficiently mediates ES cells into midbrain dopaminergic neurons within two weeks (Kawasaki *et al.*, 2000; Barberi *et al.*, 2003). In contrast to monolayer cultures, there was a striking association between Lmx1a expression and dopaminergic neuron differentiation in PA6 co-cultures. With this culture method, ES cells were continuously exposed to factors from PA6 stromal cells for around two weeks. Under these conditions, Lmx1a expression did not peak midway during differentiation, but instead increased throughout the differentiation period, with ~23% cells registering as AMP+ by day 15. Although the percentages of Lmx1a+ cells were similar at the end of both monolayer and PA6 co-culture differentiation, the occurrence of TH+ cells was more frequent in PA6 co-cultures compared to monolayer cultures. Additionally Lmx1a+ colonies were more likely to contain dopaminergic neurons than Lmx1a- colonies and Lmx1a expression was detected in TH+ neurons. Further studies done by Christian Nefzger from our laboratory have shown that FACS sorted Lmx1a+ cells from PA6 co-cultures gave rise to TH+ neurons, commonly expressing Lmx1a-GFP, at a 3 fold higher frequency compared to GABA+ neurons. In contrast, the frequency of TH+ neurons observed in terminally differentiated Lmx1a- cultures was approximately 3 times lower than GABA+ neurons. These observations indicate that the PA6 co-culture method is an effective way of generating dopaminergic neurons and in this differentiation paradigm Lmx1a is marker for TH+ neurons.

4.5 Conclusion

Based on results from this chapter, Lmx1a during differentiation under chemically defined conditions signifies the rise of a cortical progenitor population that gives rise to forebrain GABAergic neurons with a distinct cortical layer-specific identity. To our knowledge, it represents the first time Lmx1a has been associated with GABAergic neuron development. Although Lmx1a+ cells in adherent monolayer cultures express downstream targets Msx1 and Wnt1, these cells in are primed very early on and appear largely restricted to GABAergic neuron and astrocyte fates as they cannot be patterned towards a dopaminergic fate even under the influence of exogenous patterning cues after FACS separation. These findings are in line with the concept that co-expression of Lmx1a and Foxa2 is required for the specification of dopaminergic neurons *in vitro*. Depending on the culture system, Lmx1a can be a marker for GABAergic neurons under adherent monolayer or for dopaminergic neurons in a PA6 co-culture method. Taken as a whole, these data indicate that extraction of midbrain dopaminergic progenitors by flow cytometry will require at least one more reporter gene to identify the correct sub-population of Lmx1a+ cells.

CHAPTER 5

Functional characterisation of ionotropic glutamate receptors on differentiated neurons

5.1 Introduction

5.1.1 Neurotransmission

Neurons are able to communicate with one another via the release of signalling molecules known as neurotransmitters. Neurotransmitters are released from the branching axon terminals of presynaptic neurons which diffuse across a synaptic cleft and bind to postsynaptic receptors (Greenamyre, 1986; Masson *et al.*, 1999). Neurotransmitters are commonly synthesised within the presynaptic terminal and are then stored in synaptic vesicles until release. When presynaptic nerve terminals depolarise, there is a local influx of Ca^{2+} which activates calcium sensitive proteins attached to synaptic vesicles causing the membrane of the synaptic vesicles to fuse with presynaptic plasma membrane leading to the release of neurotransmitters into the synaptic cleft (Wiedenmann and Franke, 1985; Momiyama and Takahashi, 1994). Binding of neurotransmitters to postsynaptic receptors elicits cell responses. Neurotransmitters are inactivated by either enzymatic degradation or active transport back into the nerve terminal and/or glial cells by neurotransmitter transporters (Greenamyre, 1986; Masson *et al.*, 1999; Edwards, 2007).

5.1.2 Glutamate receptors

Glutamate is the principal excitatory neurotransmitter in the CNS and plays an important role in regulating differentiation, migration and survival of neurons in the developing brain (Hack and Balázs, 1994; Yano *et al.*, 1998; Meldrum, 2000). Glutamate receptors are divided into ionotropic and metabotropic subtypes. Ionotropic glutamate receptors consist of three classes, the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-

4-isoxazolepropionic acid (AMPA) and kainate receptors. These receptors are ligand gated ion channels consisting of five subunits encoded by 16 genes (Mayer, 2005). There are seven NMDA receptor subunits (NR1, NR2A-D, NR3A,B), four AMPA receptor subunits (GluR1-4) and five kainate receptor subunits (GluR5-7 and KA1,2) (Hollmann and Heinemann, 1994; Dingledine *et al.*, 1999; Mayer, 2005). The AMPA and kainate receptor subunits are structurally and pharmacologically related and are distinct from NMDA receptor subunits. Both AMPA and kainate receptors, unlike NMDA receptors, show fast kinetics (activation, deactivation and desensitisation) (Colquhoun *et al.*, 1992; Lester and Jahr, 1992). Metabotropic glutamate receptors are monomeric G-protein coupled receptors which are linked to intracellular second messenger systems. They are classified into three main classes (Groups I - III) consisting of eight receptor subtypes (mGluR1-8) (Conn and Pin, 1997).

The activation of ionotropic glutamate receptors has been linked to an influx of intracellular Ca^{2+} leading to excitotoxic neuronal death (Choi, 1988; Chen *et al.*, 1995). In the basal ganglia, which consists of the striatum, globus pallidus, substantia nigra and subthalamic nucleus, glutamate activates all three ionotropic receptor subtypes (Götz *et al.*, 1997). In PD, excitotoxicity of SNpc dopaminergic neurons is caused by high levels of glutamate activating NMDA receptors (Waxman and Lynch, 2005). Studies have shown that midbrain dopaminergic neurons expressing calbindin-D_{28K}, a calcium-binding protein, in the VTA and the dorsal tier of the SNPc are relatively spared, further supporting the mechanism that an overload of calcium leads to dopaminergic neuron degeneration (Yamada *et al.*, 1990; Lavoie and Parent, 1991; Caudle and Zhang, 1992). Furthermore,

studies involving blockade of NMDA receptors reported improved behavioural assay scores in various Parkinsonian animal models (Greenamyre and O'Brien, 1991; Ossowska, 1994; Schmidt and Kretschmer, 1997; Marino *et al.*, 2003; Johnson *et al.*, 2009). AMPA/kainate receptors also mediate glutamatergic neurotransmission in the basal ganglia (Albin *et al.*, 1992; Chatha *et al.*, 2000). However, increases in intracellular calcium due to activation of AMPA receptors are less frequently associated with excitotoxicity (Lynch and Guttmann, 2002). In preclinical studies, administration of AMPA receptor antagonists on their own did not result in anti-Parkinsonian effects, however the combination of levodopa and AMPA receptor antagonists enhanced the efficacy of levodopa treatments (Löschmann *et al.*, 1991; Löschmann *et al.*, 1992; Wachtel *et al.*, 1992; Zadow and Schmidt, 1994). These studies indicate that NMDA receptors in particular, may have a significant role in PD.

5.1.3 Wnt5a

Wnt5a has been shown to activate both non-canonical Wnt-Ca^{2+} and Wnt-JNK signalling pathways. In the Wnt-Ca^{2+} signalling pathway, Wnt binds to Fz receptors, activating calcium/calmodulin-dependent kinase II (CamKII) and protein kinase C (PKC) which leads to the release of intracellular calcium (Kohn and Moon, 2005; Inestrosa and Arenas, 2010). In the Wnt-JNK pathway, Wnt binds to Fz receptor and DV, forming a complex which interacts with G-proteins. This leads to the activation of the monomeric GTPases Rho and Rac, which in turn activates JNK (Kohn and Moon, 2005; Inestrosa and Arenas, 2010). Activation of both non-canonical signalling pathways by Wnt5a in hippocampal neurons increases post-synaptic density protein-95 (PSD-95) clustering. PSD-95 induces

rapid insertion of NMDA and AMPA receptors at the postsynaptic site (Farías *et al.*, 2009). In addition, Wnt5a modulates glutamatergic postsynaptic current by activation of both postsynaptic NMDA and AMPA receptors in CA1 pyramidal neurons (Cerpa *et al.*, 2010).

5.1.4 Aims

No previous studies have shown that different culture methods generate the same receptors on the same neuronal populations. In this study, I attempt to establish that dopaminergic neurons have multiple glutamate receptors and that these receptor populations do/do not change under different culture conditions. The aim of this chapter is to begin to functionally characterise ionotropic glutamate receptors expressed by midbrain dopaminergic neurons terminally differentiated from ES cells derived from adherent monolayer cultures or PA6 co-cultures to determine whether neurons derived from the two differentiation paradigms express similar glutamate receptor subtypes, in particular the NMDA receptors.

5.2 Methods

Unless detailed below, the experimental methods used in this chapter have been described in Chapter 2.

5.2.1 Terminal differentiation

Monolayer and PA6 co-culture differentiation with the Pitx3-eGFP cell line were initiated as previously described in 2.2.4. On day 10, both cultures were incubated with accutase for at least 15 minutes and dissociated into single cells before replating onto laminin (1

μg/cm^2) coated plates at 5 x 10^4 cells/cm^2 (monolayer) or 6 x 10^4 cells/cm^2 (PA6 co-culture) in N2B27 with FGF2 (20 ng/mL; Peprotech, Israel). After 3 days, FGF2 was withdrawn from the medium and replaced with L-ascorbic acid (200 μM; Merck, Australia) up to day 21.

5.2.2 Calcium imaging

Cultures were first washed twice with HEPES buffer (see Appendix I) and incubated with 10 μM Fluo-4AM (Molecular Probes, USA) in HEPES buffer for 30 to 60 minutes at 37°C. Following incubation, cells were again washed twice with HEPES buffer and covered with 1 mL HEPES containing 0.3% (w/v) BSA (Sigma, Australia) and 1 μM tetrodotoxin (TTX; Tocris, United Kingdom) then placed on a hotplate heated to 37°C. Cells were viewed with an Eclipse TE2000-U microscope (Nikon, Japan) coupled to a Coolsnap-*fx* low-light camera or the Nikon A1R confocal microscope (Nikon, Japan) (see Figure 45). To visualise fluorescence, cells were excited at 488 nm and emission detected at ~510 nm. Regions were drawn on up to 40 cells with neuronal morphology in a field of view using MetaFluor v6.1r5 software (Universal Imaging Co., USA) or NIS Elements Image Software (Nikon, Japan). A region drawn on an area without any cells was used to measure background intensity which was subtracted from fluorescence intensity calculated by MetaFluor (Universal Imaging Co., USA) or NIS Elements Image Software (Nikon, Japan). Time lapse images were recorded every second. For all antagonist and Wnt5a studies, calcium imaging was carried out on the Nikon A1R confocal microscope (Nikon, Japan) using the NIS Elements Image Software (Nikon, Japan).

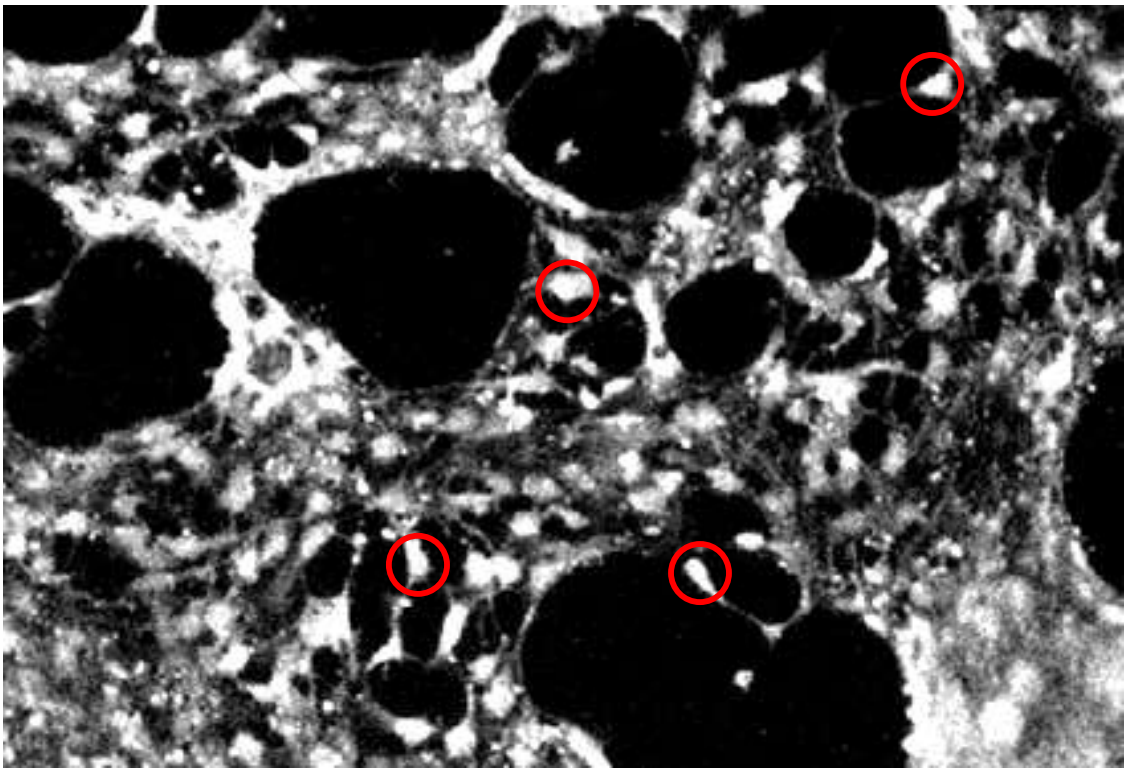

Figure 45: An example of a day 21 **c**ulture loaded with FLUO-4AM. Cells exhibiting neuronal morphology (red circles) were selected for analysis.

Cells were left to equilibrate for 10 minutes on a hotplate at 37°C before the addition of agonists to the culture well. After 90 to 120 seconds, cells were washed twice with HEPES buffer and allowed 5 minutes to equilibrate before addition of the next agonist. This process was repeated for all remaining agonists. At the end of all experiments, 30 mM KCl was added to determine viability of the cells. Findings from our laboratory have reported that endogenous GABA, which is the main inhibitory neurotransmitter in the CNS, affects glutamate mediated elevations of intracellular Ca^{2+} (Watmuff *et al.*, 2012). Therefore in all experiments, cells were incubated with 30 µM bicuculline and 1 µM CGP55845, selective $GABA_A$ and $GABA_B$ receptor antagonists respectively, to block the effect of GABA on cell responses to agonists. After calcium imaging experiments, cells were fixed as

described in 2.3 then kept in 0.1% (v/v) sodium azide in PBS until immunocytochemistry was carried out.

5.2.2.1 Antagonist studies

Antagonist studies were performed with repeated doses of 30 µM glutamate in the presence of the following antagonist concentrations: 10 µM LY235959, 20 µM NBQX and 10 µM CNQX. Antagonist concentrations used in these studies were based on other works to date (Lerma *et al.*, 1993; Traub *et al.*, 1995; Rawls *et al.*, 2007). After initial glutamate addition, antagonists were added to the well and allowed to equilibrate for 5 minutes before the final glutamate addition (see Figure 46). Experiments were performed using the combinations of antagonists: LY235959, LY235959 + NBQX and LY235959 + NBQX + CNQX to determine expression of NMDA, AMPA and kainate receptors respectively.

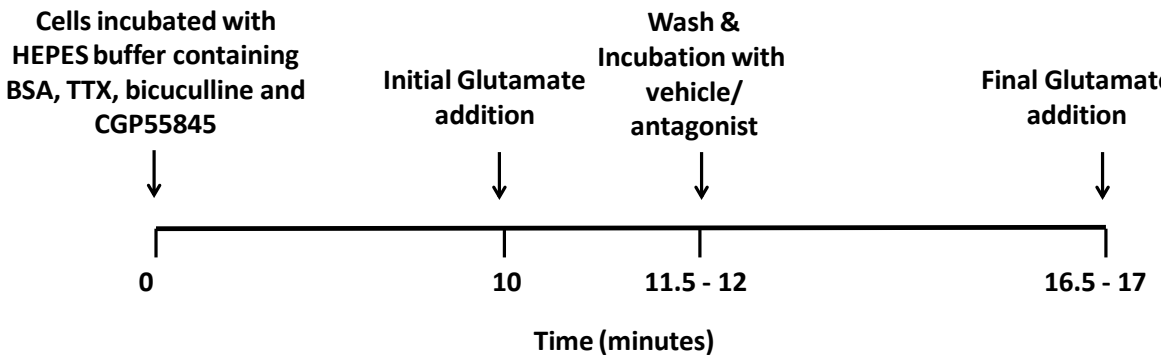

Figure 46: Schematic representation of the steps involved in antagonist studies with timeline.

5.2.2.2 Wnt5a studies

To examine the effect of Wnt5a on calcium signalling, Wnt5a vehicle or Wnt5a (100 ng/mL; R&D Systems, USA) was added to the well after initial agonist addition and incubated for 20 minutes before final agonist addition (see Figure 47). For monolayer cultures, all agonists were used at 3 µM. Concentrations used for PA6 co-cultures were 3 µM for glutamate, kainate and NMDA and 30 µM for AMPA. These agonist concentrations were unable to elicit maximal cell responses in the respective cultures and were selected to determine whether Wnt5a potentiates sub-maximal responses to glutamate receptor agonists.

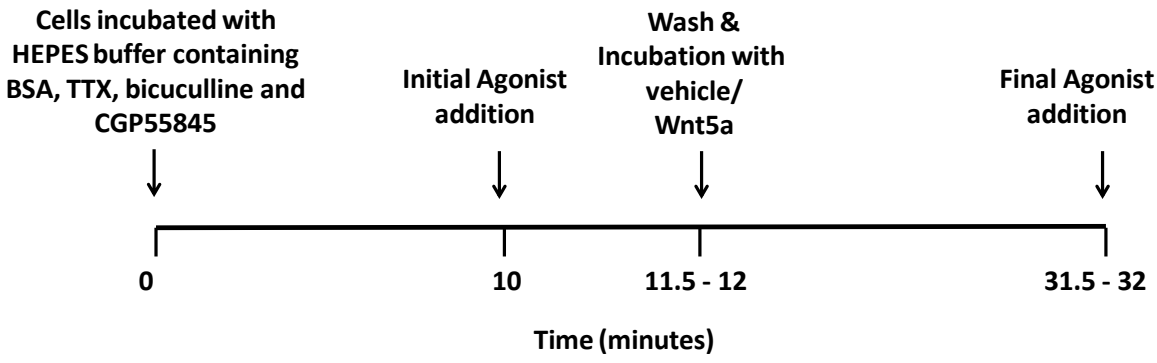

Figure 47: Schematic representation of the steps involved in Wnt5a studies with timeline.

5.2.2.4 Identification of Pitx3+ TH+ cells

24 hours prior to imaging, regions containing Pitx3-eGFP+ cells were identified and images taken with the Nikon A1R confocal microscope (Nikon, Japan). Following calcium imaging and immunocytochemistry as detailed in 5.2.2, the coordinates of the Pitx3-eGFP+ cell images were revisited using the Nikon A1R confocal microscope (Nikon, Japan). Images of TH+ cells within the same coordinates were then taken. Afterwards, images of TH+ cells were aligned with GFP+ images corresponding to the same coordinates to select for individual cells expressing both markers.

5.2.2.5 Drugs and solutions

For calcium imaging studies all agonists were purchased from Sigma (Australia). For antagonists, LY235959, NBQX, CNQX and CGP55845 were purchased from Tocris (United Kingdom) and bicuculline was purchased from Enzo Life Sciences (USA).

5.2.3 Calcium imaging data analysis

The fluorescence intensity of each selected region was calculated using either MetaFluor (Universal Imaging Co., USA) or NIS Elements (Nikon, Japan); maximum responses and the percentage of cells in culture responding to addition of agonists were then calculated for each field of view. Cells were only considered responsive to an agonist when the maximal response generated within 60 seconds after agonist addition exceeded five standard deviations above mean fluorescence intensity in the 30 seconds prior to vehicle or agonist addition (Figure 48) (Khaira *et al.*, 2011; Watmuff *et al.*, 2012). The maximum magnitude of responding cells were presented as a fraction of the pre-agonist mean baseline $[Ca^{2+}]_i$.

The fractions of cells within the population responsive to agonist addition were presented as a percentage of all selected cells within a field of view. Statistical comparisons were only performed on the responding cells.

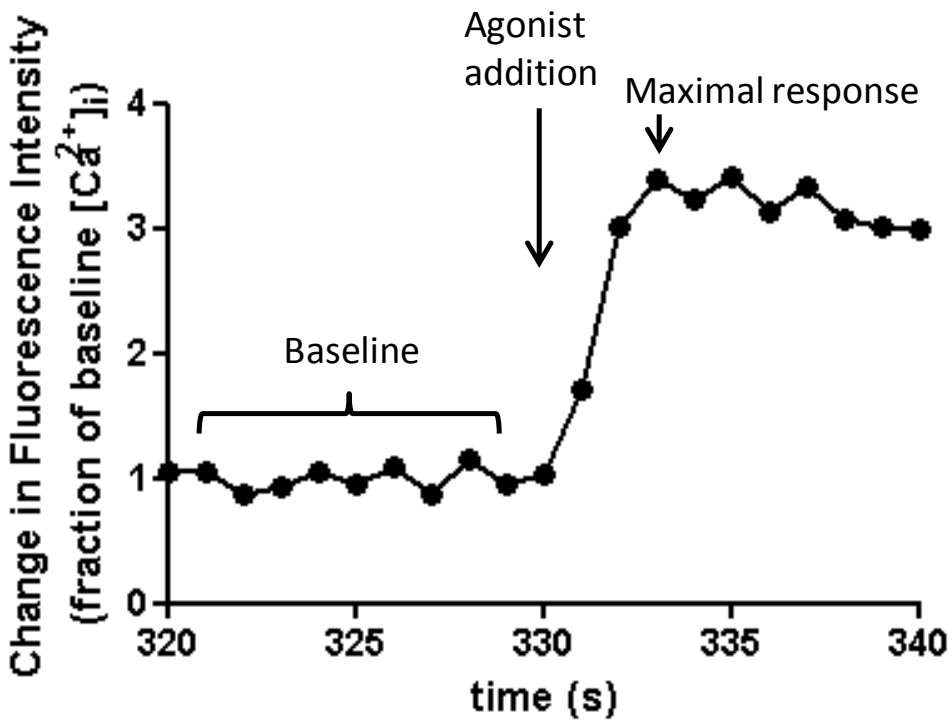

Figure 48: General response of a cell to agonist addition. A cell is considered responsive when the maximal response exceeds 5 standard deviations of the average fluorescence intensity prior to agonist addition.

5.3 Results

5.3.1 Concentration-response curves for glutamate receptor agonists

The response of neurons to glutamate receptor agonists was first investigated. Concentration-response curves were established for glutamate, AMPA, kainate and NMDA in adherent monolayer and PA6 co-cultures.

5.3.1.2 Monolayer cultures

The concentration-response curves for all agonist additions displayed typical agonist response relationship, with the magnitude of cell responses increasing with higher agonist concentrations, as seen in Figure 49. The first data points of all agonist curves represent cell responses to vehicle control. The concentration-response curves had a rank of order of agonist potency of AMPA (EC_{50} = 8 μM) = kainate (EC_{50} = 9 μM) = NMDA (EC_{50} = 9 μM) = glutamate (EC_{50} = 35 μM). The percentage of responding neurons in the population is illustrated in Figure 50. The percentage of neurons responding to each agonist increased with increasing concentrations of glutamate agonists.

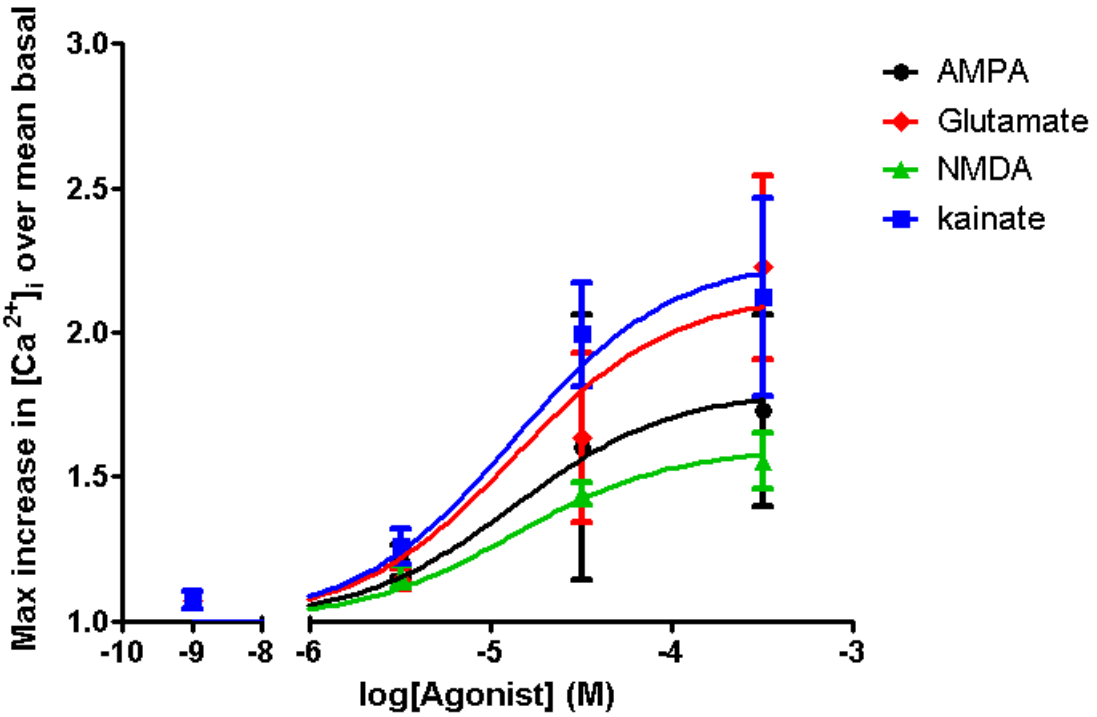

Figure 49: Concentration-response curves of adherent monolayer-derived neurons responding to glutamate receptor agonists: AMPA, Glutamate, NMDA and kainate. Results are presented as the mean ± SEM of 3 independent experiments.

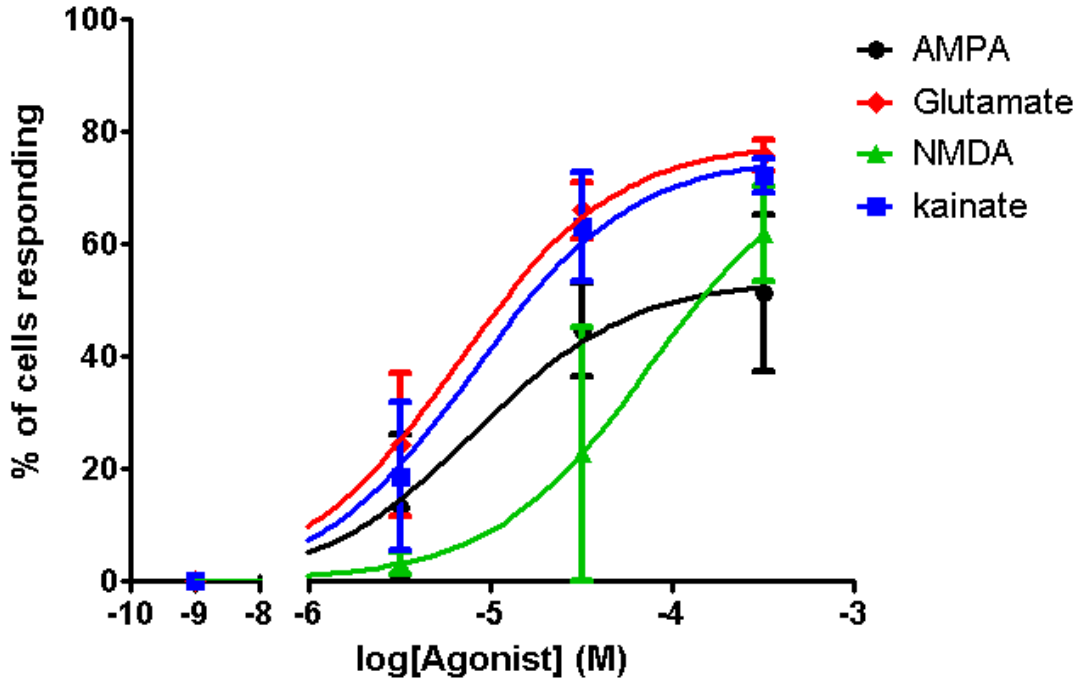

Figure 50: The percentages of adherent monolayer-derived neurons displaying elevations of intracellular Ca^{2+} in response to glutamate, AMPA, kainate and NMDA. Results are presented as the mean ± SEM of 3 independent experiments.

5.3.1.3 PA6 co-cultures

The concentration-response curves obtained for AMPA, kainate and NMDA in PA6 co-cultures also exhibited typical agonist response relationship as seen in Figure 51. For PA6 co-cultures, the concentration-response curves had rank of order of agonist potency of glutamate (EC_{50} = 8 µM) = NMDA (EC_{50} = 9 µM) = kainate (EC_{50} = 10 µM) > AMPA (EC_{50} = 118 µM). The percentage of the neuronal population responding to each agonist also increased with increasing concentrations of the glutamate agonists in PA6 co-cultures (Figure 52).

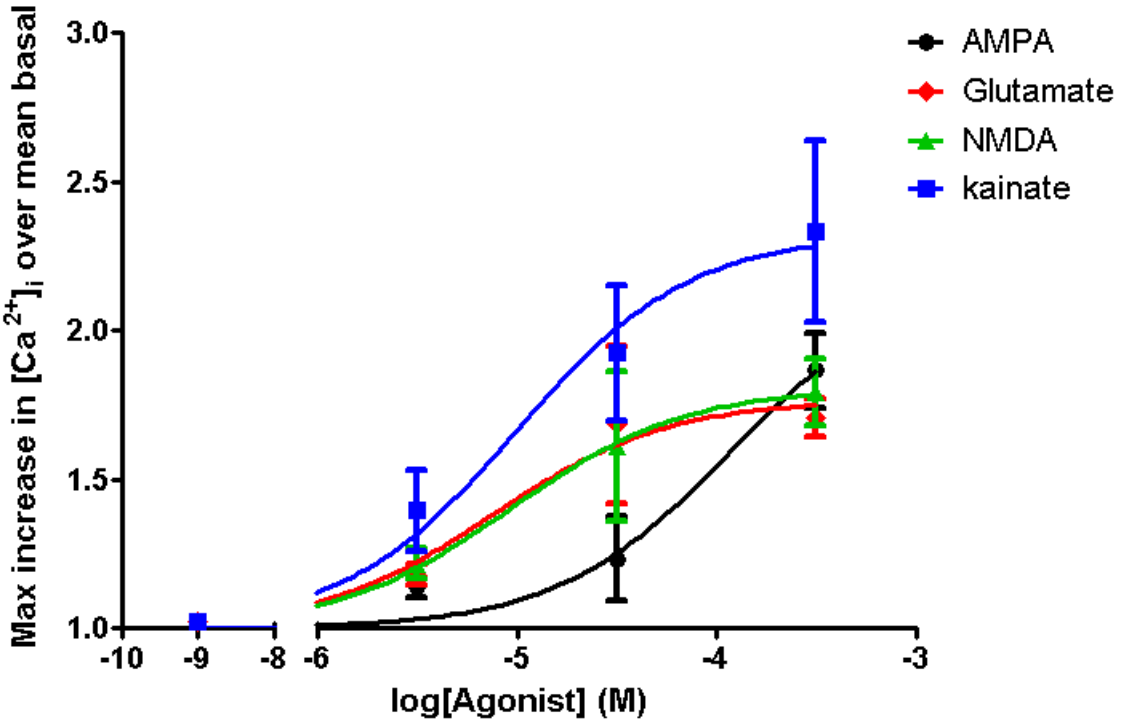

Figure 51: Concentration-response curves of PA6 co-culture-derived neurons responding to glutamate receptor agonists: AMPA, Glutamate, NMDA and kainate. Results are presented as the mean ± SEM of 3 independent experiments.

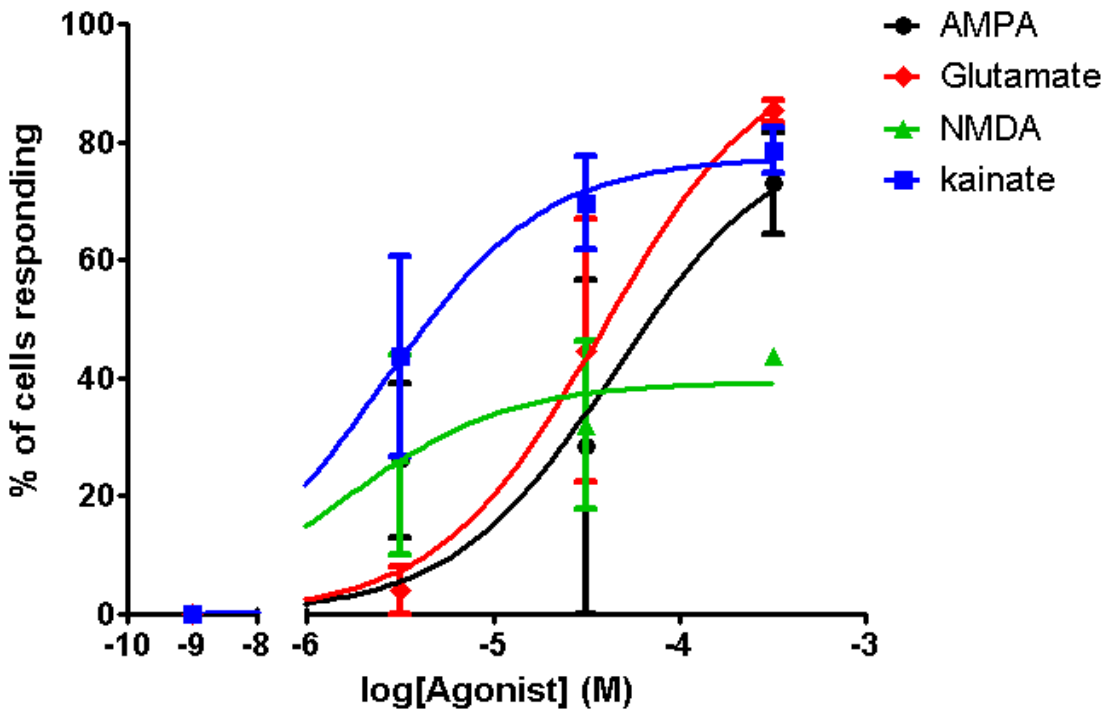

Figure 52: The percentages of PA6 co-culture-derived neurons displaying elevations of intracellular Ca^{2+} in response to glutamate, AMPA, kainate and NMDA. Results are presented as the mean ± SEM of 3 independent experiments.

5.3.2. Glutamate receptor antagonist studies

In the next study, I attempted to show an effect of glutamate receptor antagonists on the responses to glutamate on both midbrain dopaminergic and GABAergic neurons from both differentiation protocols. For this purpose, the Pitx3-eGFP cell line was used to identify Pitx3+ cells in both culture systems. Pitx3 is expressed by post-mitotic midbrain dopaminergic neurons during the late differentiation phase *in vivo* and is used as a marker for mature midbrain dopaminergic neurons (Smidt *et al.*, 2004; Watmuff *et al.*, 2012).

Initially, day 21 cultures were immunolabelled for TUJ-1 and counter stained with TOPRO-3 to determine whether co-localisation of Pitx3 and TUJ-1 occurred. In both culture systems, the majority of Pitx3+ cells also co-expressed TUJ-1 (Figure 53A + 53B). Quantification of TUJ-1+ cells in both culture systems revealed that the percentage of cells that labelled for both TUJ-1 and TOPRO-3 was not significantly different (Figure 53C). However, the number of Pitx3+ cells in PA6 cultures that also stained for TOPRO-3 and TUJ-1 was significantly higher compared to Pitx3+ cells in monolayer cultures (Figure 53D + 53E).

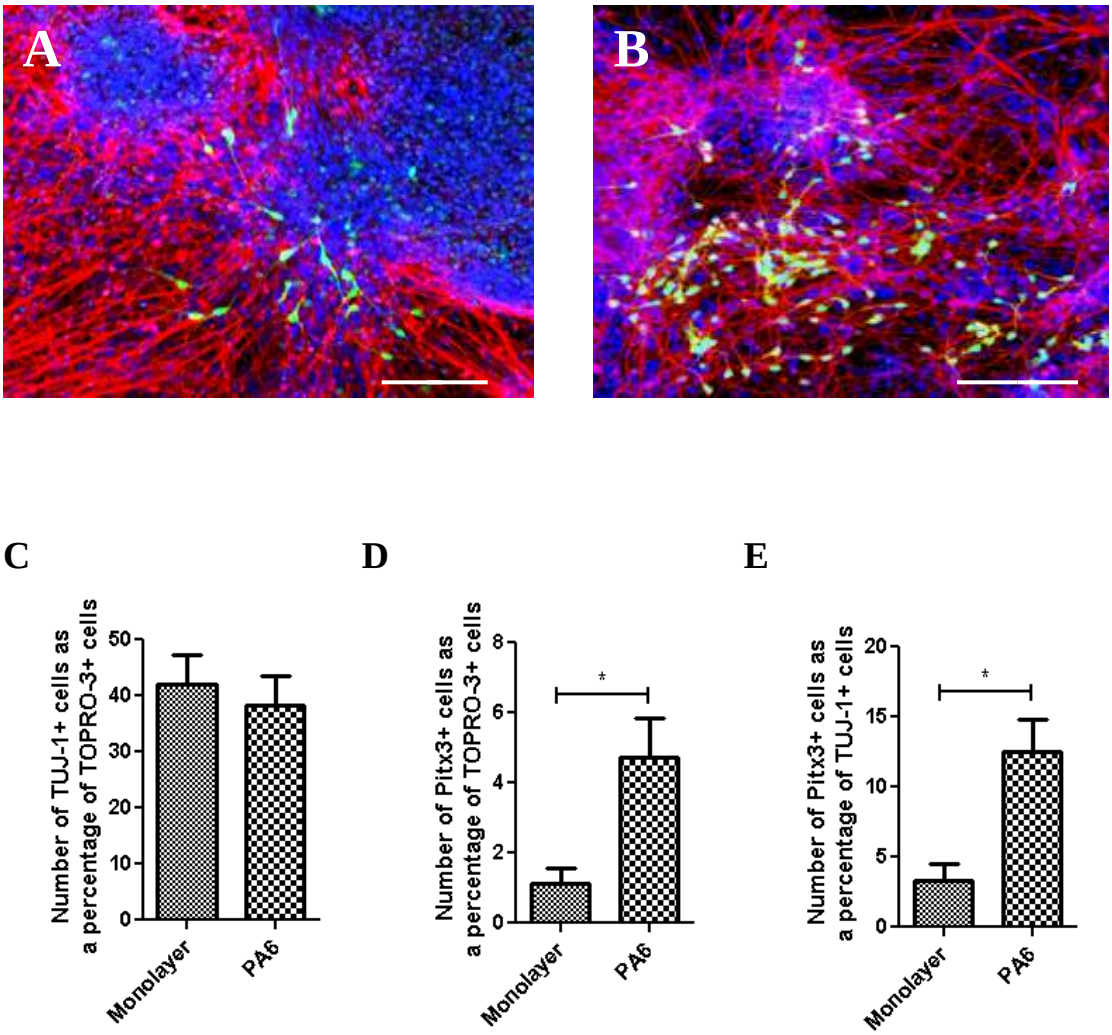

Figure 53: Pitx3-eGFP+ cells in terminally differentiated cultures. Immunolabelling of cultures for TH (red) in (A) monolayer cultures and (B) PA6 co-cultures; Pitx3-eGFP+ cells are in green. TOPRO-3 (blue) was used as nuclear counter stain. Comparison of the percentages of (C) TUJ-1+ TOPRO-3+, (D) Pitx3+ TOPRO-3+ and (E) Pitx3+ TUJ-1+ cells in both monolayer and PA6 co-cultures. Results are presented as the mean ± SEM of 3 independent experiments. * indicates $p < 0.05$ compared between monolayer and PA6 co-culture cells with Student's t-test. Scale bar = 100 µm.

Prior to calcium imaging experiments, regions containing Pitx3+ cells were first identified and images of the fields of view taken as outlined in 5.2.2.4. Post calcium imaging immunocytochemistry was performed using antibodies against TH and GAD67. The same coordinates containing Pitx3+ cells were then revisited. Previous images of Pitx3+ cells were superimposed onto images containing TH+ and GAD67+ cells to identify midbrain dopaminergic and GABAergic neurons. During experimental re-runs, only Pitx3+ TH+ cells and GAD67+ cells were selected for functional characterisation (Figure 54).

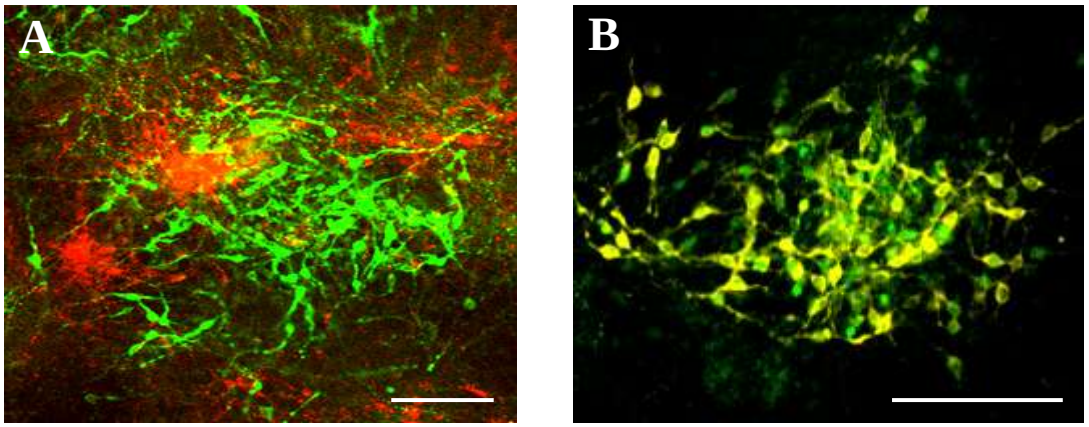

Figure 54: Immunolabelling of terminally differentiated cultures. (A) Culture immunolabelled for GAD67 (red) and TH (green). (B) Culture immunolabelled for TH (yellow). Pitx3-eGFP+ cells are in green. Scale bar = 100 µm.

For analysis of antagonist effects, peak $[Ca^{2+}]_i$ after addition of glutamate in the presence of glutamate receptor antagonists was expressed as a fraction of the initial response to glutamate addition. The antagonists used in these studies were LY235959, NBQX and CNQX. LY235959 is a competitive NMDA receptor antagonist, NBQX is an AMPA receptor antagonist which shows a 20 - 150 fold selectivity for AMPA over kainate receptors (Kew and Kemp, 2005) and CNQX is a competitive AMPA/kainate receptor antagonist which exhibits very little selectivity between AMPA and kainate receptors (Kew and Kemp, 2005). Terminally differentiated cultures were incubated with vehicle control, one (LY235959), two (LY235959 and NBQX) or all three antagonists to determine the presence of NMDA, AMPA and kainate receptors respectively.

In this study, NBQX, the AMPA receptor antagonist had the most profound effect on the response to glutamate for Pitx3+ TH+ and GAD67+ cells in monolayer cultures (one-way ANOVA post-hoc Bonferroni's test, $p < 0.001$, $n=3$) (Figure 55A + 55B). The subsequent addition of the AMPA/kainate antagonist, CNQX, did not further inhibit responses to glutamate for Pitx3+ TH+ cells. However, CNQX significantly inhibited responses to glutamate for GAD67+ cells (one-way ANOVA post-hoc Bonferroni's test, $p < 0.05$, $n=3$) (Figure 55B).

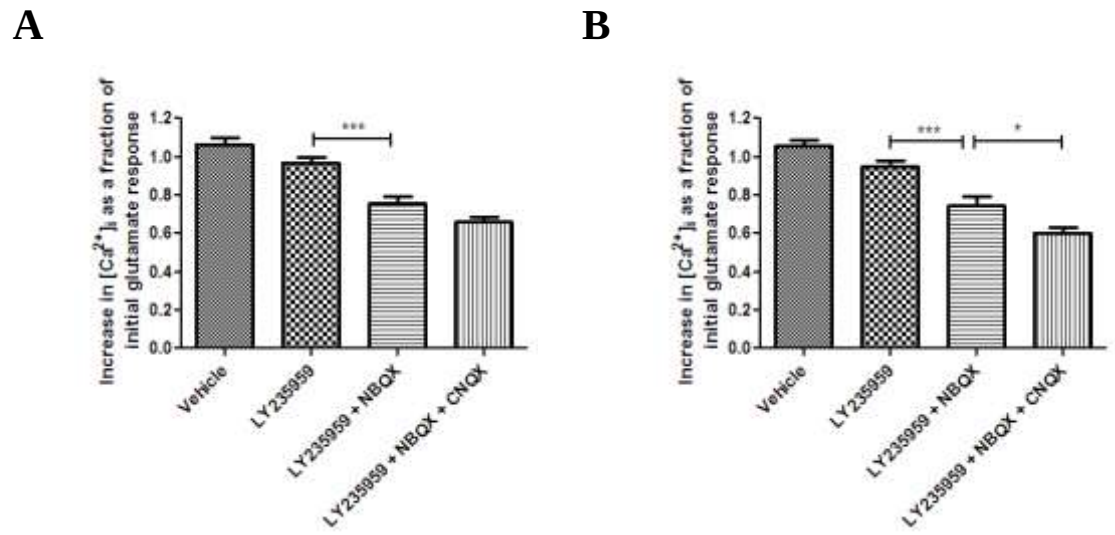

Figure 55: Effects of selective glutamate receptor antagonists on midbrain dopaminergic and GABAergic neurons derived from monolayer differentiation. Changes in intracellular Ca^{2+} of (A) Pitx3+ TH+ and (B) GAD67+ cells in response to glutamate after incubation with 10 µM LY235959, 20 µM NBQX and 10 µM CNQX. Results are presented as the mean ± SEM of 3 independent experiments. *, *** indicate p <0.05 and p <0.001, respectively, compared to vehicle or antagonist addition with one-way ANOVA plus post-hoc Bonferroni's test.

In PA6 co-cultures, LY235959, the NMDA antagonist had a significant inhibitory effect on the response to glutamate for both Pitx3+ TH+ and GAD67+ cells (one-way ANOVA post-hoc Bonferroni's test, p <0.05, n=3) (Figure 56A + 56B). However, subsequent additions of NBQX and CNQX had no significant effect on the response to glutamate for Pitx3+ TH+ cells. A significant effect on the response of GAD67+ cells to glutamate was observed with the addition of CNQX (one-way ANOVA post-hoc Bonferroni's test, p <0.05, n=3) (Figure 56B).

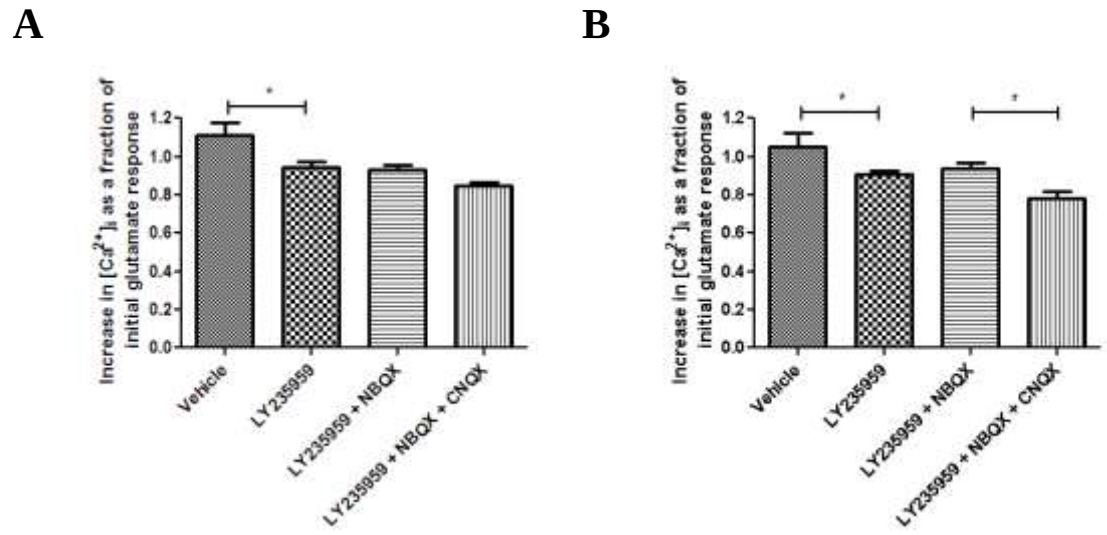

Figure 56: Effects of selective glutamate receptor antagonists on midbrain dopaminergic and GABAergic neurons derived from PA6 co-culture differentiation. Changes in intracellular Ca^{2+} of (A) Pitx3+ TH+ and (B) GAD67+ cells in response to glutamate after incubation with 10 µM LY235959, 20 µM NBQX and 10 µM CNQX. Results are presented as the mean ± SEM of 3 independent experiments. *, *** indicate $p < 0.05$ and $p < 0.001$, respectively, compared to vehicle or antagonist addition with one-way ANOVA plus post-hoc Bonferroni's test.

5.3.3. Wnt5a studies

To determine whether Wnt5a potentiated responses to sub-maximal concentrations of glutamate receptor agonists in Pitx3+ TH+ and GAD67+ cells, cultures were incubated with Wnt5a for 20 minutes. Agonist concentrations used in these studies were based on data from 5.3.1, thus concentrations were chosen that were unable to elicit maximal increases in Ca^{2+} fluorescence intensity.

Basal $[Ca^{2+}]_i$ levels after vehicle or Wnt5a incubation were compared to the basal $[Ca^{2+}]_i$ levels before incubation for both culture systems. The change in fluorescence intensity was expressed as the mean basal fluorescence intensity after vehicle or Wnt5a incubation

(post) over the mean basal fluorescence intensity prior to incubation (pre). Incubation of cells from both differentiated cultures with Wnt5a failed to increase basal $[Ca^{2+}]_i$ levels (Student's t-test, p >0.05, n=3) (Figure 57). For monolayer cultures, 3 µM each of glutamate, AMPA, kainate and NMDA were used. Wnt5a was able to potentiate significant responses to NMDA in Pitx3+ TH+ neurons (Student's t-test, p <0.01, n=3) (Figure 58A). For GAD67+ neurons, Wnt5a elicited a significant increase in Ca^{2+} fluorescence intensity in response to both glutamate and NMDA (Student's t-test, p <0.01, n=3) (Figure 58B).

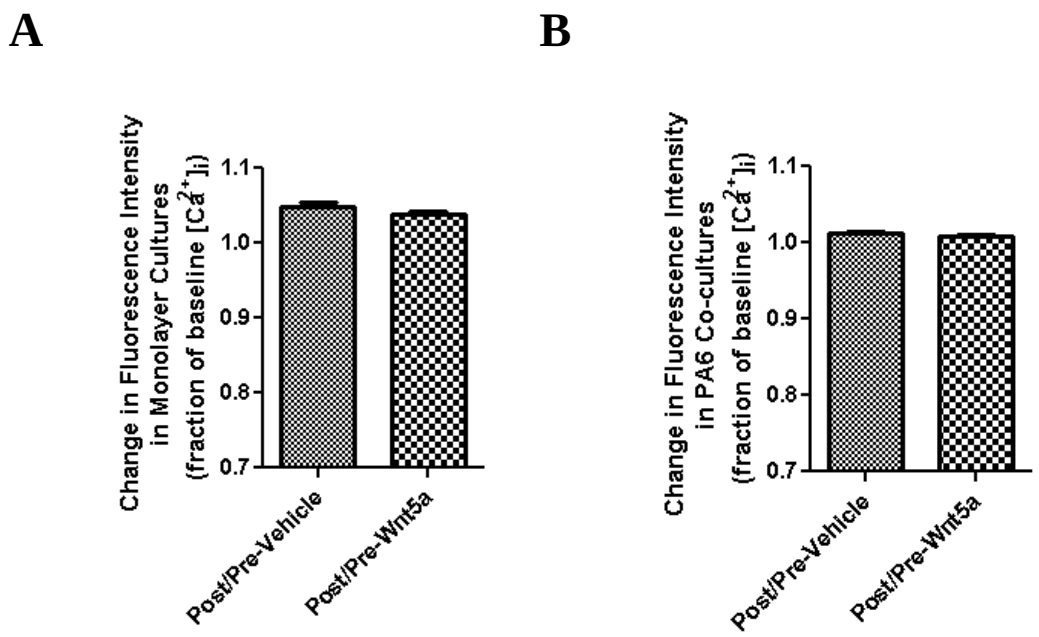

Figure 57: Effect of Wnt5a on basal intracellular Ca^{2+} levels. Changes in basal intracellular Ca^{2+} after vehicle and Wnt5a incubation in (A) monolayer cultures and (B) PA6 co-cultures. Results are presented as the mean ± SEM of at least 3 independent experiments.

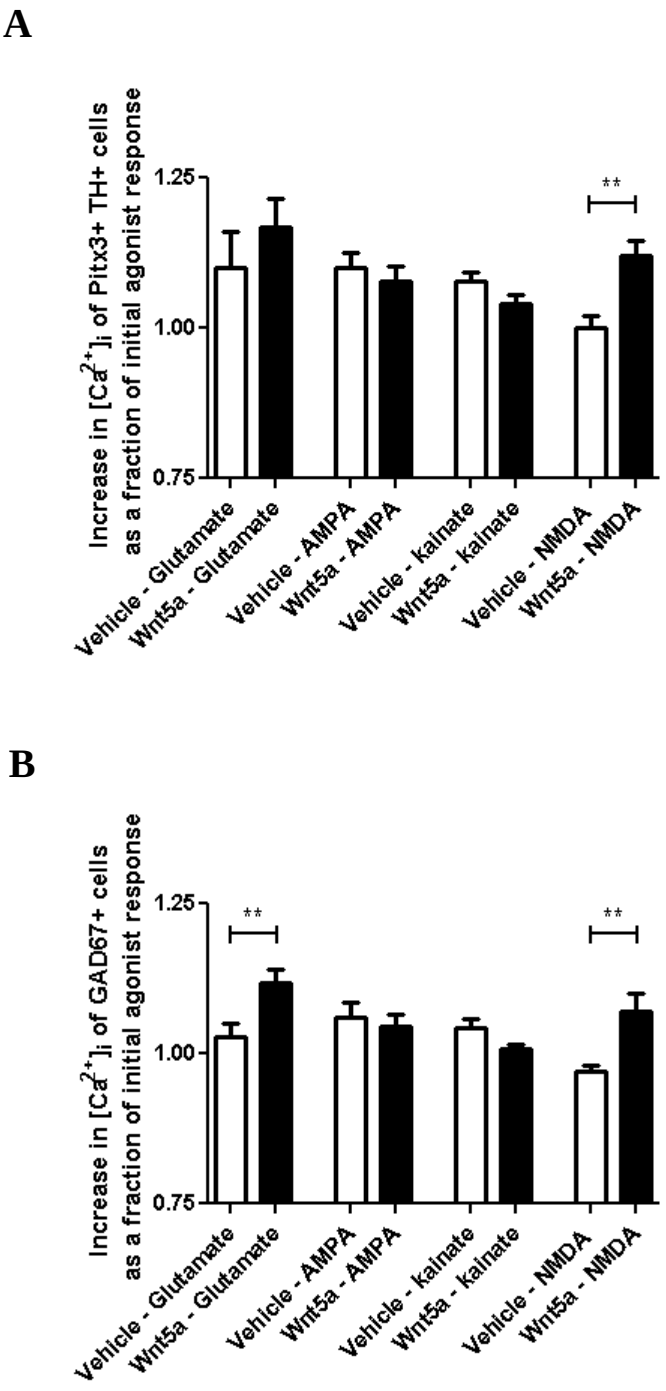

Figure 58: Effect of Wnt5a on intracellular Ca^{2+} in response to glutamate agonists in monolayer cultures. Changes in intracellular Ca^{2+} of (A) Pitx3+ TH+ and (B) GAD67+ cells in response to 3 µM glutamate, 3 µM AMPA, 3 µM kainate and 3 µM NMDA after incubation with vehicle and Wnt5a. Results are presented as the mean ± SEM of 3 independent experiments. ** indicates $p < 0.01$ compared to vehicle control with Student's t-test.

For PA6 co-cultures, 30 µM AMPA was selected as $[Ca^{2+}]_i$ elevations in response to AMPA was sub-maximal and 3 µM each of glutamate, kainate and NMDA were used. Exposure to Wnt5a significantly increased the response of Pitx3+ TH+ neurons to kainate (Student's t-test, $p < 0.01$, n=3) (Figure 59A). AMPA and kainate were able to significantly increase $[Ca^{2+}]_i$ of GAD67+ cells (Student's t-test, $p < 0.01$, n=3) after Wnt5a incubation (Figure 59B).

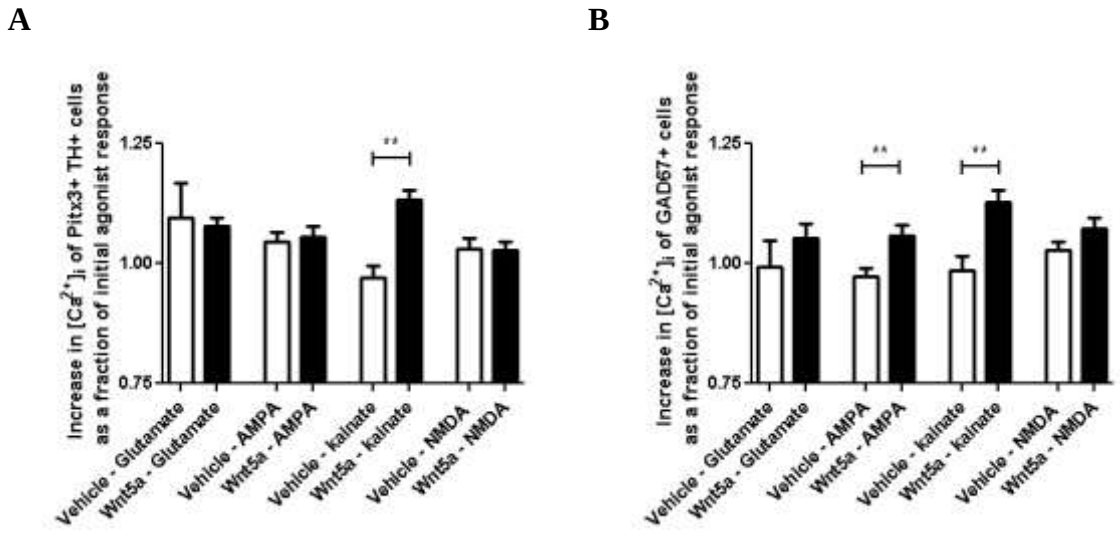

Figure 59: Effect of Wnt5a on intracellular Ca^{2+} in response to glutamate agonists in PA6 co-cultures. Changes in intracellular Ca^{2+} of (A) Pitx3+ TH+ and (B) GAD67+ cells in response to 3 µM glutamate, 30 µM AMPA, 3 µM kainate and 3 µM NMDA after incubation with vehicle and Wnt5a. Results are presented as the mean ± SEM of 3 independent experiments. ** indicates $p < 0.01$ compared to vehicle control with Student's t-test.

To determine whether Wnt5a increased the number of cells responding to agonists, the fractions of cells responding to agonists before and after Wnt5a additions were analysed. The fraction of responsive cells after incubation (post) over the fraction of responsive cells prior to incubation (pre) is presented as the change in fraction of responsive cells after incubation with vehicle or Wnt5a. In monolayer cultures, there was no significant increase in fraction of Pitx3+ TH+ or GAD67+ cells responding to 3 µM glutamate, 3 µM AMPA, 3 µM kainate and 3 µM NMDA after Wnt5a incubation compared to vehicle control (Student's t-test, $p > 0.05$, $n=3$) (Figure 60 + 61 + 62 + 63).

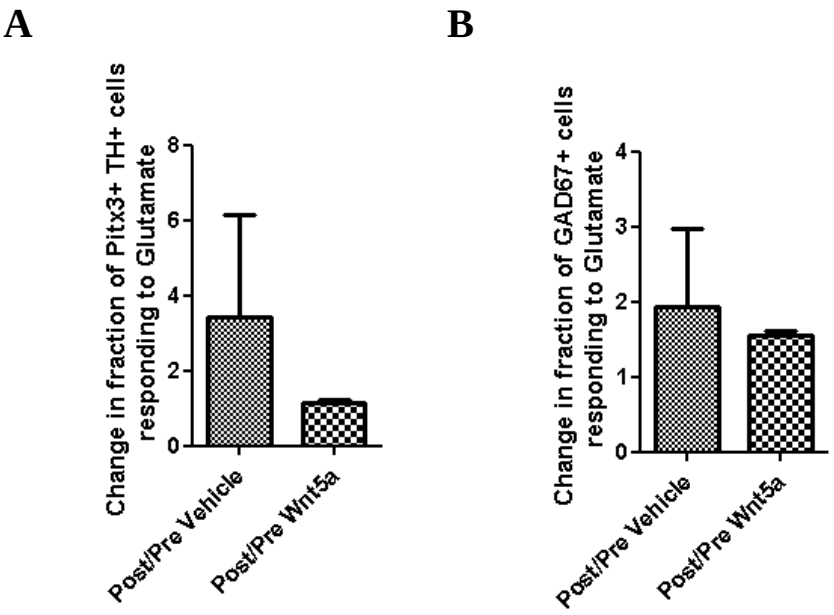

Figure 60: Effect of Wnt5a on the fraction of Pitx3+ TH+ and GAD67+ cells in monolayer cultures responding to 3 µM glutamate. Fraction of (A) Pitx3+ TH+ and (B) GAD67+ cells displaying elevation in intracellular Ca^{2+} after (post) compared to before (pre) incubation with vehicle control and Wnt5a. Results are presented as the mean ± SEM of 3 independent experiments.

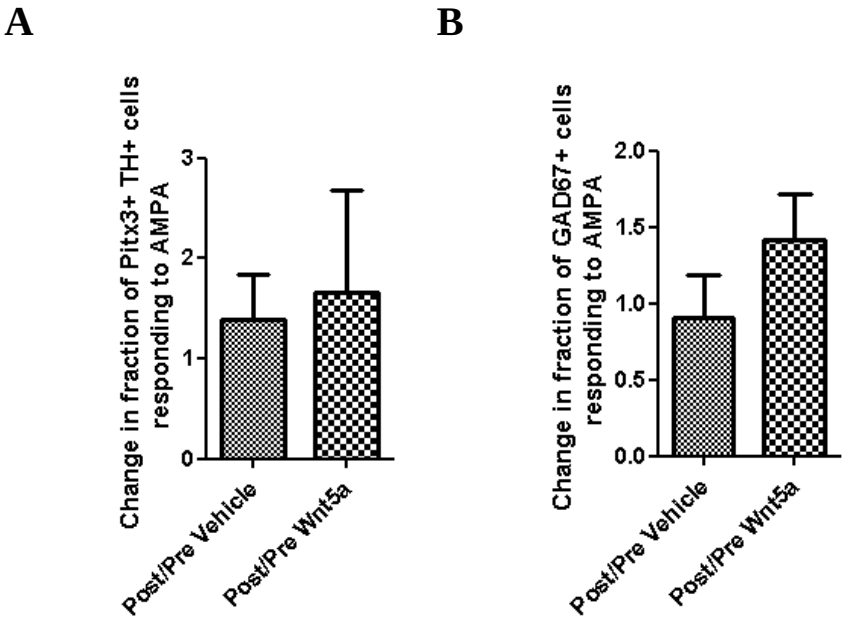

Figure 61: Effect of Wnt5a on the fraction of Pitx3+ TH+ and GAD67+ cells in monolayer cultures responding to 3 µM AMPA. Fraction of (A) Pitx3+ TH+ and (B) GAD67+ cells displaying elevation in intracellular Ca^{2+} after (post) compared to before (pre) incubation with vehicle control and Wnt5a. Results are presented as the mean ± SEM of 3 independent experiments.

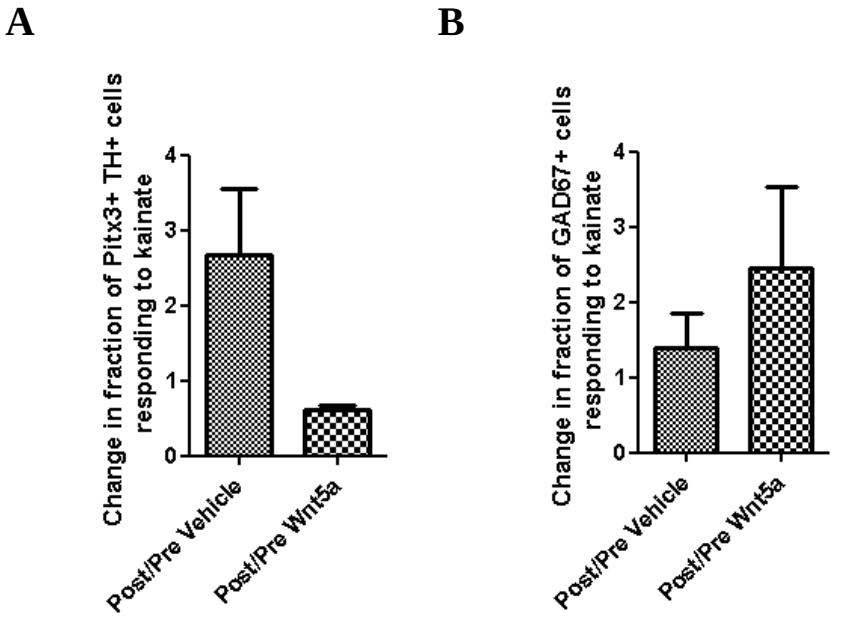

Figure 62: Effect of Wnt5a on the fraction of Pitx3+ TH+ and GAD67+ cells in monolayer cultures responding to 3 µM kainate. Fraction of (A) Pitx3+ TH+ and (B) GAD67+ cells displaying elevation in intracellular Ca^{2+} after (post) compared to before (pre) incubation with vehicle control and Wnt5a. Results are presented as the mean ± SEM of 3 independent experiments.

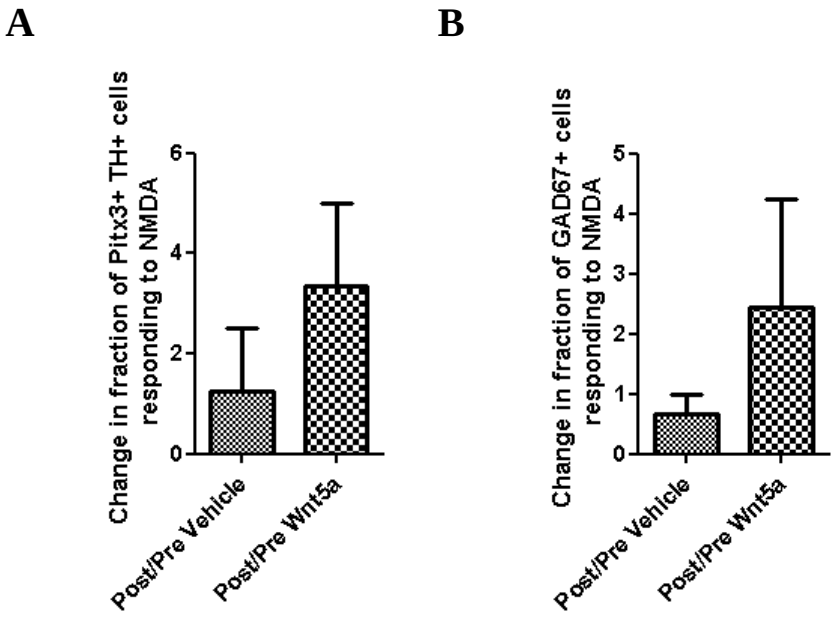

Figure 63: Effect of Wnt5a on the fraction of Pitx3+ TH+ and GAD67+ cells in monolayer cultures responding to 3 µM NMDA. Fraction of (A) Pitx3+ TH+ and (B) GAD67+ cells displaying elevation in intracellular Ca^{2+} after (post) compared to before (pre) incubation with vehicle control and Wnt5a. Results are presented as the mean ± SEM of 3 independent experiments.

Similarly in PA6 co-cultures, there were no significant changes in the fraction of Pitx3+ TH+ and GAD67+ cells responding to 3 µM glutamate, 3 µM kainate, 3 µM NMDA and 30 µM AMPA after incubation with Wnt5a compared to vehicle control (Student's t-test, $p > 0.05$, $n=3$) (Figure 64 + 65 + 66 + 67).

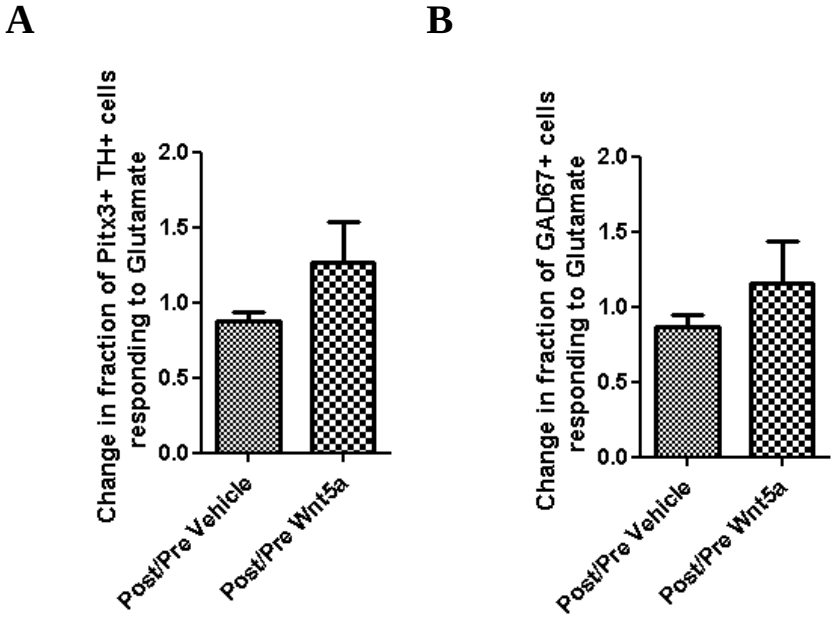

Figure 64: Effect of Wnt5a on the fraction of Pitx3+ TH+ and GAD67+ cells in PA6 co-cultures responding to 3 µM glutamate. Fraction of (A) Pitx3+ TH+ and (B) GAD67+ cells displaying elevation in intracellular Ca^{2+} after (post) compared to before (pre) incubation with vehicle control and Wnt5a. Results are presented as the mean ± SEM of 3 independent experiments.

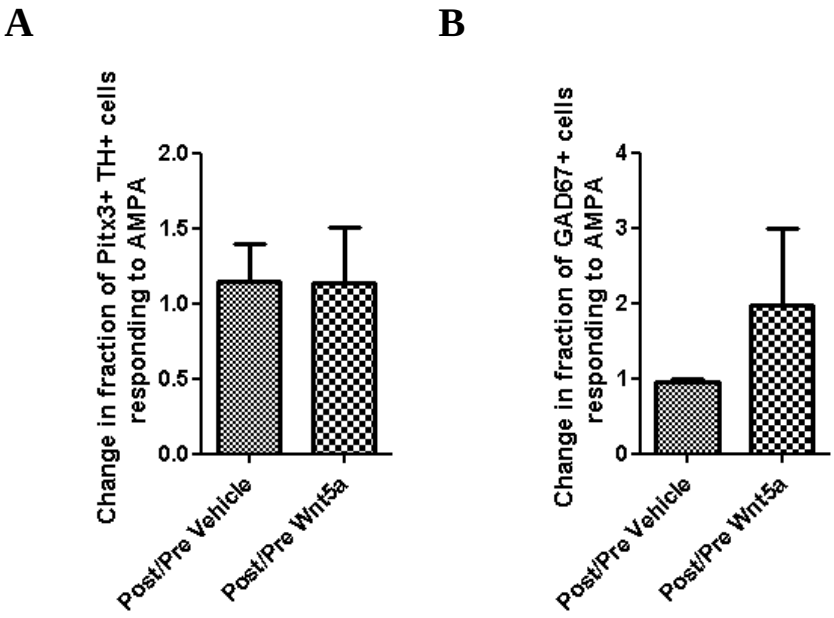

Figure 65: Effect of Wnt5a on the fraction of Pitx3+ TH+ and GAD67+ cells in PA6 co-cultures responding to 30 µM AMPA. Fraction of (A) Pitx3+ TH+ and (B) GAD67+ cells displaying elevation in intracellular Ca^{2+} after (post) compared to before (pre) incubation with vehicle control and Wnt5a. Results are presented as the mean ± SEM of 3 independent experiments.

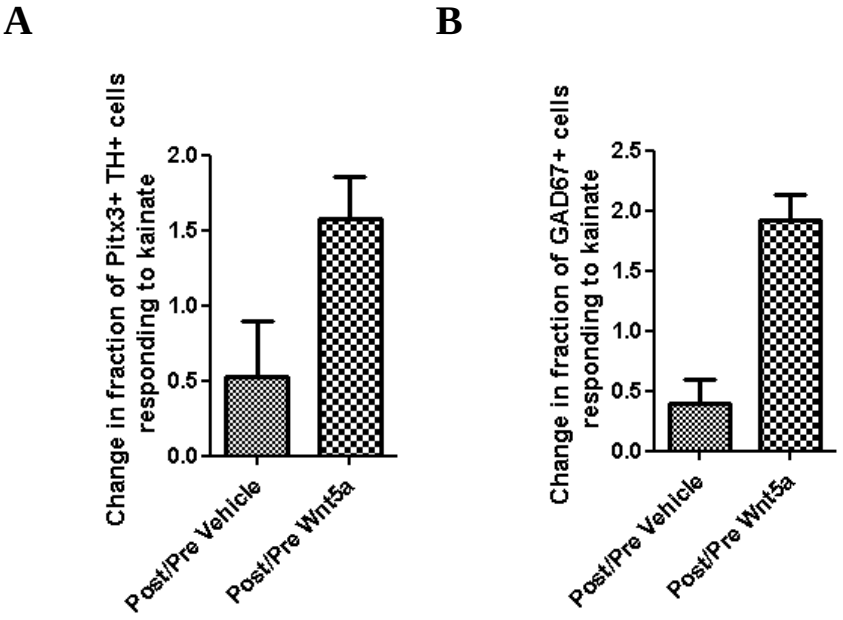

Figure 66: Effect of Wnt5a on the fraction of Pitx3+ TH+ and GAD67+ cells in PA6 co-cultures responding to 3 µM kainate. Fraction of (A) Pitx3+ TH+ and (B) GAD67+ cells displaying elevation in intracellular Ca^{2+} after (post) compared to before (pre) incubation with vehicle control and Wnt5a. Results are presented as the mean ± SEM of 3 independent experiments.

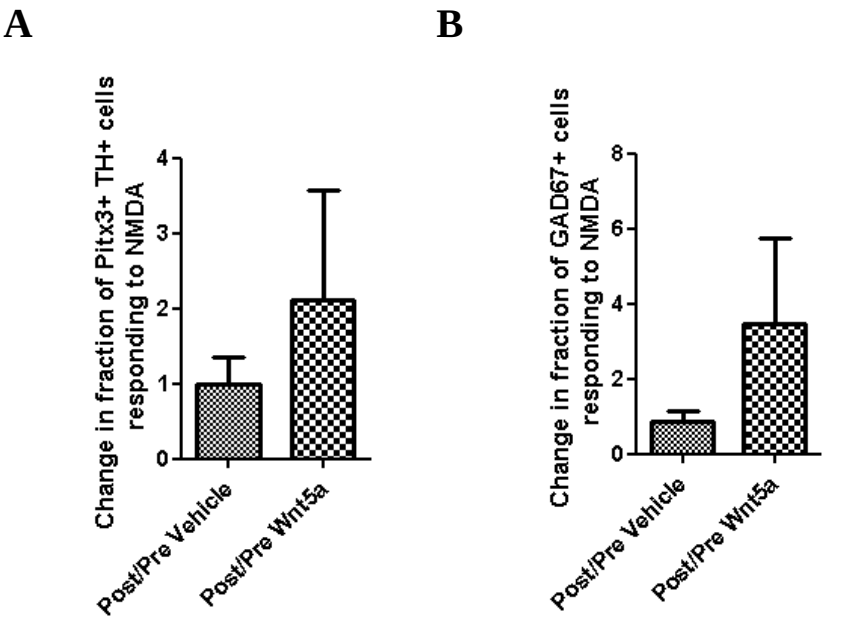

Figure 67: Effect of Wnt5a on the fraction of Pitx3+ TH+ and GAD67+ cells in PA6 co-cultures responding to 3 µM NMDA. Fraction of (A) Pitx3+ TH+ and (B) GAD67+ cells displaying elevation in intracellular Ca^{2+} after (post) compared to before (pre) incubation with vehicle control and Wnt5a. Results are presented as the mean ± SEM of 3 independent experiments.

5.4 Discussion

Neurons can be differentiated from ES cells, these neurons exhibit similar morphology and functionality to the adult neurons of the brain (Vescovi *et al.*, 1993; Smith *et al.*, 2003). In this chapter, I examined the responses of midbrain dopaminergic and GABAergic neurons derived from monolayer and PA6 co-culture differentiation protocols and partially characterised the glutamate receptors expressed by these cells. In addition, the capability of Wnt5a to modulate cell responses to sub-maximal glutamate receptor agonist addition was also investigated.

5.4.1 Responses to glutamate agonists

Differentiating cultures from both protocols were replated on day 10 with calcium imaging performed on day 21. Cells were replated at day 10 of differentiation as (1) findings from our laboratory have shown that cultures replated on day 10 produced higher numbers of TH+ neurons (Zeng *et al.*, 2011) and (2) calcium imaging studies require the selection of single cells, replating reduces the density of cells in the cultures enabling single neurons to be easily identified. Furthermore, our laboratory has also identified day 21, of monolayer differentiation, as the time point when neurons in culture are functionally mature (Watmuff *et al.*, 2012). As PA6 co-cultures contain both ES cells and stromal cells, a higher replating density was used compared to monolayer cells. Cells that displayed distinctive neuronal morphology were selected for further analysis.

In previous studies conducted in our laboratory, we have shown that glutamate agonists, including AMPA, kainate and NMDA elevate intracellular Ca^{2+} in mature TH+ neurons

derived from mES cells (Lang *et al.*, 2004; Raye *et al.*, 2007; Watmuff *et al.*, 2012). Measurement of intracellular calcium concentration using calcium imaging is used by many groups to examine cellular function (Takahashi *et al.*, 1999; Brownlee, 2000; Stosiek *et al.*, 2003; Wang *et al.*, 2008; Meyer *et al.*, 2009). The advantage of this technique is that it allows the investigation of intracellular Ca^{2+} influx of multiple neurons at the same time compared to electrophysiological techniques which usually investigate the properties of one particular neuron at a time. Agonists bind to receptors to trigger a series of events leading to an increase in intracellular Ca^{2+}, which is then visualised during imaging experiments. All calcium imaging experiments were performed in the presence of TTX, a sodium channel blocker, to abolish action potentials transmitted by axons and potentially reduce the release of neurotransmitters, particularly GABA which affects elevations of intracellular Ca^{2+} in response to agonist additions (Yoshida, 1994; Watmuff *et al.*, 2012).

Previous findings from our laboratory have shown that mature neurons derived via an EB formation method respond to glutamate agonists with a rank order of potency of glutamate=AMPA=kainate>NMDA (Lang *et al.*, 2004). In adherent monolayer cultures, the concentration-response curves obtained for glutamate agonists had a rank order of potency of glutamate=AMPA=kainate=NMDA, with the potency of NMDA higher compared to EB-derived cultures (Lang *et al.*, 2004). These data suggest that neurons derived via monolayer differentiation possibly express AMPA, kainate and NMDA receptors. Interestingly, in these monolayer cultures the addition of NMDA potentiated responses from neurons in the absence of glycine. Glycine, an inhibitory factor that acts a

co-agonist which binds at a distinct site on NMDA receptors to modulate glutamate binding (Johnson and Ascher, 1987), has been reported as a requirement for the activation of NMDA receptors (Kleckner and Dingledine, 1988) which may explain the low magnitude of responses to NMDA seen in this study. At the lowest agonist concentrations used, 25%< of the population of neurons responded to the agonist additions. However, when concentrations of the agonists were increased a 100-fold, >50% of the neurons responded to each agonist addition.

Unlike monolayer cultures, the rank order of agonist potency in PA6 co-culture derived neurons was glutamate=kainate=NMDA>AMPA. These data indicate that the rank order of potency for glutamate agonists varies in each culture system. Surprisingly, the magnitude of $[Ca^{2+}]_i$ increase in response to NMDA was similar to glutamate at 300 µM even without glycine. It is not known how, in this study, NMDA potentiates responses from cells in the absence of glycine as previous studies have reported that glycine is required to potentiate NMDA responses (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). It would be interesting to determine whether the addition of glycine can further increase the magnitude of $[Ca^{2+}]_i$ response to NMDA. Similar to monolayer cultures, increasing percentages of cells responding to each agonist addition was observed with increasing concentrations. At 300 µM, >70% of the selected population responded to glutamate, AMPA and kainate while ~40% neurons responded to NMDA. These results indicate that AMPA, kainate and NMDA receptors are possibly expressed by neurons in PA6 co-cultures. Through these early experiments we determine that mature neurons derived via varying differentiation protocols do not respond similarly to

glutamate agonists. Due to insufficient time, a future study that could be carried out is to measure the response of any particular neuron to all the glutamate agonists to determine whether more than one type of glutamate receptor is expressed.

5.4.2 Pitx3+ cells in terminally differentiated cultures

Pitx3 expression in the brain is restricted to all midbrain dopaminergic neurons and is required for proper development and survival of those neurons (Hwang *et al.*, 2003; Munckhof *et al.*, 2003; Nunes *et al.*, 2003; Smidt *et al.*, 2004; Maxwell *et al.*, 2005). The majority of Pitx3-eGFP+ cells co-localised with TUJ-1, indicating that these cells were indeed neurons. Differentiation from both protocols yielded a similar number of neuronal populations within the cultures. The percentage of Pitx3+ cells that were immunoreactive for TUJ-1 in replated PA6 cultures outnumbered the double positive cells in monolayer cultures at a ratio of 4:1. It was not surprising that Pitx3+ neurons were also more abundant in PA6 co-cultures as data from the previous chapter has shown that TH+ neurons are more frequently observed in PA6 co-cultures. Terminally differentiated cultures were immunolabelled with antibodies against TH and GAD67 after calcium imaging experiments to identify midbrain dopaminergic and GABAergic neurons. Only cells that were positive for both Pitx3-eGFP and TH were determined as *bona fide* midbrain dopaminergic neurons (Smidt *et al.*, 2004) as dopaminergic neurons in the hypothalamus and olfactory bulb as well as noradrenergic neurons express TH but not Pitx3 (Smidt *et al.*, 1997; Smidt *et al.*, 2000).

5.4.3 Effects of ionotropic glutamate receptor antagonists

To confirm the responses seen from the previous agonist studies was due to binding of agonists to their respective receptors, cells were incubated with combinations of NMDA and AMPA/kainate receptor antagonists; LY235959, NBQX and CNQX. For this study, post calcium imaging immunocytochemistry was conducted to identify midbrain dopaminergic and GABAergic neurons.

5.4.3.1. Monolayer cultures

In monolayer cultures, only NBQX had a profound effect on the response of Pitx3+ TH+ neurons to glutamate. Previous studies have shown that NMDA and AMPA receptor subunits are expressed in the rat substantia nigra (Martin *et al.*, 1993; Petralia *et al.*, 1994; Standaert *et al.*, 1994; Yung, 1998; Albers *et al.*, 1999; Chatha *et al.*, 2000). It has also been reported that almost all SNpc and VTA dopaminergic neurons in monkey brains express AMPA (GluR1, GluR2/3 and GluR4) and NMDA receptor subunits (NR1) (Paquet *et al.*, 1997). Consistent with these findings, our results indicate a predominant AMPA receptor effect on midbrain dopaminergic neurons derived from adherent monolayer differentiation. The response of GAD67+ neurons to glutamate was also profoundly affected by NBQX. Furthermore, CNQX reduced the response of these neurons to glutamate. These findings suggest that these GABAergic neurons express both AMPA and kainate receptors, and was consistent with previous findings by other groups that AMPA (Yin *et al.*, 1994; Kharazia *et al.*, 1996; Racca *et al.*, 1996; Kondo *et al.*, 2000; Namba *et al.*, 2006) and kainate (Braga *et al.*, 2003; Gisabella *et al.*, 2012) receptor subunits are expressed by GABAergic neurons located in various regions of the brain.

5.4.3.2 PA6 co-cultures

Most of the antagonist effects detected on the monolayer neurons were not observed in PA6 co-cultures, where LY235959 decreased the response of Pitx3+ TH+ neurons to glutamate but the AMPA and kainate antagonists had no further effect, indicating a population of NMDA receptors present in the midbrain dopaminergic neurons. Unlike the GAD67+ neurons in monolayer cultures, the response of GAD67+ neurons derived from PA6 co-cultures to glutamate was reduced by LY235959 but not NBQX. However, CNQX also reduced the response of these cells to glutamate. These data suggested that these GABAergic neurons express NMDA and kainate receptors, but not AMPA receptors. The presence of NMDA receptors on GABAergic neurons is consistent with previous reports that GABAergic neurons express the NR1 and NR2 receptor subunits (Plant *et al.*, 1997; Standaert *et al.*, 1999; Küppenbender *et al.*, 2000; Shiokawa *et al.*, 2010). Considering that for both culture systems, the highest increases of $[Ca^{2+}]_i$ were observed in response to agonists at 300 μM, it is possible that binding of the glutamate agonists was not selective to the ionotropic glutamate receptors.

Interestingly, there were distinct differences between the glutamate receptors expressed by midbrain dopaminergic and GABAergic neurons in monolayer and PA6 co-cultures. The differences in receptor expression appear to be restricted by differentiation method, rather than neuronal subtypes as Pitx3+ TH+ and GAD67+ neurons predominantly express AMPA receptors in monolayer cultures while both these neuronal phenotypes derived from PA6 co-cultures express NMDA receptors. However, results from the agonist studies

indicate that neurons in both culture systems express all glutamate receptors but data from antagonist studies suggest that binding of certain agonists, such as NMDA in adherent monolayer and AMPA in PA6 co-cultures, were not selective. A caveat to the antagonist experiments is that only a single agonist is used versus a combination of antagonists and that a better approach would be to use additions of the other glutamate receptors agonists and incubation with their respective antagonists. In addition, future antagonist experiments should be performed in a single culture well rather than multiple wells to determine the effect of each antagonist on the response of any particular neuron. To fully characterise the receptors expressed by these neuronal phenotypes derived from each differentiation paradigm, qPCR or reverse-transcriptase PCR would have to be performed on sorted neuronal populations to identify the subunits of each ionotropic glutamate receptor classes. However, due to time constraints, qPCR experiments and a more comprehensive functional analysis were not carried out.

5.4.4 Effect of Wnt5a on intracellular calcium

The Wnt signalling pathways are heavily involved in neurogenesis and synaptic plasticity (Inestrosa and Arenas, 2010). Recent studies have implicated Wnt5a in the development of the ventral midbrain and specifically, midbrain dopaminergic neurons (Castelo-Branco *et al.*, 2003; Schulte *et al.*, 2005; Castelo-Branco *et al.*, 2006; Andersson *et al.*, 2008; Parish *et al.*, 2008). Farias *et al.*(2009) showed that the non-canonical Wnt-Ca^{2+} signalling pathway in hippocampal neurons could be activated by the binding of Wnt5a within 20 minutes. Therefore, cultures were incubated with Wnt5a for 20 minutes to determine the effects of Wnt5a on $[Ca^{2+}]_i$ and the changes in response to glutamate receptor agonists.

Fluorescence intensities were recorded before and after Wnt5a incubations to determine whether Wnt5a affected $[Ca^{2+}]_i$ in response to sub-maximal additions of each agonist. Incubation with Wnt5a had no effect on basal $[Ca^{2+}]_i$ levels. However, significant elevations in $[Ca^{2+}]_i$ in response to certain agonists were observed after cultures were incubated with Wnt5a. In monolayer cultures, Wnt5a modulated agonist induced elevations of $[Ca^{2+}]_i$ in response to glutamate and NMDA but not AMPA and kainate. The increase in response to glutamate and NMDA was not observed in PA6 co-cultures; significant increases in responses to sub-maximal additions of AMPA and kainate after Wnt5a incubation were detected instead. Wnt5a is able increase the cycling of NMDA receptors to the membrane in CA1 pyramidal neurons (Cerpa *et al.*, 2010). The agonist elevated $[Ca^{2+}]_i$ in the presence of Wnt5a is possibly due to the increased turnover of NMDA and AMPA/kainate receptors to the membrane of neurons, but not long enough for increased synthesis. In addition, the increased $[Ca^{2+}]_i$ in response to glutamate agonists after Wnt5a incubation was consistent with the finding by Cerpa *et al.* (2010) that the potentiation of glutamatergic transmission by Wnt5a is due to postsynaptic modulation of glutamatergic currents mediated by activation of NMDA and AMPA receptors. Although responses to certain glutamate receptor agonists were increased by Wnt5a, the fraction of responding cells in either of the culture systems did not change. This further supports that Wnt5a modulates cycling of ionotropic glutamate receptors to the membrane of neurons.

5.5 Conclusion

In summary, these data indicate that midbrain dopaminergic neurons and GABAergic neurons derived via adherent monolayer and PA6 co-culture do not possess similar ionotropic glutamate receptor functional properties. Neurons respond to glutamate, AMPA, kainate and NMDA at different magnitudes depending on the culture method. Furthermore, midbrain dopaminergic and GABAergic neurons derived from different protocols express different ionotropic glutamate receptors, with the difference in receptor expression restricted by differentiation method. Wnt5a is able to potentiate responses to sub-maximal concentrations of different glutamate agonists depending on the differentiation protocol performed. These data may facilitate the derivation of midbrain dopaminergic or GABAergic neurons which are most similar, in terms of ionotropic glutamate receptor expression, to neurons in the mammalian brain. However, the significance of these findings is currently unknown.

CHAPTER 6

General Discussion

6.1 Thesis findings

The focus of this work was to understand the development of midbrain dopaminergic neurons *in vitro* and isolate early dopaminergic progenitors using genetically modified reporter mES cell lines. In recent years, the identification of key transcription factors and patterning cues has enhanced the understanding of midbrain dopaminergic neuron development *in vivo*. However, the expression of these genes during ES cell differentiation has not been previously established.

Dopaminergic neurons have been derived from mES cultures via many differentiation protocols with varying success; some methods generate high numbers of dopaminergic neurons while others rarely give rise to many TH+ neurons (Kawasaki *et al.*, 2000; Lee *et al.*, 2000b; Barberi *et al.*, 2003; Ying *et al.*, 2003b; Perrier *et al.*, 2004). In the first chapter, differentiation of reporter cell lines was performed on adherent monolayer because of its chemically defined nature, allowing the effects of Shh, BMP and Wnt agonists and antagonists to be examined. Lmx1a and Msx1 expression were determined using the Lmx1a-AMP-IRES-eGFP and Msx1-AMP-IRES-eGFP cell lines which were generated by homologous recombination in our laboratory. Experiments showed that the proportion of cells expressing these genes was comparable throughout the differentiation process and their peak expressions coincide with culture conditions that lead to peak Sox1 expression, when cultures are enriched with neural progenitors. Exposure of cultures to exogenous factors right from the start of differentiation significantly influenced Lmx1a and Msx1 expression as well as TH+ cell numbers. Most interestingly, antagonism of the BMP and Wnt pathways elevated numbers of TH+ neurons in culture although Lmx1a and Msx1

expression were unaffected, proving that expression of these genes are not indicative of number of dopaminergic neurons in monolayer cultures. This was further reflected in results from our RA experiments. However, these data are in contrast to the study by Andersson *et al.* (2006b) which reported that Lmx1a and Msx1 are key determinants of midbrain dopaminergic neurons *in vivo*. These results suggested that Lmx1a+ cells have potentially already committed to pathways of other neuronal subtypes. Upon closer examination, TH+ cells in monolayer cultures expressed low levels of Foxa2 indicating these cells are not of midbrain origin. This further supports the suggestion that the TH+ neurons are actually of forebrain identity (Björklund *et al.*, 2002). The finding that Lmx1a is limited as a marker for midbrain dopaminergic neurons in monolayer cultures is important as it demonstrates that differentiation of ES cells into neurons in monolayer cultures can give rise to other progenitors that express high levels of Lmx1a. This suggests that more than one reporter will be needed to identify progenitors of midbrain dopaminergic neurons.

In the next chapter, I investigated the expression of genes in FACS sorted Lmx1a+ and Lmx1a- cells from monolayer cultures. Genes associated with neural and midbrain dopaminergic neurons *in vivo* were examined using qPCR. The Lmx1a+ cells expressed downstream targets Msx1, Wnt1 and Lmx1b but not Foxa2, which was upregulated in the Lmx1a- cells. Lmx1a+ cells also express Nestin and were able to generate viable neurospheres. Furthermore, these cells gave rise to cultures enriched with neurons and glia. However, the presence of dopaminergic neurons was rarely observed and the neurons predominantly expressed GABA. Upon closer inspection these GABA+ neurons

expressed GAD67 and SatB2, indicating the majority of neurons generated from Lmx1a+ cells are forebrain GABAergic neurons with upper-layer identity. This represents the first time Lmx1a has been associated with GABAergic neuron development. Furthermore, Lmx1a+ cells are already committed towards a forebrain GABAergic neuron fate by day 8 of monolayer differentiation as patterning cues were unable to alter the yield of other neuronal phenotypes. Findings from our laboratory have shown that these SatB2+ GABAergic neurons are functional, although it remains to be determined if these neurons have any physiological relevance since SatB2+ neurons in the cortex are unlikely to be GABAergic (Alcamo *et al.*, 2008). It has been reported that SatB2+ GABAergic interneurons are found in the mammalian retina (Kay *et al.*, 2011) but there are no reports associating Lmx1a with eye development *in vivo*. As no other studies have reported the presence of SatB2+ GABAergic neurons, it is uncertain whether there is a direct *in vivo* equivalent of these monolayer derived Lmx1a+ cells. Even though these cells become GABAergic neurons when directly plated at low density, at high densities Lmx1a+ cells form R-NSCs, radial structures with anterior identity (Elkabetz *et al.*, 2008). The PA6 co-culture method is an established method of deriving midbrain dopaminergic neurons (Kawasaki *et al.*, 2000; Barberi *et al.*, 2003), with TH+ neurons obtained from this protocol frequently co-expressing Lmx1a, an occurrence that was rarely observed in monolayer cultures. This work has shown that Lmx1a expression is context-specific; its expression does not necessarily generate midbrain dopaminergic neurons in monolayer differentiation protocols, unlike in PA6 co-cultures. This study further highlights the need for Foxa2 expression and supports the findings of Chung *et al.* (2009) that two independent transcription factor cascades, characterised by Lmx1a-Wnt1 and Shh-Foxa2

respectively, are required for midbrain dopaminergic neuron differentiation. In addition, two recent studies have reported that only co-expression of Lmx1a and Foxa2 are indicative of midbrain dopaminergic neuron differentiation. Jaeger *et al.* (2011) showed that under standard monolayer differentiation conditions mouse epiblast stem cells as well as hES cells produced Lmx1a+ Foxa2- cells and generated cultures with only moderate numbers of TH+ cells. Modulation of FGF/ERK signalling early during differentiation followed by exposure to Shh and FGF8 facilitated the generation of Lmx1a+ Foxa2+ cells and concomitantly increased the percentage of dopaminergic neurons. Kriks *et al.* (2011) showed that dual Smad inhibition produced cultures with Lmx1a+ Foxa2- cells that rarely gave rise to TH+ cells, whereas dual Smad inhibition plus stimulation of Shh and canonical Wnt pathways (from day 3) allowed efficient generation of cultures with Lmx1a+ Foxa2+ cells that produced dopaminergic neurons at high frequency. The observation that Wnt stimulation is required on day 3 at first appears to be incompatible with the data presented here. However in common with my work, Kriks *et al.* (2011) showed that stimulation of the Wnt pathway from day 1 of differentiation prevented production of floorplate and subsequent differentiation to midbrain dopaminergic neurons.

In the last chapter, the ionotropic glutamate functional properties of ES cell cultures derived from adherent monolayer and PA6 co-culture differentiation were examined. Neurons derived *in vitro* have been reported to express ionotropic glutamate receptors (Strübing *et al.*, 1995; Lang *et al.*, 2004), however the neuronal phenotypes were not characterised. I have shown that the rank order of glutamate agonist potency for

adherent monolayer and PA6 co-cultures were not similar. Using combinations of antagonists, I have also shown functional evidence of glutamate receptor expression in midbrain dopaminergic and GABAergic neurons in both culture systems. In monolayer cultures, midbrain dopaminergic neurons predominantly expressed AMPA receptors while GABAergic neurons expressed AMPA and kainate receptors. However, in PA6 co-cultures, midbrain dopaminergic neurons and GABAergic neurons expressed NMDA receptors while kainate receptors were also present on GABAergic neurons. These results indicate that neurons generated *in vitro*, at least in adherent monolayer and PA6 co-cultures, are not entirely similar in terms of receptor expression. While expression of these receptors were consistent with previous studies (Martin *et al.*, 1993; Petralia *et al.*, 1994; Standaert *et al.*, 1994; Yin *et al.*, 1994; Kharazia *et al.*, 1996; Racca *et al.*, 1996; Paquet *et al.*, 1997; Plant *et al.*, 1997; Yung, 1998; Albers *et al.*, 1999; Standaert *et al.*, 1999; Chatha *et al.*, 2000; Kondo *et al.*, 2000; Küppenbender *et al.*, 2000; Braga *et al.*, 2003; Namba *et al.*, 2006; Shiokawa *et al.*, 2010; Gisabella *et al.*, 2012), the differences in receptor expression was restricted by differentiation method rather than neuronal subtypes. Wnt5a is unable to increase basal intracellular Ca^{2+} levels. However, cells incubated with Wnt5a displayed increased intracellular Ca^{2+} in response to certain agonists, depending on the culture method; responses to glutamate and NMDA were elevated in monolayer cultures while responses to AMPA and kainate in PA6 co-cultures were elevated.

6.2 Future directions

In the growth factor screens, additions of noggin and dkk1 increased dopaminergic neuron numbers even though these neurons were not of midbrain origin. It would be interesting to examine the combinatorial effects of both these factors on numbers of TH+ neurons. Although Shh did not have any effects on mES cell differentiation in our studies, using higher concentrations or the Shh-C25II which is ~10 times more potent than non modified N terminal Shh (Fasano *et al.*, 2010) could ventralise ES cells to produce more midbrain dopaminergic neurons. Fasano and group (2010) induced large numbers of Foxa2+ floor plate cells and suppressed anterior neuroectoderm markers by exposing hES cells to the high levels of Shh-C25II during early stages of differentiation. The dual Smad inhibition protocol can potentially be applied to mES cells as it is a method that efficiently promotes neural conversion and has recently been shown that in combination with activation of the Shh and canonical Wnt signalling pathways, high percentages of Lmx1a+ Foxa2+ dopaminergic neurons can be produced from hES cells (Kriks *et al.*, 2011).

As a rosette formation step is employed in many hES cell neuronal differentiation protocols (Zhang *et al.*, 2001; Schulz *et al.*, 2003; Gerrard *et al.*, 2005; Itsykson *et al.*, 2005; Sonntag *et al.*, 2007), it could be possible to utilise extraction of Lmx1a+ mES cells to form R-NSCs then terminally differentiate them similarly to hES cell protocols. As differentiation of R-NSCs can be directed into dopaminergic neurons upon exposure to Shh and FGF8 (Elkabetz *et al.*, 2008), it may offer an alternative way of deriving dopaminergic neurons while still using defined conditions, without the need for PA6 stromal cells.

Although the expression of Lmx1a during mES cell differentiation has been established in this study, it would be interesting to investigate the Lmx1a expression pattern during hES cell differentiation. A similar Lmx1a reporter hES cell line has been generated in our laboratory, however due to time constraints, studies on this cell line were not carried out. Experiments conducted in this study, including the growth factors screens, qPCR and FACS extraction studies, could be applied to the hES reporter cell line to determine whether Lmx1a expression in hES cells is comparable to mES cells.

Through these studies it is clear that Lmx1a on its own is insufficient to isolate midbrain dopaminergic progenitors. For this purpose, one more target gene is required to efficiently identify midbrain dopaminergic progenitors. At present, Foxa2 appears to be a good candidate to possibly generate a Lmx1a-Foxa2 reporter cell line. As Lmx1a+ Foxa2+ cells produce dopaminergic neurons at high frequency (Kriks *et al.*, 2011), FACS sorting for cells containing both these markers could potentially generate a homogenous population of midbrain dopaminergic progenitors.

Although the generation of cortical GABAergic neurons from sorted Lmx1a+ cells was unexpected, this could lead to further investigation on the role of cortical neurons in the formation of memory networks. Synaptic plasticity in the cerebral cortex has been associated with memory formation (Klintsova and Greenough, 1999). A further study should involve the generation of reporter cell lines for the different cortical layers to study the connections between cortical neurons in mediating formation and storage of memory.

The ionotropic glutamate receptors expressed by midbrain dopaminergic and GABAergic neurons were determined through pharmacological experiments using selective agonists and antagonists. However due to time constraints, the exact receptor subunits expressed by these neuronal phenotypes from monolayer and PA6 co- cultures could not be investigated. To do this, Pitx3+ cells could be FACS sorted and qPCR or reverse-transcriptase PCR performed on the isolated Pitx3+ cells. A GAD reporter system would be required to identify the ionotropic glutamate receptor subunits in GABAergic neurons. Furthermore, as Pitx3+ TH+ cells derived from the monolayer and PA6 co-culture differentiation methods respond differently to glutamate receptor antagonists, immunolabelling for markers of A8, A9 and A10 dopaminergic neurons using markers such as the G-protein-gated inwardly rectifying K^+ channel 2 (GIRK2), Calbindin and calretinin (Liang *et al.*, 1996; Thompson *et al.*, 2005; Friling *et al.*, 2009) could be performed to determine the dopaminergic cell groups of these cells.

In the long term, transplantation of midbrain dopaminergic progenitors into Parkinsonian animal models, and ultimately into human patients, should be carried out. Further optimisation of growth factors/inhibitors and a dual reporter cell line would enable enrichment of mitotic, *bona fide*, midbrain dopaminergic progenitors. DNA microarray should be performed to measure the expression levels of genes in midbrain dopaminergic progenitors and subsequently identifying specific surface markers for these cells. This would enable FACS isolation of midbrain dopaminergic progenitors without the need for genetic modifications.

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APPENDIX I

Buffers and Solutions

All buffers and solutions were purchased from Sigma (Australia):

- PBS buffer (136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 at pH 7.4)
- PBST (PBS containing 0.1% Tween 20)
- HEPES buffer (145mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM HEPES, 10mM D-glucose, 2.5 mM $CaCl_2$ at pH 7.4)
- TAE buffer (40 mM Tris-acetate, 1 mM EDTA)

APPENDIX II

STEM CELLS®

EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELLS

Lmx1a Allows Context-specific Isolation of Progenitors of GABAergic or Dopaminergic Neurons During Neural Differentiation of Embryonic Stem Cells

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Key words. embryonic stem cells • differentiation • Lmx1a • Msx1 • GABAergic neurons • dopaminergic neurons

ABSTRACT

LIM homeobox transcription factor 1 alpha (Lmx1a) is required for development of midbrain dopaminergic neurons, roof plate formation and cortical hem development. We generated a reporter ESC line for Lmx1a and used it to track differentiation and extract neural progenitors from differentiating mouse ESCs. Lmx1a$^+$ cells gave rise to functional cortical upper layer GABAergic neurons or dopaminergic neurons depending on the culture conditions employed for differentiation. Under chemically defined neurobasal conditions, ESC differentiation resulted in widespread and transient expression of Lmx1a, without the addition of exogenous factors such as sonic hedgehog (Shh), Wnts, and/or BMPs. Under neutral conditions Lmx1a-positve cells express genes known to be downstream of Lmx1a and cortical hem markers Wnt-3a and p73. The majority of these cells did not express the ventral midbrain dopaminergic marker Foxa2 or dorsal roof plate marker BMP-2. Lmx1a$^+$-Foxa2$^-$-cells were primed to become SatB2-positive GABAergic neurons and appeared to be resistant to dopaminergic patterning cues. PA6 co-culture produced a substantial population of Lmx1a$^+$ progenitors that also expressed Foxa2 and on further differentiation gave rise to dopaminergic neurons at high frequency. We conclude that Lmx1a is a useful marker for the extraction of progenitors of GABAergic or dopaminergic neurons. We caution against the assumption that it indicates dopaminergic commitment during *in vitro* differentiation of ESCs. Indeed, in monolayer culture under neurobasal conditions, with or without addition of Shh and FGF8, Lmx1a$^+$ cells were predominantly progenitors of forebrain GABAergic neurons. We obtained dopaminergic cells in large numbers only by co-culture with PA6 cells.

INTRODUCTION

Parkinson's disease (PD) and schizophrenia are common diseases of the central nervous system, the main symptoms resulting largely from a progressive loss of midbrain dopaminergic neurons in PD [1, 2] and impaired function of cortical GABAergic neurons in schizophrenia [3-6]. Derivation of these neuronal subtypes in high purity from embryonic stem cells (ESCs) could pave the way to alleviate these conditions directly, through cell transplantation strategies, or indirectly through the development of *in vitro* models suitable for screening of novel therapeutic compounds [7]. Various strategies have been used to derive mature subtypes of

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neurons from ESCs, including: culture in the presence of specific growth factors [8-10], co-culture with a stromal cell line [11, 12] or forced expression of developmental genes [13, 14]. A major obstacle that thwarts progress with cell transplantation or screening studies is the common observation that neuronal differentiation protocols produce heterogeneous populations. The cultures may contain the desired cell type, but also contain other neuronal subtypes, non-neural derivatives, undifferentiated cells and unwanted types of neural cells. Cell grafts contaminated with undifferentiated cells are unacceptable due to their predisposition to form tumours [15, 16], while for *in vitro* applications, in particular the screening of novel compounds, the presence of high numbers of unwanted cell types is likely to obscure results. The notion of extracting committed mitotic progenitors is attractive since their proliferative potential makes subsequent expansion for *in vitro* and *in vivo* applications a possibility. The derivation of ESC reporter lines via homologous recombination has the potential to enable isolation of a specific progenitor subpopulation by fluorescence activated cell sorting (FACS), and we chose to use this strategy to investigate whether we could identify midbrain dopaminergic progenitors. In recent years Lmx1a has gained prominence as a key transcription factor in dopaminergic neuron differentiation. Midbrain dopaminergic neurons arise *in vivo* in the ventral midline from Foxa2$^+$ floor plate cells established by sonic hedgehog (Shh) signalling from the notochord [17-19]. In this context Lmx1a activates downstream determinants of the dopaminergic phenotype by binding to the promoter sequences of Msx1, Lmx1b, Wnt1 and potentially other target genes [20, 21].

Although Lmx1a is prominent in dopaminergic neuron differentiation, it is also expressed in three unrelated neural tissues during development: (I) the cortical hem, a forebrain structure that gives rise to cortical and hippocampal neurons [22]; (II) the roof plate of the developing cerebellum [22, 23] and (III) the non-neurogenic roof plate of the neural tube [24, 25].

In recent studies we have shown that nominal midbrain differentiation protocols give rise to heterogeneous populations of dopaminergic and GABAergic neurons [26, 27]. In this study we investigate the phenotypic potential of Lmx1a$^+$ cells that arise during *in vitro* differentiation, in monolayers or in co-culture with stromal cells. We report that Lmx1a expression is early and widespread when ESCs are removed from maintenance medium and are subjected to neural differentiation protocols in vitro. The propensity of these cells to give rise to GABAergic or dopaminergic neuron fates is highly dependent on the culture paradigm employed for differentiation.

METHODS

Generation of targeted genetic reporter ESC lines

The methods used are described in detail in the supplementary files.

Cell culture and neural differentiation

Before differentiation E14-tg2a, Lmx1a-AMP-IRES-GFP, Msx1-AMP-IRES-GFP and Lmx1a-IRES-eGFP ESCs were maintained in DMEM supplemented with 10% FCS (ESC qualified), Pen/Strep (1000 units/ml), 2mM Glutamax, 0.1 mM β-mercaptoethanol and LIF (10^3 units/ml). Sox1-GFP ESCs (a modified version of E14-tg2a, received as a courtesy of Stem Cell Sciences) were maintained in GMEM supplemented with 10% FCS, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids, 0.1 mM β-mercaptoethanol, 2.75 g/l sodium bicarbonate and LIF (10^{-3} units/ml). Cells were passaged every other day and replated at 2.5 x 10^4 cells/cm^2.

Monolayer neural differentiation was undertaken using a modified version of a previously described protocol [28]. The logic of using a monolayer is that it offers the potential to produce an enriched culture of differentiated cells without a contaminating feeder layer. Briefly, ESC were dissociated with accutase and replated onto 0.1% (v/v) gelatine-coated plates at 4.5 x10^3 cells/cm^2 and cultured in ESC culture media for 24 hours. The culture medium was changed to N2B27 neurobasal medium and supplemented with

BMP4 (10 ng/ml), Noggin (500 ng/), Shh (200 ng/ml), SANT-1 (1 μM, Tocris Bioscience, Bristol, UK), Cyclopamine (1 μM, Sigma-Aldrich, Sydney, Australia), Wnt1 (50 ng/ml), Wnt3a (50 ng/ml), CHIR99021 (3 μM, Axon Medchem, Groningen, The Netherlands) or Dkk1 (100 ng/ml), as indicated. The N2B27 medium was supplemented with L-ascorbic acid (200 μM, Merck, Melbourne, Australia) from day 12 onwards. All cytokines were purchased from Peprotech (Rehovot, Israel) unless stated otherwise.

PA6 stromal cells (Riken, Tokyo, Japan) were maintained in α-MEM supplemented with 10% FCS and Pen/Strep (1000 units/ml). Prior to differentiation of ESCs, PA6 cells were seeded at 5 x 10^4 cells/cm^2 in maintenance medium and then switched to GMEM supplemented with 15% KSR, 2 mM Glutamax, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids and 0.1 mM β-mercaptoethanol. ESC were seeded at 100 cells/cm^2 onto PA6-containing wells. After 8 days, the medium was switched to N2B27. From day 3 of differentiation onwards medium was renewed at least every other day.

Hela cells were culture in Eagle's Minimum Essential Medium in the presence of 10% FBS (Invitorgen, Melbourne, Australia).

Immunocytochemistry

Details of is the immunocytochemical methods are included in the supplementary files.

Imaging and cell counting

Images were taken with an A1R confocal microscope (Nikon, Japan). For quantification, TH or GABA-positive neurons were counted manually on 9 random fields of view (6x magnification) taken from 3 separate experiments. To quantify $Foxa2^+/TH^+$-cells, stacks (50 μm, 2.5 μm/step) were taken at 10 x magnifications to confirm co-labelling for both markers.

As described previously [14], in order to assess cell proliferation under different growth conditions, cultured cells were stained with a nuclear dye (TOPRO-3, Invitrogen, Melbourne, Australia) and plates scanned with an Odyssey Infrared Imaging System (Li-cor Biosciences, Lincoln, USA) at 700nm. Emission values of each well were standardised to control wells.

Flow cytometry

Lmx1a-AMP-IRES-GFP and Msx1-AMP-IRES-GFP cultures were dissociated into single cells with accutase (Sigma-Aldrich, Sydney, Australia) in prepration for FACS analysis. Cells were loaded with substrate using the LiveBLAzer™-FRET B/G Loading Kit (Invitrogen, Melbourne, Australia) according to manufacturer's specifications for 2-3 hours prior to analysis. Wild-type cells submitted to the staining procedure were used as the control for flow cytometry.

The Click-iT™ EdU Alexa Fluor® 647 Flow Cytometry Assay Kit (Invitrogen, Melbourne, Australia) was used to detect dividing cells in culture according to manufacturer's specifications. Briefly, cells of the reporter line were incubated with Click-iT™ EdU (10 μM) for 2 hours, dissociated into single cells, submitted to β-lactamase staining, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X and then incubated with the Click-iT™ reaction cocktail for 30 minutes. Wild-type cells (controls for flow cytometry) were processed in the same way with the exception that they were not exposed to Click-iT™ EdU.

Cell cultures were dissociated with accutase, resuspended in PBS with 5% FCS (v/v) and run through a 40 μm strainer (BD Biosciences, Sydney, Australia) to remove clumps. Flow cytometry was performed with a FACS Canto II analyser (BD Biosciences) or a FACS Aria I (BD Biosciences) for cell sorting experiments. After FACS extraction, $Lmx1a^+$ and negative fractions were either used for RNA extraction (>1 million cells per sample) or replated onto laminin/fibronectin/poly-d-lysine (1 μg/ cm^2 each)-coated wells at 3 x10^5 cells/cm^2 (low-density) or 1.5 x 10^6 cells/cm^2 (high-density). For the monolayer differentiation paradigm, extracted cells were plated in N2B27 supplemented with FGF2 (20 ng/ml) alone or in combination with Shh (200 ng/ml) and FGF8 (100 ng/ml, Peprotech). 5 days later the medium was replaced with N2B27

supplemented with BDNF (20 ng/ml) and L-ascorbic acid (200 μM) for another 7 days. For experiments on PA6-mediated differentiation cells were seeded in PA6-preconditioned N2B27 medium supplemented with FGF2 (20 ng/ml). 2 days later cells were switched to PA6-preconditioned N2B27 media supplemented with BDNF (20 ng/ml) and L-ascorbic acid (200 μM) for another 5 days. Medium was renewed at least every other day.

RNA extraction and quantitative-PCR methods

These methods are described in the supplementary files.

Calcium imaging

Calcium imaging was undertaken as described previously [26, 29]. Briefly, cells were washed twice with HEPES buffer (containing in mM; NaCl 145; 3 KCl 5; MgSO4 1; HEPES 10; D-glucose 10; CaCl2 2.5) at pH 7.4, and incubated with FLUO-4AM (10 lM, Invitrogen) in HEPES buffer for 30 minutes at 37°C. After incubation, cells were viewed using a Nikon Eclipse Ti camera (Nikon) coupled to an A1 Nikon confocal microscope (Nikon). Cells were illuminated at 488 nm and emission was recorded at 515 nm. Cellular fluorescent intensities were captured with NIS-elements-AR software (Nikon) 30 times per second. Background fluorescence was subtracted from each neuron, and fluorescence intensity was calculated. During experiments, cultures were first exposed to vehicle control, Glutamate(30μM) and then KCl (30 mM).

Statistical analysis

For all experiments, results are presented as mean with standard error of the mean (SEM) of at least 3 independent experiments. Statistical analyses were performed using PRISM v5.00 (Graphpad Software, California, USA). All raw data were analysed by Student's t-test or one-way analysis of variance (ANOVA) with post-hoc Dunnett's test. In all cases, $p<0.05$ was considered to be statistically significant.

RESULTS

Expression of Lmx1a and Msx1 is widespread in differentiating ESC cultures under chemically defined neurobasal conditions

We used an Lmx1a-AMP-IRES-GFP ESC line to examine Lmx1a expression during neural differentiation of ESCs. The sensitive catalytic reporter β-lactamase (e.g. the ampicilin resistance gene, AMP) and enhanced green fluorescent protein (GFP) were co-expressed under control of the endogenous Lmx1a promoter (Fig. 1 A). We chose to study reporter expression in detail during monolayer differentiation [28], since this approach makes use of chemically defined conditions, without the unpredictable interference of animal serum or co-culture with a stromal cell line. During differentiation of the reporter line for 20 days AMP and GFP expression peaked on day 8 and day 10 respectively, with approximately 40% cells positive for each reporter (Fig. 1 B, C). Considering that detection of GFP by flow cytometry requires 3 orders of magnitude more molecules of GFP per cell than the catalytic reporter AMP [30], AMP expression was detected at earlier timepoints than GFP, resulting in an apparent lag in the proportion of cells expressing GFP (Fig 1C). At the peak, day 8 for AMP and day 10 for GFP, 35-40% of cells were positive for reporter expression. We observed that GFP expression peaked at day 10, a time when the percentage of AMP-positive cells was already in decline, reflecting the considerably shorter half life of AMP (~3.5 hours [30]) compared to GFP (~26 hours [31]). To further substantiate that the Lmx1a promoter was activated in 35-40% of cells we established an analogous reporter line for Msx1, the direct downstream target of Lmx1a. The expression profile of AMP in the Msx1 reporter line was indistinguishable from that obtained with the Lmx1a reporter line (Fig. 1 D), implying that the endogenous Lmx1a transcript translates into functional protein at levels sufficient for activation of downstream gene expression. To determine whether expression of Lmx1a or Msx-1 correlates with exit from the proliferative state, differentiating cultures were pulsed with DNA-base analogue EdU for 2 hours at various time points (Fig. S1

A). The overall percentage of cycling cells in the culture decreased gradually during differentiation from approximately 70% (day 0) to 10% (day 20) (Fig. S1 B). The percentage of Lmx1a-AMP$^+$ or Msx1-AMP$^+$ positive cells that incorporated EdU decreased progressively during differentiation and no significant differences were found between AMP$^+$ -positive and AMP$^-$ -negative cells (Fig. S1 C, D). This indicates that Lmx1a$^+$ and Msx-1$^+$ cells are in general a proliferating population up to day 20, and suggests that the gradual reduction in cell division over time was a consequence of contact inhibition in the 2D cultures.

Transient Lmx1a expression on day 8 is not predictive of dopaminergic neuron differentiation

To put the timecourse of expression of Lmx1a in context with the state of neural differentiation we established the timecourse for conversion of ESCs into neuroectoderm using a Sox1-GFP reporter line. 65% of cells expressed Sox1-GFP by day 4, indicating that the first stages of commitment to neural lineages had taken place. The maximum percentage of Sox1$^+$ cells were observed at day 8 (75%) but this was not significantly higher than the estimate on day 4 (Fig. 1 E). Less than 10% of cells were Lmx1a$^+$ on day 4 indicating that Lmx1a expression occurred well after neural conversion of ESCs (Fig. 1 C and E). Immunocytochemical labelling of day 8 cultures showed that Lmx1a expressing cells (GFP reporter) were positive for the neural progenitor marker nestin, but did not label for pluripotency marker Oct-4 or mature neuron marker TUJ-1 (Fig. 1 F-J). Considering the previous experience of ourselves and others [32], that monolayer differentiation produces limited numbers of dopaminergic neurons, we were surprised to see widespread expression of Lmx1a. To investigate whether Lmx1a expression on day 8 was a marker of dopaminergic progenitors, we investigated the effect of various growth factors on induction of Lmx1a expression at day 8, and investigated whether there was a correlation with the numbers of TH$^+$ cells present on day 20. We chose factors modulating pathways that have been associated with the induction of Lmx1a expression *in vivo*, namely canonical Wnt-signalling and Shh-signalling (both ventral midline), and BMP signalling (roof plate) [21, 24, 33, 34]. Pathway agonists and antagonists were added to cultures either at the start of differentiation process (day 0) or after neural conversion (day 4). After treatment with each of the factors, we allowed cultures to progress towards terminal differentiation in the presence of ascorbic acid (AA) from days 12 to 20. Results indicated that none of the factors tested induced a significant increased in the percentage of Lmx1a$^+$ cells present on day 8 of differentiation (Fig. 2 A, B). We found that stimulation of the BMP pathway or the canonical Wnt pathway reduced the percentage of Lmx1a$^+$ cells present on day 8. Decreases were much more pronounced when factors were added from day 0. In view of the fact that BMP and canonical Wnt signalling are known to antagonize neural commitment of ESCs we also investigated the percentage of Sox1-GFP$^+$ cells present on day 8 after treatment with the various factors (Fig. 2 C, D). Decreases in Lmx1a expression correlated with reductions in numbers of Sox1$^+$ cells, suggesting that agonism of BMP or canonical Wnt pathways reduced the occurrence of Lmx1a$^+$ cells indirectly, by inhibiting neural conversion. Interestingly, while none of the treatments increased Lmx1a expression at day 8, exposure to Noggin or Dkk1 (days 0 to 12) significantly increased numbers of TH$^+$ cells on day 20 (Fig. 2 G-N). We concluded that Lmx1a expression on day 8 was not predictive of subsequent development of TH$^+$- neurons by day 20. Furthermore induction of Lmx1a expression under chemically defined conditions occurred independently of signalling pathways that are known to be implicated with the induction of Lmx1a expression *in vivo* in the ventral midline or the roof plate [24, 33, 34].

It is noteworthy that inhibition of Shh signalling (either by Sant-1 or Cyclopamine) did not alter the number of TH$^+$ neurons on day 20. This was surprising because of the obligatory role of Shh during the *in vivo* differentiation of midbrain dopaminergic neurons [19, 34]. Closer examination of monolayer-derived TH$^+$- neurons indicated that the majority did not express Foxa2, a marker

present in all midbrain dopaminergic neurons [35], implying that most monolayer-derived TH^+ cells are not *bona fide* midbrain dopaminergic neurons (Fig. 2 O-Q).

Analysis of $Lmx1a^+$ and $Lmx1a^-$ cells sorted at day 8 of differentiation

To study $Lmx1a^+$ cells in more detail and to investigate which cell types they were able to give rise to, we separated $Lmx1a^+$ and negative cells on day 8 using the AMP reporter (Fig. 3 A). Plating of $Lmx1a^+$-cells produced homogenous cultures with morphology consistent with our expectation that these were neural progenitors. Immunocytochemistry supported this hypothesis, showing that approximately 80% percent of cells labelled positive for nestin, while very few TUJ-1 positive neurons could be found in these cultures (Fig. 3 B-D). $Lmx1a^-$ cells produced cultures with mixed cell morphologies and significantly fewer cells labelling for nestin (Fig. 3 E, F). Although few TUJ-1 immunoreactive cells in day 8 cultures seemed to survive dissociation and FACS separation, these cells were primarily found in the negative fraction (Fig. 3 G) providing further support that $Lmx1a^+$ cells (day 8) were primarily neural progenitors and not primitive neurons. A remarkable feature of the $Lmx1a^+$ fraction, when seeded at high density, was the formation of neural rosettes, characterised by $ZO\text{-}1^+$ foci surrounded by $nestin^+$ cells, within 3-4 days of plating (Fig. 3 H). This was rarely observed in cultures of the $Lmx1a^-$ fraction (Fig. 3 I). Interestingly, after 5 days of culture in neurobasal medium supplemented with FGF2 only 30% cells of the positive fraction still expressed Lmx1a (AMP reporter), while 5% Lmx1a positive cells could now be detected in cells grown from the negative fraction (Fig. 3 J). To investigate changes in Lmx1a expression after sorting we tracked the presence of Lmx1a-GFP positive/negative cells using confocal microscopy. Consistent with the flow cytometry results shown in Fig. 3J, a gradual down regulation of GFP expression could be observed in the positive fraction (Fig. 3 K). In the negative fraction clusters of Lmx1a positive cells could be detected after 96 hours. Retrospective analysis of white light images taken 72h after replating showed that many of the GFP^+ clusters, produced by cells which were AMP^- at day 8, originated from spherical colonies with distinctly undifferentiated morphology (Fig. 3 L, M). We hypothesize that $Oct\text{-}4^+/Lmx1a^-$ cells were present in day 8 cultures, giving rise to a second wave of $Lmx1a^+$ cells, indicative of a degree of asynchronous differentiation during monolayer culture.

The majority of $Lmx1a^+$ cells express expected downstream genes but not early dopaminergic neuron marker Foxa2 or roofplate marker BMP2

Quantitative PCR (qPCR) analysis of the Lmx1a-positive and negative fractions (AMP reporter, day 8) showed that transcript levels of Lmx1a over two orders of magnitude higher in the positive fraction compared to the negative fraction (Fig. 4 A), demonstrating that our reporter line allowed robust separation of Lmx1a expressing cells from the rest of the population. Probing for direct downstream targets of Lmx1a [20, 21] showed that the positive fraction was significantly enriched with transcripts of Wnt1, Wnt3a,Msx-1 and Lmx1b (Fig. 4 B, C). The expression level of neural stem cell/progenitor marker nestin was >2 fold higher in the positive fraction (Fig. 4 B, C) reflecting our immunocytochemical data for the distribution of nestin labelled cells in FACS separated cultures (Fig. 3 D). Roof plate marker BMP-2 and ventral marker Foxa2, which is known to be expressed in the ventral midline well before Lmx1a [34], were primarily up-regulated in the negative fraction (Fig. 4 B, C). Given the significance of Foxa2 we confirmed this finding by immunocytochemistry, showing that Foxa2 and Lmx1a-GFP expressing domains in day 8 cultures did not overlap (Fig. 4 D). The expression of cortical hem marker Wnt-3a [36] was strongly up-regulated in the positive fraction, while genes associated with multipotent rosette neural stem cells and their maintenance (BF-1, Notch-1, Jag-1, Dll-1) [37] were up regulated in the negative fraction (Fig. 4 B, C).

Lmx1a$^+$ neural progenitors primarily give rise to cortical GABAergic neurons

Since Lmx1a$^+$ cells (day8) did not highly express key markers associated with early dopaminergic progenitors or roofplate identity, we speculated that they might correspond to cells of the cortical hem. In the developing forebrain this structure is a putative signalling centre at the interface of the future hippocampus and the choroid plexus [38]. Furthermore it is the origin of a migratory population of neural progenitors that give rise to marginal zone cortical neurons [38]. In agreement with our hypothesis the Lmx1a+ cell fraction showed upregulated expression of p73, a marker expressed in the hippocampus and by cortical hem derived marginal neurons [38] (Fig. 4B, C). In order to determine the developmental potential of Lmx1a$^+$ and negative cells we terminally differentiated FACS-separated day 8 cultures. The positive fraction gave rise to cultures with highly neuronal morphology that labelled strongly for TUJ-1 (Fig. 5 A, B). Despite the fact that the plated negative fraction also contained nestin$^+$ cells that we presumed were neural progenitors (Fig. 3 D, E), at the end of the differentiation period rapidly proliferating cells with non-neural morphology dominated the cultures (Fig. 5 F-J). At this stage the terminally differentiated positive fraction contained predominantly neural cells labelling for TUJ-1, GABA, TH and GFAP, that were mostly Lmx1a-GFP$^-$ (Fig. 5 B-E). The vast majority of neurons produced by day 8 Lmx1a$^+$ cells were GABA$^+$/GAD67$^+$ GABAergic neurons (Fig. 5 K, L) that frequently labelled for upper layer cortical marker SatB2 (Fig. 5 M), while expression of TBR-1, a transcription factor found in other layers, was rare. Expression of p73 could not be detected in terminally differentiated cultures using immunocytochemistry (Fig. S2 A-E). Very few TH$^+$ cells could be detected among the GABAergic cells (Fig. 5 N). At day 8 the Lmx1a$^+$ cells, while giving rise to neurons with forebrain identity, did not yet label for SatB2 or TBR-1 (Fig. 5 O, P). When the positive fraction was exposed to the caudalizing agent retinoic acid (day4), the percentage of Lmx1a$^+$ cells by day 8 (Fig. 5 Q, R) was reduced, which implies that the untreated cells were destined for an anterior identity, whereas retinoic acid-treated promoted more posterior fates. The observation that transcripts for forebrain marker BF-1/FoxG1 were primarily up-regulated in the negative fraction by day 8 (Fig. 4 B, C) is not necessarily paradoxical as this marker is only very early expressed throughout the forebrain; by embryonic day 10.5 BF-1 expression is excluded from the dorsomedial telencephalon which includes the cortical hem [39]. Considering that Lmx1a positive cells give rise to neural rosettes (Fig. 3 H), a morphology that has been associated with multipotent neural stem cells [37] we tried to pattern extracted Lmx1a positive cells towards a dopaminergic fate by exposure to Shh and FGF8. Regardless of the absence or presence of morphogens after sorting at day 8, Lmx1a$^+$ cells produced GABAergic and dopaminergic neurons at a ratio of 20:1 implying that the day 8 Lmx1a$^+$ cells were already committed to give rise to a specific neuronal subtype (Fig. 6 A, B). To further test whether day 8 Lmx1a$^+$ positive cells had neural stem cell properties we attempted their maintenance after extraction in the presence of FGF2 and EGF, factors shown to allow the propagation of neural stem cells [40]. In agreement with our working hypothesis that the majority of the Lmx1a$^+$ cells were at a transient stage during the differentiation of SatB2$^+$ GABA$^+$ neurons, Lmx1a expression decreased rapidly during successive passages following FACS extraction (Fig. 6 C). By passage 4 cultures were Lmx1a$^-$ and had stopped proliferating. Substitution of EGF with rosette stem cell maintenance factors [37] yielded similar results (data not shown).

PA6-coculture produces Lmx1a$^+$ cells biased towards a dopaminergic neuron fate

Since monolayer differentiation, a culture method strongly associated with the occurrence of forebrain phenotypes [41] produced Lmx1a$^+$ cells which appeared to be predisposed to become GABAergic neurons, we wanted to explore the fate of Lmx1a$^+$ cells produced during PA6 co-culture, a method that has been reported to produce midbrain dopaminergic neurons at high frequency within 2 weeks [12, 42]. Analysis of Lmx1a expression under these culture conditions showed that the percentage

of Lmx1a$^+$ cells did not transiently peak on day 8, as observed for monolayer differentiation, but gradually increased to >20% positive cells by the end of the differentiation period (AMP reporter, day 15, Fig 7 A). Immunocytochemistry at day 8 showed that Lmx1a-GFP$^+$ cells were TUJ-1$^-$, nestin$^+$ and frequently labelled for ventral marker Foxa2 (Fig. 7 B-D). By day 15 of a typical differentiation experiment 70.0% ± 5.0% of the TUJ-1$^+$ colonies contained patches of Lmx1a-GFP$^+$ cells. We observed a strong correlation between the presence of Lmx1a and the occurrence of TH$^+$ neurons in these cultures, and co-expression of the two markers was observed frequently at the edges of colonies (Fig. 7 E, F). Furthermore colonies that contained ≥10 TH$^+$ neurons were more likely to be positive for Lmx1a than colonies with <10 TH$^+$ neurons ($P<0.01$) (Fig. 7 G). To verify that Lmx1a$^+$ cells produced by PA6 differentiation were committed to the dopaminergic neuron fate we performed FACS extraction. We chose to do this on day 10 since published PA6 differentiation protocols which recommend enhancement of dopaminergic differentiation by supplementation with Shh and Fgf8, recommend removal of patterning factors at approximately day 10 [42, 43]. We hypothesised that commitment to a dopaminergic fate during PA6 co-culture is likely to be completed by this time. Making use of an Lmx1a$^+$ reporter line that also constitutively expresses mCherry under control of the Rosa26 promoter, we were able to purify ESC-derived Lmx1a$^+$ and negative cells by FACS while excluding the remaining PA6 stromal feeder cells. Terminal differentiation of either fraction for 7 days produced neuronal cultures with few GFAP$^+$ glial cells (Fig. 7 H-O). Strikingly Lmx1a$^+$ cells gave rise to TH$^+$ neurons at 3 fold higher frequency than GABA$^+$ neurons (Fig. 7 P, Q). This trend was reversed for the negative fraction where GABA$^+$ neurons were produced at approximately 3 times higher frequency than TH$^+$ cells (Fig. 7 P, Q). Lmx1a-GFP expression was commonly observed in terminally differentiated cultures of the positive fraction (Fig. 7 J) which contrasted with our observations during differentiation of monolayer cultures, when substantial down-regulation of reporter expression occurred in the days following extraction (Fig. 3 K). It is also noteworthy that the few TH$^+$ cells produced after differentiation of the PA6 coculture-derived negative fraction were associated with secondary clusters of Lmx1a expressing cells that arose subsequent to FACS separation (Fig. 7 K). To substantiate that the neurons produced from extracted Lmx1a$^+$ cells (monolayer or PA6 co-culture) possess functional properties we performed calcium imaging studies. As would be expected for neuronal cells *in vivo,* stimulation with glutamate the major excitatory neurotransmitter of the central nervous system, or depolarization by KCl elicited significant elevations in intracellular calcium compared to vehicle control (in all cases $p<0.01$, Fig. S3).

DISCUSSION

Genetic reporters offer a powerful means to track differentiation of ESCs, provided that the expression of the reporter faithfully reproduces endogenous patterns of expression. We chose to use the most robust method to ensure high-fidelity expresson by targeting the reporter construct to replace exon 1 of a single allele of the gene in question. The endogenous protein is expressed by the second allele only, however this did not appear to result in haploinsufficiency. Neither the ability of the reporter ES cells to differentiate into neurons or specify either the GABAergic or dopaminergic phenotypes were affected (Fig S4). Our interest in targeting the Lmx1a gene was raised by its reported ability to identify precursors of dopaminergic neurons in the ventral midbrain [20, 44, 45]. There is considerable interest in research that can establish the best cells for use in cell therapy of Parkinson's disease. Neural stem cell implants produce a beneficial effect by way of the 'chaperone effect' but these cells do not produce new dopaminergic neurons [46]. There may be a need to implant dopaminergic neurons or their precursors, and to test this there is a need for methods to enrich cell cultures for these cells. We hypothesised that an Lmx1a reporter might allow us to track development of dopaminergic precursors and also sort them using flow cytometry, to enrich

and to allow detailed analysis of their phenotype. To this end we targeted the dual reporter, AMP-IRES-eGFP, so that we could visualise cells using GFP if expression was strong enough, but could also introduce a substrate for β-lactamase (i.e. using the AMP reporter gene) to give a very sensitive assay or FACS sorting method (Fig. 1B, 3A).

Initially we chose to study differentiation of ESCs in monolayer culture, reasoning that this approach would ultimately give the best control over differentiation, as opposed to the alternative use of differentiation in embryoid bodies [47, 48]. By using an ESC reporter line for Lmx1a, we demonstrated that depending on the culture conditions, Lmx1a$^+$ cells gave rise to GABAergic or dopaminergic neurons. To our knowledge this is the first time that Lmx1a has been implicated with GABAergic neuron differentiation *in vitro*. Indeed we observed that unless conditions were optimised for the generation of midbrain phenotypes, Lmx1a is predominantly a marker for forebrain GABAergic neurons during monolayer differentiation of ESCs.

Monolayer culture is a chemically-defined approach which has been reported to be prone to production of anterior neuron fates [41]. Under neurobasal conditions in the absence of patterning cues we observed that expression of Lmx1a was widespread and transient (Fig 1). The vast majority of neurons produced by Lmx1a$^+$ cells were GABAergic neurons that labelled for SatB2 but not TBR-1 (Fig 5), indicating an upper layer cortical identity [41, 49, 50]. Some of the Lmx1a$^+$ cells derived by monolayer culture did infact give rise to TH$^+$ neurons, albeit at very low frequency. However most of these cells were not midbrain dopaminergic neurons, as their differentiation was independent of Shh signalling and, crucially, the majority of these TH$^+$ cells were not immunopositive for Foxa2. Our results reflect previous reports that monolayer differentiation in neurobasal medium, with or without patterning factors, is a poor source of midbrain dopaminergic neurons (Gaspard et al., 2008; Konstantoulas et al., 2010). We hypothesise that monolayer derived TH+ neurons correspond mostly to forebrain dopaminergic neurons (Björklund et al., 2007). In support of this concept differentiation in the presence of either Dkk1 or Noggin increased the numbers of TH$^+$ neurons. Both Noggin, in the context of 'dual smad' inhibition in the absence of caudalizing factors, as well as Dkk1, have been reported to promote or enhance the generation of anterior neural fates from ES cells (Chambers et al., 2009; Fasano et al., 2010; Watanabe et al., 2005).

As the cortical hem is the primary site of Lmx1a expression in the developing forebrain (Chizhikov et al., 2010) it is tempting to speculate that monolayer derived Lmx1a+ cells are the *in vitro* equivalent of this structure. In support of this hypothesis; (i) the occurrence of Lmx1a+ cells (day 8) was not enhanced by factors implicated in dopaminergic neuron or roof plate differentiation (Fig. 2); (ii) consistent with the concept that Lmx1a+ cells were an anterior population, the caudalizing factor retinoic acid reduced Lmx1a expression (Fig 5P); and (iii) Lmx1a$^+$ strongly expressed cortical hem markers Wnt3a [36] and p73 [51]. This assumption is complicated by reports indicating that the cortical hem is also a site of BMP-2 expression [52]. In this study during monolayer differentiation BMP-2 expression was primarily upregulated in the negative fraction (Fig. 4 B) with only modest increases in the Lmx1a$^+$ fraction (6.5 ± 4.9 fold) compared to day 1 (Fig 4 C). Furthermore we detected high levels of Wnt-1 expression in Lmx1a$^+$ cells (Fig. 4 B, C), which has been implicated as a cortical hem marker in the chick [53] but not in mouse system [54]. Most importantly *in vivo* the cortical hem is primarily a source of p73$^+$ Cajal-Retzius cells, a type of marginal zone glutamatergic neuron [36], while our monolayer derived Lmx1a$^+$ cells predominantly give rise to p73$^-$ /GAD67$^+$/GABA$^+$ neurons with upper layer identity (Fig. 5 K-M). We surmise that these differences might be an artifact of the *in vitro* differentiation process due to the absence of one or many factors present during forebrain embryonic development. In a permissive environment, like the embryonic brain, *in vitro* derived Lmx1a+ cells (day8) might very well be capable of giving rise to cortical hem derivates. But until this possibility has been

explored it is uncertain whether there is a direct *in vivo* equivalent for monolayer derived $Lmx1a^+$ cells. Furthermore the primary differentiaton product that we obtained from Lmx1a+ cells, e.g. $SatB2^+$ GABAergic neurons, are of questionable physiological relevance since $SatB2^+$ neurons in the cortex are unlikely to be GABAergic [55]. GABAergic interneurons labelling for SatB2 have been reported in the mammalian retina [56], however there are no reports implicating *Lmx1a* with eye development *in vivo*. At this stage we are not aware of any other reports of $SatB2^+$ GABAergic neurons.

Frilling et al. reported that monolayer cultures can be coaxed to give rise to dopaminergic neurons at high frequency under the influence of forced expression of Lmx1a [13]. In this experiment Lmx1a was expressed under control of the nestin promoter which is active as early as 24 hours after neural induction. The recent study by Chung and colleagues [21] demonstrated that two independent transcription factors cascades, one characterised by Lmx1a and the second by Shh-induced Foxa2, are required for midbrain dopaminergic differentiation. This explains the observation by the former group that efficient derivation of TH^+ neurons by mis-expression of Lmx1a was only possible in the presence of Shh [57]. We showed in the present work that monolayer differentiation gives rise to cultures in which typically 40% cells endogenously expressed Lmx1a (days 8-10). While this does not translate into widespread dopaminergic differentiation, even in the presence of Shh, it marks the generation of a cortical progenitor population. We conclude that there is only a brief early period during monolayer differentiation, before the expression of endogenous Lmx1a, when forced expression of Lmx1a has the capacity to induce dopaminergic neuron specification.

We conclude that the majority of the $Lmx1a^+$ cells produced in monolayers express downstream targets Msx1, Lmx1b and Wnt1, but not Foxa2, and that these cells are primarily committed and restricted to GABAergic neuron and astrocyte differentiation. They cannot be patterened towards the dopaminergic fate and Lmx1a expression is not maintained after extraction. In contrast to monolayer culture we identified a striking association between Lmx1a expression and dopaminergic neuron differentiation in PA6 co-cultures, as $Lmx1a^+$ colonies were more likely to contain dopaminergic neurons than $Lmx1a^-$ colonies. Furthermore $Lmx1a^+$ cells (day10) expressed Foxa2 and when extracted gave rise to dopaminergic neurons at high frequency. Our findings are in line with the concept that co-expression of Lmx1a and Foxa2 is required for the specification of dopaminergic neurons *in vitro*. We show here that the PA6 co-culture method, a robust method for the differentiation of dopaminergic neurons with midbrain identity [58, 59], provides the appropriate patterning cues for the generation of Lmx1a positive dopaminergic progenitors. Interestingly even in PA6 co-culture some of the $Lmx1a^+$ cells gave rise to GABAergic neurons. Since these neurons do not express cortical marker SatB2 (Fig. S5) we believe that they possess a different regional identity compared to their monolayer-derived counterparts. This observation indicates that regardless of culture conditions and their associated regional identity, $Lmx1a^+$ cells can differentiate into GABAergic neurons. This occurs at markedly different frequencies during monolayer differentiation (ratio $GABA^+$ to TH^+ neurons - 20:1) and PA6 co-culture (ratio $GABA^+$ to TH^+ neurons - 1:3). Taken as a whole the data indicates that extraction of mid-brain dopaminergic progenitors by flow cytometry will require at least one more reporter gene to identify the correct sub-population of $Lmx1a^+$ cells. At present Foxa2 appears to be a good candidate for use in a future dual reporter system.

Lmx1a has also a pivitol role during dopaminergic neuron differentiation from human ESCs [60-62], however there is conflicting evidence about the requirement of Lmx1a and Foxa2 co-expression for the generation of this neuronal subtype. In the context of a 5-stage protocol that produced up to 20% TH^+ cells, Cai et al. (2009) noted that barely any neural progenitors co-labelled for Lmx1a and Foxa2. Two very recent studies on the other hand report that only early co-

expression of both factors is indicative of dopaminergic neuron differentiation. Jaeger et al. (2011) showed that under standard monolayer differentiation conditions mouse epiblast stem cells as well as human ESCs produced $Lmx1a^{+}/Foxa2^{-}$ cells and generated cultures with only moderate numbers of TH^{+} cells. Modulation of FGF/ERK signalling early during differentiation followed by exposure to Shh and FGF8 facilitated the generation of $Lmx1a^{+}/Foxa2^{+}$ cells and concomitantly increased the percentage of dopaminergic neurons. Kriks et al. (2011 – a study that was published after this manuscript was submitted for review) showed that dual SMAD inhibition produced cultures with $Lmx1a^{+}/Foxa2^{-}$ cells that rarely gave rise to TH^{+} cells, whereas dual smad inhibition plus stimulation of Shh and canonical Wnt pathways allowed efficient generation of cultures with Lmx1a+ Foxa2+ cells that produced dopaminergic neurons at high frequency.

CONCLUSION

In this study we show that a reporter line for Lmx1a can be employed to isolate neural progenitors biased towards the GABAergic or the dopaminergic neuron fate depending on the culture method employed for differentiation. By doing so we demonstrate the limitations of Lmx1a as a marker for dopaminergic neuron differentiation *in vitro*. Furthermore we provide convincing evidence that the widespread expression of Lmx1a during differentiation under chemically defined conditions signifies the rise of a forebrain progenitor population that gives rise to functional GABAergic neurons, albeit with aberrant marker expression. This highlights that artificial culture condition can result in cells with no physiological relevance. Nevertheless this does not negate the potential of PA6 co-culture-derived dopaminergic neurons to become valuable research tools for the study Parkinson's disease with applications ranging from *in vitro* drug screening to transplantation work. While enrichment for progenitors of either GABAergic or dopaminergic neurons was feasible, we acknowledge that the use of a single reporter was not sufficient to produce pure cultures. Optimized growth factor treatment and/or a dual reporter line for Lmx1a and a transcription factor differentially expressed in GABAergic and dopaminergic progenitors should enable isolation of considerably enriched populations. This is desirable to enable global gene expression analysis, and identification of selectively expressed surface markers, in particular of dopmaminegic neuron progenitors, to facilitate extraction from mouse and human sources without the need for genetic modifications.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Figure 1. Reporter expression during ESC differentiation in N2B27. (A) Outline of the expression construct that was coupled to the endogenous Lmx1a promoter via homologous recombination. (B) Typical flow cytometry blots for reporter detection of AMP and GFP on day 8 of differentiation. (C) Time courses for AMP and GFP expression under control of the Lmx1a promoter. (D) Comparison of AMP expression under control of the Lmx1a or Msx1 promoter. (E) Time course for Sox1-GFP expression during differentiation. (F-J) Labelling of Lmx1a-GFP cultures on day 8 for (F) Nestin and DAPI, (G) Oct-4 and DAPI and (H-J) TUJ-1 and DAPI (arrows indicate the location of cell bodies of TUJ-1 positive cells). Abbreviations used: pLmx1a, Lmx1a promoter; AMP, ampicilin resistance gene/beta-lactamase;IRES, internal ribosome entry site; GFP, green fluorescent protein; p.A., poly adenylation site; n.s., not significant. Scale bar = 100μm.

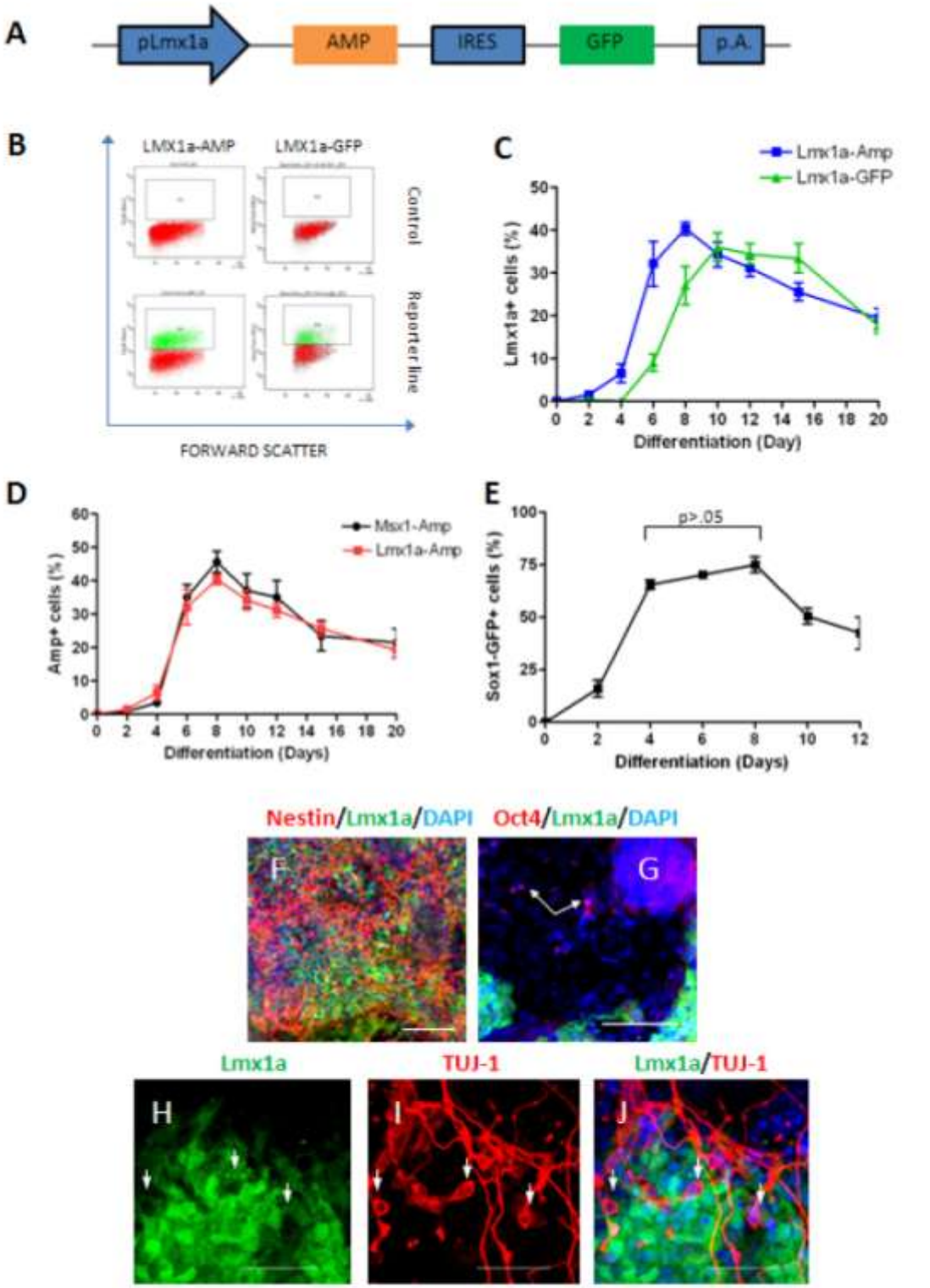

A
pLmx1a
AMP
IRES
GFP
p.A.
B
LMX1a-AMP
LMX1a-GFP
Control
Reporter line
FORWARD SCATTER
C
Lmx1a-Amp
Lmx1a-GFP
Lmx1a+ cells (%)
Differentiation (Day)
D
Msx1-Amp
Lmx1a-Amp
Amp+ cells (%)
Differentiation (Days)
E
p>.05
Sox1-GFP+ cells (%)
Differentiation (Days)
Nestin/Lmx1a/DAPI
Oct4/Lmx1a/DAPI
F
G
Lmx1a
TUJ-1
Lmx1a/TUJ-1
H
I
J

Figure 2. Effects of BMP, Shh and Wnt signalling on Lmx1a/Msx-1/Sox1 expression and dopaminergic neuron differentiation. Inhibition and stimulation of pathways was carried out with BMP-4 (agonist) and Noggin (antagonist) for BMP signalling; Shh (agonist), cylopamine (antagonist) and SANT-1 (antagonist) for Shh signalling; Wnt-1 (agonist), Wnt-3a (agonist), CHIR99021 (agonist) and Dkk-1 (antagonist) for canonical Wnt signalling. (A-D) Percentage of Lmx1a-AMP and Sox1-GFP positive cells on day 8 in response to continuous factor treatment (day 0 to day 8) or factor treatment from day 4 to day 8. (G-J) Number of TH positive cells and emission values for nuclear dye TOPRO-3 (for assessment of cell proliferation) on day 20 of differentiation in response to continuous factor treatment (day 0 to day 12) or factor treatment from day 4 to day 12. (K-N) Labelling of day 20 cultures exposed to BMP-4, Noggin or DKK-1 from day 0 to day 12 for TH and DAPI. (O) Percentage of TH positive cells that label for Foxa2 (day 20) in cultures treated with growth factors from day 0 to day 12. (P-Q) Labelling of day 20 cultures for TH and Foxa2. *, ** indicate $p < .05$ and $p < .01$, respectively, compared to control with one-way analysis of variance plus post hoc Dunnett's test. Scale bar = 100µm.

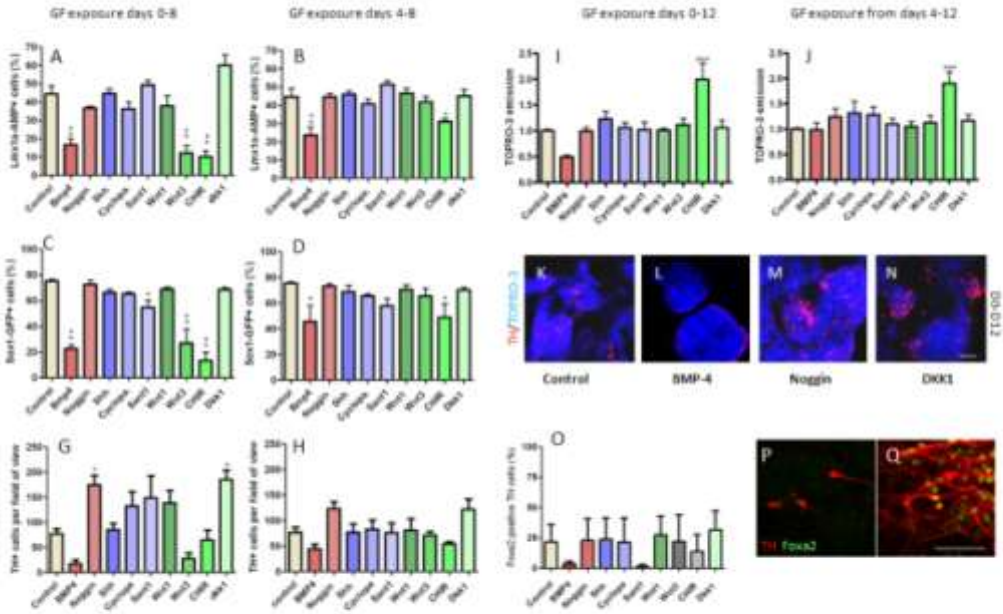


Figure 3 (A) FACS extraction and culture of Lmx1a positive cells. Typical flow cytometry blot (FACS ARIA II) for the extraction of Lmx1a-AMP positive (pos) and negative (neg) cells (day 8 of differentiation); gates were set generously to only extract cells that were unambiguously positive or negative. Note: For some experiments a Lmx1a reporter line that constitutively expresses mCherry from the Rosa26 locus was used. (B, C) Labelling of the AMP positive fraction (48 hours after extraction) for (B) nestin and (C) TUJ-1; (D) Percentage of cells labelling for Nestin in the positive and negative fraction. (E, F) Labelling of the AMP negative fraction for (E) nestin and (F) TUJ-1. (G) Percentage of cells labelling for TUJ-1 in the positive and negative fraction. (H,I) Labelling of the positive and negative fraction for ZO-1, Nestin and DAPI five days after extraction. (J) Reassessment of AMP reporter expression 5 days after FACS extraction. (K) Changes in GFP expression during the first 5 days following FACS separation. (L) White light image of cell colony with distinctly undifferentiated morphology (negative fraction/72 hours after FACS separation) (M) GFP fluorescence 96 hours after separation (same field of view as depicted in panel L). *, $p<.05$, **, $p<.01$ and ***, $p<0.001$. Scale Bar = 100µm.

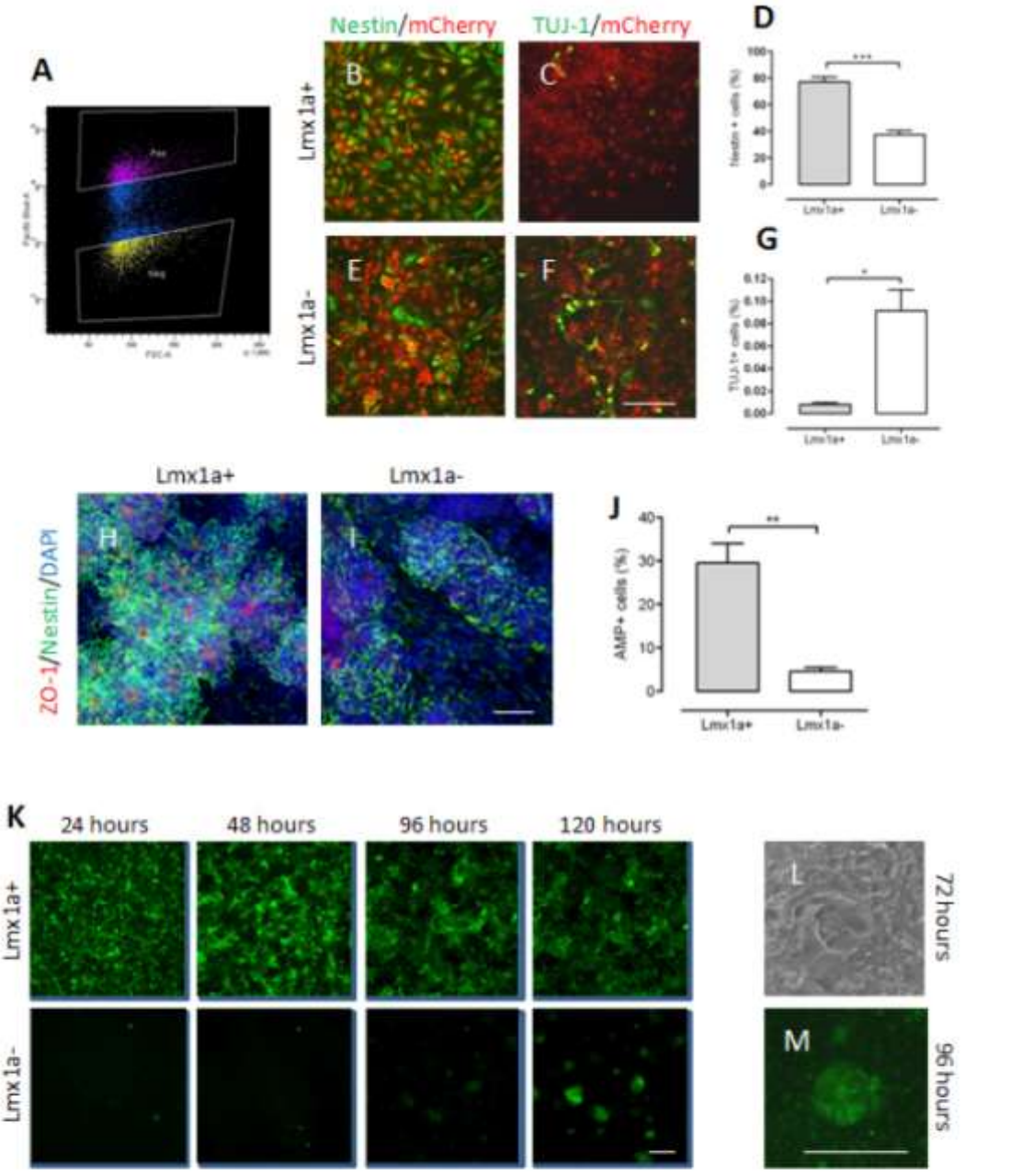
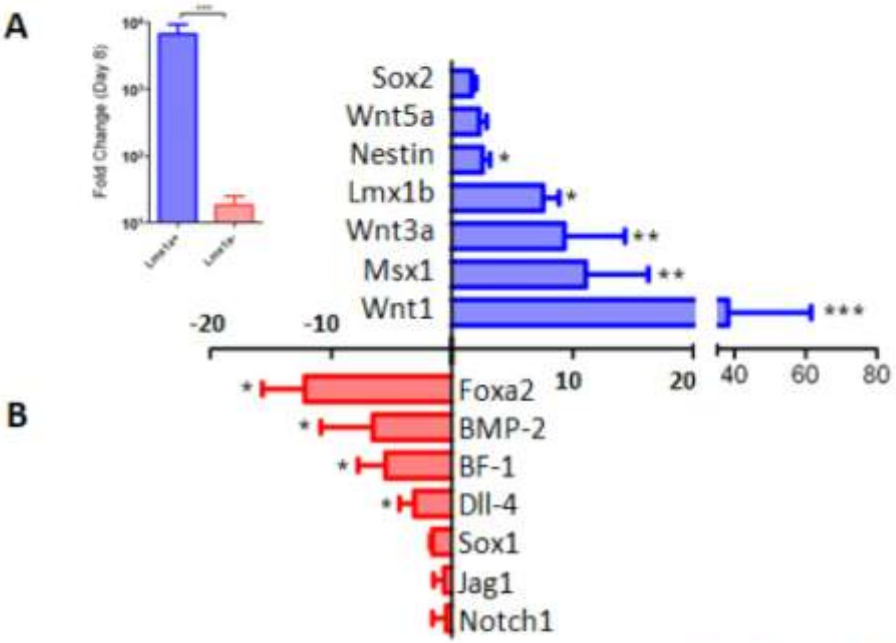

A
Nestin/mCherry
TUJ-1/mCherry
Lmx1a+
Lmx1a-
B
C
E
F
D
G
Lmx1a+
Lmx1a-
ZO-1/Nestin/DAPI
H
I
J
AMP+ cells (%)
K
24 hours
48 hours
96 hours
120 hours
L
M
72 hours
96 hours

Figure 4. Expression profiling (qPCR) of separated Lmx1a-AMP positive and negative cells (day 8 of differentiation in N2B27). (A) Fold up-regulation of Lmx1a transcript levels in the positive and negative fraction compared to day 1 cells. (B) Fold differences in expression levels for selected genes in the positive fraction compared to the negative fraction. (C) Different representation of the panel B data: Fold changes are shown compared to unsorted Lmx1a–AMP cells on day 1 of differentiation. (D) Immunolabelling of day 8 Lmx1a-AMP cultures for Foxa2 and DAPI. *, p<.05, **, p <.01 and ***, p <0.001. Scale Bar = 100µm.



C

Gene	Lmx1a+ cells	Lmx1a- cells
Sox2	1.1 ± 1.2	- 1.7 ± 0.54
Jag1	2.7 ± 1.3	3.7 ± 0.9
Wnt5a	2.9 ± 1.3	1.7 ± 0.4
BF-1	3.4 ± 1.5	21.3 ± 13.7
Dll4	3.4 ± 1.5	10.6 ± 3.1
Notch1	4.3 ± 1.5	6.0 ± 3.4
Bmp2	6.5 ± 4.9	21.1 ± 9.1
Foxa2	16.3 ± 2.5	263.8 ± 150.3
Sox1	21.6 ± 8.0	40.2 ± 6.9
Nestin	57.5 ± 25.7	31.5 ± 16.7
Lmx1b	321.0 ± 186.4	53.2 ± 35.2
Wnt3a	903.7 ± 286.5	177.8 ± 105.1
Msx1	1428.0 ± 1182.7	123.0 ± 52.7
Lmx1a	6612.52 ± 4680.69	18.41 ± 11.65
Wnt1	101303.3 ± 68276.0	7482.7 ± 7192.3

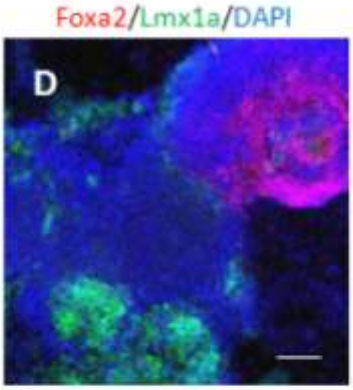


Figure 5. Characterization of monolayer derived Lmx1a+ cells. Positive and negative cells were FACS separated and terminally differentiated; panels A and F show white light images of respective cultures. Both fractions were labelled for (B, G) TUJ-1, (C, H) GABA, (D, I) TH and (E, J) GFPA; DAPI was used as nuclear counter stain. (K-N) The positive fraction was also labelled for (K) TUJ-1 and GABA, (L) GABA and SatB2, (M) GAD67 and GABA and (N) TH and GABA. (O, P) Labelling of unsorted day 8 cultures (Lmx1a reporter line) for (O) SatB2 and (P) TBR-1; DAPI was used as nuclear counter stain. (Q, R). Effect of RA treatment on the percentage of Lmx1a+ and Sox1+ cells on day 8 of differentiation. *, p<.05. Scale Bar = 100µm.

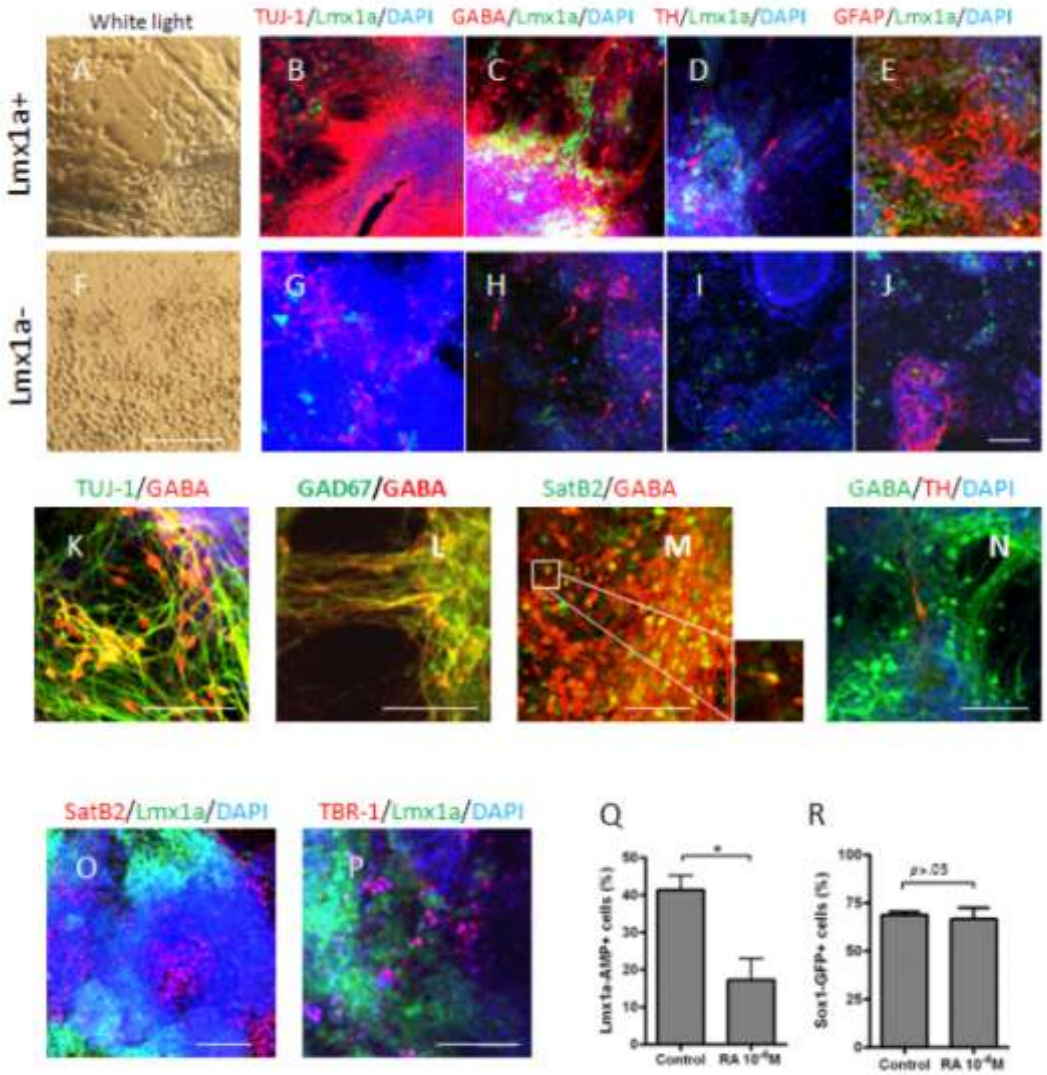


Figure 6. Patterning and maintenance of FACS separated, monolayer derived, Lmx1a+ cells. (A) Numbers of GABA+ and TH+ neurons and (B) emission values of nuclear dye TOPRO-3 (for assessment of cell proliferation) in terminally differentiated cultures after treatment with FGF2 alone or in combination with Shh and FGF8. (C) Percentage of Lmx1a-AMP+ cells during propagation in the presence of EGF and FGF2. Abbreviations used: P, Passage number.

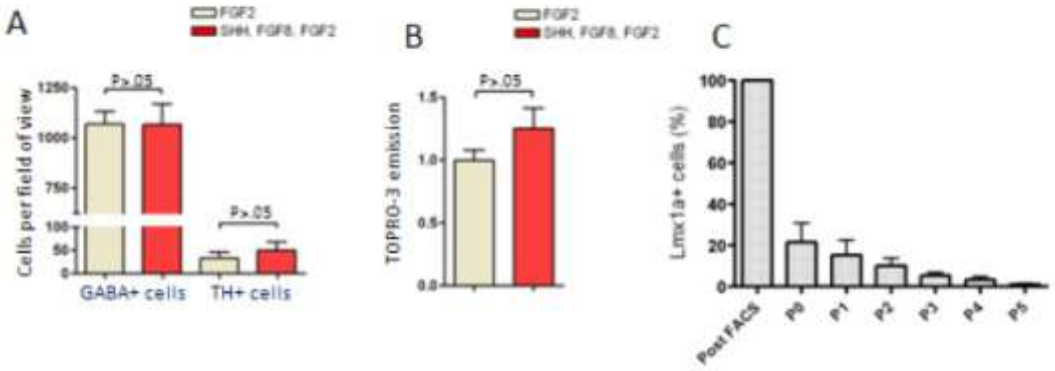


Figure 7. Lmx1a+ cells in the context of PA-6 co-culture. (A) Time course showing the percentage of Lmx1a-AMP+ cells during differentiation. (B,C) Labelling of day 8 cultures (reporter line) for (B) TUJ-1 and (C) Nestin; DAPI was used as nuclear counter stain. (D) Labelling of day 10 cells (Lmx1a reporter line) for Foxa2; DAPI was used as nuclear counter stain. (E, F) Labelling of day 15 cultures (lmx1a reporter line) for TH; DAPI was used as nuclear counter stain. (G) Percentage of TUJ-1^{+} colonies (day 15) that contain more or less than 10 TH+ cells (red bars); neighbouring bars (green) indicate the percentage of these colonies that are positive for Lmx1a reporter GFP. Panel H-O show terminally differentiated cultures of FACS extracted Lmx1a positive and negative cells labelled for (H,I) TH and TUJ-1, (J, K) TH, (L, M) GABA, (N, O) GFAP; DAPI was used as nuclear counter stain. (P) Numbers of TH positive (red bars) and GABA positive (green bars) cells per field of view in terminally differentiated cultures of FACS extracted Lmx1a positive and negative cells. Panel Q depicts TOPRO-3 (nuclear dye) emission values for terminally differentiated cultures of the positive and negative fraction as a measure of cell density for both conditions. *, $p<.05$, **, $p<.01$ and ***, $p<0.001$. Scale bar = 100μm.

Lmx1a cells in differentiating ESCs