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GENERATION OF DISEASE-RELEVANT NEURONS FROM HUMAN PLURIPOTENT STEM CELLS

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I declare that the work presented in this thesis is my own, unless otherwise stated.

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Abstract

This thesis describes investigations into exogenous factors that influence fate-choices during human pluripotent stem cell (PSC) differentiation *in vitro*. I describe novel growth factor environments and small molecule regimes that provide patterning signals enabling directed differentiation of human PSCs towards biomedically relevant neurons. I provide evidence that the TGF β growth factor Activin can promote ventral telencephalic differentiation. Small adjustments in Activin administration can produce an over-representation of forebrain derived medium spiny neurons and cortical interneurons, with significance in Huntington's disease and epilepsy respectively. Additionally, I manipulate the earliest growth factor environments of PSC differentiation to lead to broad changes in rostro-caudal patterning. Inhibiting FGF signalling leads to a midbrain-like phenotype that can be further differentiated towards a ventral midbrain dopaminergic fate, a cell type that degenerates in Parkinson's disease. Our novel manipulations and differentiation protocols provide insights into early events in human development, which would otherwise be impossible to study. This logic can also be applied to investigate diseased states during human development and ageing via use of disease-specific cell lines. The mature neurons produced may provide a tool that can be applied to large-scale drug screening assays and toxicology testing as well as having the potential for cell based therapies in future years.

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List of abbreviations

ACT	Activin A
ACTRIIA/B	Activin receptor 2 A or B
AGN	AGN193109 – RAR inhibitor
aka	Also known as
ALDH1a1-3	Aldehyde dehydrogenase 1 members 1 to 3 – aka retinal dehydrogenase (RALDH1-3)
ALK	Anaplastic lymphoma receptor tyrosine kinase
ANR	Anterior neuroectodermal ridge
APC	Adenomatous polyposis coli
ARPP21	Cyclic AMP regulated phosphoprotein
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CALB	Calbindin
cDNA	Complimentary DNA
CGE	Caudal ganglionic eminence
ChIP	Chromatin immunoprecipitation
CKI	Casein Kinase 1
CR	Calretinin
CRABP	Cellular retinoic acid binding protein
CTIP2	Coup TF interacting protein 2 – aka BCL11b
CYP26b	Cytochrome P450
d	dorsal – e.g. dCGE
dd	Double distilled
DAPI	4, 6 diamidino 2 phenylindole
DARPP32	Dopamine and cyclic AMP regulated phosphoprotein 32 – aka PPP1R1B
DISP	Dispatched
DKK	Dickkopf

DLX1/2	Distal-less homeobox 1 or 2
DMEM	Dulbecco's modified eagle medium
DMRT5	Doublesex and Mab3 related transcription factor 5 – aka dmrta2
DNA	deoxyribose nucleic acid
DRD1/2	Dopamine receptor 1 or 2
DSV	Dishevelled
DYN	Dynorphin – gene name TAC1
EB	Embryoid body
EdU	5 ethynyl 2 deoxyuridine
EGF	Epidermal growth factor
EN1/2	Engrailed 1 or 2
ENK	Enkephalin – gene name PENK
epiSC	Epiblast-derived stem cell
ER81	ETS variant gene1 – aka ETV1
ERK	Extracellular signal regulated kinase – aka MAPK, mitogen activated protein kinase
ESC	Embryonic stem cell
ETS	v-ets erythroblastosis virus E26 oncogene homologue
FACS	Fluorescence activated cell sorting
FB	Forebrain
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FOS	FBJ osteosarcoma oncogene
FOXA1/2	Forkhead box A1 or A2 – aka HNF3 β
FOXG1	Forkhead box G1 – aka BF1
FOXP1/2	Forkhead box P1 or 2
FRZ	Frizzled
FU	Fused

GABA	γ Amino butyric acid
GAD67	Glutamate decarboxylase – gene name GAD1
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GBX2	Gastrulation brain homeobox 2
GFAP	Glial fibrillary associated protein
GFP	Green fluorescent protein
GLI	GLI family zinc finger
GMEM	Glasgow minimum essential medium
GMP	Good manufacturing protocol
GRB2	Growth factor receptor bound protein 2
GSK3 β	Glycogen synthase kinase 3 beta
GSX2	GS homeobox 2 – aka GSH2
GTPase	Guanosine triphosphatase
h	Human – e.g. hESCs
HB	Hindbrain
HD	Huntington's disease
HOX	Homeobox
HuNu	Human nuclear antigen
i	inhibition – e.g. ERKi
ICM	Inner cell mass
IGF	Insulin like growth factor
IK	Ikaros – aka IKZF1
iPSC	Induced pluripotent stem cell
ISL1	Islet LIM homeobox 1
ISO	Isthmic organiser
JUN	JUN proto-oncogene
LEF	Lymphoid enhancer binding factor

LGE	Lateral ganglionic eminence
LHX6/8	LIM homeobox protein 6 or 8
LIF	Leukaemia inhibitory factor
LMX1a	LIM homeobox transcription factor 1a
m	Mouse – e.g. mESCs
MAP2	Microtubule associated protein 2
MAPK	Mitogen activated kinase
MASH1	Aschaete-scute complex homologue 1 – aka ASCL1
MB	Midbrain
MD	Monolayer differentiation
mDA	Midbrain dopaminergic
MEF	Mouse embryonic feeder
MGE	Medial ganglionic eminence
MHB	Midbrain hindbrain border
Min	Minute
MSN	Medium spiny neuron
MSX1/2	Msh homeobox 1
NEAA	Non essential amino acids
NeuN	Neuronal nuclear antigen
NGN2	Neurogenin 2
NKX2.1	NK 2 Homeobox 1 – aka TTF1
NOLZ1	Zinc finger protein 503 – aka ZNF503
NPY	Neuropeptide Y
NSC	Neural stem cell
NURR1	Nuclear receptor family 4 member 2 – aka NR4A2
OCT4	POU class 5 homeobox 1 – aka POU5F1
OTX2	Orthodenticle homologue 2

PARV	Parvalbumin
PAX6	Paired box gene 6 – aka SEY
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PD	PD0325901
PDGF	Platelet derived growth factor
PFA	Paraformaldehyde
PI3 Kinase	Phosphoinositide 3 kinase
PITX3	Paired like homeodomain transcription factor 3
PLC γ	Phospholipase C gamma
PSCs	Pluripotent stem cells
PSD95	Membrane associated guanylate kinase scaffolding protein – postsynaptic densities
PTCH	Patched
QPCR	Quantitative polymerase chain reaction
RA	Retinoic acid
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBP4	Retinoic acid binding protein 4
RLN	Reelin
RNA	Ribose nucleic acid
ROCK	RHO-kinase associated coiled coil containing protein kinase
RPM	Revolutions per minute
RTK	Receptor tyrosine kinases
RXR	Retinoid X receptor
SD	Standard deviation
SDIA	Stromal derived inductive activity

Sec	Second
SFEB	Serum free embryoid bodies
SHH	Sonic hedgehog
SIX3	Sine oculis-related homeobox 3
SMAD	SMAD, homologue to mothers against dpp
SMO	Smoothened
SN	Substantia Nigra
SOX1	SRY box containing gene 1
SPRY	Sprouty
SOS	Son of sevenless homologue
SST	Somatostatin
STRA6	Stimulated by retinoic acid gene 6
SUBP	Substance P
TCF/LEF	Transcription factor lymphoid enhancer binding factor
TGF β	Transforming growth factor beta
TH	Tyrosine hydroxylase
TUJ1	β -III-tubulin antigen
UVI	UVI3003 – RXR inhibitor
v	Ventral – e.g. vCGE
vMB	Ventral midbrain
VTA	Ventral tegmental area
WGE	Whole ganglionic eminence
WNT	Wingless type MMTV integration site family
Xt	Extra toes mutant – GLI3 knockout
ZCCHC12	Zinc finger CCHC domain containing 12
ZLI	Zona limitans intrathalamica

Chapter 1 Introduction

1.1 Thesis Introduction

In this thesis, I shall investigate the differentiation of human pluripotent stem cells (hESCs) into neurons with specific relevance to certain diseased states. I aim to optimise a differentiation scheme to produce cells that are competent to mature into a range of neuronal cell types. Via use of novel exogenous growth factor combinations, I aim to alter the cellular environment in order to specify these disease-relevant neurons. This technique makes use of the genetic networks set in place for normal development.

The focus will be on dopaminergic neuronal populations that degenerate in Parkinson's disease, GABAergic projection neurons that are lost in Huntington's disease and cortical interneurons that have been theorised to be hypoplastic in epilepsy, autism, schizophrenia and Tourette's syndrome.

1.2 Development of the Neural Tube

1.2.1 Neurulation

During gastrulation of the embryo, the three germ layers are specified; namely the endoderm, mesoderm and ectoderm. The ectoderm has the potential to form neural tissue and the epithelium, whereas the mesoderm and endoderm form the remaining cell types, e.g. blood lineages and the internal organs respectively. In the 1920s, Spemann and Mangold performed grafting studies between amphibian embryos and helped to define the Organiser region (also called the Node), a signalling region responsible for initial neural induction. Isolation and grafting of the Organiser onto a second embryo initiated formation of a second, complete neural axis (Figure 1.1 A) (Spemann and Mangold, 1924). Further work by Nieuwkoop in the 1950s and 1960s examined the induction of the Organiser and found a Fibroblast Growth Factor (FGF2) signal from the underlying tissue was critical for Organiser formation (Nieuwkoop, 1969).

Studies culturing dissociated tissue *in vitro* have demonstrated that the ectoderm has a default neural differentiation program and that strong Bone Morphogenetic Protein (BMP) signals are widely expressed to promote epithelial, rather than neural, development (Hemmati-Brivanlou and Melton, 1997). The function of the Organiser was thus described as a disinhibitory role, whereby many inhibitors of epidermal-inductive BMP signals are secreted by the Organiser. In mammalian development, the Organiser is termed the Anterior Neuroectodermal Ridge (ANR) and BMP inhibitors such as Noggin and Chordin (Anderson et al., 2002; Bachiller et al., 2000; Smith and Harland, 1992), Wnt inhibitors such as DKK1 (Glinka et al., 1997) and TGF β inhibitors such as

Follistatin (Hemmatibrivanlou et al., 1994) have all been shown to be expressed in the ANR. Importantly, neural tissue has a default anterior character and as the neural tube extends to the posterior of the embryo, further exogenous signalling is required for caudalisation of the tissue, this is termed the Activation-Transformation model (Figure 1.1 B) (neural induction followed by progressive caudalisation) (Nieuwkoop and Nigtevecht, 1954).

Upon neural induction, the neural plate separates from the epithelium and forms a neural tube; this is achieved via complex cell morphology changes (Figure 1.1 C). Cells actively divide in the neural tube and maturing cells are found at the extremity of the structure whereas naive cells are found close to the lumen in the sub ventricular zone (SVZ) (Figure 1.1 D). Understanding of cell diversification and structure formation has focused on morphogenetic signals, whereby changes in the anterior-posterior and dorso-ventral axes convey positional identities to the cells and cellular domains. The balance between proliferation and maturation sets the final size and structure of the central nervous system and the broad range of positional identities are responsible for creating the full complement of cell types of the nervous system.

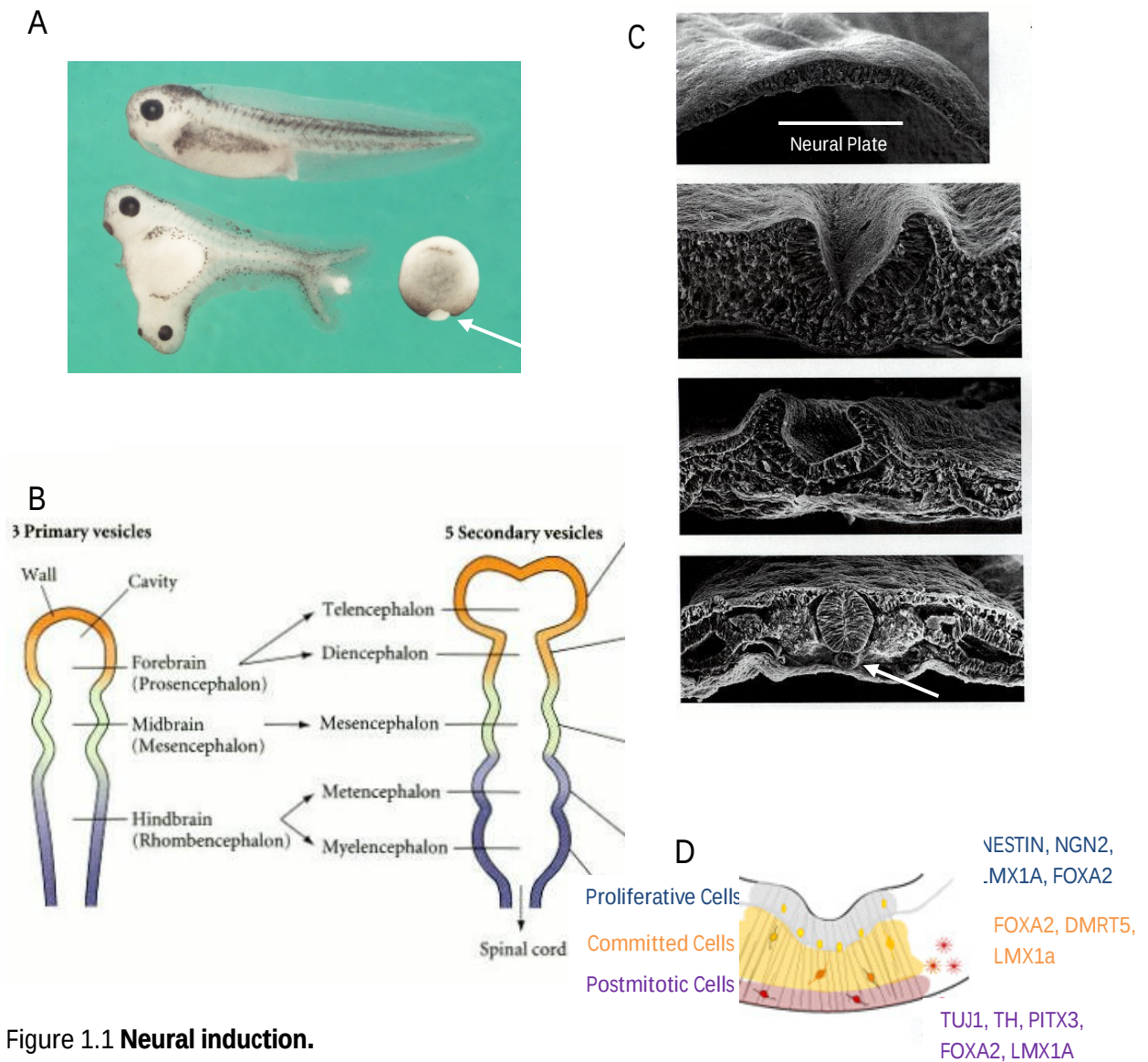


Figure 1.1 **Neural induction.**

(A) depicts a reproduction of the initial *Organiser* grafting studies of Spemann and Mangold, demonstrating that a donor *Organiser* located at the dorsal lip of the blastopore (bottom right, arrow, vegetal view) can induce an entirely new neural axis (bottom left) compared with the wild type (top). (B) shows the neural tube domain organisation in the anterior-posterior axis. More posterior/caudal tissues are thought to be posteriorised from a default anterior character. (C) shows electron microscope images of neural tube formation, whereby the underlying notochord (arrow) instructs the neural plate to separate from the epithelium and form a tube structure. Note, the tube resembles *in vitro* rosette structures, discussed Chapter 1.2.2.2. (D) is a diagram of a transverse section of the neural tube at the ventral midbrain. Depicted are the naive cell markers expressed close to the lumen of the neural tube and mature TH (dopaminergic) neurons being generated at the periphery, markers explained in Chapter 1.2.4. The SVZ is closest to the lumen and is proliferative (blue), the intermediate zone marks committed cells (yellow) and postmitotic neurons are in the marginal zone (purple) (De Robertis and Kuroda, 2004; Gale and Li, 2008; Gilbert, 1985; MmcCurie, 2000)

1.2.2 Up to date: Neural Induction and the Default Model

Since the *Xenopus* experiments discussed, certain studies have reinforced the importance of FGF signalling for neural induction in mouse embryonic stem cells as FGF deficient cells remain pluripotent (Kunath et al., 2007; Stavridis et al., 2007). It appeared that neural organiser region induction from the mouse anterior visceral endoderm might function in a parallel manner to the vegetal-pole-derived FGF induction of the Organiser region in *Xenopus* (Nieuwkoop, 1969).

Further investigations have shown that this is not the case. FGF signalling has been proven to be critical for mouse embryonic stem cells (ESCs) to exit the pluripotent state (Kunath et al., 2007) and this is supported as FGF inhibition together with Wnt agonists maintain a pluripotent ground state (Silva et al., 2008). Importantly, it was shown that this requirement for FGF signalling is no longer required after epiblast formation and that inhibition of FGF, after its requirement for epiblast induction, actually facilitates neural induction. This is witnessed via enormous ectopic neural marker expression when epiblast embryos are cultured with FGF inhibitors (Di-Gregorio et al., 2007).

It is considered that mouse cells offer a better model to study human development than lower organisms; however, there are certain differences between human and mouse ESCs. Although human cells express many of the same key pluripotency markers, their growth factor dependencies are divergent. This results in the very contentious hypothesis that human ESCs represent a later, possibly epiblast, stage of development (Greber et al., 2010; Tesar et al., 2007). This is supported by the investigations of Greber et. al. who show that inhibiting FGF signalling enhances neural differentiation from the earliest time points of human ESC differentiation – contradicting mouse ESC data (Greber et al., 2011). Contrastingly, parallel studies in other groups argue that FGF signalling is endogenously active during early human ESC differentiation. For example, FGF2 was shown to facilitate neural differentiation and its blockage inhibits this process (Cohen et al., 2010), although neural differentiation can progress in the absence of FGF signalling.

Therefore, the role of FGF signalling in early differentiation is complex and may also be influenced by species diversity. For example, mouse and human embryos use different neural fate determinants (Zhang et al., 2010) and so growth factor dependency may differ. Nonetheless, it is clear that both human and mouse systems use inhibitors of BMP and TGF β signalling pathways to initiate neural patterning in a similar disinhibitory manner to the *Xenopus* Organiser. Molecules such as Noggin, Cerberus, Lefty and Follistatin are all expressed in mammalian Organiser regions, described in the next section (Bachiller et al., 2000; Greber et al., 2011), and have therefore become important for ESC differentiation (Chambers et al., 2009), discussed in Chapter 1.6.

1.2.3 Secreted Growth Factor Signalling

Throughout development, organiser regions are responsible for establishing positional cues, enabling individual cells to sense their relative position in the embryo and develop into the correct cell type accordingly. Organiser regions are responsible for secreting soluble and lipid bound growth factor stimuli to which nearby cells are able to respond in a paracrine manner. This growth factor secretion is the basis of the morphogen model of development, whereby a cell's respective distance from an organiser is tantamount to the dosage it perceives (Figure 1.2). Thus, the gradient of a morphogen over distance (and so the morphogen concentration) instils positional identity to the responding cells.

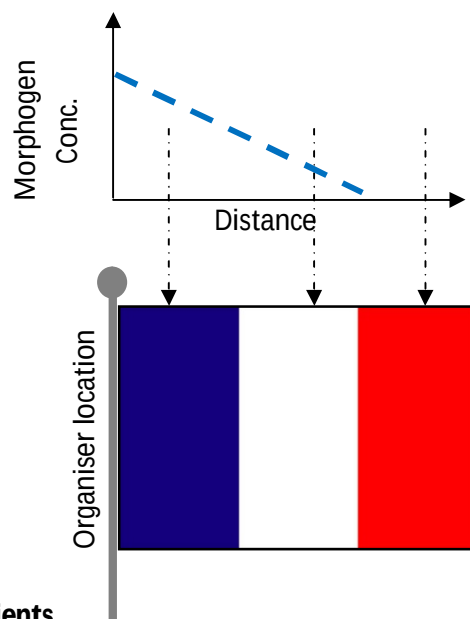


Figure 1.2 **Morphogen gradients.**

Cells in close proximity to the organiser (left) receive high doses of a diffusible signal (top). As distance from the organiser increases, dose perception reduces. This leads to the French flag hypothesis (bottom), as cells respond to the signal to produce three cell types relative to morphogen concentration, thus conveying positional identity.

In this thesis, I shall consider growth factor stimuli relevant to the anterior neural tube. The Organiser regions responsible for the major patterning events in the forebrain are described thus (and in Figure 1.3).

1. Anterior Neuroectodermal Ridge (ANR, also known as the Node or the rostral patterning centre). The ANR is initially responsible for specifying the neural tube from the neuroectoderm, see Chapter 1.2.1. Inhibitors of BMP, TGF β and WNT signalling such as Noggin, Chordin, Follistatin and Lefty are all crucial for neural fate determination but also for specifying anterior neural fates (Bachiller et al., 2000; Glinka et al., 1997; Hemmatibrivanlou

et al., 1994; Smith and Harland, 1992). Once established, the ANR has a major role in secreting FGF8 to pattern the anterior neural tissues. FGF8 has been shown to be responsible for inducing FOXG1, a crucial telencephalic fate determinant gene (Shimamura and Rubenstein, 1997).

2. Floorplate SHH secretion. SHH is a morphogen that is expressed throughout the ventral neural tube (Briscoe and Ericson, 2001; Ericson et al., 1995). Initial expression in the notochord (mesoderm derived) is responsible for subsequent neural SHH expression. SHH has been shown to act as a classic morphogen, whereby homeodomain proteins respond to SHH signals in a graded manner to set up pools of progenitor cells with distinct dorso-ventral spatial identities (Muhr et al., 2001).
3. The cortical hem and dorsal roofplate. BMP and Wnt proteins expressed in the cortical hem are required for proper development of dorsal tissues including the cortex and neural crest cells (Furuta et al., 1997; Grove et al., 1998). Throughout the neural tube, antagonistic cues from the ventral SHH and dorsal BMP/Wnt signals specify the full complement of dorso-ventral cell types (Ulloa and Briscoe, 2007).
4. The midbrain hindbrain boundary (MHB, or Isthmus/Isthmic organiser). The MHB is the boundary demarcating the posterior limit of OTX2 expression (the forebrain and midbrain homeodomain protein) and anterior limit of GBX2 expression (hindbrain homeodomain protein). These two proteins are expressed in a mutually exclusive manner. FGF8 is a major secreted factor from the MHB and is responsible for the patterning of the midbrain tissues and the hindbrain-derived cerebellum (Ye et al., 2001). FGF8 is then responsible for induction of WNT1, a second major morphogen secreted by the MHB, that is also crucial for midbrain and hindbrain development (Danielian and McMahon, 1996).
5. The Zona Limitans Intrathalamica (ZLI). The ZLI in the diencephalon secretes SHH signals and effects the proliferation, patterning and boundary formation during thalamic development (Echevarria et al., 2003). The ZLI may have more complex instructive properties through FGF signals that remain to be elucidated.

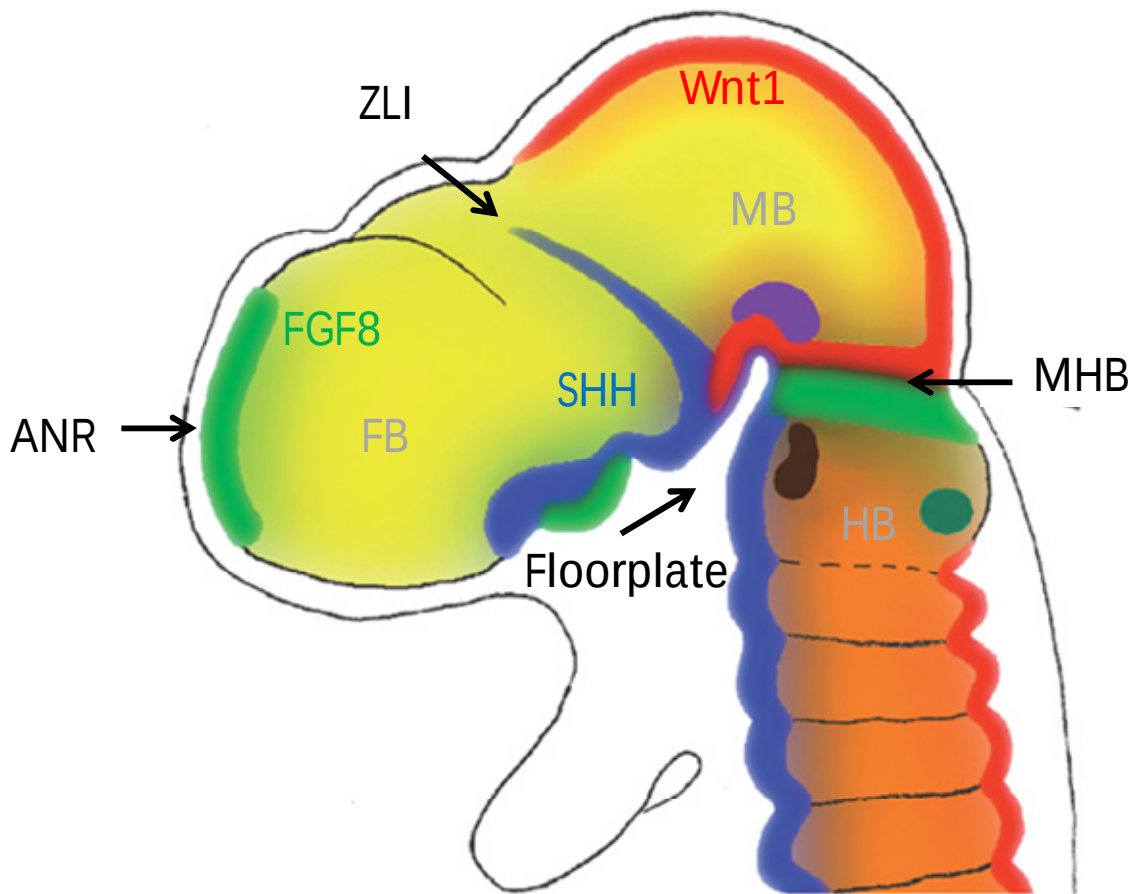


Figure 1.3 **Organiser Regions.**

Organiser regions of the developing neural tube are responsible for establishing morphogen gradients and conveying positional identity. The Organisers discussed in this thesis are the ANR at the anterior most tip of the embryo that secretes FGFs among other signals, the ZLI in the diencephalon that mainly secretes SHH, the MHB that secretes FGFs and Wnt signals, dorsal BMP and WNT secretion by the roofplate, and the floorplate that spans the ventral neural tube and secretes SHH signals. FB - forebrain, MB - midbrain and HB – hindbrain are depicted (MmeCurie, 2000; Prakash and Wurst, 2006).

1.2.3.1 Fibroblast Growth Factor Signalling

Fibroblast growth factor (FGF) signalling has been implicated in a variety of processes from proliferation and survival to patterning and terminal differentiation. Currently 22 FGF ligands have been described, with a conserved heparin sulphate binding domain. Additionally there are 4 FGF receptors; each with several splice variants. This diversity means that there is a great deal of complexity in the FGF signalling response. FGF signals are transduced via the receptor tyrosine kinase (RTK) cascade and so are akin to other RTK signal pathway activators such as PDGF, EGF, etc.

Binding of the FGF ligand leads to receptor dimerisation and autophosphorylation of the receptor intracellular domain (Figure 1.4). Phosphorylated serine and threonine residues then promote the formation of a protein complex, including GRB2 and SOS, which leads to activation of the signalling hub RAS-GTPase. Activation of RAS leads to the MAP kinase phosphorylation cascade resulting in phosphorylated ERK, which can enter the nucleus and lead to transcriptional changes in target genes such as ETS, c-FOS and JUN. Negative regulation of this cascade is promoted directly via ERK-mediated phosphatases attenuating the MAP kinase signal and also via transcriptional changes of negative regulators, such as *SPROUTY* (Kim and Bar-Sagi, 2004). FGF signalling can also effect secondary pathways such as the PI3 kinase pathway and PLC γ mediated IP3, leading to pleiotropic downstream effects.

In this project I use the FGF signalling inhibitor PD0325901. This small molecule prevents ERK phosphorylation via inhibition of kinase activity of the MAPKK/MEK molecule.

FGF signalling has a range of relevant effects during development. Firstly, FGF2 signals from the Nieuwkoop centre are pivotal for the establishment of the Spemann Organiser and so all the neural tissues (Chapter 1.2) (Nieuwkoop, 1969). Subsequent FGF8 signals in the ANR and the MHB are required for the development, proliferation and survival of forebrain (Shimamura and Rubenstein, 1997) and midbrain/hindbrain tissues respectively (Ye et al., 2001). FGF15/19 signals from the diencephalon have been shown to be complementary to ANR-derived FGF8 signals and function to promote proliferation and survival of caudal forebrain and diencephalic tissues (Borello et al., 2008). Additionally, FGF2 signals have been shown to have a caudalising effect on the embryo, aiding hindbrain/spinal cord development (Cox and Hemmati-Brivanlou, 1995). FGF8 knockout mice have an enlarged OTX2-positive forebrain/midbrain, reinforcing a caudalisation effect via FGF signals (Sun et al., 1999).

In vitro, FGF2 is routinely used for maintenance of human pluripotent stem cells (PSCs), see Chapter 1.4. Strong doses of the growth factor are used in concert with feeder cells to maintain cell pluripotency. FGF2 has also been used together with EGF to promote proliferation and prevent

differentiation of neural stem cells (NSCs) (Tropepe et al., 1999). Subsequently, differentiation ensues when the growth factors are withdrawn. FGF signalling has also been demonstrated to be critical for initial lineage commitment and differentiation. ERK knockout ESCs are not able to exit the self-renewal phase, potentially due to the lack of Nieuwkoop centre inductive capacity. This FGF dependency has been shown to be FGF4 specific (Kunath et al., 2007; Stavridis et al., 2007), but also FGF5 exhibits an epiblast-specific expression pattern.

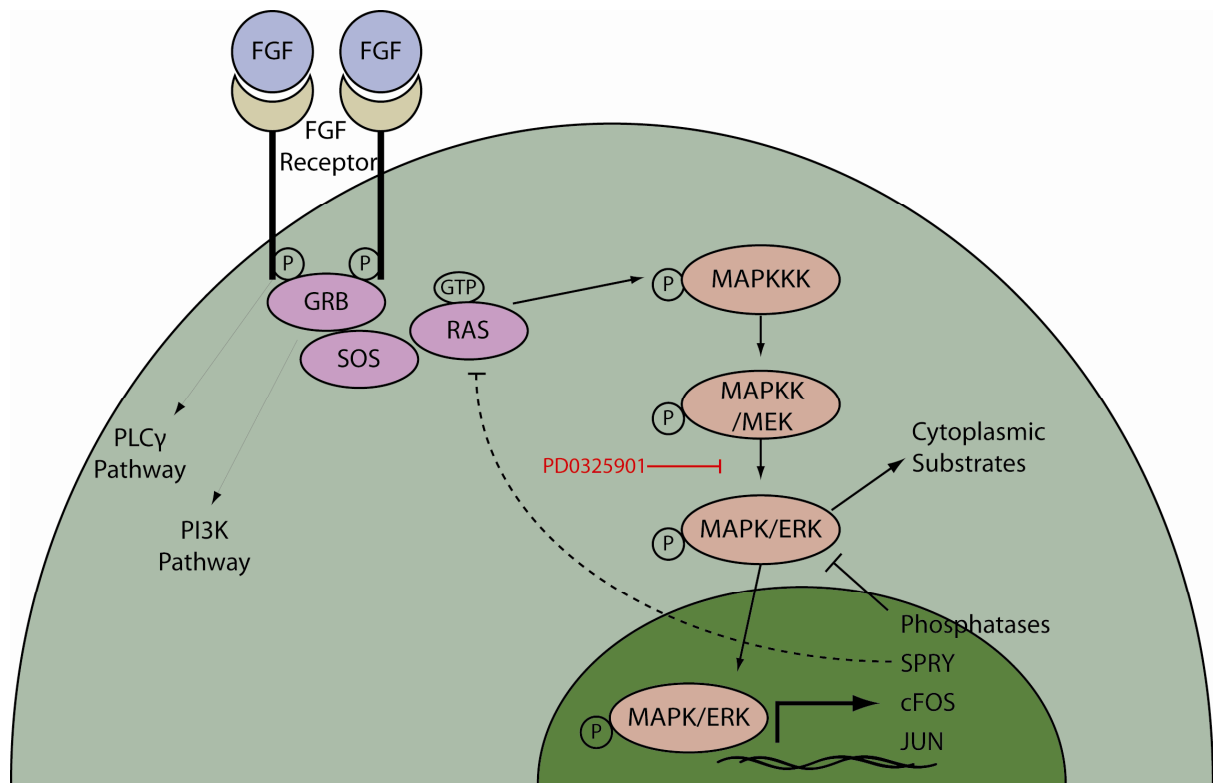


Figure 1.4 **FGF signalling.**

Secreted FGF ligands bind to FGF receptors leading to dimerisation and autophosphorylation on receptor intracellular domains. Phosphorylated receptors then recruit a complex of proteins including GRB and SOS to activate the signalling hub RAS. Activated RAS leads to a phosphorylation cascade through mitogen activated kinases leading to phosphorylated ERK; this is inhibited by the small molecule PD0325901. Phosphorylated ERK is able to enter the nucleus and alter expression of target genes, which include the negative feedback regulators SPRY and phosphatases such as DUSP. Other pathways such as PLC γ and PI3K are also stimulated after receptor autophosphorylation.

1.2.3.2 Sonic Hedgehog Signalling

Sonic hedgehog (SHH) signals in the ventral neural tube establish patterning in the dorsoventral axis via antagonising dorsal signals such as BMP (Ericson et al., 1995). Additionally, SHH has been shown to have a strong mitogenic activity and may be involved in correct tissue growth during development (Elkabetz et al., 2008; Fuccillo et al., 2006). Establishment of ventral SHH expression, e.g. in the floorplate of the neural tube, requires SHH expression in the underlying mesodermal structure the notochord.

SHH signals are mediated through a protein cascade in the cells receiving the signal (Figure 1.5), for review see (Hooper and Scott, 2005; Ingham and McMahon, 2001). SHH is a secreted glycoprotein ligand that is palmitoylated and covalently associated with cholesterol in the secreting cell. This apparent lipophilic characteristic is counterintuitive for a long-range secreted morphogen; however a dependence on the protein Dispatched (DISP), that may displace the ligand from lipid rafts, suggests one mechanism for ligand activation. Also, the possible presence of two isoforms of SHH, a full-length unmodified version in addition to palmitoylated SHH, may account for the long range effects of SHH (Ingham and McMahon, 2001). In the absence of SHH, a protein kinase complex containing Protein Kinase A (PKA), Glycogen Synthase Kinase (GSK3 β), Casein Kinase I (CKI) and Fused (FU), phosphorylates and inactivates GLI1/2 transcription factor effectors. In contrast, phosphorylation of GLI3, the negative SHH effector, leads to its cleavage and activation. Upon SHH binding to its receptor Patched (PTCH), a Smoothened (SMO)-mediated inhibitory signal is lifted and the protein kinase complex is disrupted from the microtubule cytoskeleton. The cytoplasmic pool of GLI1/2 is no longer inactivated via phosphorylation/cleavage and after translocation to the nucleus, GLI1/2 bring about positive SHH signalling effects. Active GLI signalling displaces Groucho-mediated inhibition of transcription to alter expression of the homeodomain SHH target genes (Muhr et al., 2001).

The inhibitor of SHH signalling that I use in this project is termed Cyclopamine. This molecule antagonises SHH by directly binding to SMO to enforce the negative signal state (Chen et al., 2002). We also use the small molecule Purmorphamine which is a SHH agonist. This molecule also functions via binding Smo with a similar target motif to Cyclopamine, albeit with opposing effects to the SHH signal activity (Sinha and Chen, 2006).

SHH has a number of developmental roles including tooth, hair and gonadal development. SHH signalling has been extensively studied in the developing neural tube. Signalling in the spinal cord is crucial for motor neuron specification. SHH patterns the ventral midbrain which includes the dopaminergic cells of the basal ganglia. In the forebrain SHH induces cortical interneurons subtypes

and also plays a critical role in the development of the eye. Additionally to a patterning role, SHH has a role in proliferation of neural progenitors and maintains self-renewal (Fuccillo et al., 2006).

In vitro techniques have demonstrated the mitogenic capacity of SHH, specifically at the rosette stage of differentiation (Elkabatz et al., 2008). This translates to the neural progenitor stage, when cells are cycling and when SHH has its strongest patterning effect at parallel developmental stages *in vivo*. The specification function of SHH towards ventral tissues has been employed for relevant ventral cell type differentiation, such as cortical interneurons and projection neurons of the basal ganglia (Aubry et al., 2008; Maisano et al., 2012), ventral diencephalic cells (Ohyama et al., 2005), ventral midbrain dopaminergic cells (Barberi et al., 2003; Fasano et al., 2010) and motor neurons of the spinal cord (Wichterle et al., 2002).

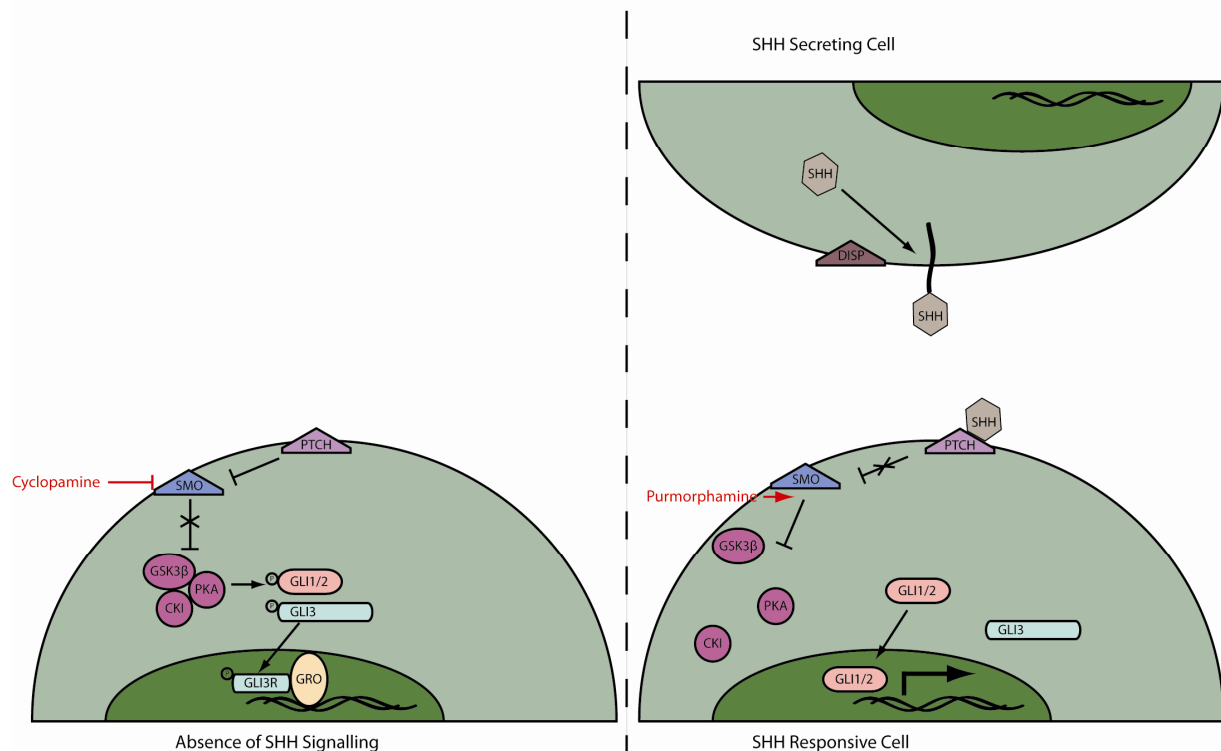


Figure 1.5 **SHH signalling.**

SHH is a secreted, palmitoylated protein that requires Dispatched (DISP) processing in the SHH producing cell. In the absence of SHH signal, Smoothened (SMO) is inhibited by Patched (PTCH), leading to an active protein kinase complex that includes GSK3β, PKC and CKI. GLI proteins are thus phosphorylated and cleaved leading to active repressors (e.g. GLI3R) or inactive SHH transducers (e.g. GLI1). Ligand binding to PTCH relieves the inhibition on SMO and results in disruption of the kinase complex. Non-phosphorylated GLI proteins remain free from cleavage, leading to active GLI1

and inactive GLI3. The result of SHH signalling is GLI1/2 mediated transcriptional changes to homeodomain factors that are grouped into two classes depending upon positive or negative responses to GLI1/2. SMO is the target of both the agonist Purmorphamine and antagonist Cyclopamine used in the study. Transcriptional changes are achieved via lifting Groucho-mediated (GRO) repression, adapted from (Fuccillo et al., 2006).

1.2.3.3 Wnt Signalling

Multiple Wnt family members exhibit conserved expression in the dorsal midline of the neural tube as well as a dorsoventral ring expression pattern in the MHB organising centre. Currently 19 Wnt family members have been described as well as a family of 10 Frizzled (FRZ) receptor proteins (Rawal et al., 2006). Wnt signalling has a role in proliferation, differentiation, polarity, migration and cell death (Prakash et al., 2006).

Wnt ligands are palmitoylated glycoproteins. There are two modes of Wnt signalling, canonical and non-canonical. During canonical Wnt signalling (Figure 1.6), Wnt binds to FRZ in the extracellular domain and promotes formation of a complex including Dishevelled (DSV) and LRP coreceptors. Upon Wnt binding, DSV disrupts a cytoplasmic protein complex that includes AXIN scaffold, GSK3 β and APC. This complex functions to promote proteosomal degradation of the transcription factor β catenin in the off state, which is present at adherens junctions and also free in the cytosol. Active Wnt signalling therefore represses degradation of β catenin, leaving the transcription factor free to enter the nucleus and bring about transcriptional changes via association with TCF and LEF transcription factors. Active signalling removes Groucho-mediated transcriptional repression of target genes. Negative feedback of this pathway occurs through modulation of GSK3 β , via transcription of molecules involved in repression such as AXIN2 and via secretion of antagonists that either sequester Wnt (e.g. Cerberus and WIF) or bind receptor molecules and occlude Wnt binding, such as DKK1. Non-canonical Wnt signalling is termed the planar cell polarity (PCP) pathway and acts through RHO and ROCK to alter the actin cytoskeleton, cell shape and cellular morphology.

Wnt, along with BMP, has been shown to have a negative effect on early neurulation of the embryo, instead favouring formation of the epithelium (Aubert et al., 2002; Glinka et al., 1997; Nieuwkoop and Nigtevecht, 1954). Contrastingly, after neural induction, Wnt signals have multiple and varied effect throughout neural tube. Wnt1 signals are important for regionalisation in the embryo, for example Wnt1 is able to induce *Engrailed* genes that are crucial for cell survival in the midbrain and the hindbrain (Danielian and McMahon, 1996). Wnt1 signals from the MHB are subsequently critical for dopaminergic neurogenesis in the ventral midbrain (Joksimovic et al., 2009; Prakash et al., 2006) and this is in part by inducing the fate determinant *Lmx1a* (Chung et al., 2009; Friling et al., 2009). Wnt signals in the dorsal telencephalon are also involved in specification and patterning of the cortex and appear to cooperate with BMP signals from the dorsal midline. Importantly, Wnt deficiencies in this tissue lead to loss of dorsal structures such as the choroid plexus (Grove et al., 1998). Different responses to different Wnt ligands are exemplified by the opposing roles of Wnt1 and Wnt5a *in vivo* as Wnt1 has been shown to promote neurogenesis and

proliferation whereas Wnt5a promotes terminal differentiation in the ventral midbrain (Castelo-Branco et al., 2003).

During *in vitro* differentiation, the lack of an active Wnt1 ligand due to its lipophilic nature has hindered investigations into its effect. This has forced the use of recombinant, homologous Wnt3a or small molecule agonists which have been used in place of Wnt1. Wnt5a, on the other hand is a soluble factor. The inhibitory effect of Wnt signalling during neural lineage commitment has been noted in differentiation studies (Aubert et al., 2002) and demonstrated via the beneficial effect of Wnt antagonists during initial neural commitment, such as DKK1 (Watanabe et al., 2005).

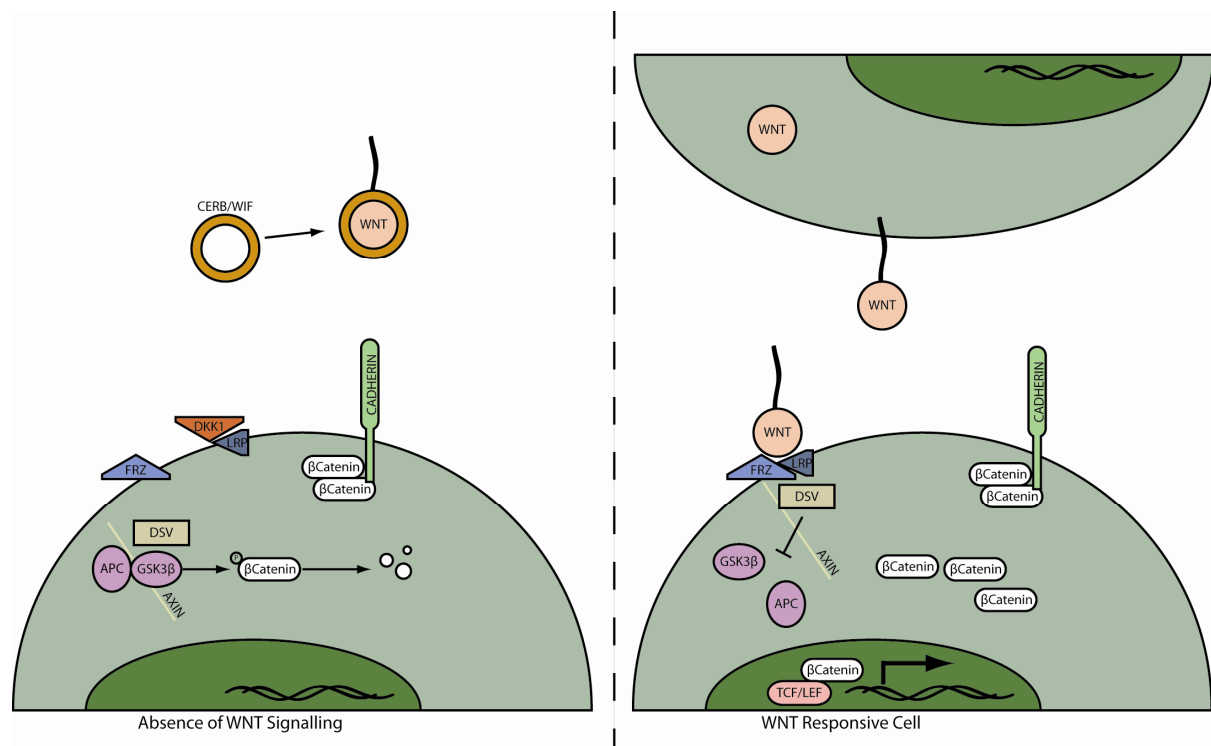


Figure 1.6 **Wnt Signalling.**

Wnt is a secreted morphogen that signals in a paracrine manner. In the absence of Wnt, a protein kinase complex containing GSK3 β and APC phosphorylate cytosolic β catenin, targeting it for proteosomal degradation. Wnt ligands bind via LRP and Frizzled (FRZ), which results in the recruitment of Dishevelled (DSV) which then disrupts the kinase complex leading to cytosolic accumulation of β catenin. β catenin then is free to enter the nucleus and bring about relevant transcriptional events in target genes with TCF and LEF transcription factors. Wnt signalling is antagonised via DKK1-mediated receptor blockade or ligand sequestration via Cerberus or WIF, adapted from (Reya and Clevers, 2005).

1.2.3.4 Retinoic Acid Signalling

Retinoic Acid (RA) signalling is chordate specific and so an evolutionarily recent acquisition. Vitamin A is absorbed from food, processed and released as retinol by such organs as the liver. Signalling primarily occurs in a paracrine manner, whereby RA processing tissues signal to neighbouring cells through exogenous RA.

Retinol, the alcohol form of RA, is present in the bloodstream after being secreted by the liver and is bound by the RA binding protein RBP4. Interactions between RBP4 and the cell surface receptor STRA6 leads to internalisation of retinol into the cytoplasm of responsive cells (Figure 1.7). Retinol is subsequently dehydrogenated to retinaldehyde via alcohol dehydrogenases and retinol dehydrogenases, both sets of enzymes being expressed ubiquitously. Retinaldehyde is then dehydrogenated once more to RA via ALDH1a1-3 enzymes. ALDH1a dehydrogenases show tissue specific expression that can predict RA-responsive tissues. RA then travels to neighbouring cells in an undefined manner, or alternatively can be bound by CRABP binding proteins cell autonomously, where it can translocate to the nucleus and bind two RA receptor families, RARs and RXRs. RA complexed to these receptors, then binds the DNA in RA response elements (RAREs) and alters transcription. For review see (Duester, 2008). Negative regulation of RA signalling occurs as RA is degraded by cytochrome enzymes, such as CYP26b, which also demonstrate discrete expression patterns in tissues negatively regulating RA signals.

The RA antagonists that I use in this project are AGN193109 and UVI3003. AGN193109 is a RAR antagonist whereas the UVI3003 small molecule is a RXR antagonist. The mechanism of these molecules is unknown, although they may function via hindering RA receptor dimerisation and also RA binding and so lead to disrupted RAR/RXR function.

The paracrine nature of RA signalling is demonstrated as mutants for ALDH1a enzymes exhibit cellular defects in adjacent tissues to those normally expressing the ALDH1a gene, e.g. the retina and the ectoderm express ALDH1a1/3 genes during eye development, however defects are witnessed in the periocular mesenchyme in mutant mice (Matt et al., 2005). The expression of *ALDH1a* genes and *Cyp26* genes can hint to RA receptive tissues during development. For example, RA has a caudalising effect in the neural tube and ALDH1a gene expression in the hindbrain demonstrates a competence to respond here. Specifically, RA has been shown to regulate homeodomain *Hox* genes to specify caudal tissues, e.g. the presence of RAREs in the promoter of *Hoxb1*, a gene that defines the hindbrain. However, more anterior in the midbrain, CYP26b expression delineates a resistance to caudalising RA signals (Maden, 2007). RA also has developmental effects in the eye, spinal cord and forebrain.

Differentiation of ESCs *in vitro* has long since used RA to direct neural differentiation from pluripotent cells, although the mechanism is not fully understood (Andrews, 1984). However, new data from Stavridis *et al.* implicates RA in mediating changes in FGF signalling activity to instruct early lineage fate choices (Stavridis *et al.*, 2010). RA has since been used in conjunction with SHH to specify motor neurons which are derived in the ventral spinal cord. The strong caudalising effect of RA together with ventralising SHH signals can drive motor neuron differentiation (Li *et al.*, 2005; Wichterle *et al.*, 2002). Also, RA has recently been implicated in differentiation of dorsal forebrain types (Shi *et al.*, 2012). Finally, RA has been shown to have a strong pro-differentiation effect, favouring terminal differentiation of neural cultures (Chatzi *et al.*, 2011).

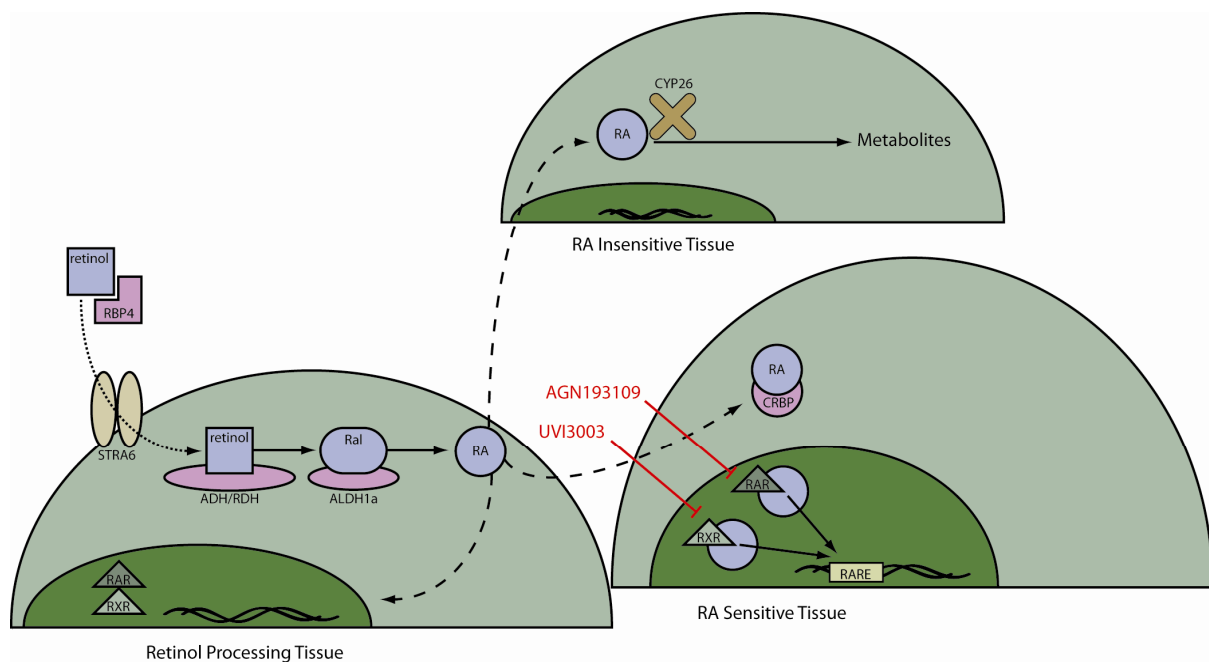


Figure 1.7 RA Signalling.

The RA precursor retinol is present in the bloodstream bound by RBP4 binding protein. RBP4 interacts with the receptor STRA6 to internalise retinol, where two rounds of dehydrogenation occur via ADH/RDH and ALDH1 dehydrogenases to produce RA. RA travels to adjacent cells in an undefined manner bound by CRBP binding proteins, where it can enter the nucleus, be coupled to RA receptors RARs and RXRs and bind promoter regions via RAREs. The antagonists used in this study are AGN193109 and UVI3003 which disrupt RA and RAR/RXR complex formation respectively. RA is degraded in unresponsive cells via cytochrome enzymes such as CYP26b, adapted from (Duester, 2008).

1.2.3.4 Activin Signalling

The TGF β superfamily of signalling molecules include BMP, TGF β , Activin, Nodal and Lefty ligand families. These signal pathways all use SMAD signal transducers; however receptor specificity and SMAD transducer subtype specificity both lead to distinct signal responses. Activin is a dimeric soluble protein ligand. There are 3 Activin and 2 Inhibin mature ligands, synthesised from common precursor proteins. Activin and Inhibin are ligands that signal through ALK2/4/7 TGF β receptors. Although the role for maintenance of pluripotency and mesodermal/visceral endodermal differentiation has been established, we describe in this work the first investigations into a role for Activin in the developing neuroectoderm.

Secreted Activin binds to TGF type II receptors, ActRIIA and ActRIIB (Figure 1.8). Specific type I receptors are then recruited, ALK2, ALK4 and ALK 7. Formation of this receptor dimer leads to type I receptor phosphorylation on intracellular serine/threonine residues. Phosphorylated and activated type I receptor is then able to interact with signal transducer molecules SMAD2/3, leading to their phosphorylation and activation. SMAD2 and SMAD3 are common for Activin and TGF β signalling whereas SMAD1/5/8 are BMP transducers. Once phosphorylated and active, SMAD2/3 bind to the coSMAD SMAD4, translocate to the nucleus and act as transcription factors via conserved DNA binding domains. These binding domains are common on the genome, once every 1024bp, therefore binding partners are required for effective transcriptional alterations. These binding partners include cFos/Jun, ATF2, TFE3, Gli3, SIP1 and most notably FOXH1 and FAST (Wrana and Attisano, 2000). Negative regulation of the pathway is achieved via secreted Activin binding proteins such as Follistatin and competing receptor binding molecules such as Inhibin, which has complementary signalling outcomes. Also, inactive receptors sequester Type I and II receptors and reduce signal potency and SMAD6 acts as a SMAD4 decoy. ERK signalling can inactivate SMAD3 and SMAD is targeted for proteosomal degradation once inside the nucleus, for review see (Massague and Wotton, 2000).

During dual SMAD inhibition we use SB431542 to inhibit Activin/Nodal/Lefty/TGF β signalling. The action of this small molecule is to inhibit the phosphorylation of the Activin-like receptors ALK4, ALK5 and ALK7 (Inman et al., 2002). This functions to inhibit mesendodermal differentiation and to disrupt pluripotency (D'Amour et al., 2005; Xu et al., 2008).

During development, Activin signalling has been shown to be a strong inducer of early mesoderm and endodermal formation (Smith et al., 1990). Concurrently, Activin signalling has a function to inhibit embryonic neural differentiation, witnessed via the presence of the Activin/TGF β inhibitor Follistatin in the Organiser region (Hemmatibrivanlou et al., 1994) and dominant negative

Activin receptors lead to enhanced neural specification (Hemmatibrivanlou and Melton, 1994). Importantly, Type II receptor double knockouts do have some abnormal forebrain development, although the authors of this study describe the phenotype as incredibly complex (Song et al., 1999). Activin and Inhibin have opposing roles, e.g. in the developing gonads with regards to promoting steroidogenesis (Woodruff and Mather, 1995). Activin has been implicated in adult neural tissues, whereby there is a large induction of Activin expression in response to injury in the adult brain (Abdipranoto-Cowley et al., 2009; Foster et al., 2004; Mukerji et al., 2007; Tretter et al., 1996).

In vitro, Activin has been shown to be a critical mediator of pluripotency along with FGF signalling. Activin signalling is important for mouse Epiblast stem cell (epiSC) and hESC maintenance, possibly representing later developmental stages than mESCs (Brons et al., 2007; Tesar et al., 2007; Xu et al., 2008). Inhibiting Activin signalling increases the efficiency of neural lineage commitment (Chambers et al., 2009); possibly analogous to Follistatin expression in the organiser region. Activin is used in mesodermal differentiation protocols due to a defined role in this developmental process (D'Amour et al., 2005). Other TGF β family members have been implicated during *in vitro* differentiation, BMPs regulate epithelial and dorsal neural cell fates in a manner similar to Wnts (Furuta et al., 1997) and TGF β molecules have been used as pro-survival molecules in postmitotic neuronal cultures (Perrier et al., 2004; Schubert et al., 1990).

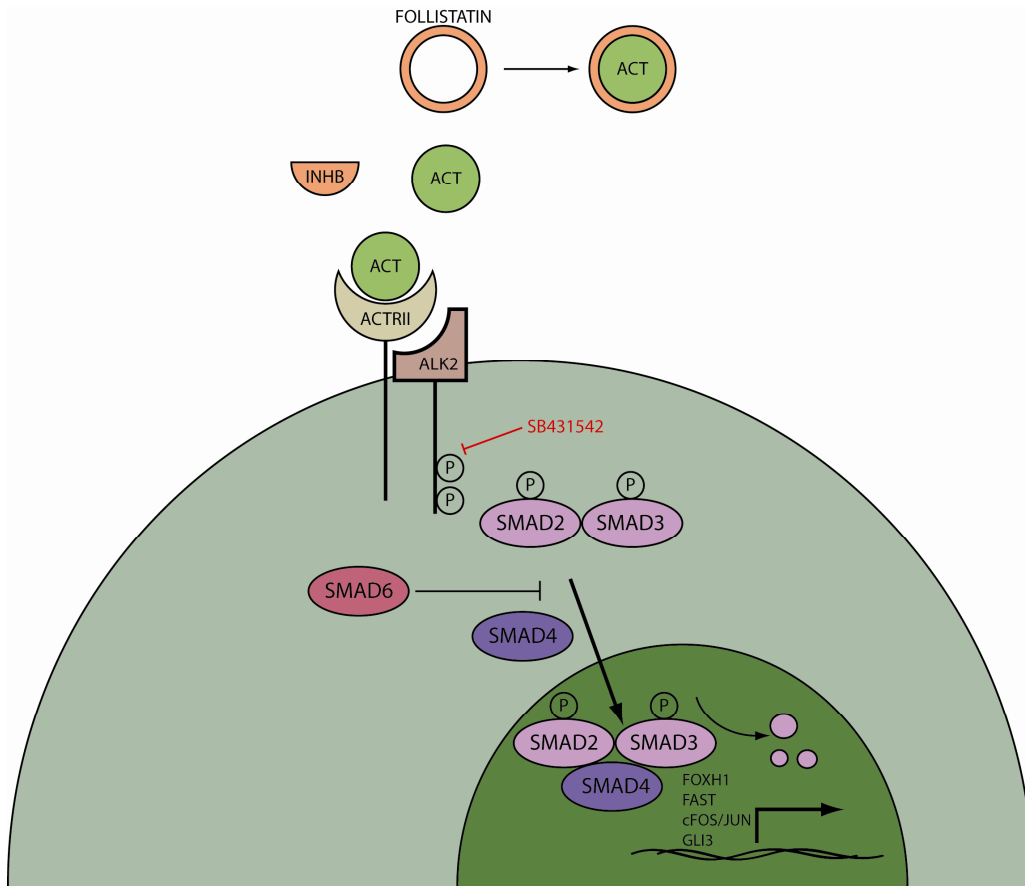


Figure 1.8 **Activin signalling.**

Soluble Activin ligand binds to type II receptor ACTRIIA or ACTRIIB leading to the recruitment of the type I receptor ALK2/4/7. Autophosphorylation of the ALK intracellular domain leads to phosphorylation of signal transducer molecules SMAD2/3, which together with the coSMAD translocate to the nucleus, bind promoter regions of target genes and alter transcription. Activin is antagonised via Inhibin signalling or via sequestration from Follistatin. SMAD6 competes with SMAD2/3 for the coSMAD and SMAD3 is actively degraded in the nucleus. The antagonist SB431542 prevents receptor phosphorylation.

1.2.4 Exogenous Signalling - Relevance to *in vitro* Differentiation

The aforementioned organiser regions (see Chapter 1.2.2) are critical for correct patterning of the developing embryo. This is demonstrated via either ectopic expression or knock-out studies which are able to disrupt normal development. For example, Spemann and Mangold were able to show that grafting a second Organiser (ANR) onto a gastrula-stage embryo was able to induce a second neural tube and body axis (Spemann and Mangold, 1924). Also, mice lacking SHH do not develop ventral structures (Chiang et al., 1996) and ectopic SHH is sufficient to specify ventral tissues in dorsal locations (Wilson and Rubenstein, 2000). These studies show the power of growth factor patterning.

Developmental timing greatly affects the outcome of a signalling cue. The stage of the embryo and the maturity of the responsive cell can lead to discrete responses. For example, early in development RA signalling has a strong caudalising effect, whereas after cells are committed to a given lineage, RA can augment maturation (Maden, 2007; Wichterle et al., 2002). A similar temporal dichotomy is present between SHH proliferative and patterning responses (Fuccillo et al., 2006). Therefore both **spatial** and **temporal** considerations are involved in growth factor application.

Although an exogenous signalling factor elicits transcription changes and a gene expression response, a critical consideration is the cell's intrinsic **competence** to respond. Exogenous signalling requires the presence of certain transcription factors and signal transducer proteins in order to elicit a growth factor response. However, certain genes have an overriding master regulator identity that is sufficient to prime the cell to further signals or be insensitive to inhibitory signals. This is exemplified in forced expression studies. Fate determinants are sufficient to drive directed differentiation to a given cell type, e.g. LMX1a directs midbrain dopaminergic cell differentiation, but only in a context dependent manner (Friling et al., 2009). Reprogramming studies have shown the potential of this technology as certain gene combinations can switch a cell fate directly, e.g. from fibroblast to neuron (Caiazzo et al., 2011; Pfisterer et al., 2011; Vierbuchen et al., 2010). Additionally, fine tuning can be performed via an intrinsic positional identity due to intrinsic cues. An example of this is the OTX2/GBX2 homeodomain signatures. Responses to FGF8 signalling in the tissues are divergent due to the inherent homeodomain expression, grafting the MHB into the diencephalon leads to ectopic midbrain whereas grafting into the hindbrain produces ectopic cerebellum (Marin and Puelles, 1994; Martinez et al., 1991).

Due to the fact that *in vitro* differentiation is a model for *in vivo* development, careful consideration must be given to developmental process. In order to direct differentiation to desired cell types, critical signalling events and cell competence to respond must be considered. For this

reason, organiser region derived growth factor stimuli are modelled and differentiation of PSCs uses such growth factor stimuli to confer position cues to the differentiating cells. Therefore, a close knowledge of the signalling pathways, the developmental outputs and the mechanisms are all required to maintain careful control over differentiation schemes.

1.2.5 Development of the Ventral Forebrain

The forebrain develops from the telencephalon, the anterior most part of the neural tube. For this reason it is under the influence of strong signalling from the ANR (Figure 1.1, Figure 1.3). The major fate determinant of the entire forebrain is the forkhead transcription factor *Foxg1* and this is directly dependent on ANR signals such as FGF8 (Shimamura and Rubenstein, 1997). *Foxg1*, and its downstream target *Six3*, are both critical for regionalisation and growth of all the forebrain structures as *Foxg1* mutant mice exhibit a reduction in size of all of the forebrain structures (Xuan et al., 1996).

The first subregionalisation of the telencephalon occurs at the pallial-subpallial boundary. This boundary demarcates the dorsal pallium, set to be the cortex, and the ventral subpallium, the basal ganglia anlage (Figure 1.9). This regionalisation has been shown to be controlled by dorsal *Pax6* expression and ventral *Gsx2* expression that form a mutually exclusive boundary (Toresson et al., 2000; Yun et al., 2001). These differences are established via differential responses to ventral SHH signals and dorsal Wnt and BMP signalling pathways. As a result, *GLI3*, the negative SHH regulator, has a dorsal high: ventral low expression profile, consistent with ventral SHH activity (Figure 1.9 B).

The subpallium is further subdivided into three domains, named the lateral, medial and caudal ganglionic eminences (LGE, MGE and CGE respectively, Figure 1.9). The LGE develops into the striatum, the brain structure that is responsible for synthesising movement cues from the cortex and transmitting the information through the basal ganglia. The MGE and the CGE are the birthplace of the cortical interneurons that make up 20-30% of the cells in the cortex. These are inhibitory GABAergic neurons that migrate tangentially to the cortex to effect proper glutamatergic cell development, proliferation, migration and, in the adult, control long term potentiation, reinforcing cell output and strengthening signals. The CGE has been described as a caudal expansion of the LGE and the MGE, sharing many common marker genes (Willi-Monnerat et al., 2008). The CGE can be divided into dorsal and ventral tissue (dCGE and vCGE respectively) exhibiting similarity to LGE and MGE accordingly, e.g. MGE and vCGE sharing expression of *NXK2.1* (Figure 1.9). The CGE is the birthplace of discrete subsets of cortical interneurons as discussed below.

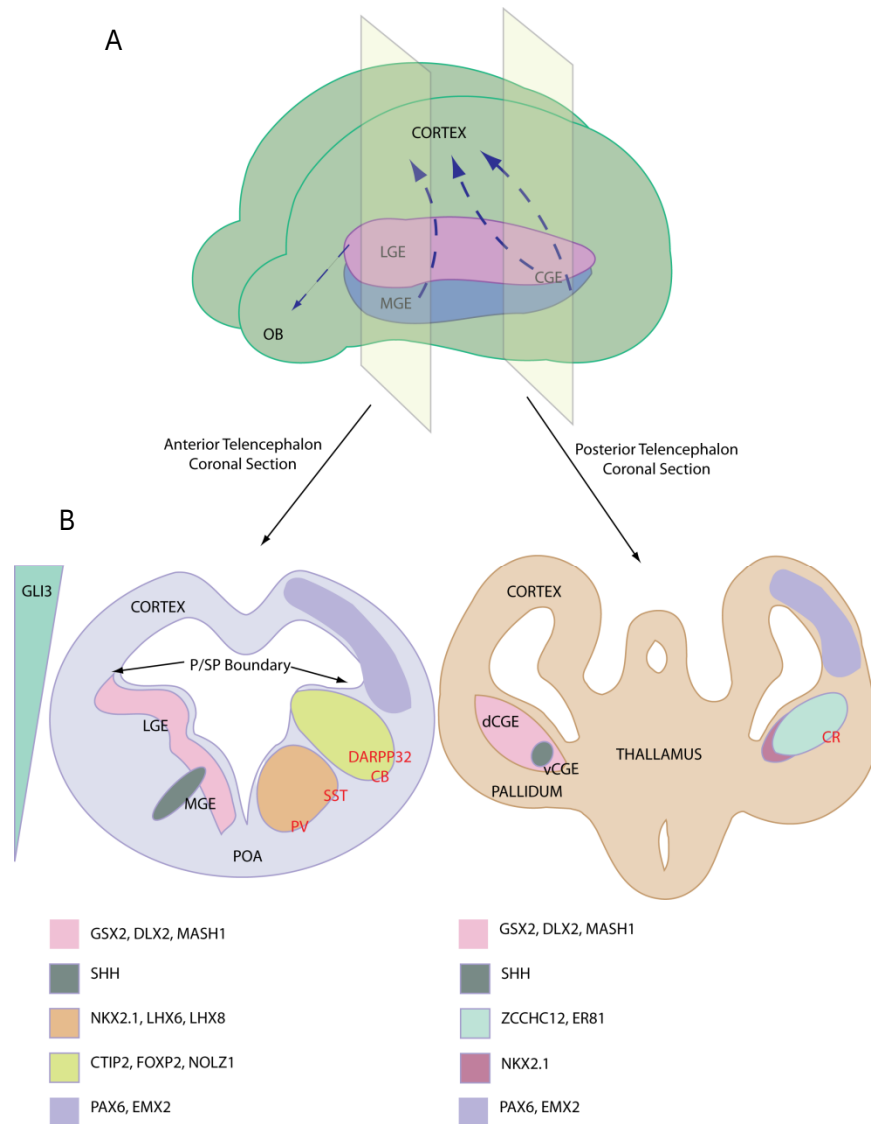


Figure 1.9 Ventral forebrain development.

(A) Depicts the location of the ganglionic eminences in the ventral forebrain. Arrows show the migration of the cortical interneurons to the dorsal tissues (blue) and the possible anterior migration of olfactory bulb interneurons cells from the dorsal LGE (dLGE). Note the theorised similarity between the CGE and the more anterior tissues. (B) Coronal sections of the telencephalon. Ventral SHH signals are abutted by dorsal GLI3 expression (left). This correlates to the boundary between ventral GSX2 and dorsal PAX6 at the pallial-subpallial (P/SP) boundary. GSX2, DLX2 and MASH1 mark the entire subpallial proliferative SVZ (pink), whereas NKX2.1 specifically marks the MGE and the ventral CGE (orange and magenta). MGE specific (LHX6/8), LGE enriched (CTIP2, FOXP2 and NOLZ1), CGE enriched (ZCCHC12, ER81) and pallial genes are highlighted in the coloured domains. RED lettering depicts the birthplace of mature neurons, not the expression domain. LGE lateral ganglionic

eminence, MGE medial ganglionic eminence, CGE caudal ganglionic eminence (d dorsal and v ventral), OB olfactory bulb, P/SP pallial-subpallial, POA preoptic area, CB Calbindin, PV Parvalbumin, SST Somatostatin, CR Calretinin. .

The entire subpallium shares ventricular zone/subventricular zone genes that are involved in patterning and proliferation. *Gsx2* is one of the first genes witnessed in the ventral forebrain and, as discussed above, promotes subpallial fate via excluding dorsal *PAX6*. *Gsx2* subsequently shares an expression pattern with *Dlx1/2* genes that are critical for proper GABAergic patterning and later migration of cortical interneurons (Long et al., 2009a). The neurogenesis gene *Mash1* (also called *Asc1*) has been shown to work in an independent pathway to *Dlx1/2* to promote development of GABAergic cells in the ventral forebrain (Long et al., 2009b).

The MGE has unique expression of *Nkx2.1* that is not present in the LGE or the dCGE. *Nkx2.1* expression is initiated by SHH signalling (Fuccillo et al., 2004) and subsequently shows a mutual dependency with SHH signalling (Sussel et al., 1999). *NKX2.1* further specifies cortical interneuron fate via promoting transcription of GABAergic interneuron fate determinant genes *Lhx6/8* (Du et al., 2008). The MGE and the cortical interneurons derived herein appear to be dependent on SHH and *Nkx2.1* expression as *Nkx2.1* knockout mice exhibit a failure to produce MGE derived cortical interneurons, suggesting a direct specification role (Anderson et al., 2001; Sussel et al., 1999; Xu et al., 2004). Another signalling pathway, FGF8, appears to be crucial for cortical interneuron determination, as FGF receptor knockout mice demonstrate a reduced *Shh* and *Nkx2.1* expression pattern (Storm et al., 2006).

Cortical interneurons are divided into subfamilies depending upon marker gene expression, birthplace data from fate-mapping experiments and isochronic-homotopic transplants (Figure 1.10). The major MGE derived interneurons are Parvalbumin (PV)-expressing and Somatostatin (SST)-expressing cells and these are dependent on *NKX2.1* expression; shown by their absence in mutant mice (Anderson et al., 2001). *NKX2.1* induces *LHX6* which is expressed in migrating MGE-derived cortical interneurons (Du et al., 2008). The different birthplaces of these two populations within the MGE have suggested differential dependencies on SHH whereby higher doses specify SST interneurons *in vivo* (Xu et al., 2010). As an aside, striatal interneurons are derived in the MGE; these are *Shh/Nkx2.1/Lhx8* dependent and typically are cholinergic rather than GABAergic (Fragkouli et al., 2009; Zhao et al., 2003).

The dCGE is the birthplace of the third major cortical interneuron subtype, Calretinin (CR) expressing cells. The dCGE appears to be specified independently from SHH signalling as SHH and *Nkx2.1* knockout mice either show an enlarged CGE or increased CR neurons (Sussel et al., 1999; Xu et al., 2010). A small number of genes have been described that can distinguish the CGE from other tissues, these include *Zcchc12* and *Er81* (Cambray et al., 2012; Willi-Monnerat et al., 2008). Other smaller subsets of cortical interneurons may be SHH/NKX2.1-dependent in the ventral CGE (vCGE) such as Neuropeptide Y (NPY) or Reelin (RLN) expressing interneurons, however, it cannot be discounted that interneurons are merely migrating through the vCGE rather than being specified therein.

In their target tissues, cortical interneurons can have varied location and morphology depending on their relative subgroup. The interneurons are enriched in certain layers of the cortex as well as cortical domains, although subtypes appear at low levels distributed throughout the cortex. The interneurons are morphologically diverse being stellate, basket-cells, bipolar or chandelier-cells forming complex local circuits. Calretinin cells, which are focused on in this thesis, are typically bipolar, bitufted and vertically orientated.

The LGE is the birthplace of the striatum and developing cells migrate radially to mature into GABAergic projection neurons termed Medium Spiny Neurons (MSN). MSNs constitute 95% of the striatum and these cells function to relay voluntary movement signals from the cortex to the globus pallidus and the substantia nigra. During development, enrichment of marker genes such as *Ctip2*, *Foxp1/2* and *Nol21* distinguish the LGE from the MGE (Arlotta et al., 2008; Chang et al., 2004; Takahashi et al., 2003a), as well as a lack of *Nkx2.1* expression.

Development of the LGE appears to be dependent on SHH signalling as expression of all of the LGE and MGE specific genes are lost in SHH null mutants in a stage dependent manner (Chiang et al., 1996). Additionally, *in vitro* differentiation techniques demonstrate that SHH signals are sufficient to generate MSNs *in vitro* (Aubry et al., 2008; Li et al., 2009). However, recent studies suggest that SHH in the forebrain merely has a role to inhibit dorsal signals via inhibiting GLI3, as compound mutants for SHH and GLI3 show a restored ventral phenotype (Aoto et al., 2002; Rallu et al., 2002). Later stage knockout of the SHH pathway also shows little effect on the LGE-derived tissue compared with MGE tissues (Machold et al., 2003; Xu et al., 2010).

Mature MSNs are characterised via a spiny and multipolar morphology. The marker gene *Darpp32* is widely used as a specific marker for MSNs; a protein that functions in responding to dopaminergic inputs (Svenningsson et al., 2004). Within the striatum, the MSNs are divided into two

pools with different projection targets and different expression profiles. One subset, the indirect pathway, projects to the globus pallidus and expresses Enkephalin and DRD2. Certain transcription factors have been implicated in development of this subtype, e.g. IKAROS (Agoston et al., 2007). The second group of MSNs, the direct pathway, projects to the substantia nigra and are typified via expression of Substance P, Dynorphin and DRD1. The overall outcome of the two projections on the movement circuitry of the basal ganglion is negative signals from the direct pathway and positive signals from the indirect pathway. Thus, a fine balance is required and disruption can lead to the diseased state. The striatum is organised into patch and matrix which may be relative to innervating dopaminergic terminals (no organisational relation to MSN subtypes), and the marker Calbindin can be used to mark the matrix neurons specifically (Gerfen, 1992).

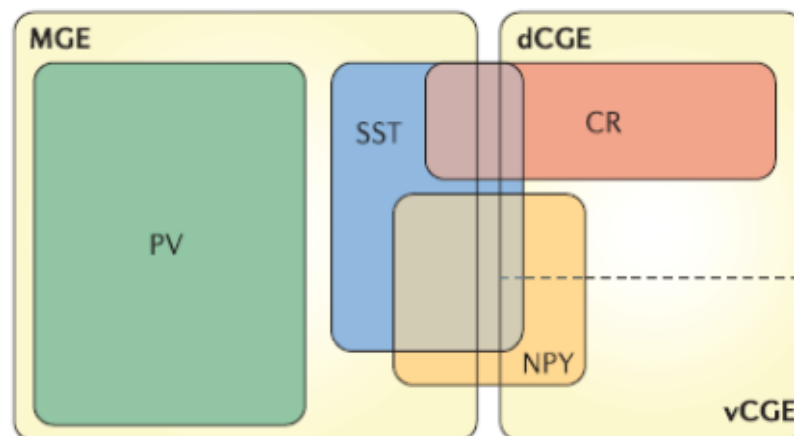


Figure 1.10 **Cortical Interneuron Progenitor Pools.**

Based on expression of marker genes (NB no functional significance) cortical interneurons can be divided into three or more subsets. These subsets have discrete birthplaces in the ventral telencephalon and this presumably correlates with different growth factor dependencies. Parvalbumin (PV) and Somatostatin (SST) are derived from the NKX2.1-positive MGE, whereas Calretinin (CR) interneurons are mostly derived from the NKX2.1-negative dCGE. Neuropeptide Y (NPY) and other smaller subsets have been less well defined (Wonders and Anderson, 2006)

1.2.6 Development of the Ventral Midbrain

The dopaminergic cells of the ventral midbrain (vMB) form from the ventral part of the mesencephalon (Figure 1.1 B). This represents the most posterior part of the *Otx2* homeobox protein expression domain. Interactions between FGF4 signalling and the OTX2/GBX2 boundary at the isthmus establishes the MHB as a signalling centre that is in close proximity to the ventral mesencephalon (Chapter 1.2.2) (Ye et al., 2001). Subsequent expression of the mesencephalic *En1/2* genes is dependent on the MHB (expressing FGF8 and Wnt1) and *En1/2* expression has been shown to be critical for vMB dopaminergic neurogenesis and survival (Alberi et al., 2004; Simon et al., 2001). The ventral part of the presumptive midbrain is under control of MHB-derived FGF8/Wnt signalling and also floorplate derived SHH signals.

In the majority of the neural tube the ventral floorplate is non-neurogenic, instead functioning in axon guidance and growth factor secretion (Charron et al., 2003; Placzek and Briscoe, 2005). In the midbrain, however, the active Wnt signalling from the nearby MHB has been shown to interact with SHH and specify the floorplate to a neurogenic fate (Joksimovic et al., 2009; Ono et al., 2007).

The progenitors in mesencephalic floorplate express key transcription factors that have been shown to be crucial for dopamine neurogenesis (Figure 1.1 D). *Foxa1/2* are SHH responsive genes that interact with neurogenesis genes such as *Ngn2* to control cell proliferation in the vMB as well as having a role in later dopaminergic specification (Ferri et al., 2007). *Lmx1a* has been shown to be necessary and sufficient to drive dopaminergic differentiation and this gene is responsive to SHH and Wnt signals (Andersson et al., 2006; Chung et al., 2009; Friling et al., 2009), demonstrated via expression of LMX1a in a complimentary manner to Wnt1 (e.g. in the roofplate). Cooperation between the genetic networks that centre on LMX1A and FOXA2 are thought to be central to the development of this vMB dopaminergic population; representing the crux of exogenous SHH and Wnt signalling (Chung et al., 2009; Lin et al., 2009). Other critical genes may be expressed in a layer specific manner, e.g. *Dmrt5* has a role in commitment and is expressed in the intermediate zone (Gennet et al., 2011).

The midbrain dopaminergic neurons (mDA) express a number of classical marker proteins. Tyrosine hydroxylase (TH) is rate limiting enzyme in the synthesis of dopamine and so a marker of dopaminergic neurons synthesising the neurotransmitter. Coexpression of TH with FOXA2, LMX1A and PITX3 (a gene that is required for mDA development (Maxwell et al., 2005; Nunes et al., 2003)) is able to demonstrate a midbrain subtype of dopaminergic cells (figure 1.1 D). This is in contrast to

other dopaminergic populations in the forebrain where TH is coexpressed with alternate markers, e.g. NKX2.1 (hypothalamus) or ISL1 (ventral thalamus) (Yan et al., 2005).

Two populations of mDA arise from the development of the midbrain floorplate, namely the substantia nigra (SN) and the ventral tegmental area (VTA) (Bjorklund and Lindvall, 1984). SN dopaminergic neurons project to the striatum and control initiation of movement and postural reflexes. The VTA dopaminergic population project to the limbic region of the prefrontal cortex and is involved in reward behaviour, cognition and motivation. Thus, degeneration of these areas has effects in these behavioural pathways.

1.3 Neurodegenerative Diseases

Stem cell based therapy for neurodegenerative diseases (see Chapter 1.4-1.6) focus on replacing a cellular deficiency. Replacing a cellular deficiency via cell therapy, grafting of foetal tissue, and regenerative medicine (stimulating recovery *in situ*) hold promise for currently incurable diseases, where surgical, medicinal and stimulatory approaches treat the symptoms but do not reverse the disease progression. In this thesis, I shall investigate three cell types that are implicated in neurodegenerative diseases, namely midbrain dopaminergic neurons that degenerate in Parkinson's disease, striatal projection neurons that degenerate in Huntington's disease and cortical interneurons that are involved in epilepsy, autism and schizophrenia.

1.3.1 Interneuronopathies

Cortical interneurons comprise 20-30% of the cells in the cortex and function to modulate cortical cell maturation, plasticity and output. Interneuronopathies are diseases resulting from a loss of interneurons and have wide ranging symptoms. A reduction of inhibitory interneurons has been theorised to lead to over-activity of the excitatory cortical neurons, and this has been associated with epilepsy and seizure in human patients (Kitamura et al., 2002). Also, autism has been linked to a reduction in GABAergic cortical interneurons, a disease characterised by frequent seizures (Hussman, 2001). Other psychiatric disorders such Tourette's syndrome, bipolar disorder and schizophrenia have also been associated with cortical interneuron deficits (Lewis, 2000).

Although the study of transplanted cortical interneurons is relatively new, there is some evidence that cell therapy may benefit the diseased state. Interneurons are born in the ventral forebrain and must undertake a major tangential migration to the dorsal cortex. In a similar manner, grafting of naive cortical interneuron progenitors is accompanied by an extensive migration capacity away from the site of grafting. This is in contrast to other forebrain cell types and promotes interneurons as a good candidate for cell therapy (Wichterle et al., 1999). Investigations into the effect of grafted interneurons on the spread of seizures demonstrated that grafted MGE-derived interneurons reduced the spread of seizures in the rodent brain (Alvarez-Dolado et al., 2006; Baraban et al., 2009; De la Cruz et al., 2011; Zipancic et al., 2010).

The extensive migratory capacity and the ability to modulate endogenous activity promote cortical interneurons as a positive source of material for putative cell replacement therapies. Many seizure-associated diseases can be pharmacologically treated; however, the subset that is drug-resistant represents an important target for cell-based therapy. A lack of understanding into interneuronopathies as well as the poorly defined nature of the symptoms means that clinical trials of

foetal tissue is yet to be applied. However, the benefits of cell-based therapies are currently being investigated in model organisms.

1.3.2 Huntington's disease

The early stages of Huntington's disease (HD) is characterised by the preferential degeneration of GABAergic striatal projection neurons (MSNs) (Reiner et al., 1988). This leads to choreatic movements, psychiatric impairments and dementia. HD typically results in fatality 17 years after diagnosis. HD is an autosomal dominant genetic disorder, characterised by amplification of a CAG trinucleotide repeat in the Huntington gene leading to misfolded protein and toxic aggregates (Macdonald et al., 1993). There is currently no cure for HD.

Intrastriatal grafting of foetal ventral forebrain tissue (both LGE and MGE-derived) into rodent (Peschanski et al., 1995) and primate (Kendall et al., 1998; Palfi et al., 1998) HD models have restored neural circuitry and demonstrated alleviation of disease symptoms. Based on the success of these *in vivo* models, clinical trials were attempted using primary human foetal forebrain, grafted into HD patients (Hauser et al., 2002; Keene et al., 2007; Reuter et al., 2008; Rosser et al., 2002). Long lasting benefits were witnessed in a small percentage of these patients; however, the results were varied and suggested a need for standardisation and further refinements (Bachoud-Levi et al., 2000a; Bachoud-Levi et al., 2000b; Bachoud-Levi et al., 2006). Nevertheless, the fact that 5 patients out of 22 demonstrated functional improvement and increased striatal metabolism calls for further studies to validate, optimise and take the technology forward.

The major drawback of the proof-of-principle investigation is the observation that the beneficial effect of the graft is temporary, as the improvements were seen to diminish after 2 years (Bachoud-Levi et al., 2006). Therefore, there may be a need for neuroprotection to avoid graft rejection or disease contraction in the grafted cells (Kordower et al., 2008). Also, investigators are not certain whether to attribute benefit to replacement of striatal projection neurons or enhancing cell support via striatal interneuron grafting, as current strategies utilise MGE and LGE tissue in the grafts. Therefore, further extensive investigations are called for.

Stem cells represent an inexhaustible source of neurons for such studies (see Chapter 1.4) and cell replacement therapies may provide a novel curative therapy to this currently incurable and fatal disease. It should be noted that as an inherited monogenic disease, other techniques to reduce the disease may be more relevant than cell replacement in the future such as gene therapy or preimplantation diagnosis (PGD) to abrogate the disease completely.

1.3.3 Parkinson's disease

Parkinson's disease is a neurodegenerative disease characterised by the preferential loss of the nigrostriatal dopaminergic system. There are a number of different genes that predict Parkinson's disease prevalence; however the disease mechanism remains poorly understood. Lewy bodies, made up of α -synuclein aggregates, are typically seen in the cells of Parkinson's disease patients prior to degeneration. Parkinson's disease leads to a loss of motor control manifested as bradykinesia, hypokinesia, resting tremors and rigidity (Lang and Lozano, 1998). Parkinson's disease is estimated to affect 4 million people worldwide (von Bohlen und Halbach et al., 2004). Palliative approaches to the symptoms include drug-based dopamine replacement therapies, such as L-DOPA administration, electrical deep brain stimulation and stereotactic surgery. However, there is currently no cure to the disease.

Cell-based therapies using foetal-derived dopaminergic progenitor cells have proven to reverse the disease in human patients. The goal is to replenish baseline dopamine levels in the striatum to control stimuli for movement from the cortex. Graft derived neurons have been shown to integrate into the host brain, survive for up to 10 years, reduce akinesia and even allow patients to withdraw from L-DOPA treatment (Piccini et al., 1999). Very successful open label trials in the 1980s (Lindvall et al., 1989; Madrazo et al., 1988) were followed by less successful double-blind placebo trials in the 1990s (Freed et al., 2001), demonstrating age related outcomes. The disparities in the results are due to contrasting patient disease state, graft tissue preparation and transplantation technique and indicate the requirement for transplant optimisation (Torres et al., 2007).

The success for a subset of these trials and the continued benefit of foetal cell grafting in rodent Parkinson models (Torres et al., 2008) provide a proof-of-principle for cell therapy in Parkinson's disease. The fact that foetal tissue is limited (as more than three embryos are required for one grafting procedure) and the delicate ethical background for this cell source calls for an alternative source of tissue. Trials in model organism suggest that striatal grafting of PSC-derived dopaminergic cells is able to reverse disease progression (Yang et al., 2008). Therefore, subsequent to optimisation of patient selection, graft procedure and ensured safety of grafts, PSC-derived dopaminergic cells provide hope for Parkinson's disease treatment in the near future.

Grafts are typically transplanted into the striatum, the target tissue of the dopaminergic neurons. This obviates any dendritic inputs to the graft cells as in a healthy situation and essentially limits this technology as a novel source of dopamine. Future studies may investigate grafting to the substantia nigra and reinnervating the striatum to completely replace the lost circuit. Foetal grafts

have also suffered from contaminating cell types, such as serotonergic cells causing graft-induced dyskinesias. Finally there is evidence that grafted tissue can contract the diseased state, as Lewy bodies have been witnessed in the graft cells of post-mortem patients (Kordower et al., 2008).

1.4 Embryonic Stem Cells

The 5-6 day old human embryo is termed the blastocyst and contains around 100 cells. At this stage there are two cell types, the trophoblast cells that progress to form the extra-embryonic cells (e.g. the placenta) and the inner cell mass (ICM), which form all the cells of the embryo. Blastocysts that are supernumerary to the IVF procedure can be donated to science whereby human embryonic stem cells (hESCs) can be derived from the ICM of these embryos. The culture of hESCs utilises the fact that ICM cells divide symmetrically to increase their numbers, while maintaining the potential to form all the cell types of the embryo. 'Capture' of the cells at the ICM stage thus confers the features that define hESCs *in vitro*;

1. The ability to self-renew – i.e. divide symmetrically to produce more ESCs and increase the culture population.
2. The cells are pluripotent – i.e. they retain the ability to develop/differentiate into any cell type of the embryo (although they are not totipotent as they cannot form trophoblast cells).

These ESC characteristics may be tested as the cells demonstrate spontaneous differentiation to all three germ lineages, can be clonally propagated, form teratomas when injected into a host and can form chimaeras and contribute to the germ line when incorporated into a host ICM (model organism only).

Since their first derivation in 1998 by James Thomson (Thomson et al., 1998), hESCs have been routinely cultured on mouse embryonic feeder cells (MEFs) in combination with exogenous FGF2 signals. This is in contrast to mouse ESCs (mESCs) which are dependent on LIF signalling and serum (Smith et al., 1988). This difference may relate to differing stages of development that the ESCs have been 'captured' from, or alternatively simply due to species differences. However, the progress made with mESCs into chemically defined, feeder-free culture conditions and an enhanced ground state via the 2iL inhibitor LIF approach (Silva et al., 2008) has not been reproduced as effectively in human cells. Culture of hESCs on MEFs is dependent on undefined factors secreted by the MEFs to maintain pluripotency. Certain studies have begun to remove the need for MEFs and utilise chemically defined media, however, these are expensive and have yet to be widely employed (Beattie et al., 2005; Xu et al., 2001; Xu et al., 2005; Yao et al., 2006).

Pluripotent stem cells (PSCs) have been derived from sources other than ICM embryos. These include adult cells that have been reprogrammed via somatic cell nuclear transfer (Wakayama et al., 1998; Wilmut et al., 2002) whereby an oocyte is able to reprogram an adult differentiated nucleus. Parthenogenesis forms PSC lines from unfertilised ova; these are homozygous for HLA and

so may be grafted to a range of patients due to reduced rejection issues. Finally, genetic manipulation can produce induced pluripotent stem cells (iPSCs) via forced expression of pluripotency factors, whereby mature cells are reprogrammed to PSCs (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). These reprogrammed cells offer a new source of PSCs that can include disease-carrying genomes and are also readily accessible. Another reprogramming regime utilises cell fusion of differentiated cells with pluripotent cells to form heterodikaryons. These are not-suitable for differentiation studies due to their genomic instability and tetraploid nature.

PSCs can be stably maintained in culture for long periods where the karyotypic stability and the lack of contaminants such as *Mycoplasma* must be monitored. The self-renewal and pluripotency properties allow hESCs to be proposed as an alternative source of tissue for cell replacement studies.

1.5 Neural Differentiation

Employing PSCs for neural differentiation has a number of applications. Firstly, the normal developmental process can be closely examined in an accessible manner, e.g. human development is ethically and logistically difficult to follow and hESC differentiation provides a model for the earliest developmental steps. Comparisons can then be made to model organism development, e.g. marker gene expression and dynamics (Nelander et al., 2009; Zhang et al., 2010). Secondly, disease-specific ESCs (and especially iPSCs) offer a tangible model for disease aetiological testing, when compared to wild-type scenarios, e.g. many human disorders cannot be modelled in animals or cannot be accessed due to early developmental stages. Thirdly, PSC-derived neurons (and other cell types) offer an unlimited supply of material for toxicology testing for new drugs and high throughput drug screening for novel medicines. *In vitro* PSCs and their differentiated progeny are amenable to genetic manipulation, enabling reporter cell lines, selectable marker cell lines and investigations into forced fate determinant expression. Lastly, PSC-derived cell progeny can be used for cell-replacement therapies, replacing a cellular deficiency in a diseased state (see Chapter 1.3).

Neural differentiation can be promoted *in culturo* via a number of different protocols. The process aims to simulate embryonic conditions that promote certain fate choices. Using information from development, these conditions can be mimicked in order to utilise genetic networks that normally bring about neural fate choices (see Chapter 1.2).

1.5.1 Embryoid Body Formation

After withdrawal of feeders and growth factors associated with pluripotency, PSCs can be grown in floating 3-dimensional aggregates termed embryoid bodies (EBs). These EBs recapitulate embryo development as the normal processes therein are thought to be imitated. EBs can be formed naturally via disassociating adherent ESC cultures or alternatively via forced aggregation techniques increase EB size homogeneity, e.g. hanging droplet-based or centrifugation-based aggregation (Burridge et al., 2007; Keller, 1995). Once committed, EBs are then plated onto the culture dish to enable better visualisation and reduce 3-dimensional limitations such as growth factor deliverance and nutrient exchange.

Exogenous factors are added to the EBs to upregulate the relative proportion of neural tissue in the inherently heterogeneous aggregate. In a manner similar to the Nieuwkoop centre, described in Chapter 1.2.1, FGF2 signals promote the generation of organiser activity and subsequent neural tissue. Zhang et. al. demonstrate that FGF2 is sufficient to derive neural cells from human EBs and, after morphology-based isolation, can produce neural cultures with 96% purity (Zhang et al., 2001).

Retinoic acid is also able to promote neural fate determination in mESC-derived EBs (Bain et al., 1995; Li et al., 1998). These studies demonstrated the first example of chemically defined differentiation media, using N2B27 based media, and also made use of selectable drug-resistance markers to reduce contaminating cell types.

In recent years, this technology has been extensively used by the laboratories of Sasai and Zhang to generate multiple neuronal cell types from hESCs. Based on the study demonstrating serum-free EB (SFEB) neural differentiation using knockout serum replacement, this technique has been employed to derive anterior neural tissues (Watanabe et al., 2007) such as pituitary (mouse) (Suga et al., 2011), retina (Osakada et al., 2009), cortex and striatum (Eiraku et al., 2008; Li et al., 2009), Purkinje cells (mouse) (Muguruma et al., 2010) and midbrain dopamine cells (Yang et al., 2008).

EB differentiation benefits from enabling simple and effective neural induction. The ability to use chemically defined media and potential bioreactor-based scalability achieved from floating culture method makes this technique attractive for large-scale pharmacological studies and high throughput screening. Drawbacks of EB-based differentiation include heterogeneity within and among EBs. Also, the 3D nature of the EBs means that there is a great deal of unknown signalling activity within the aggregate, removing some control from the researcher.

1.5.2 Coculture Differentiation

Stromal cell lines have been shown to instruct ESCs to differentiate towards neurons and even neural subtypes when grown in coculture. This inductive capacity of the stromal cells remains undefined and has been termed stromal-derived-inducing-activity (SDIA).

The PA6 cell line derived from mouse calvaria bone stromal cells can induce up to 96% neural colonies in a serum free media from mouse ESCs. ESCs alone rarely differentiated to neural tissues, demonstrating that SDIA was actively promoting neurogenesis. This has been shown to be contact dependent, as PA6-conditioned media does not contain SDIA. SDIA appears to be present on the cell surface as PFA fixed PA6 cells maintain neural inductive capacities (Kawasaki et al., 2000). Interestingly, PA6 cell neural differentiation of mESCs and hESCs leads to an overrepresentation of dopaminergic cells and so is of use to the Parkinson's disease research community (Zeng et al., 2004). This lineage selection is in the absence of exogenous growth factors and purely dependent on SDIA action. Nonetheless, progenitor cells do remain competent to respond to morphogenetic conditions as BMP administration can lead to neural crest differentiation, a dorsal cell type (Kawasaki et al., 2002). Other cell lines that contain neurogenic SDIA include the MS5 and S2 cell lines. These were shown to induce neurogenesis in primate and human ESCs and rosettes picked

from the coculture were amenable to further patterning via exogenous growth factor administration (Perrier et al., 2004).

Coculture differentiation techniques are advantageous due to the simple and cheap differentiation scheme. The specific differentiation towards dopaminergic neurons offers a low-cost differentiation alternative without use of expensive recombinant proteins. The drawbacks of coculture experiment include the use of animal-derived feeder cells. These obviate the potential for clinical applications due to failure to comply with Good Manufacturing Procedures (GMP). Even though the media may be chemically defined, the interactions between stroma and ESC are undefined with potential variability. Also, this technique may be unpredictable and the requirement for manual picking of neural rosettes in some protocols increases labour-intensiveness. Nonetheless, the action of SDIA raises pertinent mechanistic questions and may provide useful insights into neural-based and lineage-directed differentiation.

1.5.3 Monolayer Differentiation

Monolayer differentiation is the process of differentiating ESCs as a homogenous sheet of cells, adherent to the culture dish. Theoretically, the entire monolayer senses a similar condition throughout the culture, e.g. exogenous growth factor concentration, and can be manipulated with a greater degree of control by the researcher.

Neural induction is initiated after withdrawal of self-renewal conditions. The initial mESC monolayer differentiation experiments demonstrated a requirement on FGF signalling, potentially in a manner similar to the inductive action of the Nieuwkoop centre, as discussed in Chapter 1.2.1. These experiments were carried out in chemically defined media (without serum) and enable close investigations into single growth factors (Ying et al., 2003).

Recent advances on hESC monolayer differentiation have focused on SMAD inhibition. The BMP inhibitor Noggin has been shown to augment neural conversion of hESCs, potentially in a manner similar to Organiser-induced neurogenesis (Lee et al., 2007). Inhibition of other TGF β signalling via a SMAD2/3 small molecule inhibitor also promotes neural differentiation, possibly analogous to Follistatin expression in the Organiser (Smith et al., 2008). Combining BMP inhibition and TGF β inhibition, described by Chambers et. al. lead to a highly efficient, robust and synchronous differentiation to primitive neuroectoderm (Chambers et al., 2009). These cells produced patternable neural progenitors and exhibited an inherently rostral identity (Fasano et al., 2010). This novel protocol has been employed in numerous directed differentiation schemes already, such as neural crest cells (Lee et al., 2010), floorplate tissue (Fasano et al., 2010) and astrocytes (Emdad et al., 2012).

Monolayer differentiation enables a high degree of user-control during the differentiation process. Homogeneous cellular environments and forced exit from pluripotency convey replicable, homogeneous and robust experimental regimes, enabling reliable investigations into individual factors. The reduction of 3D aggregates may also accelerate differentiation compared with SFEB as remote proliferative niches are reduced. Space constraints may limit the scalability of this technique, due to required adhesion, and the cost of recombinant growth factors represents a drawback. However, media component definition and independence from animal-derived products permit future GMP uses for monolayer differentiation.

1.6 Directed Differentiation

1.6.1 Differentiation towards Cortical Interneurons

As discussed in Chapter 1.2.3, cortical interneurons are generated in the ventral forebrain before migrating to their dorsal target tissue. The MGE has been shown to be under the influence of strong SHH signals and as such, *in vitro* protocols have attempted to mimic this environment to specify cortical interneurons from PSCs.

A number of studies into the dorso-ventral axis identity of forebrain precursors *in vitro* have focused on SHH versus Wnt/BMP signals. Higher concentrations of SHH (or small molecule agonists) are able to generate *Nkx2.1* expressing ventral progenitors (MGE-specific) derived from mouse and human ESCs (Danjo et al., 2011; Li et al., 2009). Goulburn et. al. generated an *NKX2.1*-driven GFP reporter hESC cell line (Goulburn et al., 2011). After EB differentiation in optimal concentrations of RA and FGF2, sorted cells were able to differentiate into cortical interneurons *in vitro* and *in vivo* without further exogenous signalling, demonstrating the power the *NKX2.1* genetic network.

Maroof et. al. created an *Lhx6* reporter mESC line (Maroof et al., 2010). EB-based differentiation was supplemented with SHH to produce *Lhx6*-positive progenitors (Figure 1.11). The sorted cell progeny was able to migrate substantially in the host brain and differentiate into SST and PV expressing cortical interneurons *in vivo*, demonstrating the MGE-like character of these cells.

Maisano et. al. use a mESC monolayer differentiation protocol with SHH agonist to produce cortical interneuron progenitors and postmitotic neurons in chemically defined media (Maisano et al., 2012). When precursors were transplanted into a mouse model for temporal lobe epilepsy, the cells integrated effectively and demonstrated functional electrophysiological properties. This raises the potential for cell based treatment of such seizure-based disorders. Further work is required to assess an ability of these cells to reverse epileptic symptoms; however, there is growing evidence that grafting of *in vivo* derived MGE progenitors are able to reduce the spread of seizures in the mouse brain (De la Cruz et al., 2011).

Calretinin cortical interneurons are generated in the CGE, an *Nkx2.1*-negative tissue. Knocking out *Nkx2.1* *in vivo* did not reduce the number of Calretinin interneurons, suggesting an alternative, SHH-independent and undefined specification pathway for CGE derived cortical interneurons (Xu et al., 2010). Differentiation of Calretinin-positive cells has not been described and thus, further investigation into the specification of the CGE *in vivo* is required to be able to direct differentiation towards a Calretinin-positive cortical interneuron fate.

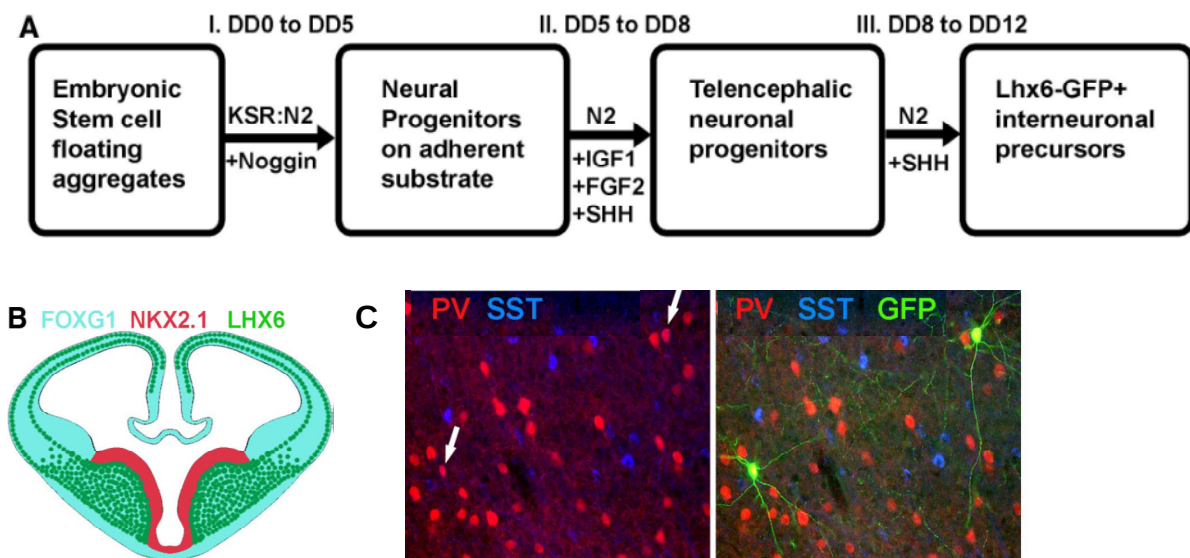


Figure 1.11 **Maroof et. al. protocol for generating cortical interneurons.**

Maroof et. al. utilise an EB-based protocol followed by SHH exposure to upregulate LHX6-driven GFP reporter expression for FACS sorting (A). These progenitors represent the migrating interneuron progenitors (B). The sorted cells were then able to differentiate in vivo into postmitotic cortical interneurons expressing markers of different subtypes including SST and PV (C) (Maroof et al., 2010).

1.6.2 Directed Differentiation of Striatal Projection Neurons

Much attention has been focused on MSNs of the striatum due to their degeneration in Huntington's disease. Putative cell replacement therapies and drug screening strategies would benefit from an effective differentiation protocol to efficiently derive MSN *in vitro*. Groups that have investigated telencephalic dorso-ventral patterning have described a precise dosage of SHH - with or without the dorsal Wnt inhibitor DKK1 - that can preferentially induce LGE-character (Danjo et al., 2011; Li et al., 2009).

Aubry et. al. utilised the MS5 coculture method to promote neurogenesis of hESCs and subsequently stimulated MSN directed differentiation via SHH and DKK1 treatment (Figure 1.12) (Aubry et al., 2008). This protocol robustly generated MSN. After transplantation into Huntington's model rats, DARPP32-positive neurons were present, however, large tumourigenic overgrowths were witnessed from the grafts, possibly due to the mitogenic effect of SHH (Fuccillo et al., 2006) or contaminating undifferentiated cells. This outcome severely limits the application of these cells *in vivo*. Other SHH based studies have shown a low SHH dosage that is able to ventralise the cellular identity to an extent similar to the LGE (Li et al., 2009) and striatal progenitors have also been reportedly generated from midbrain-directed differentiation protocols that require SHH (Ferrari et al., 2006). These studies demonstrate an induction of MSN character from SHH-based differentiation.

Danjo et. al. used mESC derived forebrain precursors, sorted for FOXG1-reporter expression, to generate transplantable MSN *in vitro*. Patterning here is also achieved via precise SHH doses. Importantly, these cells were demonstrated to be functional as they were able to integrate and project to normal MSN target tissues, the globus pallidus and the SN (Danjo et al., 2011).

The field of striatal projection neuron directed differentiation has progressed relatively slowly in recent years. In order to progress the field to complement the success of foetal-derived grafting in patients, safe protocols and *in vivo* transplantation studies are required to further advance the technology. SHH signalling (in conjunction with DKK1-mediated dorsal fate inhibition) has been employed to facilitate correct dorso-ventral patterning and specify MSNs *in culturo*. It should be noted that some *in vivo* studies have demonstrated an independence of the LGE on SHH signalling. Smo conditional knockout mice demonstrate an upregulation of Gsx2 (Xu et al., 2010) (note Gsx2 expression is higher in the LGE than the MGE). Also, SHH knockout-mediated LGE ablation is recovered in SHH-GLI3 double mutants suggesting an action for SHH in inhibiting dorsal GLI3 signals (Aoto et al., 2002; Rallu et al., 2002). Lastly, there is no evidence for a direct action of

SHH on LGE specific genes. This leaves the possibility that alternative signalling pathways actively direct LGE development, enabling greater optimisation of MSN directed differentiation.

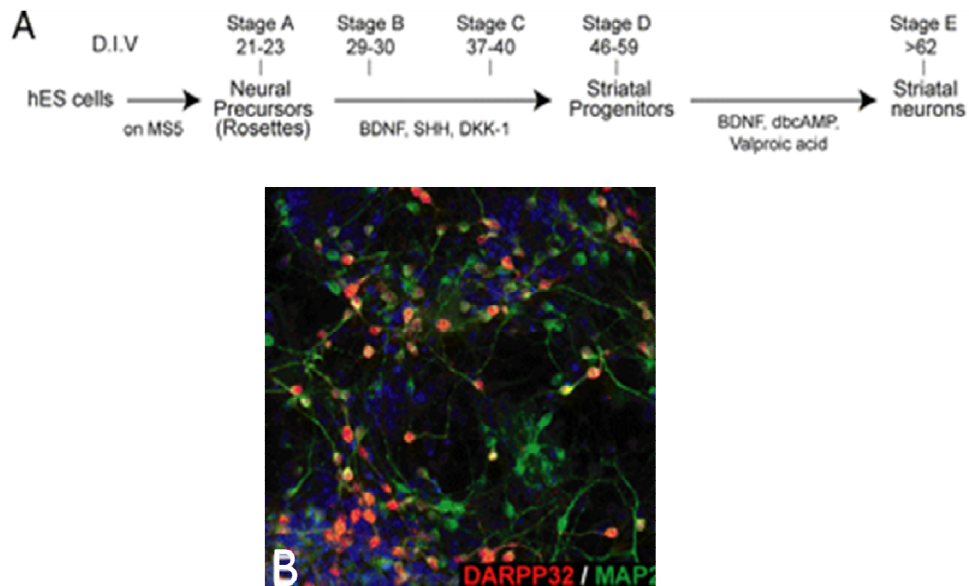


Figure 1.12 **Aubry et. al. protocol for MSN Differentiation.**

Aubry et. al. use coculture-based neural induction followed by SHH and DKK1 mediated patterning towards MSNs (A). The resultant cultures exhibit DARPP32-positive neurons (B) that can also be generated in vivo after transplantation of patterned progenitors (Aubry et al., 2008).

1.6.3 Midbrain Dopaminergic Directed Differentiation

In vitro derivation of mDA neurons has received a great deal of focus due to the association with Parkinson's disease; which affects over 4 million people worldwide (von Bohlen und Halbach et al., 2004). The success of a subset of foetal-derived transplants into patients has lead to much interest in tissue derived from differentiation of PSCs.

As described in Chapter 1.5.2, SDIA present in ESC/PA6 coculture differentiation is sufficient to generate dopaminergic neurons (Kawasaki et al., 2000). These cells are able to integrate into the striatum of rodents and express TH. Subsequent coculture, monolayer differentiation protocols and EB based protocols routinely utilised treatment with SHH and FGF8 to specify vMB identity analogous to MHB and ventral midline signalling centre proximity (Barberi et al., 2003; Kim et al., 2002; Lee et al., 2000; Perrier et al., 2004; Yan et al., 2005).

Mouse ESC differentiation has seen significant success in the last 10 years. Lee et. al. employed EB differentiation and were the first to demonstrate that SHH, FGF8 and ascorbic acid treatment could produce properly specified mDA neurons with a vMB phenotype (Lee et al., 2000). Barberi et. al. demonstrated that coculture-derived neurons with SHH and FGF8 treatment were able to repair behavioural deficits in Parkinson's disease model rodents after transplantation (Barberi et al., 2003).

Human ESC differentiation has had mixed success using PA6/MS5-based coculture, with many groups seeing results that varied depending on the cell line being used and demonstrating failure to improve behavioural deficits in disease-model rodents, for review see (Taylor and Minger, 2005). Noggin treatment was shown to augment the efficacy of coculture-based differentiation and grafted cells did reduce drug-induced rotations in Parkinsonian mice (Ben-Hur et al., 2004). Perrier et. al. employed a human ESC-derived differentiation scheme using stromal coculture with SHH, FGF8 and BDNF growth factor supplements, followed by mechanical isolation of rosette structures. The resultant neurons exhibited expression of marker genes, ultra-structural characteristics and electrophysiology of true dopaminergic neurons (Perrier et al., 2004). Yan et. al. proposed an EB-based differentiation with additional SHH and FGF8 that was able to produce dopaminergic neurons exhibiting characteristics of forebrain and midbrain relative to growth factor administration (Yan et al., 2005).

Many of the early differentiation studies using human PSCs did not investigate the exact identity of the dopaminergic cells and it became apparent that many of the TH-positive cells produced *in vitro* did not have a specific vMB phenotype, i.e. marker gene coexpression that specifically demarcates mDA neurons (FOXA2, LMX1a, and PITX3). At the outset of this project,

effective *in vitro* directed differentiation techniques for human PSC remained to be optimised and there was especially a lack of monolayer differentiation schema for dopaminergic differentiation of hPSCs.

The vast scope of the mDA directed differentiation field has enabled a high degree of understanding into the correct *in vitro* differentiation of mDA neurons. Taken together with the success of foetal and ESC-derived grafts to reverse Parkinsonian disease symptoms in model organisms, the academic landscape is set to focus on the technical challenges of transplantation. For the safest possible cell-based treatments, a detailed knowledge of all developmental/differentiation events will enable the purest cell preparations for future patient transplantations.

1.7 General Aims

I aim to develop a differentiation protocol that can produce multipotent, neural progenitors with the ability to generate many neuronal cell types.

I hypothesise that I can manipulate the extrinsic cellular environment to direct differentiation towards neuronal cell types of interest. Via altering growth factor environments to follow *in vivo* developmental conditions (e.g. proximity to morphogen secreting Organiser regions), I aim to develop protocols that enrich mDA neurons, MSNs and cortical interneurons.

I theorise that the cells derived *in vitro* are properly functional. Together with collaborators, we aim to demonstrate that ESC-derived neurons express the proper range of marker genes, show electrophysiological properties of functional neurons and show the capacity to integrate into the brain of a host animal.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Media for tissue culture

Feeder media

GMEM (Invitrogen), 10% FCS (Invitrogen), 1mM NEAA (Gibco), 1mM sodium pyruvate (Gibco), 2mM L-glutamine supplemented with Pen Strep antibiotics (Gibco) and 0.1mM β -mercaptoethanol (Gibco).

hPSC media

koDMEM (Invitrogen), 22% knockout serum replacement (Invitrogen), 1mM NEAA (Gibco), 1mM sodium pyruvate (Gibco), 2mM L-glutamine supplemented with Pen Strep antibiotics (Gibco) and 0.1mM β -mercaptoethanol (Gibco).

N2B27 (150ml)

100ml DMEM F12 (Invitrogen), 50ml Neurobasal (Invitrogen), 1ml N2 supplement (Invitrogen), 1ml B27 without Retinol (Invitrogen), 2mM L-glutamine supplemented with Pen Strep antibiotics (Gibco) and 0.1mM β -mercaptoethanol (Gibco).

MEF Conditioned Media

Feeder free media contained 50% hESC media with 8ng/ml FGF2 and 50% MEF conditioned media. Conditioned media was produced from hESC media with 8ng/ml FGF2 (Peprotech) and conditioned on inactivated MEFs for 24 hours (3ml per 6 well plate). Adding FGF2 at this stage is critical to stimulate Activin secretion from the MEFs (Greber et al., 2007). More specifically, a confluent T25 of MEFs was used to seed an entire 6 well plate. 3ml of hESC media plus FGF2 was added per well and collected 24hours later. This was repeated for 7-10 days.

2.1.2 Cell Lines Used

Human ESC lines H7 and H1 were used with permission from WiCell (Thomson et al., 1998). Cell line H7 was used for most experiments and then repeated in other cell lines.

Human iPSC lines 2F8 and 4FHuang#1 were employed in this thesis. The 2F8 line was a kind gift from Austin Smith's group. The 4FHuang#1 cell line was generated in our lab by Kuo Hsuan Chang.

2.2 Methods

2.2.1 Cell Culture

All cell cultures were maintained in 37°C and 5% CO₂ (Sanyo). Work was carried out under a laminar flow hood (Nuair) and all surfaces were disinfected with 70% ethanol and following work adhered to sterile techniques. Cells were grown on tissue cultures plastics (Falcon) or glass coverslips (VWR) and coated as stated.

2.2.1.1 MEF Cell Culture

MEF cells were maintained in feeder media (Chapter 2.1) and passaged using 0.025% trypsin. Cells were washed with PBS and 1ml of trypsin was added to each T25 flask (Sigma) followed by a 2 min incubation at 37°C. Cells were washed off the plate via titration with feeder media to quench the trypsin and centrifuged at 1300rpm for 2.5 mins (Eppendorf 5702). MEFs were routinely passaged at ratios of 1 in 8.

MEFs were inactivated using 10µg/ml Mitomycin C (Sigma) for 2-3 hours and one confluent T25 flask was used to fill a 6well plate of inactivated feeders.

2.2.1.2 hPSC Culture

Human PSCs were seeded on inactivated feeder cells in hPSC media (Chapter 2.1) supplemented with 8ng/ml FGF2 (Peprotech). Half the media was changed every day. Cells were passaged every 5 days as follows. hPSCs were washed with PBS once and then treated with 0.02% EDTA for 2 mins at room temperature. EDTA was aspirated and hPSC media was added. Cells were manually dissociated using a serological pipette and scraping/titration, maintaining small cell clusters. One third of a confluent well from a 6well plate was centrifuged at 1300rpm for 2.5 mins (Eppendorf 5702), resuspended and added to fresh inactivated MEFs at 1/2, 1/3 and 1/6 of these cells (i.e. 1/6, 1/9 and 1/18 of the original well). The lowest well was ready for passaging in 5 days routinely.

H7 cells, 2F8 and 4FHuang cells were most robust and withstood passaging as small colonies whereas H1 cells were easiest to maintain as larger, slow growing colonies.

2.2.1.3 Cell Freezing and Thawing

For storage of cells at low passage numbers, cells were passaged as normal and frozen in 10% DMSO with hESC or Feeder media accordingly. Freezing was performed slowly with an overnight step at -70°C before being placed in liquid nitrogen storage tanks. Thawing of cells was performed as rapidly as possible with cells being centrifuged immediately at 1300rpm for 2.5 mins and DMSO containing media aspirated. Cells were resuspended and plated at appropriate ratios.

2.2.2 Monolayer Differentiation

2.2.2.1 Monolayer Differentiation of human PSCs

Monolayer differentiation was initiated with as many hPSCs as possible to reduce expansion time in feeder free conditions. hPSCs were passaged as usual but were subject to preplating for 45 mins on gelatin coated plates to remove MEFs due to differential adhesive dynamics (0.1% gelatin coating for 20 mins). hPSCs still in suspension were then transferred to matrigel coated plates (diluted 1in15 in koDMEM and coated for 1 hour at 37°C) and grown to confluency in feeder free media containing MEF-conditioned hPSC media. Once 100% confluent, the media was changed to N2B27 defined media and this time point was taken as day 0. H7 differentiation; The TGFβ antagonist SB431542 (10μM, Tocris) was added to the media from day 0 until day 5. BMP antagonists Noggin (100ng/ml, R&D) and Dorsomorphin (200nM, Tocris) were added from day 0 until day 9. Passage 1 was performed at day 9-12 using 0.02% EDTA as above. Cells were split 2:3 onto fibronectin coated plates and kept as relatively large cell clusters. Passage 2 at day 19-22 was performed with EDTA as above, whereas cells were split 1:1 onto poly-L-lysine and laminin coated plates, but titration was more vigorous than passage 1. Once the cells were neuronal, at around day 25, BDNF (10ng/ml, Peprotech), GDNF (10ng/ml, Peprotech) and ascorbic acid (0.2mM) were added to aid cell survival.

Fibronectin coating was performed with 15μg/ml fibronectin (Millipore) in PBS for 2 hours at 37°C. Poly-L-lysine and laminin coating was performed via 2 hour incubation of 0.002% poly-L-lysine (Sigma) and then 2 hour incubation of 10μg/ml laminin (Sigma), both at 37°C.

H1, 2F8 and 4FHuang cell lines differentiated more rapidly and so were treated with SB431542 for 4 days, Noggin and Dorsomorphin for 8 days and were passaged at day 9 directly onto poly-L-lysine and laminin coated plates, without the fibronectin coated plate stage.

Activin (R&D) was added during the relevant time period at either 10ng/ml (Chapter 3, Figure 3.1) or 25ng/ml (Chapter 4, figure 4.1). The SHH antagonist Cyclopamine was used in some experiments as stated (1μM, Sigma).

For ERK inhibitor treatment dual SMAD inhibition was halted as ERK inhibition was performed from day 3 until day 7 (PD0325901, 1μM, Axon). SHH C24II (200ng/ml, R&D) supplemented with SHH agonist Purmorphamine (1μM, Merck) was then added to the media from day 7 until passage 1 at day 11 (ratio of 1:2) and then SHH, Purmorphamine and FGF8 (100ng/ml, Peprotech) was added to the media from day 10 until day 16, (Figure 5.1).

2.2.2.2 Monolayer Differentiation of mouse ESCs (Serafi Cambray)

Briefly, mouse ESCs were maintained on gelatin coated plates (0.1%) in media containing 10% FCS and LIF. Passaging of mESCs was performed using 0.025% trypsin, as with MEFs, (Chapter 2.2.1.1). Monolayer differentiation was performed in Neurobasal Medium (Invitrogen) supplemented with B27 (1:50, Invitrogen) at low density (7,500 cell per cm²) on gelatin coated plates. Cells were passaged on day 5 using trypsin onto poly-D-lysine and laminin coated plates and Activin was added at this time point. See (Cambray et al., 2012).

2.2.2.3 Monolayer differentiation of mouse epiSCs (Ines Jaeger)

Mouse epiSCs were maintained in N2B27 (N2 1:100, retinol free B27 1:50) supplemented with FGF2 (12ng/ml) and Activin (20ng/ml). MD was initiated in N2B27 without growth factors, with or without ERK inhibitor from day 0 on fibronectin coated plates.

2.2.3 Chromosome Counting

hPSCs were routinely tested for karyotypic stability. Colchicine was added to the cells to arrest cell division (1µg/ml, Sigma) and then cells were trypsinised as with MEFs, above. Cells were centrifuged and resuspended in 0.5ml of 0.075M KCl for 10 mins at 37°C to allow the cells to swell. Cells were centrifuged and supernatant was aspirated. Cells were fixed in fresh methanol : acetic acid (3:1 v/v) for 30 mins at 4°C. Cells were washed twice in fixative before being dropped from 30cm height onto coverslips, air dried and fixed with mounting media containing DAPI (Vectashield, Vectalabs). Images were taken on the Leica SP5 confocal microscope. Counting of metaphase spreads was done using Image J software on at least 20 cells per Karyotyping. Chromosome counts were performed on spreads where there was no overlap seen, aiding separation. The number of abnormal cells increased with passage number. For example, H7 cells displayed around 30% abnormal cells after 115 passages in total. For this reason, cells were expanded and frozen at early passage numbers and only used between passages 40 and 90.

2.2.4 Immunocytochemical Staining

2.2.4.1 Immunocytochemical staining and Immunohistochemical Staining

Cell cultures were washed 1 time in PBS to remove floating cells and fixed in 3.7% PFA for 15 mins at room temperature, either on coverslips (VWR) or in tissue culture plates (Falcon) or as floating sections. Cells were then stored at 4°C for a maximum of two weeks in PBS or processed immediately for immunostaining. Fixed cells were permeabilised in PBST (PBS+0.3% Triton-X-100) via three washes, each for 10 mins. Cells were then blocked in PBST containing 3% BSA and 1% normal donkey serum for 20 mins at room temperature. Primary antibodies were then added in blocking solution overnight at 4°C or 2 hours at room temperature. Cells were washed three times in PBST for

5 mins each and then secondary antibodies were added in blocking solution for 1 hour at room temperature (1 in 200 Alexafluor secondary antibodies, 488/555/594/647nm, Invitrogen). Cells were washed once in PBST containing 1 in 2000 DAPI (final concentration 1 µg/ml, Molecular Probes) for 10 mins and then twice more in PBST for 10 mins each. Finally cells were mounted and fixed in mounting media (DAKO).

A list of antibodies used in this thesis is presented in Chapter 7.2.

For FOXG1 immunostaining, cells were washed in PBS one time, fixed in methanol acetone (1:1) which was prepared fresh at -20°C for 10 mins and dried for 5-10 mins. Blocking and antibody staining was done as above but without Triton-X-100 for all solutions.

Images were taken on a SP5 confocal microscope (Leica). Counting was performed on random fields of view from north, south, east and west of biological duplicates using Image J software. At least 1000 cells were counted manually from each replicate. More specifically, at least 4 random fields of view were selected per replicate without using the SP5 viewfinder to capture images. Images were confocal sections to aid individual cell visualisation. Quantification was performed using the Image J cell counting software, whereby counted cells may be marked concurrently. DAPI was always counted as well as specific markers to ensure sufficient cell numbers.

2.2.4.2 EdU Detection

ClickIT EdU staining was done as per the manufacturer's instructions (Invitrogen). Cells were fixed and permeabilised as normal; ClickIT reaction cocktail was added to the cells for 30 mins at room temperature protected from the light. Colabelling with primary antibody staining was then performed on the EdU stained cells as per Chapter 2.2.4.1.

2.2.5 Quantitative PCR

2.2.5.1 RNA Extraction

For RNA extraction, cell cultures were washed with PBS and RNA was harvested using 500 µl TRI Reagent (Sigma). Cell lysates were then stored at -70°C for up to one month. Prior to RNA purification, lysates were incubated at room temperature for 5 mins.

RNA was purified as per the manufacturer's instructions. Briefly, 100 µl of chloroform was added to the lysate followed by 30 secs vigorous shaking. After 2 mins at room temperature, the

mixture was centrifuged at 12,000 x g for 15 mins at 4°C. The upper aqueous phase was then separated transferred to a new eppendorf. 250µl of isopropanol was then added to the supernatant, followed by mixing and 5 mins incubation at room temperature. The mixture was then centrifuged at 12,000 x g for 15 mins at 4°C. The supernatant was removed and the pellet was washed in 70% ethanol by centrifugation at top speed for 15 mins at 4°C. RNA was then resuspended in 10-20µl of dd water. RNA was periodically tested for degradation on a 1% agarose gel.

2.2.5.2 Reverse Transcription

RNA concentration was measured on a Nanodrop spectrophotometer ND 1000 (Labtech) and 2µg was taken to a new tube for reverse transcription in a final volume of 10µl dd water. RNA was heated to 70°C for 5 mins with 1µl random primer mix (3µg/µl, Invitrogen) and then allowed to cool for 5 mins at 4°C. Reverse transcription mix containing 4µl 5x first strand buffer, 1µl dNTP (10mM), 1µl DTT (0.1M Invitrogen), 1µl RNase OUT (Invitrogen) and 1µl superscript III Reverse Transcriptase (Invitrogen). Reverse transcription was performed in a PCR machine with the following steps: (25°C for 5 mins), (42°C for 10 mins), (50°C for 30 mins), (55°C for 10 mins), (70°C for 15 mins) and (4°C for 10 mins). cDNA was then diluted 10x with dd water.

2.2.5.3 Quantitative PCR

Quantitative PCR was performed using SYBR MESA green mastermix. Mastermix containing 10µl MESA green (2x, Eurogentech), 7.8µl dd water and 0.1µl of each primer pair (100µM, all custom made oligonucleotides, Sigma) was aliquoted to relevant wells of a 96 well plate (Biorad). cDNA was aliquoted accordingly using 2µl of the 10x diluted mix, above. QPCR was performed on a Biorad Chromo4 machine. The PCR program was as follows: (94°C for 4 mins), [(94°C for 30 secs) + (60°C for 15 secs) + (72°C for 30 secs)] repeated 40 times. Melting curve analysis was performed from 65°C to 95°C with a read every 0.2-1°C.

All qPCR data is relative to three reference genes (GAPDH, β -ACTIN and CYCLOPHILIN) and data was analysed using Opticon Monitor 3 software (Biorad) and Microsoft Excel. All qPCR data is normalised to the basal condition and error bars represent the SD of biological duplicates. Data was excluded if Δ CT was above 30 cycles.

All primers were designed using the primer Blast software (NCBI). Primers were resuspended in dd water to 100µM. Primer pairs were tested on cDNA from human foetal whole brain tissue with serial dilutions to test efficiencies. All annealing temperatures were 60°C.

A list of all primers used in these works is presented in Chapter 7.1.

2.2.6 FACS Analysis (Ines Jaeger)

Mouse differentiating cells were washed one time with PBS and then trypsinised. Single cells were resuspended in PBS with 10% FCS, washed twice with PBS and then fixed in 2% PFA in suspension for 15 mins at 4°C. Cells were washed and then resuspended in sorting buffer (Chapter 2.1). Cells were strained using cell strainer caps to ensure single cell suspension (BD Falcon) and then analysed on a BD LSRII (Becton Dickinson Biosciences). Data was analysed on BD FACS Divaaria software (Becton Dickinson Biosciences) and FlowJo (TreeStar).

2.2.7 Transplantation of hPSC-derived neural Precursors (Sophie Precious)

All animal work was carried out under the UK Animals Act 1986. Adult female Sprague-Dawley rats (Harlan) were maintained lesioned with 45nM quinolinic acid unilaterally in the striatum. All surgery was performed under isoflurane anaesthesia. Immunosuppression was achieved with Cyclosporin A at 10mg/kg i.p. (Sandimmun) starting one day prior to transplantation. Animals were sacrificed with barbiturate overdose.

Activin-treated neural precursors were transported to Cardiff at 37°C in plates with surplus media. Cells were rinsed with PBS and dissociated using Accutase for 2 mins at 37°C (PAA Laboratories). The cells were centrifuged at 1000rpm for 3 mins, resuspended in 2ml N2B27, counted and the volume adjusted to 200,000cells/ μ l. 400,000 cells were transplanted into the striatum of each adult lesioned rat, i.e. 2 μ l.

Chapter 3 Activin Accelerates Neural Differentiation and Specifies a Cortical Interneuron Fate

3.1 Introduction

Differentiation of human pluripotent cells (hPSCs) provides an invaluable tool for biomedical research. Firstly, it provides a model system to study normal and diseased human development, a process that would otherwise be ethically and logistically impossible to access. Secondly, cells produced via *in vitro* differentiation can provide large amounts of material for new drug toxicology testing or novel drug screening strategies. Lastly, the use of foetal tissue to reverse neurodegenerative diseases has proven to have some, albeit varied, efficacy (Bachoud-Levi et al., 2000a; Bachoud-Levi et al., 2006; Freed et al., 2001; Lindvall et al., 1989; Madrazo et al., 1988). Using pluripotent stem cell derived cultures in place of foetal tissue provides ethically more agreeable and also limitless supplies of such tissues that may prove to have similar therapeutic effects.

The goal of *in vitro* differentiation is to recreate the *in vivo* developmental regimes that are programmed into the cells' genetic responses. A close understanding of the developmental decisions made by cells *in vivo* and the inductive cues leading to these decisions can allow the researcher to recreate exogenous cellular environments and bring about similar choices to follow specific developmental pathways. For example, understanding the three dimensional arrangement of tissues and the relative proximity of organiser regions (Chapter 1.2), one can estimate the relevant conditions that a cell may be exposed to during normal development. Additionally, *in vitro* differentiation may provide new information about development, for example, novel genes can be implicated during differentiation and relayed back to *in vivo* development.

The differentiation process can be performed in three ways *in vitro* (see Chapter 1.5). The first technique for differentiation of human pluripotent stem cells is coculture with mouse stromal cells, which have been shown to promote differentiation in a directed manner via an undefined signalling activity (Barberi et al., 2003; Kawasaki et al., 2000; Perrier et al., 2004). Embryoid body (EB) formation is the second method for hPSC differentiation, whereby 3D aggregates of PSCs differentiate in a manner resembling the developing gastrula (Keller, 1995). The drawback of these techniques are that there are undefined signalling events from the stromal cells or within the EB, leading to reduced experimental control, increased heterogeneity and lower reproducibility when testing individual variables.

The third technique, Monolayer differentiation (MD), involves the seeding of cells in a monolayer and sustaining the adherent cells in a chemically defined media. This allows all the cells

to be exposed to similar morphogen concentrations throughout the culture as determined by the investigator. For this study, I aim to adapt and optimise the dual SMAD inhibitor protocol in our hands (Chambers et al., 2009), see Chapter 2. This protocol efficiently gives rise to proneural populations with increased homogeneity across the culture due to effective, forced and synchronous entry to the neural lineage via SMAD inhibition; mimicking the manner hypothesised for *in vivo* development (Anderson et al., 2002). A number of BMP and TGF β inhibitors (such as Noggin, Chordin and Follistatin) are present at the Spemann's Organiser and are believed to be responsible for the initial induction of the neuroectoderm in the developing gastrula (Chapter 1.2) (Bachiller et al., 2000; Spemann and Mangold, 1924).

I then will employ the neural precursor cells produced in this protocol for investigations into novel growth factor environments. I shall examine the effect of exogenous Activin A treatment (referred to as Activin hereafter). This molecule has been implicated as a pro-survival factor during *in vitro* differentiation (Schubert et al., 1990) and also in adult brains after exposure to insult (Foster et al., 2004; Mukerji et al., 2007; Tretter et al., 1996; Wu et al., 1999). However, Activin has not been studied in early neural developmental/differentiation paradigms. Interestingly, the expression of Activin subunits and their receptors have been described to be widespread in the anterior neuroectoderm (Feijen et al., 1994; Foster et al., 2004), with phosphorylated and active SMAD2/3 signalling transducers seen in the ventral forebrain at early developmental stages (Feijen et al., 1994; Maira et al., 2010).

Activin is a member of the TGF β superfamily and signals via binding to Activin Type II receptors recruiting Type I receptors via dimerisation. This binding leads to phosphorylation and activation of SMAD2 and SMAD3 transducers which together with the coSMAD, SMAD4, translocate to the nucleus and alter gene expression (see Chapter 1.2).

I shall investigate the effect of Activin treatment on anterior neural cell types, notably the ventral telencephalon. Cortical interneurons are the GABAergic inhibitory cells making up 20-30% of the cortex, whereby excitatory glutamatergic cells make up the remainder of the population. Cortical interneurons are required for proper modulation of cortical glutamatergic cell output (Wang et al., 2004), e.g. long term potentiation, and are key during development for glutamatergic cell proliferation, migration and establishing cortical circuitry (Hensch and Stryker, 2004; Owens and Kriegstein, 2002). There is interest in producing cortical interneurons *in vitro* for cell therapy as their improper functioning and cellular deficiencies have been implicated in disease (epilepsy, autism and schizophrenia, Chapter 1.3). Also their propensity to migrate away from the site of transplantation and then modulate cortical activity provides added value to transplantation studies because of the

ability to functionally integrate into the host; thus showing potential to replace a cell deficiency in the disease state (Alvarez-Dolado et al., 2006; Martinez-Cerdeno et al., 2010).

In vivo, cortical interneurons are produced in the ventral telencephalon during development before they migrate tangentially and dorsally to the cortex (Chapter 1.2.4). The ventral telencephalon can be divided into three domains, namely the lateral ganglionic eminence (LGE) which forms the striatal projection neurons, the medial ganglionic eminence (MGE) which is the major site of interneurogenesis and the caudal ganglionic eminence (CGE), which is a second birthplace of cortical interneurons and is a caudal continuation of the two more rostral tissues. Interneurons can be divided into three major cell subtypes; Parvalbumin (PV) and Somatostatin (SST) expressing cells that are produced in the MGE and induced/specified by SHH signalling and NKX2.1 expression and also Calretinin (CR) expressing neurons that are produced in the dorsal CGE (dCGE) through undefined inductive signals (Wonders and Anderson, 2006).

I will investigate SHH and Retinoic acid (RA) signalling pathways in this chapter; see Chapter 1.2. Canonical SHH signalling acts to ventralise the cells and also has a strong mitogenic effect. Active SHH signalling removes a repression on the system to allow GLI1/2 transcription factors to become active and alter gene expression in the nucleus (Fuccillo et al., 2006). RA can act as a caudalising growth factor in the neural tube, however, RA signalling can also function as a pro-differentiation factor; this depends on spatiotemporal setting (Maden, 2007). Exogenous retinol is converted to retinaldehyde which is subsequently converted to RA via the ALDH1a enzyme family. RA then binds receptor proteins in the nucleus to alter gene expression.

Activin signalling has been described to be detrimental to the earliest stages of neural differentiation, whereby dominant negative receptors augment the efficiency of neural lineage fate choice (Hemmatibrivanlou and Melton, 1994), reinforcing the presence of the Activin antagonist Follistatin in the Anterior Neuroectoderm Ridge (Hemmatibrivanlou et al., 1994) and the use of SB421543 antagonist in neural differentiation paradigms (Chambers et al., 2009). There is little evidence of an effect of Activin signalling in anterior neural tissues (Song et al., 1999), even though Activin subunits, its receptors and activated SMAD signal transducers are witnessed in the developing forebrain (Feijen et al., 1994; Maira et al., 2010). For this reason, we set out to investigate an effect of Activin stimulation on anterior neural precursors in culture and hypothesised a negative effect of activating this signal pathway on neural differentiation.

The investigation into activin function in human ESC differentiation was performed in conjunction with mESC differentiation and will be cited as such, Serafi Cambray (Cambray et al., 2012).

3.2 Results

3.2.1 Optimisation of the Differentiation Protocol

Prior to MD, hPSCs were maintained on mouse feeder cells and in media containing knockout serum replacement and 8ng/ml of FGF2. The morphology of the pluripotent cells was confirmed with large nuclear to cytoplasmic ratio and multiple visible nucleoli (figure 3.1 A). Cells were routinely tested for karyotypic stability (figure 3.1 B) and screened to remain free from mycoplasma contamination. Immunostaining confirmed the maintenance of pluripotency markers OCT3/4 and TRA-1-81 (figure 3.1 C). Colonies were manually passaged every 5 days using EDTA disaggregation, maintaining consistent colony size, cell morphology and pluripotency.

To initiate differentiation, human pluripotent cells were preplated for 45 minutes on gelatin coated plates to remove contaminating feeder cells and then grown to confluency on matrigel coated plates in MEF conditioned media. When confluent, MEF-conditioned media was removed and differentiation was initiated in RA-free N2B27 media with the dual SMAD inhibitors (figure 3.1 D-E). To validate the differentiation regime, I tested the levels of pluripotency and differentiation markers at the mRNA and protein levels. I observed a rapid downregulation of pluripotency markers within 3 days of differentiation and a subsequent upregulation of the neural lineage markers PAX6 and SOX1 (figure 3.1 F-L). PAX6 is the major neural fate determinant in human neurulation, meaning its upregulation is pivotal in this differentiation scheme (Zhang et al., 2010).

Interestingly, I observed a dissimilar rate of differentiation for varying cell lines. Rosette structures represent immature neural progenitors forming two dimensional neural tube structures. These were observed at day 18-25 in the H7 hESC line whereas the H1 cell line and the iPS cell lines displayed rosettes and the first neurite outgrowths from day 8-10. The H7 cell line required an additional passage onto fibronectin coated plates from day 9 to day 19 in order to provide additional space and to prevent cells being lost via detaching from the plate as the cells proliferated. This diversity has been described between human pluripotent stem cell lines and so may be an expected feature when using multiple cell lines (Allegrucci and Young, 2007; Osafune et al., 2008). To test the effect of Activin on proneural cells, I added the signalling factor to the media at the rosette stage (figure 3.1 D-E).

With this functional neural inductive MD protocol for hPSCs *in vitro*, I have a model amenable to investigations into developmental patterning cues. This chemically defined monolayer differentiation enables undefined growth factors to be investigated and implicated in development

and also new differentiation protocols to be optimised with a view to producing neurons with clinical significance.

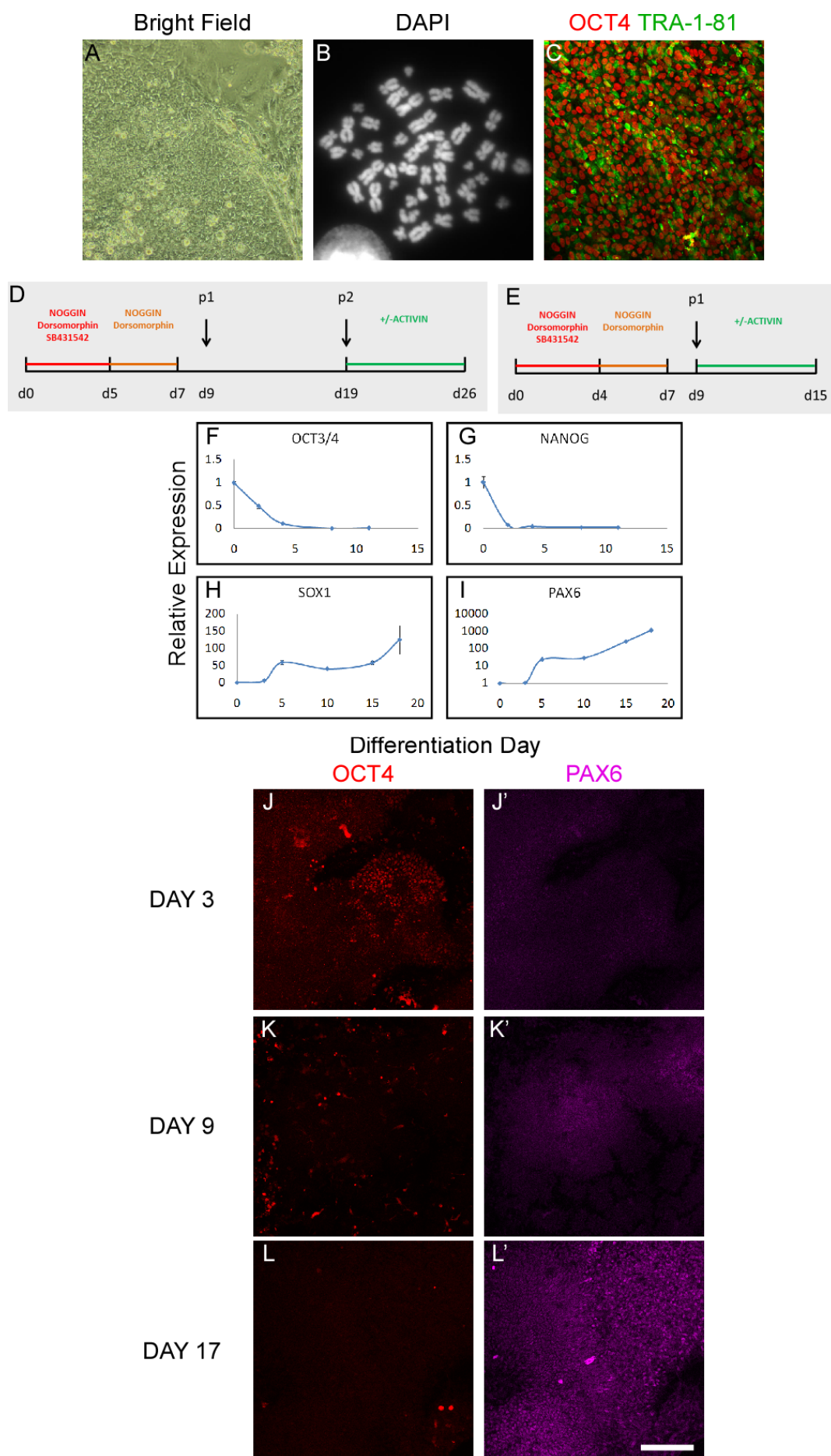


Figure 3.1 Human ESC culture and neural differentiation.

(A) Bright field image of hESC colonies on feeder cells in culture. Morphology was monitored to maintain a small cytoplasmic to nuclear ratio and multiple, visible nucleoli. Image taken using 20x magnification.

(B) Metaphase spread of hPSC, used to determine the karyotypic stability of the cells over multiple passages. Scale bar represents 10 μ m.

(C) Immunostaining for OCT4 and TRA-1-81 in H7 hPSCs in culture, confirming the expression of key pluripotency factors. This was repeated in all cell lines used. Scale bar represents 75 μ m.

(D) Differentiation scheme for H7 hESC line (see Chapter 2). Rosettes were visible after around day 18.

(E) Differentiation scheme for H1 hESC and all iPSC lines (see Chapter 2). Rosettes were seen around day 9.

(F-I) Quantitative PCR to demonstrate gene transcription dynamics for pluripotency factors OCT4 and NANOG and neural lineage fate determinants PAX6 and SOX1, NB log scale for PAX6. Error bar represents SD of biological duplicates.

(J-L) Immunostaining to demonstrate the activation of pan-neural marker PAX6 as well as the downregulation of the pluripotency associated factor OCT4 through time during H7 MD. Scale bar represents 100 μ m.

3.2.2 Activin treatment accelerates neuronal differentiation

Treating cells with 10ng/ml of Activin at the neural progenitor cells had a striking effect on differentiation (figure 3.2 A). After 6 days of activin exposure there was a clear change in the cell phenotype. Based on morphology, the number of cells displaying neurite extensions was increased in the treated cultures compared with the controls, suggesting an accelerated terminal differentiation. To test this, I immunostained cells 6 days after Activin treatment for Nestin and TUJ1 to delineate neural progenitors and postmitotic neurons respectively (figure 3.2 B-C). Counting data demonstrated a significantly increased presence of postmitotic cells in Activin treated cultures (figure 3.2 D).

The pro-differentiation effect of Activin was reinforced via investigating the number of cells progressing through the cell cycle. The incorporation of the nucleotide analogue EdU reveals progression through S phase as EdU is assimilated in newly synthesised DNA. Cells were treated with Activin for 6 days and then exposed to EdU in the media for 1 hour. Detection of EdU incorporation revealed a significant difference between the Activin treated ($12.0 \pm 2\%$) and control cells in which two-times the number of cells appeared to be actively cycling ($27.5 \pm 6\%$) (Figure 3.2 E-F).

Therefore, I have observed an accelerated differentiation after cells at the proneural stage were treated with Activin, as demonstrated by an increased representation of postmitotic TUJ1-positive neurons and a decrease in cycling cells incorporating EdU.

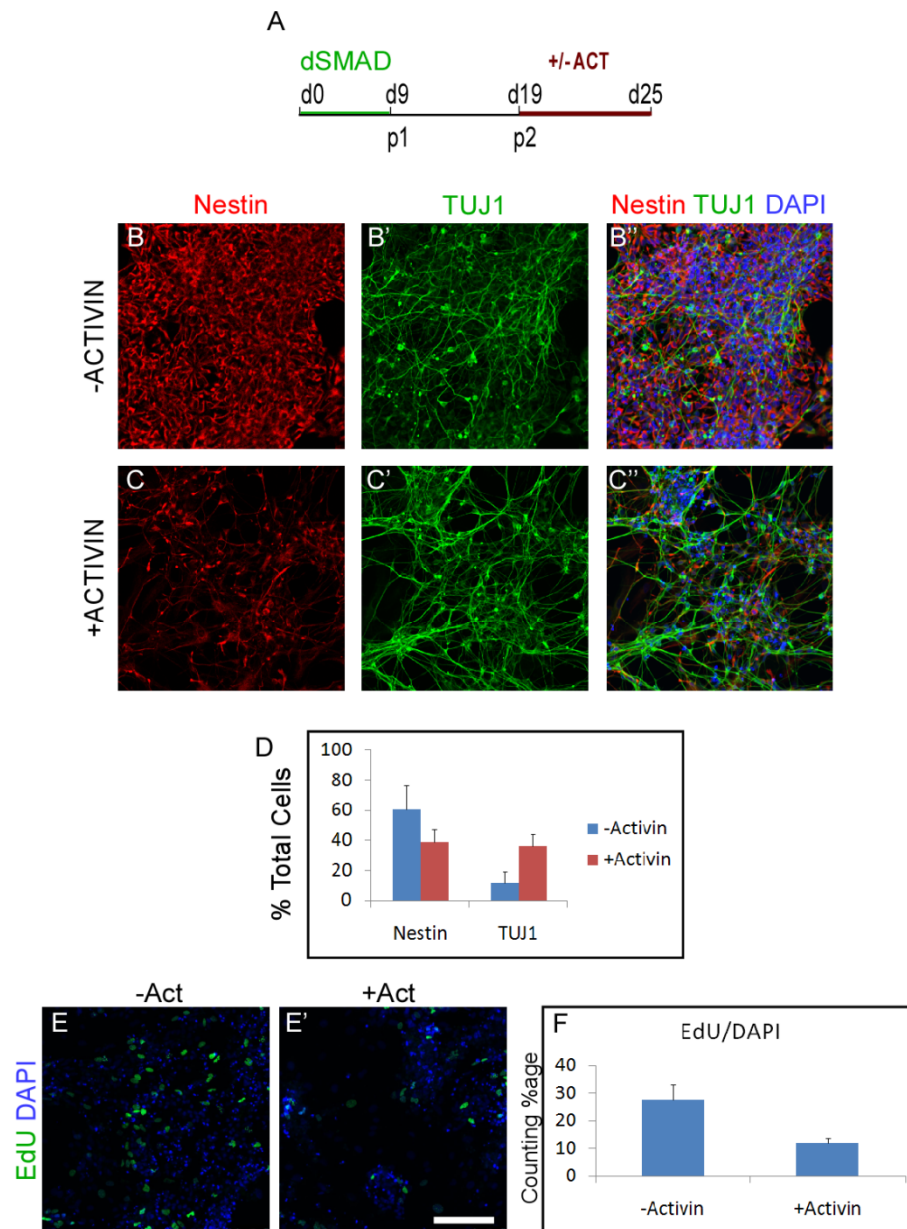


Figure 3.2 Affect of exogenous Activin on rate of neural differentiation.

(A) Differentiation scheme for H7 cells.

(B-C) Immunostaining to show Nestin (red, neural precursors) and TUJ1 (green, postmitotic neurons) after 6 days of Activin treatment from day 19 H7 MD. DAPI staining (blue) shows all nuclei. Scale bar represents 75µm.

(D) Counting data to show proportion of cells that are TUJ1-positive and Nestin-positive in the plus or minus Activin conditions - biological triplicates. Basal condition shows (60.5 ± 16%) Nestin and (12.1 ± 7%) TUJ1. Activin condition shows (38.9 ± 9%) Nestin and (36.1 ± 8%) TUJ1.

(E) Nucleotide analogue EdU incorporation into actively cycling cells. Cells were treated with EdU for 1 hour after 6 days of Activin exposure from day 19. Scale bar represents 100µm.

(F) Counting data of EdU incorporation into total cells (DAPI) - biological and experimental duplicates.
Basal condition ($27.5 \pm 5\%$), Activin condition ($12.0 \pm 2\%$).

3.2.3 Activin accelerates terminal differentiation via inhibiting SHH signalling

The SHH signal has a role in ventralising cells throughout the neural tube (Ericson et al., 1995), but it also has a known mitogenic action and has been shown to promote renewal of rosette-stage cells (Elkabetz et al., 2008; Fuccillo et al., 2006). This effect is demonstrated as the presence of Cyclin D1, a defined SHH target in neural precursors (Kenney and Rowitch, 2000), is detected in the rosette structures in SHH treated cultures (figure 3.3 A-B). In Activin treated cultures, rosettes are rarely present and there is only diffuse Cyclin D1 staining (figure 3.3 C) and so we sought to investigate a link between SHH and Activin signalling.

Addition of 200ng/ml of SHH (supplemented with 1 μ m purmorphamine, see Chapter 2) at the proneural stage lead to faithful upregulation of SHH target genes *PTCH1* and *GLI1* seen via qPCR analysis (figure 3.3 D-E). Addition of Activin had little effect on the expression of these genes compared with the control cultures. However, when added in combination with SHH, Activin reduced the overall upregulation of *PTCH1* and *GLI1* (figure 3.3 D-E), suggesting an inhibition of SHH signalling-mediated stimulation.

To explain this reduced SHH signalling we investigated the involvement of the SHH repressor protein GLI3. Positive Activin signalling can lead to dissociation of GLI3 from the Activin effector molecule SMAD2, enabling GLI3 to negatively regulate SHH signalling (Liu et al., 1998). To test whether Activin can inhibit SHH signalling and accelerate differentiation in this way, we used the mESC GLI3 knockout cell line, Xt (figure 3.3 F). Importantly, SHH target genes *Ptch1* and *Gli1* consistently show high levels of expression in this cell line compared to wild-type cells, indicating hyperactivity of the SHH pathway (data not shown, Serafi Cambray).

Immunostaining revealed that Activin treatment of these cells was not able to bring about the upregulation of TUJ1 seen in the wild-type cells (figure 3.3 G), suggesting a requirement of GLI3-mediated repression of SHH signalling. To inhibit the action of SHH in the Xt cells, we added the SHH signalling antagonist cyclopamine to replace the absent GLI3. Although the cyclopamine treatment did lead to a greater number of TUJ1-positive cells, this was not to the same extent as Activin plus cyclopamine-treated cells, suggesting incomplete substitution of Activin treatment by cyclopamine (figure 3.3 G) (Serafi Cambray).

In conclusion, Activin signalling may repress SHH signalling, as demonstrated by the reduction in SHH-mediated target gene stimulation. This reduction appears to be dependent on GLI3 mediated repression as the effect is abrogated in Xt mESC-derived proneural cells. However, there appears to be additional mechanisms to the Activin action as cyclopamine-mediated SHH inhibition cannot fully recapitulate the effect of Activin alone.

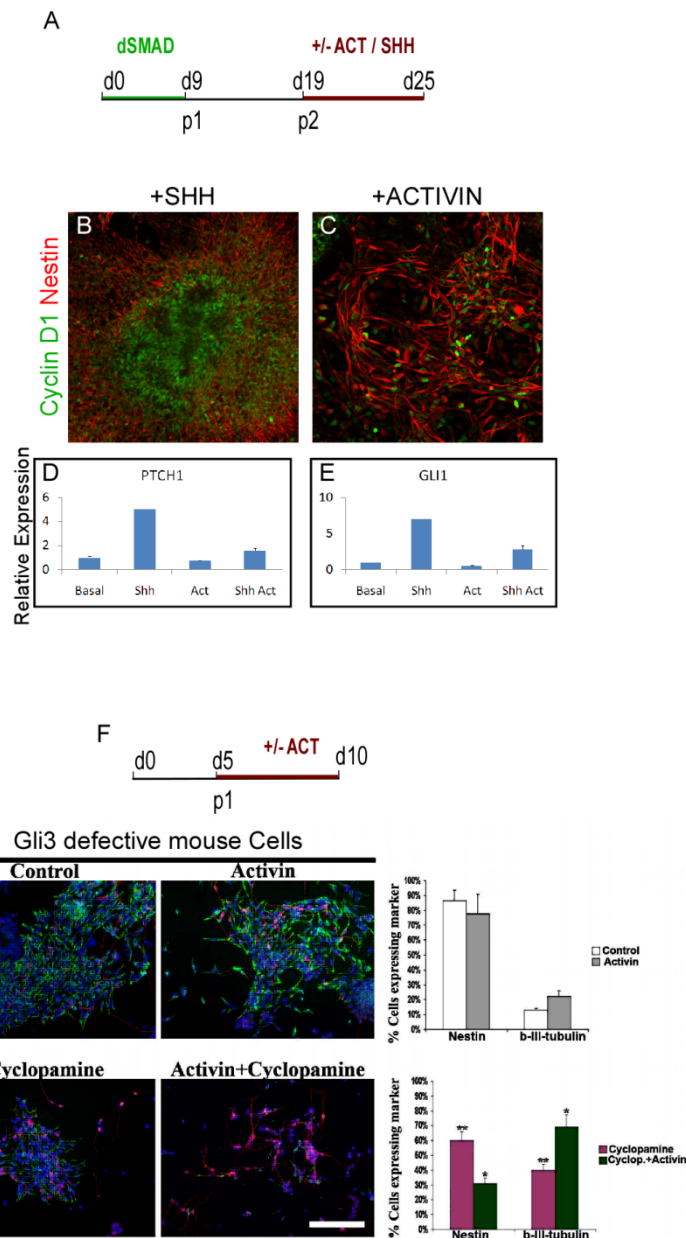


Figure 3.3 F-G
Experiments
done by Serafi
Cambray

Figure 3.3 **Activin accelerates differentiation via reducing SHH signalling.**

(A) Differentiation scheme for H7 hESC MD.

(B-C) Immunostaining for Cyclin D1 (green, a SHH responsive gene) and Nestin (red, neural precursor cells) after 6 days of Activin treatment from day 19 H7 MD. Scale bar represents 75µm.

(D-E) QPCR data from cDNA of cells treated for 6 days with Activin and/or SHH from day 19.

(F) Differentiation scheme for Xt mESCs (Serafi Cambray).

(G) Immunostaining and counting data from GLI3 knockout mouse ESC-derived neural progenitors. Cells were treated with Activin and/or cyclopamine for 5 days from day 5 of MD. Counting data (right) for Nestin:TUJ1 ratio suggests the degree of terminal differentiation in the different culture conditions. Counting data, [basal (86.7 ± 7%) Nestin (13.3 ± 2%) TUJ1], [Activin (78.3 ± 5%) Nestin

($21.7 \pm 4\%$) TUJ1], [cyclopamine ($60.0 \pm 4\%$) Nestin ($40.0 \pm 4\%$) TUJ1], [cyclopamine plus Activin ($31.5 \pm 3\%$) Nestin ($70.6 \pm 13\%$) TUJ1]. Scale bar represents 50 μ m. Figure part reproduced with kind permission from S. Cambray (Cambray et al., 2012).

3.2.4 Activin sensitises proneural cells to the differentiation signal RA

In the developing telencephalon, transcription of the RA processing *ALDH1a* genes is controlled via FOXH1 (Silvestri et al., 2008), an Activin signal transducer. For this reason I investigated whether Activin had an effect on RA processing to explain the maturation effect.

QPCR analysis revealed that expression of *ALDH1a* genes were upregulated in cells treated with Activin for 6 days (figure 3.4 A-C). Additionally, I observed a reduction in the expression of *CYP26b1*, an RA degrading enzyme. Therefore, the cells are in a primed state to respond to retinol in the media (figure 3.4 B-D).

To further explore this role of Activin via RA signalling, I used small molecule inhibitors that antagonise the nuclear RA receptors, RAR and RXR (AGN193109 and UVI3003 respectively) (Duester, 2008). I performed staining for Nestin versus TUJ1 after treatment with Activin, exogenous retinol and inhibitors to investigate the differentiation status of the cultures. RA was added to the media at day 19 of H7 MD via use of B27 with retinol. Interestingly, in cultures lacking exogenous retinol, the accelerated differentiation after addition of Activin was abrogated as seen by the high proportion of Nestin-positive cells (figure 3.4 E). On addition of Activin in media containing retinol, the number of TUJ1-positive cells was significantly increased as the cells rapidly differentiated (figure 3.4 E). Lastly, addition of the RAR inhibitor (AGN), but not the RXR inhibitor (UVI), reversed this effect, suggesting a signalling cascade dependant on RA-RAR binding (figure 3.4 E-H).

Therefore, the observed acceleration of differentiation seen after addition of Activin appears to be dependent on RA signalling through RAR-dependent transcriptional changes. The data supports a role for Activin in sensitising proneural cells to RA signalling via upregulating the molecules required for processing the RA signal.

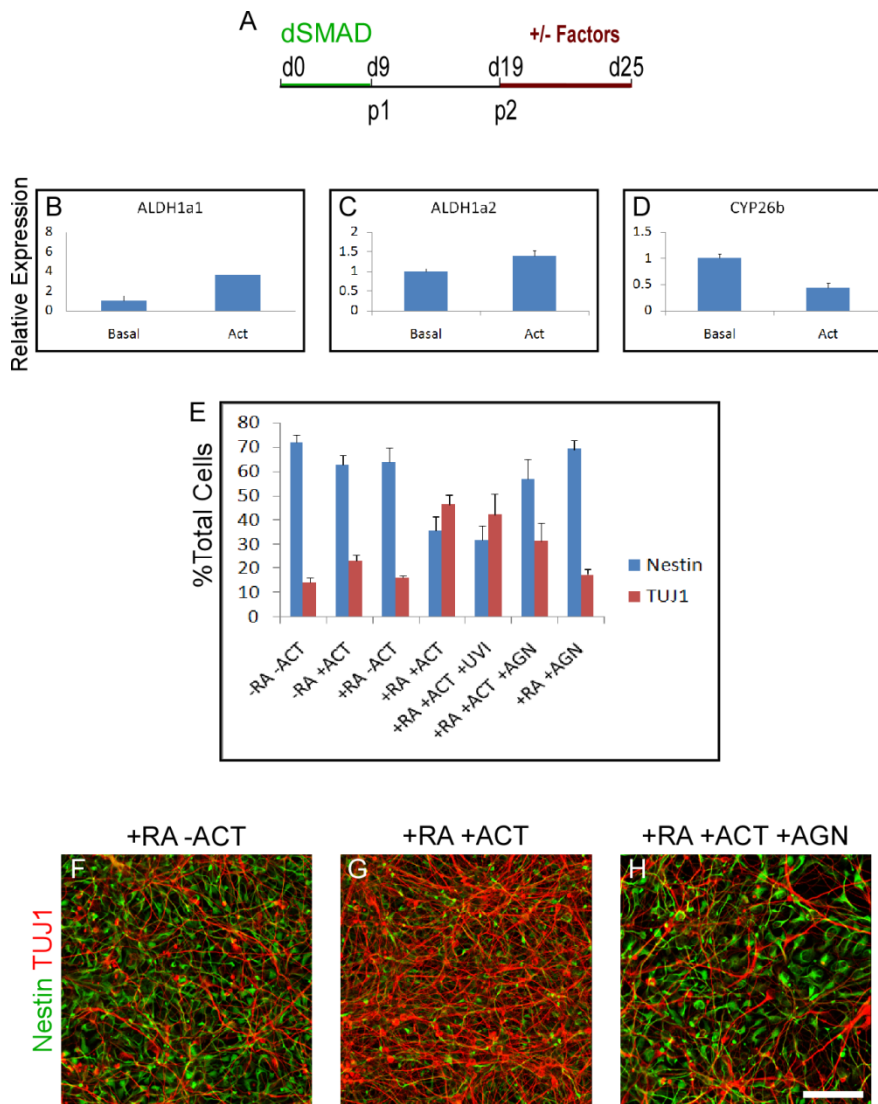


Figure 3.4 **Activin accelerates differentiation via sensitising the cells to RA signalling.**

(A) Differentiation scheme for H7 MD, factors added include Activin, retinol within the B27 supplement and RA receptor inhibitors.

(B-D) QPCR analysis of 6 day Activin-treated neural cultures at day 25 of H7 MD. cDNA analysis suggests an upregulation of RA processing enzymes and a downregulation of RA degrading enzymes.

(E) Counting data to represent the proportion of TUJ1 and Nestin-positive cells in cultures treated with Activin, RA, RAR inhibitor (AGN) and RXR inhibitor (UVI) – biological duplicates. Cells were exposed to conditions for 6 days after passage 2 of H7 MD – biological duplicates. [-RA -ACT (72.1 ± 3%) Nestin (14.3 ± 2%) TUJ1], [-RA +ACT (62.6 ± 4%) Nestin (23.0 ± 3%) TUJ1], [+RA -ACT (64.0 ± 5%) Nestin (16.0 ± 1%) TUJ1], [+RA +ACT (35.9 ± 5%) Nestin (46.6 ± 4%) TUJ1], [+RA +ACT +UVI (32.0 ± 6%) Nestin (42.5 ± 8%) TUJ1], [+RA +ACT +AGN (57.1 ± 8%) Nestin (31.6 ± 7%) TUJ1], [+RA +AGN (69.1 ± 4%) Nestin (17.5 ± 2%) TUJ1].

(F-H) Representative immunostaining to show the differentiation state (Nestin green and TUJ1 red) of neural cultures at the time of counting analysis from (D). Scale bar represents 50µm.

3.2.5 Activin promotes specification towards a Caudal Ganglionic Eminence fate

Having produced postmitotic neurons *in vitro*, I investigated the identity of these cells to test whether a specific neuronal subtype was enriched following the addition of exogenous Activin (figure 3.5 A). Differentiated cells produced via the dual SMAD inhibitor protocol are thought to have a telencephalic character (Fasano et al., 2010) and a number of neuronal subtypes are derived within the telencephalon, including dorsal glutamatergic neurons and ventral GABAergic cells.

First I validated the rostral identity of the proneural cells produced via the Chambers dual SMAD protocol (Chambers et al., 2009). *FOXG1* marks the prosencephalon during early embryogenesis and is critical for telencephalic and optic vesicle regionalisation and development (Xuan et al., 1996). Expression data suggests an increase of *FOXG1* (figure 3.5 B) concomitant with neural fate determinants *SOX1* and *PAX6* (figure 3.1 H-I). I also see the majority of the cells stain positive for *FOXG1* prior to the rosette stage at day 17 of H7 differentiation (figure 3.5 C).

QPCR analysis after 6 days of Activin exposure demonstrated that the treated cells downregulate markers for the dorsal pallium such as *PAX6* (figure 3.5 D). Contrarily, *GSX2* and *DLX2*, makers of the ventral telencephalon, are upregulated, consistent with a subpallial fate. In support of this observation, the expression of *GAD67*, a gene responsible for the production of GABA, is also upregulated. Upregulation of genes associated with the dorsal CGE such as *ZCCHC12* and *ER81* suggests a bias towards this region within the *GSX2/DLX2* positive ventral telencephalon. I also see a 3.8 fold upregulation of *Calretinin* in Activin treated cultures, demarcating the major interneuron cell type produced in the dorsal CGE (figure 3.5 D).

Immunocytochemistry of the cells revealed that the number of Calretinin-positive neurons in the Activin-treated cultures was vastly upregulated compared with the control condition; with $79.5 \pm 11\%$ of the H7 neural cells expressing this interneuron marker (figure 3.5 E). These cells coexpressed *TUJ1* (figure 3.5 E-I), demonstrating they were postmitotic neurons, they colabelled with GABA, and appeared to have the bipolar morphology typical of the dCGE derived cortical interneurons (figure 3.5 J-K). These results can be recapitulated in three independent cell lines, demonstrating a consistent effect of Activin across cell lines with more than 60% Calretinin-positive neurons (figure 3.5 F-I).

In conclusion, the data supports a directed differentiation towards a dCGE fate after Activin treatment. Upregulation of markers specific to the dCGE and an increase in the number of cells positive for Calretinin with an appropriate morphology suggests that there was a specification of Calretinin interneurons in the Activin treated cultures.

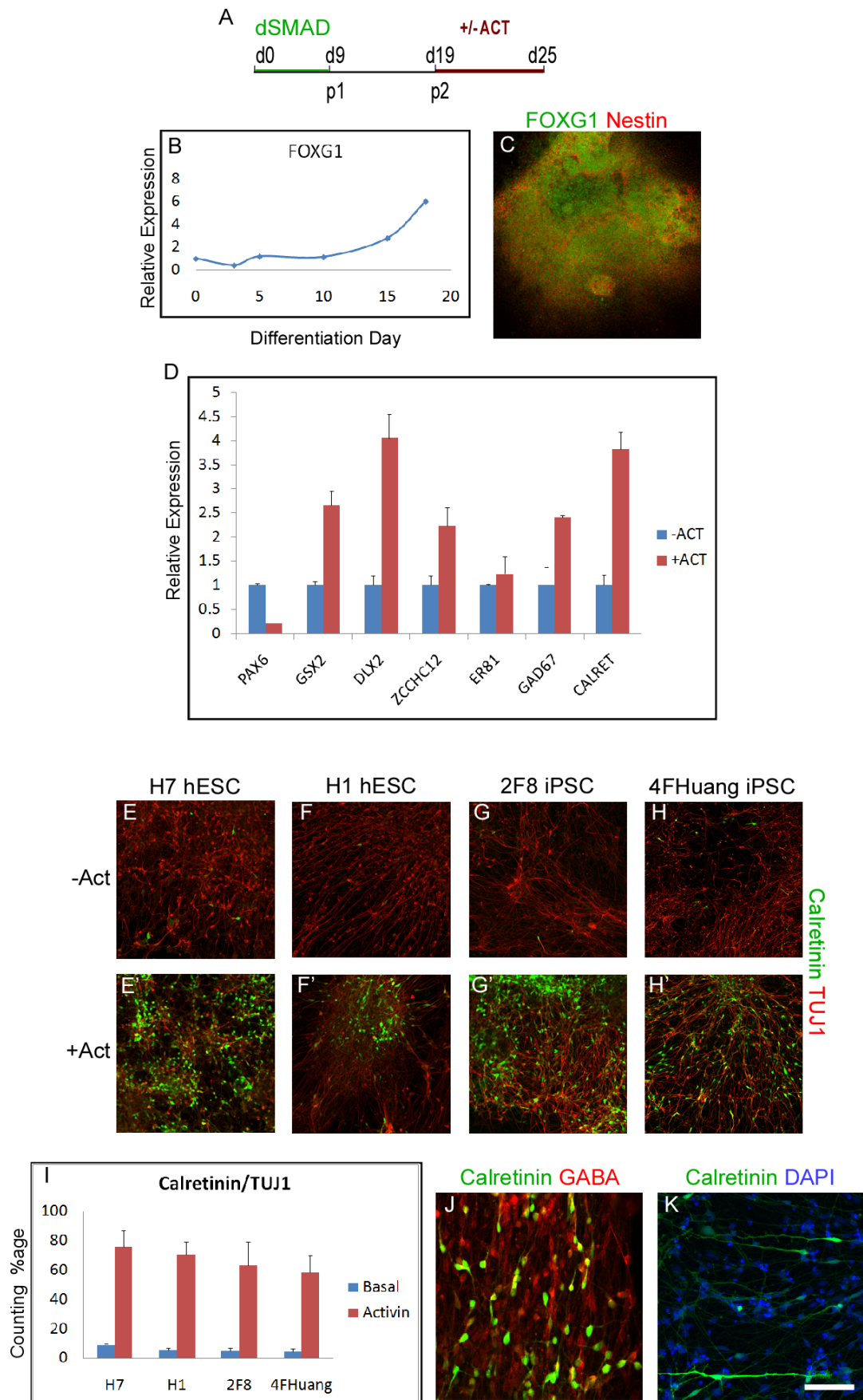


Figure 3.5 Activin directs differentiation towards a CGE-like fate.

(A) Differentiation scheme for H7 MD.

(B) QPCR analysis of basal MD conditions to show expression of the telencephalic marker FOXG1 during early neural lineage commitment.

(C) Immunostaining for FOXG1 (green) and Nestin (red) demonstrates a telencephalic character in the proneural cells at day 17 of H7 MD. Scale bar represents 100µm.

(D) QPCR analysis of neuronal cultures with or without Activin treatment. Dorsal telencephalic markers were downregulated, such as PAX6. General subpallial markers such as GSX2 and DLX2 were robustly upregulated. dCGE enriched markers ZCCHC12 and ER81 were upregulated after Activin exposure. Mature markers of CGE-derived cortical interneurons (Calretinin) and general GABAergic neurons (GAD67) showed a significant upregulation after 6 days of Activin treatment at day 28 of MD compared to the basal condition.

(E-H) Immunostaining of cultures treated for 6 days with or without Activin for Calretinin-positive neurons, Calretinin (green) marks dCGE-derived cortical interneurons and TUJ1 (red) marks all neurons. Scale bar is 100µm.

(I) Counting data demonstrates the proportion of Calretinin staining in all hESC (H7 and H1) and hiPSC (2F8 and 4FHuang) lines tested – biological duplicates. H7 basal ($8.9 \pm 1\%$) Activin ($76.0 \pm 11\%$). H1 basal ($5.4 \pm 1\%$) Activin ($70.3 \pm 9\%$). 2F8 basal ($4.9 \pm 2\%$) Activin ($63.2 \pm 16\%$). 4FHuang basal ($4.4 \pm 2\%$) Activin ($58.7 \pm 11\%$).

(J) Calretinin positive cells (green) coexpressed GABA (red), reinforcing the interneuron phenotype. Scale bar is 50µm.

(K) Calretinin (green) neurons exhibited the characteristic bipolar morphology of CGE-derived cortical interneurons. Scale bar represents 50µm.

3.3 Discussion

In this chapter I have described a novel role of the signalling molecule Activin during human pluripotent stem cell differentiation; namely regulating the balance of progenitor cell proliferation versus terminal differentiation and also specifying the terminal neuronal fate choice. The significance of this molecule in forebrain patterning is supported by the expression of receptor proteins throughout the mouse telencephalon (Feijen et al., 1994) and activated SMAD2/3 signal transducers that are evident at an appropriate time of mouse development (Maira et al., 2010).

I have employed and developed a differentiation scheme for hPSCs. I am able to maintain hPSCs in the pluripotent state for an extended period in culture and then, via an adapted version of the dual SMAD inhibitor MD protocol (Chambers et al., 2009), I am able to initiate neural differentiation efficiently with a strong bias towards telencephalic precursors (figure 3.1 F-L and figure 3.5 B-C). The fact that the majority of the neural precursors produced via dual SMAD inhibition have a rostral identity is in keeping with previous studies (Fasano et al., 2010), and this may be explained by the use of high doses of Noggin, a known head inducer (Bachiller et al., 2000). The highly efficient production of neuroepithelial cells via the dual SMAD protocol is important here to maintain homogeneity in proneural cultures, as non neural cells may respond to Activin in other well described manners, e.g. to remain pluripotent (Beattie et al., 2005) or to initiate mesodermal differentiation (Smith et al., 1990).

I have implicated Activin signalling in balancing the fate of neural precursor cells. I see a strong pro-differentiation effect after Activin treatment at the expense of neural progenitor cell maintenance. Cross-talk between Activin and SHH via the GLI3 repressor molecule appears to be key to this balance, as SHH mitogenic signals are reduced by the Activin signal. Inherent in the pro-differentiation effect I see is that Activin has a role in sensitising cells to extracellular retinol, implementing terminal differentiation. This bimodal action of promoting RA-induced maturation, together with the inhibition of mitotic SHH signals, implicates Activin as an important regulator in the differentiation process. This coordination role of Activin may be critical in normal development to properly control tissue growth and developmental timing as tissues switch from growth to maturation. The timing of Activin signal activation may thus be crucial.

I demonstrate that this pro-differentiation effect is consistent with fewer cycling cells in the Activin treated cultures via EdU incorporation (figure 3.2 E). It is noteworthy that, as the cells exit the cell cycle and terminally differentiate, fewer cells are undergoing cell division. For this reason, in the neuronal cultures, there are fewer cells present in total, e.g. as seen in figure 3.2 B-C. Also, the morphology of the Activin treated cells is interesting; it is clear that there is a strong signal to mature

due to the absence of rosette structures and also the elongated morphology of the Nestin-positive cells, suggestive of post mitotic cultures. Nestin marks progenitor cells, but figure 3.2 C and figure 3.3 C both show that the cells are rapidly and actively maturing as the treated cells exhibit a more neuronal morphology than the cells in the basal condition.

The outcome of Activin treatment can be partially mimicked via the addition of cyclopamine, to inhibit Shh signalling (Chen et al., 2002), and 10 μ M exogenous RA in mouse cultures, Serafi Cambray (Cambray et al., 2012). In these conditions, a similar pro-differentiation effect is observed and confirms the bimodal mechanism of Activin treatment observed in this study. However, there remain possible effects of Activin that have not been addressed here, for example, SMAD3 has been shown to alter the cell cycle via p27^{kip} (Garcia-Campmany and Marti, 2007), adding further functional levels to Activin prodifferentiation effects. Also, cyclopamine/RA treatment cannot recapitulate the specification towards CGE. Therefore, further investigations are required to determine the direct and indirect outcomes of Activin signalling leading to the effects described in this chapter.

Activin has a documented survival-promoting effect after insult in the adult brain (Foster et al., 2004; Mukerji et al., 2007; Tretter et al., 1996; Wu et al., 1999). I infer that the enhanced differentiation *in vitro* that I observe is not due to augmented survival of neurons as parallel work in mouse cells demonstrated similar levels of Annexin V and Casapase3 staining in treated and control cells (Serafi Cambray (Cambray et al., 2012)).

Activin also performs a role in specifying terminal differentiation in our cultures. I see an upregulation of genes associated with the molecular profile of the dCGE within the ventral telencephalon. This tissue is the birthplace of calretinin interneurons that migrate to the cortex. In four human PSC lines, I see over 60% of TUJ1-positive cells colabelled for Calretinin after six days of treatment. These cells costained for GABA and appeared to be bipolar, which is an identifiable characteristic of the CGE-derived cortical interneurons. Previous work producing ventral telencephalic cells in culture has focused on SHH patterning (Maroof et al., 2010). However, it has been shown that knockout of SHH signalling *in vivo* can lead to an increase in the Calretinin population (Xu et al., 2004; Xu et al., 2010). Unlike the MGE-derived interneurons that are dependent on SHH signalling and its target NKX2.1 (Sussel et al., 1999; Xu et al., 2004; Xu et al., 2010), this study suggests that the dCGE may be independent of SHH signalling, but instead be influenced by growth factors such as Activin and other undefined signals to produce Calretinin-positive neurons.

The mouse cell work demonstrated a consistent downregulation of NKX2.1 after Activin treatment (Serafi Cambray) (Cambray et al., 2012), further promoting dCGE-type cells at the expense of the NKX2.1-positive ventral population. A downregulation of NKX2.1 was not observed in human cultures, which may be explained in a number of ways. Firstly, it has been proposed that human differentiating cells have endogenous high levels of WNT and so have a default dorsal character, whereas mouse cells have higher endogenous SHH levels and so more ventral phenotype (Li et al., 2009). Therefore, Activin may downregulate NKX2.1 that is already present in mouse cultures, whereas in human cells, the ventralisation from dorsal character may have little effect on an already low NKX2.1 expression. Alternatively, having been exposed to dual SMAD inhibitors, the human cells may have a different endogenous signalling activity from the mouse cultures and so respond differently to exogenous Activin due to intrinsic factor dissimilarities. Lastly, there may just be species differences between mouse and human development, whereby homeobox genes have been employed to promote different outcomes during divergent evolution; i.e. Activin and NKX2.1 may have a different relationship in human and mouse development.

The overrepresentation of Calretinin in our neuronal cultures suggests a cortical interneuron phenotype; however, Calretinin is expressed promiscuously in many areas of the brain. There is a lack of many good markers for CGE derived interneurons, nevertheless, the cells produced here colabel for GABA, are not double positive for REELIN (consistent with cajal-retzius cells, data not shown) and appear to be bipolar in morphology, reinforcing dCGE-derived interneuron identity.

To test the functionality, electrophysiological studies were performed on the mouse ESC-derived neurons. Not only did cells appear to be functional with firing action potentials and relevant ion channels, but the Activin treated cells displayed a higher proportion of postmitotic neurons than control cells, demonstrated via ability to fire multiple action potentials after depolarising current. This work reinforces our findings implicating Activin signalling in driving maturation, (Antonios Dougalis and Serafi Cambray (Cambray et al., 2012)). In addition to the electrophysiological data, Activin-treated, murine neural progenitor cells were transplanted into the somatosensory cortex of adult mice (Graham Little and Serafi Cambray), a major target tissue of calretinin interneuron migration. GFP-labelled transplanted cells were able to migrate away from the site of the graft and appeared as vertically orientated, bipolar cells; reminiscent of true Calretinin positive cortical interneurons. This reinforces the identity and functionality of the mouse neurons produced *in vitro* as well as showing an ability of the cells to survive, migrate away from the graft and integrate *in vivo*.

In vivo, Activin expression has been linked to areas of the adult brain after seizure (Foster et al., 2004). In light of our current study, it may prove interesting to investigate *de novo*

interneurogenesis in recovery or protection from seizure after such overstimulation. The authors see an increase in interneurons associated with recovery and revisiting this work in light of our findings may provide new insights into the mechanism of recovery.

Our results infer a novel signalling factor in the development of the CGE and the first directed differentiation protocol towards Calretinin-positive cortical interneurons. It will prove interesting to go back to *in vivo* models to investigate a potential signalling centre for Activin secreting cells and expression data. The CGE has been defined relatively recently and this current work will help to understand the nuances of the development of this tissue. The current work provides a platform for new marker discovery and the study of development, especially in the human system where direct *in vivo* investigations are impossible. The functionality of the cells also opens up possibilities for disease modelling with a potential view to cell therapies for interneuronopathies, as well as drug screening and toxicology tests. Cortical interneurons have been associated with epilepsy, schizophrenia and autism and potential cell therapies can be further studied using protocols such as this. For example, transplantation of ESC-derived cortical interneurons can be used to investigate the reduced spread of seizures, similar to studies using foetal-derived tissues (De la Cruz et al., 2011). The role of Activin in dorsoventral patterning may initiate further investigations in other tissues along the rostro-caudal axis.

Chapter 4 Activin Promotes Differentiation of hPSCs towards a Striatal Projection Neuron Fate

4.1 Introduction

Medium spiny neurons (MSNs) are the major projection neurons of the basal ganglia and are found in the striatum, within the ventral forebrain. MSNs are marked by the protein DARPP32 and comprise 95% of the cells in the striatum (Ouimet et al., 1984). MSNs receive inputs from glutamatergic cortical neurons, thalamic cells and mesencephalic dopaminergic neurons and subsequently project to the globus pallidus and the substantia nigra (reviewed (Gerfen, 1992)). These circuits are involved in voluntary movement related to memory, associations, emotion and reward. Multiple diseases are implicated in this circuit such as schizophrenia, Parkinson's disease and most notably Huntington's disease which is characterised by an early preferential degeneration of the striatum and specifically the MSNs (Lange et al., 1976; Reiner et al., 1988).

During development, the striatal anlage is represented by a bulge in the ventral telencephalon and termed the lateral ganglionic eminence (LGE) due to its anterior-lateral position relative to the medial and caudal ganglionic eminences (MGE and CGE respectively). The MGE and CGE produce populations of cortical interneurons that migrate tangentially and dorsally to cortical target tissues, whereas the progenitors from the LGE mature radially to form the striatal projection neurons. The dorsal LGE is also the birthplace of a small population of interneurons that migrates rostrally from the LGE towards the olfactory bulb (Waclaw et al., 2009). MSNs can be tracked through development using a number of marker genes. *GSX2* expression marks the subventricular zone cells of the entire ventral telencephalon and *DLX1/2* has a similar expression pattern and is thought to be involved in the same transcriptional networks (Corbin et al., 2000). The LGE is distinguished from the MGE via a lack of the MGE marker *NKX2.1* and expression of striatal-enriched genes such as *CTIP2* (Arlotta et al., 2008) (which is maintained in mature MSNs), *NOLZ1* (Chang et al., 2004) and *FOXP1/2* (Takahashi et al., 2003a; Takahashi et al., 2003b). Critically, these striatal-enriched genes are not exclusive to the striatum and are often expressed in the cortex. However, a lack of ventral *NKX2.1* and dorsal *PAX6*, together with the combination of these striatal expressed genes can be used to reinforce a striatal positional character (Flames et al., 2007; Long et al., 2009b). As discussed in the previous chapter, the CGE is a caudal extension of the LGE and the MGE and shares similarities with the two tissues in a dorsal or ventral manner respectively, e.g. *GSX2*, *DLX2* and *NKX2.1* expression (Flames et al., 2007).

The mature MSNs are most specifically marked via *DARPP32* expression (dopamine and c-amp regulated phosphoprotein, which is involved in dopaminergic signal transmission). The cells are morphologically distinct; given that they are multipolar, with spiny projections forming synapses with many cells and cell types along their axons. Spiny boutons along the neurite projections form synapses with glutamatergic inputs, whereas the shaft and bouton necks typically forms connections with dopaminergic afferents. The *DARPP32*-positive MSNs are subdivided into two pools depending on projection targets, whereby those connecting to the external segment of the globus pallidus express *DRD2* and *Enkephalin* and those projecting to the ventral midbrain express *DRD1*, *Substance P* and *DYNORPHIN*. These two circuits have opposing functional outputs in the basal ganglia circuitry and there is a reliance on a fine balance between the two to coordinate motor control stimuli. Thus degeneration of the striatum in Huntington's disease upsets this balance within the signalling loop. Additionally, the MSNs within the striatum are organised into a patch and matrix formation, whereby the patches form around innervations from the midbrain dopamine system. The marker Calbindin can be used to discriminate matrix MSNs from patch neurons (Gerfen, 1992).

The signalling factors that will be discussed in this chapter are Activin and SHH (Chapter 1.2). Activin is a TGF β family member and binding of the ligand to type II receptors (ActRIIA and ActRIIB) recruits type I receptors ALK2, 4 and 7. This leads to SMAD2/3 transducer phosphorylation which recruits the coSMAD, SMAD4. This complex then translocates to the nucleus to alter gene expression. SHH is the major hedgehog ligand. Binding of ligand to PTCH releases SMO-mediated signal inhibition, leading to active signalling and results in GLI1/2 mediated transcriptional alterations (see Chapter 1.2).

There is currently no cure for Huntington's disease. Grafts of foetal ventral forebrain tissue (both MGE and LGE) have demonstrated proof of principle for cell replacement therapies in patients with Huntington's disease, where some functional recovery has been shown (Bachoud-Levi et al., 2000a; Bachoud-Levi et al., 2006; Gaura et al., 2004). Therefore, there is an interest in producing MSNs from hPSCs as a limitless supply of cells for putative cell replacement strategies. Additionally, *in vitro* derived MSNs enable Huntington's disease processes to be studied and screens for novel therapeutic drugs to be performed.

In vitro differentiation of MSNs has focused on ventralising forebrain progenitors using low doses of SHH (Danjo et al., 2011; Li et al., 2009) and additional inhibition of dorsal cell types using the WNT antagonist DKK1 (Aubry et al., 2008). As described in the previous chapter, SHH signalling is crucial to maintain *NKX2.1* expression and in turn specify MGE cell types. SHH has been implicated in LGE specification and induction of *GSX2* in these paradigms, however, this specifier role remains

contentious in the literature, as exemplified by the relative difficulty in producing DARPP32-positive neurons in culture and the observation that LGE tissue remains intact after knockout of SHH/SMO (Machold et al., 2003; Xu et al., 2010). Concurrently, the use of SHH in these protocols leads to a high mitotic index in the cultures as demonstrated by large tumour formation after grafting the cells into the striatum of model animals (Aubry et al., 2008; Fuccillo et al., 2006).

In chapter 3, I have implicated the TGF β signalling molecule Activin in dCGE development. The Activin receptor molecules are expressed throughout the forebrain and the active phosphorylated signal transducer SMAD2 is present in the LGE as well as the CGE (Feijen et al., 1994; Maira et al., 2010). For this reason and also the fact that the LGE and dCGE have many genes in common, I decided to investigate the ability of exogenous Activin exposure to enrich an LGE character and produce striatal MSNs. Hitherto, Activin has been implicated in survival of neurons (Schubert et al., 1990) and in neurogenesis after injury in the adult brain (Abdipranoto-Cowley et al., 2009; Foster et al., 2004; Mukerji et al., 2007; Tretter et al., 1996; Wu et al., 1999) but had little role suggested in neural developmental processes.

Thus, there is a paucity of knowledge of the developmental patterning of the LGE and subsequent MSNs. In this chapter, I suggest that human pluripotent stem cells can be effectively specified towards a MSN phenotype using an optimised exposure of the growth factor Activin A compared with Chapter 3. I use the dual SMAD inhibitor protocol (Chambers et al., 2009) to produce forebrain precursors and subsequently show that prolonged Activin exposure specifies cells towards a striatal phenotype. Additionally, we show the cells are functional through transplantation studies in Huntington's model rats (in collaboration with Sophie Precious).

4.2 Results

4.2.1 Activin Enriches an LGE Progenitor Phenotype

As described in the previous chapter, cells exposed to the modified dual SMAD inhibitor protocol (Chambers et al., 2009) exhibit anterior positional identity. This is demonstrated by the expression of *FOXP1* at the neural progenitor stage, a critical early marker for forebrain development (Shimamura and Rubenstein, 1997) (figure 4.1 A, figure 3.1 and figure 3.5).

To investigate the effect of Activin on LGE progenitor marker expression, I treated early forebrain precursors at day 10 of H7 MD with 25ng/ml of Activin (figure 4.1 B), an earlier and higher Activin dosage compared with the interneuron differentiation protocol in chapter 3. After 8 days of treatment, RNA was harvested from Activin treated and control cells and qPCR was performed on the samples (figure 4.1 C). Similar to the previous chapter, I observed around a 4 fold increase in *GSX2* and *DLX2* transcripts compared with the basal condition. Concurrently, there was an upregulation of striatal-enriched genes, *CTIP2* (40 fold), *FOXP2* (7 fold) and *NOLZ1* (2.3 fold). Activin induces a small downregulation of dorsal markers, e.g. *PAX6* (0.6 fold).

Immunocytochemical studies depicted that *GSX2*, *DLX2*, *CTIP2* and *FOXP2* were all enriched in the Activin treated cultures, significantly different from the basal condition (figure 4.1 D). $50 \pm 12\%$ of the Activin-treated cultures expressed the key striatal marker *CTIP2*, with $25 \pm 12\%$ expressing *DLX2* and $16 \pm 7\%$ staining positive for *FOXP2*.

The striatal marker upregulation could be due to an active patterning role on naive cells, or alternatively a specific proliferation of LGE subtype progenitors already present in the cultures. To test this I performed nucleotide analogue EdU incorporation studies. I confirmed the Activin-induced reduction on total proliferation of neural progenitors, seen in chapter 3 (figure 4.1 F, figure 3.2 D-E). When cells were colabelled for *GSX2*, counting data demonstrated that the reduced EdU incorporation was similar within the *GSX2*-positive population compared with the entire culture (figure 4.1 E-F). This suggests that the prodifferentiation effect of Activin is not cell-type specific and that the increase in LGE marker expression is due to a patterning function of Activin.

Finally I looked at the gene dynamics of Activin treatment to infer direct target genes of Activin signalling. Cells treated with Activin at day 12 for 12 hours already exhibited a marked upregulation of *GSX2*, *DLX2* and *CTIP2* and a further 12 hours of Activin exposure lead to upregulation of *FOXP2* and *NOLZ1* (figure 4.1 G). This short-term effect of Activin hints to rapid transcriptional changes and may suggest direct versus indirect signalling targets.

Therefore, Activin treatment of early forebrain neuroepithelial precursors can upregulate the expression of a subset of transcription factors that are expressed in the developing striatum. This patterning role appears to be consistent with downregulation of dorsal cortical markers, implicating Activin signalling in dorso-ventral patterning of the telencephalon. It is important to consider the mutual exclusivity of ventral GSX2 expression and dorsal PAX6 expression, which set up the pallial-subpallial boundary (Toresson et al., 2000).

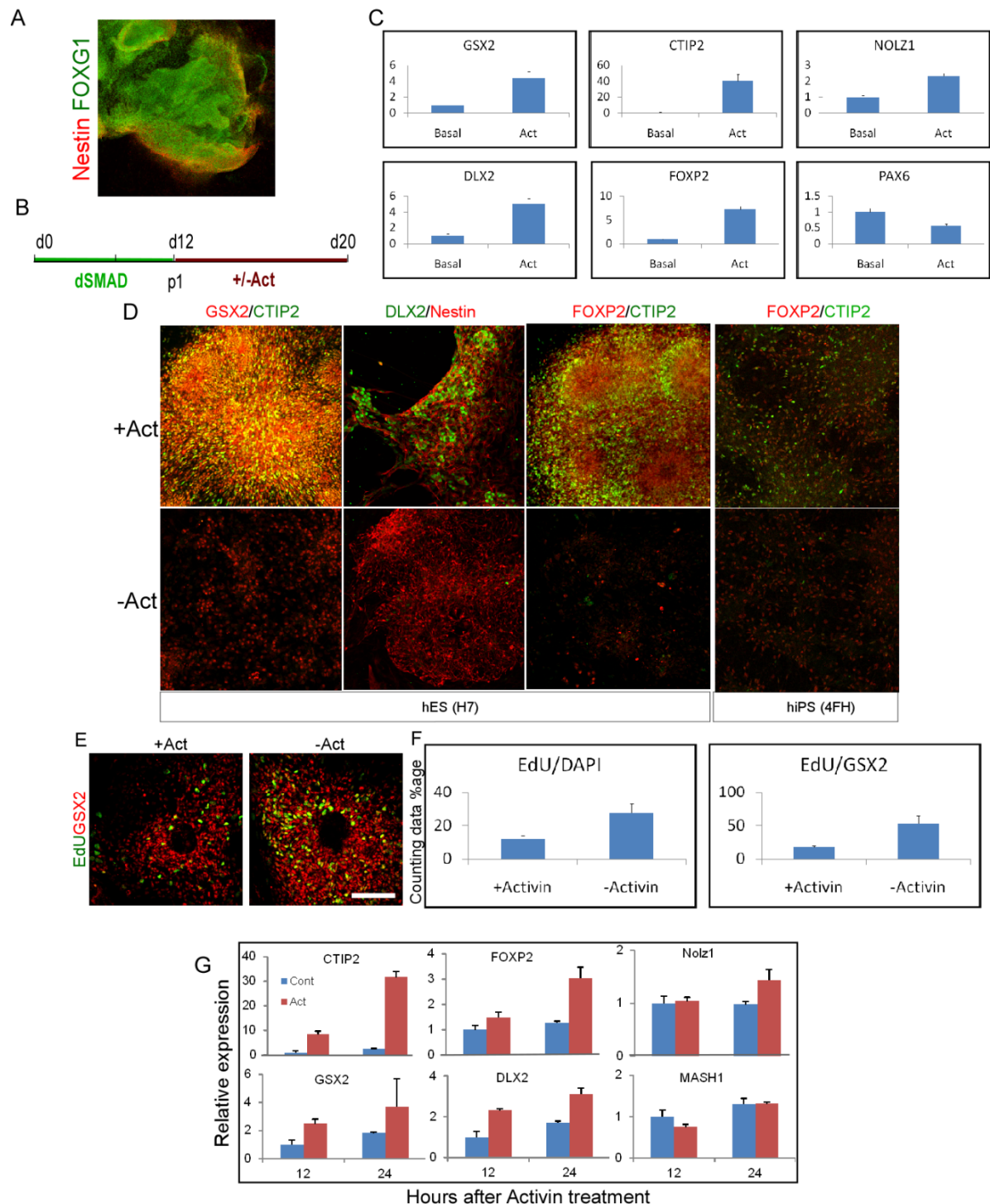


Figure 4.1 Activin patterning effect on telencephalic precursor cells.

(A) Immunostaining for FOXG1 (green) and Nestin (red) at day 18 of untreated H7 MD. Scale bar represents 250µm.

(B) Differentiation scheme to test the effects of Activin on striatal lineage choice in H7 MD.

(C) QPCR analysis of telencephalic progenitor markers at day 20 of H7 MD treated with Activin from day 12. General markers for the subpallium, GSX2 and DLX2, and striatal specific markers CTIP2, FOXP2 and NOLZ1 are all upregulated whereas pallial specific marker PAX6 is downregulated.

(D) Antibody staining at day 22 H7 MD and day 18 4FHuang MD with or without Activin treatment from day 12 and day 8 respectively, for Nestin, GSX2, DLX2, CTIP2 and FOXP2. Scale bar represents 100µm.

(E) Nucleotide analogue EdU incorporation and chemical staining (green) together with GSX2 (red) immunostaining from day 25 cultures. EdU was added to the media for 1 hour. Scale bar represents 75µm.

(F) Counting data for EdU incorporation into the entire cell population (DAPI staining not shown) and within the GSX2-positive subpopulation – biological duplicates. EdU/DAPI basal ($27.5 \pm 6\%$) Activin ($12.0 \pm 2\%$). EdU/GSX2 basal ($53 \pm 12\%$) Activin ($18.1 \pm 2\%$)

(G) QPCR analysis of RNA harvested from cells treated with Activin from day 12 for 12 hours and 24 hours.

4.2.2 Activin Functions in a discrete pathway from SHH to upregulate LGE phenotype

In chapter 3, we proposed that Activin promoted differentiation in part via suppressing SHH signalling (figure 3.3 C-D). Previous studies specifying LGE progenitors have all used precise doses of SHH to upregulate an LGE phenotype (Aubry et al., 2008; Danjo et al., 2011; Li et al., 2009). Therefore, I investigated whether the upregulation of LGE-specific markers by Activin treatment was acting independently from the ventralising morphogen SHH.

To infer a direct patterning effect of SHH and Activin treatment, I investigated the dose response of ventral telencephalic gene expression to the two morphogens. Increasing the dose of SHH lead to increased expression of established SHH target genes from the MGE, namely *NKX2.1* and *LHX8* (figure 4.2 A). Conversely, this was not the case for either early subpallial domain genes *GSX2* or *DLX2* or striatal specific markers *CTIP2* and *FOXP2*. Similar dose response experiments with Activin showed a complimentary effect, whereby MGE-specific markers showed no clear dose response to Activin. However, striatal-specific markers responded in a dose-dependent fashion (figure 4.2 B). General subpallial markers *GSX2* and *DLX2* showed a similar upregulation in response to Activin, reinforcing the results from Chapter 3.

To test whether Activin was acting independently from SHH, I treated cultures with the SHH antagonist cyclopamine in combination with Activin. Consistent with its function, 1 μ M cyclopamine treatment lead to downregulation of SHH target genes *GLI1* and *PTCH1* (figure 4.2 C), whereas Activin treatment had little effect on endogenous SHH signalling. The Activin-dependent upregulation of *CTIP2* and *GAD67* (a marker of mature subpallial GABAergic neurons) was not affected via cyclopamine addition (figure 4.2 C), suggesting that inhibiting SHH has little effect on Activin mediated expression changes of these genes.

Finally, I investigated whether there was a synergistic effect of Activin and SHH treatment (figure 4.2 D). SHH lead to augmented *NKX2.1* expression, to which additional Activin treatment had little effect. This is in contrast to the antagonism of the two growth factors described in figure 3.3. *GSX2* and *CTIP2* were upregulated after Activin treatment, and additional SHH had no significant synergistic effect on this response. *GAD67* expression was further increased in the SHH and Activin condition, consistent with the SHH mediated induction of GABAergic interneurons derived in the MGE, in addition to Activin-mediated induction.

Therefore, I can suggest that the transcriptional changes observed 6 days after Activin treatment appear to be independent of the SHH signalling pathway. Here I argue that LGE and striatal-enriched genes are not under the control of SHH compared with MGE-specific genes, but instead show a dose response to exogenous Activin treatment.

4.2.3 Activin treatment produces medium spiny neurons *in vitro*

Having established that Activin treatment can produce an enrichment of LGE-like progenitors *in vitro*, I continued differentiation to investigate whether the upregulation of LGE-enriched transcription factors can lead to post-mitotic MSN specification. I maintained Activin exposure to the endpoint and neuronal cultures were supplemented with retinol containing media for maturation (see Chapter 3), BDNF (10ng/ml), GDNF (10ng/ml) and ascorbic acid (200nM) to aid cell survival (figure 4.3 A).

RNA was harvested at day 36 and qPCR analysis revealed that many striatal enriched genes were upregulated in the Activin-treated condition (figure 4.3 B). *DARPP32*, *ARPP21*, *Calbindin* and *GAD67* are all expressed in the mature projection neurons of the striatum. The upregulation of these genes compared to the basal condition suggested an enrichment of a striatal character in our cultures. I saw upregulation of genes representing the two subsets of MSNs, namely the *Substance P* (*TAC1* gene) subpopulation as well as the *DRD2/Enkephalin* subpopulation. Dopaminergic neurons can induce *DARPP32* expression. However I observe a reduction in dopaminergic cells, shown by a downregulation of the dopamine biosynthesis gene *TH* upon Activin treatment. Also, the cortical glutamatergic neuron marker *vGLUT1* was similar in the Activin treated and the control cultures, consistent with *PAX6* expression at the progenitor stage.

Immunostaining of the cultures revealed numerous multipolar cells reactive for *DARPP32* with branching axons (figure 4.3 C-D). *DARPP32*-positive cells were seen in four independent hPSC lines after Activin treatment and rarely witnessed in basal conditions. *DARPP32* cells stained positive for mature striatal marker *CTIP2* (figure 4.3 E). In turn, the *CTIP2*-positive cells were *GAD65/67*-positive (figure 4.3 F), indirectly suggesting that the *DARPP32* cells are GABAergic. *Calbindin* positive cells were overrepresented in the Activin treated cultures and many of these cells coexpressed *CTIP2* (figure 4.3 G). *DARPP32*-positive cells also expressed the late neuronal markers *MAP2* and *NeuN*, demonstrating that they are postmitotic neurons (figure 4.3 H). Counting data for *DARPP32* versus *NeuN* revealed that early Activin addition at day 9 lead to the highest numbers of *DARPP32* neurons, with up to 35% of neurons being *DARPP32*-positive. Later Activin treatment had a less dramatic effect on MSN specification (figure 4.3 I).

Consistent with their *in vivo* morphology, *DARPP32*-positive cells appeared to have spines along their processes and the post-synaptic marker *PSD95* accumulated along their processes, demonstrating the presence of synapses (figure 4.3 J-L). *DARPP32* staining is weaker in spines than along the dendrites, meaning *PSD95* and *DARPP32* overlap is difficult to visualise (Ouimet and

Greengard, 1990). Finally, Enkephalin and Substance P staining was observed within the CTIP2 population, suggesting that both striatal MSN subpopulations were present (figure 4.3 M-N).

In conclusion, prolonged Activin treatment enriches the neuronal cultures for striatal projection neuron features. Expression of a panel of genes as well as characteristic staining and morphology suggest that I specify a large proportion of GABAergic medium spiny neurons.

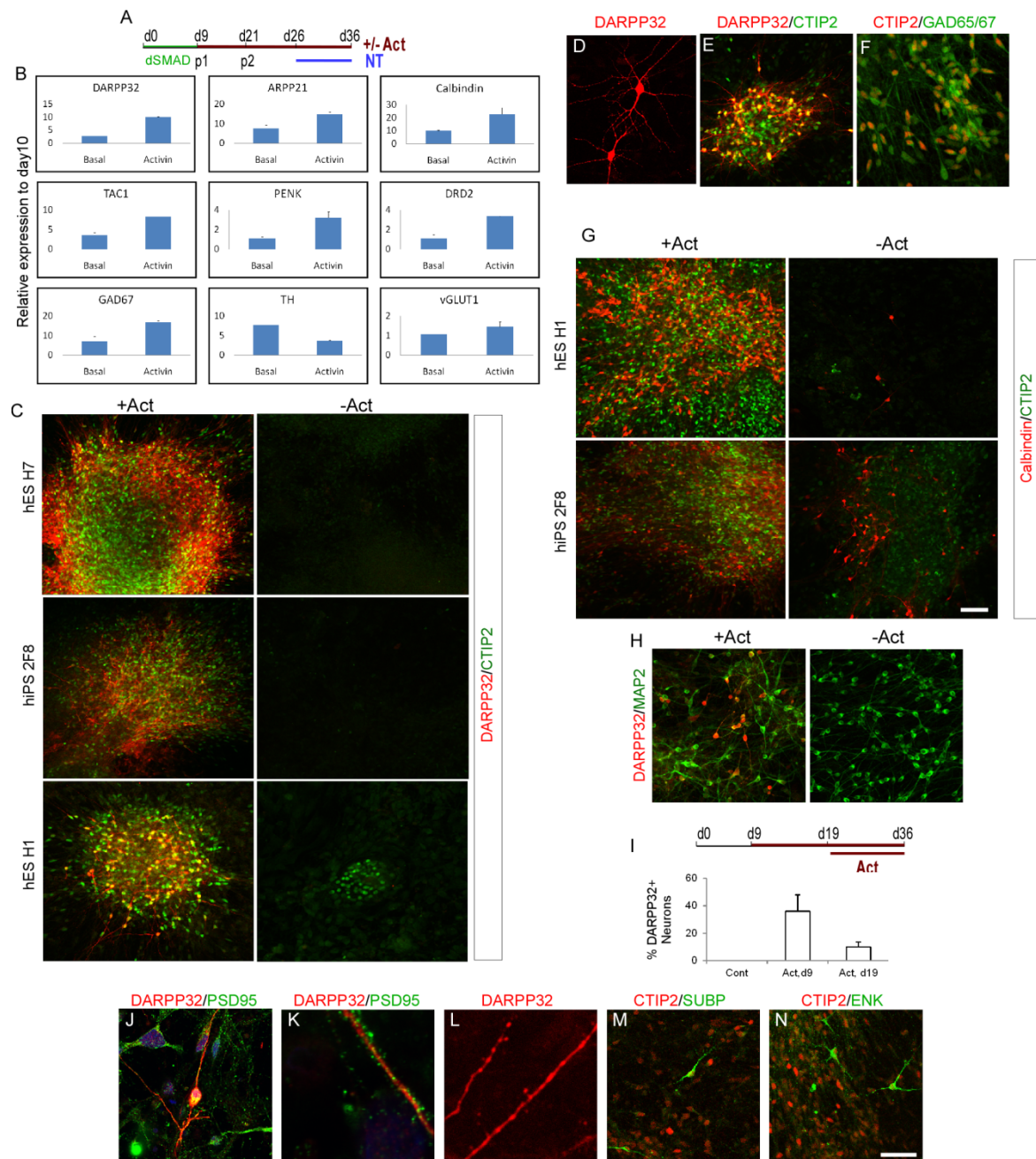


Figure 4.3 Effect of Activin patterning on neuronal cultures.

(A) Differentiation scheme whereby Activin was added from day 9 H7 MD until the end point at day 35. NT (neurotrophins, BDNF, GDNF and Ascorbic Acid) were added to aid cell survival in mature neuronal cultures. See Chapter 2.

(B) QPCR analysis of expression differences between Activin treated and untreated cultures relative to day 10, RNA was harvested at day 35. *DARPP32*, *ARPP21* and *Calbindin* mark the MSN population. *TAC1* (Substance P gene), *PENK* (Pro Enkephalin) and *DRD2* distinguish the two MSN subtypes. *GAD67*, *TH* and *vGLUT1* represent different neuronal types in the cultures, GABAergic, dopaminergic and glutamatergic respectively.

(C)Immunostaining for DARPP32 and CTIP2 in three hPSC lines at day 36 of H7 MD. Scale bar represents 75µm.

(D)Immunostaining to show DARPP32-positive cell morphology after Activin treatment. Scale bar represents 30µm.

(E)Antibody staining showing DARPP32 and CTIP2 expression in the same cells in Activin treated cells. Scale bar represents 75µm.

(F)Immunostaining showing CTIP2 expression within the GAD65/67-positive GABAergic cell population. Scale bar represents 30µm.

(G)Immunostaining to demonstrate CTIP2 and Calbindin coexpression from day 36 H7 MD. Scale bar represents 75µm.

(H-I)Immunostaining of DARPP32 and MAP2 (neurons) and counting data for percentage of DARPP32-positive cells within the neuronal population – biological duplicates. Activin addition at a later stage resulted in fewer DARPP32-positive neurons. Scale bar represents 50µm. Basal (0%) Day 19 Activin ($10.6 \pm 4\%$) Day 9 Activin ($35.7 \pm 13\%$).

(J-L)Immunostaining for DARPP32 and PSD95 demonstrating spiny morphology and post synaptic marker localisation on the DARPP32-positive cell projections. Scale bar represents 40µm in J, 25µm in K and 7.5µm in L.

(M-N)Immunostaining to demonstrate the presence of both MSN subtypes in the CTIP2-positive cell population, via Enkephalin and Substance P colocalisation. Scale bar represents 50µm.

4.2.4 Activin-Induced LGE progenitors give rise to functional MSNs in animal models of Huntington's disease

To test the functionality of the MSNs produced from hPSCs, I dissociated day 18-20 H7 progenitor cells that had been treated with Activin for 6-8 days and grafted them into quinolinic-acid lesioned adult rats (in collaboration with Sophie Precious).

Cells continued to mature *in vivo*, as demonstrated via diminishing Nestin staining seen 4 weeks, 8 weeks and 16 weeks after grafting (figure 4.4 A-C). Nestin staining was present throughout the grafts at 4 weeks but was completely absent after 16 weeks. Importantly, proliferation was low and there was no evidence of rosette-like structures, typifying continued growth of contaminating undifferentiated cells. Subsequently, we saw no tumours or neural overgrowths in the 16 week rats (n=8). This is in contrast to previous studies in which teratoma formation hugely impacted upon the therapeutic potential of *in vitro* derived MSNs (Aubry et al., 2008).

DARPP32 staining was evident in small numbers 8 weeks post transplant, however after 16 weeks, a large proportion of human nuclei (graft tissue) were positive for DARPP32 ($49 \pm 5\%$) (Figure 4.4 D-F). FOXP2 staining was also present throughout the graft cells (figure 4.4 G-I). The DARPP32-positive cells appeared to have processes within and extending outside the graft region (figure 4.4 J). Calbindin staining complimented DARPP32 staining, as this mature marker was present in small numbers after 8 weeks, but many of the graft cells stained positive 16 weeks post transplantation, $35 \pm 8\%$ (figure 4.4 K-L). GABA staining within the HuNu positive graft cells (figure 4.4 M) together with FOXP2 and Calbindin staining; suggest that the Activin treated precursors matured into correctly specified MSNs *in vivo*.

I stained for other cell types in the 16 week grafts. I saw an accumulation of GFAP-positive glia around the graft, possibly indicating a host response to the transplant procedure (figure 4.4 N). Interestingly, I observed TH fibres innervating the graft, suggesting functional interactions between the graft and the host dopaminergic system (figure 4.4 O). I observed a small number of Calretinin-positive cells in the graft (figure 4.4 P), consistent with Calretinin interneuron specification by Activin, presented in chapter 3.

Therefore, Activin pre-patterned LGE progenitors are able to mature into DARPP32-positive neurons *in vivo*. After 16 weeks of maturation *in situ* the graft coexpressed GABA, Calretinin and FOXP2 suggesting true striatal phenotype. Also, innervations from host tissues suggest functionality of the graft cells.

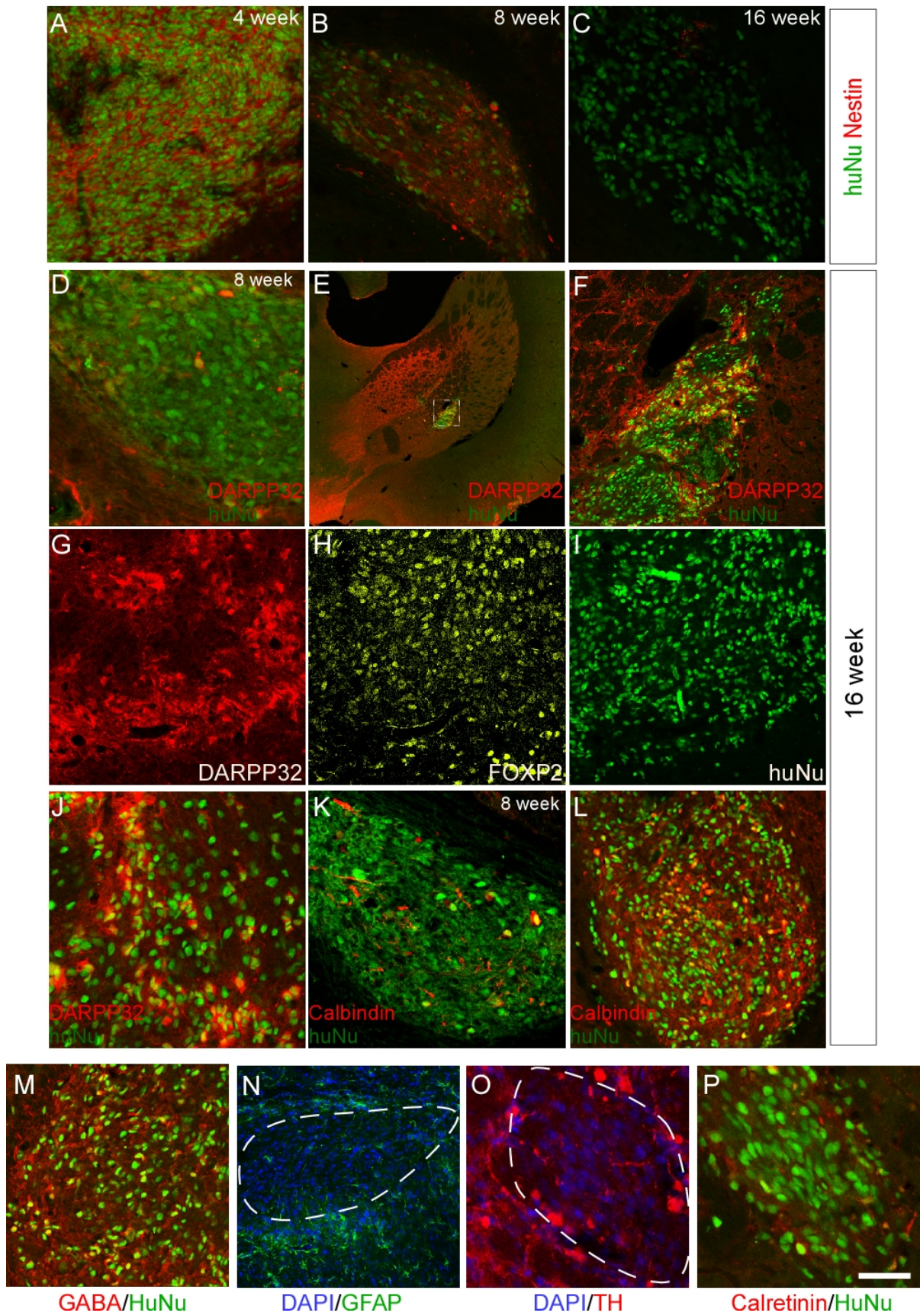


Figure 4.4 **Activin treated progenitors mature *in vivo* to produce DARPP32-positive neurons.**

(A-C) Immunostaining to show Nestin-positive progenitors in the grafted HuNu-positive cells, from 4 to 16 weeks after grafting. Scale bar represents 60µm.

(D-J) Immunostaining for DARPP32 cells within the graft at 8 weeks and 16 weeks. FOXP2 staining was also present in the grafted DARPP32-positive cells in 16 week grafts. Scale bar represents 250µm (E), 50µm (D), 75µm (F), 75µm (G-I), 50µm (J).

(K-L) Calbindin staining in the graft at 8 week and 16 week post grafting. Scale bar represents 35µm (K) and 60µm (L).

(M-P) Immunostaining to depict other cell types in the graft in 16 week grafts. White line represents graft, determined from adjacent sections. Many graft cells are GABAergic, and some express Calretinin (see Chapter 3). GFAP-positive glia accumulate around the graft and there is evidence for TH dopamine cell innervations of the graft (O). Scale bar represents 75µm (M), 100µm (N), 50µm (O-P).

4.3 Discussion

In this chapter I have described a novel role for Activin signalling in patterning hPSCs to MSNs *in vitro*. Previous studies have demonstrated expression of Activin signalling pathway components and phosphorylated SMAD2 in the developing LGE (Feijen et al., 1994; Maira et al., 2010), but have not investigated the developmental role of Activin further. In Chapter 3 we proposed a novel role for Activin in the induction of the dCGE, a tissue that has a number of genes in common with the LGE. Therefore, I adapted our protocol to enrich striatal MSNs, a cell type of much interest due to its degeneration in Huntington's disease, using hPSCs as a model to investigate development.

Compared with the CGE directed protocol that was described in Chapter 3, I found that an earlier, longer and more concentrated exposure of Activin to the telencephalic precursors lead to a greater upregulation of LGE-specific genes, rather than CGE-specific genes. These differences in CGE and LGE enrichment may infer a proximity to putative organiser regions secreting Activin, i.e. the LGE requires a higher dose of Activin and may be closer to the morphogen secreting tissue. Alternatively the tissues may respond with different inherent sensitivity to the morphogen present in abundance, as a lower dose is sufficient to produce Calretinin-positive interneurons compared with MSNs.

The difficulty in producing mature striatal projection neurons from *in vitro* differentiation protocols is demonstrated via the paucity of published articles. Also, primary LGE tissue quickly loses its character with current *in vitro* expansion/culture techniques. DARPP32-positive neurons have been produced from hPSCs in just three studies, Aubry *et al.* (Aubry et al., 2008), Li *et al.* (Li et al., 2009) very recently Ma *et al.* (Ma et al., 2012). There has been one recent study using mPSCs (Danjo et al., 2011). This is in contrast to the significant interest in these cells due to their degeneration in Huntington's disease and the evidence of functional recovery from cell replacement strategies (Bachoud-Levi et al., 2000a; Bachoud-Levi et al., 2006; Gaura et al., 2004). All of the published differentiation protocols use precise doses of exogenous SHH to pattern the precursors towards an LGE fate. However, the use of feeder layers (Aubry et al., 2008) or EB differentiation (Li et al., 2009; Ma et al., 2012) present a degree of uncertainty into the precise signalling events. Also, as discussed in the previous chapter, work in SHH deficient mice argues that SHH signalling is dispensable for dCGE and LGE development (Machold et al., 2003; Xu et al., 2010). Whereby SHH deficient mice (conditional *Smo* knockout) lack MGE-specific *NKX2.1* expression but upregulate LGE-enriched *GSX2* expression (Xu et al., 2010). In addition to this, the loss of ventral telencephalic structures in *Shh* null mice is reversed with GLI3-SHH double knockout mice (Rallu et al., 2002). One can argue that SHH

treatment is permissive for LGE development, potentially via reducing GLI3 repression and allowing LGE determination in a passive manner. Alternatively, SHH may be required in a precise and early spatiotemporal manner during development of lateral tissues. NB Contaminating ventral interneuron fates are often present in the neuronal cultures after SHH-based MSN directed differentiations (Danjo et al., 2011).

In this Chapter, I propose Activin as an instructive factor, capable of driving striatal differentiation. This is shown via rapid upregulation of striatal relevant genes as well as a dose dependent patterning effect. Contrarily, zebrafish model organisms have provided evidence that SMAD2/3 signalling is sufficient to induce SHH signalling in the ventral forebrain (Muller et al., 2000; Rohr et al., 2001). However, I see no such upstream activation of SHH via Activin treatment in our cultures.

I observed an upregulation of numerous markers of the LGE after 6 days of Activin treatment at the protein and RNA levels (figure 4.1). Interestingly, this occurs in a dose-dependent manner with Activin treatment and also is unaffected with cyclopamine-mediated SHH repression (figure 4.2). These data argue that Activin has a specific role to pattern telencephalic progenitors *in vitro* that is novel and independent of SHH signalling. Scans of promoter regions of genes involved in striatal development have shown an enrichment for SMAD2/3 binding (e.g. *CTIP2*, *GSX2*, *FOXP2*, *MEIS1*, *DARPP32*) and these would be interesting to investigate further for direct activation after Activin treatment (rVista alignment). However, it is also possible that intermediary signals are responsible for the observed Activin-mediated effects. One such mediator may be FGF signalling (Gutin et al., 2006), also implicated by Danjo *et. al.* (Danjo et al., 2011), where FGF8 versus FGF15/19 signalling has been proposed to induce anterior LGE rather than posterior fates respectively. Interestingly, Activin binding has been shown in the FGF15/19 promoter and Activin effects may potentially be mediated through FGF signalling (Guzman-Ayala et al., 2009). ChIP and luciferase promoter studies would prove informative to delineate a direct or indirect action of Activin.

I do not witness a downregulation of *NKX2.1* expression in the Activin and SHH treated culture compared to SHH alone (figure 4.2 D). This is in contrast to the observation that Activin antagonises SHH and its target genes when both factors are exogenously added to the proneural cultures (figure 3.3 D-E). One explanation of this might be the different stages of the cells; whereby Activin/SHH is added to the cells at day 19 in Chapter 3 and day 11 in Chapter 4. Signal factor responses are known to be context dependent and influenced by transcription factor expression state. Alternatively, the mitogenic action of SHH described in Chapter 3 and in Cambray *et. al.* may be preventing terminal differentiation at later stages which is antagonised by supplementing Activin;

whereas at earlier time points, SHH may have a more prominent patterning effect (Cambray et al., 2012; Fuccillo et al., 2006).

Continued Activin treatment can bring about DARPP32-positive neurons in our cultures. These cells are neuronal, costain for CTIP2, have a branching, spiny morphology and appear to create synapses throughout the length of their processes (figure 4.3). I use CTIP2 as an intermediary marker to propose the DARPP32 cells are GAD65/67 positive and are representing both Enkephalin and Substance P expressing subpopulations. This step is made necessary due to antibody species conflicts. Thus it appears that simple Activin treatment alone is sufficient to promote DARPP32-positive MSN overrepresentation in our cultures. I add neurotrophins to the neuronal cultures to aid survival and BDNF has been proposed to promote MSN differentiation (Baydyuk et al., 2011; Ivkovic et al., 1997; Nakao et al., 1995). However, I have also observed DARPP32 staining in the absence of neurotrophin addition. The prodifferentiation role of Activin presented in Chapter 3 may lead to upregulation of MSN markers compared with the basal condition simply due to terminal differentiation state. However, I have continued differentiation to day 90 and mature basal cultures continue to show rare DARPP32 staining.

Importantly, the prodifferentiation effect that I see from Activin treatment (figure 3.2 and figure 4.1) has proved to be hugely beneficial for our transplantation studies compared with other studies. When cells were transplanted at day 18 of differentiation, the large decrease in proliferation meant that tumourigenesis/neuroblastogenesis/glioblastogenesis was absent in striatal engraftments. After engraftment of 400,000 cells, we saw an average of 122,705 cells making up the graft. This demonstrates a lack of proliferation but also the cell death involved with the transplant procedure. In contrast, other studies with cells prepared using mitogenic SHH either produced large tumours (Aubry et al., 2008) or continued to grow 35 fold, depicting large graft volume (Ma et al., 2012). Alternatively, the use of the dual SMAD inhibitor protocol may reduce contaminating undifferentiated hPSCs, and the lack of growth may simply be due to increased homogeneity in the neural precursor population before Activin treatment.

I observed faithful differentiation of LGE-like human progenitors into DARPP32 neurons in the transplants, presumably due to further endogenous instructive factors *in vivo*. Conservative counts demonstrated that 49% of all HuNu cells were DARPP32-positive after 16 weeks *in situ* (figure 4.4). This *in vivo* maturation process is slower than *in vitro* differentiation due to suboptimal, adult brain environments with reduced support cells in the lesioned striatum. This is more rapid than primary human foetal tissue that can take 6 months to exhibit MSN character after grafting. A lower proportion of the grafted cells were Calbindin-positive (35%), mirroring the *in vivo* state where only

matrix derived MSNs, outside the striosomes, express Calbindin. Interestingly, Calretinin-positive neurons were present in the grafts, reinforcing the role of Activin in dCGE development, our findings in Chapter 3. The low numbers of Calretinin-positive interneurons is presumably due to the site of the graft in the striatum disfavours CGE differentiation. CTIP2 staining could not be performed on the graft as the rat derived antibody cannot be visualised in rat tissues due to cross-reaction of host immunoglobulins.

Our preliminary data suggests that the MSNs produced via this Activin protocol are functional. *In vitro*, I see presence of synaptic boutons and synaptic marker staining along the length of the DARPP32 processes, characteristic of MSNs and suggesting functional synapse formation. Electrophysiology was performed on these cells and the results show functional neurons with action potentials and GABAergic features consistent with MSNs (Jessica Risner, data not shown). Additionally, the interaction of the graft with the host dopaminergic system *in vivo* after transplantation, suggests that the Activin-derived MSNs are able to integrate into the lesioned rat striatal system.

Studies are ongoing into the functional recovery of the Huntington's model animals after grafting. Previous investigations have shown that both MGE tissue and LGE tissue convey benefits when grafted into the striatum of lesioned animals and clinical studies have used whole ganglionic eminence (WGE) grafts (Precious and Rosser, 2012). Therefore, it will be informative to compare the behavioural recovery after an Activin-based protocol and the exciting recovery published by Ma et al. (who use a SHH-based protocol and presumably contain a subset of interneurons, similar to WGE grafting) (Ma et al., 2012).

In conclusion, I have implicated a new player in the differentiation of MSNs *in vitro*. I utilise hPSC differentiation to model development and aid discovery of developmental mechanisms. Activin may thus be involved in development of this tissue and should be further investigated *in vivo*. SMAD2/3 ChIP assays would prove interesting to suggest a direct inductive capacity of Activin on striatal genes. The simple differentiation protocol presented here will impact on the field to improve current differentiation regimes and may be used for future cell replacement studies, drug screening and toxicology tests for striatal tissues.

Chapter 5 Temporal Control of FGF Signalling Specifies a Midbrain Dopaminergic Cell Fate

5.1 Introduction

The dopaminergic neurons of the ventral midbrain have received much attention due to their significance in Parkinson's disease. Midbrain dopaminergic cells are present in two populations; the neurons of the ventral tegmental area (VTA) are involved in the mesolimbic pathway that functions in emotion and reward behaviour. The substantia nigra (SN) forms the nigrostriatal pathway that functions to control postural reflexes and initiation of movement (Bjorklund and Lindvall, 1984). Degeneration of the nigrostriatal pathway is implicated in Parkinson's disease for which there is currently no cure. After the merits of foetal tissue derived therapeutic approaches, hPSCs are being viewed as a potential source of dopamine cells for Parkinson's disease cell therapy (see Chapter 1.3).

The differentiation of correctly specified midbrain dopaminergic (mDA) neurons from hESCs *in vitro* has proved difficult. Dopaminergic neurons, which are marked by the expression of the rate limiting enzyme for dopamine biosynthesis tyrosine hydroxylase (TH), are present in multiple locations in the brain and subgroups can be demarcated by coexpression of marker genes. Midbrain specific markers such as FOXA2, LMX1A and PITX3 help to identify dopaminergic neurons with a ventral midbrain identity. The best results from *in vitro* differentiation have come from transgene overexpression, whereby forced expression of fate determinants such as LMX1A, NURR1 and PITX3 can produce mDA neurons, however, this can be context dependent (Andersson et al., 2006; Chung et al., 2005; Friling et al., 2009; Kim et al., 2003; Roybon et al., 2008). For example the timing of the transgene induction must be during a critical window in which the cells are competent to respond to such effects (Friling et al., 2009). I set out to investigate the extrinsic signalling requirements of differentiating hESCs to be properly specified towards this cell fate.

During development, it has been demonstrated that the differentiating neuroectoderm initially has an anterior, forebrain-like bias, but becomes progressively posteriorised to form the remaining neuroectodermal domains; the activation/transformation model (Nieuwkoop and Nigtevecht, 1954). In this model, activators instruct cells to become neural with an intrinsically rostral identity. Further transformer factors caudalise the cells to produce subsequent posterior neural tissues. As discussed in earlier chapters, cells that are produced from the dual SMAD inhibitor protocol intrinsically have a rostral identity, presumably due to the effect of the forebrain inducer

molecule Noggin (Chambers et al., 2009; Fasano et al., 2010). Therefore I sought to manipulate the extrinsic signalling cues to specify a more caudal, midbrain phenotype.

In this chapter, I will focus on fibroblast growth factor (FGF) signalling. The family of FGF ligands signal through the receptor tyrosine kinase pathway, in which binding of a ligand to the receptor leads to phosphorylation cascades through ERK kinases leading to phosphorylated ERK, which effects transcriptional alterations of target genes (see Chapter 1.2.2).

FGF signalling is known to be required for telencephalic development and is expressed in the anterior neur ectodermal ridge, the rostral tip of the developing embryo (Paek et al., 2009). Specifically, FGF8 signalling has been demonstrated to be critical in inducing *FOXP1* expression, a key transcription factor for forebrain development (Shimamura and Rubenstein, 1997). Additionally, the role of FGF2 in posterior spinal cord development is well documented and exogenous FGF2 can induce caudalisation of the neural tube (Cox and Hemmati-Brivanlou, 1995).

With regards to the midbrain, after formation of the neural tube, FGF (possibly FGF4) and Wnt signalling is vital for normal formation of the Midbrain Hindbrain Border (MHB) (Olander et al., 2006; Shamim et al., 1999; Ye et al., 2001). Subsequent FGF8 signals from the MHB have a morphogenetic function in neural fate specification in the proximal tissues, i.e. ventral midbrain and cerebellum (Marin and Puelles, 1994; Martinez et al., 1991). However, in contrast to forebrain and spinal cord discussed above, preceding MHB formation there are low levels of ERK phosphorylation in the presumptive midbrain (Corson et al., 2003; Lunn et al., 2007).

In vitro it has been shown that ERK signalling is required for ESCs to exit the self-renewal phase, as demonstrated by the inability of ERK2 knockout cells to differentiate into neural or mesodermal lineages while maintaining pluripotency (Kunath et al., 2007; Stavridis et al., 2007). Conversely, FGF2 is applied to neural progenitor cells and pluripotent cells to maintain proliferative, multipotent state and differentiation can only ensue when FGF2 is withdrawn (Tropepe et al., 1999; Xu et al., 2005; Zhang et al., 2001). For later specification of mDA cells, many protocols routinely use FGF8 in conjunction with the ventralising factor SHH, in an attempt to mimic endogenous morphogen conditions of the ventral midbrain where MHB-derived FGF8 has a proliferative role as well as a patterning effect (Barberi et al., 2003; Kim et al., 2002; Lee et al., 2000; Marin and Puelles, 1994; Perrier et al., 2004). Therefore, FGF signalling is very much context dependent and can lead to pleiotropic effects.

These dynamic spatiotemporal patterns of FGF signalling and the detrimental effect described on early neural fate choice (Di-Gregorio et al., 2007) lead us to investigate the action of

exogenous FGF in differentiating hESCs. I aimed to examine whether recapitulating the ERK activity seen during midbrain development would bring about specification of true mDA neurons in our cultures.

This work has been carried out in conjunction with parallel studies of mouse epiblast stem cell (epiSC) cultures by Ines Jaeger and will be cited as such. The work has been published as an efficient way to produce mDA neurons and has since been recapitulated in two similar studies, see Chapter 5.3 (Jaeger et al., 2011).

5.2 Results

5.2.1 Effects of ERK Inhibition after Neural Induction

Human ESCs were differentiated using the modified dual SMAD protocol, as described in Chapter 2, followed by treatment with 200ng/ml recombinant human SHH and 100ng/ml FGF8. Under these conditions dopaminergic neurons were present in large numbers in the neuronal cultures, as confirmed by the expression of the rate limiting enzyme in dopamine biosynthesis, TH (Figure 5.1 A-B). However, when looking at the markers for *in vivo* positional identity of these dopaminergic neurons, the number of TH cells coexpressing mDA specific markers, such as FOXA2, was very low. Further investigation into the relative *in vivo* positional identity of these cells revealed many TH-positive cells coexpressed NKX2.1 and ISL1, characteristic of the dopamine populations of the hypothalamus and the thalamus respectively (Marin et al., 2005) (figure 5.1 A-B).

To recapitulate *in vivo* ERK-phosphorylation status described in Chapter 5.1, I treated the cells with the small molecule ERK inhibitor PD0325901 (henceforth referred to as PD), inhibiting phosphorylation and so activation of ERK. PD was added on day 3 of monolayer differentiation after exit from the pluripotent state, as ERK signalling is required for exit from the pluripotent state (Kunath et al., 2007) (figure 3.1, figure 5.1 C). It is worth noting that this may, or may not, be a mouse specific phenomenon and requires more detailed analysis in hPSCs (Cohen et al., 2010; Greber et al., 2011). When the cells were treated with PD from day 3 until day 7, qPCR analysis revealed that neural differentiation was supported and accelerated as seen by early upregulation of neural lineage makers PAX6 and SOX1 compared with non-PD treated cultures. Cells did not revert to the pluripotent state as they demonstrated similarly low expression of pluripotency markers (figure 5.1 D-F).

In differentiating mouse cells, accelerated neural conversion after PD treatment was confirmed using the SOX1-GFP mouse epiSC line, which expresses GFP driven by the transcriptional promoter of the early pan-neural marker SOX1. Cells treated with ERK inhibitor upregulated the SOX1:GFP at earlier time points compared with the basal condition, with peak expression at day 3 in treated cells and day 5 in control cultures (figure 5.1 G-H, Ines Jaeger).

Immunocytochemical analysis of the cells at the neural progenitor stage, day 11 of H1 MD, revealed a striking difference between the PD treated and the control cells. The proportion of Nestin positive progenitors that coexpressed the forebrain/midbrain marker OTX2 was greater in the treated cultures (figure 5.2 A). Additionally, the key ventral midbrain fate determinants FOXA2 and DMRT5 were similarly enriched in the PD treated cultures (figure 5.2 B-C).

Gene expression was analysed by qPCR for the PD-treated and control neural progenitors. The upregulation of the forebrain/midbrain marker OTX2, and the transcription factors FOXA2 and DMRT5 seen at the protein level was mirrored at the mRNA level at day 7-11 of H7 MD (figure 5.2 D-F). *LMX1a* and *MSX1* are additional transcription factors central to mDA development and these genes also appear to have an early upregulation in the PD treated cultures (figure 5.2 G-H).

Interestingly, *FOXP1* which is critical for telencephalic development is significantly downregulated at the transcriptional level (figure 5.2 I) and the downregulation of *SIX3*, also marking the forebrain domain, reinforces this result (figure 5.2 J). Additionally, the hindbrain homeobox marker *GBX2* shows a striking downregulation (figure 5.2 K).

Next I investigated the effect of ERK inhibition on the active signalling pathways in the PD treated populations. As one might expect, the addition of PD leads to lower *FGF8* expression within the cultures (figure 5.2 L). The expression of *SHH* and *WNT1* suggest changes in the activity of endogenous signalling within the cultures (figure 5.2 M-N) and the action of these pathways is supported by known target gene upregulation; *FOXA2* for SHH signalling and *LMX1a/LEF1* for active WNT signalling (figure 5.2 F, G, O). These qPCR results indicate an early stimulation of Wnt signalling concurrent with ERK inhibition as well as a sensitisation to SHH signals upon PD withdrawal.

These data suggest that during the proneural phase of monolayer differentiation, treatment with the ERK inhibitor brings about gross changes in prominent signalling pathways and also fate determinant expression profiles. Importantly, I see an increase in the number of cells expressing key ventral midbrain fate determinants as well as an increase in SHH and Wnt1 signalling, a combination central to mDA development.

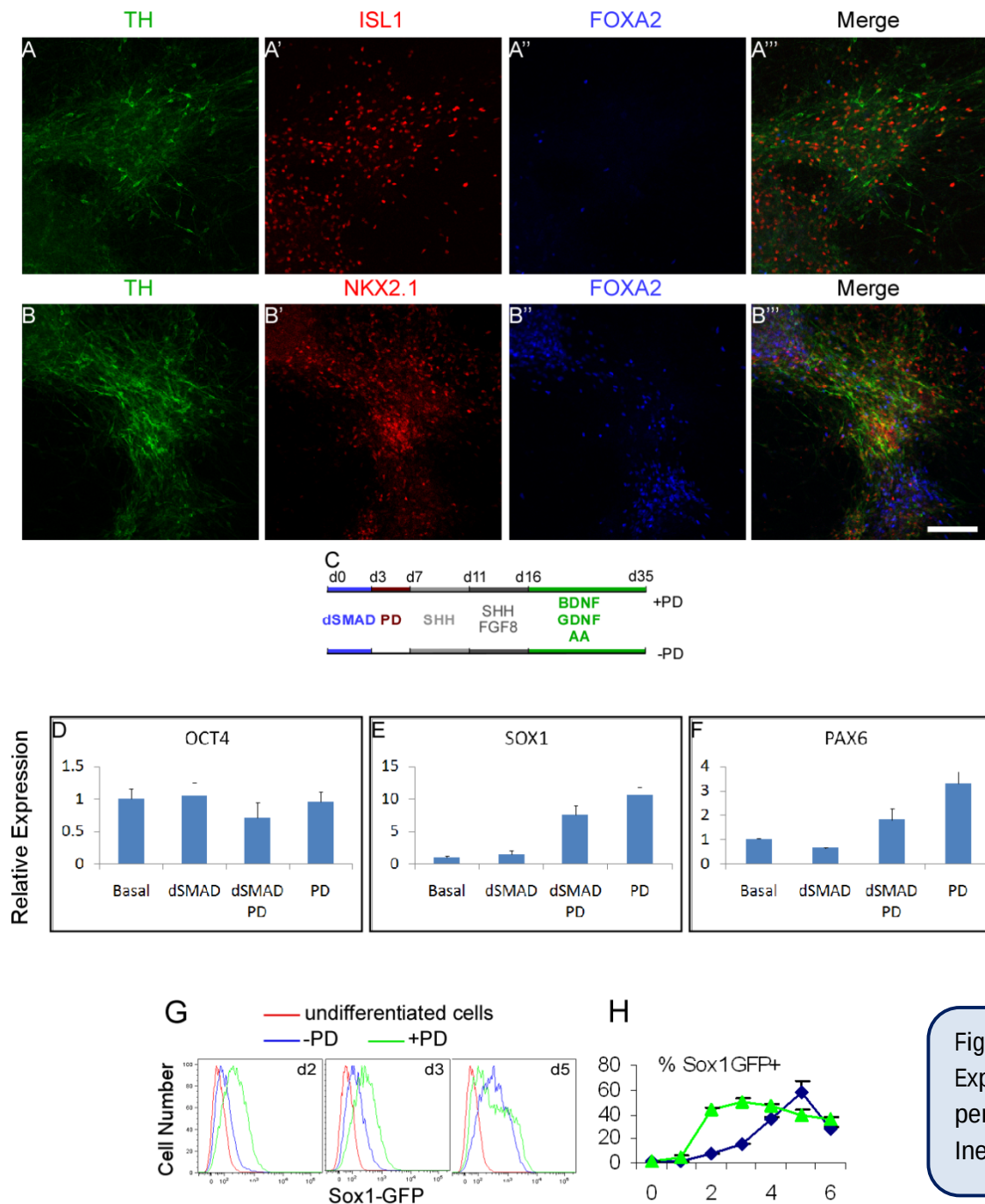


Figure 5.1 G-H
Experiments
performed by
Ines Jaeger

Figure 5.1 **Basic mDA differentiation protocol and early events after ERKi.**

(A-B)Immunostaining of d35 neuronal cultures for dopaminergic (TH green) subtype markers; showing (A) characteristic thalamic (ISL1 red) and (B) hypothalamic (NKX2.1 red) staining and midbrain staining (FOXA2 blue). Cells were differentiated with the dual SMAD inhibitor protocol and treated with SHH and FGF8 similar to the published protocol (Chambers et al., 2009). Scale bar represents 75µm.

(C)Represents the ERKi-based differentiation protocol for H7 MD.

(D)QPCR of early neural differentiating cultures after ERKi. RNA was collected at day 7 after treatment with SMADi (dual SMAD inhibitors) followed by PD-supplemented (ERK inhibitor) or basal media.

(E)FACS sorting of SOX1:GFP reporter mouse epiSCs after N2B27-based MD with or without PD from day 0. Reproduced with kind permission from Ines Jaeger.

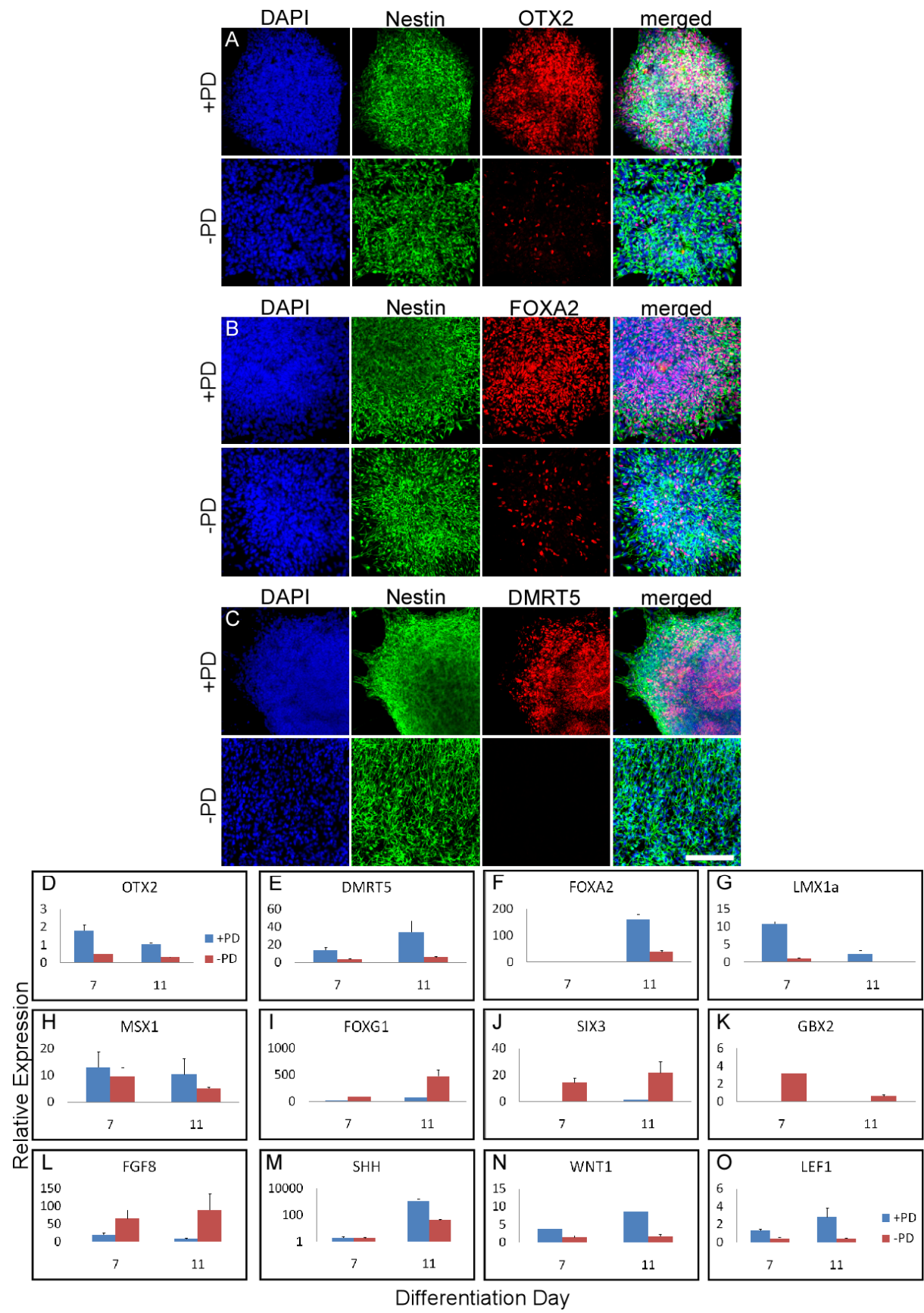


Figure 5.2 Effects of ERK inhibition on neural progenitor phenotype.

(A-C) Immunostaining of PD treated or non-treated progenitors at day 11 of H1 MD for Nestin (green) costained with OTX2, FOXA2 and DMRT5 (red). Scale bar represents 75µm.

(D-O) QPCR analysis of ERKi mediated transcriptional changes early during differentiation relative to day 3 cultures before ERKi. Anterior-posterior markers (D, I-K), dorso-ventral markers (E-H) and signalling pathways (L-O). RNA was collected from day 7 and 11.

5.2.2 Formation of Ventral Midbrain Dopaminergic Neurons

To investigate the phenotype of the postmitotic neurons generated after early ERK inhibition, I continued monolayer differentiation to completion using the classic signalling conditions for mDA differentiation; i.e. treatment with SHH (supplemented with 1 μ m purmorphamine) and FGF8 followed by maturation with neurotrophins; BDNF, GDNF and AA (figure 5.1 C). Therefore, I aimed to mimic *in vivo* ERK status described in Chapter 5.1 by activating FGF signalling later during differentiation, at the time when cells are patterned by signals from the MHB.

Having seen an upregulation of midbrain specific markers at the proneural stage, I looked at the identity of the postmitotic dopaminergic neurons via immunocytochemistry. Colabelling of TH with FOXA2 suggested that many of the dopaminergic neurons in the PD treated cultures were specified towards a ventral midbrain positional identity (figure 5.3 A). This is in contrast with the basal condition where FOXA2/TH colabelled cells were very seldom observed, notwithstanding expression of these proteins in neighbouring cells. Counting data for the two conditions demonstrates that there is an increase in the total number of TH-positive cells in the PD-treated cultures ($53 \pm 14\%$) compared to the control cultures ($27.9 \pm 9\%$). Crucially, the number of these TH cells coexpressing FOXA2 is significantly higher with PD treatment ($77.9 \pm 4\%$) than without ERK inhibition ($7.3 \pm 4\%$) (Figure 5.3 B-C).

In the PD-treated cultures, immunocytochemistry revealed that many TH-positive neurons displayed double staining for PITX3 and LMX1a, both key transcription factors expressed in mature mDA neurons in addition to FOXA2 (figure 5.3 D-E). Other dopaminergic cell types were also present in these cultures, as NKX2.1 and ISL1 staining was evident in the TH population (figure 5.3 F-G). However, the increase in FOXA2/TH-positive colabelled cells via PD treatment may be at the expense of the NKX2.1/TH-positive population, suggested by the decrease in this staining characteristic of hypothalamic dopaminergic cells (figure 5.3 H).

Thus, the data supports that, for the first time in our hands, I have been able to produce dopaminergic neurons that are adequately specified towards a ventral midbrain phenotype. Coexpression of ventral midbrain specific transcription factors suggests that precise regulation of ERK activity allows us to bring about early lineage fate choices, that later enrich for mDA neurons in culture.

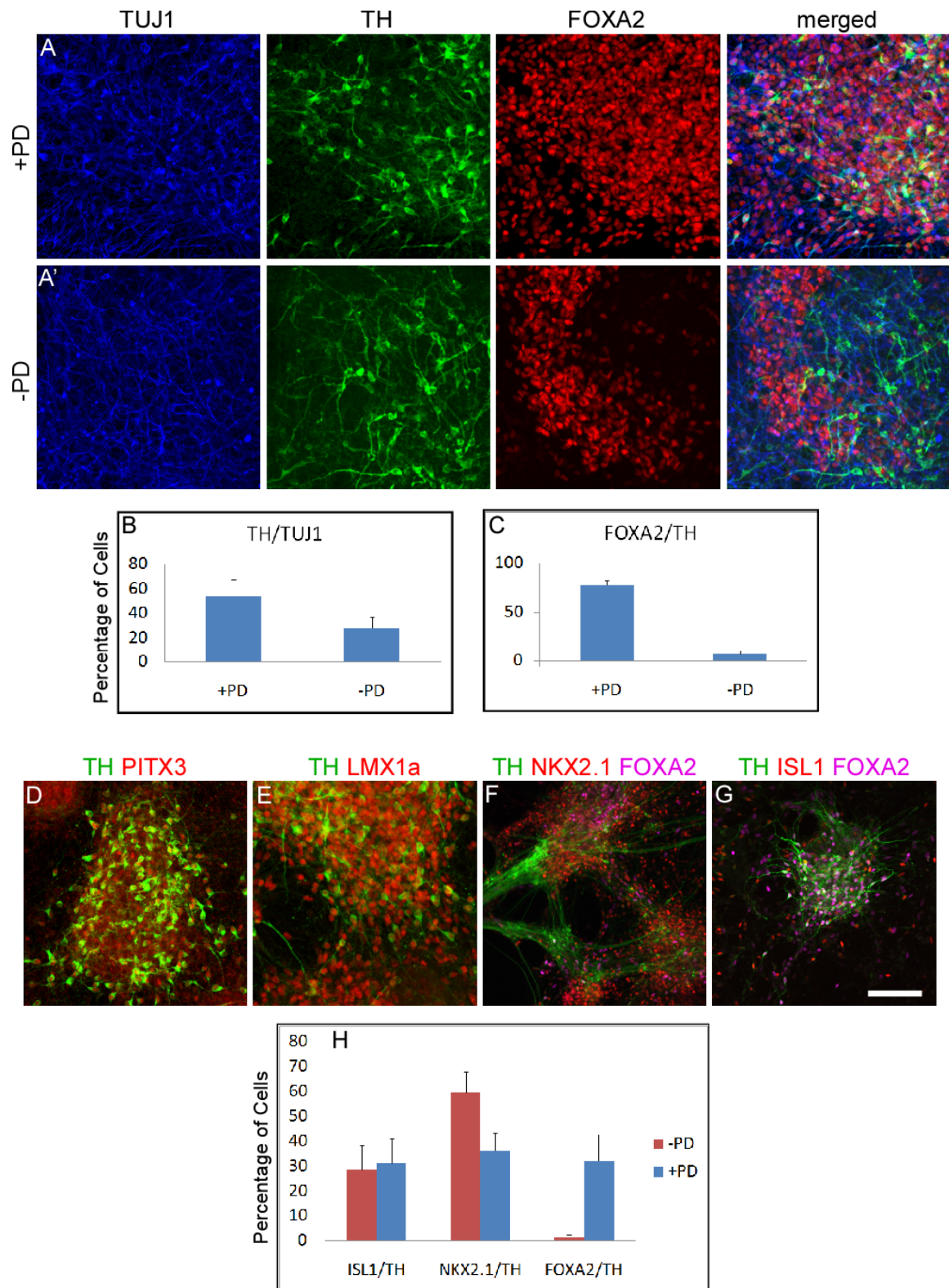


Figure 5.3 **Phenotype of postmitotic cultures after early ERKi-based terminal differentiation.**

(A) Immunostaining to depict neurons (TUJ1, blue), dopamine cells (TH, green) and floorplate cells (FOXA2, red) in day 35 cell cultures with or without early ERK inhibition. Scale bar represents 75µm.

(B-C) Cell counting data for dopaminergic cells in the neuronal population (TH/TUJ1) and midbrain dopamine cells within the dopamine population (FOXA2/TH) with or without ERK inhibition (note this data is an average from experimental replicates). TH/TUJ1 -PD ($27.9 \pm 9\%$) +PD ($53.3 \pm 14\%$). FOXA2/TH -PD ($7.3 \pm 4\%$) +PD ($77.9 \pm 4\%$).

(D-E) Immunostaining for mature cell markers of mDA neurons (PITX3 and LMX1a, red). Scale bar represents $50\mu\text{m}$.

(F-G) Immunostaining to determine proportions of dopaminergic subtype prevalence, depending on marker costaining, after early ERK inhibition. Scale bar represents $75\mu\text{m}$.

(H) Counting data to depict the relative proportions of dopaminergic neuron subtypes from day 35 immunostained cultures (note this data is from one experiment and error bars represent SD of random fields). ISLET/TH -PD ($28.4 \pm 10\%$) +PD ($31.2 \pm 10\%$). NKX2.1/TH -PD ($59.3 \pm 8\%$) +PD ($36.3 \pm 7\%$). FOXA2/TH -PD ($1.3 \pm 1\%$) +PD ($32.1 \pm 10\%$).

5.3 Discussion

In this chapter, I have presented data supporting a central role of ERK signalling in the differentiation of hESCs towards midbrain dopaminergic neurons. For the first time, I was able to produce dopaminergic neurons expressing marker genes that suggest a correct, ventral midbrain positional identity by controlling ERK activity in a stage-specific manner.

The dual SMAD inhibitor protocol has been shown to accelerate exit from self-renewal and entry to the neural lineage in a more homogenous manner than previous techniques (Chambers et al., 2009; Chiba et al., 2008). This may provide a high degree of competency to respond to growth factor signals in a synchronous manner and ERK inhibition further strengthens the neural lineage choice as early markers of neural lineages are further upregulated (figure 5.1 D-H). This observation complements studies in hESCs by Greber et al. where FGF inhibition was shown to facilitate neural lineage fate choice (Greber et al., 2011).

Inhibiting ERK signalling after initial neural induction leads to a sharp increase of signalling factors and endogenous fate determinants that have a key role in the developing ventral midbrain. Parallel work in mESCs demonstrated that the effect of ERK inhibition was primarily due to inhibiting the FGF receptor tyrosine kinase pathway. Using a second inhibitor, PD173074 that specifically blocks ERK signalling at the FGF receptor, we found the same outcome was seen compared with ERK inhibition using PD0325901 (Ines Jaeger) (Jaeger et al., 2011). Thus, the effect of inhibiting ERK is primarily due to inhibiting the ERK-mediated FGF signalling pathway and a change in subsequent signalling environments leads to interesting questions about the presumptive *in vivo* positional identity of the cells in culture.

In model organism development, FGF8 signalling can induce *Foxg1* expression, which in turn is responsible for regional specification in the forebrain (Shimamura and Rubenstein, 1997). More caudally, the homeodomain gene *Gbx2* marks the hindbrain and spinal cord (Ye et al., 2001) and *Gbx2* has been shown to be induced by strong FGF8 signals from the MHB (Liu et al., 1999). Thus, FGF signalling may directly specify these domains meaning early ERK inhibition may lead to a reduction of *GBX2*-positive cells as well as *FOXG1*-positive cells directly. Taken together with the fact that *OTX2* expression is upregulated, the lack of *FOXG1* and *GBX2* demonstrates a homeodomain genetic environment that is permissive for midbrain patterning. Also, it has been demonstrated that Wnt1 signalling can induce *OTX2* expression (Prakash et al., 2006), and the upregulation of *WNT1* seen via PD treatment may provide further *OTX2* specification.

FGF signals from the ANR have been shown to be mutually exclusive and opposing from dorsal, cortical Wnt and BMP signals (Shimogori et al., 2004). Therefore, an initial downregulation of dominant FGF8 signalling via ERK inhibition may be permissive for more posterior domains. Notably, in frogs, Noggin is a known head-inducer (Bachiller et al., 2000) expressed in the Spemann organiser. Treating cultures with Noggin downregulates BMP4 (Anderson et al., 2002; Ohkubo et al., 2002), possibly explaining the anterior nature of the cells after the dual SMAD protocol via an upregulation of FGF signalling.

Importantly, the observed upregulation of *SHH* transcripts and target genes immediately after PD withdrawal hints that the cells are primed to respond to SHH signalling. SHH morphogen signals have been demonstrated to have a ventralising role throughout the neural tube and oppose dorsal Wnt and BMP signals (Ericson et al., 1995). Wnt signals are central to roofplate development; however, the ventral midbrain is a unique tissue where development is dependent on both factors. This explains the expression of Wnt target genes both in roofplate and ventral midbrain such as *Lmx1a* (Andersson et al., 2006). The activity of both signalling networks leads to the proposed two regulatory loops: SHH-FOXA2 (Perez-Balaguer et al., 2009) and WNT1-LMX1a (Chung et al., 2009; Prakash et al., 2006). It is contentious whether these loops are interconnected or have opposing roles (Joksimovic et al., 2009).

Having been exposed to early ERK inhibition and subsequent patterning factors SHH and FGF8, I observed authentic postmitotic midbrain dopamine neurons. This effective mDA patterning in the PD-treated cultures may be at the expense of the more anterior NKX2.1-positive population because of reduced FGF signalling. It should be noted that the percentage of FOXA2/TH cells in Figure 5.3 H is inconsistent with Figure 5.3 C. This is because the data from Figure 5.3 H is from one biological experiment, rather than replicates, highlighting the potential variability for *in vitro* monolayer differentiation.

The induction of fate determinant transcription factors is the functional outcome of the user-mediated exogenous growth factor control. Upregulation of these key developmental genes is the ultimate goal to harness the transcriptional networks leading to proper differentiation of intended cell types. Thus, the early expression of genes such as *FOXA2*, *LMX1A* and *DMRT5* are critical for the ERK inhibitor differentiation mechanism (Gennet et al., 2011; Lin et al., 2009). The importance of upregulation of LMX1a and FOXA2 is exemplified by their inclusion in the factors necessary for direct reprogramming of fibroblasts to mDA neurons and hint to a master regulatory role (Caiazzo et al., 2011; Pfisterer et al., 2011).

I use known target genes of growth factors and the expression of the endogenous proteins to infer dominant and active signalling pathways in our cultures. This is relevant to compare the two conditions, however, further experiments into the mechanisms of ERK inhibition would benefit from improved readout techniques, such as TOPFLASH TCF Wnt reporter assays and luciferase experiments to evaluate active signalling pathways.

In vivo data supports a lack of FGF signalling in the early developing midbrain. Knockout of FGF8 has been shown to increase OTX2 expression domains and reduce GBX2-positive tissues (Storm et al., 2006; Sun et al., 1999). Further *in vivo* investigations into specific FGF ligand signalling, relevant knockout studies and expression data in light of the current work will be illuminating and not only help to explain the results seen here, but further define the mechanisms of mDA development.

Recent studies support our findings, in that early exposure of cells to SHH, Wnt agonists and FGF8 leads to efficient generation of properly patterned mDA neurons (Kirkeby et al., 2012; Kriks et al., 2011). Whereas, Fasano et al. (Fasano et al., 2010) demonstrate that early SHH exposure is sufficient to induce floor plate tissue, Kriks et al. (Kriks et al., 2011) provide evidence that the GSK3 β inhibitor CHIRON, a Wnt signalling agonist, is critical at these early time points to specify an mDA character. Interestingly, this study replicates the importance of Wnt signalling in inducing LMX1A/FOXA2 coexpression seen in TH-positive cells. Also, they provide evidence that FGF8 is detrimental for OTX2 expression, that dual SMAD inhibition alone upregulates a cortical phenotype and that SHH and FGF8 treatment alone specifies hypothalamic tissue types. Without considerations into the initial anterior-posterior positional identity of differentiating cells, we and others now show that mDA fate determination is ineffective.

The strategy described in this chapter represents an important step in the production of mDA cells *in vitro*. Understanding the *in vivo* processes that bring about ventral midbrain development allows us to recreate these pathways in culture. Thus, inhibition and reactivation of ERK in differentiating cells upregulates key fate determinants relevant for ventral midbrain development and enables specification of properly patterned mDA neurons. The functionality of these cells has been supported in further studies; firstly the cells exhibit proper electrophysiological properties of functional neurons and a pacemaker properties consistent with mDA neurons (Jessica Janiczek) (Jaeger et al., 2011) and also, the cells have been grafted into Parkinsonian mice, shown to differentiate correctly and integrate into the host striatum (data not shown, Ines Jaeger and Sophie Precious).

Chapter 6 Discussion

6.1 Summary

In this thesis, I have provided evidence for the validation of human pluripotent stem cell (hPSC) differentiation towards disease-relevant neurons. I have adapted the highly effective neural inductive capabilities of the novel dual SMAD inhibitor protocol from Chambers *et al.* into a scheme based on N2B27 (Chambers *et al.*, 2009; Ying *et al.*, 2003). This has the benefit of being simpler to employ and potentially is quicker than KSR-based strategies (Gerrard *et al.*, 2005; Shi *et al.*, 2012). Consequently, I have demonstrated the effective generation of PAX6-positive, SOX1-positive neural precursor cells. Additionally, I confirm the highly rostral identity of the progenitors produced herein via investigations into the positional information of the cells (Chambers *et al.*, 2009; Elkabetz *et al.*, 2008; Fasano *et al.*, 2010) and discuss the role of Noggin and other SMAD inhibitors in preferentially specifying a rostral neural identity.

This technology allows further investigations into signalling pathways in a controlled manner. The monolayer character of this protocol in chemically defined, xeno-free N2B27 media enables the researcher to have close control over the factors exposed to the differentiating hPSCs. Subsequently, via regarding *in vivo* developmental information as a parallel guide for *in vitro* differentiation, it is possible to implicate growth factors or signalling environments that are able to preferentially produce mature neurons of special interest relating to neurodegenerative diseases. Synthesis of *in vivo* information to control cellular patterning in the anterior-posterior and the dorso-ventral axes provides effective and directed cell patterning. Here I have focussed on Calretinin-positive cortical interneurons which have been implicated in seizure, epilepsy and schizophrenia, DARPP32-positive medium spiny neurons that degenerate in Huntington's disease and midbrain dopaminergic neurons that degenerate in Parkinson's disease.

Novel growth factors and new spatiotemporal roles for known growth factors can be closely investigated using the chemically defined MD in this thesis. Data gained *in vitro* can be used to further define *in vivo* development, i.e. the flow of information can be two way. This becomes especially relevant for human development, with difficulty accessing early developmental events. For example, I investigate Activin signalling in neural development which has previously not been described. This data can then be relayed to the *in vivo* paradigm in future studies.

I use a panel of genetic markers to track differentiation and imply positional information within our cultures via immunocytochemical staining and quantitative PCR. Readouts for major signalling pathways allow us to (1) deduce relative activity of endogenous signalling pathways, (2) to

investigate the subsequent effects on critical intrinsic fate determinants and (3) come up with inferences relative to the developing embryo. In collaboration with others, we argue the neurons produced are functional, firstly via electrophysiological data, hinting to synapse formation and ion channels and secondly via integration into host brains, suggesting proper functionality of *in vitro* derived neurons and speculations into future cell replacement therapies.

6.2 Context

This work has benefitted from the novel dual SMAD inhibitor protocol as the effective and synchronous nature of the cultures enable detailed analysis of exogenous factors with a high degree of confidence, reproducibility and robustness. The low heterogeneity reported via this technique has since been utilised in numerous settings to study the *in vitro* differentiation of a number of neuronal/proneural cell types, see Chapter 1.5.3 (Emdad et al., 2012; Fasano et al., 2010; Lee et al., 2010). This technology is enabling many researchers to study hPSC differentiation and the number of studies is set to increase rapidly. Also, this protocol produces neurons more rapidly than previous protocols, as dual SMAD inhibition initiates differentiation forcibly and the monolayer character reduces the formation of proliferative niches.

I present evidence for new protocols to produce three disease-relevant neuronal subtypes. I generate Calretinin cortical interneurons with an efficiency of more than 60% in culture. I produce medium spiny neurons that develop *in vivo* to make up 49% of grafted cells. And I produce more than 75% authentic midbrain TH cells within our cultures. All produced in chemically defined, xeno-free media and more rapidly than previous protocols.

These works signify the first Calretinin targeted differentiation protocol. The field of cortical interneurons remains relatively novel due to complexity of subpopulations, migration capacities and final widespread location. Current differentiation studies have been based on SHH to reproduce MGE-like character; however, Calretinin-positive dCGE tissue appears to be independent from SHH signalling (Xu et al., 2010). Therefore the implication of Activin signalling in these works provides a novel platform for future work both *in vitro* and *in vivo*.

I demonstrate a chemically defined, xeno-free and rapid induction of MSN fate with Activin signalling. This is in contrast to previous feeder cell-based strategies (Aubry et al., 2008). During preparation of this work, Ma *et. al.* published an EB, SHH-based regime that produces MSNs that can repair Huntington's behavioural symptoms in disease model mice (Ma et al., 2012). This provides validation for cell-based therapies for Huntington's disease and also reinforces the interest in the field that we exhibit. Our work is the first SHH-free scheme (potentially reducing proliferation of grafted cells) and I provide arguments that Activin is the first factor to exhibit direct induction of LGE-specific genes. It remains a pertinent question whether grafting LGE or MGE tissue is optimal for behavioural recovery of Huntington's disease symptoms as both tissues have displayed recovery in animal models (Precious and Rosser, 2012). Comparing an Activin-based protocol and the Ma *et. al.* SHH-based protocol (which produces some MGE-like cells) will prove informative in this facet.

I also demonstrate an efficient scheme to produce mDA neurons with the correct regional identity. These cells will enhance behavioural studies in disease model animals and bring the field closer to clinic. Other studies since this work was published have also produced dopaminergic neurons with correct midbrain identity (Kirkeby et al., 2012; Kriks et al., 2011). These works recreate the early requirement of Wnt signalling. Excitingly the patterned precursors have been utilised for functional recovery in two rodent models of Parkinson's disease and also demonstrated appropriate *in situ* differentiation in primate models.

Thus the three schemes reported in this thesis represent novel, relevant and important advances in the field of human PSC differentiation.

6.3 General Concepts

The use of hPSCs to model disease and produce cells for pharmaceutical and biomedical applications relies heavily on the parallels inferred from *in vivo* development. Differentiation is a model for normal development and exogenous factors are used in a manner to mimic positional cues and instructive events within the embryo. Human development is a process that is unobtainable *in utero* and so hPSCs provide an invaluable tool to study this process. I use analogies from mouse development and the limited data from *in vivo* investigations to propose factors critical for human development.

As such, I employ the dual SMAD inhibitor protocol that functions to specify neural tissue. This is based on work over the last 50 years implicating TGF β inhibitors and other inhibitors of epithelial induction in this patterning event. As such I produce tissue that is characteristically anterior due to the activation transformation model, whereby caudal tissue is produced from, and subsequent to, anterior neural tissue induction.

In chapter 5, I am able to effectively alter the positional identity of these cells at early time points. Using the ERK inhibitor protocol that is presented, I am able to control endogenous signalling within the cultures to promote specific developmental domains, namely the mesencephalon. This is shown to be crucial for differentiation of midbrain dopaminergic cells, as without this regionalisation step, midbrain TH cells are sparingly produced and the cells retain diencephalic/telencephalic character.

In chapters 3 and 4, I make use of the anterior positional identity of the progenitor cells produced from the dual SMAD inhibitor protocol to investigate novel signalling events in the ventral forebrain. Activin signalling has not been studied in detail during development, but active signal transducers have been shown in the LGE and CGE (Feijen et al., 1994; Maira et al., 2010), prompting this work. It is perplexing that this previous work was not further investigated but new stem cell technologies may have enabled access to these previously inaccessible developmental events.

Development has been proposed to progress in the three stages; regionalisation, specification and terminal differentiation (Gale and Li, 2008). This may translate to *in vitro* differentiation as; competence, commitment and maturation. In this thesis, the three chapters address these processes. By controlling FGF/ERK signalling, I am able to alter the regionalisation/competence of the precursors, influencing further differentiation. After this, I use different growth factors to alter specification/commitment towards differentiation lineages. This is addressed in chapters 3 and 4 whereby Activin is shown to promote specific fates within the ventral

telencephalon. Also in chapter 5 SHH and FGF8 treatment confers this specification/commitment step for mDA neurons. Lastly, maturation and survival has been promoted in these studies using neurotrophins to aid survival but also in a new manner using Activin-mediated terminal differentiation (with or without RA).

It is important to understand that the reductionist approach applied here may become superseded by whole genome techniques in the future. Inspecting individual genetic pathways can provide caveats and misleading results as well as providing a partial understanding of the cellular environment. Techniques such as microarrays, ChIP-on-chip and ChIP-seq allow entire gene panels to be elucidated, e.g. SMAD2/3 ChIP-seq would provide a list of genes that are under the control of Activin signalling and may provide further evidence for CGE or LGE developmental relevance and a direct action. Future studies will rely more heavily on such bioinformatic approaches.

It will be interesting to investigate Activin action *in vivo*. Having described its relevance using our *in vitro* model, Activin signal origins (such as Organisier regions) and other responsive tissues can be investigated in the developing embryo. It will be interesting to see if Activin effects maturation in all cells and is ubiquitous in late stage neural development or whether it is more specific to the telencephalon and is unique in specifying subpallial differentiation.

In sum, all of the *in vitro* differentiation work is inherently linked to *in vivo* development. The use of signalling molecules relates to active signalling *in vivo* and transcription factor marker information is taken from developmental studies. Thus, in order to create neurons of interest in culture, developmental processes must be closely adhered to.

6.4 The Future

The disease-relevant neurons produced via these three novel protocols have the potential to be used in several ways. Firstly, the development of human and mouse embryos can be closely investigated outside the uterus. This enables normal development to be tracked, individual genes to be tested, knocked-out, knocked-down and knocked-in, as well as cellular environments to be investigated, such as inhibitors or novel signals. For example, the gene responsible for Activin and its negative counterpart Inhibin, *INHBA*, can be altered at the dose level within the cells to potentially limit or promote MSN/Calretinin interneuron differentiation *in vitro*.

Secondly, the diseased state can be understood. Comparing mutant genes or disease specific iPSCs to the wild-type scenario will allow a greater characterisation of the disease process, especially if this is a developmental impairment. Human PSC differentiation can thus enable advancement of our understanding of diseases, deficiencies, mutations and infections. For example, it may be interesting to test FGF signalling and point mutations present in the wider human population to investigate early events that affect ventral midbrain development, potentially predisposing individuals to Parkinson's disease.

Neurons produced *in vitro* can be of use to biotechnology companies and pharmaceutical industries in two ways. Firstly, new drugs can be tested in a preclinical manner as a first port of call for toxicology screening. This reduces the need for animal testing, solving ethical hurdles, but also greatly reducing cost. The second manner is drug discovery. The use of reporter genes will allow small molecule libraries to be screened to investigate candidate compounds. For example, the ERK inhibition protocol can be used to produce progenitor cells that will enable screening for novel compounds that promote midbrain dopaminergic differentiation or survival. These drugs may then be used *in vivo* to promote endogenous recovery or slow disease progression for Parkinson's disease from resident adult neural stem cells pools or remaining mDA neurons respectively.

It is possible that the functionality of the cells will prove sufficient to reverse neurodegenerative disease models. Cell replacement therapy with these *in vitro* derived cells of interest may then be employed to treat currently incurable diseases in patients. Transplant studies of human foetal tissue have provided evidence for some efficacy in reversing Parkinson's and Huntington's diseases. These proof-of-principle studies have shown variation and the success is still contentious. However, a number of patients have benefited and shown functional recovery and also model organisms show robust recovery after cell grafting. This implicates hPSCs as a source of limitless neurons for cell therapy. Additionally, these cells may be engineered or patient derived to abrogate the need for immunosuppression. Much work is needed to understand and prove the

efficacy of foetal tissue transplants, but assuming the correct site, patient, disease stage, graft cell stage and other criteria are found where cell therapy can be functional, hPSC-derived neurons, such as the DARPP32 cells, Calretinin cells and the midbrain TH cells presented here, constitute a superior source of cells for therapy.

These differentiation processes may be applied to produce many cell types in the laboratory, such as other disease-relevant cells. Early domain specification, subsequent regionalisation based on *in vivo* understanding and consequent maturation may produce any given cell type. Neurons generated in this manner will be valuable to diseases that have an *undefined cause*; as monogenetic diseases such as Huntington's may be addressed in other ways. It is the opinion of the author that this technology from PSCs will remain valid and more functional than direct reprogramming to given cell types (Caiazzo et al., 2011; Pfisterer et al., 2011; Vierbuchen et al., 2010). Direct generation from other cell types will suffer from lack of cell numbers, efficiency and instability of genomic material, limiting its clinical relevance; however, it still remains an interesting academic exercise.

More speculatively, these cells may be used to form cell circuits *in vitro*, similar to basal ganglia circuits. For example, when combined with glutamatergic differentiation protocols, cells can be placed on a chip in a circuit such that an ON state stimulates a glutamatergic projection cell to fire, GABAergic projection cell firing can constitute an OFF signal, dopaminergic projections can synthesise the two signals to produce a final OUTPUT signal and GABAergic interneurons can add layers of complexity to the information processing. This may result in biological computer chips that can store and process large amounts of information as the complexity increases, but also enables electrophysiological studies into synapse functionality and cell to cell interactions. For example, the effect of the site of a synapse can be tested (cell soma versus dendrite extremities).

With a clinical view, the protocols presented here should be taken forward to further optimise efficiency, reduce heterogeneity and augment safety, so that if the biomedical field becomes ready, hPSC-derived neurons are ready for treatment of incurable diseases.

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Chapter 8 Appendices

8.1 Primers used for qPCR

Note all primers listed require 60°C annealing temperature.

Marker Category	GENE	Forward Primer	Reverse Primer	Amplicon
housekeeping				
	GAPDH	atgacatcaagaagggtggtg	cataccaggaaatgagcttg	177bp
	CYCLOPHILIN	ggcaaatgctggaccaaacac	ttcctggacccaaaacgctc	147bp
	B ACTIN	tcaccaccacggccgagcg	tctccttctgcatcctgtcg	351bp
pluripotency				
	OCT3/4	ttctggcgccggttacagaacca	gacaacaatgaaaatcttcaggaga	218bp
	NANOG	gcttgcttgccttgaagca	ttcttgactgggacctgtc	256bp
	SOX2	catggcaatcaaaatgtcca	tttcacgtttgcaactgtcc	119bp
epiblast				
	FGF5	tgt gtc tca ggg gat tgt agg	gaa aac gct ccc tga act tg	125bp
	GBX2	ctcgctgctgccttctc	gccagtcagattgtcatccg	169bp
	OTX2	tgccaaaaagaagacatctcca	aagctgggctccagatagacac	137bp
pan neural				
	MASH1	gtcctgtcgcccaccatctc	ccctccaacgccactgac	251bp
	NESTIN	agcaggagaaacagggcctac	ctctggggtcctaggaattg	218bp
	NGN2	tcaagaagaccgtagactgaagg	gtgagtgccagatgtagtgtg	169bp
	SOX1	ggaaggatcatgtccgaggcc	acttgtccttcttgagcagcg	138bp
	TUJ1	catggacagtgtccgctcag	caggcagtcgcagttttcac	175bp
ap axis				
	EMX1	accggaggacaaagtacaaac	tagtcattggagggtgacatcg	148bp
	EN1	gactcgacagcagcctctc	gcctggaactccgccttg	179bp
	EN2	cggctcgtggtgtttctaacc	aggtcctacaagtcgccgg	79bp

FOXG1	tggcccatgtcgcccttct	gccgacgtggtgccgttgta	77bp
HOXB4	commercially obtained from qiagen	qt00236390	
IRX2	gcccttctacggcaactacac	aattgtgacacctacttgcattg	297bp
LDB1	cgatgtgaagatgtcagtgggctg	atccctatccagcatggtgcc	133bp
SIX3	caactgggttaagaaccggc	ttaccgagaggatggagggtg	218bp
dv axis			
DLX2	actaccctggtaccaccagac	tctgctctcagtctctggcgagttctc	186bp
GSX2	tcactagcacgcaactcctg	ttttcacctgcttctccgac	117bp
FOXA2	att gct ggt cgt ttg ttg tg	tgt acg tgt tca tgc cgt tc	189bp
PAX6	aacagacacagccctcacaaca	cggaactgaactggaactgac	275bp
NKX2.2	cagaaccaccgctacaagatga	gctgtaggcagaaaaggggaatg	186bp
NKX2.1	cgcatccaatctcaaggaat	tgtgccagagtgaagtttg	170bp
NKX6.1	caaggatccattttgttgaca	ggaaccagaccttgacctgact	181bp
PAX2	caggcatcagagcacatc	gtcacgaccagtcacaac	165bp
ISL1	aagcgcaggaagagagactg	ccaagagaccaggatttca	125bp
PAX7	ctcctcaggtgatgagcatcttg	agataatcaacagcagtctggccg	271bp
HELT	accaaggacaggaagtacgc	gctgataggagaaatccggc	118bp
dopaminergic			
LMX1B	cttaaccagcctcagcgact	tcaggaggcgaagtaggaac	118bp
MSX1	actcctcaagctgccagaagat	ttacggttcgtctgtgtttgc	156bp
DMRTA2	cta cct ccg gat ccc tct tc	tct cgg att atc ctg cca tc	152bp
TH	gagtacaccgccgaggagattg	gcggatatactgggtgcactgg	279bp
NURR1	tcggcagagttgaatgaatg	gaaattaaaggtggacagtgt	140bp
LMX1A	gagaccacctgcttctaccg	gcccgcataacaaactcatt	122bp
PITX3 NEW	ctgttgacaggacgcactagcc	cctctggaaggtcgctcttag	278bp
signalling			
ALDH1A1F	ccagacttacctgtctactc	gctcctcctcagttgcaggat	127bp
ALDH1A2F	ctcgctgcagctcctgccg	cacttgctctcctgtggctg	233bp

AXIN2 2F	gctttaacttgaagggg	gatgacgaacatggtcaac	271bp
CYP26F	cgcgacaagagctgaagct	ccaacaaatcgcttgaaca	148bp
DKK1 1F	aaggttctgttctccg	ccttctccacagtaacaacgc	172bp
FGF8	tctgcctccaagcccaggaactg	cggctgagctgatccgtcac	91bp
GLI1	tgaggcccttcaaagccc	gtatgacttccggcacccttc	101bp
GLI3 2F	tcccccacagcaactattc	gtttctcctggcttgaag	153bp
GRG4/TLE4	cagtatccccatcagccagttcc	gctggactaatcggggcatc	265bp
LEF1 2F	gtccaagaatgacagctgcc	tgggttttcaacaagcttcc	122bp
PTCH1 2F	ttcgctctggagcagatttcaag	gcttttaatccaccgcgaag	167bp
RARB F1	tggatcaattacaggcttttagc	gatctccctgcactgaatg	187bp
SHH	ccaattacaaccccgacatc	agtttactcctggccactg	133bp
SPRY1	acaaatccttgcttaggctcag	gaaatccttttcggacaatcc	168bp
STRA6 SERAFI	ggactactcctatggcagctg	gccaggcaggcgtggtacag	123bp
WNT1	cgaccgtattctccgagatg	tgggcgatttctcgaagtag	267bp

telencephalon

CALRETININF	ccttacctgcacctggccga	ccagagcctttccttgccttc	143bp
COUPTF 2 SERAFI	ggagaagctcaaggcactgca	cctgcaagctttccacatggg	119bp
CTIP2	ctccgagctcaggaaagtgc	tcattttacctgcaatgttctcc	129bp
CUX1	cttgaaagggcaaccagag	acctctatggcctgtccac	140bp
FOXP2 1F	aatgtgggagccatacgaag	gcctgccttatgagattgc	137bp
GAD67F	cgtcttcgacccatcttctg	cgcagatcttgagcccagtt	141bp
REELIN	atgtggaggtcgtcctagtaagc	ggaaagtgggtacactcgg	272bp
SATB2	caacgcaactaataatcatctccc	gagaaagggtgagaaccgg	291bp
TBR1	agcagcaagatcaaaagtgagc	atccacagacccctcactag	149bp
VIP1	gttagtaacatctcagaagaccctg	aactcttctggaagtcggg	170bp

of interest

FAF1	ttacaagtatccccgttcgc	atcggtgattgttcttccg	173bp
OSBPL9	ttcgtgctggactacaatgc	aaggctgtcgtcctcatc	134bp
PCNA	ggccagagctcttcccttac	gcctccaacaccttcttgag	193bp

SOX21	cgagtggaaactgctcacag	cggtacttgtagtcgggggtg	102bp
SPATA6	gaggaggatttctggccttc	atcctgagtggtgcatttcc	106bp
striatal immature			
BRN4 F1	ataactagtaggggatcctcaccg	tggaactgagaatgctgtagg	70bp
DLX5 1F	gccggagacagagacttcac	tcaaacactcctgtcatcgc	213bp
DLX6 1F	accattcccagactgacgag	ttcctgattccagttcaggg	221bp
EBF1 F1	aatgtaagcaaggtggacgc	tcaaggtctaagccggacac	177bp
HELIOS 2F	tctgtttggggaggacagac	gtgcttagctgggtcctacc	268bp
IK1 F1	acatgtccaagtttcaggg	cattcgtaaactcctccgac	230bp
NOLZ1 1F	acattttgcaccccgagtac	ggagtacggcttgaaactcg	281bp
striatal mature			
ADORA 2F	cctgtgtacatgtgagcagcc	acacagggttcacctgac	175bp
ARPP21 1F	ggaagctggttgacgatgtgtc	ggcttctgtcgttctacgcc	138bp
CALB F1	atcaggacggcaatggatac	taagagcaagatccgttcgg	168bp
CHRM4 2F	acgaacgatctttgccattc	tgttgacgtagcagagccag	140bp
DARPP 4F	ttggaaaatccagaaaaccg	ctggtagaagccggtgagag	210bp
DRD1 F1	ttgtcatctccttggtgtg	aggccaccagatgttacag	120bp
DRD2 F1	ctgagggtccactaaaggag	cattcttctctggtttggcg	193bp
PDYN 2F	tggcacactctatctcgctg	accgagtcaccacttgaac	245bp
PENK 1F	gctgtccaaaccagagcttc	tctggctccatgggataaag	159bp
TAC1 2F	tgggggtgaaaattcaaaaag	ggagtttccttcttttccg	234bp
cge and mge			
CRABP1 1F	acttatcctgacgtttggcg	tattggtaggggaaaagggc	164bp
ER81 2F	tggagactatttgcttggg	ggcctatgactcagtttggc	294bp
LHX8 F1	ggaaacgcttcccctatttc	tgcgaccaagagtctgtacg	107bp
ZCCHC12 1F	aattctgtgcagctgattgc	caggggacgaactctgaaac	158bp

8.2 Antibodies used for Immunostaining

ANTIBODY	species	company	dilution	reactivity in vitro	in vivo m/rat
5HT	m	abcam	1/50	good	
AMPK B2	rb	custom	1/1000	ok, background	
AMPK P172	rb	custom	1/100	no specific signal	
BRDU	rat	abcam	1/100	no signal (ok serafi)	
CALBINDIN	rb	swant	1/500	very bright	good
CALRETININ	m	swant	1/500	good	good
CALRETININ	gt	swant	1/1000	bad	bad
CALRETININ	rb	synaptic systems	1/500	good	good
CASPASE3	rb	cell signalling	1/100	good	good
COUP TF1	m	r&d	1/100	good	good
COUP TF2	m	r&d	1/100	good	good
CTIP2	rat	abcam	1/500	good	good
DARPP32	rb	sc	1/200	good	good
DARPP32	m	bd		very poor	bad
DAT	rb		1/250		
DLX2	rb	millipore	1/300	good-background	bad
DMRT5	rb	custom	1/1000	overexpressed protein only	
DRD2/3	rb	chemicon	1/200	no good	
ENK	rb	immunostar	1/400	weak	?
ER	rb	sc	1/200	some background	
FOXA2	gt	sc	1/200	very good	good
FOXP1	rb	abcam	1/200	very good	good
FOXP2	gt	abcam	1/100	good	good
FOXG1	rb	abcam	1/250	bad	
GABA	rb	sigma	1/500	good, lots background	ok
GAD67	m	millipore	1/500	poor	
GAD65/67	rb	sigma	1/200	lots background	
VGAT	gt	sc	1/100	no good	

GEPHRIN	m	synaptic systems	1/200	good, background	good
GFAP	m	dako	1/100	good	good
GFP	rb	invitrogen	1/500	good	
GFP	m	invitrogen	1/500	good	
GIRK	rb	alamone	1/200	unspecific	ok
GSH2	rb	lifespan	1/500	tonnes of background	bad
HUNU	m	millipore	1/250	very good	very good
IKAROS	rb	gift mathias lab	1/100	no signal in vivo	bad
ISL1	rat	dshb	1/10	good	
KI67	rb	novocastra	1/500	good	good
LIM1/2	m	dshb	1/10	no signal yet	
MAP2 ABC	m	sigma	1/500	good	
MEIS2	gt	sc	1/500	ok i think	good
MSX1/2	ch	dshb	1/40	no signal	
NANOG	rb	abcam	1/500	bad, no signal	
NESTIN	m	neuromics	1/300	very good	good graft
NESTIN	m	bd	1/300	very good	
NESTIN	gt	sc	1/500	ok, not as good	
NESTIN	ch	neuromics		bad, i think (ines says good)	
NESTIN	m	dshb	1/100	bad	
NESTIN	rb	millipore	1/500	very good	good graft
NEUN	m	millipore	1/250	good, very late	
NKX2.1 (TTF1)	rb	abcam	1/1000	good, but stopped working	
NUAK1	rb	custom	1/100	no specific signal	
NURR1	rb	sc	1/200	ok, background and faint	
OCT4	gt	sc	1/500	very good	
OLIG2	gt	r&d	1/200	good	
OTX2	rb	millipore	1/300	good	
OTX2	gt	r&d	1/500	very good	
OTX2	gt	sc	1/100	good	

PARVALBUMIN	rb	swant	1/50	not sure	
PAX2	rb	covance	1/100	good	good
PAX5	gt	sc	1/100	unsure maybe ok	
PAX6	m	dshb	1/1000	very good	ok
PCNA	m	dako	1/200	bad	
PH3	rb	abcam	1/250	very good	good
PITX3	rb	zymed	1/400	bad	
PITX3	gt	sc	1/50	?	
PSD95	m	thermo scientific	1/200	very good	maybe ubiquitous
REELIN	m	mbi	1/200	no signal yet	
SATB2	m	abcam	1/10	good	good
SOMATOSTATIN	rb	millipore	1/200	?	
SOX1	rb	abcam	1/200	ok, unsure of specificity	good
SUBP	rb	immunostar	1/500	good	not sure
TBR1	rb	abcam	1/500	good	good
TBR2	rb	abcam	1/300	very good	good
TH	rb	pelfreeze	1/500	very good	good
TH	sh	pelfreeze	1/500	useless	
TH	m	millipore	1/500	good	good
TRA-1-60	m	santa cruz	1/100	very good	
TRA-1-81	m	bd	1/100	very good	
TUJI	m	sigma	1/500	good	
TUJI	rb	covance	1/1000	good	
TUJI	m	covance	1/500	good	