

# Directed differentiation of mouse embryonic stem cells for transplantation in Huntington's disease

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at Cardiff University

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**Dedicated to the memory of my Grannies,  
for endless inspiration and belief.**

## Summary

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The research presented in this thesis focused on neural precursors derived from mouse ES cells (also called neural embryoid bodies, NEBs) in particular attempting to direct their differentiation towards specific telencephalic precursors, with a view to providing an alternative donor cell source to primary foetal tissue for the generation of DARPP-32 positive medium spiny neurons (MSNs) for neural transplantation in Huntington's disease. Neural induction of mouse ES cells was addressed, with further directed differentiation by addition of factors known to be important in the neural tube for development of specific telencephalic precursors. Subsequent neuronal differentiation of precursors was assessed both *in vitro* and *in vivo*.

**Chapter 3** characterised the neural and forebrain features of NEBs up to day 8 in neural induction medium, using a reporter cell line, in which *LacZ* was tagged to the early forebrain marker, *Foxg1*. Addition of FGF2 but not the Wnt inhibitor DKK-1, increased the forebrain population within the overall cell population. Day 8 NEBs, subjected to neuronal differentiation conditions, yielded  $\beta$ -III-tubulin positive neurons but no DARPP-32 expression.

In **Chapter 4**, NEBs cultured for either 8 or 16 days were compared for expression of neural markers and then transplanted into the QA lesioned rat striatum to assess their potential to generate mature phenotypes *in vivo*. Day 8 NEBs generated teratomas by 2 weeks post-transplantation. At 6 weeks post-transplantation, day 16 NEBs generated heterogeneous grafts with graft-derived neurons and glia, but no graft-derived DARPP-32 expression.

Expression of dorso-ventral telencephalon markers in NEBs was investigated in **Chapter 5**, drawing comparisons to expression in the mouse (E14) developing telencephalon. The effects of the SHH agonist, purmorphamine, and the SHH antagonist, cyclopamine, were used in an attempt to enrich the forebrain precursors generated in Chapter 3 for ventral telencephalic-like precursors. Although gene expression changes were demonstrated, no distinct dorso-ventral patterns were shown.

*In vitro* neuronal differentiation of day 16 NEBs was analysed in **Chapter 6**, in particular looking for expression of the MSN marker DARPP-32. Cultures of cells from the lateral ganglionic eminence (LGE) (the origin of MSNs) were used to determine if certain factors could increase the yield of DARPP-32 positive neurons. Addition of BDNF yielded the highest proportion of DARPP-32 from LGE. However, when this condition was applied to neuronal differentiation cultures of day 16 NEBs there was no effect on the expression of GABA, DARPP-32 or FoxP1, all markers of MSNs.

In **Chapter 7**, day 16 NEBs, with and without addition of purmorphamine, were transplanted into the QA lesioned mouse striatum. All resulting grafts were small, but analysis revealed densities of neuronal and striatal markers in day 16 NEB-derived grafts to be similar to E14-derived grafts.



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## Abbreviations

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AChE	Acetylcholinesterase
ADF+	Advanced DMEM/F12 plus components for neural induction
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
AP	Anterior-posterior
APC	Adenomatous polyposis coli
BDNF	Brain-derived neurotrophic factor
bHLH	Basic helix-loop-helix
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CDM	Chemically defined medium
ChAT	Choline acetyltransferase
CMFDG	5-chloromethylfluorescein di- $\beta$ -D-galactopyranoside
CsA	Cyclosporin A
CTIP2	COUP TF1-interacting protein 2
CTX	Cortex
DA	Dopamine
DAB	Diaminobenzidine
DARRP-32	Dopamine and cyclic adenosine 3' 5' - monophosphate-regulated phosphoprotein, 32kDa
DCX	Doublecortin
DKK-1	Dickkopf-1
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
Dsh	Dishevelled
E	Embryonic day
<i>E. coli</i>	<i>Escherichia coli</i>
EB	Embryoid body
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
ES	Embryonic stem
FACS	Fluorescence activated cell sorting

FCS	Foetal calf serum
FGF	Fibroblast growth factor
GABA	Gamma-amino butyric acid
GAD	Glutamate decarboxylase
GFAP	Glial fibrillary acidic protein
GMP	Good manufacturing practice
GP	Globus pallidus
GSK-3 $\beta$	Glycogen synthase kinase 3 $\beta$
H	Height
HBSS	Hank's balanced salt solution
HD	Huntington's disease
HTT	Huntingtin
ICM	Inner cell mass
IddU	Iododeoxyuridine
IMDM	Iscove's modified Dulbecco's medium
iPS	Induced pluripotent stem
IT-15	Interesting transcript 15
IVF	<i>in vitro</i> fertilisation
JNK	Jun N-terminal kinase
KSR	'Knock-out' serum replacement
L	Lateral
LB	Luria-Bertani
LGE	Lateral ganglionic eminence
LIF	Leukaemia inhibitory factor
MACS	Magnetic activated cell sorting
MAPK	Mitogen-activated protein kinase
MEM	Non-essential amino acids
MGE	Medial ganglionic eminence
MOPS	3-(N-Morpholino)-propane sulfonic acid
MOR1	$\mu$ -opioid receptor
MSN	Medium-sized spiny neuron
NEB	Neural embryoid body
NeuN	Neuronal nuclei
NPA	Nitropropionic acid
P	Postnatal day
PBS	Phosphate buffered saline

PD	Parkinson's disease
PFA	Paraformaldehyde
PGD	Pre-implantation genetic diagnosis
PLL	Poly-L-lysine
PS	Penicillin/Streptomycin
QA	Quinolinic acid
QPCR	Semi-quantitative RT-PCR
RA	Retinoic acid
RALDH2	Retinaldehyde dehydrogenase 2
RAR	Retinoic acid receptor
RBP	Retinol binding protein
RT-PCR	Reverse transcription polymerase chain reaction
RXR	Retinoic X receptor
SHH	Sonic hedgehog
SN	Substantia nigra
STN	Subthalamic nucleus
SVZ	Sub-ventricular zone
TBS	Tris-buffered saline
TCF	T-cell factor
TE	Tris-EDTA
TH	Tyrosine hydroxylase
TNS	Tris-non-saline
TXTBS	Triton X-100 TBS
UHDRS	Unified HD rating scale
VGLUT1	Vesicular glutamate transporter 1
VZ	Ventricular zone
WGE	Whole ganglionic eminence
Wnt	Wingless
X-gal	5-Bromo-4-chloro-3-indolyl galactopyranoside
YAC	Yeast artificial chromosome

## Contents

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### *Chapter 1: Introduction*

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1.1	Huntington's disease	1
	<i>1.1.1 Clinical Huntington's disease</i>	2
	<i>1.1.2 Pathology of Huntington's disease</i>	3
1.2	The normal adult striatum	5
	<i>1.2.1 DARPP-32 expression in the striatum</i>	7
1.3	Development of research models for Huntington's disease	9
1.4	Transplantation in Huntington's disease	12
1.5	Embryonic stem cells	15
	<i>1.5.1 ES cells for transplantation in Huntington's disease</i>	16
1.6	Neural development	23
	<i>1.6.1 Factors involved in telencephalon development</i>	25
	<i>1.6.2 Foxg1 expression in the developing telencephalon</i>	31
	<i>1.6.3 Dorso-ventral organisation of the developing telencephalon</i>	33
1.7	Directed differentiation of ES cells	38
1.8	Aims of this thesis	45

### *Chapter 2: Materials and Methods*

---

2.1	<i>in vitro</i> methods	46
	<i>2.1.1 Mouse ES cell culture</i>	46
	<i>2.1.2 Freezing and thawing mouse ES cells</i>	46
	<i>2.1.3 Formation and maintenance of NEBs</i>	47
	<i>2.1.4 Differentiation of NEBs</i>	48
	<i>2.1.5 Dissection and preparation of primary mouse cells</i>	49
	<i>2.1.6 LGE glia expansion cultures</i>	50
	<i>2.1.7 LGE glia co-cultures</i>	50
	<i>2.1.8 LGE neuronal differentiation cultures</i>	51
	<i>2.1.9 Immunocytochemistry</i>	51
	<i>2.1.10 X-gal assay</i>	52
	<i>2.1.11 Microscopy</i>	52
2.2	Molecular methods	54
	<i>2.2.1 RNA extraction and quality check</i>	54

2.2.2	<i>cDNA synthesis</i>	54
2.2.3	<i>RT-PCR</i>	54
2.2.4	<i>QPCR</i>	55
2.2.5	<i>Primer sequences</i>	55
2.2.6	<i>Transformation and inoculation of plasmid DNA</i>	55
2.2.7	<i>Midi prep</i>	56
2.2.8	<i>DNA digest</i>	56
2.2.9	<i>Transfection</i>	56
2.3	<i>in vivo methods</i>	58
2.3.1	<i>Animal care, anaesthesia and immunosuppression</i>	58
2.3.2	<i>Quinolinic acid lesion</i>	58
2.3.3	<i>Unilateral striatal grafts</i>	59
2.3.4	<i>Perfusions and sectioning</i>	59
2.3.5	<i>Cresyl violet staining</i>	60
2.3.6	<i>Acetylcholinesterase histochemical staining</i>	60
2.3.7	<i>Immunohistochemistry on free-floating sections</i>	60
2.3.8	<i>Photomicroscopy and analysis of grafts</i>	61
2.4	<i>Statistical analysis</i>	62

### *Chapter 3: The Forebrain character of NEBs*

---

3.1	Summary	63
3.2	Introduction	64
3.3	Experimental Procedures	66
3.4	Results	68
3.5	Discussion	79

### *Chapter 4: in vivo characterisation of NEBs (xenograft model)*

---

4.1	Summary	85
4.2	Introduction	86
4.3	Experimental Procedures	88
4.4	Results	90
4.5	Discussion	99

## *Chapter 5: Generation of ventral telencephalon-like precursors*

---

5.1	Summary	105
5.2	Introduction	106
5.3	Experimental Procedures	108
5.4	Results	110
5.5	Discussion	131

## *Chapter 6: in vitro differentiation: Looking for DARPP-32*

---

6.1	Summary	137
6.2	Introduction	138
6.3	Experimental Procedures	140
6.4	Results	142
6.5	Discussion	160

## *Chapter 7: in vivo characterisation of NEBs (allograft model)*

---

7.1	Summary	167
7.2	Introduction	168
7.3	Experimental Procedures	170
7.4	Results	172
7.5	Discussion	183

## *Chapter 8: General discussion*

---

8.1	<i>In vitro</i> neural induction of mouse ES cells	188
8.2	Transplantation of mouse ES cells	190
8.3	Directed differentiation	192
8.4	Is generation of MSNs from ES cells achievable?	193
8.5	Mouse-to-mouse allografts	195
8.6	Where next?	195

	<i>Bibliography</i>	197
--	---------------------	-----

	<i>Appendices</i>	a-l
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# Chapter 1

## Introduction

*Neural transplantation in neurodegenerative diseases currently employs primary foetal striatal tissue for cell replacement and circuitry repair. In Huntington's disease cell loss occurs predominantly in the medium spiny neuron population of the striatum. The specificity of cell loss makes transplantation a viable therapeutic option. Ethical and logistical issues surrounding the use of primary foetal tissue indicate the need for an alternative donor cell source. The requirement of any alternative donor source is to have the capability to generate the mature phenotype that is lost in the disease process. Thus, cells need to be responsive to inductive developmental cues and the major challenge for the use of any donor cell is the specificity of the precise terminal phenotype. Here, Huntington's disease is used as a model for striatal transplantation and this thesis attempts to identify the signals required for directing differentiation of embryonic stem cells in order to generate medium spiny striatal projection neurons.*

### 1.1 Huntington's disease

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Huntington's disease (HD) was first described by George Huntington in 1872, when he detailed a condition referred to as 'hereditary chorea' in *The Medical and Surgical Reporter: A Weekly Journal (Philadelphia)* (Huntington, 2003). Huntington was a physician in East Hampton, Long Island and observed that this condition was confined to 'certain and few families' and was passed on between generations, rarely skipping a generation, with a tendency to manifest itself only in adult life, gradually developing over a number of years and increasing in severity. The choreic movements may be described as 'dance-like' with a 'continual jigger' while the sufferer is awake, but not while asleep, and have the potential to affect all voluntary muscles. Huntington also reported that those diagnosed had a tendency towards insanity and



suicide. At this early date little was known about the pathology of HD and the chorea was thought to be due to a 'functional derangement' of the cerebellum.

It was over 100 years later when the gene responsible for this hereditary condition was first mapped and found to reside on the short arm of chromosome 4 (Gusella et al., 1983). A further decade on, in 1993, a new gene, *IT-15* (interesting transcript 15), now known as 'huntingtin', was isolated and cloned by the *Huntington's Disease Collaborative Research Group*, and a mutation of the huntingtin (*HTT*) gene was found on exon 1 of the short arm of chromosome 4 (HDCRG, 1993). This mutation was found to be an unstable expansion of the tri-nucleotide repeat CAG which codes for glutamine, and HD may also be referred to as a poly-glutamine repeat disorder. Tri-nucleotide repeats, as a 'mechanism of mutation', were first recognised in 1991 in Kennedy disease and fragile X syndrome, followed by myotonic dystrophy in 1992 and then HD in 1993 (Harper, 1997).

### *1.1.1 Clinical Huntington's disease*

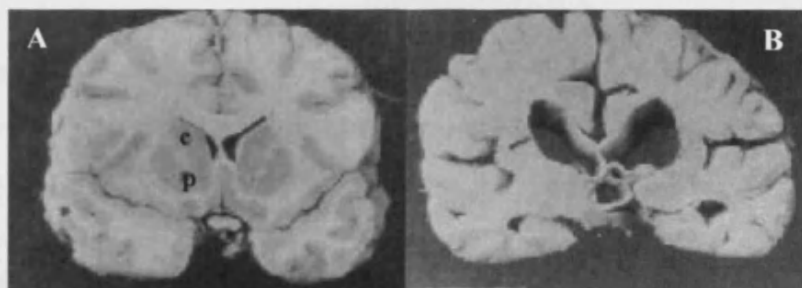
Huntington's disease affects one in 10,000 individuals in most European populations (Harper et al., 1991). Age of onset of the disease is usually within the third and fourth decades of life, however there have been juvenile as well as late-onset cases reported. Disease onset is progressive and death occurs within 15-30 years.

HD is an inherited autosomal dominant disorder which is characterised by neurodegeneration in the form of extensive atrophy of the striatal projection neurons. The loss of these cells leads to a variety of symptoms which can be put into three different categories: motor disturbances, cognitive impairment and psychiatric disorders. Motor symptoms include involuntary choreic movements, dystonia, dysphasia, dysarthria, bradykinesia, apraxia and eye movement abnormalities; cognitive symptoms include executive impairment, memory loss and progressive dementia; psychiatric symptoms include irritability, depression, anxiety, apathy, personality changes and, rarely, psychosis. HD is a progressive neurodegenerative disease and, as time goes on, the associated symptoms get progressively worse. Pneumonia is the leading cause of death in HD patients, and others include choking, nutritional deficiencies and suicide (Lanska et al., 1988; Harris and Barraclough, 1994).

In normal individuals up to 35 repeats of the CAG triplet code may be carried and when the number of repeats is increased above 35 this is considered a 'disease-causing allele'. However, CAG repeat lengths between 36 and 39 are regarded as 'variable penetrance', with repeat lengths greater than 39 invariably leading to disease state within a normal life span (Rubinsztein et al., 1996). Longer repeat lengths, typically greater than 70, are associated with juvenile onset of the disease. There has been shown to be an inverse correlation between the number of repeats and the age of onset of the disease, suggesting that the greater the number of CAG repeats the younger the age of onset (Andrew et al., 1993). The number of repeats has also been shown to correlate with the severity and progression of the disease on a population basis, although it is not the only determinant of these factors.

### *1.1.2 Pathology of Huntington's disease*

The mechanism by which the genetic mutation causes HD is unknown. HD pathology affects widespread areas in the brain (Rosas et al., 2003), but it is predominantly the striatum that is affected. The characteristic pathology is the selective loss of the medium-sized spiny neurons (MSNs) of the caudate nucleus and putamen, collectively called the 'neostriatum', and there is also a degree of cortical degeneration which may be primary or secondary to the striatal degeneration (Figure 1.1). The dysfunction and severe atrophy of the striatum causes a disruption to the basal ganglia circuitry and this is largely responsible for the symptoms associated with the disease.



**Figure 1.1.** (A) Coronal section through a normal adult brain, showing the caudate nucleus (c) and the putamen (p). (B) Coronal section through an HD brain, showing the atrophy in the caudate nucleus and putamen as well as the cerebral cortex. (Bates et al., 2002).

Vonsattel et al., graded HD brains according to striatal loss, with a link between extent of atrophy and stage of clinical impairment (Vonsattel et al., 1985). The post-mortem brain of an HD sufferer may be 10-20% lighter than that of an age-matched control (Gutekunst et al., 2002).

Another characteristic of the HD brain is the presence of insoluble neuronal intranuclear inclusions formed by the mutant Htt. These have been detected in post mortem brains of HD patients, in both the striatum and the cortex (Difiglia et al., 1997). It is not yet known what role these aggregates play in the course of the disease, if any. One proposal is that they are causative of the cell death associated with HD (Yang et al., 2002). Introduction of mutant Htt to cultured striatal neurons induced cell death which could be protected by addition of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) (Saudou et al., 1998). However, Saudou et al (1998) demonstrated that the presence of intranuclear inclusions did not correlate with cell death, and suggested that the aggregates might be acting to protect the cell from htt-induced death, which was further demonstrated by Arrasate et al (2004) who showed that cells with aggregate formation demonstrated improved cell survival. In addition, a mouse model of HD with widespread aggregation, displayed no neuronal degeneration or dysfunction, further indicating a protective mechanism (Slow et al., 2005). It has also been proposed that aggregates might be toxic in one cell location but protective in another (Arrasate et al., 2004), and that the timing of aggregate formation might predict whether the subsequent effects are toxic or beneficial (Gong et al., 2008).

One leading suggestion concerning the development of the disease is that the HD mutation causes the subsequent damage via a gain-of function of the mutant htt protein. The insertion of exon 1 of the *HD* gene carrying an expanded CAG repeat, resulted in a mouse model with a reduction in the size of various brain structures including the striatum, and a progressive neurological phenotype including symptoms such as dyskinesia, tremor and involuntary stereotypic movements (Mangiarini et al., 1996).

It is also suggested that loss-of-function of normal *Htt* may contribute to the HD phenotype. Mice homozygous for the targeted mutation of the *Htt* gene are embryo-lethal, while those that are heterozygous survive to term and adulthood (Nasir et al., 1995). Stereological analysis of the heterozygous mutant brains revealed a loss

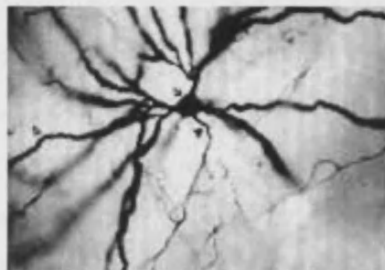
of neurons in the globus pallidus (GP) and subthalamic nucleus (STN) with an increase in apoptosis in these areas (O'Kusky et al., 1999).

## 1.2 The normal adult striatum

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The striatum is made up of the caudate nucleus and the putamen (see Figure 1.1), and forms part of the basal ganglia along with the STN, GP and substantia nigra (SN). It receives inputs from the cerebral cortex, SN, thalamus and brain stem, and sends outputs to the GP and SN.

The striatum is both anatomically and neurochemically heterogeneous (Prensa et al., 1999), and comprises a variety of neurons which can be subdivided according to size, density of spines, target destination of axons and utility of neurotransmitters and neuropeptides. The MSNs (Figure 1.2) constitute 95% of the striatal neuronal population in the rodent, whilst the aspiny interneurons of the striatum make up most of the remaining 5%, with non-MSN projection neurons representing a small percentage (Freeman et al., 1995).



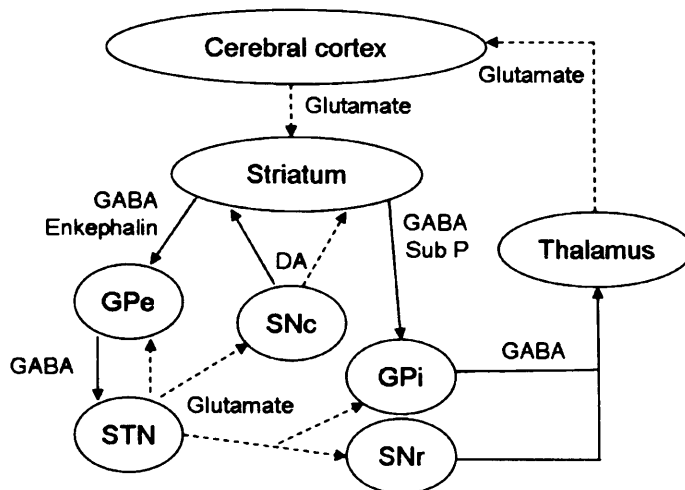
**Figure 1.2.** A medium-sized spiny neuron, with branched, spiny dendrites.  
Courtesy of Professor P. Harper (Institute of Medical Genetics, Cardiff University).

MSNs have a medium-sized cell body, branched dendrites that are densely spiny, and they are the projection neurons of the striatum, which can be distinguished from the interneurons by their expression of the phosphoprotein dopamine and cyclic adenosine 3', 5'- monophosphate-regulated phosphoprotein, 32kDa (DARPP-32). DARPP-32 is found in the majority of striatal neurons projecting to the SN and those projecting to the GP, but it is absent from major types of interneuron. Morphologically MSNs are a homogeneous population; however they are biochemically heterogeneous (Graybiel, 1990).

MSNs are the predominant projection neurons of the striatum and use gamma-aminobutyric acid (GABA), which is an inhibitory transmitter, as their principal neurotransmitter (Kita and Kitai, 1988) along with one or more neuropeptides (Reiner and Anderson, 1990). These GABAergic neurons can project to the GP, internal (GPi) and external (GPe) segments (striatopallidal neurons) and the SN pars compacta and reticulata (striatonigral neurons). The neuropeptides involved are enkephalin, substance P and dynorphin, with the latter two largely co-existing in striatal projection neurons (Reiner and Anderson, 1990). The majority of striatal projection neurons expressing enkephalin project to the GP, while the majority of neurons that co-express substance P and dynorphin project to the SN (Gerfen and Young, III, 1988). The presence of these neuropeptides in addition to GABA has been shown to be important in communication between the striatal projection neurons and their target neurons.

Striatal inputs from the SN are dopaminergic and those from the cortex and thalamus are non-dopaminergic, primarily glutamatergic. The glutamatergic afferents provide excitatory inputs to the MSNs. The inputs and outputs of the striatum are shown in Figure 1.3. Dopaminergic projections from the SNc act on the D1 and D2 dopamine receptors of the GABAergic MSNs. Projection to the D1 receptors results in activation of the direct pathway to the GPi via neurons expressing GABA and substance P. The indirect pathway is activated as a result of dopaminergic projections acting on the D2 receptors which causes inhibition of those striatal MSNs expressing GABA and enkephalin which project to the GPe.

The aspiny interneurons, or 'local circuit' neurons, comprise medium and large cells with smooth dendrites. They may be split into three categories; i) those that use acetylcholine as a neurotransmitter, ii) those that contain the calcium-binding protein parvalbumin and also express glutamate decarboxylase (GAD) and use GABA as a neurotransmitter, and iii) those that contain somatostatin, neuropeptide Y and NADPH diaphorase (Kawaguchi, 1993).



**Figure 1.3.** Inputs and outputs of the striatum. Excitatory projections are represented with dashed lines and inhibitory projections with solid lines. Abbreviations: DA - dopamine; GPe - external segment of globus pallidus; GPi - internal segment of globus pallidus; SNc - substantia nigra pars compacta; SNr - substantia nigra pars reticula; STN - subthalamic nucleus; Sub P - substance P. (Adapted from (Bates et al., 2002) Bates G, Harper P, Jones L., Huntington's Disease, *Third Edition*.).

One level of compartmentalisation in the striatum is the characteristic striosome (patch)-matrix organization which is based on the expression of neurochemical markers. Acetylcholinesterase (AChE) positive neurons are distributed throughout the striosome-matrix compartments, with AChE-poor areas comprising the striosomes and AChE-rich areas comprising the matrix. The matrix compartment also contains neurons expressing the 28kD calcium-binding protein calbindin as well as somatostatin-positive fibres. Calbindin and parvalbumin have been shown to label striatonigral projection neurons. Calbindin labels the striatonigral matrix neurons whereas parvalbumin labels the striatonigral striosome neurons (Gerfen et al., 1985).

### 1.2.1 DARPP-32 expression in the striatum

DARPP-32 is a phenotypic marker of the MSNs of the adult striatum that distinguishes them from the other striatal cells. It is the most commonly used MSN marker and in the rodent brain it has been demonstrated that more than 90% of all MSNs express DARPP-32 (Ouimet et al., 1998; Anderson and Reiner, 1991). However, it is important to note that expression of DARPP-32 is not absolute in

defining a MSN, but is currently one of the only measures available in distinguishing mature MSNs from other striatal neurons.

Further markers of MSNs are continuously being sought, including those expressed in MSN precursors. The transcription factor COUP TF1-interacting protein 2 (CTIP2) has been shown to be expressed exclusively in striatal MSNs (Arlotta et al., 2008). In the adult mouse striatum, all DARPP-32 positive cells were shown to co-express CTIP2 (Arlotta et al., 2008). Conversely however, in neurons derived from human ES cells, CTIP2 has been shown to co-label with the dorsal telencephalic markers TBR1 and vesicular glutamate transporter 1 (VGLUT1) (Li et al., 2009). Another potential candidate is FoxP1, which has also been shown to co-label with CTIP2 (Arlotta et al., 2008), and was shown to be upregulated during development in the striatum between the ages embryonic day (E) 12 and E16 (Kelly et al., in preparation). FoxP1 expression has been detected by *in situ* hybridization from E13.5 in the developing mouse brain, and its expression persists into adulthood, with expression in the projection neurons but not interneurons of the adult striatum (Ferland et al., 2003; Tamura et al., 2004).

In the embryonic rat caudate nucleus it has been demonstrated by immunohistochemistry, that DARPP-32 immuno-reactive cells are first detectable at E14 (Foster et al., 1987). At this age only very few DARPP-32 positive cells are present, although both the number of DARPP-32 positive cells and the intensity of immuno-fluorescent labelling increase rapidly and by postnatal day 0 (P0) the pattern of DARPP-32 immuno-reactivity is nearly that seen in the adult striatum.

In the mouse brain DARPP-32 mRNA is undetectable at E14 and at P0 both DARPP-32 mRNA and protein are present in very small amounts (Ehrlich et al., 1990). Levels of both then increase dramatically throughout the first 3-4 postnatal weeks, with the mRNA reaching adult levels before the protein. Ehrlich et al (1990) propose that induction of DARPP-32 gene transcription occurs primarily during the last week of gestation, since DARPP-32 cells are largely present at birth, and MSN production peaks at E15 and is almost complete by P1-2 in both the mouse and rat (Marchand and Lajoie, 1986; Sturrock, 1980).

Immunohistochemical analysis reveals that there is an overlap of DARPP-32 immuno-reactive neurons and dopaminergic neurons containing dopamine D1 receptors (Walaas and Greengard, 1984), which coincides with the observation that DARPP-32 phosphorylation is regulated by dopamine D1 receptor activation (Ouimet

et al., 1998). Further, the ontogeny of DARPP-32 and pre- and post-synaptic components of the dopamine D1 synapse follow a similar time course which further suggests a role for DARPP-32 in regulating dopamine D1 receptor mediated signal transduction (Ehrlich et al., 1990).

The HD paradigm, in which degeneration in the striatum is a focal point at which to aim to replace the cells lost, offers a disease model suitable for striatal transplantation. Since it is the DARPP-32 positive MSNs that are predominantly lost in HD, it is these cells that we endeavour to replace.

### 1.3 Development of research models for Huntington's disease

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Models of HD are required to further our understanding of the progression of the disease, and to give an insight into the potential of therapeutic strategies for repair, including transplantation. Excitotoxic lesions are widely used to generate models of HD. They are useful for studying behavioural aspects, as well as investigating cell-based therapies of the disease, reviewed in (Dunnett and Rosser, 2004). This method has been shown to reproduce many of the histological, neurochemical and behavioural features of the disease. One such toxin is quinolinic acid (QA) which, when administered directly to the striatum, causes striatal atrophy with predominant loss of MSNs (Schwarcz et al., 1983; Schwarcz and Kohler, 1983). QA is toxic to the GABAergic MSNs and spares the cholinergic interneurons and the aspiny interneurons containing somatostatin, neuropeptide Y and NADPH diaphorase (Beal et al., 1986). The selective striatal cell loss observed with the QA lesion resembles that seen in the human condition and this model is currently the most widely used for transplantation studies in HD. Another toxin is 3-nitropropionic acid (NPA), which is administered systemically in low doses and over a period of weeks causes striatal cell loss (Borlongan et al., 1997). It functions to disrupt mitochondrial energy metabolism, which causes the striatal damage and induces functional impairments. For transplantation studies it is a less useful model than the QA lesion because it results in a less stable lesion, which is bilateral and therefore leaves no control side, and there is more variability in lesions within experiments.



The identification of the mutation of the gene causing HD has led to the generation of genetic models of the disease for research. Genetically modified models of HD have been generated in *Drosophila melanogaster* (Warrick et al., 1998), *Caenorhabditis elegans* (Faber et al., 2002), zebrafish (Schiffer et al., 2007) and non-human primates (Yang et al., 2008), as well as rodents. In terms of cell-replacement strategies for repair of function and circuitry, rodents offer the most attractive model due to both ease of use in transplantation and behavioural studies, and their complex brain organisation.

Models of HD differ phenotypically according to behaviour and neuropathology and can therefore be used to look at different aspects of the disease. There are both knock-in and transgenic rodent models of HD. The knock-in models of HD are those in which an expanded CAG repeat is introduced into the endogenous mouse *Htt* gene. They include the poly-Q (polyglutamine) models which differ according to the number of CAG repeats, such as the Q92 and Q111 models (Wheeler et al., 1999). The knock-in models show few behavioural deficits and no cell loss but there is the presence of neuronal intranuclear inclusions.

Transgenic models are those in which either a portion of the human HD gene or the full length gene is inserted randomly into the genome. These include the R6/2 model which contains the HD promoter, exon 1, and about 155 polyglutamine repeats (Mangiarini et al., 1996). R6/2 mice show reduced numbers of striatal neurons and overall brain atrophy (Stack et al., 2005), as well as learning impairments and progressive motor deterioration. Polyglutamine aggregates in the form of neuronal intranuclear inclusions and cytoplasmic aggregates have been reported in the R6 models (Davies et al., 1997), and as with most of the transgenic mouse models, there is a rapid progression of the HD phenotype which largely resembles juvenile onset. Transplantation into the R6/2 striatum resulted in graft survival with a degree of integration, although improvements in the behavioural phenotype were only marginal (Dunnett et al., 1998). Other transgenic mouse models include the YAC (yeast artificial chromosome) mouse models, YAC72 and YAC128, which contain the entire human *HD* gene, its regulatory elements and 72 or 128 polyglutamines, respectively (Hodgson et al., 1999; Slow et al., 2003). The BACHD model (bacterial artificial chromosome) contains the human *Htt* locus with a modified exon 1, and displays progressive striatal and cortical atrophy with expression of neuronal intranuclear inclusions (Gray et al., 2008).

The majority of transgenic models of HD are mouse models although a rat model of HD has recently been generated (von Horsten et al., 2003). This model has a truncated htt cDNA fragment with 51 CAG repeats under the control of the native rat htt promoter. The homozygous transgenic rat has been shown to exhibit phenotypes which appear to represent the late-onset and slow progression of adult HD. Neuronal intranuclear inclusions have been reported from 12 months of age (von Horsten et al., 2003) and a decrease in striatal neuronal numbers is observed at 12 months (Kantor et al., 2006).

*In vitro* models of HD have been developed to investigate the intracellular pathology associated with the disease and can be useful for screening potential drug candidates. Cell culture models of HD provide the opportunity to study the distribution of *Htt* within cells and the role it plays in different cellular compartments. These models have been used to investigate the aggregation of both wild-type and mutant *Htt*. Various cell culture models of HD have been generated by transfection of full-length *Htt* with normal or expanded polyglutamine repeats, resulting in both transient expression (Trottier et al., 1995; Cooper et al., 1998) and stable, long-term expression (Lunkes and Mandel, 1998). Following transient transfection with full-length *Htt*, the protein was found to be localized diffusely in the cytoplasm, whereas with N-terminal fragments of htt, aggregates were found in both the cytoplasm and the nucleus (Cooper et al., 1998). With the stable cell model, it was found that mutant *Htt* expression leads to formation of inclusions in both the cytoplasm and the nucleus, and this was dependent on time and the length of polyglutamine repeats (Lunkes and Mandel, 1998). It is suggested here that it is a truncated N-terminal fragment of *Htt* which is involved in inclusions in the cytoplasm at early time points and in the nucleus at later time points. A truncated *Htt* fragment has also been implicated in HD pathogenesis (Difiglia et al., 1997).

Cell lines have been established from mouse embryos of knock-in models of HD. Trettel et al (2000) generated striatal cells from HdhQ111 mice, compared these with wild-type cells and found that the mutant protein causes toxicity and disrupts striatal cell homeostasis (Trettel et al., 2000). An alternative approach, for generation of human embryonic stem (ES) cell lines, involves donation of blastocysts following *in vitro* fertilisation (IVF) and pre-implantation genetic diagnosis (PGD). PGD allows detection of embryonic genetic defects and can result in availability of embryos for derivation of ES cell lines. A blastocyst donated for research, in which the paternal

donor was affected with HD, was used to derive a human ES cell line which carries 44 CAG repeats (Mateizel et al., 2006). This cell line expressed markers of undifferentiated human ES cells, formed embryoid bodies when subjected to suspension culture, and was able to differentiate into cells of the three germ layers, ectoderm, endoderm and mesoderm (Mateizel et al., 2006). More recently, two human ES cell lines carrying CAG repeats of 37 and 51 have been derived following PGD, and have been shown to undergo neural differentiation with formation of neurospheres and subsequent generation of neurons and astrocytes (Niclis et al., 2009).

The recent discovery of induced pluripotent stem (iPS) cells, where pluripotency-linked factors are introduced to fibroblasts to reprogram them so they express pluripotency markers and behave like ES cells, offers a novel cell source (Takahashi and Yamanaka, 2006; Lowry et al., 2008; Yu et al., 2007; Takahashi et al., 2007; Park et al., 2008). iPS cells do not exhibit chromosomal abnormalities and can differentiate to produce cells of the three embryonic germ layers. Disease-specific iPS cells have been generated, including a human iPS cell line for HD that has 72 CAG repeats (Park et al., 2008).

Models of HD have proved useful for the study of pathogenesis of the disease, symptomatic aspects and therapeutic strategies. Cell culture models of HD allow analysis and characterisation of cells affected by the disease and allows specific cells to be targeted for therapeutic strategies. In terms of neurodegeneration, HD offers a model with a focal point of cell loss (predominantly the striatum) making transplantation an attractive therapeutic approach.

## 1.4 Transplantation in Huntington's disease

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There is currently no known cure for HD and only limited symptomatic treatments are available. Various therapeutic strategies are being explored targeting the cellular pathology, including neuronal protection, knock-down of mRNA and cell replacement by transplantation of foetal neural precursors. Transplantation aims to replace the neurons that are lost and reconstruct the circuitry that is affected by the

disease. Primary foetal striatal tissue offers a source of MSN precursors and is currently the 'gold standard' for transplantation in HD.

Preclinical studies have investigated the optimum parameters for transplantation into the HD brain, such as age of the developing embryo, region of the developing brain for dissection and tissue preparation (Olsson et al., 1995; Fricker et al., 1997; Watts et al., 1997). It has been reported that whole ganglionic eminence (WGE) grafts into the lesioned striatum in rat generate a higher proportion of striatal-like tissue, as determined by AChE and DARPP-32 immunohistochemistry, when derived from gestational ages E14 and E16, and not older donor ages (Fricker et al., 1997), but functional recovery of animal recipients of these grafts was only improved when the donor tissue was younger (E14) rather than older (E16) (Watts et al., 1997). Xenotransplantation studies of human foetal neural precursors into rodent models of HD have provided information which suggests that human foetal tissue between 8-10 weeks of gestational age is optimal for harvesting striatum for transplantation (Rosser and Dunnett, 2003; Freeman et al., 1995). However, to date, no systematic studies looking at donor age of human foetal tissue have been carried out.

Comparison of grafting the WGE versus the lateral ganglionic eminence (LGE) or medial ganglionic eminence (MGE) indicate that the LGE grafts contain a greater proportion of striatal-like cells, confirmed by staining with DARPP-32 (Pakzaban et al., 1993), and a much higher proportion of AChE-positive tissue is seen. The LGE represents the major source of striatal projection neurons (Olsson et al., 1998) and the striatal interneurons appear to come predominantly from the MGE with a smaller proportion originating in the LGE (Olsson et al., 1998; Marin et al., 2000). Fate mapping studies revealed that cells originating in the LGE migrate both anteriorly and ventrally, giving rise to projection neurons of the striatum, nucleus accumbens and olfactory tubercle, whilst the majority of cells originating in the MGE migrate dorsally and generate interneurons expressing GABA, parvalbumin and somatostatin (Wichterle et al., 2001). Grafts comprising both LGE and MGE have been shown to be heterogeneous with respect to striatal phenotype (Olsson et al., 1995). In these grafts, there are regions with striatum-like tissue, called 'P regions' which express DARPP-32 and AChE. There are also 'NP regions' which contain neurons usually found in adjacent forebrain regions such as the cortex and GP (Olsson et al., 1995; Graybiel et al., 1989).

Human foetal neural precursor cells for replacement are currently obtained from collection of fetuses following elected termination of pregnancy under full ethical consent of the maternal donors (Polkinghorne, 1989; Boer, 1994). Upon retrieval of foetal tissue, the developing striatum is dissected out and tissue may be 'hibernated' for up to a week before transplantation, without a reduction in viability (Hurelbrink et al., 2000).

Clinical trials for cell-replacement therapy in HD using foetal tissue are ongoing and have demonstrated proof-of-principle, but consistent efficacy has not yet been achieved. Bilateral human foetal striatal grafts in symptomatic HD patients have been shown to survive and show typical morphology of developing striatal grafts, as seen in post mortem evaluation of one patient (Hauser et al., 2002). Assessments of safety reveal that human foetal striatal grafts do not accelerate disease progression (Rosser et al., 2002), and patients exhibiting no graft benefits appear to decline similarly to non-grafted patients (Bachoud-Levi et al., 2006). Hauser et al (2002) reported a decrease in the unified HD rating scale (UHDRS) score at 12 months post-transplantation, indicating an improvement in neurological assessment. More convincing evidence of efficacy was demonstrated by a group in Creteil, France, who reported on five patients at six years post-transplantation, who received foetal neural transplants (Bachoud-Levi et al., 2006). They used UHDRS, neuropsychological tests and MRI and determined that there are benefits both clinically and in terms of brain activity, which provide a short term improvement and stability for the patient.

A recent post mortem study, ten years post-transplantation, demonstrates graft survival but also suggests degeneration of grafted neurons (Cicchetti et al., 2009) compared with a patient from the same cohort who died 18 months post-transplantation (Freeman et al., 2000). In the more recent report, the authors demonstrate survival of tissue with expression of striatal projection neurons and striatal interneurons as well as synaptic connections between transplanted neurons and host-derived dopaminergic and glutamatergic neurons, although there is also evidence of some graft damage (Cicchetti et al., 2009).

Another recent post-mortem analysis study, at six months post-transplantation, reported graft-derived DARPP-32, NeuN, calretinin, somatostatin and astrocytes, as well as innervation of host-derived TH fibers (Capetian et al., 2009). There was also the presence of more immature precursors including doublecortin (DCX), Sox2 and Ki67 (Capetian et al., 2009). This study gives an indication of graft morphology at an

early time point post-transplantation. However, we are unable to draw any direct comparisons between data from the various studies because there are different protocols being applied at different centres, and this therefore reinforces the need for controlled multi-centre trials.

Foetal tissue as a source for cell replacement is limited and brings about both practical and ethical constraints. For transplantation in Parkinson's disease (PD) it has been shown that there is a requirement for several fetuses per transplant. It is likely that this is also the case for clinical transplantation in HD and if so, would pose logistical and quality control issues in terms of retrieving several fetuses of the same gestational age, as well as manipulation and storage of tissue under new Good Manufacturing Practice (GMP) guidelines (EU Directive).

There is an urgent need for an alternative supply of tissue. Possible alternatives include stem cells, such as ES cells, neural stem cells and mesenchymal stem cells, xenogenic tissue (for example, porcine), genetically engineered cells and most recently iPS cells (Takahashi and Yamanaka, 2006). The emergence of iPS cells might prove useful for both research and clinical applications, since they bypass the use of embryos and would enable generation of patient-specific cell lines therefore avoiding administration of immuno-suppression. Potential alternative cell sources have been investigated, although the majority of research has focussed on neural stem cells and ES cells. Neural stem cells are attractive because they are already lineage restricted, but they do have limited capacity for proliferation (Zietlow et al., 2005). ES cells have a greater proliferation potential, are more plastic, and can be directed down the various different lineages. There is now over a decade of work on ES cells for the purpose of achieving an alternative cell supply and it is likely that outcomes found for ES cells will be pertinent for the differentiation of other alternative cell sources, making ES cells a good area on which to focus.

## 1.5 Embryonic stem cells

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ES cells offer an alternative source of cells for transplantation in neurodegenerative disorders such as HD. The characteristic features of these cells are their capacity for unlimited self-renewal and proliferation and their pluripotency

which gives them the potential to generate any cell type (Evans and Kaufman, 1981; Martin, 1981). ES cells are derived from the inner cell mass (ICM) of the developing blastocyst and have the potential to generate cells from any of the three primary germ layers of the embryo; ectoderm, endoderm and mesoderm. This makes them an attractive source of cells for therapeutic strategies in a range of diseases. In HD, since it is the MSNs of the striatum that are predominantly lost, and the optimum 'cell-type mix' that will ultimately be necessary for restoration of function is not yet known, the MSNs are likely to be the cells we wish to generate for replacement and this, therefore, is the current focus.

Mouse ES cell lines have been derived, including those with insertion of reporter genes, to investigate effects on gene expression within neural induction culture systems and determine effects of addition of factors. More recently human ES cell lines have also been derived (Thomson et al., 1998). Studies have shown similarities and differences between mouse and human ES cell lines in terms of ES cell culture conditions and subsequent neural induction protocols, reviewed in (Wobus and Boheler, 2005).

### *1.5.1 ES cells for transplantation in Huntington's disease*

ES cells offer a potentially inexhaustible supply of cells with the potential for use in cell replacement strategies. One of the main caveats is obtaining the specific phenotype required, which in the case of HD is MSNs. Even foetal neural precursors, taken from the developing striatum, which have been exposed to the temporal and spatial cues necessary for induction of the desired phenotype, do not go on to generate the full complement of striatal projection neurons and interneurons when transplanted into the adult striatum. Long-term *in vitro* expansion of both mouse and human foetal neural progenitors prior to transplantation resulted in reduced survival following a long-term post-transplantation period (Zietlow et al., 2005). It has been reported that there are changes in gene expression between early (8 weeks) and late (20 weeks) *in vitro* expansion times of human foetal neural progenitors, with upregulation of genes associated with apoptosis after prolonged time in culture (Anderson et al., 2007). Further, the long-term expanded progenitors showed reduced transplant size as well as reduced neurogenic potential (Anderson et al., 2007). For ES cells, the predominant

requirement is to direct the differentiation both in the initial *in vitro* stage, and then in the subsequent maturation stage following transplantation into the adult striatum.

There has been some degree of success in generating specific neuronal phenotypes from ES cells for replacement in neurodegenerative disease, for example PD. Transplantation of ES cell-derived neural precursors has predominantly focused on obtaining dopaminergic neurons and the general principles of directing differentiation of these cells applies for cell types throughout the developing brain. Dopaminergic neurons have been reported *in vivo* in graft-derived cells post-transplantation (Wernig et al., 2004; Ferrari et al., 2006; Lee et al., 2007), with one case demonstrating long-term survival and function of these neurons (Rodriguez-Gomez et al., 2007). Table 1.1 outlines transplantation studies that have employed neural induction of ES cell-derived precursors. Few studies have been carried out involving transplantation of ES cell-derived neural precursors into HD models and of those that have been reported only the QA-lesioned striatum model has been employed.

Mouse ES cell-derived precursors subjected to RA-induced suspension culture and then transplanted into the QA-lesioned striatum rat model generated GABA and AChE immuno-positive neurons following 6 weeks survival (Dinsmore et al., 1996). A study looking at the proportion of neural precursors, dividing cells and neurons prior to transplantation revealed that the greater the proportion of neural precursors (as determined by nestin immuno-reactivity) and dividing cells (using BrdU), the greater the potential to generate tumours (Dihne et al., 2006). When the proportion of dividing precursor cells was lower and that of mature neurons was greater, grafts generated neurons, astrocytes and oligodendrocytes, and there were no teratomas formed. Transplantation of cell populations greater than 95% nestin and 42% BrdU positive resulted in 70% tumours; 42% nestin, 16% BrdU and 13%  $\beta$ -tubulin resulted in 17% tumours; and 14% nestin, 5.9% BrdU and 76%  $\beta$ -tubulin resulted in no teratoma formation (Dihne et al., 2006).

Human ES cell-derived neural precursors induced in defined neural induction medium generated DCX immuno-positive neuroblasts, neurons and astrocytes, but no DARPP-32 immuno-positive neurons and no tumours at six weeks post-transplantation in a QA-lesioned rat striatum (Joannides et al., 2007b). A similar study looked at neural induction of human ES cell-derived precursors with addition of Sonic Hedgehog (SHH) and Dickkopf-1 (DKK-1) and after six weeks survival no DARPP-



32 was found but there were teratomas (Aubry et al., 2008). Increased neural induction time with addition of BDNF, dbcAMP and valproic acid prior to transplantation, resulted in generation of some DARPP-32 immuno-positive neurons and no teratomas (Aubry et al., 2008). Longer-term survival up to 21 weeks post-transplantation of these latter neurally induced precursors revealed regions of DARPP-32 immuno-positive cells but there was also the observation of large, overgrown grafts that compressed the host brain (Aubry et al., 2008).

A recent study of human ES cell-derived precursors generated in neurobasal medium with bFGF and the BMP inhibitor noggin, cultured for more than four weeks expressed 80% nestin-positive cells at transplantation (Nasonkin et al., 2009). Relatively low numbers of cells were transplanted into the non-lesioned rat striatum and differentiation was analysed at 1.5, 3 and 6 months. Nestin and DCX decreased over time post-transplantation (44% reduced to 1% for nestin expression; 52% reduced to 5% for DCX expression), while  $\beta$ -III-tubulin expression increased over time (20% increased to 86%), and although there was no DARPP-32 expression at three months, nearly a third of grafted cells were DARPP-32 positive at six months, demonstrating the need for long-term differentiation to achieve DARPP-32 expression (Nasonkin et al., 2009).

Thus far in HD research, transplantation of ES cell-derived neural precursors has resulted in good survival in the QA-lesioned host brain, with good integration and neuronal generation in some instances even into DARPP-32 immuno-positive neurons. However, the teratoma formation reported is a concern that needs to be addressed as urgently as directing the differentiation towards the desired phenotype.

**Table1.1.** Transplantation studies of neurally induced ES cell-derived precursors

	Study	Experimental Paradigms			Notes	Results
		Cells	Host	Protocols		
Neurally induced ES cells into QA lesioned striatum	Dinsmore et al., 1996	Mouse ES cells (lines E14 & D3)	QA lesioned striatum, rat, CsA	Free-floating, RA-induced neuronal differentiation	$3 \times 10^5$ - $10^6$ cells transplanted. Survival: 6 weeks.	Neurons expressing GABA, AChE, NSE & neuron specific tubulin, but no <i>in vivo</i> cell numbers reported.
	Dihne et al., 2006	Mouse ES cells (i)eGFP line derived from transgenic C57L/6J mice expressing eGFP under influence of chicken $\beta$ -actin promoter (ii)R1 line	QA lesioned striatum, mouse	EB formation in absence of LIF; neural induction in insulin/ transferrin/selenium chloride/fibronectin, then expanded in B27 & FGF2	$\sim 100,000$ cells/ $\mu$ l transplanted. Survival: 1, 4 & 16 weeks.	The higher the proportion of neural precursors (nestin) and dividing cells (BrdU), the greater the risk of tumours. 56.9% NF-200 neurons. Grafted cells expressing $\beta$ -tubulin and GAD65/67, also astrocytes & oligodendrocytes.
	Joannides et al., 2007	Human ES cells (i)H9 (ii)HUES9	QA lesioned striatum, rat, CsA	Defined neuralizing medium	100,000-250,000 cells transplanted. Survival: 6 weeks.	Grafted cells expressing DCX, NeuN, GFAP, but no DARPP-32.
	Song et al., 2007	Human ES cells Miz-hES1	QA lesioned striatum, rat, CsA	Co-culture on PA6 stromal cells (generate high proportion of nestin-positive cells)	20,000 cells in $2 \mu$ l transplanted. Survival: 3 weeks.	Improved apomorphine rotations at 1, 2 & 3 weeks compared with sham group. Grafted cells expressing nestin, Tuj1, DCX & GAD. No NeuN, MAP2 or DARPP-32. No tumours.

	Study	Experimental Paradigms			Notes	Results
		Cells	Host	Protocols		
QA lesioned striatum	Aubry et al., 2008	Human ES cells (i) SA-01 (ii) H9	QA lesioned striatum, immuno-competent rat	Rosettes in N2 & BDNF; addition of SHH/DKK-1; differentiation in BDNF/dbcAMP/valproic acid	50,000-200,000 cells transplanted. Survival: 4-6 weeks.	Transplantation in early stages of neural induction resulted in teratomas & no DARPP-32. Transplantation of later stage cells resulted in no teratomas, and grafts contained clusters of DARPP-32 & calbindin neurons.
			QA lesioned striatum, immuno-competent rat	Neural induction as above. Cells at the stage which gave best <i>in vivo</i> outcome at 4-6 weeks.	50,000 cells transplanted. Survival: 13-21 weeks.	Large overgrown grafts that compressed the host brain. Regions of graft-derived DARPP-32 cells throughout the graft, often calbindin-negative, unlike short-term grafts. Very few TH neurons, no AChE & few GFAP cells.
Unlesioned	Nasonkin et al., 2009	Human ES cells BG01	Unlesioned striatum, nude rats	Neurobasal with 7.5% KSR/bFGF/noggin for 3 days. Then colonies picked from MEFs onto gelatin in neurobasal with bFGF/noggin for 4 weeks	15000 cells transplanted. Survival 1.5, 3 and 6 months.	High nestin expression prior to grafting (80%). Nestin and DCX expression decreased overtime <i>in vivo</i> , $\beta$ -tubulin expression increased overtime. No GAD at 6 months; no DARPP-32 at 3 months but 30% DARPP-32 at 6 months; Some calretinin & parvalbumin at 6 months. No teratomas or graft overgrowth.
Other cells into QA	Bosch et al., 2004	Immortalised striatal neural stem cell line ST14A	QA lesioned striatum rat	Sequential RA then KCl treatment, pre-differentiated to GABAergic phenotype	9000 cells per striatum (6 graft sites). Survival: various time points.	Maintained GABAergic phenotype 3 days post-transplantation. At 1 & 5 weeks after grafting, reduction in apomorphine rotations, compared with ungrafted rats.

	Study	Experimental Paradigms			Notes	Results
		Cells	Host	Protocols		
Different models; <i>in vivo</i> differentiation of neurally induced ES cells	Brustle et al., 1997	Mouse ES cells (line J1), DIG-labeled	Intrauterine transplant (E16-18), telencephalic vesicle, rat	Embryoid body formation, insulin-transferrin medium	0.1-1x10 <sup>6</sup> cells injected. Survival: 0-15 days after birth.	Grafted cells migrated & integrated into various brain regions including striatum, cortex & thalamus. Neurons, astrocytes & oligodendrocytes detected. Undifferentiated ES cells & non-neural tissue. Used M2 & M6 antibodies.
	Deacon et al., 1998	Mouse ES cells (i) D3 (ii) E14TG2a	Rat 6-OHDA unilateral nigrostriatal lesion, CsA; Mice unlesioned	1.5x10 <sup>6</sup> cells in 5ml DMEM +/- RA (0.5mM), no LIF	Rats: 100,000-300,000 cells; Mice: 60,000 cells. Survival : 2-4weeks.	RA+ grafts: rats 2/5 grafts survived, mice 4/4 grafts survived. RA- grafts: rat 17/26 grafts survived, mice 3/3 grafts survived. In rat host, no migration of donor cells away from graft site. TH & 5-HT neurons & GFAP. Weak AChE in RA+ but none in RA- grafts. Variability between graft size & appearance. Heterogeneous tissue types, with significant volume negative for neuronal markers.
	Kim et al., 2002	Mouse ES cells (stable Nurr1 line, pCMV driving expression of Nurr1)	6-OHDA lesioned rat, immuno-suppressed	Neural induction, includes addition of SHH, FGF8 & FGF2/FGF4.	480,000 cells transplanted. Survival: 4 & 8 weeks.	Grafted cells expressed TH, serotonin & GAD67, at 4 & 8 weeks. No Ki67 cells. Grafted neurons developed functional synapses & electro-physiological properties like mesencephalic neurons. Improvement in rotations, cylinder & paw-reaching. No teratomas detected. Used M2 to detect grafted cells.

	Study	Experimental Paradigms			Notes	Results
		Cells	Host	Protocols		
Different models; <i>in vivo</i> differentiation of neurally induced ES cells	Wernig et al., 2004	TauEGFP ES cells (neuron-specific enhanced GFP)	Embryonic (E16.5) rat, cerebral ventricles	EB formation 5-7 days	Transplanted $2-4 \times 10^5$ cells. Survival: 1 & 14 days after birth.	Cells migrated to various brain regions incl. hippocampus, thalamus, neocortex, & striatum. Immature neural cells; bipolar, multipolar & pyramidal neurons; GAD65/67, & TH. Synaptic integration of donor & host. Ectopic expression of region-specific markers Bfl, Dlx, En1 & Pax6. TauEGFP didn't allow analysis of non-neuronal cell types. Teratomas but no undifferentiated ES cells.
	Ferarri et al., 2006	Cyno1 primate parthoenogenetic ES cell line	6-OHDA rat, immuno-suppressed	SHH/FGF8, then BDNF/AA, differentiation in BDNF/GDNF/dbcAMP/AA. Progenitors day28 (pES); differentiated neurons day42 (dES).	100,000 cells over 2 sites. Survival: 12 weeks.	No sig difference in TH positive cells between pES & dES grafts; 80% improvement in D-amphetamine rotations with dES; 50% with pES. Patches of very dense TH axons, with NeuN, NF70 & Foxg1. Expression of DARPP-32 & calbindin. Heterogeneous graft morphology with non-neural cells.
	Lee et al., 2007	Human ES cells (i)H1; (ii)H9; (iii)RUES1eGFP Non-human primate ES cells (i)Cyno1	<i>In ovo</i> : stage 16-17 chick spinal cord; <i>In vivo</i> : adult rat spinal cord	Rosettes exposed to FGF8/SHH, then RA/SHH. Differentiation in GDNF, BDNF & AA.	Rat; 40,000 cells per side, bilateral. Survival: 1 day, 2 and 6 weeks.	<i>In vitro</i> : FGF8/SHH resulted in forebrain but not hindbrain markers. RA/SHH reduced forebrain & increased hindbrain. <i>In ovo</i> : survival of graft-derived motoneurons. <i>In vivo</i> : Graft survival up to 6 weeks. Expression of motoneuron precursors at 1 day, then decrease at 2 & 6 weeks. Increase in mature neurons over time eg ChAT.

## 1.6 Neural Development

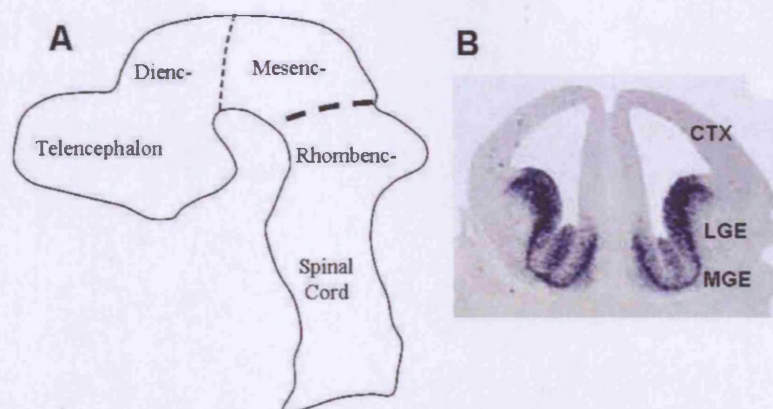
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Currently there is no protocol for robust, efficient and reproducible differentiation of MSNs either *in vitro* or *in vivo*. Central to the development of new protocols for directing the differentiation of cells to specific phenotypes is the understanding of signals that are important in their development in the brain. In this section neural development is reviewed, with the focus being on the developing telencephalon, the origin of the putative striatum.

In early mouse embryonic development, following fertilization, the ICM and primitive endoderm are formed within the trophectoderm. Just prior to gastrulation, at E5.5 to E6, the anterior-posterior axis is formed as the distal visceral endoderm (DVE) migrates towards the anterior pole and gives rise to the anterior visceral endoderm (AVE). The AVE primarily functions to repress posterior gene expression involved in induction of mesoderm, which is as a result of expression of the genes *DKK-1* and *Lefty1*, which act to antagonise Wnt and TGF- $\beta$  signalling in the epiblast. During gastrulation the three germ layers of the gastrula are formed; endoderm, mesoderm and ectoderm. It is at this stage of development that generation of the nervous system begins. Endogenous signals are released from the underlying mesoderm along the midline of the developing brain, and in response to these signals the ectoderm thickens and forms the neural plate. The neural plate folds inwards to generate the neural groove (E7.5), and eventually the folds meet and give rise to the neural tube, a process known as neurulation (E8.5). Patterning of the neural tube at this stage is regulated, in part, by the anterior neural ridge (ANR). As the neural tube develops it bends, resulting in flexures and this gives rise to vesicles along the tube: the telencephalon and diencephalon make up the forebrain, the mesencephalon the midbrain and the rhombencephalon the hindbrain (Figure 1.4; A) (Rubenstein et al., 1998; Lu et al., 2001; Wilson and Houart, 2004; Takaoka et al., 2007).

The developing telencephalon, at the most rostral point of the neural tube, can be divided into the pallium (the dorsal-most region), which gives rise to the cerebral cortex, and the subpallium (the ventral-most region), which forms the striatum and is also the origin of cells that go on to populate the olfactory bulb, GP and cortex. The subpallium comprises the LGE and MGE (Figure 1.4; B). In the human striatum, the MGE starts to appear a day before the LGE, at Carnegie stages 14 and 15 respectively

(Freeman et al., 1995). The MGE gives rise to the amygdaloid body and also the GP and ventral pallidum, whilst the LGE is the foetal primordium which gives rise to the caudate and putamen (Deacon et al., 1994; Sturrock, 1980). Striatal projection neurons originate in the LGE and make up nearly 90% of LGE neurons. The majority of telencephalic interneurons are generated in the ventral telencephalon and then migrate to the region that they populate in the adult brain. Those from the LGE go on to populate the cortex, olfactory bulb and striatum, while those from the MGE will migrate to the cortex, GP and also the striatum (Wilson and Rubenstein, 2000; Anderson et al., 1997b; Anderson et al., 1997a; Marin et al., 2000).



**Figure 1.4.** The distinct regions of the developing mouse brain. The folding of the neural plate and then the neural groove results in the formation of the neural tube, which comprises the four regions of the developing brain: telencephalon, diencephalon, mesencephalon and rhombencephalon (A). Coronal brain section of mouse embryonic day (E) 12.5, showing the dorsal/pallial (CTX) and ventral/subpallial (LGE and MGE) regions of the developing telencephalon. Expression of the *Dlx2* gene showing the position of cells in the LGE and MGE (B). Abbreviations: CTX - cortex; LGE - lateral ganglionic eminence; MGE - medial ganglionic eminence.

During telencephalic development, positioning of progenitor cells within the neural tube accounts for the distinct regionalization seen in the adult brain. The molecular mechanisms involved in forebrain development are complicated and not yet fully elucidated. Signalling centres produce secreted molecules and morphogens which generate transcription factors at various levels, which in turn induce patterning within the neural tube according to their graded levels of expression. It is the expression of combinations of transcription factors at certain times and in specific regions that is responsible for this organisation within the developing neural tube

(Lupo et al., 2006). Dorsal and ventral regions differ with respect to the secreted molecules present in that region and the level at which cells are exposed to these molecules, imposing a degree of regional identity on the cells.

### *1.6.1 Factors involved in telencephalon development*

There are various groups of factors involved in the patterning of the telencephalon. Members of the fibroblast growth factor (FGF) family are one such group. FGF8 is expressed in the ANR and as well as being involved in cell proliferation and cell survival it has been shown to have a role in rostro-caudal patterning and regulates expression of the rostral forebrain marker *Foxg1* (Shimamura and Rubenstein, 1997; Ye et al., 1998; Storm et al., 2006). Interestingly, FGF8 beads inhibit expression of caudal forebrain markers *Otx2* and *Emx2* (Crossley et al., 2001). Expression of FGF8 is not the only factor involved in rostral forebrain formation since in FGF8 null mutant mice the telencephalon is still present although it is small and has patterning abnormalities (Storm et al., 2006; Wilson and Rubenstein, 2000; Shanmugalingam et al., 2000). Loss-of-function studies reveal a role in ventralisation of the telencephalon as there is a loss of the MGE and LGE structures in the absence of FGF8, which coincides with a reduction of ventral gene expression including *Nkx2.1* and *Dlx2*, and an expansion of dorsal gene expression including *Pax6* (Storm et al., 2006). Expression of *Dlx2*, but not *Nkx2.1* has been shown to increase with increased FGF8 (Crossley et al., 2001).

One factor required for maintaining FGF8 expression is the signalling protein sonic hedgehog (SHH) (Ohkubo et al., 2002). SHH is a morphogen secreted from the notochord, which underlies the posterior structures of the brain and is thought to extend anteriorly as far as the posterior diencephalon, and the prechordal plate, which underlies the telencephalon (Rubenstein et al., 1998). It is important in embryonic development and patterning, as well as cancer progression and stem cell renewal, and is required for the generation and development of the mammalian telencephalon, giving rise to distinct neural progenitor subtypes in the ventral neural tube (Jessell, 2000). SHH has been shown to elicit its effect in a concentration-dependent gradient, for example in the patterning of the spinal cord the generation of motoneurons and



interneurons arise in distinct dorso-ventral progenitor domains according to the levels of expression of this morphogen (Jessell, 2000; Altaba et al., 2003). Both the level and duration of SHH expression are important for specification of ventral cell fates (Stamataki et al., 2005).

Expression of SHH is observed in the ventral telencephalon from E11.5 in the developing brain (Kohtz et al., 1998). It plays an essential role in patterning of the forebrain in particular ventralising activity (Kohtz et al., 1998; Ericson et al., 1995; Chiang et al., 1996) and is both necessary and sufficient to induce expression of ventral forebrain markers (Chiang et al., 1996). In mice lacking SHH expression there is a complete absence of ventral forebrain structures, accompanied by an ectopic expression of dorsal forebrain markers in the ventral telencephalon (Chiang et al., 1996), whereas over-expression of SHH results in the dorsal expansion of ventral-specific genes (Goodrich et al., 1997). Culture of telencephalon explants treated with SHH express ventral forebrain markers such as *Nkx2.1* (Ericson et al., 1995), and misexpression of SHH in the cortex results in ectopic expression of *Dlx2* and *Nkx2.1* (Kohtz et al., 1998).

SHH expression initiates a pathway involving patched and smoothed such that when hedgehog binds patched, patched is blocked and smoothed is de-repressed. This activates Gli (glioma-associated oncogene homolog 3) which induces the target gene expression of the SHH pathway (Figure 1.5).



**Figure 1.5.** The SHH induction pathway for target gene expression. When SHH binds Patched, Patched is blocked and Smoothened is freed. The Gli repressor (GliR) becomes activated to the Gli activator (GliA) and this induces target gene expression of Hedgehog. Abbreviations: SHH – sonic hedgehog; Gli – glioma-associated oncogene homolog.

There are three members of the Gli family of zinc finger-containing transcription factors, which are homologues of the *drosophila cubitus interruptus* (Ci), namely Gli1, Gli2 and Gli3. Gli transcription factors have been shown to regulate SHH-dependent gene expression. Stamataki et al (2005) showed that incremental

changes in SHH concentration result in a gradient of Gli activity (Stamatakis et al., 2005). Gli1 and Gli2 proteins generally promote ventral patterning in response to SHH signalling. At low levels of SHH signalling, the Gli3 protein is cleaved from an activator into a repressor form that results in promotion of dorsal patterning. Inhibition of the Gli3 repressor formation appears to be the primary role of SHH in telencephalic patterning, and in *SHH*<sup>-/-</sup>; *Gli3*<sup>-/-</sup> double mutants there is substantial rescue of ventral telencephalic patterning (including expression of *Nkx2.1*) (Gulacsi and Anderson, 2006).

Retinoic acid (RA) is the biologically active form of vitamin A. It has been implicated in survival, proliferation, specification and differentiation during forebrain development (Haskell and LaMantia, 2005; Marklund et al., 2004). RA is generated by members of the retinaldehyde dehydrogenase family, it elicits its effects through retinoic acid receptors (RAR) and retinoid X receptors (RXR), and subtypes ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) of both receptor groups can be found in the developing striatum. RAR $\beta$  is selectively expressed in the LGE and RXR $\gamma$  is primarily expressed in the LGE with low levels in the MGE and no expression in the CGE. *RXR $\gamma$ 1*<sup>-/-</sup> mutant mice exhibit specific reduction of choline acetyltransferase (ChAT) expression in the striatum, with no change in expression in GABA and TH (Saga et al., 1999). Loss of RAR $\beta$  in *RAR $\beta$* <sup>-/-</sup> mice results in reduction of striatal-enriched tyrosine phosphatase mRNA, a gene found to be regulated by RA, in the striatum (Liao et al., 2005), and loss of the compartmental structure of the rostral striatum, with a reduction in DARPP-32 positive neurons as well as dynorphin,  $\mu$ -opioid receptor (MOR1) and TH, compared with wild-type mice (Liao et al., 2008).

RA is required for forebrain development (Schneider et al., 2001; Ribes et al., 2006), and its expression is maintained in forebrain regions throughout adult life (Haskell and LaMantia, 2005). Retinoid signalling has been shown to maintain FGF8 and SHH expression in the forebrain (Schneider et al., 2001; Haskell and LaMantia, 2005), and in the absence of retinoid signalling, expression of FGF8 and SHH is lost (Schneider et al., 2001).

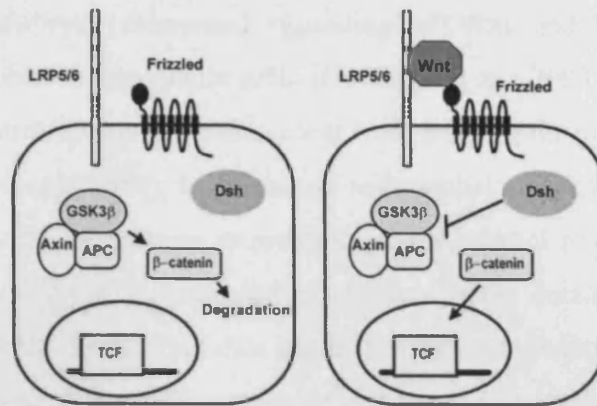
The LGE appears to be a source of retinoid signalling, and retinoids from the glia of this region have been implicated in striatal neuronal differentiation (Toresson et al., 1999). In the developing chick telencephalon, RA has been shown to specify intermediate character. Blocking RA in chick embryos blocks expression of *Meis2*,

which is expressed in progenitor cells in the intermediate zones of the telencephalon (Marklund et al., 2004). In *Gsx2* mutant mice, where development of the telencephalon is perturbed and DARPP-32-expressing neurons are reduced, addition of exogenous RA results in an increase in the number of DARPP-32 positive neurons (Waclaw et al., 2004), thus RA is implicated in the regulation of DARPP-32 in the developing brain.

*In vitro* LGE cultures have been shown to be responsive to both RAR and RXR signalling, showing up-regulation of striatal neuron differentiation (Toresson et al., 1999). Supplementation of RA to LGE cultures induces an increase in DARPP-32 expression (Toresson et al., 1999). This was observed at different RA concentrations ( $10^{-7}$ M and  $10^{-8}$ M) and was specific to the LGE, with no increase in DARPP-32 expression detected in MGE or cortical cultures.

Wingless (Wnt) protein family of ligands are a class of secreted glycoproteins that regulate  $\beta$ -catenin activity and therefore gene transcription and have been shown to be important during development. Intracellular Wnt signalling can be divided into three pathways; the canonical pathway which involves the activation of  $\beta$ -catenin; the planar cell polarity pathway which involves jun N-terminal kinase (JNK); and the calcium pathway which involves calcineurin. The latter two are non-canonical pathways which are  $\beta$ -catenin-independent reviewed in (Huelsken and Behrens, 2002). It is the canonical pathway that is involved in telencephalon development.

In the canonical Wnt signalling pathway (Figure 1.6), Wnt binds to the membrane-bound receptor Frizzled which activates the intracellular component Dishevelled (Dsh). This destabilizes the complex formation between axin and glycogen synthase kinase  $3\beta$  (GSK- $3\beta$ ) which in turn decreases the phosphorylation of  $\beta$ -catenin and prevents its degradation.  $\beta$ -catenin accumulates in the cytoplasm and is then translocated to the nucleus where it binds and activates target transcription factors of Wnt signalling such as T-cell factors (TCF). This regulates the expression of a variety of Wnt target genes such as c-myc and cyclin D1. In the absence of Wnt the cytoplasmic  $\beta$ -catenin joins the axin and GSK- $3\beta$  complex where it becomes phosphorylated and subsequently degraded, preventing its accumulation and translocation into the nucleus for gene activation.



**Figure 1.6.** The canonical Wnt signalling pathway for target gene expression. When Wnt binds Frizzled, Dsh is activated and inhibits formation of the axin-GSK-3 $\beta$  complex. This prevents the break-down of  $\beta$ -catenin and permits its translocation to the nucleus where it induces target gene expression. Abbreviations: APC - adenomatous polyposis coli; Dsh - Dishevelled; GSK3 $\beta$  - glycogen synthase kinase 3 $\beta$ ; TCF - T-cell factor. (Taken from *Frontiers in Bioscience* 9 (Hsieh, 2004).

Using a reporter line where a *LacZ* gene is under the control of  $\beta$ -catenin/TCF responsive elements, the canonical Wnt signalling pathway has been shown to be active in the pallium but not the subpallium of the mouse telencephalon at E11.5 and E16.5 (Maretto et al., 2003; Backman et al., 2005). More recently, a reporter line using *LacZ* tagged to *Axin2* (a negative feedback regulator of the Wnt pathway which is expressed in response to Wnt signalling) revealed Wnt signalling activity in the cortex and up to 5% of cells in the subventricular zone, at E14.5 in the mouse brain (Kalani et al., 2008). Inhibition of Wnt signalling has been reported to be necessary for telencephalic generation (Houart et al., 2002). In order to look at the effects of canonical Wnt signalling in the telencephalon before neurogenesis, Backman et al (2005) generated a mouse line with a conditional Cre/loxP system to inactivate or activate  $\beta$ -catenin before or after neurogenesis in the telencephalon. The loss of  $\beta$ -catenin in the mouse telencephalon prior to the onset of neurogenesis leads to down-regulation of the dorsal markers *Emx1*, *Emx2* and *Ngn2* (Backman et al., 2005). Interestingly, expression of the dorsal telencephalic marker *Pax6* was not down-regulated in this model at this time. Analysis of the subpallial markers *Dlx2*, *Mash1* and *Gsx2* pre-neurogenesis revealed their ectopic expression in the pallium of the inactivated- $\beta$ -catenin mutant. The expression of the MGE marker *Nkx2.1* remained the same in both wild-type and mutant animals.

In chick embryos sequential signalling of Wnt and FGF results in the specification of dorsal telencephalic cells (Gunhaga et al., 2003). During embryonic mouse development Wnt signalling is evident from E6.5, in the posterior region of the embryo (ten Berge et al., 2008). In the mouse telencephalon, activation of  $\beta$ -catenin in the subpallium results in reduced expression of the ventral markers *Nkx2.1*, *Dlx2*, *Gsx2* and *Mash1*, along with expanded expression of the dorsal markers *Pax6* and *Ngn2* (Backman et al., 2005). This data implicates a role for canonical Wnt signalling in the specification of telencephalic cells by repressing induction of ventral character. Analysis of the expression of dorsal and ventral markers in the inactivated- $\beta$ -catenin mutant following onset of neurogenesis revealed that there was no shift in the dorsal-ventral specification of cells of the telencephalon, as was observed pre-neurogenesis (Backman et al., 2005).

Loss of  $\beta$ -catenin in the ventral telencephalon caused reduced growth of the MGE with reduced proliferation of cells in this region but, although neuronal numbers were reduced, the differentiated fate of cortical interneurons and subcortical cholinergic neurons from this region was largely unaltered (Gulacsi and Anderson, 2008).

Bone morphogenetic proteins (BMPs) belong to the TGF- $\beta$  family of secreted growth factors. They are secreted by the roof plate, spread ventrally and influence the induction of dorsal neural cell types in a concentration-dependent manner. Signalling of FGF appears to be necessary for inhibition of BMP expression and the subsequent neural induction in the neural tube (Wilson and Rubenstein, 2000). Increased BMP4 expression by implantation of BMP4-soaked beads results in a reduction of both FGF8 and SHH expression in the telencephalon (Ohkubo et al., 2002), and in the absence of RA there is a reduction in BMP4 expression (Wilson and Houart, 2004). Forebrain specification was repressed in telencephalic explant cultures treated with BMPs as shown by inhibition of *Foxg1* expression, as well as *Nkx2.1* and *Dlx2* (Furuta et al., 1997).

For normal neural development it is essential to have the right levels of the right molecules in the right region so that the appropriate levels of transcription factors are generated and subsequently cells are correctly specified according to their

location, which then ensures the correct balance of mature phenotypes following differentiation.

### 1.6.2 *Foxg1* expression in the developing telencephalon

The earliest known marker of the telencephalon is *Foxg1* (forkhead box G1), formerly known as Brain factor 1 (BF-1) (Tao and Lai, 1992). *Foxg1* is a member of the winged helix family of transcription factors and in mammals was first identified in the developing rat brain with an expression pattern restricted to the telencephalon of the forebrain (Tao and Lai, 1992). This initial study revealed high levels of *Foxg1*, detected by *in situ* hybridization, from E10 in the developing rat telencephalon and reported the absence of expression in the neighbouring diencephalon (Tao and Lai, 1992). From this it was suggested that *Foxg1* might have a role in telencephalon development including establishing boundaries between the forebrain subdivisions.

Xuan et al (1995) generated mice with a null mutation in the *Foxg1* gene. By replacing most of the *Foxg1* coding sequence with a *LacZ* and neomycin cassette, the expression of the  $\beta$ -galactosidase enzyme is under the control of the *Foxg1* promoter. X-Gal histochemistry revealed *Foxg1* expression in the neural tube from as early as E8.5 and E9.0, and at this stage there was no difference in *Foxg1* expression between wild-type and *Foxg1*<sup>-/-</sup> mice. From E10.5 the size of the telencephalon was greatly reduced compared with the wild-type and this was further exacerbated by E12.5 (Xuan et al., 1995; Martynoga et al., 2005).

In the *Foxg1*<sup>-/-</sup> mutant telencephalon there is a significant reduction in the expression of FGF8 at E10.5, as compared with wild-type (Martynoga et al., 2005). This reduction is not due to an increase in apoptosis in this region, since apoptosis was shown to decrease in the *Foxg1*<sup>-/-</sup> mutant telencephalon at this age (Martynoga et al., 2005). BMP expression is increased (indicated by immunohistochemistry for the tyrosine-phosphorylated forms of Smad-1, 5 and 8; direct effectors of BMP signalling) at E11.5 in the *Foxg1*<sup>-/-</sup> mutant telencephalon, but not at the earlier time point of E10.5 where the reduction of FGF8 was observed and Wnt signalling does not appear to be affected (Martynoga et al., 2005).

Furthermore, Xuan et al., (1995) showed that it is the ventral telencephalon that is more severely affected than its dorsal counterpart, and at E12.5 the ganglionic

eminences were not present in the *Foxg1*<sup>-/-</sup> mutants. In addition they reported the presence of the dorsal telencephalic markers *Emx2* and *Pax6*, but not the ventral telencephalic markers *Dlx1* and *Dlx2*. Analysis of region-specific markers of the ventral telencephalon, comparing wild-type and *Foxg1*<sup>-/-</sup> mutant telencephalon, reveals the absence of *Nkx2.1* and *Mash1* from the ventral region at E9.5, and *Gsx2* at E10.5 (Martynoga et al., 2005). This is consistent with earlier findings that report the absence of expression of the ventral markers *Nkx2.1*, *Dlx1/2* and *Gsx2* throughout the telencephalon at E12.5 (Xuan et al., 1995; Dou et al., 1999). In addition, there is an increase in expression of dorsal markers, such as *Pax3*, whose expression reveals an expanded dorsal region in the *Foxg1*<sup>-/-</sup> mutant, and *Pax6*, whose expression expands throughout the telencephalon rather than being restricted to only the dorsal-most region (Martynoga et al., 2005). Studies in the developing cortex have reported that *Foxg1* suppresses the production of the earliest born neurons and in *Foxg1*<sup>-/-</sup> mutants these neurons are over-generated (Hanashima et al., 2004).

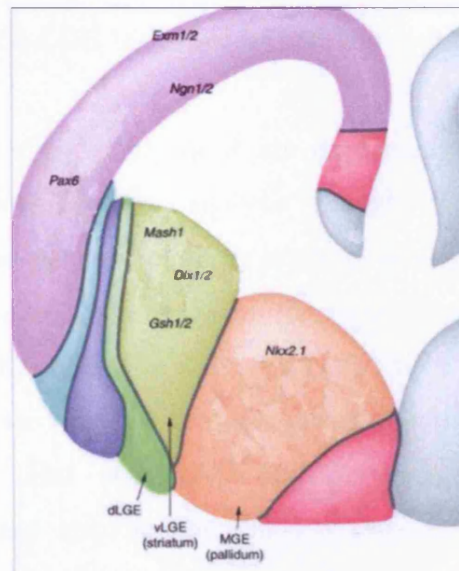
Analysis of cell proliferation by labelling with bromodeoxyuridine (BrdU) revealed that only cells of the dorsal telencephalon, and not the ventral telencephalon, are actively proliferating which suggests that it is the lack of proliferating precursor cells in the ventral telencephalon that is responsible for the absence of this region. A more recent study (Martynoga et al., 2005) introduced a BrdU and iododeoxyuridine (IdU) double-labelling protocol to determine total cell cycle time and the length of S-phase in the cell cycle. They reveal an increase in cell cycle time in the *Foxg1*<sup>-/-</sup> mutant telencephalon however they did not observe this increase in ventral regions (Martynoga et al., 2005) as reported previously (Xuan et al., 1995).

Recently *Foxg1* has been shown to co-ordinate the signalling pathways of SHH and Wnt/ $\beta$ -catenin, which are independently required for development of the subpallial and pallial telencephalon, respectively (Danesin et al., 2009). Danesin et al (2009) demonstrate that *Foxg1* inhibits dorsal identity via the inhibition of the Wnt/ $\beta$ -catenin pathway, and confirm the requirement of *Foxg1* for ventral telencephalic identity, but reveal that *Foxg1* and SHH act independently in their induction of ventral fates.

The studies mentioned here support *Foxg1* as an integral part of forebrain development and reveal its requirement for the expression of ventral telencephalic markers.

### 1.6.3 Dorso-ventral organisation in the developing telencephalon

As mentioned earlier, the developing telencephalon can be divided into the dorsal/pallial portion which is the developing cortex, and the ventral/subpallial portion which is made up of the MGE and LGE. There are distinct boundaries between the dorsal and ventral regions, and between the two ventral subdivisions, the LGE and MGE, in terms of gene expression (Figure 1.7). In the dorsal telencephalon *Pax6*, *Ngn1/2* and *Emx1/2* genes are expressed; in the ventral telencephalon *Gsx2*, *Mash1*, *Dlx1/2* and *Nkx2.1* are expressed. *Nkx2.1* is specific to the MGE; *Gsx2*, *Mash1* and *Dlx1/2* genes are expressed predominantly in the LGE although they are not necessarily specific to this domain.



**Figure 1.7.** Coronal schematic section through the telencephalon showing the dorsal and ventral domains defined by distinct gene expression patterns. Dorsal telencephalic markers include *Emx1/2*, *Ngn1/2* and *Pax6*, and ventral telencephalic markers include *Mash1*, *Dlx1/2*, *Gsh1/2* (now *Gsx1/2*) and *Nkx2.1*. Figure adapted from (Schoorjans and Guillemot, 2002).

*Pax6* is a paired homeodomain gene essential for the proper development of cortical progenitors and its expression is restricted to proliferating precursors in the developing cortex (Toresson et al., 2000). *Emx1* is a marker of the dorsal telencephalon and its expression domain is further limited to the dorsal region of the cortex, with no expression in the ventral portion of the cortex. *Neurogenin 1* (*Ngn1*) and *neurogenin 2* (*Ngn2*) genes encode basic helix-loop-helix (bHLH) transcription



factors that are necessary for determining dorsal telencephalic phenotype. *Ngn1/2* are expressed throughout the cortex along with *Pax6*.

*Pax6* expression is first observed in the developing forebrain at E8.0 (Stoykova and Gruss, 1994). *Pax6* mutant mice, also known as the *Small eye (Sey)* mutation, have patterning defects in the forebrain with abnormal development involving a shift in the cortical-striatal boundary (Stoykova et al., 1997) and expression of other dorsal markers such as *Ngn1/2* and *Emx1* are down-regulated in the cortical region (Toresson et al., 2000; Stoykova et al., 2000). Ventral markers of the LGE, *Dlx1/2* (Stoykova et al., 1996; Stoykova et al., 2000; Toresson et al., 2000), *Mash1* (Toresson et al., 2000; Stoykova et al., 2000) and *Gsx2* (Toresson et al., 2000) show ectopic expression across this boundary into more dorsal regions of the *Pax6* mutant telencephalon, and expression of the MGE marker *Nkx2.1* expands dorsally into the LGE and shifts the LGE-MGE border (Stoykova et al., 2000).

The neurogenins *Ngn1* and *Ngn2* are expressed exclusively in the dorsal telencephalon and loss-of-function analysis reveals their requirement for the maintenance of the dorsal-ventral boundary in the developing telencephalon (Fode et al., 2000). In the absence of *Ngn* expression the ventral marker *Mash1* is ectopically expressed in the dorsal telencephalon, resulting in dorsal cells acquiring a ventral fate. This subsequently alters the specification of a subset of cortical neurons to express the ventral markers *Dlx1*, *Dlx2* and GAD 65/67, which is expressed by ventral telencephalic neurons and encodes the enzyme required for biosynthesis of the neurotransmitter GABA.

In the wild-type developing forebrain, expression patterns of the ventral gene *Gsx2* and the dorsal gene *Pax6* are complementary. Further, these two genes have been shown to cross-repress each other in order to generate the cortical-striatal (pallial-subpallial) boundary and correct regional expression of each is dependent on the other. The regions of the pallium and subpallium that are immediately proximal to this boundary have been termed the ventral pallium and the dorsal LGE (dLGE), and in the dLGE expression of *Pax6* and *Gsx2* overlaps at E10.5 in the mouse (Yun et al., 2001). The pallial-subpallial boundary (PSB), a source of a subgroup of Cajal-Retzius cortical neurons and olfactory bulb interneurons, requires both *Pax6* and *Gsx2* for correct expression of PSB-specific genes (Carney et al., 2009).

*Gsx2* (formerly *Gsh2*) is a homeobox gene (Hsieh-Li et al., 1995) with expression first detected in the developing forebrain between E9 and E10 and during early telencephalic development it is restricted to the prospective LGE. It is essential for the early expression of a number of genes that mark various aspects of specification and differentiation of the LGE (Corbin et al., 2000). *Gsx2* is also essential for specification of distinct cell populations and the establishment of the molecularly defined boundary between the developing cortex and the LGE.

Loss of *Gsx2* in *Gsx2*<sup>-/-</sup> mutants results in loss of *Mash1* and *Dlx2* (Szucsik et al., 1997; Corbin et al., 2000; Toresson et al., 2000), accompanied by expansion of dorsal markers *Pax6* and *Ngn2* into the LGE (Corbin et al., 2000; Toresson et al., 2000). Overall there is a reduction in LGE size from E12 (Szucsik et al., 1997), reduction in striatum size at E18.5 (Wang et al., 2009) and reduced expression of striatal projection neurons which originate in the LGE (Deacon et al., 1994; Olsson et al., 1998). Interestingly the decreased *Dlx2* is more pronounced in LGE than MGE, and expression of the MGE specific marker *Nkx2.1* is not affected by the loss of *Gsx2* (Szucsik et al., 1997). From these loss-of-function studies it has been established that *Gsx2* is required for the early expression of *Mash1*, *Dlx1* and *Dlx2* in the ventral telencephalon, and promotes expression of these ventral genes while repressing dorsal character via cross-repression with *Pax6*. In the *Gsx2*<sup>-/-</sup>;*Pax6*<sup>-/-</sup> double mutant there is rescue of the telencephalic defects reported in the single mutants of these genes (Corbin et al., 2003).

Gain-of-function studies with *Gsx2* revealed no ectopic expression of *Dlx2* or *Mash1*, nor inhibition of the dorsal markers *Pax6* and *Ngn2*, but it did cause ectopic expression of the retinol binding protein (RBP) normally expressed in LGE radial glial cells, which is not lost in the *Gsx2*<sup>-/-</sup> mutant (Corbin et al., 2000). In *SHH* knockout mice the striatum is lost (Chiang et al., 1996) along with loss of *Gsx2* and RBP in the forebrain (Corbin et al., 2000). In addition, mis-expression of *SHH* by injection of a *SHH*-expressing retrovirus *in vivo* resulted in ectopic expression of *Gsx2*. Taken together this indicates that *Gsx2* is a downstream target of *SHH* signalling (Corbin et al., 2000).

*Mash1*, also called *Ascl1* (Mouse Genome Informatics) is a basic helix-loop-helix (bHLH) gene which has been shown to be essential for proper development of the ventral telencephalon (Casarosa et al., 1999). In contrast to the dorsal bHLH genes

*Ngn1/2* which are expressed only in the dorsal telencephalon, *Mash1* expression is seen throughout the ventral telencephalon in a complementary pattern to *Ngn1/2* (Fode et al., 2000). Analysis of mutant mice that lack *Mash1* (*Mash1*<sup>-/-</sup>) reveals defects in the developing telencephalon including a reduction in size of the MGE and loss of early born striatal neurons (Casarosa et al., 1999). Expression of *Gsx2* in the *Mash1*<sup>-/-</sup> mutant is similar to that in the wild-type at E12.5, but by E18.5 there is an increase in *Gsx2*-expressing cells indicating a potential repressive function of *Mash1* on *Gsx2* at a later developmental time point (Wang et al., 2009).

Expression of the dorsal marker *Ngn2* in the *Mash1*<sup>-/-</sup> mutant is similar to that in the wild-type (Wang et al., 2009), whereas in the *Gsx2*<sup>-/-</sup> mutant, dorsal character expands ventrally with expression of *Ngn2* (Corbin et al., 2000; Wang et al., 2009) and *Pax6* (Corbin et al., 2000) observed in the LGE.

*Gsx1*, a gene closely related to *Gsx2*, is also expressed in the ventral telencephalon and has been implicated in partially compensating ventral telencephalon patterning in *Gsx2*<sup>-/-</sup> mutants (Wang et al., 2009). In the *Gsx2*<sup>-/-</sup> mutant, expression of *Gsx1* and *Mash1* is similar in the LGE. DARPP-32 expression is absent and calbindin expression is maintained in both *Gsx2*<sup>-/-</sup>; *Mash1*<sup>-/-</sup> and *Gsx2*<sup>-/-</sup>; *Gsx1*<sup>-/-</sup> double mutants. The similarities in mutant phenotypes suggest that *Mash1* is necessary for the role of *Gsx1* in striatal compensation in *Gsx2*<sup>-/-</sup> mutants (Wang et al., 2009).

Analysis of the striatal neuronal marker FoxP1 at E18.5 reveals a slight reduction of expression in the *Mash1*<sup>-/-</sup> mutant, a more marked reduction of expression in the *Gsx2*<sup>-/-</sup> mutant and a severe reduction in the *Gsx2*<sup>-/-</sup>; *Mash1*<sup>-/-</sup> double mutant (Wang et al., 2009). A similar pattern is seen with DARPP-32, the striatal projection MSN marker, with the *Gsx2*<sup>-/-</sup>; *Mash1*<sup>-/-</sup> double mutant exhibiting a complete loss of DARPP-32 expression in the striatum (Corbin et al., 2000; Wang et al., 2009). Interestingly expression of the striatal-matrix marker calbindin is reduced in the *Mash1*<sup>-/-</sup> striatum and slightly increased in both the *Gsx2*<sup>-/-</sup> and *Gsx2*<sup>-/-</sup>; *Mash1*<sup>-/-</sup> striatum (Corbin et al., 2000; Wang et al., 2009).

*Distal-less* homeobox (*Dlx*) 1 and 2 genes have almost identical expression patterns in the developing brain (Bulfone et al., 1993). Bulfone et al looked at the expression at E12.5 and observed that in the telencephalon, both *Dlx1* and *Dlx2* were

found to be expressed in the MGE, the LGE and the septum, and the only difference in expression in these areas was the intensity of the hybridization signal, which was always greater for *Dlx1*. *Dlx1/2* expression patterns suggest a role in striatal development and evidence supports a role in differentiation of the basal ganglia and migration of cells from the subcortical telencephalon to the neocortex (Anderson et al., 1997a; Anderson et al., 1997b). Single mutants of *Dlx1* and *Dlx2* have almost no forebrain defects, but double *Dlx1/2* mutants reveal marked abnormalities in the development of the striatum, specifically LGE (Nery et al., 2003; Anderson et al., 1997b).

Expression of these genes begins when progenitors move from the ventricular zone (VZ) into the sub-ventricular zone (SVZ) and later these *Dlx*-expressing cells migrate away from this proliferative zone to various regions of the brain and *Dlx* expression is switched off when the cells differentiate (Nery et al., 2003). *Dlx2*-expressing cells are highly migratory, following which they differentiate into interneurons (Anderson et al., 1997a). Overexpression of *Dlx2* can induce telencephalic progenitors to express GAD65/67 (Stuhmer et al., 2002a). Analysis of mice with the *tauLacZ* reporter gene inserted into the *Dlx2* locus shows migration of *Dlx2*-expressing cells firstly to the cortex from E13.5 onwards and later to the olfactory bulb and hippocampus (Nery et al., 2003). Another fate-mapping study supports the presence of *Dlx* cells expressing GABA in the cortex (Stuhmer et al., 2002b). Further *Dlx1/2* double mutants display a reduction in cortical GABAergic interneurons (Anderson et al., 1997a) and a loss of later born striatal matrix neurons that are believed to be derived from the SVZ (Anderson et al., 1997b).

*Nkx2.1*, also known as thyroid transcription factor 1 (TTF1) (Mouse Genome Informatics) is another homeodomain-containing transcription factor. It is expressed in the medial subdivision of the WGE, is required for the development of this region and functions to repress LGE character in the MGE. Expression of *Nkx2.1* is induced by SHH (Ericson et al., 1995). Inhibition of SHH results in reduced expression of *Nkx2.1* in the MGE (Gulacsi and Anderson, 2006) with dorsalisation of the ventral telencephalon (Chiang et al., 1996) and overactivation of SHH signalling results in the dorsal expansion of *Nkx2.1* (Goodrich et al., 1997). Loss of Gli3 however does not cause dorsalisation of the MGE suggesting that the SHH-induced *Nkx2.1* expression is independent of Gli3.

In the absence of *Nkx2.1*, in *Nkx2.1<sup>-/-</sup>* mice, the MGE fate converts to that of the LGE, with some MGE-like cells expressing DARPP-32 (Sussel et al., 1999). Generation of *Nkx2.1<sup>-/-</sup>* mutants with the *Dlx2 tauLacZ* reporter gene revealed loss of *Dlx2* early in development but not later (Nery et al., 2003). The *Nkx2.1<sup>-/-</sup>* mutant results in loss of GABA and calbindin positive neurons from the cortex, and also loss of striatal cholinergic cells (Sussel et al., 1999). In *Nkx2.1<sup>-/-</sup>;Gsx2<sup>-/-</sup>* double mutant mice the combined loss of these medial and lateral ventral telencephalon genes results in more profound effects on regional patterning of the MGE and LGE (Corbin et al., 2003).

In the knockouts of *Dlx1/2*, *Mash1* and *Nkx2.1* a reduction in cortical interneurons is observed (Anderson et al., 1997a; Anderson et al., 1997b; Casarosa et al., 1999; Sussel et al., 1999; Marin et al., 2000). The cortical interneurons of *Gsx2* mutants are unaffected and it is the olfactory bulb interneurons that are altered in this case (Corbin et al., 2000). This suggests a role for *Gsx2* in olfactory bulb interneuron differentiation and migration. These factors are essential for establishment of regional specification during development and also the subsequent identity of mature neurons that originate in the specified regions. The window of expression and balance between these dorsal-ventral transcription factors are important in ensuring the identity of cells in the correct region.

## 1.7 Directed differentiation of ES cells

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Mouse ES cells *in vitro* are maintained in their self-renewing, pluripotent state in the presence of leukaemia inhibitory factor (LIF) and upon withdrawal of this cytokine ES cells lose this undifferentiated state and begin to differentiate. For *in vitro* differentiation of ES cells, different induction conditions are applied depending on the cell type of interest. Mouse ES cells can be induced to differentiate into a neural lineage (Bain et al., 1995; Okabe et al., 1996). The original approach towards promoting neural cell populations involved culture of mouse ES cells in bacteriological dishes where they grow as free-floating multicellular aggregates termed embryoid bodies (EB) (Bain et al., 1995). In this study EBs were cultured for

4 days without RA followed by 4 days with RA in medium without LIF or  $\beta$ -mercaptoethanol, and the initial cell-seeding density was arbitrarily determined as a 'quarter of a confluent flask'. When these aggregates were dissociated and plated as a monolayer, cells developed a neuronal appearance and 38% were immuno-reactive for the neuronal marker  $\beta$ -III-tubulin, whereas untreated aggregates generated no  $\beta$ -III tubulin positive neurons.

Suspension culture of EBs for 4 days followed by attachment to substrate and change of culture medium to DMEM/F12 supplemented with insulin, transferrin, selenium and fibronectin (ITSF medium) for 6 days resulted in 84% cells showing immuno-reactivity for the neural precursor marker nestin (Okabe et al., 1996). These factors have a role in cell survival with insulin in particular shown to be specific for survival of nestin-positive cells. Addition of basic FGF to the culture medium resulted in increased proliferation of nestin-positive cells and subsequent differentiation in the absence of basic FGF revealed a threefold decrease in nestin-positive cells and three- to four-fold increase to 60% in MAP2-positive neurons (Okabe et al., 1996).

It has been shown that mouse ES cells will survive, proliferate and differentiate in serum-free medium (Johansson and Wiles, 1995), and can yield neuroectodermal cells under these conditions (Ying et al., 2003). This indicates that the intermediate step of EB generation prior to neural precursor cell (neural lineage) induction can be by-passed. Direct transfer of mouse ES cells to suspension culture with no serum, but bovine serum albumin (BSA), synthetic lipids, LIF, transferrin and insulin, collectively termed chemically defined medium (CDM), results in differentiation as neural embryoid bodies (NEB) with up-regulation of neuroectoderm and telencephalic precursor marker Pax6 (Wiles and Johansson, 1999). This study showed that with addition of activin the aggregates generate both mesoderm and Pax6 in parallel, but a dose response of BMP4 reveals that Pax6 expression is inversely related to BMP4 concentration.

Continued investigations into the serum-free (CDM) suspension culture system have shown the importance of an optimal cell-seeding density for suspension cultures from ES cell colony dissociation (Bouhon et al., 2005; Watanabe et al., 2005). The neurogenic potential of the suspension cultures has been reported following immunocytochemistry identifying nestin, sox1 and RC2 immuno-positive cells following 8 days in CDM and when these cells are dissociated and plated onto substrate they are seen to express  $\beta$ -III tubulin (Bouhon et al., 2005). Fluorescence

activated cell sorting (FACS) analysis reveals 83% nestin positive cells at day 2 and a decrease to 67% by day 8 in CDM suspension culture. With addition of FGF2 to the medium the percentage of nestin positive cells at day 8 is more similar to that seen at day 2 in CDM alone. Cultures beyond day 8 are dependent on FGF2 for maintenance (Bouhon et al., 2005). Markers of the developing neural tube were seen to up-regulate between day 4 and day 8 in CDM culture, including *Pax6* (dorsal telencephalon marker) and *Gsx2* (ventral telencephalon marker) (Bouhon et al., 2005).

Wnt signals have been shown to inhibit neural differentiation of EBs *in vitro* (Aubert et al., 2002). Within EBs, activation of the Wnt pathway is necessary for establishing the anterior-posterior axis (ten Berge et al., 2008). Addition of exogenous Wnt induces posterior character with mesendodermal differentiation, while inhibiting Wnt signals induces anterior character with neuroectodermal differentiation (ten Berge et al., 2008).

In the serum-free suspension culture system expression of Wnt3a is not detected at day 2 but is detectable from day 4 through to day 8 (Bouhon et al., 2005). Supplementing CDM with BMP, LiCl (to mimic Wnt signalling), an FGF receptor antagonist and a Hedgehog antagonist reduced the percentage of neural precursors as shown by immunocytochemistry with Sox1 (Bouhon et al., 2005). Using an ES cell line where the reporter GFP is tagged to Sox1, separate addition of Wnt3a, Nodal (a member of the TGF- $\beta$  ligand superfamily) and BMP each causes a decrease in Sox1-positive cells (Watanabe et al., 2005). The anti-Nodal agent, LeftyA, and the anti-Wnt reagent DKK-1, together caused an increase in Sox1 positive cells, but individually there was only a small non-significant increase and anti-BMP reagents did not have an effect on the Sox1 population (Bouhon et al., 2005). Interestingly, a study looking at the effect of Sox1 on telencephalic neural progenitor cells (from E12-E16 mouse ganglionic eminences) reported that neurosphere formation was not compromised with reduced or absent Sox1 expression, however subsequent differentiation resulted in a significant reduction of  $\beta$ -III-tubulin positive neurons, compared to wild-type derived cultures (Kan et al., 2007).

Addition of DKK-1 during days 0-5 of NEB culture caused an increase in the percentage of Foxg1 positive cells (from 15% to 30%), and this increase was counteracted by addition of Wnt3a (Watanabe et al., 2005). DKK-1 did not affect the percentage of cells positive for TH, 5-HT and Tuj1. With addition of both DKK-1 and LeftyA, Foxg1 positive cells increased to 35%. Late addition of Wnt to serum-free

suspension cultures did not significantly alter the *Foxg1* population, but within the *Foxg1* population there was a reduction of ventral telencephalic markers *Nkx2.1* and *Gsx2*, and an increase in the percentage of the dorsal telencephalic marker *Pax6* (Watanabe et al., 2005).

Analysis of rostral markers revealed that SHH addition to serum-free suspension culture increased *Nkx2.1* positive cells and decreased *Pax6* and *Emx1* positive cells, and did not have a significant effect on *Gsx2* and *Mash1*, without affecting the *Foxg1* population (Watanabe et al., 2005). This indicates that SHH does not have an effect on anterior-posterior patterning but does alter dorsal-ventral patterning within the forebrain population. Inhibition of SHH in defined serum-free medium with the SHH antagonist cyclopamine resulted in increased expression of the dorsal markers *Emx1* and *Pax6*, and decreased expression of the ventral markers *Dlx1*, *Gsx2* and *Nk2.1*, without altering the nestin neural precursor population (Gaspard et al., 2008).

Exposing ES cell cultures to signals indicative of regionalisation and specification can direct their differentiation towards a specific phenotype. Addition of SHH and RA, which induced ES cells to differentiate into motoneurons (Wichterle et al., 2002) has provided proof-of-principle for this strategy of applying knowledge of developmental biology to ES cell differentiation. Further, Soundarajan et al (2006) found that treatment with a SHH agonist and RA (both at  $10^{-6}$ M), induced ES cells to differentiate into functional motoneurons specific to the medial motor column (Soundararajan et al., 2006). Functional GABAergic and glutamatergic neurons have been generated in defined serum-free culture conditions with a greater proportion of glutamatergic neurons generated in the presence of cyclopamine (Gaspard et al., 2008). Together, this demonstrates the potential to direct ES cells to functional mature neuronal phenotypes.

Time and length of exposure have also been implicated as important factors when treating cultures to direct neural differentiation. Early exposure of EBs to high RA concentrations ( $5 \times 10^{-7}$ M) has been shown to promote neural differentiation of ES cells (Bain et al., 1995), but also results in ablation of the forebrain markers *Foxg1* and *Otx2* and induction of caudal markers (Irioka et al., 2005; Watanabe et al., 2005). Later addition results in only suppression of forebrain markers alongside induction of caudal markers (Watanabe et al., 2005). This supports the theory of specific time windows for addition of factors in influencing the fate of neural precursor cells.



When added to cultures of mouse ES cells, RA induces concentration dependent effects on neural differentiation. *Ngn2* expression is enhanced by high RA concentrations ( $>10^{-7}$ M), whilst at lower concentrations, mesodermal (*Brachyury*) and endodermal (*Pdx1*) markers are detected. It has also been demonstrated that higher RA concentrations induce a dorsal phenotype, while lower RA concentrations induce a more ventral phenotype, as indicated by expression of ventral genes such as *Nkx2.2* (Okada et al., 2004). This induction of ventral neural progenitors with low RA concentrations occurred as a result of increased expression of the N-terminus of SHH protein, and the effect was abolished upon addition of the SHH antagonist cyclopamine (Okada et al., 2004).

Notably, expression of the ES cell specific marker Oct-3/4 is shown to persist following four days in CDM, albeit to a lesser degree than the same time in the presence of serum, as shown by the use of an ES cell line where *LacZ* expression is under the control of the *Oct-3/4* promoter (Bouhon et al., 2005). Oct-3/4 expression in serum-free suspension culture down-regulates over time as shown by RT-PCR (Bouhon et al., 2005) and immunocytochemistry (Watanabe et al., 2005), however RT-PCR does still reveal evidence of a faint band at day 8, and immunocytochemistry indicates that a very low percentage of cells are still expressing Oct-3/4 (Bouhon et al., 2005; Watanabe et al., 2005).

The mouse ES cell culture system offers a robust model that is easy to manipulate, enabling integration of reporter systems for analysis of genes of interest in response to signalling cues. Although there are some differences in the maintenance of mouse ES cells compared to human ES cells, their fundamental properties are transferable, including the capacity to respond to developmental cues. Mouse ES cells require LIF in order to maintain their self-renewal and pluripotency characteristics, and in the presence of LIF and serum or serum-replacement, can be maintained *in vitro* indefinitely. Human ES cells are insensitive to LIF and until recently were reliant upon the presence of feeder layers for maintenance (Thomson et al., 1998). Some of the shared characteristics of mouse and human ES cells include expression of the ES cell marker Oct-3/4, telomerase activity and the capacity to form teratomas containing derivatives of the three germ layers (Thomson et al., 1998; and reviewed in Wobus and Boheler, 2005). Human ES cells exhibit a slower growth rate than mouse ES cells and are more susceptible to cell death upon dissociation. One study looked at

addition of a Rho-associated kinase (ROCK) inhibitor, an inhibitor of apoptosis, to attempt to overcome this problem of cell death, and demonstrated that dissociation-induced apoptosis was significantly reduced compared to untreated human ES cells (Watanabe et al., 2007). Further, when they applied the ROCK inhibitor to serum-free suspension culture for neural induction, they were able to generate 30% Foxg1 positive cells, 95% of which expressed the dorsal telencephalic marker Pax6 (Watanabe et al., 2007).

Various methods have been utilised for neural lineage derivation from human ES cells, including co-culture on stromal cells, which induces formation of neural rosettes, with subsequent culture of cells attached to substrate (Perrier et al., 2004; Aubry et al., 2008); stromal cell co-culture followed by suspension culture and then further differentiation on substrate (Park et al., 2005); and direct transfer of ES cells to defined neuralizing medium under substrate and feeder-free conditions (Joannides et al., 2006; Joannides et al., 2007a). Stromal-feeder induction of neural precursors from human ES cells yields a decrease in expression of ES cell markers, such as Oct-3/4, with an increase in both Pax6 and MAP2 expression over time (Perrier et al., 2004). Neural rosettes following sequential application of SHH, FGF8, BDNF, ascorbic acid, GDNF, dibutyryl cAMP and TGF $\beta$ 3, resulted in 30-50%  $\beta$ -III-tubulin positive neurons, with 64-79% of these expressing TH and 1-2% expressing GABA (Perrier et al., 2004). Application of a similar protocol of sequential addition of factors, but with the notable inclusion of DKK-1 and exclusion of FGF8 and GDNF, resulted in 22% MAP2 positive neurons, of which 36% expressed GABA, 53% expressed DARPP-32, 10% expressed calbindin and less than 2% expressed TH (Aubry et al., 2008).

Culture of human ES cells in suspension with defined human neuralizing medium which included human serum albumin, transferrin, insulin, as well as amino acids and lipids, resulted in expression of immature neural markers including Sox1 and Pax6 (Joannides et al., 2007b; Joannides et al., 2007a). Following 14 days terminal neuronal differentiation of these cells at day 25, 95% of the population expressed  $\beta$ -III-tubulin, and at day 70, 60% expressed  $\beta$ -III-tubulin (Joannides et al., 2007b). Further analysis of the day 70 derived neurons revealed that 55% expressed GABA and 34% expressed glutamate (Joannides et al., 2007b).

A novel method for neural induction of human ES cells uses two inhibitors of SMAD signalling (activated in the BMP signalling pathway), noggin and SB431542, which have previously been shown to enhance neural induction (Lee et al., 2007;

Elkabetz et al., 2008; Smith et al., 2008), in order to avoid the use of stromal feeder layers (Chambers et al., 2009). Addition of both inhibitors resulted in an increase in Pax6 expression to 80% of the total population by day 7 in culture. There was also an increase in other neural markers Otx2, Foxg1 and Sox1, with a decrease in Oct-3/4 expression. The generation of such protocols helps to lead the way for more specific induction of ES cells to lineages whilst concomitantly avoiding potential contamination from feeder cells and associated medium components.

One issue regarding the neural induction protocols with ES cells is that differentiation is not exclusively neural and the resulting population is often heterogeneous containing multiple cell types. The continued presence of undifferentiated ES cells in the culture system makes this cell source less attractive as a prospect for transplantation due to their potential to form teratomas. To reduce the risk of teratoma formation cell populations could be subjected to purification using techniques such as FACS, or magnetic activated cell sorting (MACS), or integration of DNA plasmids with antibiotic resistance linked to a specific gene, into an ES cell line, which would enable selection of cells expressing the gene of interest. Another strategy involves increased differentiation of ES cell-derived precursors in culture prior to transplantation in order to encourage down-regulation of pluripotency markers, and with addition of the patterning factors known to be required for correct neural developmental, specifically directing cells towards the desired phenotype.

However, this is not the only issue that needs to be overcome prior to clinical translation, and of equal importance is the reliable and reproducible production of a defined phenotype that not only has the capacity to survive, but also to function following transplantation into the brain. Since primary foetal tissue has provided the proof-of-principle for cell replacement studies and is the current 'standard', it is important, when assessing alternative cell sources, to refer to this 'standard' and be able to draw comparisons.

## 1.8 Aims of this thesis

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It is evident from the research outlined above that transplantation into the neurodegenerative HD brain is a viable strategy for repairing circuitry and function, and although it is unlikely to be the sole therapy responsible for a cure, it has the potential to alleviate some of the symptoms associated with the disease. The experiments in this thesis will explore ES cell-derived neural precursors as an alternative cell source for transplantation in HD. Information obtained from developmental biology which gives an insight into the development of the cell type of interest will be applied in order to attempt to direct ES cell differentiation and generate specified neural precursors, which have the capacity to differentiate into the desired mature MSN phenotype that is lost in HD.

Chapter 3 addresses forebrain characterisation and specification of NEBs including addition of factors known to be important in the developing neural tube. In Chapter 4 NEBs following different times in neural induction culture are transplanted into the QA-lesioned rat striatum model of HD in order to assess survival and differentiation *in vivo*. NEBs are then further treated with both a SHH agonist and antagonist in Chapter 5 in order to determine the effects of this morphogen on the dorsal-ventral patterning of these cells. Following this, in Chapters 6 and 7, the mature phenotypes of the NEBs after differentiation *in vitro* and *in vivo* (in the mouse QA-lesion model) are investigated to confirm or deny the presence of the MSN marker DARPP-32 as well as other neuronal markers.

# *Chapter 2*

## *Materials and Methods*

### *2.1 In vitro methods*

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#### *2.1.1 Mouse ES cell culture*

ES cells were maintained in ES cell culture medium which consisted of Iscove's modified Dulbecco's medium (IMDM) (*Gibco, Invitrogen, Paisley, Scotland, UK*), supplemented with 15% 'knock-out' serum replacement (KSR) (*Gibco*), 1% penicillin/streptomycin (PS) (*Gibco*), non-essential amino acids (MEM) (1mM) (*Gibco*), L-glutamine (2mM) (*Gibco*),  $\beta$ -mercaptoethanol (0.1mM) (*Sigma, Gillingham, Dorset, UK*) and leukaemia inhibitory factor (LIF) (produced 'in-house' as described in Appendix 5).

ES cells were maintained on 10cm tissue culture treated petri-dishes (*Nunc, Thermo Fisher Scientific, Loughborough, Leicestershire, UK*) coated with 0.1% gelatin in Ultrapure water (*Chemicon International, Chandlers Ford, Southampton, UK*). Medium was changed daily and cultures were passaged when sub-confluent (every 2-3 days) using 0.1% trypsin/EDTA (*Gibco*). For passaging, ES cell culture medium was aspirated off and cells were washed with phosphate buffered saline (PBS) pH 7.4 (*Gibco*), then lifted off culture dishes using 0.1% trypsin/EDTA, incubated at 37°C for 2-3 minutes and this enzymatic reaction was stopped by addition of ES cell culture medium. Cells were harvested from the culture dishes and collected by centrifugation at 1000rpm for 3 minutes. The resulting pellet was resuspended in ES cell culture medium followed by re-plating according to the desired ratio for the split, usually  $2 \times 10^6$  cells per 10cm plate.

#### *2.1.2 Freezing and thawing mouse ES cells*

For freezing ES cells were washed with PBS, incubated at 37°C for 2-3 minutes in 0.1% trypsin/EDTA at 37°C, harvested from the culture dishes following addition of ES cell culture medium and centrifuged at 1000rpm for 3 minutes. Frozen

stocks were made by freezing cells at the desired density, usually  $2 \times 10^6$  cells/ml, in 10% dimethyl sulfoxide (DMSO) (*Sigma*) in ES cell culture medium, in cryovials using a cryochamber with isopropanol, at  $-80^\circ\text{C}$ .

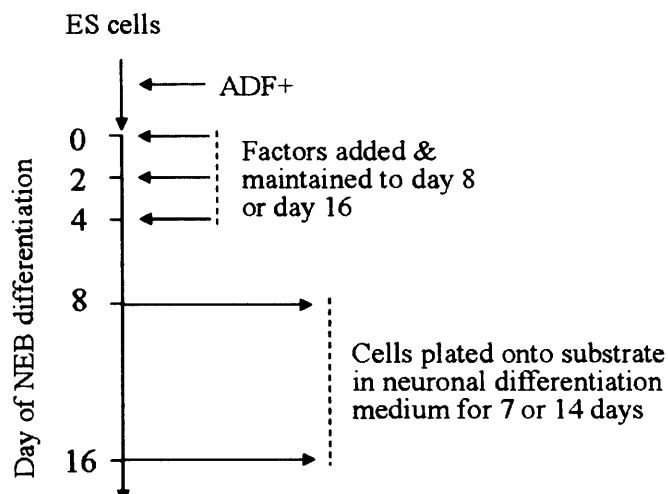
Frozen ES cells were removed from  $-80^\circ\text{C}$ , thawed rapidly in a water-bath at  $37^\circ\text{C}$  for 2-3 minutes and transferred to a tube containing ES cell culture medium before being centrifuged at 1000rpm for 3 minutes. The pellet was re-suspended in fresh ES cell culture medium and the cells were plated on gelatin-treated petri-dishes for culture.

### 2.1.3 Formation and maintenance of NEBs

ES cells were harvested using trypsin-EDTA (as described above) and a single-cell suspension was made. To remove any serum and LIF, cells were washed in 10ml of PBS and then a cell count was performed. For the cell count 10 $\mu\text{l}$  of cell suspension was taken, transferred to a haemocytometer with a glass cover-slip and viewed under the microscope. Cells in the centre square of the haemocytometer were counted and the number of cells per  $\mu\text{l}$  was calculated as well as total cell number.

Cells were re-suspended in a chemically defined medium (CDM), ADF+, which consisted of Advanced DMEM/F-12 (ADF) (*Gibco*), 1 x lipid concentrate (*Gibco*), PS, glutamine (2mM), 12.5mg/ml transferrin (final concentration 150 $\mu\text{g/ml}$ ) (*Sigma*), 10mg/ml insulin (final concentration 14 $\mu\text{g/ml}$ ) (*Sigma*), and  $\beta$ -mercaptoethanol (0.1mM). Cells were plated on untreated bacteriological grade culture dishes (*Sterilin*, *Caerphilly*, *UK*) at a density of  $5 \times 10^4$  cells/ml and this was referred to as day 0. ADF+ was changed every 2 days by collecting NEBs into falcon tubes (*Sterilin*), harvesting a pellet of cells by centrifugation at 500rpm for 3 minutes, followed by resuspending cells in ADF+ and plating onto fresh bacteriological dishes.

For some of the studies the ADF+ medium was supplemented with factors to analyse their effects on the NEB cultures. FGF2 (*R&D Systems*, *Abingdon*, *Oxfordshire*, *UK*) was resuspended in PBS and used at 1, 5, 10 and 20 ng/ml. DKK-1 (*R&D Systems*) was resuspended in PBS and used at 10, 100 and 1000 ng/ml. Purmorphamine (*Calbiochem*, *Merck*, *Hull*, *UK*) was resuspended in DMSO and used at 0.5, 1, 2 and 5 $\mu\text{M}$ . Cyclopamine (*Calbiochem*) was resuspended in methanol and used at 0.1, 0.5, 1 and 2 $\mu\text{M}$ .



**Figure 2.1. Schematic representation of NEB differentiation.**

ES cells are transferred into ADF+ chemically defined medium, termed 'day 0'. Medium is replaced every two days and addition of different factors in the different studies was at day 0, day 2 or day 4. Following initiation of growth factor addition, this factor addition was maintained through to day 8 or day 16, when cells were plated onto substrate for differentiation and analysis.

#### 2.1.4 Differentiation of NEBs

Glass cover-slips were first coated with poly-L-lysine (PLL) (0.1mg/ml in distilled H<sub>2</sub>O) (*Sigma*) overnight at 4°C in 24-well plates (*Iwaki, Sterilin*) and then washed with distilled H<sub>2</sub>O and allowed to dry. Then a 30µl drop of laminin (*Sigma*) (0.1mg/ml in distilled H<sub>2</sub>O) was added to each cover-slip and left for 45 minutes at 37°C. Immediately prior to plating down the cells, the laminin was removed, but the cover-slips were not allowed to dry.

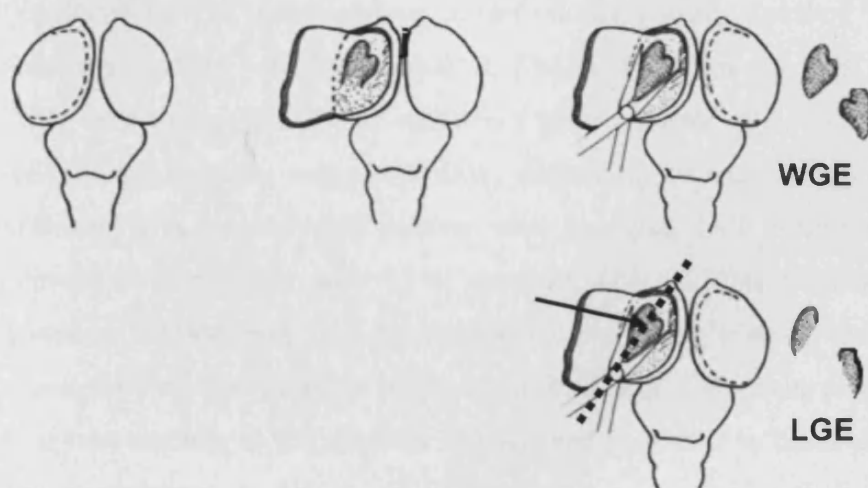
Cells were harvested, washed in PBS and incubated with accutase (*PAA Laboratories, Farnborough, Hampshire, UK*) at 37°C for 15 minutes. A single-cell suspension was generated by gently triturating the cells and neuronal differentiation medium was added to stop the enzymatic reaction. The cell suspension was centrifuged at 1000rpm for 3 minutes, the pellet was resuspended in neuronal differentiation medium (DMEM/F-12, 1% PS, 2% B-27 and 1% FCS) and cells were counted as described previously.

Cells were plated onto the PLL/laminin cover-slips or onto LGE glial cultures (preparation described in section 2.1.2 & 2.1.3), at a known density, usually 25,000 cells/cm<sup>2</sup>, in 30µl of neuronal differentiation medium. (The seeding density is lower

for the differentiating NEBs than the LGE neuronal differentiation cultures because the NEBs continue to proliferate for the first few days following plating and it becomes difficult to visualise individual cells for subsequent counts and analysis.) Cells were allowed to adhere for 2-3 hours at 37°C and then 500µl of neuronal differentiation medium was added to each well before returning the plate to 37°C. Cells were left to differentiate for 7-14 days and medium was discarded and replaced with fresh neuronal differentiation medium every 2-3 days.

### 2.1.5 Dissection and preparation of primary mouse cells

Mouse embryos at embryonic day (E) 14 were collected in Hank's Balanced Salt Solution (HBSS) (*Gibco*), the brains were removed and the striatal eminences were dissected out, as shown in figure 2.2. Dissections were carried out using a dissecting microscope in a laminar flow hood.



**Figure 2.2 Dissection of the striatal eminences.**

Following removal of the brain, a longitudinal cut is made along the cortex, close to the midline, which is opened up to reveal the striatal primordium. To remove the WGE a superficial cut is made, removing the heart-shaped structure. To remove the LGE a vertical cut is made down the midline of the WGE to divide the LGE and the MGE, and the lateral side is collected by a superficial cut underneath (arrow). (Figure adapted from (Dunnett and Bjorklund, 1992).

The dissected tissue pieces were washed in HBSS, then transferred to a solution of 0.1% trypsin (*Worthington, Lakewood, New Jersey, USA*) in 0.5% DNase (*Sigma*) in HBSS for 20 minutes at 37°C. Trypsin inhibitor (*Sigma*) and DNase were



added and the tissue was incubated for a further 5 minutes at 37°C. The tissue was then washed in DMEM/F-12 (*Gibco*), centrifuged at 1000rpm for 3 minutes and the remaining pellet was resuspended in 200 µl DMEM/F-12 and triturated 10-15 times with a 200 µl Gilson pipette to generate a single cell suspension. Cells were counted using a haemocytometer and trypan blue (0.4% trypan blue solution) (*Sigma*) exclusion, in order to assess cell viability. 10µl of cell suspension was taken, diluted with DMEM/F-12 and trypan blue, and transferred to a haemocytometer with a glass cover-slip and viewed under the microscope. Cells in the centre square of the haemocytometer were counted and the number of cells per µl was calculated as well as total cell number, taking the dilution factor into account:

$$\text{cells counted/squares counted} \times \text{dilution factor} \times 10 = \text{cells}/\mu\text{l}$$

### 2.1.6 LGE glia expansion cultures

For generation of LGE glial cultures, cells were resuspended at a density of 200cells/µl in T25 tissue-culture treated flasks (*Iwaki, Sterilin*) with 10ml glial expansion medium, which consisted of; DMEM/F-12, 1% PS, 10% fetal calf serum (FCS), glutamine (2mM) and epidermal growth factor (EGF) (20ng/ml), and the medium was changed every 2-3 days, depending on cell density. When 80-90% confluency was reached, glial cultures were passaged. Cells were washed with PBS, followed by incubation with 0.1% trypsin-EDTA at 37°C for 2-3 minutes. Glial expansion medium was added to inhibit the enzymatic reaction and the cells were harvested by centrifugation at 1000rpm for 3 minutes. Cells were resuspended in glial expansion medium at the required density, and re-seeded in tissue-culture flasks for further expansion.

When glial cultures reached passage 4, stocks were made by harvesting cells and freezing at the required density in 10% DMSO in glial expansion medium, in cryovials using the cryo-chamber.

### 2.1.7 LGE glia co-cultures

Glial cells were plated onto coverslips 2-3 days prior to the beginning of co-culture with neuronal plate-downs. Frozen glial cells were removed from the -80°C storage box, thawed rapidly in a water-bath at 37°C, for 2-3 minutes and transferred to a tube containing glial expansion medium before being centrifuged at 1000rpm for 3

minutes. The pellet was re-suspended in fresh glial expansion medium and cells were plated at a density of 40,000 cells/cm<sup>2</sup> onto 13mm diameter glass cover-slips, which had been coated with PLL. Cells were plated in 30µl drops onto the cover-slips and allowed to adhere for several hours before each well was flooded with 500µl glial expansion medium.

24 hours before co-culture, the medium in the wells was replaced with 500µl glial differentiation medium, which was DMEM/F-12 with 1% PS, 10% FCS and glutamine (2mM).

### 2.1.8 LGE neuronal differentiation cultures

For generation of LGE neuronal differentiation cultures, cells were resuspended in neuronal differentiation medium and seeded onto either PLL-coated glass cover-slips or LGE glia co-cultures. When plated onto PLL-coated cover-slips, cells were seeded at 100,000 cells/cm<sup>2</sup> in 30µl drops and were allowed to adhere for 2-3 hours before the wells were flooded with 500µl neuronal differentiation medium. When plated onto LGE glia co-cultures on cover-slips, cells were seeded at 100,000 cells/cm<sup>2</sup> in 500µl suspension, to prevent the glia cultures from drying out.

Cultures were maintained at 37°C in humidified 5% CO<sub>2</sub> and 95% atmospheric air, and differentiation medium was replaced with fresh medium every 2-3 days.

In Chapter 6 differentiation medium was supplemented with factors to determine their effects on the survival and yield of DARPP-32 positive neurons. Brain-derived neurotrophic factor (BDNF) (*Peprotech, New Jersey, USA*) was resuspended in PBS and used at 50 and 100 ng/ml. RA (*Sigma*) was resuspended in DMSO and used at 10, 100 and 1000 nM.

### 2.1.9 Immunocytochemistry

Following differentiation of cells for the required time period, differentiation medium was removed, cells were washed in PBS and fixative solutions were added. Cells were fixed in either 4% formaldehyde in PBS for 20 minutes, 0.2% glutaraldehyde in 4% formaldehyde in PBS for 15 minutes, or methanol at -20°C for 10 minutes, depending on the antibodies to be used, and were subsequently washed 3 times in PBS.

Appendix 3 lists the antibodies used and details the appropriate blocking sera, optimal concentrations and secondary antibodies to be applied.

Cells were first permeabilised with 100% ethanol for 2 minutes, followed by 3 washes in PBS. To prevent non-specific antibody binding, a blocking serum was applied for 2 hours (3% normal serum in a 3% bovine serum albumin (BSA) in PBS solution). Primary antibody was then added at the appropriate dilution, in 3% normal serum in 3% BSA in PBS, and cells were incubated overnight at 4°C. Where double-labelling was performed, both primary antibodies were added at the same time, ensuring they were raised in different species. The primary antibody was then removed, cells were washed 3 times with PBS and secondary antibody was added, in 3% normal serum in 3% BSA in PBS, and incubated for 2 hours at room temperature and in the dark. The secondary antibody was washed off with PBS and Hoechst nuclear stain was added for 10 minutes. Cover-slips were rinsed in PBS, mounted onto glass microscope slides using PBS:glycerol (1:1), sealed with clear nail varnish and stored at 4°C in the dark.

### *2.1.10 X-gal assay*

Incubation of Foxg1-lacZ ES cell-derived NEB cultures with 5-Bromo-4-chloro-3-indolyl galactopyranoside (X-gal) yields a blue product, allowing visualisation of Foxg1-lacZ positive cells within the population. Cells were harvested, plated onto PLL/laminin substrate and partially fixed in 1% formalin for 5-10 minutes. Following PBS wash X-gal staining solution (see Appendix 2b) was added and cultures were incubated at 37°C for 2-4 hours and then overnight at 4°C. X-gal staining solution was discarded, cells were fixed in 4% formalin for 15 minutes and washed 3x in PBS. Cells were counterstained with eosin solution, which was diluted 1:10 in distilled water, for 10 minutes.

### *2.1.11 Microscopy*

Cells were visualised under UV fluorescence using a Leitz microscope. UV was used to detect the Hoechst positive nuclei and obtain a total cell count within a grid placed randomly on the coverslip with a magnification of 40X. Cells were then checked for counterstaining with antibodies under fluorescence using different UV wavelengths (560nm-red; 494nm-green; 346nm-blue). The grid was placed randomly

over 5 different fields per cover-slip in order for cell counts to be performed, and replicates of 3-4 coverslips were counted per condition. Images were processed using Optronics MagnaFire Software and Adobe Photoshop.

## 2.2 Molecular methods

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### 2.2.1 RNA extraction and quality check

Cells were harvested (as described previously), washed in PBS and RNA was extracted using the Qiagen RNeasy Mini kit, RNase-free DNase set and QiaShredder (all *Qiagen, West Sussex, UK*). Following extraction, the amount of RNA was determined using NanoDrop and 500ng RNA was run on a formaldehyde gel.

Formaldehyde gel was made as a 1.7% agarose gel with addition of formaldehyde from 39% stock solution and 10X 3-(N-Morpholino)-propane sulfonic acid (MOPS) buffer, which consisted of 200mM MOPS pH7.0, 80mM sodium acetate and 10mM ethylene diamine tetra acetic acid (EDTA) pH8.0.

The extracted RNA was mixed with RNA loading buffer heated to 65°C for 10-15 minutes, cooled on ice and then loaded onto the gel. The gel was run at 100V for 40 minutes in 1X MOPS solution, and visualized using ethidium bromide and AlphaImager 2200 Multi-image Light Cabinet.

### 2.2.2 cDNA synthesis

For first strand synthesis, DNase-treated RNA was incubated with random primers (*Invitrogen*) and 10mM dNTP mix for 5 minutes at 65°C. Following a brief chill on ice, 5X first strand buffer, 0.1M DTT and RNase Out RNase Inhibitor (all *Invitrogen*) were added and incubated for 2 minutes at 25°C. Finally, Superscript II (*Invitrogen*) was added and incubated at 25°C for 10 minutes, then 42°C for 50 minutes and then 70°C for 15 minutes. cDNA was used for PCR reactions.

### 2.2.3 RT-PCR: Reverse Transcription – Polymerase Chain Reaction

For PCR amplifications, the basic PCR mix used was: cDNA from first strand synthesis, 10X Bioline NH<sub>4</sub> Reaction Buffer (*Bioline, London UK*), 50mM MgCl<sub>2</sub>, 10mM dNTP, BioTaq DNA polymerase (*Bioline*), oligo pair at 10pmol each and water (*Sigma*), to make it a 25µl reaction. The reaction conditions used were initial denaturation step at 95°C for 1 minute, with a cycle of denaturing at 95°C for 45 seconds, annealing at 60°C for 1 minute and extension at 72°C for 1 minute. The final extension was at 72°C for 10 minutes. PCR products were analysed by electrophoresis on a 1% agarose gel and visualised with ethidium bromide.

### 2.2.4 QPCR

For QPCR reactions the fluorescent probe SYBR Green (*Bioline*) was used. For each cDNA sample to be analysed 3 replicate wells were prepared with 1 µl cDNA (diluted with water at 1:20), 10 pmol of each of the oligo pairs of interest, 10 µl SYBR Green mastermix and water (*Sigma*) to give a 20 µl reaction mix. Opticon Monitor 3 software was used for QPCR analysis. Amplification conditions used were 95°C for 15 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Melt curves were generated from readings every 0.5°C between 53°C and 95°C. Amplification of the target transcript sequence was quantified using relative quantification, where the ratio between the Ct value of the target transcript and that of  $\beta$ -actin was determined.

### 2.2.5 Primer sequences

Primer pairs were generated using *Mouse Genome Informatics* and *Ensembl* for sequence information, and *Primer 3 Input* for primer design. All QPCR primers were designed to; melt at 60°C, be a sequence of 18-22 base pairs in length and generate a transcript of 80-120 base pairs. Primer pair sequences are listed in Appendix 4.

### 2.2.6 Transformation and inoculation of plasmid DNA

Plasmid DNA was added to TOP10 *E. coli* chemically competent cells (*Invitrogen*) and left on ice for 20 minutes. This underwent a heat shock at 42°C for 1 minute and was then transferred to ice for a further 2 minutes before 1 ml cultivation medium (*Invitrogen*) was added. The bacterial suspension was then transferred to a bacteriological tube (*Falcon, Becton Dickinson Labware, New Jersey, USA*) and left to shake at 37°C for 30 minutes. Subsequently, 100 µl bacterial suspension was streaked onto a plate of selective agar (ampicillin resistant) and left at 37°C overnight.

An individual colony was picked using an inoculation loop, and transferred to a bacteriological tube with 5ml Luria-Bertani (LB) media (*DIFCO; Becton Dickinson*) and ampicillin (150 µg/ml) (*Sigma*), and left to shake at 37°C overnight.

### 2.2.7 Midi prep

A colony from the 'master plate' was picked and inoculated in LB media plus ampicillin (150µg/ml) and left to shake at 37°C overnight. From this bacterial suspension, a glycerol stock was made by combining 600µl bacterial suspension with 400µl LB media:glycerol (1:1). The remainder of the bacterial suspension was centrifuged and a Midi prep was performed on the resulting pellet using the Qiagen Midi prep kit, according to manufacturer's instructions. A sample of the resulting DNA was run on a 0.8% agarose gel and visualized with ethidium bromide. The DNA was stored at -20°C in Tris-EDTA (TE) at 1µg/µl.

### 2.2.8 DNA digest

DNA was digested for an analytical check by incubation with the appropriate restriction enzyme, 10X BSA and 10X Buffer (*New England Biolabs, Ipswich, MA, USA*), at 37°C overnight. At this stage, a sample of the DNA was run on a 1% agarose gel against undigested DNA, and visualized with ethidium bromide.

### 2.2.9 Transfection

For transfection, COS (monkey renal epithelial) cells were used. Cells were maintained on T25 tissue culture treated flasks in DMEM supplemented with 10% FCS, L-Glutamine (2mM) and 1% PS. Medium was changed every 2-3 days and cells were passaged at 70-80% confluence. Briefly, medium was aspirated off, cells were washed in PBS, trypsin-EDTA was added and cells were incubated at 37°C for 2-3 minutes. Cells were harvested, resuspended in COS culture medium and centrifuged at 1000rpm for 3 minutes. The pellet was resuspended in COS culture medium and cells were re-seeded according to desired ratio for split, usually  $2 \times 10^6$  cells per T25 flask. Cells were transfected at 70% confluency and 2-4 hours before transfection, culture medium was changed to that containing no PS.

Transfection reagent FuGENE 6 (*Roche, West Sussex, UK*) was used according to instructions in the kit. FuGENE 6 was added to Opti-MEM (*Gibco*) and left at room temperature for 5 minutes. Then plasmid DNA was added and left for a further 15 minutes at room temperature and then more Opti-MEM was added. The media was aspirated off the cells and 'transfection mix' was added and left at 37°C for 2-3 hours. Following this incubation period, Opti-MEM was added and the cells

were left at 37°C overnight. After 24 hours, the transfection mix was discarded and replaced with COS cell culture medium.



## 2.3 *In vivo* methods

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### 2.3.1 *Animal care, anaesthesia and immunosuppression*

All animal experiments were performed in compliance with local ethical guidelines and approved animal care according to the UK Animals (Scientific Procedures) Act 1986 and its subsequent amendments. Adult female Sprague-Dawley rats (*Harlan, UK*) weighing 200-250g at the start of the experiment were used, and were housed in cages of up to four in a natural light-dark cycle with access to food and water *ad libitum*.

All surgery was performed under gaseous isoflurane anaesthesia. Anaesthesia was induced in an induction chamber with isoflurane and oxygen, and maintained by passive inhalation of isoflurane (1-2l/min) and a mixture of oxygen (0.8l/min) and nitrous oxide (0.4l/min). Animals were recovered in a warmed recovery cage and received analgesia by paracetamol dissolved in drinking water (2mg/ml) for 3 days following surgery.

In experiments involving xeno-transplantation into adults, animals were immunosuppressed with daily intraperitoneal injections of Cyclosporin A (CsA, Sandimmun, 10mg/kg) (*Novartis, Hampshire, UK*) commencing the day prior to transplantation and continuing for the duration of the experiment.

### 2.3.2. *Quinolinic acid lesion*

Quinolinic acid (QA) was dissolved in 0.1M phosphate-buffer to make a 90mM solution. Animals received a unilateral injection of 45nmol QA into the right striatum.

For QA lesions in rats, a Hamilton syringe attached to a pump and cannula was used to infuse 0.75µl of QA solution at two sites for 3 minutes per site and 1.5 minutes each at two heights. The stereotaxic coordinates from bregma were +0.4/+1.4 mm anterior-posterior (AP) and -3.2/-2.4 mm lateral (L). The heights (H) below dura were -5.0 mm and -4.0 mm. Following QA infusion, the needle was left at the lesion site for a further 3 minutes before being withdrawn. The incision was sutured and the animals were administered a subcutaneous injection of 5ml saline glucose into the scruff and an intramuscular injection of 0.15ml diazepam into the upper leg.

For QA lesions in mice 0.5µl QA was infused at one site for 4 minutes. The stereotaxic coordinates from bregma were +0.9mm AP, -1.8mm L and -2.7mm below dura. The needle was left at the lesion site for a further 4 minutes before being withdrawn, the incision was sutured and animals were administered a subcutaneous injection of 0.5ml saline glucose into the scruff and an intramuscular injection of 0.03ml diazepam into the upper leg.

### *2.3.3 Unilateral striatal grafts*

For transplants into adult rat brain 2µl of cell suspension (250,000cells/µl) were delivered at a rate of 1µl/minute with 1 minute each at two heights, using a Hamilton syringe. Grafts were placed ipsilateral to the side of the lesion using the stereotaxic coordinates +0.6 mm AP and -2.8 mm L from bregma, at two heights -5.0 mm and -4.0 mm below dura. Following grafting, the needle was left at the graft site for a further 2 minutes before being withdrawn. The incision was sutured and the animals were administered a subcutaneous injection of 5ml saline glucose into the scruff.

For transplants into adult mouse brain 2µl of cell suspension (125,000cells/µl) were delivered at a rate of 1µl/minute with 1 minute each at two heights, using a Hamilton syringe. Grafts were placed ipsilateral to the side of the lesion using the stereotaxic coordinates +0.9 mm AP and -1.8 mm L from bregma, at two heights -2.7 mm and -2.4 mm below dura. Following grafting, the needle was left at the graft site for a further 2 minutes before being withdrawn. The incision was sutured and the animals were administered a subcutaneous injection of 0.5ml saline glucose into the scruff.

### *2.3.4 Perfusions and sectioning*

Animals were terminally anaesthetised by intraperitoneal administration of 0.5ml sodium pentobarbital (Euthatal) and transcardially perfused with a prewash solution, pH 7.3 for 2 minutes followed by 1.5% paraformaldehyde solution, pH 7.3 (PFA) for 3 minutes. The brains were removed, post-fixed in 1.5% PFA overnight and transferred to 25% sucrose in prewash solution until they sank.

Brains were sectioned coronally at 40  $\mu$ m thickness using a freezing-stage microtome. Sections were stored in 0.2% sodium azide in Tris-buffered saline (TBS), in 24-well plates at 4°C.

### 2.3.5 Cresyl violet staining

Brain sections (1 in 12 series) were mounted onto glass microscope slides, previously double-subbed with 1% gelatin, and allowed to dry. Sections were subjected to 10 minutes in increasing levels of alcohol from 70%, to 95% and then 100%, followed by xylene for 1 hour and then decreasing alcohols for 10 minutes from 100% to 95% to 70% and then distilled water, before incubation in the cresyl violet stain for 10 minutes. Stained sections were rinsed in water, dehydrated in 70% and 95% alcohol, then decolourised in 2.5% acetic acid in 95% alcohol for 2-5 minutes, until the desired colour was achieved, further dehydrated in 95% and 100% alcohol and then cleared in xylene before coverslips were mounted using DPX mountant.

### 2.3.6 Acetylcholinesterase (AChE) histochemical staining

Brain sections (1 in 12 series) were mounted onto glass microscope slides, previously double-subbed with 1% gelatin, and allowed to dry at 37°C overnight. Sections were transferred to glass koplins jars with incubation solution for 3 hours at 37°C. The incubation solution comprised acetylthiocholine iodide and ethopropazine in a stock solution of copper sulphate, glycine and sodium acetate in distilled water. Following the incubation sections were thoroughly washed 4 x 3 minutes in distilled water and then transferred to sodium sulphide solution for 30 seconds to 2 minutes for the brown colour to develop. Sections were then allowed to dry overnight, and were then dehydrated in 70%, 95% and 100% alcohol, cleared in xylene and coverslips were mounted with DPX mountant.

### 2.3.7 Immunohistochemistry on free-floating tissue sections

1 in 12 series of brain sections were washed in TBS and then quenched with 10% hydrogen peroxide and 10% methanol in distilled water for 5 minutes followed by three 5 minute washes in TBS, pH 7.4. Blocking solution of 3% appropriate serum in 0.2% Triton X-100 in TBS (TXTBST) was added for 1 hour, and then, without

washing, block was discarded and primary antibody was added at the appropriate concentration (see Appendix 3) in 1% serum in TXTBS and incubated overnight at room temperature. Negative controls were performed in parallel, where the primary antibody was not added and sections remained in blocking solution. Sections were washed 3 times in TBS before addition of a biotinylated secondary antibody at 1:200 dilution in 1% serum in TBS for 2 hours. The secondary antibody solution was washed off by 3 washes in TBS and streptavidin ABC (A and B both at 1:200 dilution in 1% serum in TBS; prepared 30 minutes before addition) was added for a further 2 hours. The sections were washed 3 times in TBS followed by twice more in 0.05M tris non saline (TNS) pH 7.4 and positive staining was visualised using either diaminobenzidine (DAB) at 0.5mg/ml in TNS with 12µl hydrogen peroxide, which reveals the cells as brown colour, or the vector SG kit (*Dako, Glostrup, Denmark*) made up in TNS, which reveals the cells as a grey colour. Following the appropriate colour change, sections were washed twice in TNS before being mounted on gelatinised glass microscope slides, allowed to dry and then dehydrated in increasing levels of alcohol, cleared in xylene and coverslips were mounted using DPX mountant.

### 2.3.8 Photomicroscopy and analysis of grafts

Cells expressing markers of interest were counted using a Leitz light microscope and Olympus CASTgrid stereology software. Images were processed using Optronics MagnaFire Software and Adobe Photoshop.

Estimate of graft volume was calculated using the formula:  $(\Sigma a * M) / f$

where: a = area (mm<sup>2</sup>), M = section thickness and f = frequency of sampled sections (1:12).

Estimate of total number of cells per animal was calculated using the formula:

$$\Sigma c * [\Sigma a / (\Sigma n * a)] * f$$

where: c = cells counted, a = area, n = total number of grids counted within the area and f = frequency of samples sections.

## 2.4 Statistical analysis

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Statistical analyses were performed using Minitab 15 statistical software. Parametric data were analysed using two-sample t-tests, or one- or two-factor analysis of variance (ANOVA) with Tukey-Kramer *post-hoc* comparisons when appropriate, to compare differences between pairs of mean values.

## Chapter 3

### *The forebrain character of NEBs*

#### 3.1 Summary

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This chapter looks at characterising *in vitro* differentiation of mouse ES cells under defined neural induction conditions that are currently used as ‘standard’ in our lab. Mouse ES cells have been shown to generate neural precursors *in vitro* when cultured in CDM and default to a forebrain character without addition of further exogenous factors. This study utilised the mouse ES cell line Foxg1Z, in which the expression of the reporter gene *LacZ* is under the control of the *Foxg1* promoter; *Foxg1* is the earliest known marker of the forebrain. This enables detection of *Foxg1*-expressing cells using X-gal or antibodies against  $\beta$ -galactosidase. Neural induction of Foxg1Z ES cells using standard neuralising conditions yielded an increase in percentage of *Foxg1* positive cells over days in culture up to day 8. Addition of FGF2 resulted in a further increase in *Foxg1* positive cells, with 20ng/ml FGF2 added at day 4 yielding the highest *Foxg1* population, but Wnt inhibition with DKK-1 did not alter the proportion of *Foxg1* positive cells. Interestingly, FGF2 addition did not significantly increase nestin or Otx2 expression in neural induction cultures. When cultures were differentiated for 7 days  $\beta$ -III-tubulin immuno-positive neurons were generated, including GABAergic neurons but not DARPP-32 positive neurons, and there were no GFAP immuno-positive astrocytes. Addition of FGF2 resulted in increased  $\beta$ -III-tubulin yield following 7 days neuronal differentiation. In summary, neural induction cultures of mouse ES cells resulted in a proportion of *Foxg1* positive cells, which could be increased by addition of FGF2, and these precursors went on to generate neurons. These results are at odds with some previous studies in the literature and there are a number of possible explanations for this including discrepancies in culture conditions, which may have differing effects on subsequent gene expression.

## 3.2 Introduction

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ES cells have become a widely researched cell source due to their potential to generate any cell from the three germ layers, namely ectoderm, mesoderm and endoderm. With regards to neurodegenerative diseases, such as HD, and cell-replacement strategies, ES cells offer an alternative source of cells to primary foetal neural tissue which is currently used as standard. In addition, mouse ES cells offer a robust culture system that is easily employed in conjunction with genetic manipulation including incorporation of reporter systems to aid analysis of genes of interest.

*Foxg1* expression is restricted to the telencephalic neuroepithelium and the nasal half of the retina and optic stalk, and has been implicated in defining the compartmental boundary between the telencephalon and diencephalon (Tao and Lai, 1992). The *Foxg1Z* mouse ES cell line (used in this study, a gift from J. Quinn and D. Price), was derived from *Foxg1Z* mice and offers a useful tool for the analysis of *Foxg1* expressing cells. *Foxg1Z* mice were generated by replacing most of the coding sequence of *Foxg1*, which is contained in a single exon, with a *LacZ* and neomycin cassette. The *LacZ* sequence, with a nuclear localization signal was fused in-frame to the first 13 amino acids of *Foxg1*, so that expression of the  $\beta$ -galactosidase enzyme is under the control of the *Foxg1* promoter (Xuan et al., 1995). This enables visualisation of *Foxg1* positive cells using the X-gal assay or antibodies against  $\beta$ -galactosidase. *Foxg1-LacZ* knock-in reporter ES cells have previously been used to show induction of *Foxg1* expression in neural induction protocols of mouse ES cells using stromal feeder layers with serum replacement medium followed by culture in N2 and bFGF with later addition of SHH, FGF8 and BDNF (Barberi et al., 2003). Further, GABAergic neurons generated using this method were demonstrated to be of forebrain identity by assessment of  $\beta$ -galactosidase histochemistry (Barberi et al., 2003).

During embryonic development the presence of certain endogenous factors in the neural tube are important for the generation of the telencephalon. Members of the FGF family are involved in cell proliferation and cell survival, and have a role in patterning within the telencephalon including regulation of *Foxg1* (Shimamura and Rubenstein, 1997; Storm et al., 2006). Wnt proteins have been shown to be involved

in the regulation of neural development, reviewed in (Michaelidis and Lie, 2008) and Wnt signalling inhibition is reported to be necessary for telencephalic generation (Houart et al., 2002; Backman et al., 2005; Gulacsi and Anderson, 2008).

Mouse ES cells cultured in defined serum-free suspension without the addition of exogenous factors yield neural precursor cells, although different proportions of various markers are reported, including nestin positive cells ranging from 67-90% and Sox1 positive cells ranging between 60-70% (Ying et al., 2003; Bouhon et al., 2005; Watanabe et al., 2005; Smukler et al., 2006; Okabe et al., 1996). Addition of FGF2 to neural induction cultures has been reported to result in a greater proportion of neural precursors following 8 days in culture, with an increase in nestin-positive cells (Okabe et al., 1996; Bouhon et al., 2005), Sox1-positive cells (Ying et al., 2003) and an upregulation of *Foxg1* expression (Bouhon et al., 2006).

The Wnt inhibitor DKK-1 causes an increase in *Foxg1* positive cells when added early (days 0-5) to mouse ES cell-derived neural induction cultures (Watanabe et al., 2005). This increase in *Foxg1* can be counteracted by the concurrent addition of the Wnt3a protein with DKK-1. Late addition (days 6-10) of Wnt3a does not significantly alter the *Foxg1* population, although it does result in an increase in expression of the dorsal telencephalic marker *Pax6* and a decrease in expression of the ventral telencephalic markers *Nkx2.1* and *Gsx2* within the *Foxg1* population (Watanabe et al., 2005). Therefore specific time points for addition of factors to the neural induction suspension culture system are important for the induction of neural precursor cells expressing appropriate markers.

In this chapter neural induction of Foxg1Z ES cells is analysed following 8 days in serum-free suspension culture. Neural character is assessed within culture populations, in particular the proportion of Foxg1 expression, via the *LacZ* reporter assay. Following addition of factors to the culture system, including the mitogen FGF2 and the Wnt inhibitor DKK-1, expression of *Foxg1* was analysed in the precursor population. Expression of other neural precursor markers such as nestin and Otx2 was also assessed. Cultures at day 8 were transferred to neuronal differentiation medium for 7 days and subsequently analysed for expression of neuronal and astrocyte markers.



### 3.3 Experimental Procedures

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#### *Cell culture*

The mouse ES cell line Foxg1-LacZ (Foxg1Z) (a gift from J. Quinn and D. Price, University of Edinburgh, Edinburgh, UK) was used in this study. NEBs were derived from the Foxg1Z mouse ES cell line as described in Chapter 2. Briefly, ES cells were transferred from ES cell culture medium to CDM (ADF+ medium) at a density of 50,000 cells per ml and medium was replaced every 2 days. To determine the effects of addition of factors, FGF2 was added at 1, 5, 10 and 20 ng/ml. DKK-1 was added at 10, 100 and 1000 ng/ml.

#### *X-Gal assay*

Cells were plated onto PLL/laminin treated cover-slips at a density of 50,000 cells per 30µl drop on each cover-slip. Cells were fixed 2-4 hours after plating, on adhering to the substrate, in 1% formalin for 5-10 minutes, followed by washing in PBS and incubation in X-gal staining solution (see Appendix) at 37°C for 2-4 hours and then overnight at 4°C. Following the overnight incubation, X-gal was removed and cells were fixed in 4% formalin for 15 minutes, washed in PBS and counterstained with eosin for 10 minutes.

#### *Immunocytochemistry*

Cells were fixed 2-4 hours after plating, on adhering to the substrate, or after 7 days in neuronal differentiation medium. Fluorescent immunocytochemistry was performed according to the protocol detailed in Chapter 2. Primary antibodies used were anti-β-galactosidase (1:1000), anti-nestin (1:400), anti-Otx2 (1:500), anti-β-III-tubulin (1:1000); anti-GFAP (1:100), anti-GABA (1:500) and anti-DARPP-32 (1:20,000). (For antibody details see Appendix 3).

#### *Gene expression analysis*

Cells were harvested, RNA extracted and cDNA was generated. QPCR analysis of *axin* expression was performed using cDNA dilutions and the SYBR green assay. (For primer sequences see Appendix 4).

### *Quantification*

Cells expressing markers of interest were visualised and counted under UV fluorescence using a Leitz microscope. Hoechst positive nuclei were detected and cells immuno-labelled with the marker of interest were counted, as described in Chapter 2. Images were processed using Optronics MagnaFire Software and Adobe Photoshop.

Statistical analyses were performed by two-sample t-tests, and one-factor ANOVA with Tukey-Kramer *post-hoc* comparisons when appropriate, using Minitab 15 software.

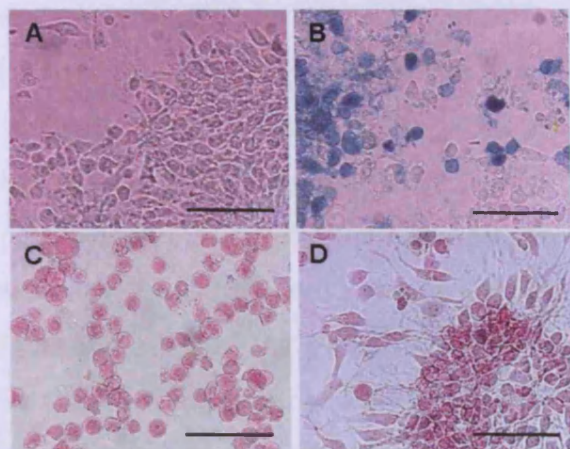
### 3.4 Results

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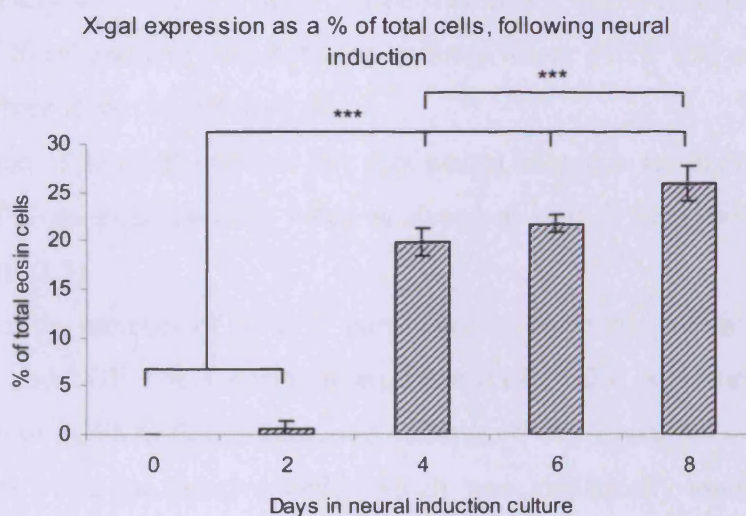
#### *Foxg1 expression in NEBs*

To determine the proportion of *Foxg1Z* positive cells in the culture and to assay the effects of factors on the proportion of *Foxg1*-positive cells, incubation with 5-Bromo-4-chloro-3-indolyl galactopyranoside (X-gal) which yields a blue product was employed. *Foxg1Z* ES cell-derived NEBs (*Foxg1Z* NEBs) were cultured in ADF+ neural induction medium for up to 8 days and were analysed at different time points within this time window. Within cultures there were clusters of cells that were negative (Figure 3.1; A) along side both individual cells and clusters of cells that were positive (Figure 3.1; B) for X-gal. Undifferentiated *Foxg1Z* ES cells were negative for X-gal (Figure 3.1; C), as were CGR8.8 NEBs (a mouse ES cell line without the *LacZ* reporter) following the 8 days in ADF+ (Figure 3.1; D).

The proportion of X-gal positive cells present in cultures at different days following neural induction was expressed as a percentage of the total cell number as determined by eosin counterstaining (Figure 3.2). Analysis was performed on NEBs from day 0 through to day 8 of neural induction. There was a highly significant difference in the proportion of X-gal positive cells with increasing time in neural induction culture (time,  $F_{4,15} = 117.31$ ,  $p < 0.001$ ), with no positive cells identified at day 0 and the greatest proportion of X-gal positive cells evident at day 8. The percentage of positive cells at day 2 ( $0.68 \pm 0.60\%$ ) (mean  $\pm$  SEM) was not significantly different from that at day 0 (0.00). The percentage of positive cells at day 4 ( $19.89 \pm 1.43\%$ ) was statistically significant from that at day 0, day 2 and also day 8 ( $25.91 \pm 1.78\%$ ). At day 6 ( $21.75 \pm 0.90\%$ ) the percentage was not statistically significant from that at either day 4 or 8, but was significantly different from that at day 0 and day 2.



**Figure 3.1.** Foxg1Z NEBs were harvested, dissociated and plated onto substrate. After 2-4 hours cells had adhered to the substrate and were partially fixed, followed by incubation in X-gal staining solution (blue) and subsequent counterstaining with eosin (pink). Within cultures there were NEBs present with very few or no X-gal positive cells (A), interspersed with regions exhibiting patches of X-gal positive cells (B). Photomicrographs A and B are different areas of the same culture plate-down. Undifferentiated Foxg1Z ES cells (C) and CGR8.8 NEBs (D) exhibited no X-gal positive cells. Scale bars = 50µm.



**Figure 3.2.** Foxg1Z NEBs were harvested, dissociated and plated onto substrate for analysis at days 0, 2, 4, 6 and 8 following neural induction. After adhering to the substrate cells were partially fixed and then incubated in X-gal staining solution followed by counterstaining with eosin. X-gal positive cells were counted and are represented as a percentage of total eosin stained cells. Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM. There was a significant difference in X-gal positive cells with varying days in culture. Significant *post-hoc* differences are indicated with brackets. (\*\*\*) $p < 0.001$ .

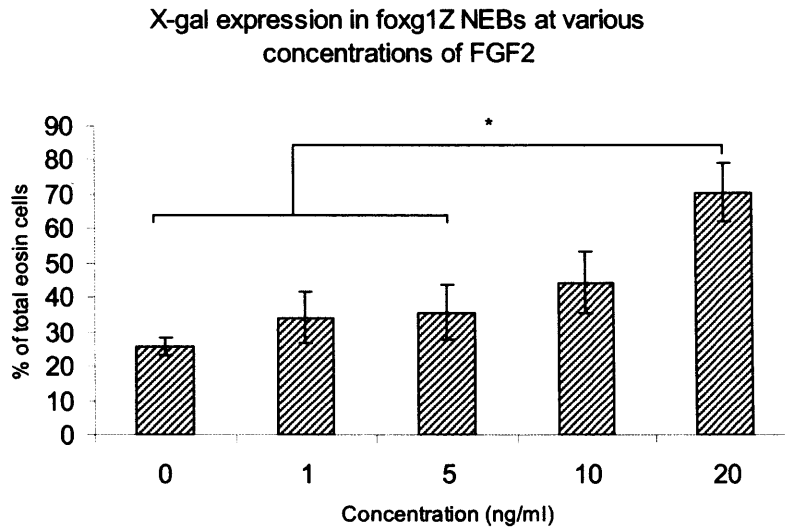
*Effect of FGF on Foxg1 expression*

Addition of increasing concentrations of FGF2 to neural induction cultures on day 4 resulted in an increase in the percentage of X-gal positive cells in the cultures (Figure 3.3). The increase was statistically significant overall (concentration,  $F_{4,15} = 5.57$ ,  $p < 0.05$ ). The percentage of X-gal positive cells in untreated cultures ( $25.73 \pm 2.71\%$ ) was not significantly lower than those treated with 1ng/ml ( $34.17 \pm 7.34\%$ ), 5ng/ml ( $35.61 \pm 8.05\%$ ) and 10ng/ml ( $44.39 \pm 8.92\%$ ) FGF2, but was significantly lower than those treated with 20ng/ml FGF2 ( $70.67 \pm 8.51\%$ ). There was no significant difference between those cultures treated with 10ng/ml and 20ng/ml FGF2.

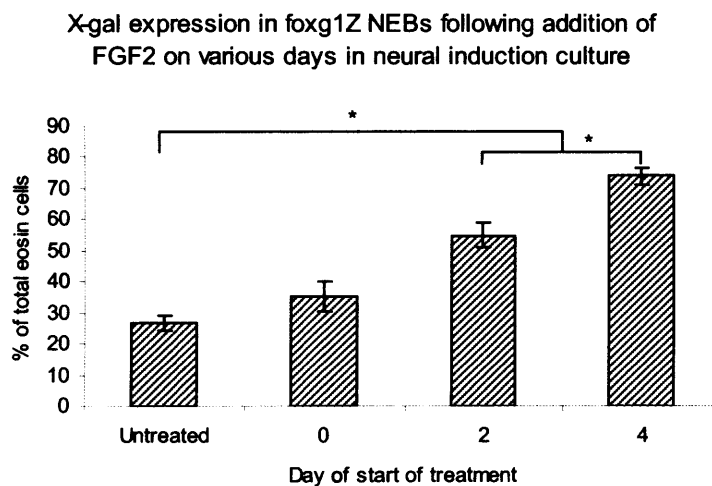
20ng/ml FGF2 was added to neural induction cultures on either day 0, 2 or 4 and was maintained in cultures until day 8 when cells were analysed. The percentage of X-gal positive cells was significantly increased the later the initial addition of FGF2 (day,  $F_{3,12} = 33.89$ ,  $p < 0.05$ ) (Figure 3.4). Untreated cultures ( $26.56 \pm 2.49\%$ ) were not significantly different from cultures treated with FGF2 from day 0 ( $35.24 \pm 4.92\%$ ), but were significantly different from those cultures treated from day 2 ( $55.01 \pm 3.88\%$ ) and day 4 ( $74.10 \pm 2.78\%$ ). There was also a significant difference in the percentage of X-gal positive cells between cultures where FGF2 was added from day 2 and those where it was added from day 4.

Addition of 20ng/ml FGF2 at day 4 of neural induction resulted in the greatest proportion of X-gal-positive cells when analysed at day 8, compared to untreated cultures (Figure 3.5).

FGF8 is the member of the FGF family known to be involved in telencephalon development, and FGF 2 is a synthetic equivalent of FGF8. Addition of increasing concentrations of FGF8 to neural induction cultures on day 4 resulted in an increase in the percentage of X-gal positive cells, which was statistically significant overall (concentration,  $F_{3,12} = 7.46$ ,  $p < 0.05$ ) (Figure 3.6). The percentage of X-gal positive cells in untreated cultures ( $23.11 \pm 0.86\%$ ) and those treated with 25ng/ml FGF8 ( $23.28 \pm 2.57\%$ ) was significantly lower than those treated with 50ng/ml FGF8 ( $52.85 \pm 6.74\%$ ), but there was no significant difference between those cultures treated with 50ng/ml and 100ng/ml FGF8 ( $36.44 \pm 7.43\%$ ).

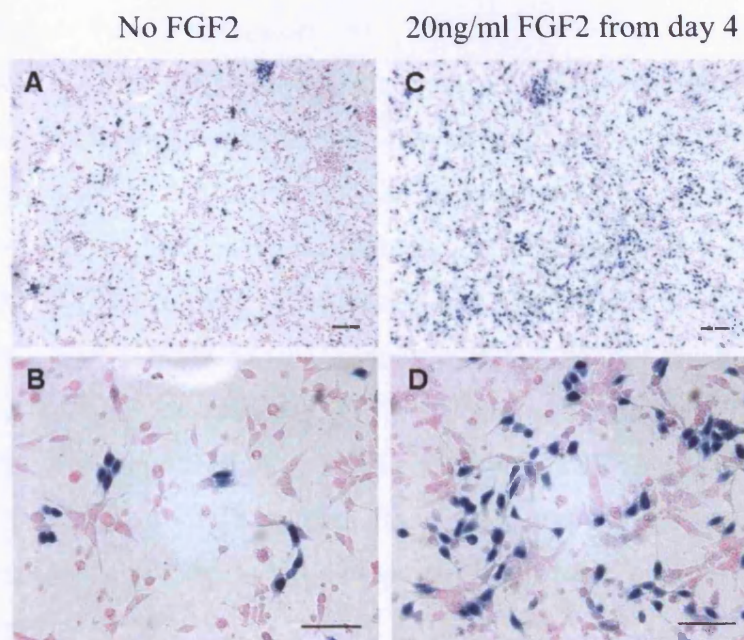


**Figure 3.3** FGF2 (1, 5, 10 and 20 ng/ml) was added to Foxg1Z NEBs at day 4 and cells were harvested, dissociated and plated onto substrate for analysis at day 8. X-gal positive cells were counted and are represented as a percentage of total eosin stained cells. Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM. There was a significant increase in X-gal positive cells with increasing FGF2 concentration. Significant *post-hoc* differences are indicated with brackets. (\* $p < 0.05$ ).

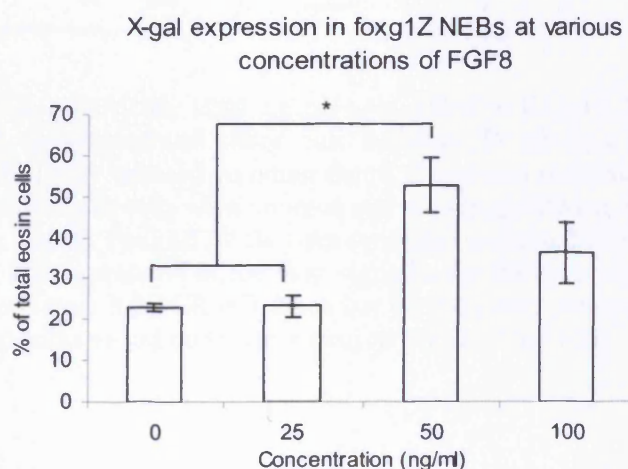


**Figure 3.4.** Foxg1Z NEBs were cultured in neural induction medium and addition of 20ng/ml FGF2 was initiated on either day 0, 2 or 4, and maintained through to analysis at day 8. X-gal positive cells were counted and are represented as a percentage of total eosin stained cells. Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM. There was a significant difference in the percentage of X-gal positive cells, depending on the initial day of FGF2 addition. Significant *post-hoc* differences are indicated with brackets. (\* $p < 0.05$ ).





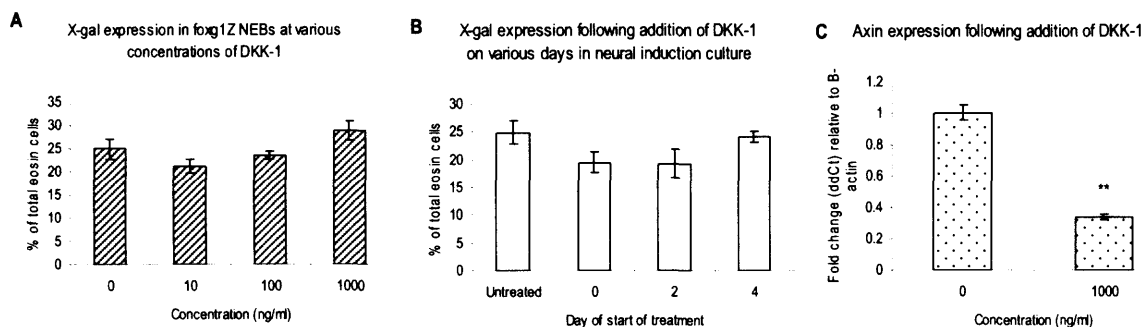
**Figure 3.5.** Foxg1Z NEBs were cultured in the absence or presence of 20ng/ml FGF2 from day 4 and at day 8 were harvested, dissociated and plated onto substrate. After 2-4 hours cells had adhered to the substrate; they were partially fixed, followed by incubation with X-gal (blue) and subsequent eosin counterstaining (pink). Foxg1Z NEB cultures in the absence of FGF2 are shown to express few X-gal-positive cells (A and B). Cultures treated with 20ng/ml FGF2 reveal a greater proportion of X-gal-positive cells (C and D). Photomicrographs A and B are different magnifications of the same culture, and photomicrographs C and D are different magnifications of the same culture. Scale bars = 200µm (A and C); 100µm (B and D).



**Figure 3.6.** FGF8 (25, 50 and 100 ng/ml) was added to Foxg1Z NEBs at day 4 and cells were harvested, dissociated and plated onto substrate for analysis at day 8. X-gal positive cells were counted and are represented as a percentage of total eosin stained cells. Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM. There was a significant increase in X-gal positive cells with increasing FGF8 concentration. Significant *post-hoc* differences are indicated with brackets. (\* $p < 0.05$ ).

### Effect of DKK-1 on *Foxg1* expression

The Wnt inhibitor DKK-1 was added to neural induction cultures to determine its effects on the forebrain population. Addition of DKK-1 did not result in a statistically significant difference in the percentage of X-gal positive cells (concentration,  $F_{3,12} = 3.45$ ,  $p = \text{n.s.}$ ) (Figure 3.7; A); (untreated  $24.89 \pm 2.13\%$ ; 10ng/ml DKK-1  $21.21 \pm 1.35\%$ ; 100ng/ml DKK-1  $23.45 \pm 0.91\%$ ; 1000ng/ml DKK-1  $28.84 \pm 2.19\%$ ). Earlier addition of 1000ng/ml DKK-1 did not result in a significant difference in X-gal positive cells (day,  $F_{3,11} = 2.19$ ,  $p = \text{n.s.}$ ) (Figure 3.7; B); (untreated  $24.89 \pm 2.13\%$ ; from day 0  $19.51 \pm 1.86\%$ ; from day 2  $19.30 \pm 2.66\%$ ; from day 4  $24.03 \pm 0.97\%$ ). DKK-1 addition from day 4 did alter the expression of the Wnt signalling pathway component, axin, compared to untreated cultures (Figure 3.7; C) (concentration,  $t_2 = 19.54$ ,  $p < 0.01$ ).

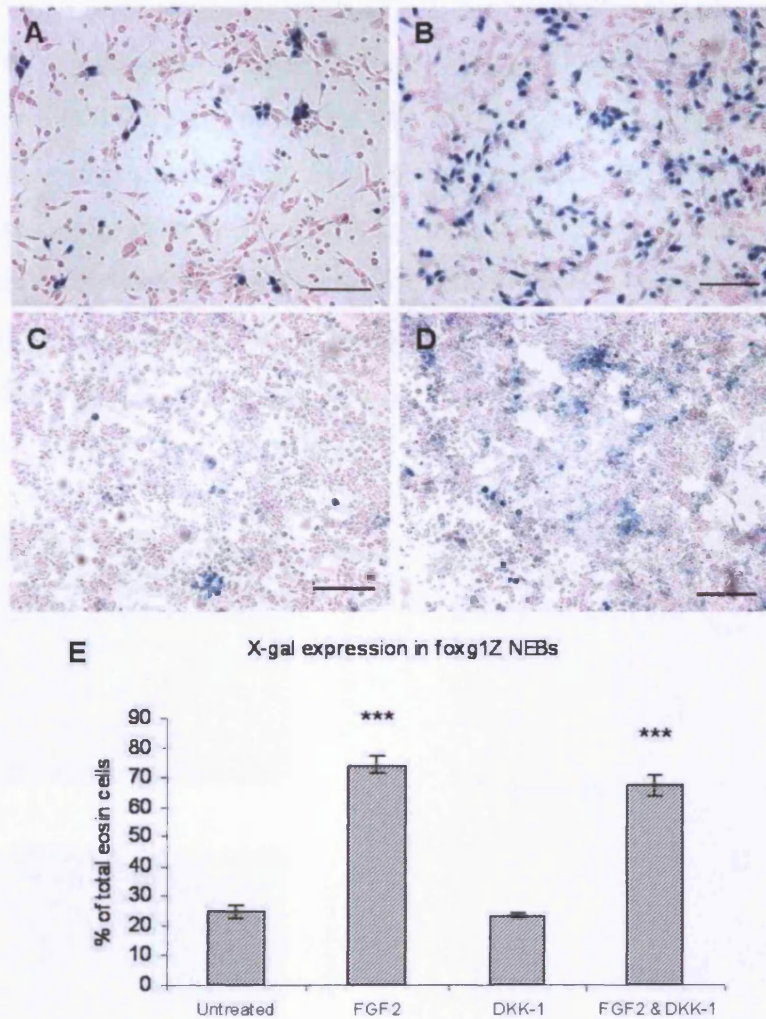


**Figure 3.7.** DKK-1 (10, 100, 1000 ng/ml) was added to *Foxg1Z* NEBs at day 4, and cells were harvested, dissociated and plated onto substrate for analysis at day 8 (A); addition of 1000ng/ml DKK-1 was initiated on either day 0, 2 or 4 and maintained through to analysis at day 8 (B). X-gal positive cells were counted and are represented as a percentage of total eosin stained cells (A and B). *Foxg1Z* NEBs were untreated or treated with 1000ng/ml DKK-1 from day 4 to day 8 and expression of the Wnt signalling pathway component axin was analysed using semi-quantitative RT-PCR (C). Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM. (\*\* $p < 0.01$ ).

The effect of DKK-1 dose response on X-gal positive cells in NEB cultures was initially analysed in the absence of FGF2. To determine if there was an additive effect of these two factors 100ng/ml DKK-1 was added in conjunction with 20ng/ml FGF2 at day 4 of neural induction and cells were plated for analysis at day 8 (Figure 3.8). Statistical analysis confirmed a highly significant difference between the percentage of X-gal positive cells yielded from untreated and treated cultures (treatment,  $F_{3,12} = 114.60$ ,  $p < 0.001$ ). Cultures treated with 20ng/ml FGF2 and 100ng/ml DKK-1 ( $67.44 \pm$



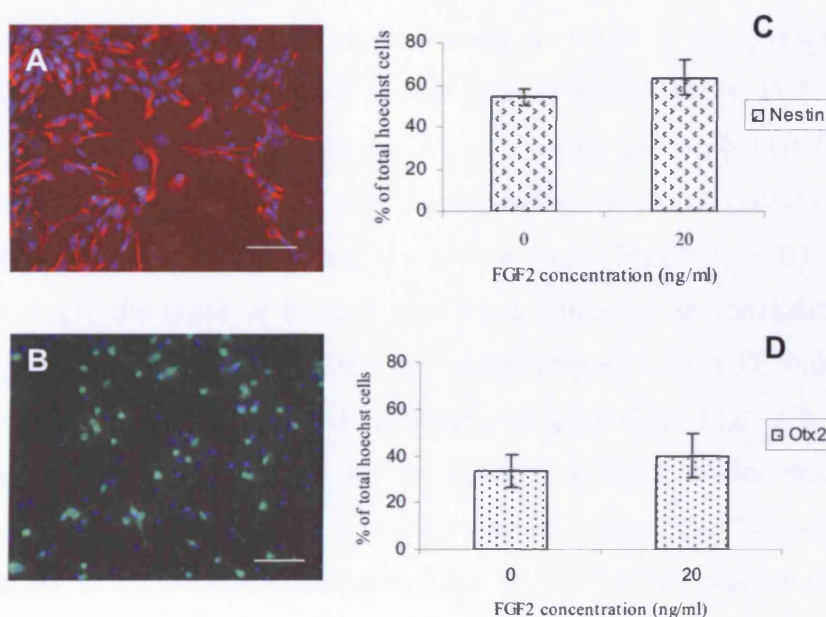
3.53%) expressed significantly more X-gal than untreated cultures ( $24.89 \pm 2.13\%$ ) and DKK-1 treated cultures ( $23.45 \pm 0.91\%$ ), but did not differ significantly from those cultures treated with FGF2 alone ( $74.09 \pm 2.78\%$ ).



**Figure 3.8.** Foxg1Z NEBs were maintained in neural induction medium with no additional factors (A), 20ng/ml FGF2 at day 4 (B), 100ng/ml DKK-1 at day 4 (C) or 20ng/ml FGF2 and 100ng/ml DKK-1 at day 4 (D), and were plated onto PLL-coated coverslips at day 8 for analysis with the X-gal assay (blue) and eosin counterstain (pink). Scale bar = 100μm. Cultures treated with FGF2 alone or both FGF2 and DKK-1 have significantly more X-gal positive cells than untreated cultures and there is no significant difference between untreated cultures and those treated with DKK-1 alone (E). Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM. (\*\*\*) $p < 0.001$ .

### Further neural analysis of *Foxg1Z* NEBs

Neural specification of *Foxg1Z* NEBs following 8 days in neural induction medium was further characterised by analysis of the neural precursor markers nestin, and *Otx2*, with or without the addition of FGF2. Immunocytochemistry using antibodies against nestin and *Otx2* was performed on day 8 NEBs that had been plated for 4 hours and fixed as precursors (Figure 3.9; A and B). Counts were performed and represented as a percentage of Hoechst positive cells (Figure 3.9; C and D). NEB cultures with no FGF2 treatment were  $54.53 \pm 4.02\%$  nestin positive at day 8, which was not significantly different from FGF2 treated cultures which were  $63.46 \pm 8.45\%$  nestin positive (concentration,  $t_5 = 1.62$ ,  $p = \text{n.s}$ ) (Figure 3.9; A and C). Analysis of *Otx2* expression revealed no significant difference between untreated cultures ( $33.34 \pm 6.79\%$ ) and FGF2 treated cultures ( $39.84 \pm 9.52\%$ ) (concentration,  $t_4 = 0.56$ ,  $p = \text{n.s}$ ) (Figure 3.9; B and D).



**Figure 3.9.** *Foxg1Z* NEBs were maintained in neural induction medium either untreated or with addition of 20ng/ml FGF2 from day 4, harvested and dissociated at day 8 and plated onto PLL-coated coverslips for 4 hours before fixation. Cultures were immuno-labelled for nestin (red) (A and C) or *Otx2* (green) (B and D) and counterstained with Hoechst nuclear stain (blue in A and B). Graphs represent immuno-positive cells as a percentage of total Hoechst cells counted. Each bar on the graph represents a mean of 4 (C) and 3 (D) different neural induction cultures and error bars represent SEM. Scale bars = 50μm.

*Neuronal differentiation of Foxg1Z NEBs*

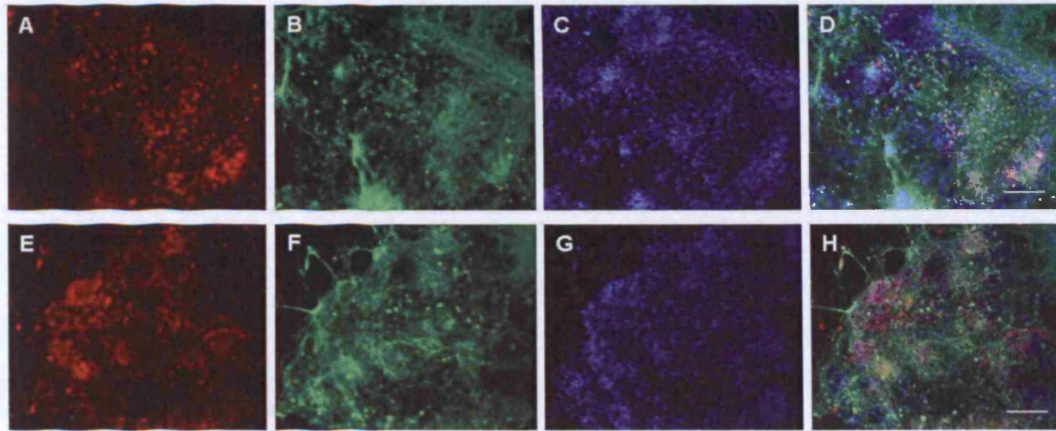
To look at the neuronal differentiation of ES cell-derived NEBs, Foxg1Z NEBs were harvested, dissociated, plated onto substrate and allowed to differentiate for 7 days in the presence of 2% B27 growth supplement and 1% FCS, and in the absence of any additional growth factors (such as FGF2). Foxg1Z NEBs continue to express  $\beta$ -galactosidase following differentiation (Figure 3.10; A and E; Figure 3.13; A) and are shown to co-express the neuronal marker  $\beta$ -III tubulin (Figure 3.10; B, D, F and H; Figure 3.13; A). There were significantly more  $\beta$ -galactosidase/ $\beta$ -III-tubulin double-labelled cells in cultures with addition of 20ng/ml FGF2 to ADF+ at day 4 ( $34.42 \pm 4.19\%$ ) compared with untreated cultures ( $17.49 \pm 4.34\%$ ) (concentration,  $t_5 = 2.81$ ,  $p < 0.05$ ).

Foxg1Z NEBs cultured for 8 days in ADF+ medium without the addition of FGF2 generated  $31.66 \pm 2.33\%$   $\beta$ -III-tubulin immuno-positive neurons whilst those cultured in the presence of 20 ng/ml FGF2 yielded  $37.80 \pm 1.29\%$   $\beta$ -III-tubulin immuno-positive neurons, following subsequent neuronal differentiation for 7 days, which was a significant difference (concentration,  $t_{12} = 2.31$ ,  $p < 0.05$ ) (Figure 3.13; B).

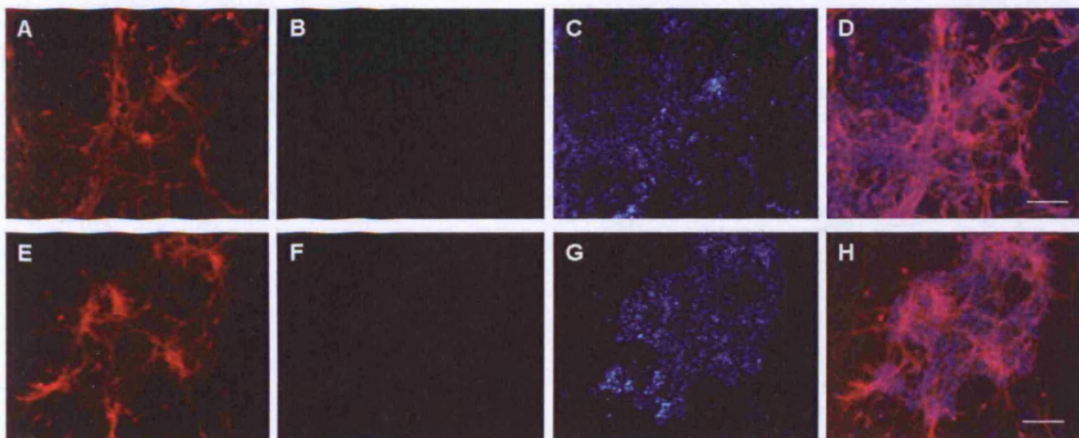
The presence of GFAP positive astrocytes (Figure 3.11; B, D, F and H) was very rare both without FGF2 addition ( $0.05 \pm 0.04\%$ ) and with FGF2 addition in ADF+ medium ( $0.14 \pm 0.13\%$ ), and the difference in the 2 conditions was not statistically significant (concentration,  $t_3 = 1.09$ ,  $p = \text{n.s}$ ) (Figure 3.13; B).

To assess the types of neurons generated following neuronal differentiation, cultures were further immuno-labelled for GABA (Figure 3.12; A-D) and DARPP-32 (Figure 3.12; E-H). Untreated neural induction cultures yielded  $12.84 \pm 1.74\%$  GABA positive neurons, as a percentage of total Hoechst positive nuclei, which was not significantly different to  $15.52 \pm 3.16\%$  yielded from FGF2 treated cultures, (concentration,  $t_3 = 0.74$ ,  $p = \text{n.s}$ ) (Figure 3.12; B). DARPP-32 was never detected in the cultures either with or without FGF2 addition to ADF+ medium (Figure 3.13; B).

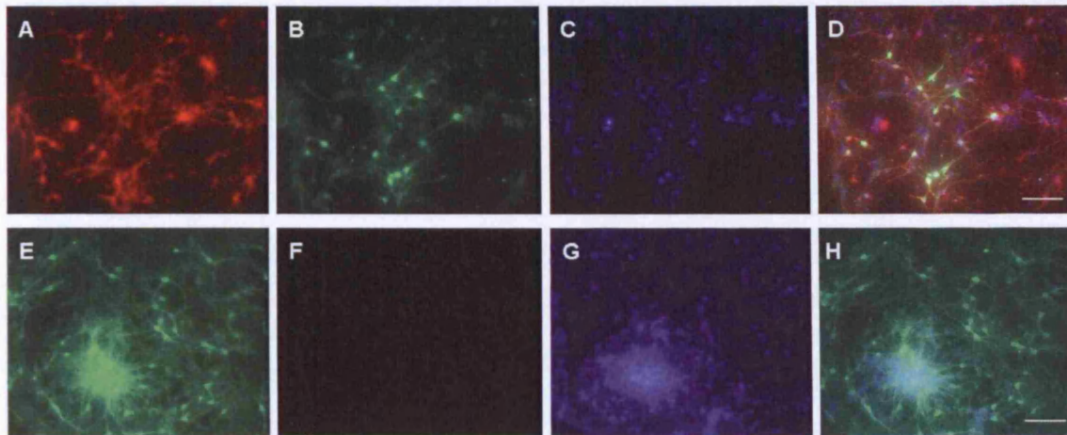




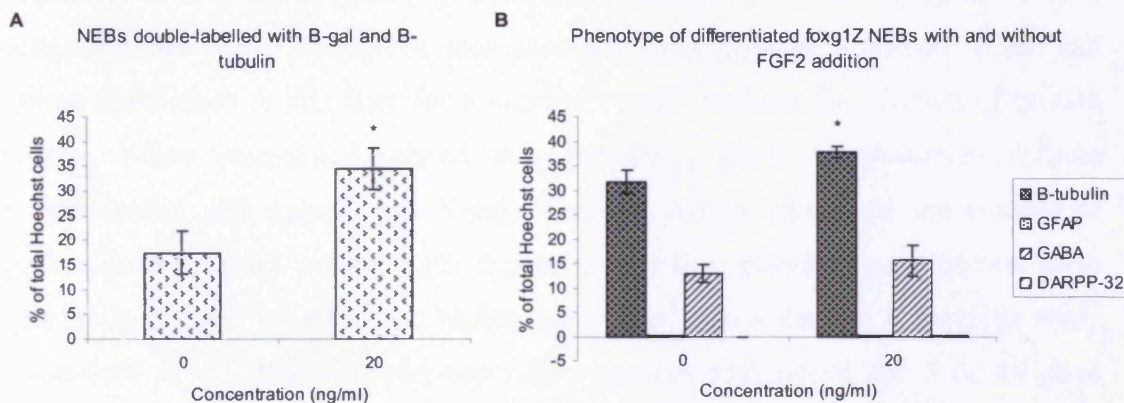
**Figure 3.10.** Foxg1Z NEBs were harvested, dissociated and plated at day 8 following neural induction in either untreated medium (A-D) or FGF2 treated medium (E-H) and were differentiated for 7 days in B27 and FCS with no additional growth factors. Cultures were triple-labelled for  $\beta$ -galactosidase (red) (A and E),  $\beta$ -III tubulin (green) (B and F) and Hoechst nuclear stain (blue) (C and G). Photomicrograph D is a merged image of A, B and C; and photomicrograph H is a merged image of E, F and G. Scale bars = 100 $\mu$ m.



**Figure 3.11.** Foxg1Z NEBs were harvested, dissociated and plated at day 8 following neural induction in either untreated medium (A-D) or FGF2 treated medium (E-H) and were differentiated for 7 days in B27 and FCS with no additional growth factors. Cultures were triple-labelled for  $\beta$ -III-tubulin (red) (A and E), GFAP (green) (B and F) and Hoechst nuclear stain (blue) (C and G). Photomicrograph D is a merged image of A, B and C; and photomicrograph H is a merged image of E, F and G. Scale bars = 100 $\mu$ m.



**Figure 3.12.** Foxg1Z NEBs were harvested, dissociated and plated at day 8 following neural induction with addition of FGF2 from day 4, and were plated for neuronal differentiation for 7 days in B27 and FCS with no additional growth factors. Cultures were triple-labelled for  $\beta$ -III-tubulin (red) (A), GABA (green) (B) and Hoechst nuclear stain (blue) (C); or  $\beta$ -III-tubulin (green) (E), DARPP-32 (red) (F) and Hoechst nuclear stain (blue) (G). Photomicrograph D is a merged image of A, B and C; and photomicrograph H is a merged image of E, F and G. Scale bars = 100 $\mu$ m.



**Figure 3.13.** Foxg1Z NEBs were maintained in ADF+ neural induction medium with or without the addition of 20ng/ml FGF2 at day 4. On day 8 NEBs were harvested, dissociated and plated onto substrate and were allowed to differentiate for 7 days in neuronal differentiation medium.  $\beta$ -galactosidase and  $\beta$ -III-tubulin double-labelled cells are represented as a percentage of total Hoechst-positive nuclei (A).  $\beta$ -III-tubulin, GFAP, GABA and DARPP-32 immuno-positive cells were counted and are represented as a percentage of total Hoechst-positive nuclei. Each bar on the graphs represent a mean of at least 3 different neural induction cultures and error bars represent SEM. (\* $p < 0.05$ ).

### 3.5 Discussion

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#### *Expression of X-gal in Foxg1Z NEBs*

Serum-free neural induction suspension culture of mouse ES cells results in generation of neural precursor cells (Wiles and Johansson, 1999; Ying et al., 2003; Bouhon et al., 2005; Watanabe et al., 2005). Over time in serum-free suspension culture there is an induction of neurogenicity, including upregulation of the neuroectodermal marker Sox1 (Bouhon et al., 2006; Watanabe et al., 2005) and the neural precursor marker nestin (Bouhon et al., 2005), with a decrease in ES cell markers such as Oct-3/4 (Bouhon et al., 2005; Watanabe et al., 2005; Bouhon et al., 2006).

The mouse ES cell line Foxg1Z enables analysis of expression of the early forebrain marker *Foxg1* via use of the *LacZ* reporter gene and an X-gal assay or antibodies against the enzyme  $\beta$ -galactosidase (Xuan et al., 1995). Foxg1Z ES cells cultured in the above conditions spontaneously form aggregates termed NEBs, and can be maintained in this state for a number of days without the addition of growth factors. When dissociated, plated onto substrate, fixed as precursors without differentiation, and stained with X-gal, blue cells can be visualised and counted as shown above. Foxg1Z positive cells increased over time in culture as expected, up to 25.91% at 8 days, which was a higher percentage than shown in a previous study (Watanabe et al., 2005). Feeder-dependent cultures maintained for 5 or 10 days resulted in less than 2% Foxg1 positive cells. This was increased to 11% when cultures were feeder-free and in suspension for the initial 5 days, and further increased to 15% when all 10 days were feeder-free and in suspension (Watanabe et al., 2005).

Different studies looking at neural induction protocols of mouse ES cells apply different protocols as outlined in Table 3.1, which indicates the need for caution when drawing comparisons. Differences in composition of neural induction culture medium include the use of 5% knock-out serum replacement (KSR) in the 'Watanabe' medium, which contains lipids, insulin and transferrin, along with other components (Price et al., 1998), compared with the additional supplementation of these 3 components to the ADF+ medium used in this study. KSR, unlike FCS, does not contain any undefined growth factors and has a stable composition. The concentration



of lipids in the ‘Watanabe’ medium is nearly a third lower than in ADF+ and the concentrations of insulin and transferrin are 28 and 187.5 times less, respectively, than supplemented in ADF+ medium. ‘Bouhon’ medium comprises insulin at half the concentration in ADF+ and transferrin is double that in ADF+. Insulin and transferrin are important for cell survival, with insulin in particular, showing specificity for survival of neural precursor cells (Okabe et al., 1996). The ADF+ serum-free suspension culture system used here appears to encourage induction of *Foxg1* precursors more than other protocols.

	Watanabe (2005)	Bouhon (2005)	This study (ADF+)
Cell lines	46CSoxGFP (gene targeting E14 cell line (129/Ola derived))	E14 (129/Ola derived)	Foxg1Z (C57Bl6/J derived)
Substrate for ES cell culture	Gelatin	Feeders (MEFs), then gelatin for 2 passages	Gelatin
Medium	Base	GMEM 5% KSR	IMDM:F12 (1:1) BSA
	Lipid	1 x lipid concentrate	1 x lipid concentrate
	Insulin	7µg/ml	14µg/ml
	Transferrin	150µg/ml	75µg/ml
	Other	Thioglycerol	β-mercaptoethanol

**Table 3.1.** Comparison of medium components in neural induction protocols.

Discrepancies between protocols regarding effects of addition of factors on gene expression may be attributed to use of different cell lines, differences in culture media or different culture conditions. ‘Baseline’ *Foxg1* expression is variable between this study and Watanabe et al; not only were there differences in culture conditions, namely medium recipes, but also the cell lines employed were different. For this study the Foxg1Z line was used, while Watanabe used the 46CSoxGFP cell line (Ying et al., 2003; Watanabe et al., 2005). When working with mouse ES cell lines, and drawing comparisons between different studies, it is important to keep in mind the differences that may arise due to background strains of the mice used when cell lines were derived from the ICM. Different ES cell culture media may contain a different complement of factors which include signals that affect ES cell pluripotency and growth curves, and may have effects on subsequent ES cell differentiation. Different culture medium recipes for differentiation of ES cells may contain varying levels of signalling factors. A consequence of this might be affects on ‘baseline’ gene expression and also the outcome of supplementing medium with additional factors of

interest, which may only influence neural induction under specific conditions. The use of feeder layers in maintenance of ES cells may also add to the differences in the presence of various signalling factors in the culture medium which in turn might impose modifications on the fate of cells.

#### *Effect of FGF and DKK-1 in Foxg1Z neural induction culture*

FGF8 is necessary for normal telencephalic development and *in vivo* studies have shown a relationship between expression of FGF8 and *Foxg1*, where loss of *Foxg1* results in reduced FGF8 expression in the developing telencephalon (Martynoga et al., 2005). Neural plate explants incubated with FGF8-soaked beads were shown to extensively express *Foxg1* in the area surrounding the beads compared with the PBS-soaked beads where no *Foxg1* was expressed (Shimamura and Rubenstein, 1997). Loss-of-function studies with FGF8 mutant mice revealed that *Foxg1* expression was reduced in the neural plate (9-10 somite stage) and also at the later gestational stage E9.0 (Storm et al., 2006).

Increasing concentrations of FGF8 added to *Foxg1Z* NEB cultures resulted in increased expression of *Foxg1/X-gal* positive cells after 8 days, with the highest yield following addition of 50ng/ml FGF8. A similar increase in *Foxg1/X-gal* positive cells was demonstrated with addition of FGF2, the synthetic equivalent of FGF8, and for subsequent studies FGF2 was used.

Increasing concentrations of FGF2 added to *Foxg1Z* NEB cultures increased the expression of *Foxg1/X-gal* positive cells after 8 days from 26% to 71%, which is consistent with previous studies that showed via semi-quantitative analysis, using RT-PCR, that treatment at day 4 with 20ng/ml FGF2 induced expression of *Foxg1* at day 8 (Bouhon et al., 2006). Supplementing culture medium with 20 ng/ml FGF2 at earlier culture days did not yield higher expression of *Foxg1* than when treated at day 4. Members of the FGF family elicit their effects via FGF receptors. FGFR1 expression is not observed in mouse ES cells but following initiation of neural induction it can be detected from day 2 with increasing expression through to day 8 (Bouhon et al., 2005). The absence of FGF receptors in early neural induction cultures might explain the lack of response to early exogenous FGF signals in culture, since the appropriate receptors are not yet detectable.

Treatment of mouse ES cells with the FGF inhibitor PD184352, which inhibits the MEK component of the mitogen-activated protein kinase (MAPK) cascade, was



shown to inhibit early neural differentiation (Gaulden and Reiter, 2008). Treatment of mouse ES cells with a different FGF inhibitor, SU5402, which inhibits tyrosine kinase activity of FGFR1, has been reported not to alter early neural differentiation (Gaulden and Reiter, 2008; Smukler et al., 2006). Together this suggests that MAPK activation may be important for ES cell neural induction, but via an FGFR1-independent mechanism. However other studies looking at inhibition of FGF signalling through FGFR1, using SU5402, contradict this finding and show a reduction in Sox1 positive neural precursor cells in both adherent and suspension cultures (Ying et al., 2003; Bouhon et al., 2005), which highlights discrepancies between culture systems and responses to inductive or inhibitive supplements. Factors other than FGFs that activate the MAPK cascade include insulin-like growth factors (IGF) (Pera et al., 2001). IGFs and insulin are members of a family of cytokines that are structurally and functionally related and IGF signals have been shown to be necessary for neural induction *in vivo* (Pera et al., 2001).

Addition of FGF2 to the ADF+ culture system did not significantly alter the expression of the neural precursor marker nestin or Otx2. Otx2 is not only expressed in the developing telencephalon, but also the diencephalon and mesencephalon (Boncinelli et al., 1993). Previous findings report that FGF2 increases nestin positive cells at day 8, when added at day 4, but it causes a decrease in Otx2 expression at day 8 (Bouhon et al., 2005). These differences might be attributable to differences in cell lines, culture medium and culture conditions, all of which are inconsistent between the study here and the Bouhon study (detailed in Table 3.1).

Analysis at day 8, following addition of FGF2 at day 4, revealed that there were fewer neural precursors, as determined by expression of nestin (63%), than *Foxg1*-expressing cells (74%). Nestin expression is restricted to the neural precursor population including those cells throughout the CNS which are pre-determined to generate neurons, astrocytes and oligodendrocytes. One explanation for the lower proportion of nestin-expressing cells than *Foxg1*-expressing cells is that some of the cells are beginning to differentiate further and therefore nestin is being switched off. Cells were only analysed at day 8 in this study and the peak expression of nestin might occur a few days prior to this. Although *Foxg1* is expressed in the forebrain progenitor cells, it is not restricted to the proliferating population and is found in post-mitotic cells derived from the telencephalic neuroepithelium (Dou et al., 1999). *Foxg1*

expression has been shown in differentiating and mature neurons of the cortex (Ariani et al., 2008).

During mouse development Wnt signalling is evident from E6.5 in the posterior region of the embryo and in embryoid body development it is required for the establishment of the anterior-posterior axis (ten Berge et al., 2008). Inhibition of Wnt signalling within embryoid bodies promotes anterior expression and induces neuroectodermal differentiation (ten Berge et al., 2008). Here, addition of the Wnt inhibitor DKK-1 did exhibit an effect on the Wnt signalling pathway, although there was no effect on the expression of *Foxg1* in the ADF+ neural induction system, both when added at day 4 at a range of concentrations, and when 1000ng/ml was added on different starting days and maintained through to day 8. This was contradictory to a previous study which showed a significant upregulation of *Foxg1* expression from 15% to 35% in the presence of 1000ng/ml DKK-1 between days 0 to 5 (Watanabe et al., 2005). In untreated neural induction cultures Wnt3a has been shown to be expressed from day 4 and its expression is maintained, albeit at a lower level, through to day 8 (Bouhon et al., 2005). The same cultures express DKK-1 at day 4 and day 6 at a similar intensity, but at day 8 expression is reduced dramatically (Bouhon et al., 2005). Although the presence of endogenous Wnt in neural induction cultures might implicate supplementation with Wnt inhibitors to allow for direction towards a neural lineage, the concomitant presence of endogenous DKK-1 in these cultures might be enough to inhibit alternative cell fates.

#### *Neuronal differentiation of Foxg1Z NEBs*

Allowing NEBs to adhere to substrate and differentiate for 7 days in the presence of B-27 growth supplement and FCS, and in the absence of exogenous growth factors such as FGF resulted in generation of  $\beta$ -III-tubulin positive neurons with very few astrocytes. Further analysis revealed that a proportion of the neurons were positive for GABA, which is the principal neurotransmitter of the striatal MSNs. The expression of  $\beta$ -III-tubulin and GABA is consistent with previous *in vitro* differentiation analysis at the same time points (Bouhon et al., 2006). Although there was expression of GABA, there was no expression of the MSN marker DARPP-32 at this stage.

DARPP-32 expression has not been reported from mouse ES cells following either *in vitro* or *in vivo* differentiation. However, transplantation of human ES cell-

derived neural precursors has produced DARPP-32 positive cells following short (4-6 weeks) and long-term (13-21 weeks) survival, but only when precursors were 'late' stage and not 'early' stage induced *in vitro* (Aubry et al., 2008). The day 8 precursor cells here are likely to be too immature in terms of their gene expression profile and are therefore not yet capable of generating the mature marker DARPP-32. Later stage precursors, and those that have undergone further patterning to specify their fate, would be more likely to generate DARPP-32.

Analysis at day 8, with FGF2 treatment from day 4, revealed that 74% of the population were expressing *Foxg1/LacZ*. Following 7 days under neuronal differentiation conditions, 34% of the forebrain population expressed the immature neuronal marker  $\beta$ -III-tubulin, and nearly 40% of the total population expressed  $\beta$ -III-tubulin. In neural induction of mouse ES cells, cells do not respond synchronously (Ying et al., 2003), and therefore it is likely that there are precursor cells as well as those that have begun to differentiate and those that are further differentiated. Due to the heterogeneity of the cell population at day 8, it is highly probable that there are cells of other lineages present, and of the neuronal lineage, there are likely to be phenotypically different neurons characteristic of different regions of the CNS.

### Conclusions

Results presented in this chapter demonstrate the use of mouse ES cells and reporter gene systems in neural induction assays. Forebrain specification of NEBs can be up-regulated by addition of FGF2 at a specific time point, but there was no effect on *Foxg1/LacZ* expression following addition of the Wnt inhibitor DKK-1 in the ADF+ culture system. Mature neurons can be yielded from these cultures following differentiation, but further specification of cells is required to generate the DARPP-32 phenotype.

Importantly, this chapter highlights some discrepancies between different culture systems employed and demonstrates the need for caution when interpreting and comparing results between the different systems. Previous work within this group has only analysed gene expression of neurally induced ES cells at relatively early time points (Bouhon et al., 2005). It has been reported that addition of FGF2 is necessary for continued culture beyond day 8 (Bouhon et al., 2005). Subsequent chapters will look at cultures beyond day 8, with analysis of later stage precursors.

## **Chapter 4**

# *In vivo characterisation of NEBs (xenograft model)*

### 4.1 Summary

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Neural transplantation of mouse ES cell-derived neural precursors (NEBs) into excitotoxic lesion models of HD has shown that these cells have the ability to generate neurons *in vivo*. Furthermore, they have been shown to generate AChE and GABAergic neurons. However, to date DARPP-32 positive neurons generated from these cells *in vivo* has not been reported. Here, mouse ES cell-derived neural precursors generated from the Foxg1Z cell line were subjected to neural induction for 8 and 16 days, and were transplanted into the QA-lesioned rat striatum. The day 8 NEB grafts developed large cell masses and survival time was only two weeks post-transplantation. All animals in the day 16 NEB group had surviving grafts after six weeks post-transplantation. Day 16 NEB grafts appeared to be comprised of areas of both neural and non-neural tissue. Since use of the mouse specific markers, M2 and M6, was unsuccessful in our study, only those cells expressing *Foxg1* and the *LacZ* reporter gene could be positively identified as donor cells. Analysis of these cells showed that grafted cells had generated neurons and glia, and further neuronal analysis showed differentiation of donor cells into calbindin and parvalbumin positive neurons, but not AChE or DARPP-32 positive neurons.

## 4.2 Introduction

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Cell replacement strategies for neurodegenerative diseases such as HD offer great potential for the repair of damaged circuitry and replacement of the lost cells. Primary neural stem cells taken from the developing striatum at the appropriate developmental age have been shown to generate beneficial results in both rodent models and human patients (Watts et al., 1997; Bachoud-Levi et al., 2006). However, for clinical studies this source of tissue is in limited supply and as well as the ethical issues surrounding use and collection of the tissue, there are also difficulties with coordination of tissue collection, the amount required for transplantation and the logistics of the transplantation itself.

ES cells offer an alternative cell source for replacement because of their pluripotency and self-renewal characteristics. However, one caveat associated with the use of ES cells is their uncontrolled proliferation, with the continued presence of ES cells following differentiation increasing the potential for teratoma formation, which is clearly not acceptable for clinical transplantation.

Neural transplantation of ES cell-derived neural precursors into the QA lesioned-striatum model of HD has to date been limited, which is in part due to difficulties in generating the desired phenotype for repair in HD ie MSNs. Studies have reported generation of neuronal phenotypes, such as AChE and GABAergic neurons, following transplantation of mouse ES cell-derived neural precursors into the rat lesioned striatum (Dinsmore et al., 1996). Transplantation of pre-differentiated GABAergic neurons derived from an immortalised striatal neural cell line (ST14A) have been shown to maintain their phenotype and also elicit a beneficial effect on reducing the number of apomorphine rotations (Bosch et al., 2004).

Precursors derived from both human and non-human-primate ES cells have been shown to generate mature neurons expressing DARPP-32 following transplantation into the rat striatum (Aubry et al., 2008; Ferrari et al., 2006). Non-human primate ES cells subjected to sequential addition of SHH and FGF8 then BDNF and ascorbic acid were transplanted at a progenitor (pES) stage (day 28) and as differentiated (dES) neurons (day 42) into the striatum of 6-OHDA lesioned rats (Ferrari et al., 2006). The pES cells generated very large grafts with heterogeneous morphology whereas the dES grafts were significantly smaller and exhibited no

overgrowth. D-amphetamine-induced rotations at twelve weeks post-transplantation were improved in 50% of the pES group and 80% of the dES group, and interestingly there was no difference in numbers of TH positive cells within the grafts of the two groups, which also expressed DARPP-32 within TH-positive regions (Ferrari et al., 2006). Aubry et al (2008) employed N2 and BDNF followed by SHH and DKK-1 for neural induction of human ES cells and reported teratoma formation and no DARPP-32 expression when cells were transplanted at early culture time points (29 days *in vitro*). Later stage cells (59 days *in vitro*) resulted in no teratomas and expression of DARPP-32 positive neurons. However, following longer survival post-transplantation grafts showed overgrowth, as well as DARPP-32 positive neurons (Aubry et al., 2008).

A longitudinal transplant study of human ES cells neurally induced in the presence of bFGF and noggin, grafted into the non-lesioned striatum resulted in no overgrowth or teratoma formation (Nasonkin et al., 2009). At three months there was no DARPP-32 expression but by six months 30% of graft-derived cells were DARPP-32 positive. To date, differentiation of mouse ES cell-derived precursors into DARPP-32 positive neurons following transplantation has not been reported.

The previous chapter involved the characterization of Foxg1Z NEBs up to 8 days in a neural induction protocol. In this chapter Foxg1Z NEBs were maintained in culture in ADF+ for either 8 or 16 days and transplanted into the QA lesioned striatum model. Oct-3/4 expression has been reported in neurally induced mouse ES cells at day 8 (Bouhon et al., 2005; Watanabe et al., 2005), albeit at low levels, but due to its presence at this time point (also shown here; Figure 4.1), longer term cultures were analysed and two cell groups were taken forward; short term (day 8) and long term (day 16).

The original aims were to analyse the potential of these cells and characterise their phenotypic differentiation *in vivo* at six weeks post-transplantation, in particular looking for differentiation to striatal phenotypes. Due to adverse events the group that received the day 8 NEB grafts were sacrificed at two weeks post-transplantation. The group that received the day 16 NEB grafts did not exhibit signs of ill-health and survived the six week experimental period. Morphological analysis was carried out on both grafted groups, but more detailed analysis of specific cell phenotypes generated *in vivo* was only carried out on the day 16 NEB grafted group.

### 4.3 Experimental Procedures

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#### *Cell Culture and Immunocytochemistry*

The mouse ES cell line *Foxg1-lacZ* was used in this study. Foxg1Z NEBs were derived from the Foxg1Z mouse ES cell line as described in Chapter 2. Briefly, ES cells were transferred from ES cell culture medium to ADF+ medium at a density of 50,000 cells per ml, medium was replaced every 2 days and FGF2 (20ng/ml) was added from day 4. Cells were plated onto PLL/laminin treated cover-slips at a density of 50,000 cells per 30 $\mu$ l drop on each cover-slip. Cells were fixed 2-4 hours after plating, on adhering to the substrate. Fluorescent immunocytochemistry was performed according to the protocol detailed in Chapter 2. Primary antibodies used were anti-Oct-3/4 (1:100), anti-Nestin (1:400) and anti-Pax6 (1:50).

#### *Neural Transplantation*

Adult female Sprague-dawley rats received a unilateral QA lesion of the right striatum. Two-three weeks after lesioning rats received transplants of Foxg1Z NEBs of either 8 days or 16 days following neural induction (n=9 for each group) into the lesioned striatum. Lesions and grafts were carried out as described in Chapter 2.

On the day of grafting, cells were harvested and dissociated following incubation with accutase, as detailed in Chapter 2. Cells were counted and resuspended at 250,000 cells per  $\mu$ l and 2 $\mu$ l cell suspension was grafted per animal. From the day prior to grafting, animals received immunosuppression in the form of daily intra-peritoneal injections of cyclosporin A (10mg/kg) for the duration of the experiment.

#### *Histology and Immunohistochemistry*

Animals were intended to survive for six weeks however the 'day 8 NEB' graft group were sacrificed at two weeks post-transplantation due to adverse events and as a requirement of our licensing protocols. The 'day 16 NEB' graft group were sacrificed at six weeks post-transplantation as originally planned. Animals were transcardially perfused and brains were taken for histological analysis. Brains were sectioned coronally on the freezing-stage microtome at 40 $\mu$ m. 1:12 series' were processed for Nissl staining using cresyl violet, histochemical staining for AChE, and

further 1:12 or 1:24 series' were processed for immunohistochemistry with anti-M2 (1:50), anti-M6 (1:50), anti- $\beta$ -galactosidase (1:1000), anti-GFAP (1:2000), anti-NeuN (1:4000), anti-DCX (1:500), anti-DARPP-32 (1:10,000) (a gift from Paul Greengard), anti-FoxP1 (1:500), anti-calbindin (1:20,000) and anti-parvalbumin (1:4000).

The immunohistochemical staining protocol was the same for each antibody, as described in Chapter 2. For double-labelling, the anti- $\beta$ -galactosidase was always processed first and visualised using DAB (brown), and then the second antibody was processed and visualised using the Vector SG kit (blue). The only exception to this was for the AChE stain, where the anti- $\beta$ -galactosidase was processed first and visualised using the Vector SG kit, since the colour reaction for the AChE stain yields a brown product.

Lesion-only animals were generated at a later time-point for comparison of immunohistochemical stains.

### *Quantification*

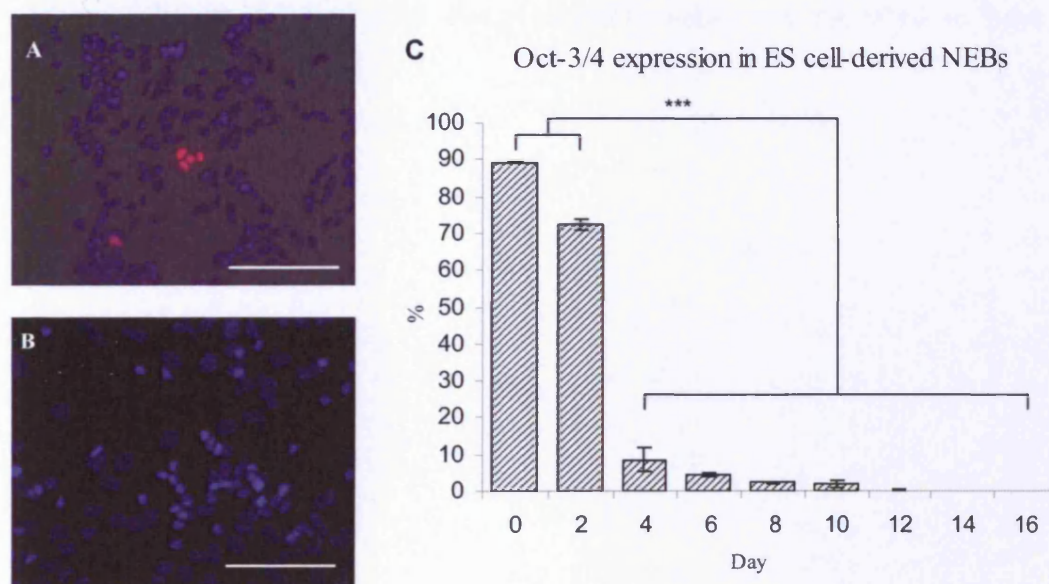
Cells expressing markers of interest were counted using a Leitz light microscope and stereology software. Graft volume was determined as described in Chapter 2. Statistical analyses were performed using two-sample t-tests and one-way ANOVA with Tukey-Kramer *post-hoc* comparisons when appropriate, using Minitab 15 software.



## 4.4 Results

### *In vitro* characterisation of NEBs pre-transplantation

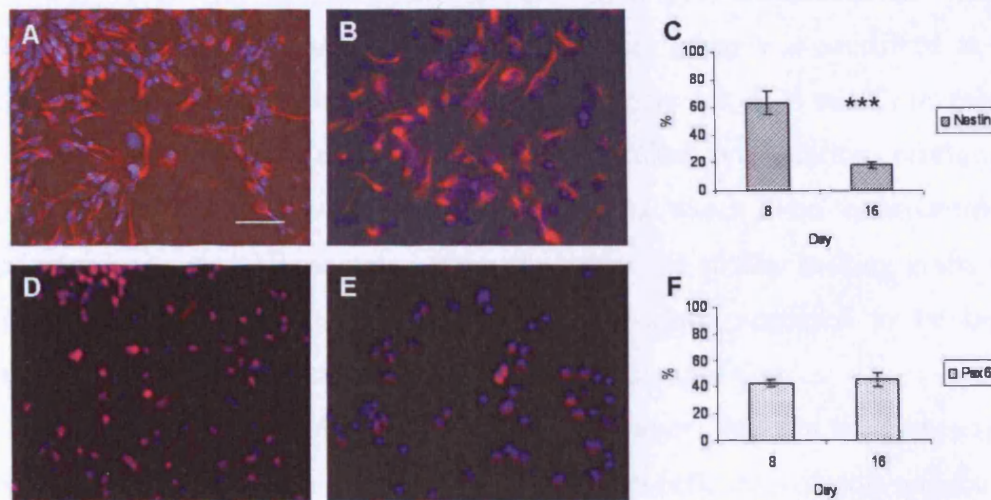
Expression of the ES cell marker Oct-3/4 in NEBs decreased over time in ADF+ neural induction culture (Figure 4.1). There was a highly significant difference between Oct-3/4 expression and day of neural induction culture (day,  $F_{8,27} = 115.81$ ,  $p < 0.001$ ). The proportion of cells that expressed Oct-3/4 on day 0 of the neural induction protocol was  $89.25 \pm 0.26\%$ ; at day 4 the proportion was  $8.43 \pm 3.21\%$  and at day 8 it was  $2.25 \pm 0.31\%$ . The detection of 2% Oct-3/4 positive cells still present in the cultures at day 8 prompted a longer time-point for analysis. Oct-3/4 expression has ‘switched-off’ by day 16 in the neural induction culture, suggesting there are no longer ES cells present.



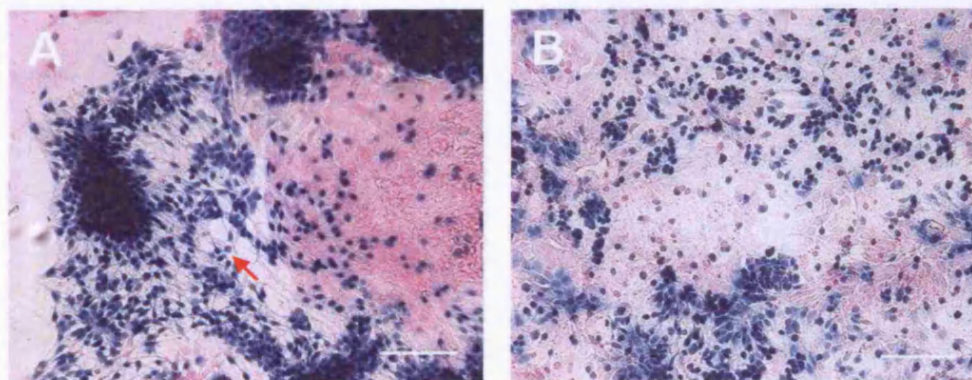
**Figure 4.1.** Foxg1Z NEBs were harvested, dissociated and plated onto substrate every two days following initiation of the neural induction protocol. Cultures were fixed after 4 hours and immuno-labelled for the undifferentiated ES cell marker Oct-3/4 (red) and Hoechst nuclear stain (blue) (A and B). Oct-3/4 immuno-positive cells were counted and are represented as a percentage of total Hoechst stained cells (C). At day 8 few Oct-3/4 positive cells were present (A) and at day 16 there were no Oct-3/4 positive cells detected (B). Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM. Significant *post-hoc* differences are indicated with brackets. (\*\*\*) $p < 0.001$ . Scale bars = 100 $\mu$ m.

Foxg1Z NEBs were analysed following 8 and 16 days in neural induction culture for the expression of nestin; a neural precursor marker and Pax6; a marker of the developing dorsal telencephalon. Cultures were dissociated, plated onto PLL/laminin substrate and fixed after 4 hours. There was a highly significant difference in nestin expression between NEB cultures at day 8 ( $63.46 \pm 8.45\%$ ) (mean  $\pm$  SEM) and at day 16 ( $18.07 \pm 2.33\%$ ) (day,  $t_4 = 8.67$ ,  $p < 0.001$ ) (Figure 4.2; A, B and C). Pax6 expression did not differ significantly from day 8 ( $42.89 \pm 2.87\%$ ) to day 16 ( $45.16 \pm 5.18\%$ ) (day,  $t_5 = 0.41$ ,  $p = \text{n.s}$ ) (Figure 4.2; D, E and F).

Following *in vitro* differentiation of day 8 and day 16 NEBs for 7 days in the presence of FCS and B-27, incubation with X-gal revealed Foxg1-positive cells. Day 8 differentiated cells (Figure 4.3; A) exhibited large clusters of cells including those with neuronal appearance. Within these cultures there was also the presence of large clusters of X-gal-negative cells which were not neural in appearance. Day 16 differentiated cultures (Figure 4.3; B) exhibited a more even distribution of Foxg1-positive cells interspersed with Foxg1-negative cells, and appeared to have less heterogeneous morphology.



**Figure 4.2.** Foxg1Z NEBs were harvested, dissociated and plated onto substrate at day 8 (A and D) and day 16 (B and E) of neural induction. Cultures were fixed after 4 hours and immuno-labelled with nestin (red) (A - C) or Pax6 (red) (D - F) and Hoechst nuclear stain (blue). Nestin and Pax6 immuno-positive cells were counted and are represented as a percentage of total Hoechst cells (C and F). The neural precursor marker nestin is more highly expressed in day 8 NEBs compared with day 16 NEBs, and there is no difference in expression of the developing forebrain marker, Pax6 between day 8 and day 16 NEB cultures. Each bar on the graphs represents a mean of 3 different neural induction cultures and error bars represent SEM. (\*\*\*) $p < 0.001$ . Scale bar = 50 $\mu$ m.



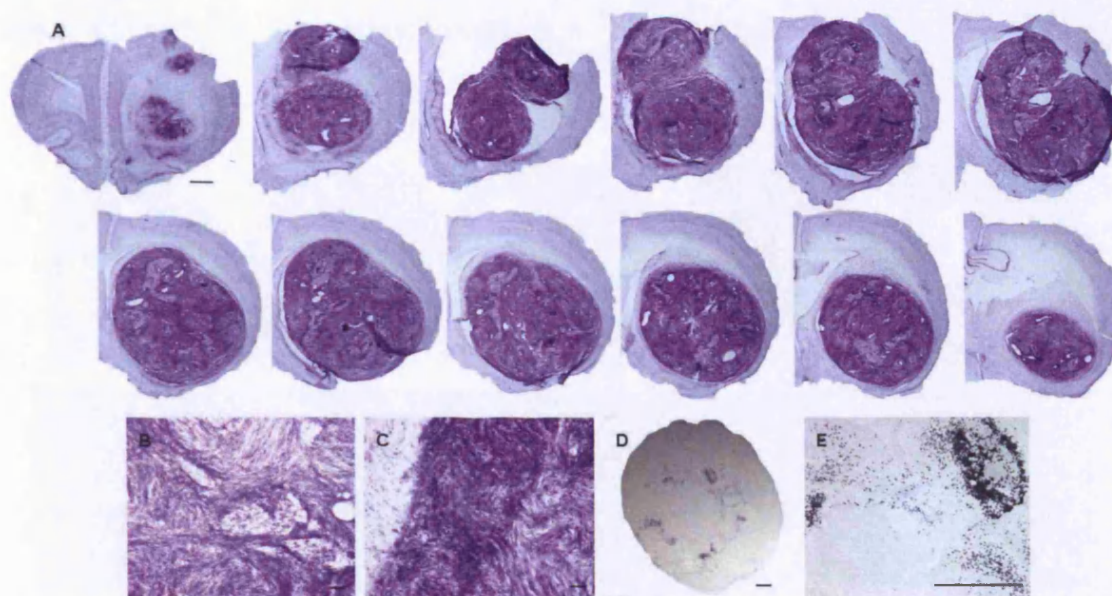
**Figure 4.3.** Foxg1Z NEBs were harvested, dissociated and plated onto substrate at day 8 and day 16, and allowed to differentiate for 7 days in neuronal differentiation medium. Cultures were incubated with X-gal staining solution (blue) and counterstained with eosin (pink). X-gal staining of day 8 cultures revealed clusters of blue cells including those showing neuronal morphology (red arrow). Within these cultures there were also large clusters of X-gal-negative cells (A). Day 16 cultures revealed a more even distribution of X-gal-positive cells throughout the platedown (B). Scale bars = 100 $\mu$ m.



*Day 8 NEB graft morphology*

Analysis of day 8 NEB grafts at six weeks post-transplantation was not possible due to the observation of ill-health and this group was sacrificed at two weeks post-transplantation. Nissl-stained sections (Figure 4.4; A, B and C) reveal that these grafts exhibited what appeared to be uncontrolled proliferation, resulting in overgrowth and a cell mass with well-defined borders, which filled the striatum and pushed into the cortex. All animals within this group had similar looking grafts with 'swirling' patches of tissue throughout, some of which appeared to be highly organised and were predominantly of non-neural cell composition.

The mouse neuron and mouse glia specific markers, M2 and M6 respectively, that were intended for use in order to identify grafted cells, proved non-specific and no graft-derived cells were labelled. The *LacZ* reporter incorporated in the Foxg1Z cell line enabled the use of the  $\beta$ -galactosidase antibody to detect Foxg1-positive-graft-derived cells (Figure 4.4; D and E). Within these 'teratoma-like' grafts there are patches of dense  $\beta$ -galactosidase immunoreactivity alongside regions with no immuno-positive cells. Graft volume was estimated using cell counts of  $\beta$ -galactosidase positive cells and the mean graft volume for the day 8 NEB grafts was  $336.32 \pm 129.16\text{mm}^3$  (mean  $\pm$  SEM). There was huge variability in graft size between animals with the range of volumes from 19.24 to  $962.32\text{mm}^3$ .



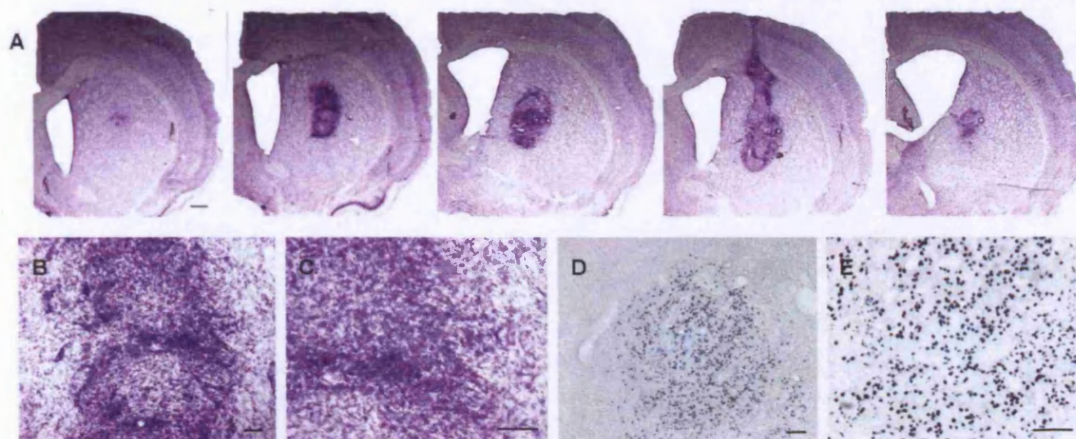
**Figure 4.4.** A representative 1:12 series, anterior to posterior, showing morphology of day 8 NEB grafts by Nissl staining with cresyl violet, at two weeks post-transplantation (A). Scale bar = 500 $\mu$ m. Photomicrographs B and C show higher power images of the Nissl stain. Scale bars = 100 $\mu$ m. The cell mass does not integrate into the host brain, but appears to grow as an isolated mass, with ‘swirling’ patches throughout and well-defined borders. Photomicrographs D and E show staining for  $\beta$ -galactosidase, which reveals  $\beta$ -galactosidase-dense regions alongside regions with no immunopositive cells. Scale bars = 500 $\mu$ m.

#### *Day 16 NEB grafts morphology*

Surviving grafts were seen in all animals that were transplanted with day 16 NEBs, as revealed by Nissl-stained sections (Figure 4.5; A, B and C) and  $\beta$ -galactosidase immunoreactivity (Figure 4.5; D and E). There are cell-dense regions present throughout the graft which are interspersed with less dense regions with a more even distribution of  $\beta$ -galactosidase immunopositive cells throughout the graft and fewer ‘less-dense’ regions, when compared with the day 8 NEB grafts. The borders of the day 16 NEB grafts are less well-defined than those of the day 8 NEB grafts (Figure 4.4; A and C, and Figure 4.5; A and B), which indicates a degree of integration of the day 16 NEB grafts into the host brain, rather than the distinctly isolated cell mass of the day 8 NEB grafts. Estimating the graft volume using the number of  $\beta$ -galactosidase positive cells revealed a range from 13.14 to 56.5 mm<sup>3</sup> with a mean value of  $21.87 \pm 4.63$  mm<sup>3</sup>. Day 16 NEB grafts exhibited a less heterogeneous appearance than day 8 NEB grafts. They had less non-neural tissue and



their appearance was more similar to grafts observed with primary striatal donor tissue, although they were larger in volume.



**Figure 4.5.** A representative 1:12 series of Nissl staining using cresyl violet, showing graft morphology of day 16 NEB grafts at six weeks post-transplantation (A). Scale bar = 500µm. Photomicrographs B and C show higher power Nissl-stained images which reveal an even distribution of cells throughout the graft. Photomicrographs D and E show β-galactosidase immuno-reactivity within the graft, showing even distribution of β-galactosidase immunopositive cells. Scale bars = 100µm.

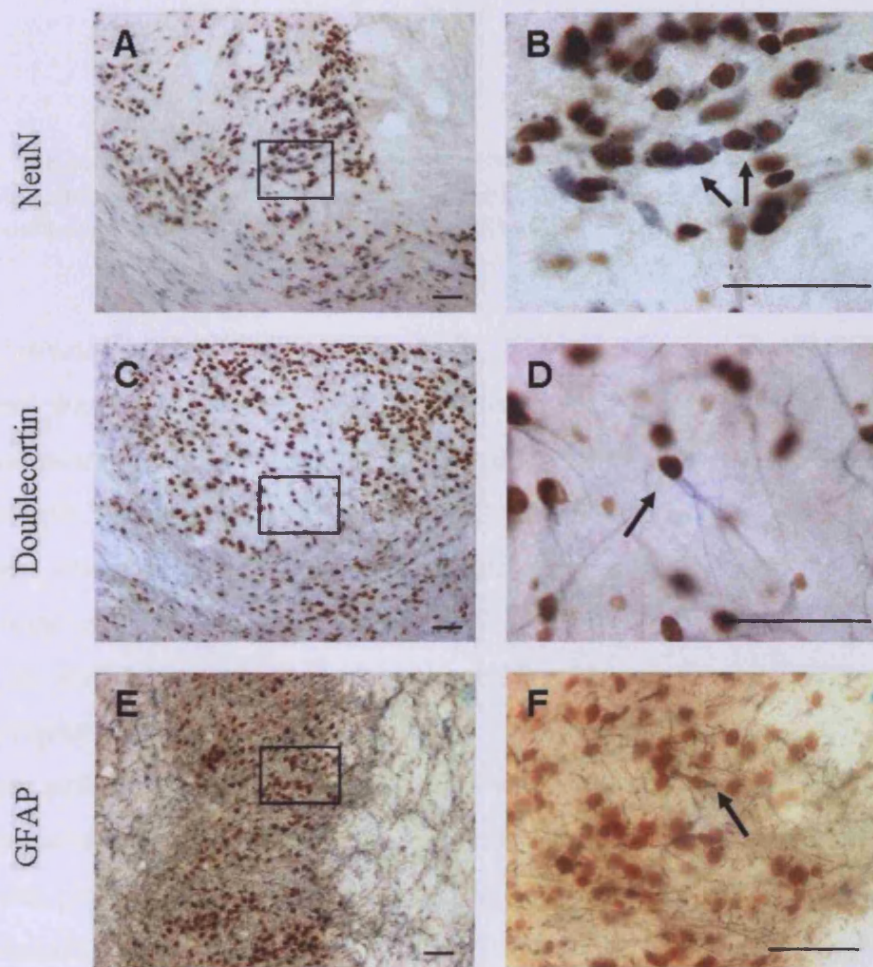
#### *Neural analysis of Day 16 NEB grafts*

Grafts were analysed for the expression of the neuronal markers DCX, which labels immature neurons, NeuN, which labels the nuclei of more mature neurons and also the glial marker GFAP. Expression of the striatal markers DARPP-32, calbindin and parvalbumin was also assessed. Due to the lack of specificity when staining with both M2 and M6 the only graft-derived cells which can be identified with confidence and analysed for expression of markers of interest are those that are immunopositive for β-galactosidase.

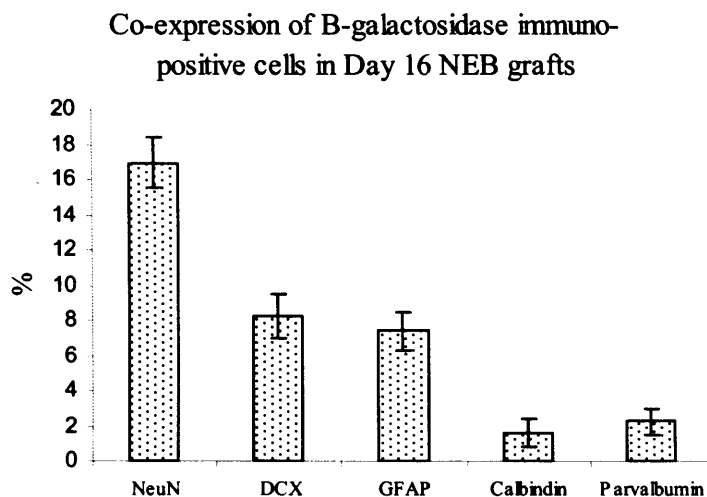
β-galactosidase-positive cells were seen to double label with NeuN throughout the graft (Figure 4.6; A and B) in all animals, and there was a proportion of NeuN immunopositive cells that did not double label. Between animals the numbers of NeuN positive cells as a percentage of β-galactosidase positive cells ranged from 10 – 25% with a mean of  $16.95 \pm 1.44\%$  (mean  $\pm$  SEM) (Figure 4.7). DCX positive cells were identified within the graft and were seen to double label with β-galactosidase (Figure 4.6; C and D). There was variability in number of co-labelled cells between



animals, ranging from 4-16%, with a mean of  $8.24 \pm 1.29\%$  (Figure 4.7). All grafts exhibited some degree of immunoreactivity to the astrocyte marker GFAP with double labelling with  $\beta$ -galactosidase (Figure 4.6; E and F), within a range of 3 – 12% ( $7.38 \pm 1.12\%$ ) (Figure 4.7) across the group, but not all GFAP positive cells within the graft area were positive for  $\beta$ -galactosidase.



**Figure 4.6.** NeuN (blue)/ $\beta$ -galactosidase (brown) staining reveals NeuN positive cells within the graft seen to double label with the  $\beta$ -galactosidase graft-derived cells, as shown with the arrow (A and B). DCX (blue)/ $\beta$ -galactosidase (brown) staining shows some expression of DCX co-expressed with  $\beta$ -galactosidase (shown with the arrow) (C and D). GFAP (blue)/ $\beta$ -galactosidase (brown) staining shows double-labelling of the astrocyte marker with grafted cells, within the graft area (shown with the arrow) (E and F). Scale bars = 50 $\mu$ m.



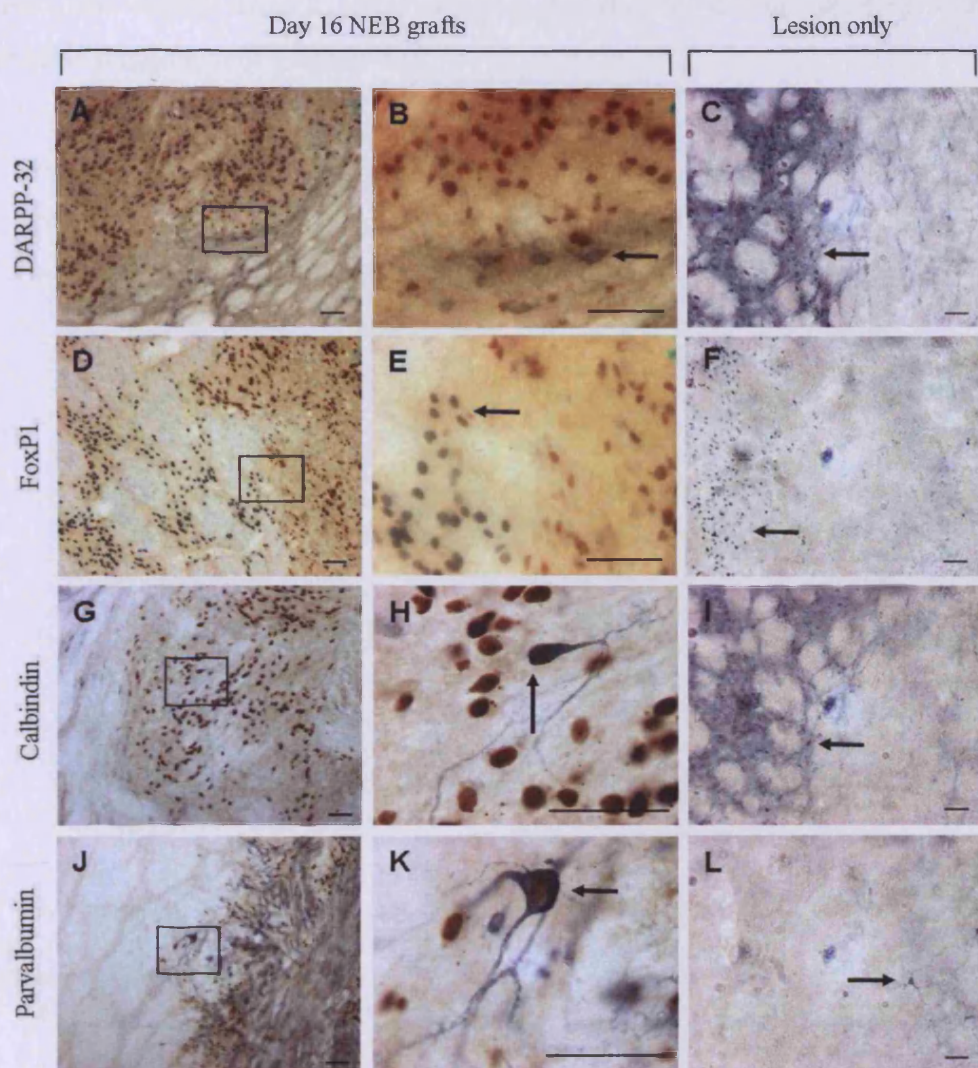
**Figure 4.7.** The percentage of  $\beta$ -galactosidase-positive cells expressing the neuronal markers NeuN, DCX, calbindin and parvalbumin, and the glial marker GFAP, in day 16 NEB grafts at six weeks post-transplantation. Error bars represent SEM.

#### *Analysis of striatal markers within the grafts*

There was no DARPP-32 immunoreactivity seen within the graft area, very few immuno-positive cells were seen at the periphery of the graft and there was no co-localisation with  $\beta$ -galactosidase (Figure 4.8; A and B). Sections stained for FoxP1 showed some immuno-reactive cells at the graft periphery and within the graft area itself, but none of these cells were seen to double label with  $\beta$ -galactosidase (Figure 4.8; D and E). Expression of both DARPP-32 and FoxP1 were seen at the periphery of the lesion (Figure 4.8; C and F).

Other striatal neuronal markers assessed were calbindin and parvalbumin. Few calbindin positive cells were seen within the grafts and there was a degree of double-labelling with  $\beta$ -galactosidase (Figure 4.8; G and H) although this was not the case with all animals. The situation was similar when sections were stained with parvalbumin, where few immunopositive cells were seen to double label with  $\beta$ -galactosidase (Figure 4.8; J and K) but this was not evident in all animals. The range for co-localisation of  $\beta$ -galactosidase with calbindin was 0-5% with a mean of  $1.62 \pm 0.77\%$ , and with parvalbumin the range was 0-6%, with a mean value of  $2.26 \pm 0.76\%$  (Figure 4.7). Calbindin expression was seen at the periphery of the lesion (Figure 4.8; I) and a few parvalbumin positive cells could be seen within the lesion, but they were very scarce (Figure 4.8; L).



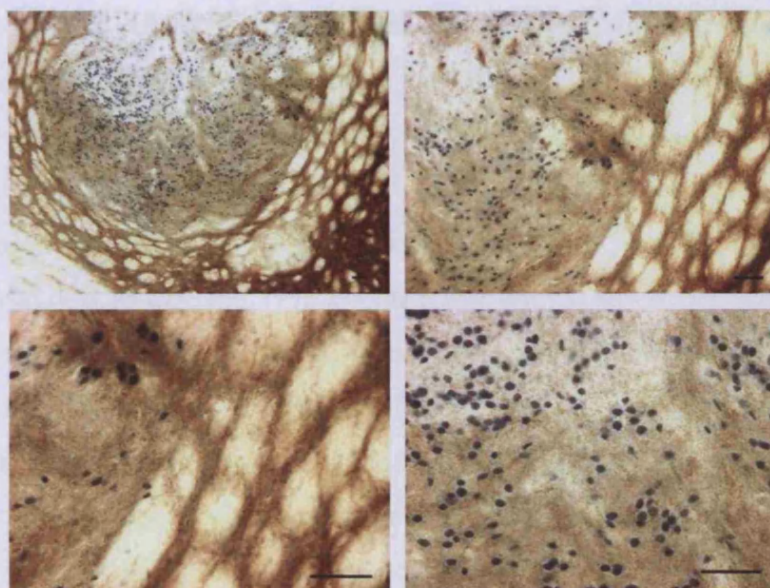


**Figure 4.8.** Striatal markers analysed within day 16 NEB grafts at six weeks post-transplantation. Sections were stained for DARPP-32 (A-C), FoxP1 (D-F), calbindin (G-I) and parvalbumin (J-L). Photomicrographs in column 3 are sections from lesion-only brains (C, F, I and L). DARPP-32 immuno-reactive cells (blue) were observed at the periphery of the graft area as shown with the arrow in B. No  $\beta$ -galactosidase (brown) positive cells were seen to double label with DARPP-32 (A and B). FoxP1 positive cells (blue) were also seen at the edge of the graft area (arrow in D), and again there was no co-expression of FoxP1 with the graft-derived  $\beta$ -galactosidase (brown) (C and D). Calbindin staining revealed some calbindin-positive cells (blue) within the graft area, and some of these cells were seen to double label with  $\beta$ -galactosidase (brown) (E and F). Parvalbumin staining showed a small proportion of the  $\beta$ -galactosidase-graft-derived cells double-labelling (G and H). Scale bars = 50 $\mu$ m.



AChE identifies the characteristic patch-matrix structure of the striatum, outside the grafted area however, there is no evidence of the patch-matrix within the graft, since the AChE histochemical staining revealed no double-labelling with  $\beta$ -galactosidase positive cells (Figure 4.9).

#### AChE



**Figure 4.9.** Photomicrographs of a representative day 16 NEB graft at various magnifications following AChE histochemical staining.  $\beta$ -galactosidase (blue) and AChE (brown) show no co-localisation. The AChE identifies the patch-matrix structure of the striatum outside the grafted area however there is no evidence of the patch-matrix within the graft. Scale bars = 50 $\mu$ m.

## 4.5 Discussion

### *In vitro expression of precursors pre-transplantation*

Oct-3/4 is a member of class V of the POU (Pit, Oct, Unc)-domain transcription factor family and is expressed by pluripotent cells (Niwa et al., 2000). It is expressed in ES cell lines derived from the ICM when the cells are in an undifferentiated state and when ES cells begin to differentiate the expression of Oct-3/4 rapidly down regulates. When ES cells are subjected to our neural induction protocol (ADF+), expression of Oct-3/4 is seen to decrease over time from 89.25% at day 0 to 2.25% at day 8. Bouhon et al report that Oct-3/4 expression at day 4 is lower

in CDM culture than when cells are cultured in 10% serum (Bouhon et al., 2006). After 8 days in CDM with and without addition of FGF2 at day 4, RT-PCR revealed a faint band representing Oct-3/4 expression relative to Gapdh (Bouhon et al., 2005) and cell counts following immunocytochemistry revealed 2.43% cells still expressing Oct-3/4 at day 8 (Bouhon et al., 2006). Oct-3/4 expression in cultures in this study was analysed using fluorescent immunocytochemistry with the Oct-3/4 antibody and is consistent with the evidence of Oct-3/4 positive cells in cultures at day 8, albeit a low proportion of cells. The few cells that still express Oct-3/4 are an issue that needs to be addressed before cells after this short period in CDM culture can be used for transplantation. After 16 days in ADF+ culture Oct-3/4 expression is shown to have ‘switched off’.

Expression of nestin following neural induction in ADF+ significantly decreased from day 8 to day 16. This is consistent with a report that showed nestin expression in neurally induced ES cells decreases over time in culture from day 0 to day 8, with a further decrease by day 12 (Bouhon et al., 2005). Analysis of nestin-positive cells present in neural induction culture prior to transplantation revealed that the lower the proportion of nestin-positive cells, the lower the risk of teratoma development (Dihne et al., 2006). Addition of FGF2 to CDM cultures has been shown to maintain the proportion of nestin-positive cells over time, up to day 8, when compared with CDM alone and the same study also reported that NEBs older than day 8 depend on exogenous FGF2 (Bouhon et al., 2005). Pax6 expression, a marker of dorsal telencephalic cells, was not significantly different between day 8 and day 16 cultures. Bouhon et al., (2005) reported an increase in Pax6 expression between day 4 and day 8 in CDM, but expression beyond day 8 was not analysed.

#### *Day 8 NEB graft survival and morphology*

During post-mortem analysis there is a need for detection of transplanted cells in order to distinguish between graft-derived and host-derived phenotypes generated following implantation, subsequent survival time and *in vivo* differentiation. When grafting cells of mouse origin into the host rat brain this identification can prove difficult since there are very few mouse specific markers available. M2 and M6 markers which are reported to specifically label mouse neurons and mouse astrocytes respectively, were initially used in our study, but proved unreliable and unsuccessful when tested using our current protocols.

The Foxg1Z ES cell line used in this study enables the detection of cells expressing *LacZ* and therefore *Foxg1*, by using antibodies against the enzyme  $\beta$ -galactosidase. Only those cells immunopositive for  $\beta$ -galactosidase can be positively identified as donor cells, and thus, due to lack of any other reliable marker, only these cells were further characterised. It is therefore not always straight forward to draw conclusions regarding whether or not a single-labelled cell is graft or host-derived.

The number of  $\beta$ -galactosidase immuno-positive cells in the day 8 NEB grafts varied greatly between animals. Histological analysis showed that all animals in the group exhibited grafts with massive overgrowth, as shown in figure 4.4, and due to signs of ill-health observed in the animals, the group was sacrificed at the early time point of two weeks. The difference in graft survival time between the two graft groups, and the fact that the day 8 NEB grafts suffered the uncontrolled proliferation and generation of 'teratoma-like' masses, make it difficult to draw comparisons between them.

The potential of ES cells to develop teratomas following transplantation is one major down-fall in their consideration as an alternative cell source for clinical applications. The appearance of non-neural-like regions of cells within the day 8 NEB grafts in this study, and the overgrowth observed indicates generation of teratomas, which can be recognised by the presence of a range of differentiated tissue types, representative of the three germ layers (Przyborski, 2005). Mouse ES cell-derived EBs implanted into the embryonic rat brain and left to survive until 2 weeks after birth have been shown to migrate to various brain regions and generate neural cell types (Brustle et al., 1997). Benninger et al (2000) generated EBs from ES cells using neurobasal medium supplemented with B27 and following 8 days in these culture conditions the cells were grafted into the adult mouse striatum where graft-derived cells differentiated into neurons, glia and oligodendrocytes (Benninger et al., 2000). Furthermore, donor cells have shown some degree of expression of region-specific markers such as *Foxg1*, *Dlx* and *Pax6* (Wernig et al., 2004). However along with these encouraging observations for the potential of these cells to develop into desired phenotypes, other findings, within the same host brains, include undifferentiated ES cells, non-neural tissue clusters (Brustle et al., 1997), including epithelium, muscle and cartilage (Benninger et al., 2000), and teratomas (Wernig et al., 2004).

*Day 16 NEB graft survival and morphology*

At six weeks post-transplantation, during which time daily immunosuppression was administered, all animals receiving day 16 NEB grafts had what appeared to be healthy, surviving grafts. The Nissl staining showed that these grafts did not cause distortion of the brain and they did not show signs of over-growth. There was an indication though that the grafts comprised heterogeneous cell types of both neural and non-neural derivation. However, this finding is not remarkable since non-neural differentiation has previously been reported following neural transplantation, as outlined earlier (Brustle et al., 1997; Benninger et al., 2000; Wernig et al., 2004).

Graft volume was variable within the day 16 NEB graft group and looked comparable to graft sizes from a previous study where mouse ES cell-derived precursors were grafted into the adult rat QA lesioned striatum (Dinsmore et al., 1996), although this study reported no cell numbers or graft volumes. Primary rat striatal tissue grafted into the rat lesioned striatum (450,000 - 500,000 cells grafted) yielded grafts with a range of volumes that are around 20 times smaller than that reported in this chapter, and interestingly, MGE-derived grafts were smaller than LGE-derived grafts (Pakzaban et al., 1993; Olsson et al., 1995).

*Phenotype of Foxg1Z cells in day 16 NEB grafts*

Due to the problem of reliably identifying all donor cells, further graft analysis considers those cells that are  $\beta$ -galactosidase positive. Donor cells were shown to generate immature and more mature neurons, shown by co-expression of  $\beta$ -galactosidase with the markers DCX and NeuN, respectively, and also glial cells, shown by co-expression with GFAP. Analysis of striatum-specific neurons revealed expression of donor-derived calbindin and parvalbumin, but neither DARPP-32 nor AChE. Co-expression of  $\beta$ -galactosidase and FoxP1 was not observed indicating the absence of graft-derived striatal precursor cells.

The 28 kD calcium-binding protein calbindin is expressed in projection neurons originating in the striatal matrix compartment. The presence of calbindin immunopositive cells within the day 16 NEB grafts indicates striatonigral projection neurons characteristic of the matrix compartment of the striatum (Gerfen, 1992). Parvalbumin immunoreactivity is evidence of striatal medium aspiny neurons and interneurons within the graft.

There are only a handful of reports on the survival and phenotype of mouse ES cell-derived precursors transplanted into the QA lesioned striatum. Dinsmore et al (1996) transplanted mouse ES cell-derived precursors that were grown as free-floating spheres with the addition of RA. Following six weeks *in vivo*, they found neurons within the graft area that were positive for neuron-specific enolase (NSE), neuron-specific tubulin, GABA and AChE (Dinsmore et al., 1996).

Bosch et al (2004) used the immortalised striatal neural stem cell line, ST14A and following sequential treatment with RA and KCl, and pre-differentiation to a GABAergic phenotype, cells were transplanted into the lesioned rat striatum. They observed that three days post-transplantation cells maintained their GABAergic phenotype, and at both one and five weeks post-transplantation apomorphine-induced rotations were reduced in grafted animals compared with non-grafted controls, suggesting a degree of benefit from GABAergic pre-differentiated neurons transplanted into QA-lesioned rats (Bosch et al., 2004).

Although there have been no reports on the generation of DARPP-32 positive neurons following transplantation of mouse ES cell-derived precursors, this has been shown for human NEBs (Aubry et al., 2008; Nasonkin et al., 2009). Transplantation of neurally induced human ES cell-derived precursors involving long-term addition of BDNF, Shh and Dkk-1, gave rise to grafts containing DARPP-32 and calbindin positive cells, following six weeks *in vivo* (Aubry et al., 2008). Nasonkin et al (2009) cultured human ES cells in FGF2 and noggin for four weeks and then transplanted into the unlesioned rat striatum. At three months post-transplantation there was no graft-derived DARPP-32 but at six months 30% of grafted cells were DARPP-32 positive (Nasonkin et al., 2009).

The absence of DARPP-32 within the grafts in our study might be because the cells were not exposed to all the necessary differential cues while in culture prior to transplantation, and also the *in vivo* maturation period might not have been long enough to encourage induction of more mature neuronal phenotypes such as DARPP-32.

## Conclusions

Work presented in this chapter supports the evidence that ES cells have the potential to be beneficial for neural transplantation. It is clear however that further controlled directed differentiation of these cells is required in order to remove

unwanted cell populations and achieve the desired striatal progenitor population. Analysis of the day 16 NEB grafts reveals that when transplanted these cells have the ability to generate neurons, a low proportion of which exhibit striatal markers. The presence of striatal neurons such as calbindin and parvalbumin is an encouraging result and longer term *in vivo* differentiation of these cells might generate increasing numbers of striatal-like cells including DARPP-32 expressing neurons.

Cells originating from the developing striatum that are transplanted for cell replacement in HD, have been exposed to the necessary complement of factors and cues to steer them towards the desired phenotype. Even these cells, when grafted into lesion models, do not appear to generate all the cell types in the necessary proportions to achieve the desired functional recovery. It is ambitious to suppose that *ex-vivo* derived NEBs, with initial differentiation *in vitro* and subsequent maturation *in vivo* in the adult striatum, will be sufficiently induced to generate the required cell identities, and it is understandable therefore that a complex of factors will need to be added to NEBs in order to generate LGE-like progenitor cells.

# Chapter 5

## *Generation of ventral telencephalon-like precursors*

### 5.1 Summary

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NEBs cultured under standard neuralising conditions without attempting directed differentiation showed some evidence, in Chapter 4, of differentiation typical of striatal neuronal markers. Others have reported similar cells in these conditions to express gene markers of the developing telencephalon, and more specifically markers which are characteristic of specific dorso-ventral regions of the telencephalon. Here, analysis of such markers in NEBs at day 8 and day 16 following standard neural induction culture, using semi-quantitative RT-PCR, showed that expression levels of all markers were lower than their comparative levels in the cells of the dorsal or ventral telencephalon. Addition of a range of concentrations of the SHH agonist purmorphamine resulted in reduced expression of dorsal markers *Pax6* and *Ng2*, and low concentrations (0.5µM) resulted in an increase in ventral markers *Nkx2.1* and *Dlx2*. Analysis of SHH-pathway components revealed that expression of SHH and Patched was higher in NEBs at both day 8 and day 16 than expression in the E14 mouse ventral telencephalon. Treatment of NEBs with various concentrations of the SHH antagonist cyclopamine did not significantly alter expression of dorsal markers, but caused a decrease in expression of ventral markers *Nkx2.1*, *Dlx2* and *Gsx2*. Interestingly, *Mash1* expression was not changed.

Fluorescent immunocytochemistry indicated that the dorsal marker *Pax6* was reduced with purmorphamine treatment and increased with cyclopamine treatment, at both day 8 and day 16. The MGE marker *Nkx2.1* was increased at day 8 with purmorphamine and decreased with cyclopamine, and at day 16 this response was more substantial. *Mash1* expression was reduced by addition of purmorphamine at day 8 and day 16, and was shown to increase with cyclopamine treatment at both days.

In summary, although gene expression changes in NEBs were demonstrated in response to purmorphamine and cyclopamine, this was not consistent for all the dorso-ventral genes analysed and no conclusive patterns were identified.



## 5.2 Introduction

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The developing telencephalon expresses markers that are characteristic of distinct regions of origin and are responsible for the subsequent generation of mature phenotypes present in the adult forebrain. The dorsal-most region of the telencephalon gives rise to the cortex, whilst the ventral-most region is the origin of cells that go on to populate the striatum, cortex, GP and olfactory bulb. It is the LGE of the ventral portion that is predominantly the origin of DARPP-32-positive MSNs.

Mouse NEBs cultured in serum-free suspension medium have been reported to express genetic markers of the developing telencephalon, and in addition, markers typical of specific regions along the dorsal-ventral axis (Bouhon et al., 2005; Watanabe et al., 2005; Gaspard et al., 2008). Supplementation of neural induction cultures with factors known to be important for development of the neural tube, in particular patterning of cells, has proved fruitful in generating such precursors (see section 1.6 Introduction).

SHH is a morphogen released from the prechordal plate, which underlies the telencephalon (Rubenstein et al., 1998) and it acts in a concentration-dependent gradient within the developing neural tube (Jessell, 2000). Addition of a range of doses of SHH (3-300nM) to serum-free suspension cultures of mouse ES cells decreased expression of the dorsal telencephalon markers *Pax6* and *Emx1*, whilst increasing expression of the ventral telencephalon markers *Gsx2* and *Nkx2.1* within the *Foxg1* population, which was not significantly changed (Watanabe et al., 2005). Treatment of neural induction cultures of mouse ES cells with the SHH antagonist cyclopamine has been reported to increase expression of the dorsal telencephalon markers *Pax6*, *Emx1* and *Emx2*, and decrease expression of the ventral telencephalon markers *Gsx2* and *Nkx2.1* (Gaspard et al., 2008).

A sonic hedgehog agonist, purmorphamine, was discovered by Wu et al, (2002), who screened a 'heterocycle combinatorial library' for small molecules with osteogenesis-inducing activity. Purmorphamine showed significant activity in the alkaline phosphatase (ALP) enzymatic assay, which is an osteoblast specific gene highly expressed during differentiation (Wu et al., 2002). Wu et al (2004) investigated how purmorphamine induced bone differentiation and found that the same genes that

are induced by Hedgehog are also upregulated by purmorphamine and, when Hedgehog signalling was blocked, the effect of purmorphamine disappeared (Wu et al., 2004). More recently, it has been shown that purmorphamine directly targets smoothened in the SHH signalling pathway and has opposing activity to smoothened inhibitors (Sinha and Chen, 2006; Briscoe, 2006).

Following the generation of optimal numbers of forebrain precursors from mouse ES cells in neural induction culture with the addition of 20ng/ml FGF2 at day 4, as demonstrated in Chapter 3, the next step was to further specify the forebrain precursors to enrich for ventral telencephalon-like precursors. This chapter analysed gene expression of NEBs relative to expression in corresponding regions of the developing mouse brain at gestational age E14. The SHH agonist purmorphamine was added to cultures at day 4 at various concentrations in order to determine if there was a dose-response effect on NEBs with analysis at day 8 and at day 16, and markers indicating dorso-ventral patterning in the developing telencephalon were analysed. Upon further analysis, endogenous SHH levels were found to be higher than anticipated and subsequently cultures were treated with the SHH antagonist cyclopamine and analysed for dorso-ventral specification.

Analysis of purmorphamine and cyclopamine treatments revealed changes in expression of both dorsal and ventral markers, and some patterns of expression were observed. However, this was not entirely consistent across all the markers analysed and there was no conclusive pattern regarding effects on dorso-ventral patterning. Semi-quantitative RT-PCR and fluorescent immunocytochemistry analysis were carried out on untreated and treated NEBs, looking at markers indicative of specification along the dorso-ventral telencephalic axis, comparing expression with that in the developing telencephalon.

## 5.3 Experimental Procedures

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### *Cell Culture*

The mouse ES cell line Foxg1-lacZ was used in this study. NEBs were derived from the Foxg1Z mouse ES cell line as described in Chapter 2. Briefly, ES cells were transferred from ES cell culture medium to CDM (ADF+ medium) at a density of 50,000 cells per ml, medium was replaced every 2 days and FGF2 (20ng/ml) was added from day 4.

For analysis of effects of the SHH agonist purmorphamine, the range of concentrations 0.5, 1, 2 and 5 $\mu$ M was used, and was added from day 4.

For analysis of effects of the SHH antagonist cyclopamine, the range of concentrations 0.1, 0.5, 1 and 2 $\mu$ M was used, and was added from day 4.

### *Dissection of mouse forebrain regions*

Control samples for gene expression analysis were taken from the E14 mouse forebrain. Cortex, WGE, LGE and MGE were dissected from E14 mouse embryos as described in Chapter 2. Each region was dissected, washed and finely chopped before RNA extraction for gene expression analysis. Tissue from the same regions was also enzymatically dissociated and plated down onto substrate for immunocytochemistry (as described in Chapter 2).

### *Gene expression analysis*

NEBs were analysed at days 8 and 16. RNA was extracted for subsequent cDNA synthesis and QPCR analysis was then carried out using the SYBR Green assay, as detailed in Chapter 2.

Primer pairs used were  *$\beta$ -actin*, *Pax6*, *Ngn2*, *Emx2*, *Nkx2.1*, *Dlx2*, *Mash1*, *Gsx2*, *Shh*, *Gli3* and *Patched1* (see Appendix 4 for sequences). Opticon Monitor 3 software was used and the differential gene expression of different samples was measured relative to the expression of  *$\beta$ -actin* (as described in Chapter 2).

*Immunocytochemistry*

NEBs were harvested, dissociated and plated onto PLL/laminin treated cover-slips at a density of 50,000 cells per 30µl drop on each cover-slip, as described in Chapter 2. Cells were fixed 2-4 hours after plating on adhering to the substrate. Fluorescent immunocytochemistry was performed according to the protocol detailed in Chapter 2. Primary antibodies used were anti-Pax6 (1:50), anti-Nkx2.1 (1:200) and anti-Mash1 (1:200).

*Microscopy and Statistical Analysis*

Cells were visualised under UV fluorescence using a Leitz microscope. Hoechst positive nuclei were detected and cells immuno-labelled with the marker of interest were counted, as described in Chapter 2. Images were processed using Optronics MagnaFire Software and Adobe Photoshop.

Statistical analyses were carried out using Minitab 15 statistical software. One-way ANOVA with Tukey-Kramer *post-hoc* comparisons when appropriate were performed.

## 5.4 Results

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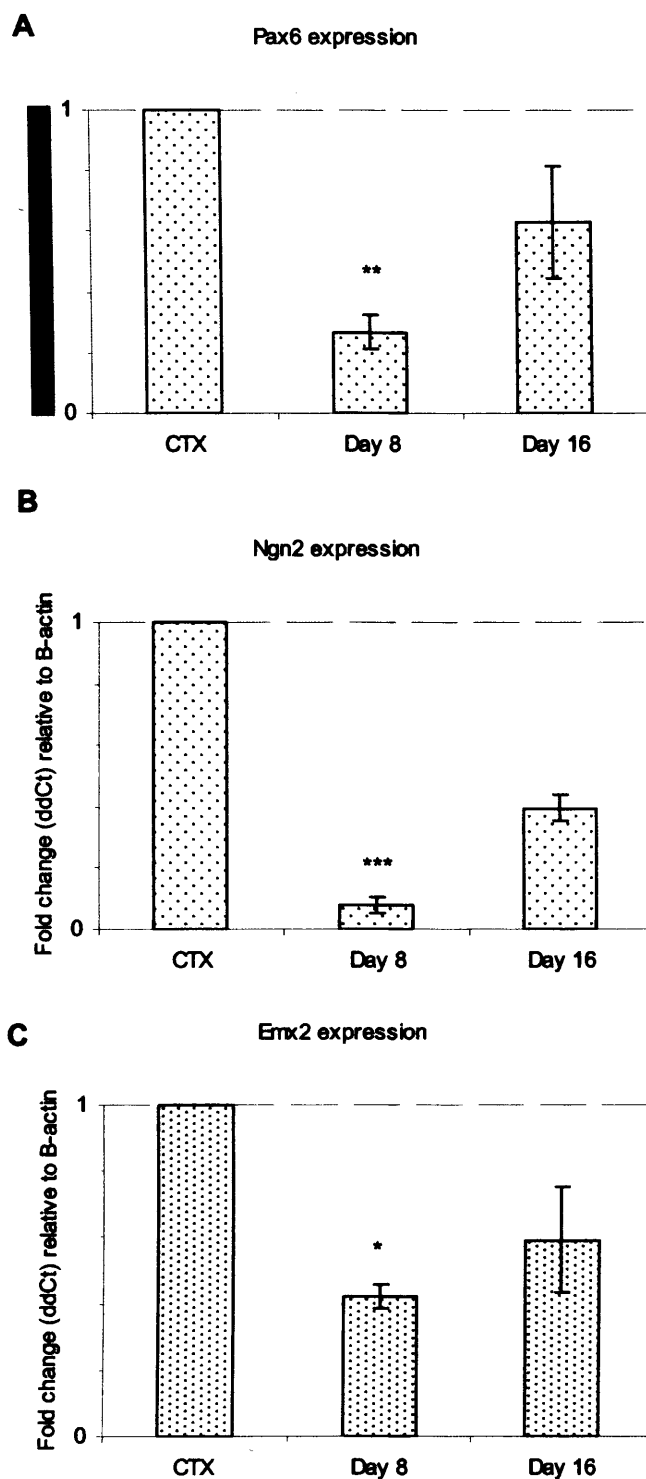
### *Dorsal-ventral telencephalic gene expression in NEBs*

NEBs were analysed for gene expression following 8 and 16 days of the ADF+ neural induction protocol, using semi-quantitative RT-PCR. Expression of the dorsal telencephalon markers *Pax6*, *Ngn2* and *Emx2* in ES cell-derived NEBs were analysed relative to developing mouse cortex at E14 (Figure 5.1). *Pax6* expression in NEBs was significantly different from that in the E14 mouse cortex ( $F_{2,9} = 17.25$ ,  $p < 0.01$ ) (Figure 5.1; A). *Post-hoc* analysis confirmed that expression at day 8 ( $0.27 \pm 0.05$ ) was significantly lower than cortical expression, but at day 16 ( $0.63 \pm 0.18$ ) there was no statistical significance. There was a highly significant difference in *Ngn2* expression between E14 cortex and NEBs ( $F_{2,9} = 251.68$ ,  $p < 0.001$ ) (Figure 5.1; B), with NEB cultures at day 8 ( $0.08 \pm 0.03$ ) having significantly lower expression than the cortex. Expression of *Ngn2* at day 16 ( $0.39 \pm 0.05$ ) was not significantly different from E14 cortex, and there was no significant difference between day 8 and day 16. *Emx2* expression revealed a significant difference between NEBs and cortex ( $F_{2,9} = 9.92$ ,  $p < 0.05$ ) (Figure 5.1; C); expression was statistically significant between cortex and NEB cultures at day 8 ( $0.42 \pm 0.06$ ), but not at day 16 ( $0.59 \pm 0.16$ ).

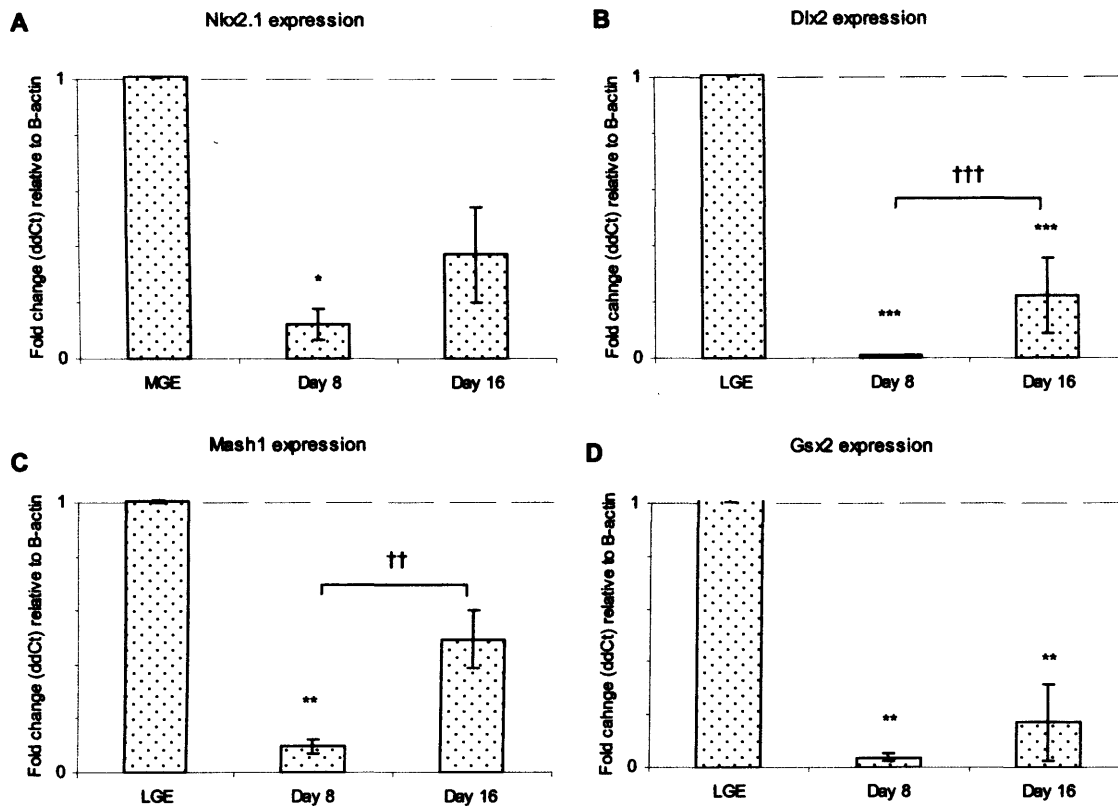
The ventral telencephalon can be subdivided, and for the purpose of this study we have used the MGE and LGE divisions. In the developing telencephalon, *Nkx2.1* expression is restricted to the MGE and is a specific marker of this region. There was a significant difference in expression of *Nkx2.1* between that in mouse E14 MGE and day 8 NEBs ( $0.12 \pm 0.05$ ), but not day 16 NEBs ( $0.37 \pm 0.17$ ) ( $F_{2,9} = 7.99$ ,  $p < 0.05$ ) (Figure 5.2; A).

Markers of the LGE include *Dlx2*, *Mash1* and *Gsx2*, and although their expression is not exclusive to the LGE (as *Nkx2.1* expression is to the MGE) they are widely used as markers for this region, but caution is necessary when attempting to draw definitive conclusions. *Dlx2* expression (Figure 5.2; B) was significantly lower than E14 LGE at both day 8 ( $0.01 \pm 0.00$ ) and day 16 ( $0.22 \pm 0.13$ ), and there was also a significant difference in expression between day 8 and day 16 NEBs, with greater expression at day 16 ( $F_{2,9} = 42.36$ ,  $p < 0.001$ ). Expression of *Mash1* (Figure 5.2; C) was significantly lower than LGE at day 8 ( $0.10 \pm 0.03$ ), but not at day 16 ( $0.49 \pm$

0.11); also *Mash1* expression at day 16 was significantly higher than that at day 8 ( $F_{2,9} = 17.45$ ,  $p < 0.01$ ). *Gsx2* expression (Figure 5.2; D) revealed significantly lower expression than LGE at both day 8 ( $0.04 \pm 0.01$ ) and day 16 ( $0.17 \pm 0.15$ ) ( $F_{2,9} = 22.08$ ,  $p < 0.01$ ).



**Figure 5.1.** Expression of dorsal markers *Pax6* (A), *Ngn2* (B) and *Emx2* (C) was analysed using semi-quantitative RT-PCR on cDNAs prepared from E14 mouse cortex and NEBs harvested at day 8 and day 16 of neural induction in ADF+ with 20ng/ml FGF2 present from day 4. Gene expression is shown relative to  $\beta$ -actin. The broken line crossing the y-axis at 1 represents relative expression in the E14 mouse cortex. Each bar on the graphs represents a mean of 3 different neural induction cultures and error bars represent SEM. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).



**Figure 5.2.** Expression of ventral markers *Nkx2.1* (A), *Dlx2* (B), *Mash1* (C) and *Gsx2* (D) was analysed using semi-quantitative RT-PCR on cDNAs prepared from E14 mouse MGE, LGE and NEBs harvested at day 8 and day 16 of neural induction in ADF+ with 20ng/ml FGF2 present from day 4. Gene expression is shown relative to  $\beta$ -actin. *Nkx2.1* expression in NEBs was determined relative to that in MGE, whilst *Dlx2*, *Mash1* and *Gsx2* expression in NEBs was determined relative to that in LGE. The broken line crossing the y-axis at 1 represents relative expression in the E14 mouse MGE (A) and LGE (B-D). Each bar on the graphs represents a mean of 3 different neural induction cultures and error bars represent SEM. \* = significant difference from MGE/LGE control. † = significant difference between day 8 and day 16 NEB groups. (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001, ††† $p$ <0.01).



*Effect of the SHH agonist purmorphamine on dorsal-ventral expression*

To determine if NEBs are responsive to the SHH agonist purmorphamine, ADF+ neural induction medium was supplemented with a range of purmorphamine concentrations and expression of SHH-pathway components was analysed (Figure 5.3). Purmorphamine was added at day 4 and NEBs were harvested for analysis at day 8 or day 16. At both day 8 and day 16 expression of *SHH* (Figure 5.3; A) was shown to increase following addition of purmorphamine and this was similar for *Patched* expression (Figure 5.3; B). Expression of *Patched* was always higher at day 16 than at day 8. *Gli3* expression was reduced slightly following addition of purmorphamine (Figure 5.3; C).

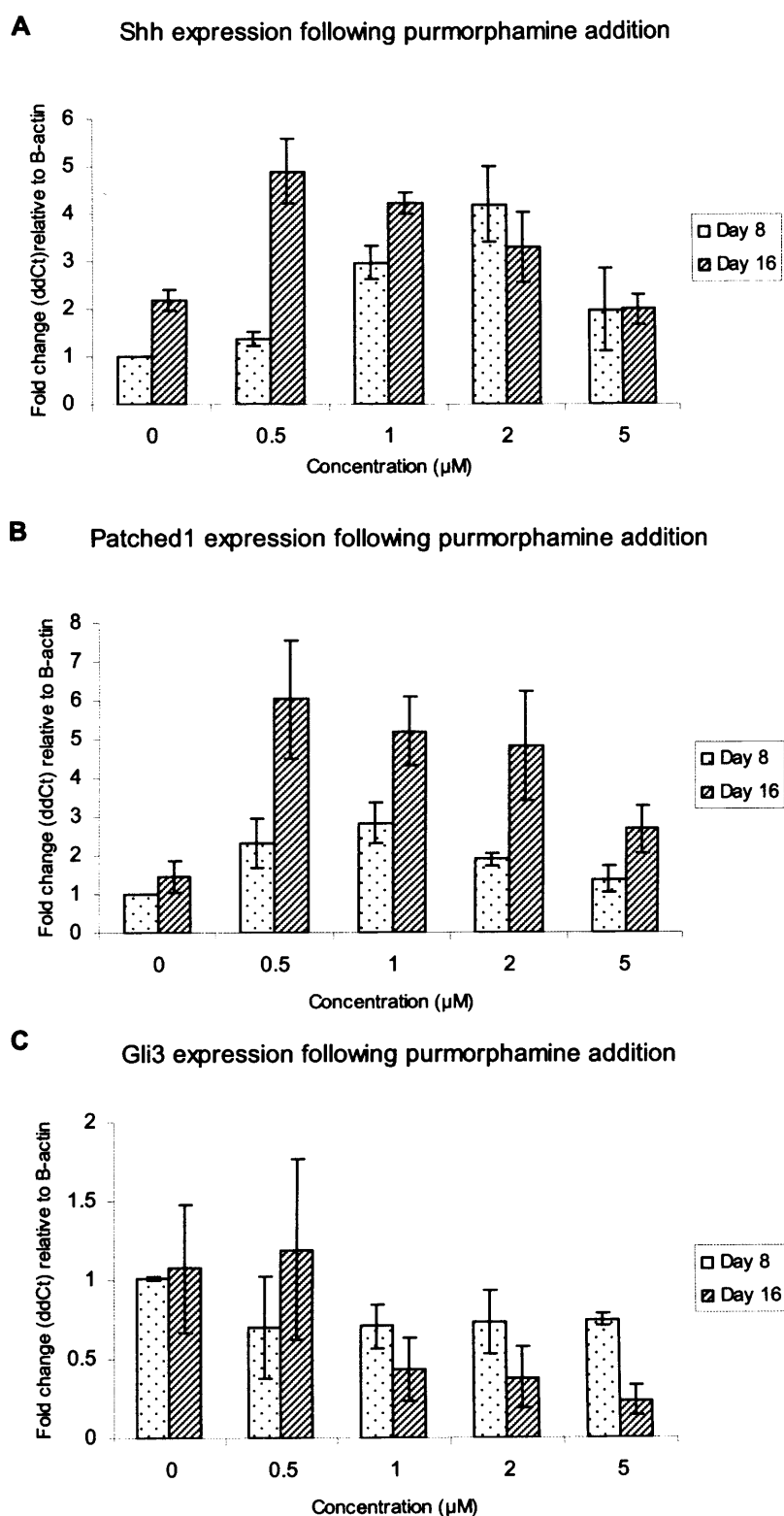
Expression of dorsal and ventral telencephalic markers in NEB cultures were analysed in response to the addition of purmorphamine, and analysis was compared relative to E14 cortex or MGE/LGE (expression relative to E14 WGE is shown in Appendix 6). *Pax6* expression was shown to decrease with addition of purmorphamine (Figure 5.4; A). There was no significant difference between the day of analysis (two-way ANOVA,  $F_{1,30} = 2.35$ ,  $p = \text{n.s.}$ ) however there was a significant difference between concentrations ( $F_{4,30} = 4.48$ ,  $p < 0.05$ ). At day 16, NEBs treated with 0.5, 1.0 or 2.0  $\mu\text{M}$  purmorphamine had significantly lower expression of *Pax6* than untreated cultures. Expression of *Ng2* (Figure 5.4; B) revealed a similar pattern to that of *Pax6*. There was no significant difference between days ( $F_{1,30} = 0.04$ ,  $p = \text{n.s.}$ ), but there was a significant difference between concentrations ( $F_{4,30} = 3.93$ ,  $p < 0.05$ ), with 1.0 and 2.0  $\mu\text{M}$  purmorphamine yielding significantly lower expression in NEBs at day 16 than untreated cultures on the same day.

*Nkx2.1* expression in NEBs following purmorphamine addition is shown relative to that in E14 mouse MGE (Figure 5.5; A). There was a significant difference in expression between day 8 and day 16 ( $F_{1,30} = 10.41$ ,  $p < 0.01$ ) and concentration ( $F_{4,30} = 5.41$ ,  $p < 0.01$ ). With 0.5  $\mu\text{M}$  purmorphamine there was significantly more *Nkx2.1* expression at day 16 than at day 8. In day 16 cultures there appeared to be an increase in expression with 0.5  $\mu\text{M}$  purmorphamine, although this was not significant; expression then declined, and 2  $\mu\text{M}$  purmorphamine yielded significantly lower expression than untreated cultures. *Dlx2* expression in NEBs following addition of purmorphamine is shown relative to LGE (Figure 5.5; B). There was a significant difference in expression between days ( $F_{1,30} = 47.02$ ,  $p < 0.001$ ), with significantly higher expression at day 16 than at day 8 following addition of 0.5 and 1.0  $\mu\text{M}$

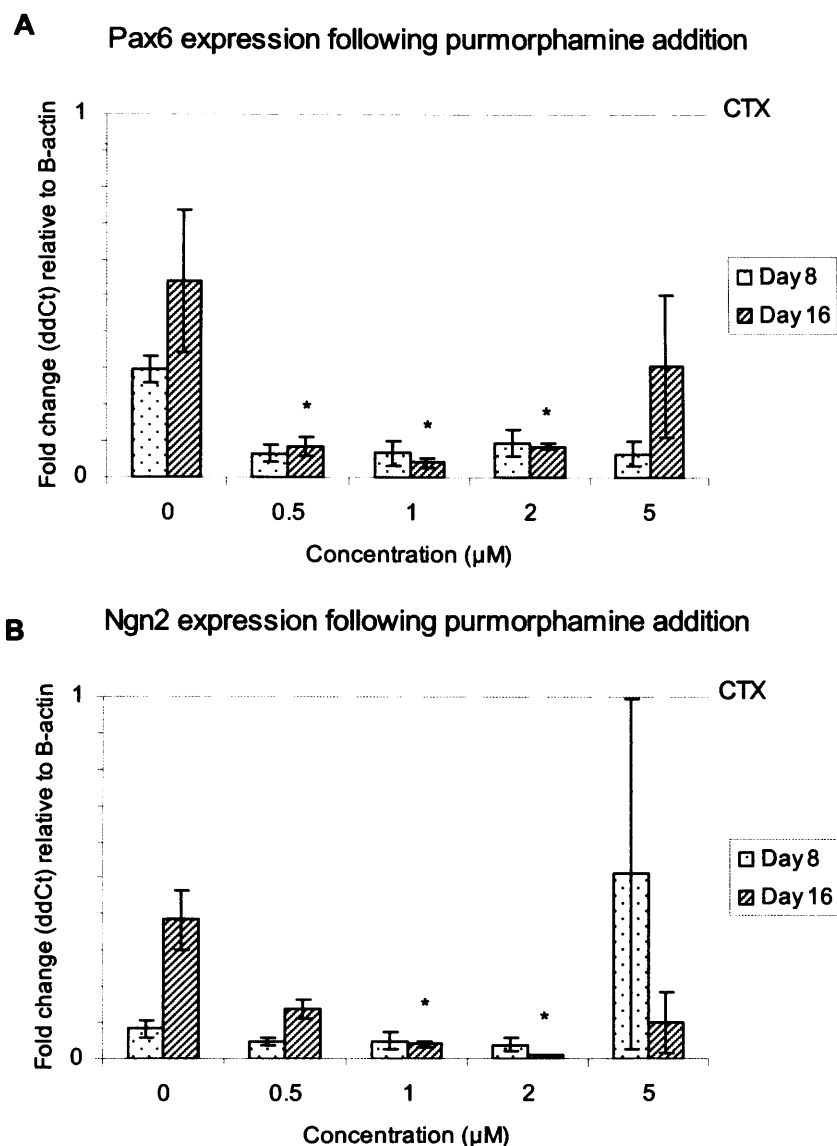
purmorphamine. There was also a significant difference in expression between concentrations ( $F_{4,30} = 5.39$ ,  $p < 0.01$ ), with a significant decrease in expression following addition of  $2\mu\text{M}$  purmorphamine at day 16. Although there was no significant increase in *Dlx2* expression with purmorphamine treatment, at day 16 addition of  $0.5\mu\text{M}$  resulted in a slight increase. Expression of *Mash1* in NEBs following addition of purmorphamine is shown relative to LGE (Figure 5.5; C). There was a significant difference in expression between day 8 and day 16 ( $F_{1,30} = 47.63$ ,  $p < 0.001$ ), with expression at day 16 significantly higher than at day 8 following addition of  $0.5$  and  $2\mu\text{M}$  purmorphamine. There was also a significant difference in expression between concentrations ( $F_{4,30} = 4.28$ ,  $p < 0.05$ ), with  $1\mu\text{M}$  purmorphamine yielding significantly less expression than untreated cultures at day 16. *Gsx2* expression in NEBs following addition of purmorphamine is shown relative LGE (Figure 5.5; D). There was no significant difference between days ( $F_{1,30} = 0.00$ ,  $p = \text{n.s.}$ ), but there was a significant difference between concentrations ( $F_{4,30} = 3.44$ ,  $p < 0.05$ ).

NEBs exhibited more of a response to purmorphamine following 16 days in neural induction culture than 8 days, when analysing dorsal and ventral telencephalic markers. Increasing concentrations of purmorphamine yielded tendencies towards a decrease in expression of dorsal telencephalic genes *Pax6* and *Ngn2* (Figure 5.4). At day 8 expression of the MGE marker *Nkx2.1* appeared unchanged as purmorphamine concentration was increased, whereas at day 16 there appeared to be an increase in expression following addition of  $0.5\mu\text{M}$  purmorphamine, which then decreased with higher concentrations (Figure 5.5; A). Expression of *Dlx2*, *Mash1* and *Gsx2* were not changed at day 8 with purmorphamine addition (Figure 5.5; B, C, and D respectively). At day 16, *Dlx2* expression increased following addition of  $0.5\mu\text{M}$  purmorphamine and decreased with higher concentrations, similar to the response seen in *Nkx2.1* expression. There was not a big response in *Gsx2* expression at day 16, although there was a tendency towards a decrease and expression of *Mash1* showed a decrease with increasing concentrations of purmorphamine.

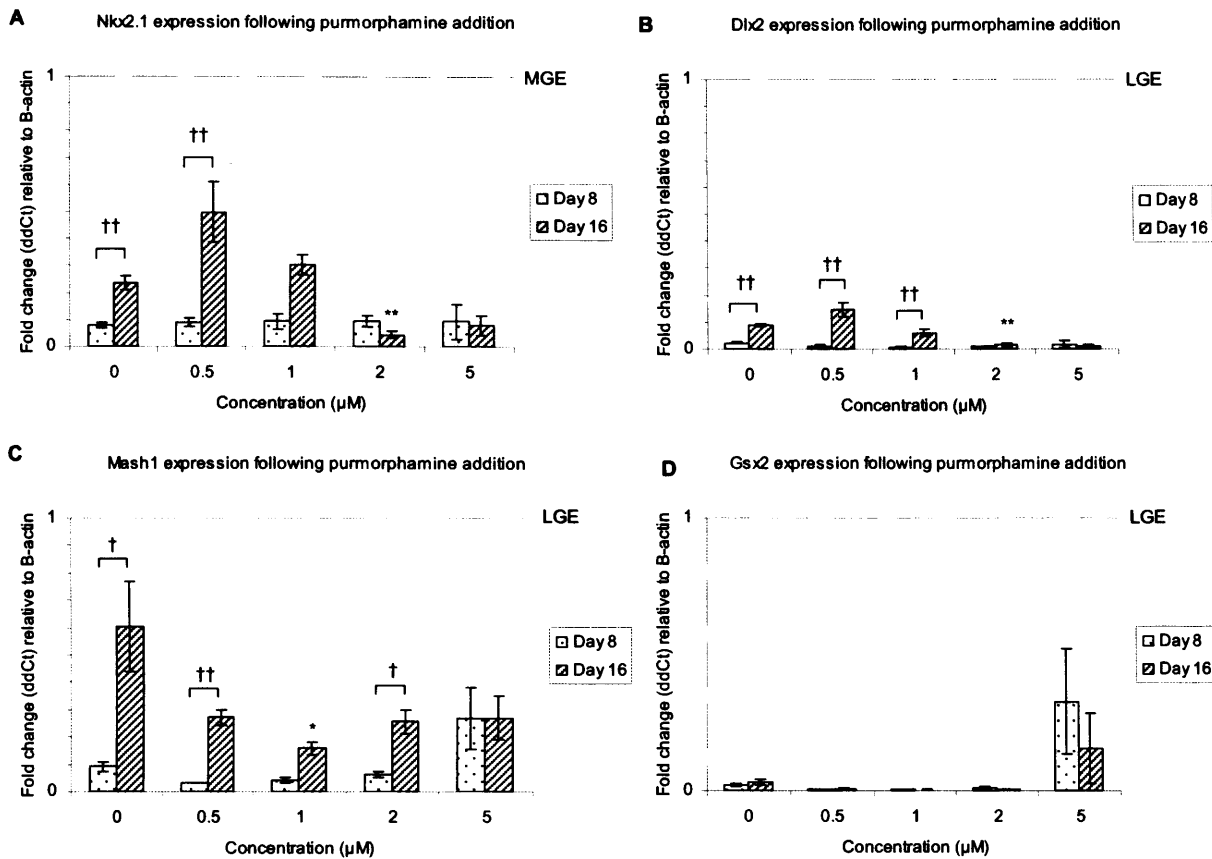
One point of note was that long-term treatment of cultures with high concentrations of purmorphamine ( $2$  and  $5\mu\text{M}$ ) resulted in formation of crystal-like structures within the culture medium. In these cultures, cell numbers were reduced when compared with untreated cultures and those treated with lower purmorphamine concentrations ( $0.5$  and  $1\mu\text{M}$ ).



**Figure 5.3.** Dose-response of expression of components of the SHH pathway. Expression of *SHH* (A), *Patched1* (B) and *Gli3* (C) was analysed using semi-quantitative RT-PCR on cDNAs prepared from NEBs at day 8 and day 16 of neural induction in ADF+ with 20ng/ml FGF2 and different concentrations of the SHH agonist purmorphamine present from day 4. Gene expression is shown relative to  $\beta$ -actin. Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM.



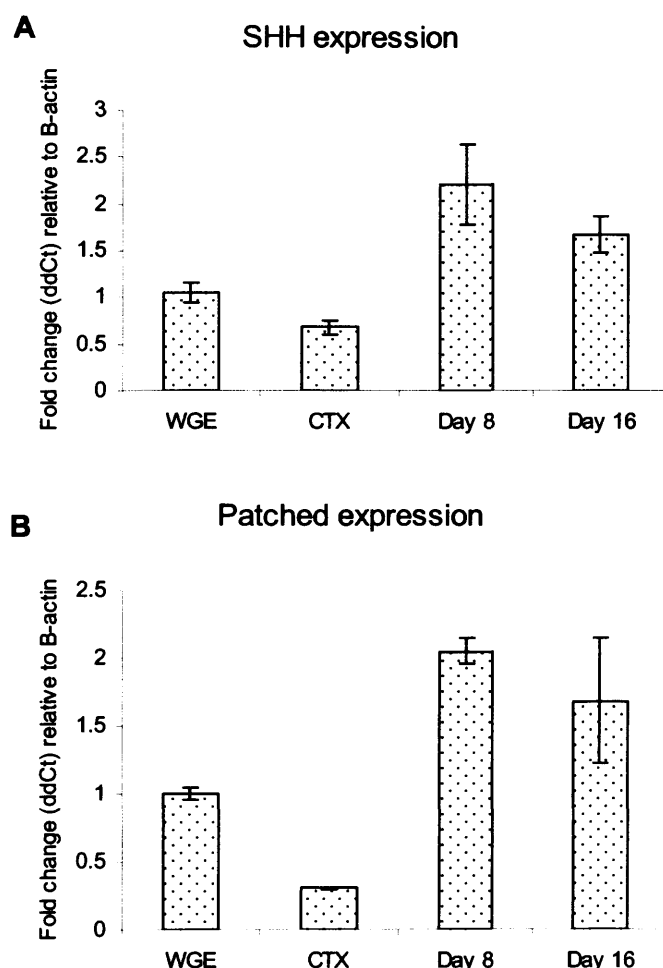
**Figure 5.4.** Expression of dorsal markers *Pax6* (A) and *Ngn2* (B) was analysed using semi-quantitative RT-PCR on cDNAs prepared from NEBs at day 8 and day 16 of neural induction in ADF+ with 20ng/ml FGF2 and different concentrations of the SHH agonist purmorphamine present from day 4. Gene expression is shown relative to  $\beta$ -actin. The broken line crossing the y-axis at 1 represents relative expression in E14 mouse cortex. Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM. (\* $p < 0.05$ ).



**Figure 5.5.** Expression of the ventral markers *Nkx2.1* (A), *Dlx2* (B), *Mash1* (C) and *Gsx2* (D) were analysed using semi-quantitative RT-PCR on cDNAs prepared from NEBs at day 8 and day 16 of neural induction in ADF+ with 20ng/ml FGF2 and different concentrations of the SHH agonist purmorphamine present from day 4. Gene expression is shown relative to  $\beta$ -actin. The broken line crossing the y-axis at 1 represents relative expression in the E14 mouse MGE (A) and LGE (B-D). Each bar on the graphs represents a mean of 3 different neural induction cultures and error bars represent SEM. Brackets indicate significance between the day 8 and day 16 NEBs at a particular concentration. \* = significant difference from WGE control. † = significant difference between NEB groups. (\* $p$ <0.05, \*\* $p$ <0.01, † $p$ <0.05, †† $p$ <0.01).

### Endogenous expression of *SHH*-pathway components in NEBs

Since the response of NEBs to the addition of purmorphamine was not ‘clean’ across the range of dorso-ventral markers analysed, interpretation of results was not always straightforward. Therefore expression of *SHH* and *Patched1* were analysed in NEBs, relative to their expression in the E14 mouse dorsal and ventral telencephalon, to determine if there was endogenous SHH present in the NEB cultures (Figure 5.6). As expected, analysis using semi-quantitative RT-PCR showed that there was less *SHH* and *Patched1* in the cortex than in the WGE. Interestingly the expression of both *SHH* and *Patched1* in NEBs at day 8 and day 16 was greater than seen in the WGE.



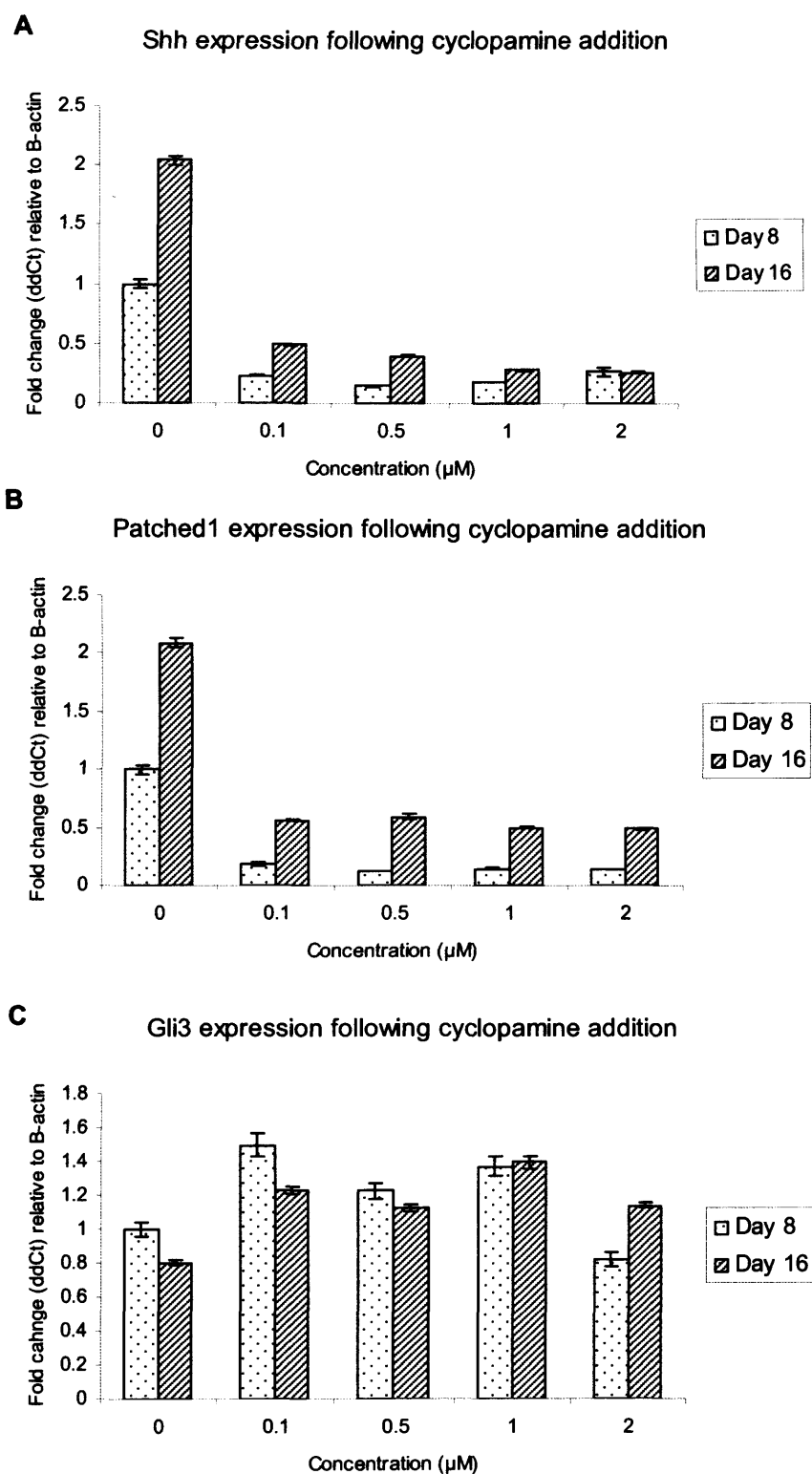
**Figure 5.6.** Expression of *SHH* (A) and *Patched1* (B) was analysed using semi-quantitative RT-PCR on cDNAs prepared from E14 mouse WGE, CTX and ES cell-derived NEBs harvested at day 8 and day 16 of neural induction in ADF+ with 20ng/ml FGF2 present from day 4. Gene expression is shown relative to  $\beta$ -actin. Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM.

*Effect of the SHH antagonist cyclopamine on dorsal-ventral expression*

To determine if NEBs are responsive to the Shh antagonist cyclopamine, ADF+ neural induction medium was supplemented with a range of cyclopamine concentrations and expression of SHH-pathway components was analysed (Figure 5.7). Cyclopamine was added at day 4 and NEBs were harvested for analysis at day 8 or day 16.

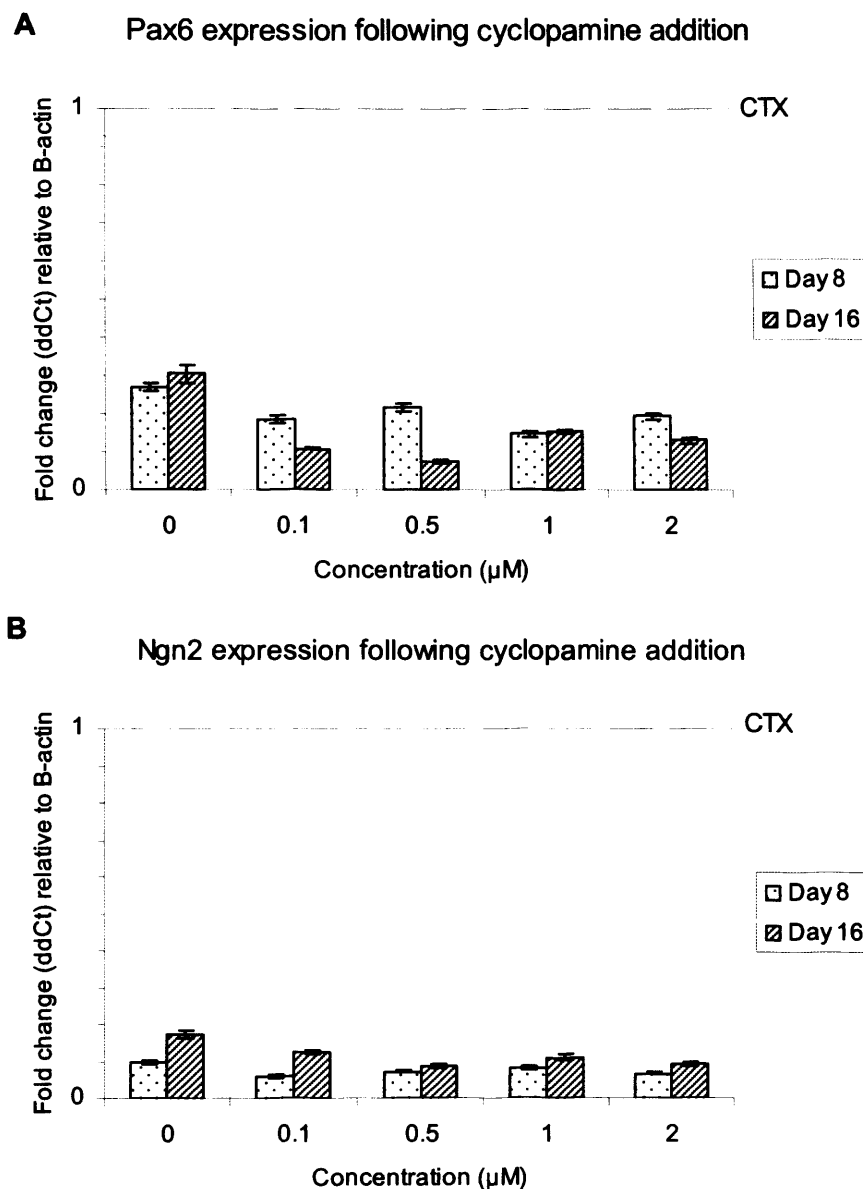
Expression of dorsal and ventral telencephalic markers in NEB cultures were analysed in response to the addition of cyclopamine, and analysis was compared relative to E14 cortex or MGE/LGE (expression relative to E14 WGE is shown in Appendix 6). Expression of the dorsal marker *Pax6* (Figure 5.8; A) did not change significantly with addition of cyclopamine relative to day ( $F_{1,30} = 2.15$ ,  $p = \text{n.s.}$ ) or concentration ( $F_{4,30} = 1.40$ ,  $p = \text{n.s.}$ ). *Ngn2* expression (Figure 5.8; B) was also not altered significantly in the presence of cyclopamine with respect to day ( $F_{1,30} = 4.91$ ,  $p = \text{n.s.}$ ) or concentration ( $F_{4,30} = 1.19$ ,  $p = \text{n.s.}$ ).

Expression of the MGE specific marker *Nkx2.1* was significantly reduced following addition of cyclopamine (Figure 5.9; A). There was a significant effect between days ( $F_{1,30} = 17.29$ ,  $p < 0.01$ ), and concentration ( $F_{4,30} = 20.39$ ,  $p < 0.01$ ), with addition of 0.1, 0.5, 1.0 and 2.0  $\mu\text{M}$  cyclopamine resulting in significantly lower expression of *Nkx2.1* than untreated cultures at both day 8 and day 16. *Dlx2* expression was significantly reduced in the presence of cyclopamine (Figure 5.9; B). There was a significant effect between days ( $F_{1,30} = 10.10$ ,  $p < 0.05$ ) and concentration ( $F_{4,30} = 5.74$ ,  $p < 0.05$ ). Post-hoc analysis confirmed significance at day 16, with all cyclopamine concentrations yielding significantly lower *Dlx2* expression. Expression of *Mash1* following addition of cyclopamine (Figure 5.9; C) was not significantly different with respect to days ( $F_{1,30} = 4.35$ ,  $p = \text{n.s.}$ ) or concentration ( $F_{4,30} = 0.30$ ,  $p = \text{n.s.}$ ). There was also no significant difference in *Gsx2* expression in the presence of cyclopamine (Figure 5.9; D) with respect to days ( $F_{1,30} = 4.26$ ,  $p = \text{n.s.}$ ) or concentration ( $F_{4,30} = 3.32$ ,  $p = \text{n.s.}$ ).

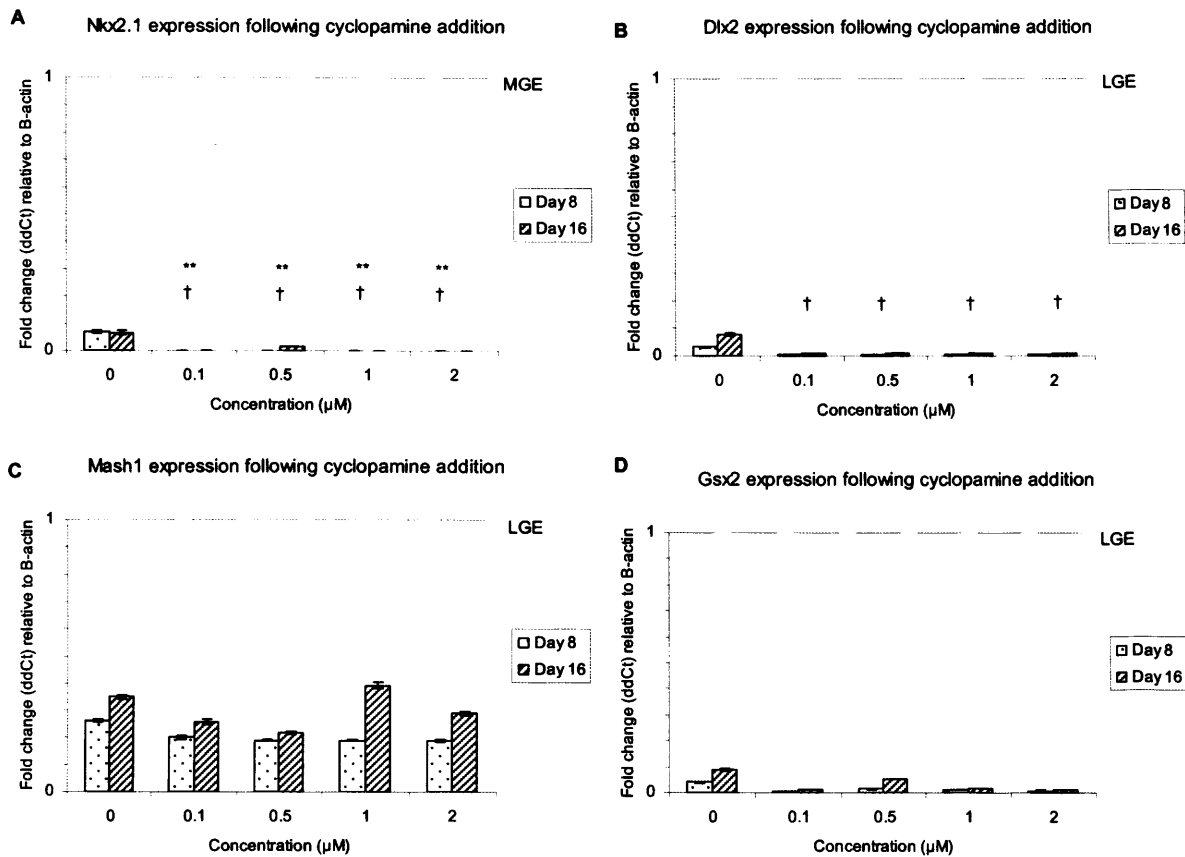


**Figure 5.7.** Dose-response of expression of components of the SHH pathway. Expression of *SHH* (A), *Patched1* (B) and *Gli3* (C) was analysed using semi-quantitative RT-PCR on cDNAs prepared from NEBs at day 8 and day 16 of neural induction in ADF+ with 20ng/ml FGF2 and different concentrations of the SHH antagonist cyclopamine present from day 4. Gene expression is shown relative to  $\beta$ -actin. Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM.





**Figure 5.8.** Expression of *Pax6* (A) and *Ngn2* (B) was analysed using semi-quantitative RT-PCR on cDNAs prepared from NEBs at day 8 and day 16 of neural induction in ADF+ with 20ng/ml FGF2 and different concentrations of the SHH antagonist cyclopamine present from day 4. Gene expression is shown relative to  $\beta$ -actin. The broken line crossing the y-axis at 1 represents relative expression in the E14 mouse CTX. Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM.



**Figure 5.9.** Expression of the ventral markers *Nkx2.1* (A), *Dlx2* (B), *Mash1* (C) and *Gsx2* (D) were analysed using semi-quantitative RT-PCR on cDNAs prepared from NEBs at day 8 and day 16 of neural induction in ADF+ with 20ng/ml FGF2 and different concentrations of the SHH antagonist cyclopamine present from day 4. Gene expression is shown relative to  $\beta$ -actin. The broken line crossing the y-axis at 1 represents relative expression in the E14 mouse MGE (A) and LGE (B-D). Each bar on the graphs represents a mean of 3 different neural induction cultures and error bars represent SEM. Brackets indicate significance between the day 8 and day 16 NEBs at a particular concentration. \* = significant difference to untreated NEBs at day 8. † = significant difference to untreated NEBs at day 16. (\*\* $p < 0.01$ , † $p < 0.05$ ).

*Fluorescent immunocytochemistry of dorsal-ventral markers*

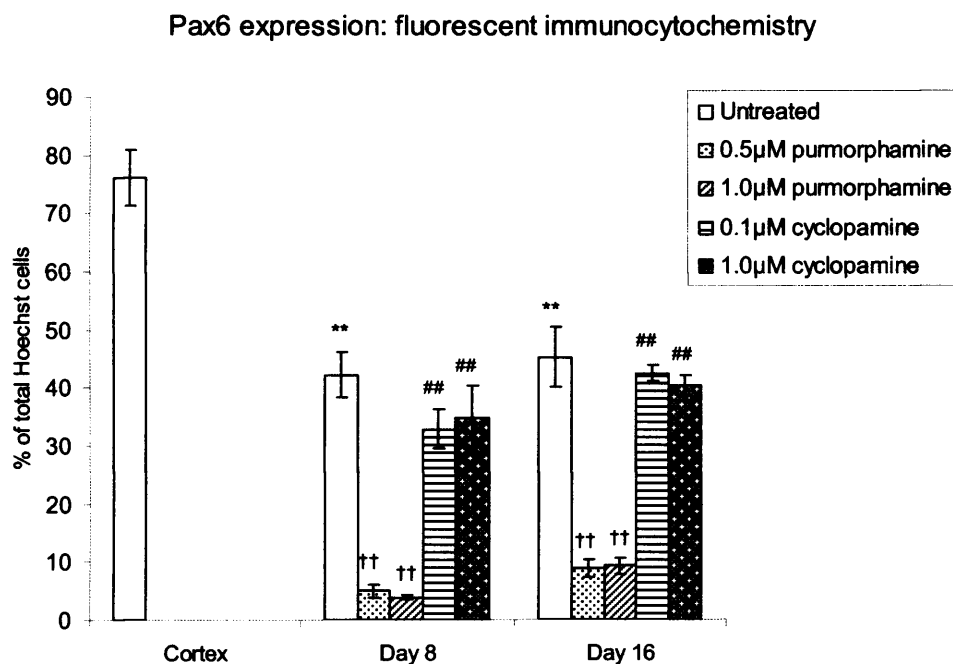
Fluorescent immunocytochemistry, using antibodies against the dorsal telencephalon marker Pax6 (Figure 5.10 and 5.11), the MGE marker Nkx2.1 (Figure 5.12 and 5.13) and the LGE marker Mash1 (Figure 5.14 and 5.15), was performed to further analyse the effects of purmorphamine and cyclopamine with respect to untreated cultures and the appropriate regions of the developing mouse brain.

Fluorescent immunocytochemistry revealed that the proportion of Pax6-expressing cells was significantly lower in NEBs than in E14 cortex ( $F_{2,9} = 16.42$ ,  $p < 0.01$ ) (Figure 5.11), but there was no significant difference between day 8 and day 16 NEBs. Following purmorphamine treatment expression of Pax6 decreased significantly compared with untreated NEBs at day 8 ( $F_{4,15} = 62.95$ ,  $p < 0.001$ ), and at day 16 ( $F_{4,15} = 62.95$ ,  $p < 0.001$ ). There was no significant difference in Pax6 expression between NEBs treated with 0.5  $\mu$ M purmorphamine and 1.0  $\mu$ M purmorphamine. Cyclopamine treatment resulted in significantly more Pax6-positive cells than purmorphamine treated NEBs, but there was no significant difference between 0.1  $\mu$ M and 1.0  $\mu$ M cyclopamine treated NEBs, and no significant difference from untreated cultures.

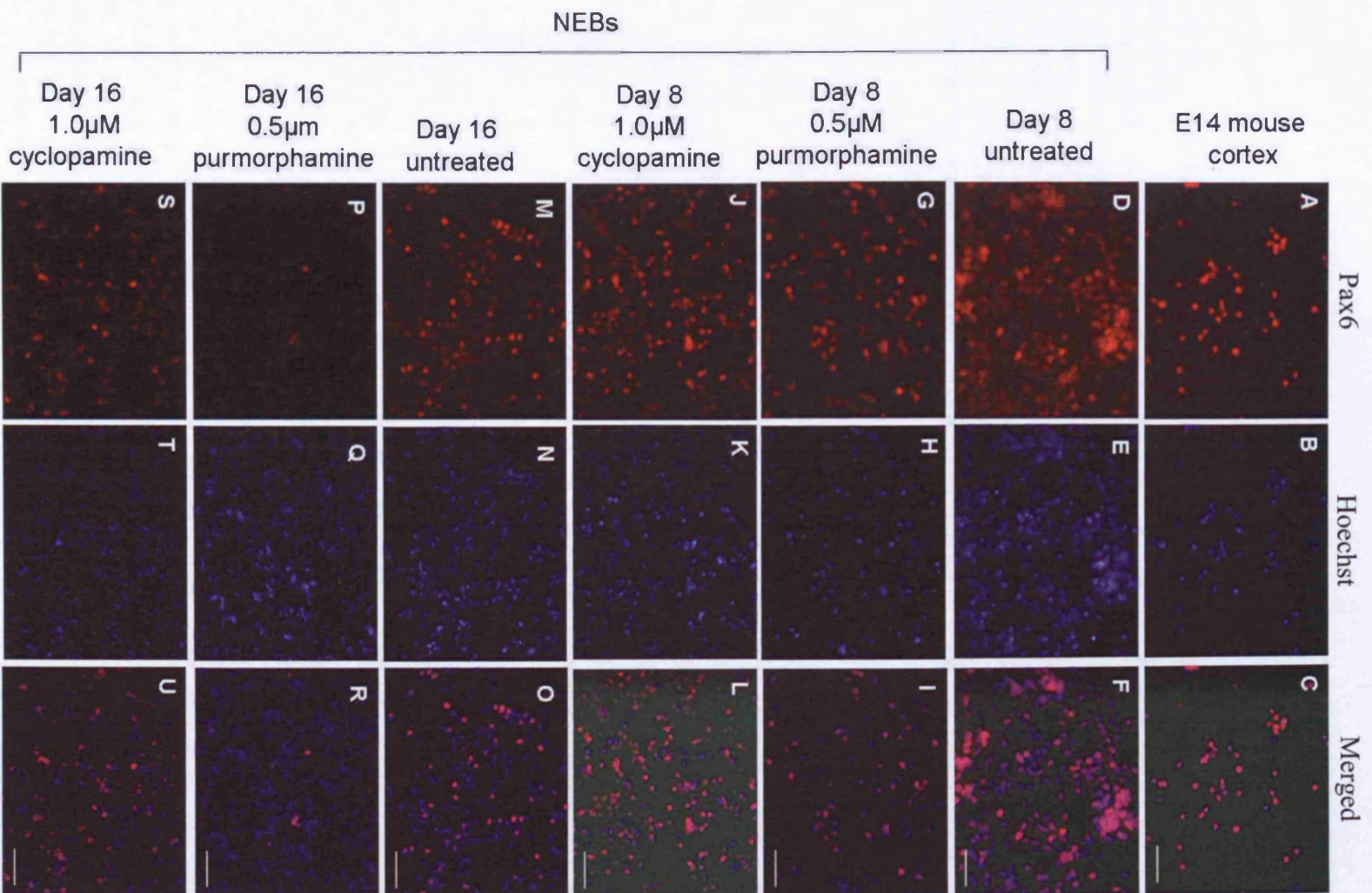
The proportion of Nkx2.1 immuno-positive cells was significantly lower in NEBs than in E14 MGE ( $F_{2,9} = 22.79$ ,  $p < 0.01$ ) (Figure 5.13). There was no significant difference between untreated NEBs and those treated with purmorphamine or cyclopamine at day 8 ( $F_{4,15} = 2.89$ ,  $p = \text{n.s.}$ ) or day 16 ( $F_{4,15} = 2.88$ ,  $p = \text{n.s.}$ ).

There were significantly more Mash1 immuno-positive cells in E14 LGE than in NEBs at day 8 and day 16, and there were significantly more at day 16 than at day 8 ( $F_{2,9} = 97.51$ ,  $p < 0.001$ ) (Figure 5.15). There was no significant difference between untreated NEBs and those treated with purmorphamine or cyclopamine at day 8 ( $F_{4,15} = 2.25$ ,  $p = \text{n.s.}$ ) or day 16 ( $F_{4,15} = 1.32$ ,  $p = \text{n.s.}$ ).

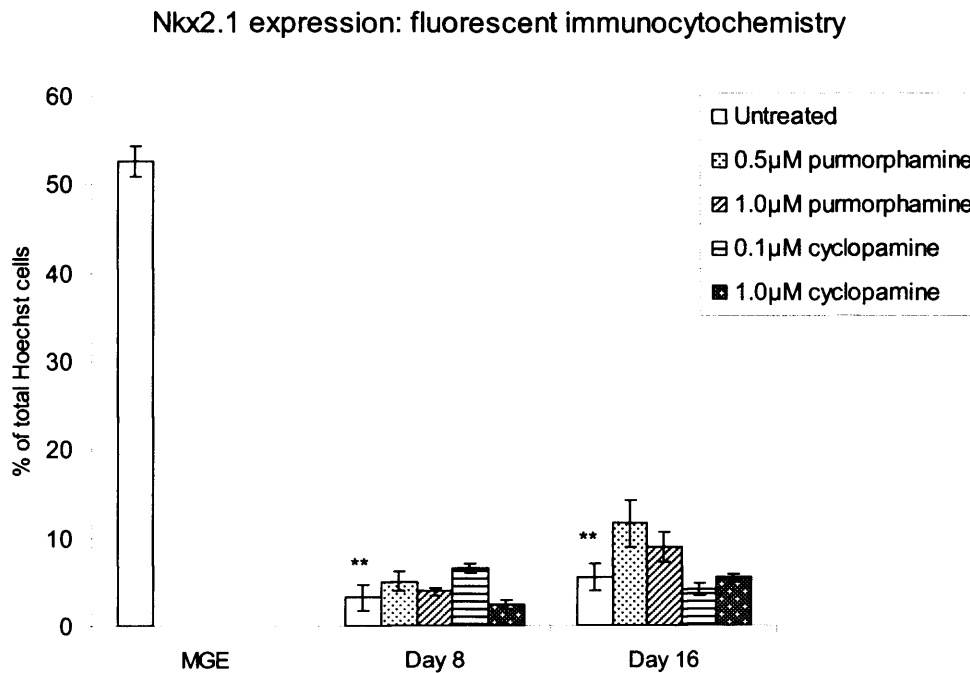
**Figure 5.10 (overleaf).** Pax6 expression using fluorescent immunocytochemistry. E14 mouse cortex (A-C) was dissected and dissociated for plating. NEBs cultured in ADF+ and 20ng/ml FGF2 were taken at day 8 following no additional treatment (D-F), treatment with 0.5 $\mu$ M purmorphamine (G-I) or treatment with 1.0 $\mu$ M cyclopamine (J-L), or at day 16 following no additional treatment (M-O), treatment with 0.5 $\mu$ M purmorphamine (P-R) or treatment with 1.0 $\mu$ M cyclopamine (S-U). Cells were harvested, dissociated and plated onto PLL/laminin substrate. After 2-4 hours, when cells had adhered to the substrate; they were fixed and double-labelled for Pax6 (red) and Hoechst nuclear stain (blue). The third column is a merged image of the first two photomicrographs in each row. Scale bars = 50 $\mu$ m.



**Figure 5.11.** Pax6 expression using fluorescent immunocytochemistry. E14 mouse cortex was dissected and dissociated for plating. NEBs cultured in ADF+ and 20ng/ml FGF2 were taken at day 8 or day 16 following no additional treatment, or treatment with 0.5 $\mu$ M purmorphamine, 1.0 $\mu$ M purmorphamine, 0.1 $\mu$ M cyclopamine or 1.0 $\mu$ M cyclopamine. Cells were harvested, dissociated and plated onto PLL/laminin substrate. After 2-4 hours, when cells had adhered to the substrate; they were fixed and double-labelled for Pax6 and Hoechst nuclear stain. Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM. (\* indicates significant difference from E14 cortex; † indicates a significant difference from untreated cultures at the same day; # indicates a significant difference from other treated cultures at the same day). (\*\*p<0.01, ††p<0.01, ##p<0.01).

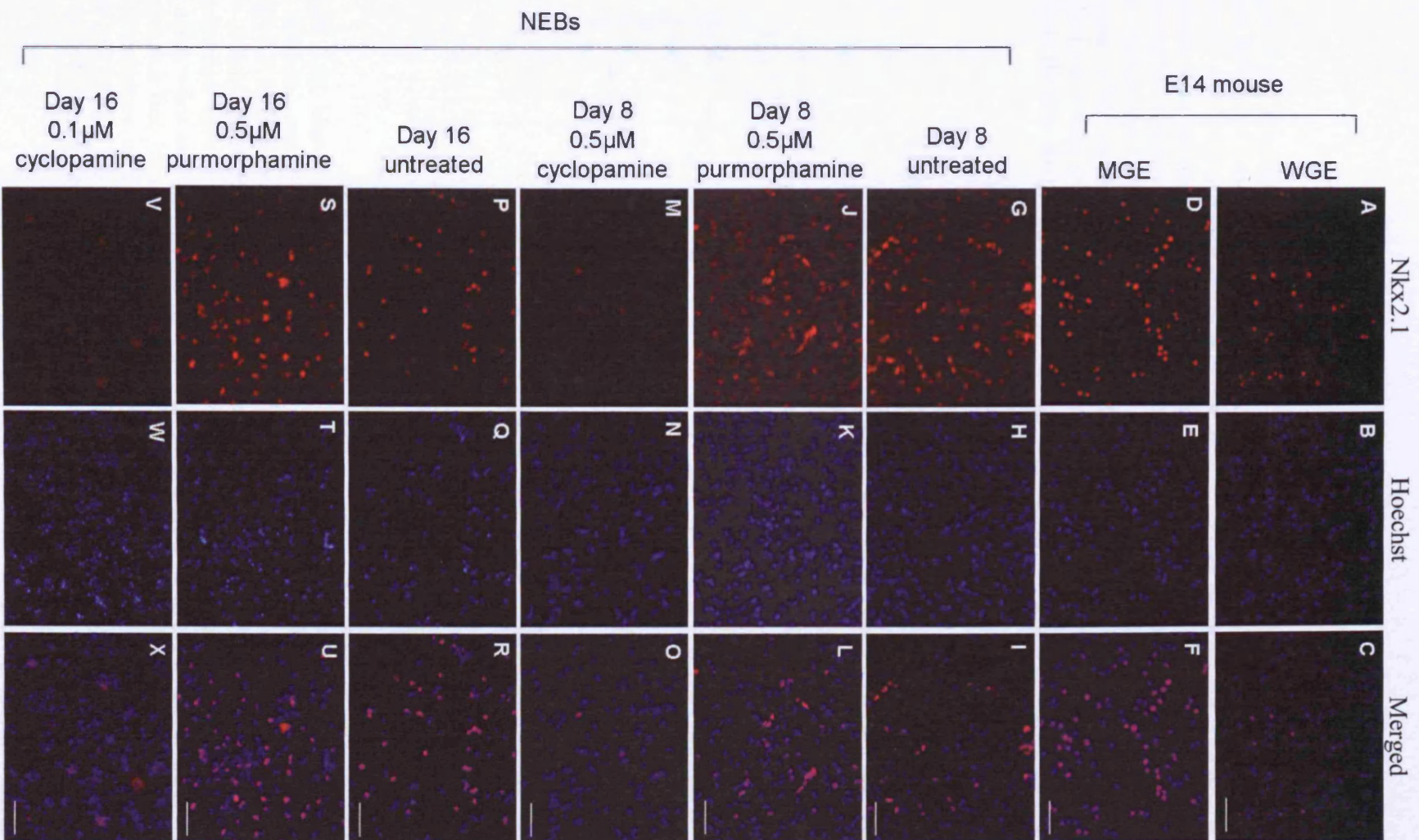


**Figure 5.12 (overleaf).** Nkx2.1 expression using fluorescent immunocytochemistry. E14 mouse WGE (A-C) and MGE (D-F) were dissected and dissociated for plating. NEBs cultured in ADF+ and 20ng/ml FGF2 were taken at day 8 following no additional treatment (G-I), treatment with purmorphamine (J-L) or treatment with cyclopamine (M-O), or at day 16 following no additional treatment (P-R), treatment with purmorphamine (S-U) or treatment with cyclopamine (V-X). Cells were harvested, dissociated and plated onto PLL/laminin substrate. After 2-4 hours cells had adhered to the substrate; they were fixed and double-labelled for Nkx2.1 (red) and Hoechst nuclear stain (blue). The third column is a merged image of the first two photomicrographs in each row. Scale bars = 50µm.

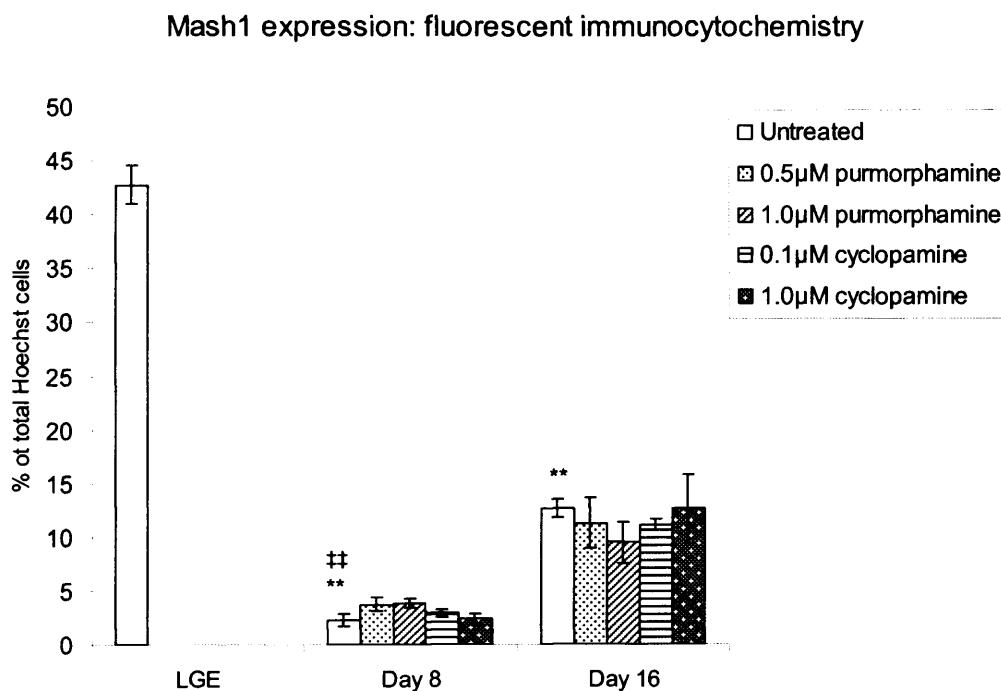


**Figure 5.13.** Nkx2.1 expression using fluorescent immunocytochemistry. E14 mouse MGE was dissected and dissociated for plating. NEBs cultured in ADF+ and 20ng/ml FGF2 were taken at day 8 or day 16 following no additional treatment, or treatment with 0.5µM purmorphamine, 1.0µM purmorphamine, 0.1µM cyclopamine or 1.0µM cyclopamine. Cells were harvested, dissociated and plated onto PLL/laminin substrate. After 2-4 hours, when cells had adhered to the substrate; they were fixed and double-labelled for Nkx2.1 and Hoechst nuclear stain. Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM. (\* indicates significant difference from E14 MGE). (\*\*p<0.01).



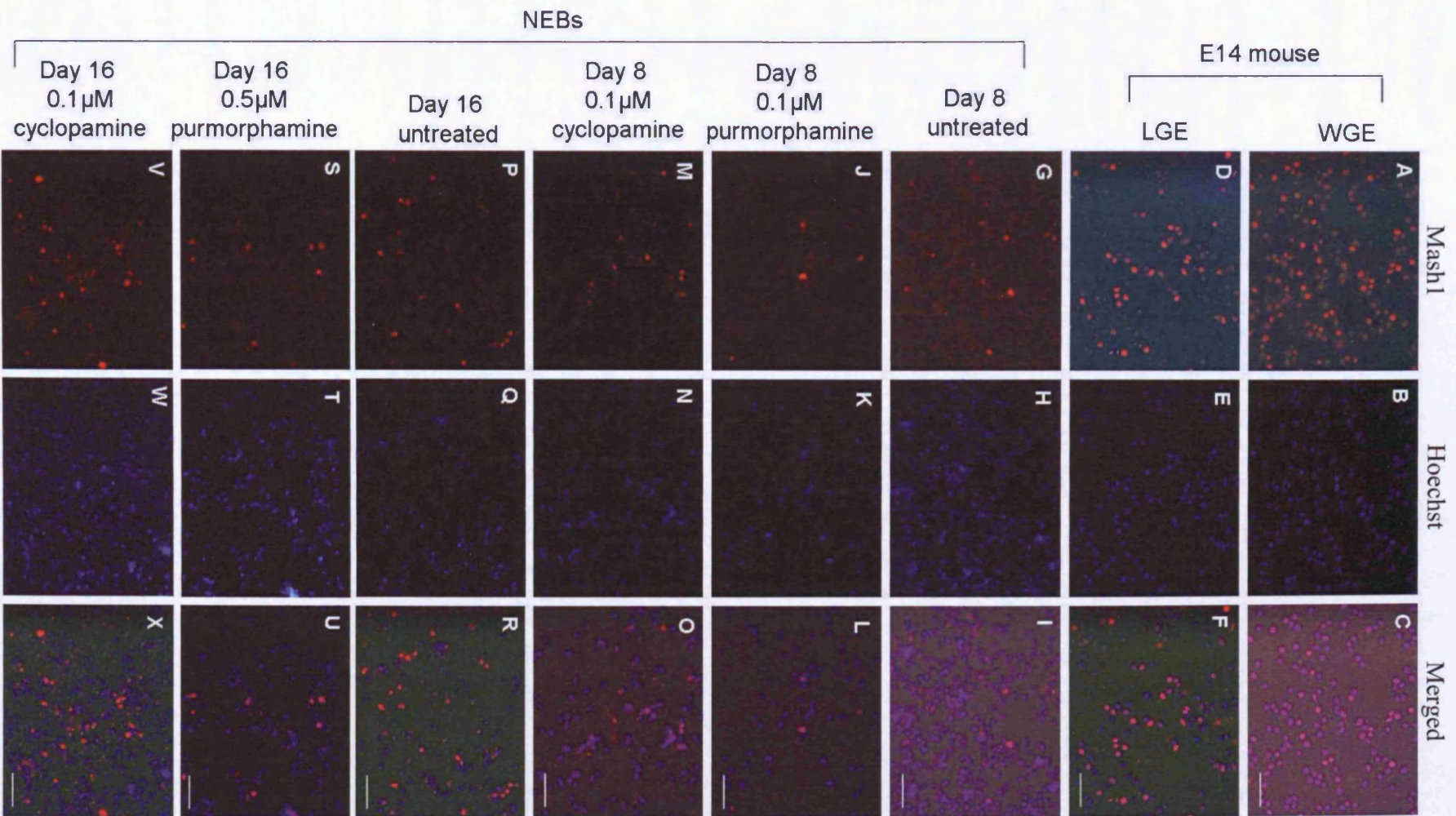


**Figure 5.14 (overleaf).** Mash1 expression using fluorescent immunocytochemistry. E14 mouse WGE (A-C) and LGE (D-F) were dissected and dissociated for plating. NEBs cultured in ADF+ and 20ng/ml FGF2 were taken at day 8 following no additional treatment (G-I), treatment with purmorphamine (J-L) or treatment with cyclopamine (M-O), or at day 16 following no additional treatment (P-R), treatment with purmorphamine (S-U) or treatment with cyclopamine (V-X). Cells were harvested, dissociated and plated onto PLL/laminin substrate. After 2-4 hours cells had adhered to the substrate; they were fixed and double-labelled for Mash1 (red) and Hoechst nuclear stain (blue). The third column is a merged image of the first two photomicrographs in each row. Scale bars = 50µm.



**Figure 5.15.** Mash1 expression using fluorescent immunocytochemistry. E14 mouse LGE was dissected and dissociated for plating. ES cell-derived NEBs cultured in ADF+ and 20ng/ml FGF2 were taken at day 8 or day 16 following no additional treatment, or treatment with 0.5µM purmorphamine, 1.0µM purmorphamine, 0.1µM cyclopamine or 1.0µM cyclopamine. Cells were harvested, dissociated and plated onto PLL/laminin substrate. After 2-4 hours, when cells had adhered to the substrate; they were fixed and double-labelled for Nkx2.1 and Hoechst nuclear stain. Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM. (\* indicates significant difference from E14 LGE; † indicates a significant difference between untreated cultures at day 8 and day 16). (\*\*p<0.01, ††p<0.01).





## 5.5 Discussion

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### *Dorso-ventral expression in NEBs*

Following culture in neural induction medium, NEBs express genes which give an indication of their position along the dorso-ventral axis within the neural tube. In comparison to expression in the developing mouse E14 cortex, expression of the dorsal telencephalic marker genes *Pax6*, *Ngn2* and *Emx2* were all lower in untreated neurally induced NEBs at day 8. At day 16, expression of these 3 markers did not differ significantly from that in the cortex. Expression of the ventral markers *Nkx2.1*, *Dlx2*, *Mash1* and *Gsx2* were also lower in NEBs at day 8 than in cells of their regional position within the neural tube, MGE or LGE. However, at day 16 expression of *Nkx2.1*, *Mash1* and *Gsx2* in NEBs were not significantly different from expression in the MGE (*Nkx2.1*) and LGE (*Mash1* and *Gsx2*). This indicates a more mature precursor at day 16 which more closely resembles *in vivo* telencephalic precursors than the earlier day 8 precursors.

Bouhon et al (2005 and 2006) reported that neurally induced mouse ES cells following 8 days in CDM express the dorsal marker *Pax6* and ventral markers *Gsx2* and *Dlx1* (Bouhon et al., 2005; Bouhon et al., 2006), using semi-quantitative RT-PCR. Fluorescent immunocytochemistry revealed Pax6-positive cells with few *Gsx2* and *Nkx2.1*-expressing cells following 10 days in serum-free suspension culture (Watanabe et al., 2005). A more recent study using CDM showed an increase in expression of *Dlx1* and *Nkx2.1* from day 7 through to day 14 using RT-PCR, and with fluorescent immunocytochemistry revealed a low proportion of Pax6-expressing cells, with few *Gsx2*-expressing cells (Gaspard et al., 2008).

### *Addition of the SHH agonist, purmorphamine to NEBs*

The response of SHH pathway components to different concentrations of purmorphamine at day 8 analysis was more typical of the expected dose response than analysis at day 16. Duration of SHH signalling, as well as concentration, has been shown to influence the expression of transcription factors which subsequently specify different neuronal subtypes. Chronic exposure results in desensitization of cells to SHH which is caused by upregulation of Patched; increasing Patched levels decreases responsiveness of cells to Hedgehog (Taipale et al., 2002; Dessaud et al., 2007).

Dessaud et al (2007) reported that SHH concentration and duration of exposure are proportional to each other, and demonstrated that chick neural cells convert different SHH concentrations into time-limited periods of signal transduction.

Analysis of dorso-ventral markers revealed that purmorphamine treatment at day 4 of the ADF+ neural induction protocol did not appear to elicit a profound response in NEBs by day 8, however there was more of a response when NEBs were analysed after 16 days. Purmorphamine resulted in down-regulation of the dorsal markers *Pax6* and *Ngn2*, which was consistent with a decrease in Pax6-expressing cells following addition of Shh protein at day 4 and analysis at day 10 (Watanabe et al., 2005). At low purmorphamine concentrations there was an increase in expression of the ventral marker *Nkx2.1* at day 16, but at increasing purmorphamine concentrations *Nkx2.1* expression was reduced. Watanabe et al observed that with addition of the Shh protein at low concentrations *Nkx2.1* expression was increased, although not significantly, and at higher concentrations expression reached a plateau (Watanabe et al., 2005). Expression of *Dlx2* was shown to increase at low purmorphamine concentrations, although at increasing purmorphamine concentrations the expression declined, and *Mash1* expression decreased with increasing purmorphamine concentrations, as did expression of *Gsx2*, although base-line expression of *Gsx2* was much lower than *Mash1* when compared relative to their expression in the E14 LGE. It has previously been reported that addition of increasing concentrations of SHH protein did not have a significant effect on *Gsx2*-expressing cells (Watanabe et al., 2005).

Although addition of purmorphamine resulted in a degree of up- or down-regulation of some dorso-ventral genes in the direction expected, this was not consistent across all the markers analysed. As a result, there was no distinctive pattern of expression in purmorphamine-treated NEBs, with respect to dorso-ventral patterning. Purmorphamine directly targets *smoothed* in the Hedgehog signalling pathway and it has been shown that *Patched*, upstream of *smoothed*, is not required for purmorphamine activity (Sinha and Chen, 2006). Moreover, in cells lacking *smoothed*, purmorphamine was not able to induce the Hedgehog signalling pathway (Sinha and Chen, 2006). Analysis of neurally induced ES cells following 8 and 16 days in ADF+ demonstrated relatively high expression of *Patched* when compared with mouse E14 WGE. As a result of high levels of *Patched* there would be subsequently low levels of *smoothed* which is the target of purmorphamine.

During development, one role of SHH is to inhibit the Gli3 repressor that plays a role in dorsal patterning, and induce the Gli3 activator (Gulacsi et al., 2006). With low levels of SHH signalling Gli3 is converted from the activator form to the repressor form, which induces a switch from ventral patterning to dorsal patterning. Since the level of SHH is definitive in this decision in the developing neural tube, this could equally be the case when treating neural induction cultures with various concentrations of SHH, and might be one explanation for the mixed responses in expression of genes of the dorso-ventral axis.

Ventral patterning in the telencephalon does not solely rely on Hedgehog signalling, but a combination of activities of a number of different signalling centres. SHH is involved in maintaining normal expression patterns of FGF8 and BMP4 in the developing telencephalon (Ohkubo et al., 2002; Hayhurst et al., 2008). Changes in SHH signalling early in development results in changes in other signalling centres, so altering SHH expression in NEBs might have a knock-on affect on other signals and modify subsequent gene expression according to these signals, as well as SHH.

Another hypothesis for the limited response of NEBs to purmorphamine treatment is the presence of endogenous SHH activity. Semi-quantitative RT-PCR comparing gene expression in NEBs relative to that in E14 WGE revealed higher expression of SHH in both day 8 and day 16 NEBs than in WGE. ES cells cultured in CDM culture have been shown to express low levels of SHH at day 6, with an increase at day 8 (Bouhon et al., 2005). A different study, also culturing mouse ES cells in CDM, showed induction of SHH expression at day 4 with an increase through to day 14 (Gaspard et al., 2008). Although initiation of SHH expression in neurally induced ES cells, as well as its continued presence in culture (showing an increase over time), has been reported, it has not previously been demonstrated relative to that in the developing mouse striatum.

Since chronic administration of high concentrations of purmorphamine resulted in the formation of crystal-like structures within the culture medium and cell numbers were reduced in these cultures when compared with untreated cultures and those treated with lower purmorphamine concentrations (0.5 and 1  $\mu$ M), data from the high concentrations (2 and 5  $\mu$ M) needs to be analysed with caution. The cells that survived in culture appeared healthy, but further analysis is required to determine what population of cells remained, i.e. forebrain population.

*Addition of the SHH antagonist, cyclopamine to NEBs*

Cyclopamine is an antagonist of the Hedgehog signalling pathway, inhibiting smoothened activity by influencing the balance between the active and inactive forms of smoothened. Treatment of neurally induced ES cells with cyclopamine resulted in a significant decrease in expression of the ventral markers *Nkx2.1*, *Dlx2* and *Gsx2*. This was consistent with previous findings which revealed abolishment of *Dlx1* and *Nkx2.1* (using RT-PCR analysis) and *Gsx2* (using fluorescent immunocytochemistry) (Gaspard et al., 2008). Interestingly here, expression of the ventral marker *Mash1* was not significantly reduced following addition of cyclopamine, however expression of this gene has not been reported in other neural induction studies, either in untreated cultures or those following addition of SHH agonists and antagonists.

Dorsal markers *Pax6* and *Ng2* were not significantly altered following addition of cyclopamine in ADF+ culture from day 4 with analysis at day 8 and day 16, which was not consistent with findings that reported a significant increase in *Pax6*-expressing cells at day 14, with cyclopamine treatment from day 2 to day 10 (Gaspard et al., 2008).

Semi-quantitative RT-PCR analysis resulted in the observation of relatively high levels of deviation between cultures, with samples from 3 different neural induction cultures taken for analysis of gene expression. Generation of neural precursors from ES cell cultures involves harvesting ES cells and then transfer to neural induction medium. The 'state' of ES cells in terms of their proliferation capacity and colony structure, immediately prior to harvesting, might result in the generation of more diverse NEB cultures, with different rates of proliferation and aggregation that might impact on subsequent cell patterning. There is a need to minimise these differences in order to maintain consistency and reproducibility between neural induction cultures.

*Dorso-ventral expression in the developing mouse telencephalon*

The ventral genes *Dlx2*, *Mash1* and *Gsx2* show similar expression in the telencephalic vesicles of the developing mouse, however their roles in influencing other precursors and their destined mature phenotypic fates differ. *Gsx2* expression is required for subsequent expression of various genes necessary for specification and differentiation of the LGE (Corbin et al., 2000). Mice lacking *Gsx2* expression fail to express *Mash1* and *Dlx2* (Szucsik et al., 1997; Corbin et al., 2000; Toresson et al.,

2000), demonstrating a requirement for *Gsx2* expression for the expression of these other ventral genes. Neuronal phenotypes lost in the *Gsx2* mutant are the striatal projection neurons which originate in the LGE (Deacon et al., 1994; Olsson et al., 1998) and the olfactory bulb interneurons (Corbin et al., 2000), whereas the cortical interneurons remain unaffected.

*Dlx1/2*-expressing cells are found in the LGE and MGE (Liu et al., 1997), and these cells are highly migratory prior to differentiation (Anderson et al., 1997a). Fate-mapping studies have reported *Dlx*-expressing cells in the hippocampus, olfactory bulb (Nery et al., 2003), and cortex, where they co-express GABA (Stuhmer et al., 2002b). Mice lacking *Dlx1/2* expression show reduced expression of cortical GABAergic interneurons and also striatal matrix neurons (Anderson et al., 1997a; Anderson et al., 1997b).

*Mash1* expression is also observed throughout the ventral telencephalon (Fode et al., 2000). Mice lacking expression of *Mash1* exhibit reduced expression of cortical interneurons (Casarosa et al., 1999), FoxP1-expressing striatal neuronal precursors, DARPP-32-expressing MSNs and calbindin-expressing striatal matrix neurons (Wang et al., 2009).

Taken together, these developmental loss-of-function studies demonstrate that although expression of these genes is geographically similar, they each play different roles in development and respond to various signals differently.

*Gsx2* expression (at a certain level) might be required in neurally induced ES cells for induction of expression of other ventral genes such as *Mash1* and *Dlx2*. However, since *Gsx2* is a downstream target of SHH signalling (shown by mis-expression of SHH resulting in ectopic *Gsx2* expression) (Corbin et al., 2000) and SHH expression is relatively higher in NEBs than in E14 WGE, perhaps over-expression of SHH has negative effects on *Gsx2* expression.

Heterogeneity of the neural induction cultures might account for the mixed response of gene expression to purmorphamine and cyclopamine, making results difficult to interpret. It is not uncommon in neurally induced ES cells to observe an array of dorso-ventral position-specifying markers that taken together do not produce a predominant pattern of expression, indicating the lack of a predominant precursor-type within the culture population. In neurally induced human ES cells a range of dorso-ventral markers have been shown to up- or down-regulate over time, which individually appear to be interesting, nevertheless failing to demonstrate a

predominant pattern overall (Nasonkin et al., 2009). It is also important to remember that the heterogeneity within cultures is not just restricted to the neural precursor population, but there are almost certainly other cell lineages present as well, which might impact on the patterning and fate of the neural precursors.

### Conclusions

Neurally induced mouse ES cells are responsive to treatment with both purmorphamine and cyclopamine. Interestingly, data in this chapter demonstrates that SHH is endogenously expressed in these neural precursors at relatively high levels, when compared to mouse WGE. Although no distinctive patterns were revealed following treatment of mouse ES cell neural induction cultures with either purmorphamine or cyclopamine, a low purmorphamine concentration supplemented through to day 16 resulted in increased expression of *Dlx2* and the MGE specific marker *Nkx2.1*, indicating an increase in ventral telencephalon precursors. Further analysis of these precursors following differentiation *in vitro* and *in vivo*, which will be assessed in the next two chapters, will demonstrate the fate of these cells as mature phenotypes and determine subsequent effects that purmorphamine might have on cell maturation.

# **Chapter 6**

## *in vitro differentiation:*

### *Looking for DARPP-32*

#### 6.1 Summary

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DARPP-32 is a marker of terminally differentiated MSNs and is currently the most commonly used marker for MSNs of the striatum, which are lost in HD. These neurons originate in the LGE region of the ventral telencephalon. Various studies have shown that *in vitro* culture of LGE cells yield a lower-than-expected proportion of DARPP-32 positive neurons. This proportion can be increased by a number of different methods, including addition of BDNF, RA or co-culture with LGE-derived glia. The rationale in this chapter was to determine conditions for terminal differentiation of MSNs using cells which are already specified (LGE), and then apply these conditions to neuronal differentiation cultures of NEBs. DARPP-32 expression in LGE neuronal differentiation cultures was shown to increase with the addition of BDNF and with co-culture on LGE-derived glia, but not with the addition of RA, and neuronal proportion was not altered. The highest proportion of DARPP-32 positive neurons was yielded following addition of BDNF to the neuronal differentiation cultures. Expression of the striatal marker FoxP1 was not altered in the various conditions but DARPP-32 positive neurons expressed as a percentage of FoxP1 was increased with BDNF. Day 16 NEBs, with or without addition of the SHH agonist purmorphamine, were allowed to differentiate for a further 7 or 14 days in neuronal differentiation medium with or without addition of BDNF, in order to determine if DARPP-32 positive neurons could be generated. Neuronal yield was unaffected in the various treatments, with no effect of purmorphamine. Within the neuronal population, GABAergic yield was not affected and DARPP-32 positive neurons showed a slight increase following BDNF addition at both 7 and 14 days, but this was not significant. FoxP1 expression was also not affected with the various treatments. This suggests that day 16 NEBs are not optimally specified to be responsive to the same cues as LGE-derived precursors.



## 6.2 Introduction

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DARPP-32 is at present the most commonly used marker of the striatal projection MSNs, which are the predominant neurons of the adult striatum. More than 90% of MSNs in rodents are DARPP-32 positive (Ouimet et al., 1998). In the developing brain, striatal projection neurons originate in the LGE region of the ventral telencephalon, making this an obvious choice for use as an *in vitro* cell culture model for optimising DARPP-32 output in terminal neuronal differentiation cultures.

*In vitro* expansion of E14 mouse LGE in the presence of FCS and EGF, followed by differentiation in the absence of these factors resulted in generation of  $\beta$ -III-tubulin positive neurons which, although they were largely GABAergic, were not seen to express DARPP-32 (Skogh et al., 2001). Differentiation of LGE-derived neurons in homotopic co-culture, on monolayers of LGE-derived glia, resulted in up to 15% DARPP-32 positive neurons, in comparison to co-culture on MGE-derived glia, which yielded 7% DARPP-32 (Skogh and Campbell, 2003).

The LGE has been reported to be a source of retinoid signalling and retinoids from LGE glia have been implicated in striatal neuronal differentiation (Toresson et al., 1999). RA has been implicated in forebrain development (Schneider et al., 2001; Haskell and LaMantia, 2005; Marklund et al., 2004; Ribes et al., 2006) and in regulation of DARPP-32 expression in the developing brain (Waclaw et al., 2004). *In vitro* E14 mouse LGE cultures have shown an increase in DARPP-32 expression in response to addition of exogenous RA (Toresson et al., 1999), which is similar to the increase in DARPP-32 expression seen following homotopic co-culture of LGE-derived neurons with LGE-derived glia (Skogh and Campbell, 2003).

Addition of brain-derived neurotrophic factor (BDNF) has been reported to promote the survival of DARPP-32 positive neurons, as well as increase neurite outgrowth and the number of branching points on the neurites (Nakao et al., 1995). Differentiating neuronal cells treated with BDNF have been shown to be multipolar rather than bipolar, and they display enhanced neuronal fates, as demonstrated by altered cell morphology (Lachyankar et al., 1997). BDNF has also been shown to increase expression of DARPP-32 in E15 mouse striatal neuronal differentiation cultures and moreover, has been shown to regulate DARPP-32 transcription in MSNs (Ivkovic et al., 1997).

To date there is very little literature demonstrating the *in vitro* terminal differentiation of ES cell-derived neural precursors with the subsequent generation of DARPP-32 positive neurons. Human ES cell-derived neural precursors cultured for 50 days with addition of BDNF, SHH and DKK-1, and terminally differentiated for a further 10 days in the absence of SHH and DKK-1, generated 22% MAP2-positive neurons, of which 36% expressed GABA and 53% expressed DARPP-32 (Aubry et al., 2008). Interpretation of these data requires a degree of caution since DARPP-32 positive neurons are also GABAergic neurons and co-expression would be expected in the DARPP-32 positive neurons. This therefore reinforces the requirement for more reliable markers of MSNs. Mouse ES cell-derived neural precursors have been shown to generate  $\beta$ -III-tubulin positive neurons (Bouhon et al., 2005; Bouhon et al., 2006; Dihne et al., 2006) including a proportion of GABAergic neurons (Bouhon et al., 2006).

DARPP-32 is currently the most commonly used marker of striatal MSNs and it is restricted to terminally differentiated neurons, therefore identification of other markers including those expressed in MSN precursors are being sought. FoxP1 is one such marker which was shown to upregulate in the developing mouse striatum from E12 through to E16 (Kelly et al., in preparation). Expression of FoxP1 is maintained to adulthood, and in the striatum, is restricted to the striatal projection neurons and not the interneurons (Ferland et al., 2003; Tamura et al., 2004).

In this chapter, the rationale was to use foetal striatal cells as a model system to explore cues to induce terminal differentiation of MSNs from already-specified precursors, and then apply these conditions to neuronal differentiation cultures of mouse ES cell-derived neural precursors, with the aim to generate MSNs. E14 LGE-derived neurons were analysed using immuno-fluorescence to assess the effects of various treatments in neuronal differentiation culture on the neuronal phenotype, in particular expression of DARPP-32 and also the earlier-onset striatal marker FoxP1. Mouse ES cell-derived neural precursors, following 16 days in ADF+ neural induction culture, were subjected to the same treatments as the LGE cultures in order to determine the neuronal phenotypes yielded, again, looking for DARPP-32 positive neurons. Neural induction cultures were untreated or purmorphamine treated, to determine if further specification prior to neuronal differentiation affected subsequent expression of DARPP-32, GABA and FoxP1.

## 6.3 Experimental Procedures

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### *Dissection and dissociation of mouse E14 LGE*

LGE was dissected from E14 mouse forebrain and dissociated to generate a single-cell suspension, as described in Chapter 2. Briefly, tissue was incubated in trypsin and DNase for 20 minutes at 37°C followed by a further 5 minute incubation in trypsin inhibitor. The tissue was then washed in DMEM/F-12, centrifuged at 1000rpm for 3 minutes, the remaining pellet was resuspended in 200µl DMEM/F-12 and triturated 10-15 times to generate a single cell suspension which was counted using a haemocytometer.

### *Generation of LGE-derived glia*

E14 mouse LGE cells were seeded in glial expansion medium (DMEM/F-12, 1% PS, 10% FCS, 2mM glutamine and 20ng/ml EGF) in tissue-culture treated flasks. Medium was changed every 2-3 days and cultures were passaged using trypsin-EDTA when 80-90% confluency was reached, and re-seeded for further expansion. Glial cultures at passage 4 were used for co-culture studies.

24 hours before plating LGE cells or day 16 NEBs for neuronal differentiation, LGE glia were resuspended in glial differentiation medium (DMEM/F-12, 1% PS, 10% FCS and 2mM glutamine) and plated onto PLL-coated glass cover-slips.

### *Generation and maintenance of ES cell-derived NEBs*

The mouse ES cell line *Foxg1-lacZ* was used in this study. NEBs were derived from the *Foxg1Z* mouse ES cell line as described in Chapter 2. Briefly, ES cells were transferred from ES cell culture medium to ADF+ medium at a density of 50,000 cells per ml, medium was replaced every 2 days and 20ng/ml FGF2 was added from day 4. Neural induction cultures were untreated (ADF+ plus 20ng/ml FGF2 at day 4) or treated with the SHH agonist purmorphamine (ADF+ plus 20ng/ml FGF2 and 0.5µM purmorphamine at day 4) prior to differentiation.

*Plating cells and neuronal differentiation*

On the day of plating NEBs were harvested and dissociated as described in Chapter 2. Cells were plated onto either PLL/laminin treated cover-slips or LGE-derived glia, at a density of 50,000 cells. 2-4 hours after plating, on adhering to the substrate, cells were flooded with 500 $\mu$ l differentiation medium (DMEM/F-12, 1% PS, 2% B-27 and 1% FCS). Cells plated onto LGE-derived glia were flooded immediately after plating to prevent cultures from drying out. Cells were allowed to differentiate for 7 or 14 days, and medium was replaced every 2-3 days. To determine the effects of addition of factors neuronal differentiation medium was supplemented with factors including BDNF at 50ng/ml and RA at 100nm.

*Immunocytochemistry*

Following 7 or 14 days in neuronal differentiation medium cells were fixed. Fluorescent immunocytochemistry was performed according to the protocol detailed in Chapter 2. Primary antibodies used were anti- $\beta$ -III-tubulin (1:1000), anti-GFAP (1:1000), anti-DARPP-32 (1:20,000), anti-FoxP1 (1:500) and anti-GABA (1:500).

*Microscopy and Statistical analysis*

Cells were visualised under UV fluorescence using a Leitz microscope. Hoechst positive nuclei were detected and cells immuno-labelled with the marker of interest were counted, as described in Chapter 2. Images were processed using Optronics MagnaFire software and Adobe Photoshop.

Two-sample t-tests or one- and two-factor ANOVA with Tukey Kramer *post hoc* comparisons when appropriate, were performed using Minitab 15 statistical software.

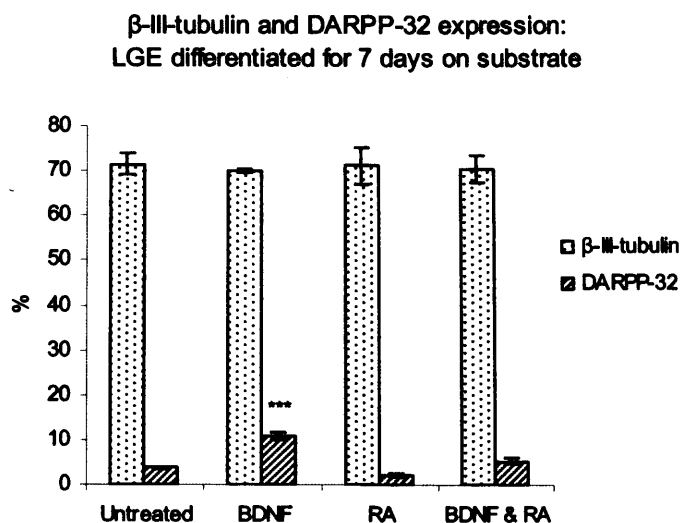
## 6.4 Results

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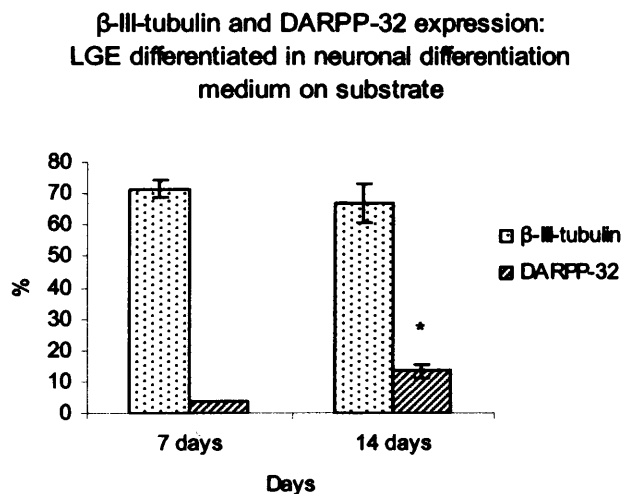
### *DARPP-32 expression in LGE-derived neurons*

Since the LGE is the origin of DARPP-32 positive MSNs of the adult striatum, this region of the developing forebrain was used as a 'standard' to assess the effects *in vitro*, of various treatments in neuronal differentiation cultures on the expression of DARPP-32. LGE was dissected from E14 mouse forebrain, plated onto PLL/laminin substrate and allowed to differentiate for 7 days. Cultures were fixed and triple-labelled for  $\beta$ -III-tubulin, DARPP-32 and the nuclei marker, Hoechst. For analysis of effects of BDNF and RA on neuronal ( $\beta$ -III-tubulin) and DARPP-32 yield, neuronal differentiation medium was supplemented with 50ng/ml BDNF and/or 100nm RA, or left untreated. Overall total of Hoechst positive nuclei was not different between cultures which suggests there were no differences in proliferation or cell death between cultures. There was no significant difference in the proportion of  $\beta$ -III-tubulin positive neurons with the different treatments (treatment,  $F_{3,12} = 0.06$ ,  $p = \text{n.s}$ ) (Figure 6.1). The proportion of  $\beta$ -III-tubulin positive neurons that co-expressed DARPP-32 was significantly higher in cultures treated with BDNF ( $10.93 \pm 0.88\%$ ) than untreated cultures ( $3.74 \pm 0.11\%$ ), and those cultures treated with RA ( $2.29 \pm 0.47\%$ ) or treated with both BDNF and RA ( $5.27 \pm 0.68\%$ ) (treatment,  $F_{3,12} = 38.90$ ,  $p < 0.001$ ).

Since expression levels of DARPP-32 in MSNs *in vivo* is initially low compared to adult levels, we increased the neuronal differentiation culture time *in vitro* to determine if this would increase DARPP-32 expression. When LGE cultures were allowed to differentiate for 14 days in untreated neuronal differentiation medium, generation of  $\beta$ -III-tubulin positive neurons was not significantly changed from that at day 7 (day,  $t_4 = 0.74$ ,  $p = \text{n.s}$ ) (Figure 6.2). However the yield of DARPP-32 positive neurons as a percentage of  $\beta$ -III-tubulin positive neurons was significantly increased following 14 days differentiation ( $13.38 \pm 2.37\%$ ) compared with 7 days differentiation in untreated neuronal differentiation medium ( $3.74 \pm 0.11\%$ ) (day,  $t_4 = 4.70$ ,  $p < 0.05$ ).



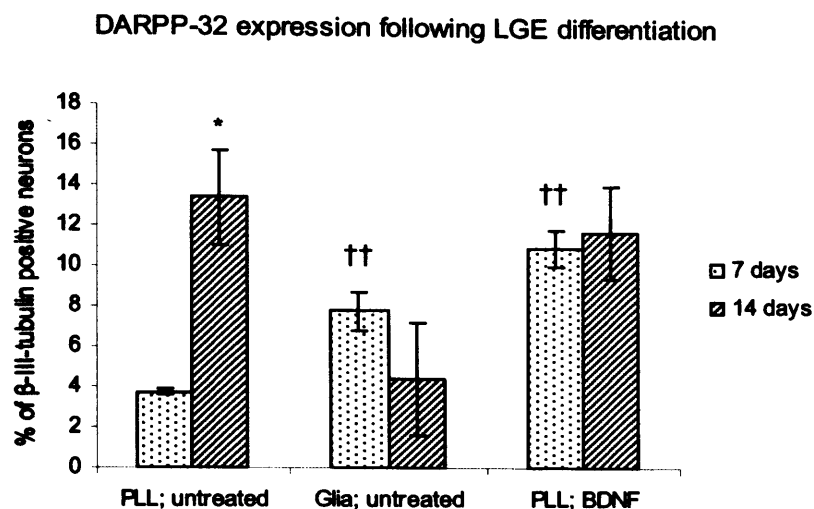
**Figure 6.1.  $\beta$ -III-tubulin and DARPP-32 expression in LGE differentiation cultures.** Mouse E14 LGE was dissected, dissociated and plated onto PLL/laminin substrate in neuronal differentiation medium and allowed to differentiate for 7 days. Differentiating cultures were: i) untreated (neuronal differentiation medium alone), ii) treated with 50ng/ml BDNF, iii) treated with 100nm RA, or iv) treated with 50ng/ml BDNF and 100nm RA. Following neuronal differentiation for 7 days, cultures were fixed and triple-labelled for  $\beta$ -III-tubulin, DARPP-32 and Hoechst nuclear marker.  $\beta$ -III-tubulin positive neurons are represented as a percentage of total Hoechst cells and DARPP-32 positive neurons are represented as a percentage of  $\beta$ -III-tubulin positive neurons. Each bar on the graph represents a mean of 3 different LGE dissections and error bars represent SEM. (\*\*\*) $p < 0.001$ .



**Figure 6.2.  $\beta$ -III-tubulin and DARPP-32 expression in LGE differentiation cultures.** Mouse E14 LGE was dissected, dissociated and plated onto PLL/laminin substrate in neuronal differentiation medium and allowed to differentiate for 7 or 14 days. Cultures were fixed and triple-labelled for  $\beta$ -III-tubulin, DARPP-32 and Hoechst nuclear marker.  $\beta$ -III-tubulin positive neurons are represented as a percentage of total Hoechst cells and DARPP-32 positive neurons are represented as a percentage of  $\beta$ -III-tubulin positive neurons. Each bar on the graph represents a mean of 3 different LGE dissections and error bars represent SEM. (\* $p < 0.05$ ).

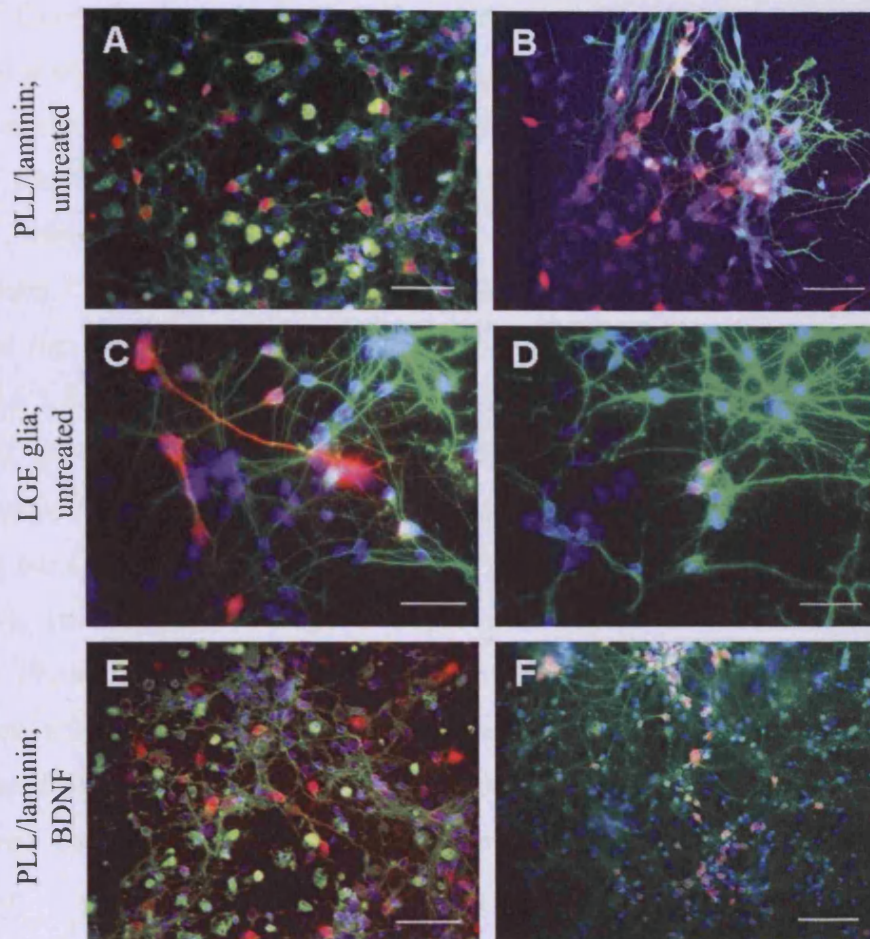
DARPP-32 yield from LGE neuronal cultures using different differentiation conditions, including plating substrate and BDNF addition to medium, was analysed (Figure 6.3). Following 7 days in neuronal differentiation medium there was a significant difference in DARPP-32 expression between differentiation treatments (treatment,  $F_{2,9} = 26.13$ ,  $p < 0.01$ ); DARPP-32 expression was not significantly higher in LGE cultures plated on PLL/laminin substrate and treated with 50ng/ml BDNF than those plated on LGE-derived glia and left untreated; but both these conditions resulted in significantly higher DARPP-32 expression than cultures plated on PLL/laminin substrate and left untreated. Following 14 days differentiation there was no significant difference in DARPP-32 expression between the different differentiation treatments (treatment,  $F_{2,9} = 4.05$ ,  $p = \text{n.s.}$ ). When LGE was plated on PLL/laminin substrate in neuronal differentiation medium with addition of 50ng/ml BDNF, there was no significant difference in DARPP-32 yield between day 7 ( $10.93 \pm 0.88\%$ ) and day 14 ( $11.68 \pm 2.30\%$ ) (day,  $t_4 = 0.31$ ,  $p = \text{n.s.}$ ), and similarly, when LGE was plated on LGE-derived glia in untreated neuronal differentiation medium there was no significant difference in DARPP-32 yield between day 7 ( $7.77 \pm 0.96\%$ ) and day 14 ( $4.42 \pm 2.82\%$ ) (day,  $t_4 = 1.82$ ,  $p = \text{n.s.}$ ).

Photomicrographs of LGE cultures that were subjected to the various differentiation treatments and subsequent fluorescent immunocytochemistry are shown in Figure 6.4, demonstrating  $\beta$ -III-tubulin and DARPP-32 expression.



**Figure 6.3. DARPP-32 expression in LGE differentiation cultures.** Mouse E14 LGE was dissected, dissociated and plated onto PLL/laminin substrate or LGE glia, and allowed to differentiate for 7 or 14 days. Neuronal differentiation medium was either left untreated or 50ng/ml BDNF was added. Cultures were fixed and triple-labelled for  $\beta$ -III-tubulin, DARPP-32 and Hoechst nuclear stain. DARPP-32 positive neurons are represented as a percentage of  $\beta$ -III-tubulin positive neurons. Each bar on the graph represents a mean of 3 different LGE dissections and error bars represent SEM. (\* = significance between days 7 and 14 with each treatment; † = significance between treatments at either day 7 or day 14). (\* $p$ <0.05, †† $p$ <0.01).



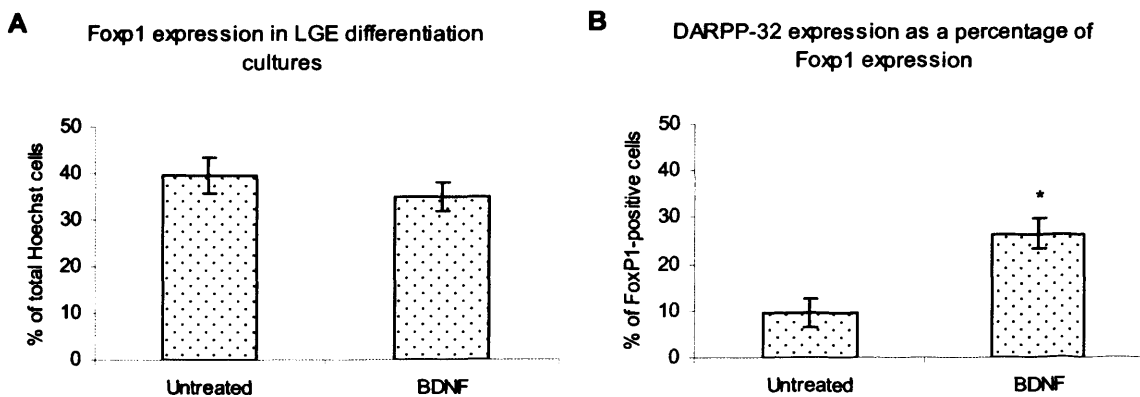


**Figure 6.4. DARPP-32 expression in LGE differentiation cultures.** Mouse E14 LGE was dissected, dissociated and plated onto substrate or LGE glia and allowed to differentiate for 7 or 14 days in neuronal differentiation medium. LGE was plated on PLL/laminin substrate and differentiated for 7 days (A) or 14 days (B); or plated on LGE-derived glia and differentiated for 7 days (C) or 14 days (D) in untreated neuronal differentiation medium. BDNF (50ng/ml) was added to neuronal differentiation medium and cultures were differentiated for 7 days on PLL/laminin substrate (E and F). Cultures were fixed and triple-labelled for  $\beta$ -III-tubulin (green), DARPP-32 (red) and Hoechst nuclear stain (blue). Scale bars = 50 $\mu$ m (A-E) and 100 $\mu$ m (F).

### Co-expression of FoxP1 and DARPP-32 in LGE-derived neurons

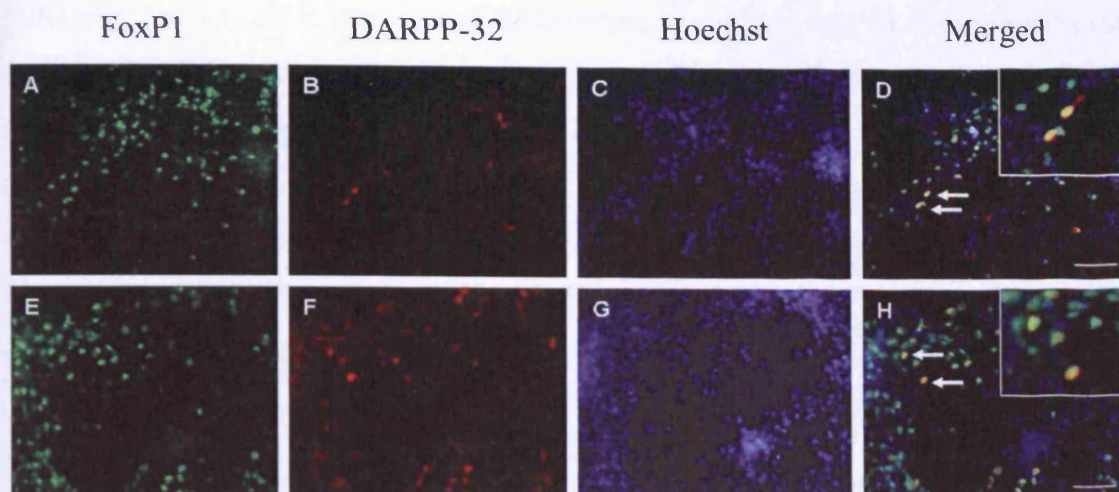
Currently the most commonly used marker for the striatal MSNs is DARPP-32, and it only labels mature neurons. Ongoing work in our group focuses on finding novel markers for these cells including markers of MSN precursors. One such marker, which was identified by a study of striatal genes during embryonic development, is FoxP1, which was shown to label striatal precursor cells (Kelly et al., in preparation). Following 7 days in neuronal differentiation medium on PLL/laminin substrate, there was no significant difference in FoxP1 expression between untreated LGE cultures ( $39.59 \pm 3.80\%$ ) and those treated with 50ng/ml BDNF ( $35.10 \pm 3.15\%$ ) (treatment,  $t_5 = 0.30$ ,  $p = \text{n.s}$ ) (Figure 6.5; A). DARPP-32 positive neurons, expressed as a percentage of FoxP1 positive cells, were significantly higher when cultures were treated with 50ng/ml BDNF ( $26.36 \pm 3.23\%$ ) than when cultures were untreated ( $9.51 \pm 3.16\%$ ) (treatment,  $t_7 = 3.73$ ,  $p < 0.05$ ) (Figure 6.5; B).

Photomicrographs of LGE differentiation cultures (untreated and treated with 50ng/ml BDNF for 7 days) that were labelled for FoxP1 and DARPP-32 using immuno-fluorescence are shown in Figure 6.6. All DARPP-32 positive neurons observed co-expressed FoxP1, but not all FoxP1 positive cells were DARPP-32 positive.



**Figure 6.5. FoxP1 and DARPP-32 expression in LGE differentiation cultures.** Mouse E14 LGE was dissected, dissociated and plated onto PLL/laminin substrate and allowed to differentiate for 7 days in neuronal differentiation medium either untreated or with addition of 50ng/ml BDNF. Cultures were fixed and triple-labelled for FoxP1, DARPP-32 and Hoechst nuclear stain. FoxP1 positive cells are represented as a percentage of Hoechst positive nuclei (A) and DARPP-32 positive neurons are represented as a percentage of FoxP1 positive cells (B). Each bar on the graph represents a mean of 3 different LGE dissections and error bars represent SEM. (\* $p < 0.05$ ).

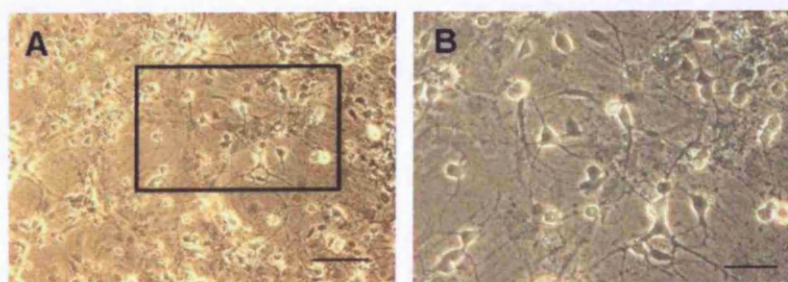




**Figure 6.6. FoxP1 and DARPP-32 expression in LGE differentiation cultures.** Mouse E14 LGE was dissected, dissociated, plated onto substrate and allowed to differentiate for 7 days in neuronal differentiation medium in the absence of growth factors (A-D) or in the presence of 50ng/ml BDNF (E-H). Cultures were fixed and triple-labelled for FoxP1 (green), DARPP-32 (red) and Hoechst nuclear stain (blue). Arrows (D and H) indicate cells co-expressing FoxP1 and DARPP-32, and insets (D and H) show higher magnifications of double-labelled cells. Scale bars = 50µm.

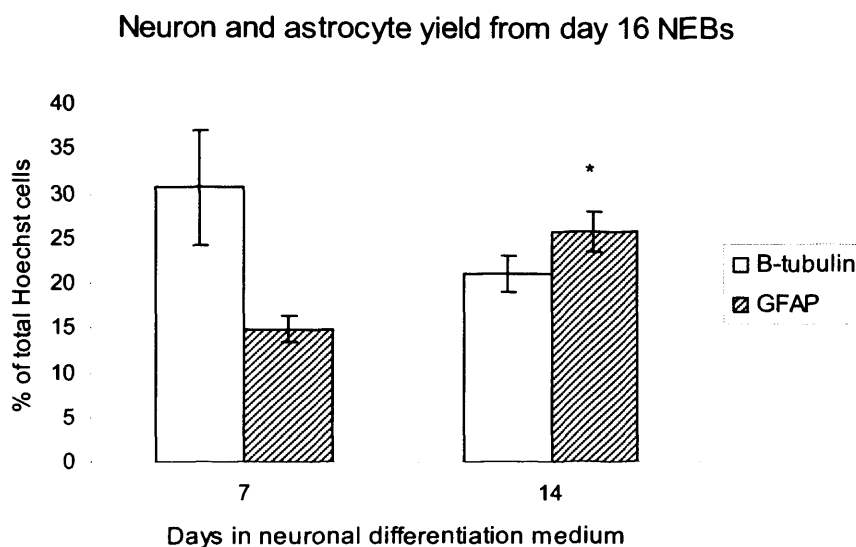
#### *Neuronal differentiation of day 16 NEBs*

NEBs following 16 days in ADF<sup>+</sup> neural induction medium with the addition of 20ng/ml FGF2 at day 4, can generate neurons when cultured in neuronal differentiation medium for 7 days, in the presence of FCS and B27 (Figure 6.7).



**Figure 6.7. Phase contrast photomicrographs of day 16 NEBs following 7 days neuronal differentiation.** NEBs were harvested, dissociated and plated on PLL/laminin substrate following 16 days in ADF<sup>+</sup> with addition of 20ng/ml FGF2 from day 4. Following 7 days differentiation in neuronal differentiation medium cultures were fixed for analysis. Cultures did not appear overgrown as was the case with day 8 NEB differentiating cultures. Photomicrograph B is a higher powered image of A, as indicated by the box in A. Scale bars = 100µm (A) and 50µm (B).

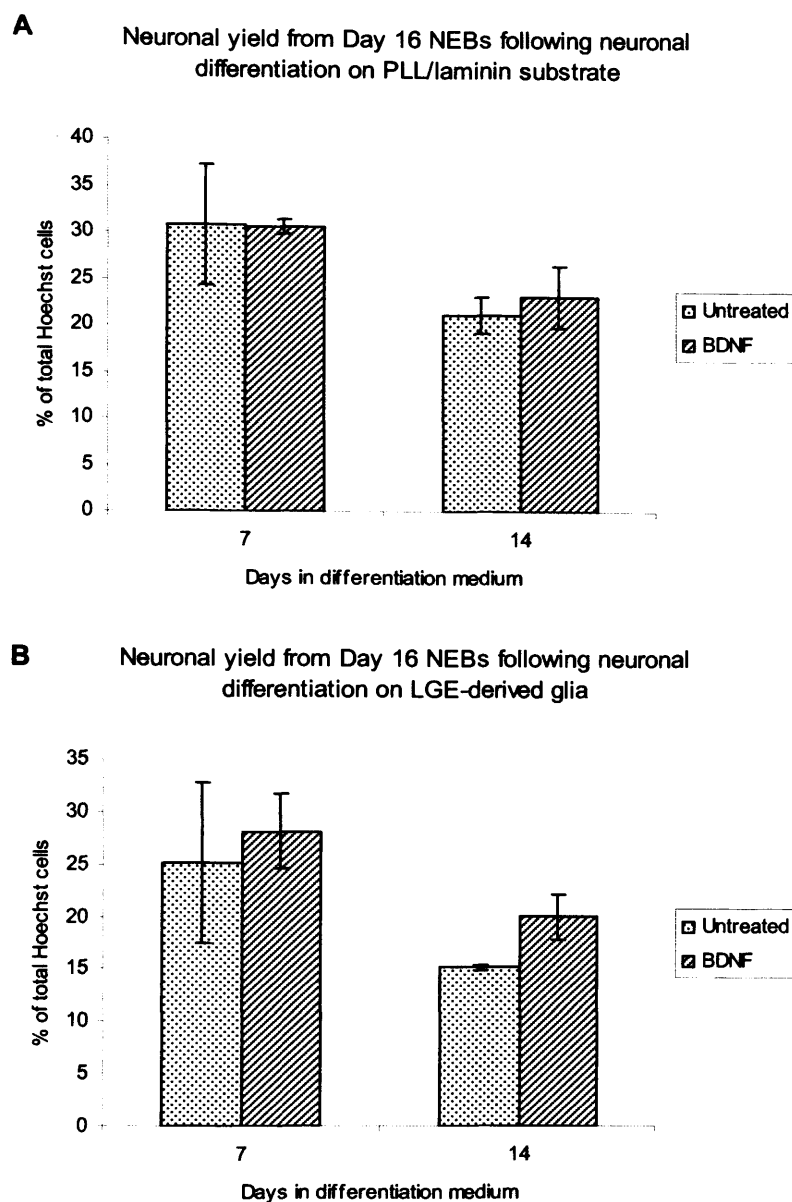
Fluorescent immunocytochemistry of day 16 ES cell-derived neural precursors following 7 and 14 days in neuronal differentiation medium on PLL/laminin substrate, confirmed the presence of  $\beta$ -III-tubulin positive neurons and GFAP positive glia (Figure 6.8). Although there appeared to be proportionally fewer neurons at day 14 ( $21.20 \pm 2.01\%$ ) than at day 7 ( $30.71 \pm 4.89\%$ ) this did not prove to be statistically significant (day,  $t_5 = 2.95$ ,  $p = \text{n.s}$ ) (Figure 6.8). The proportion of GFAP-positive cells was significantly higher at day 14 ( $25.84 \pm 2.24\%$ ) than at day 7 ( $14.92 \pm 1.48\%$ ) (day,  $t_5 = 4.01$ ,  $p < 0.05$ ).



**Figure 6.8. Neuron and astrocyte yield from day 16 NEBs.** NEBs following 16 days in neural induction medium were harvested, dissociated and plated onto PLL/laminin substrate allowed to differentiate for 7 or 14 days in neuronal differentiation medium. Cultures were fixed and triple-labelled for  $\beta$ -III-tubulin, GFAP and Hoechst nuclear stain.  $\beta$ -III-tubulin and GFAP positive cells are represented as a percentage of total Hoechst cells. Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM. (\* $p < 0.05$ ).

Neuronal yield of day 16 NEBs was analysed under conditions previously used for induction of DARPP-32 in LGE cultures (Figure 6.9; A and B). Since the proportion of  $\beta$ -III-tubulin positive neurons is calculated as a percentage of total Hoechst cells, neuronal yield from NEBs plated on PLL/laminin substrate and those plated on top of LGE-derived glia is not directly compared because the total Hoechst cell count is not equal due to the presence/absence of the glia. When expression of DARPP-32 is calculated as a percentage of  $\beta$ -III-tubulin positive neurons, the two plating conditions can be compared.

There was no significant difference in the proportion of  $\beta$ -III-tubulin positive neurons when cultures were plated on PLL/laminin substrate, after 7 days differentiation without BDNF ( $30.71 \pm 4.89\%$ ), 7 days with BDNF ( $30.58 \pm 0.69\%$ ) 14 days without BDNF ( $21.20 \pm 2.01\%$ ) and 14 days with BDNF ( $23.13 \pm 3.39\%$ ) (treatment x day,  $F_{1,12} = 1.39$ ,  $p = \text{n.s.}$ ) (Figure 6.9; A). There was also no significant difference in the proportion of  $\beta$ -III-tubulin positive neurons when cultures were plated on LGE-derived glia, after 7 days differentiation without BDNF ( $25.08 \pm 7.67\%$ ), 7 days with BDNF ( $28.12 \pm 3.56\%$ ) 14 days without BDNF ( $15.18 \pm 0.31\%$ ) and 14 days with BDNF ( $20.03 \pm 2.24\%$ ) (treatment x day,  $F_{1,12} = 1.22$ ,  $p = \text{n.s.}$ ) (Figure 6.9; B).



**Figure 6.9. Neuronal yield from day 16 NEBs.** NEBs following 16 days in neural induction medium were harvested, dissociated and plated onto PLL/laminin substrate (A) or LGE-derived glia (B). Cultures were allowed to differentiate for 7 or 14 days in neuronal differentiation medium in the presence or absence of 50ng/ml BDNF. Cultures were fixed and double-labelled for  $\beta$ -III-tubulin and Hoechst nuclear stain.  $\beta$ -III-tubulin positive cells are represented as a percentage of total Hoechst cells. Each bar on the graph represents a mean of 3 different neural induction cultures and error bars represent SEM.

*Generation of striatal neurons from day 16 NEBs*

Addition of low concentrations of the SHH agonist purmorphamine to ES cell-derived neural precursor induction cultures resulted in a slight increase in expression of some ventral telencephalic markers; *Nkx2.1* and *Dlx2*, detailed in Chapter 5. In this chapter, 'purmorphamine treated' precursors have been subjected to the neuronal differentiation protocol in parallel with 'untreated' precursors, to determine if the untreated and treated precursors respond differently to the various neuronal differentiation treatments and generate different proportions of striatal neuronal phenotypes.

Since DARPP-32 expression has not previously been reported following *in vitro* neuronal differentiation of NEBs, GABA (the principal neurotransmitter of striatal MSNs), which has been demonstrated following *in vitro* neuronal differentiation of NEBs (see section 6.2), was included for additional analysis.

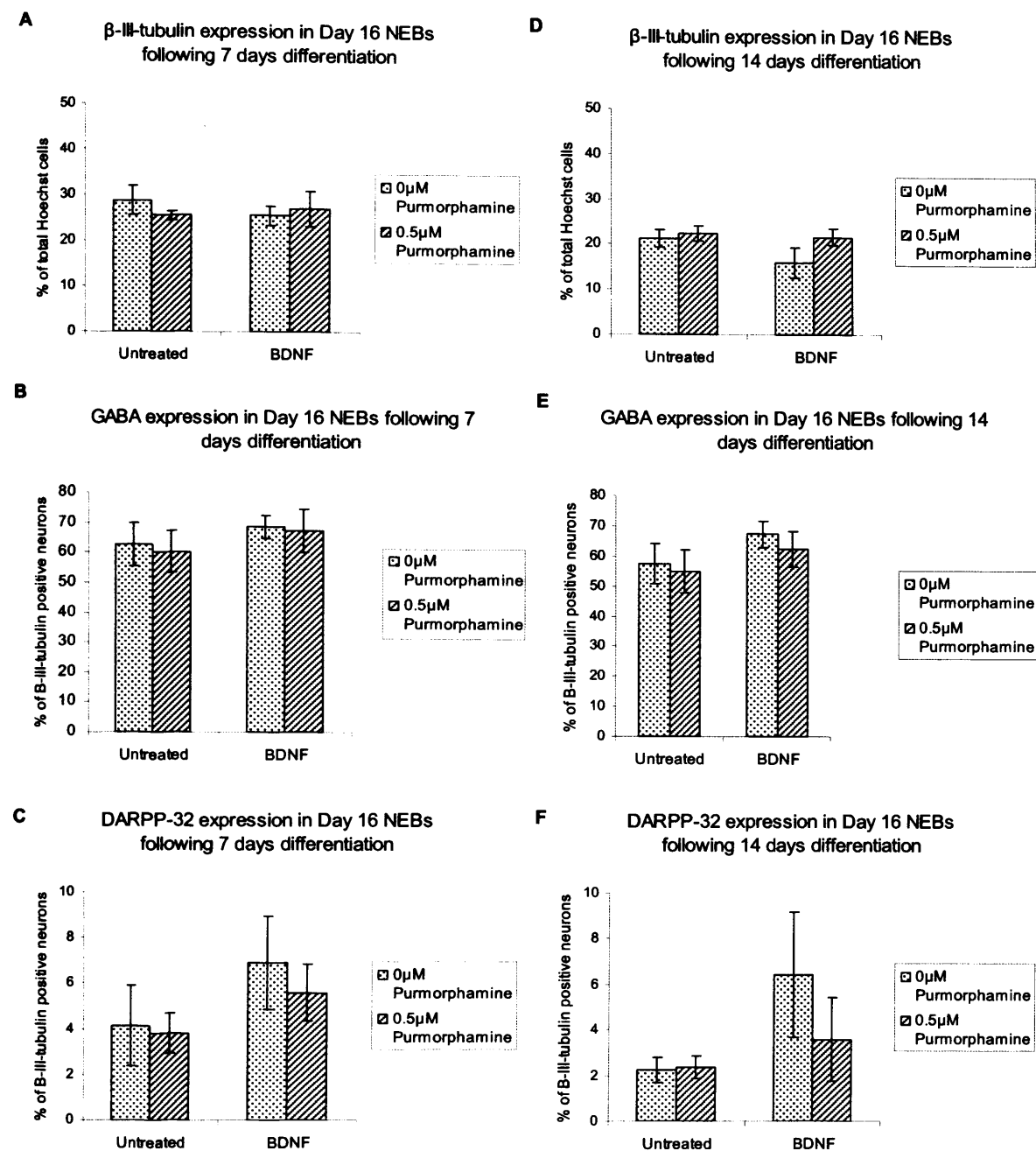
Expression of  $\beta$ -III-tubulin positive neurons was not significantly different in day 16 precursors following 7 days in neuronal differentiation medium, with respect to addition of purmorphamine to neural induction medium (purmorphamine,  $F_{1,12} = 0.11$ ,  $p = \text{n.s.}$ ) or addition of BDNF to neuronal differentiation medium (BDNF,  $F_{1,12} = 0.14$ ,  $p = \text{n.s.}$ ) (Figure 6.10; A). There was also no significant difference in expression of  $\beta$ -III-tubulin positive neurons in day 16 precursors following 14 days differentiation, with respect to addition of purmorphamine to neural induction medium (purmorphamine,  $F_{1,12} = 2.10$ ,  $p = \text{n.s.}$ ) or addition of BDNF to neuronal differentiation medium (BDNF,  $F_{1,12} = 1.68$ ,  $p = \text{n.s.}$ ) (Figure 6.10; D).

Fluorescent immunocytochemistry of day 16 ES cell-derived neural precursors following 7 and 14 days differentiation in neuronal differentiation medium on PLL/laminin substrate, revealed the presence of GABAergic neurons (Figure 6.10; B and E; and Figure 6.11). There was no significant difference in the proportion of GABA positive neurons as a percentage of  $\beta$ -III-tubulin neurons when cultures were plated on PLL/laminin substrate, after 7 days differentiation with respect to purmorphamine concentration (purmorphamine,  $F_{1,12} = 0.09$ ,  $p = \text{n.s.}$ ) or differentiation treatment (BDNF,  $F_{1,12} = 0.99$ ,  $p = \text{n.s.}$ ); untreated precursors without BDNF ( $62.75 \pm 7.28\%$ ), purmorphamine treated precursors without BDNF ( $60.18 \pm 6.95\%$ ), untreated precursors with BDNF ( $68.52 \pm 3.82\%$ ) and purmorphamine treated precursors with BDNF ( $67.26 \pm 7.10\%$ ) (Figure 6.10;B). There was also no significant difference in the proportion of GABA positive neurons when cultures were plated on

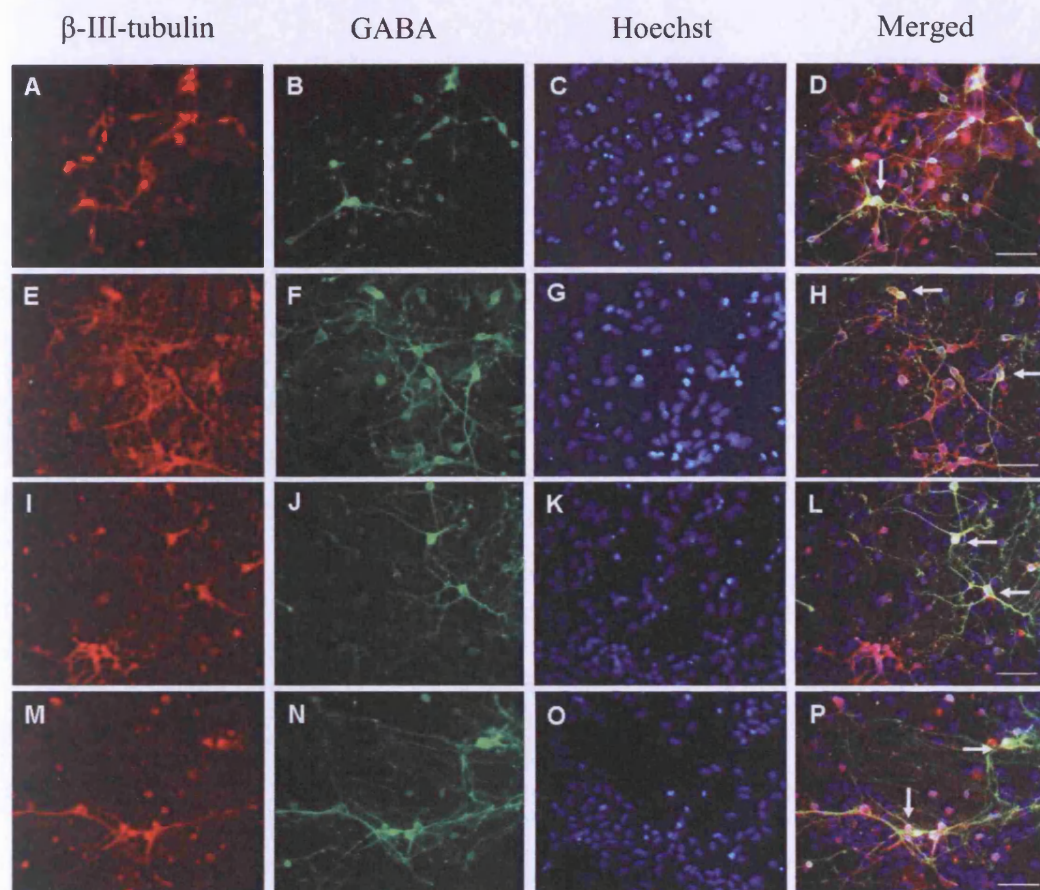
PLL/laminin substrate and allowed to differentiate for 14 days, with respect to addition of purmorphamine (purmorphamine,  $F_{1,12} = 0.35$ ,  $p = \text{n.s.}$ ) or differentiation treatment (BDNF,  $F_{1,12} = 2.03$ ,  $p = \text{n.s.}$ ); untreated precursors without BDNF ( $57.42 \pm 6.73\%$ ), purmorphamine treated precursors without BDNF ( $54.88 \pm 7.05\%$ ), untreated precursors with BDNF ( $67.24 \pm 4.51\%$ ) and purmorphamine treated precursors with BDNF ( $62.53 \pm 5.92\%$ ) (Figure 6.10;E). The proportion of GABAergic positive neurons was not significantly different between the 7 day and 14 day neuronal differentiation cultures (day,  $t_{21} = 0.99$ ,  $p = \text{n.s.}$ ).

Fluorescent immunocytochemistry of day 16 ES cell-derived neural precursors following 7 and 14 days differentiation in neuronal differentiation medium on PLL/laminin substrate, revealed the presence of  $\beta$ -III-tubulin positive neurons co-expressing DARPP-32 (Figure 6.10; C and F; and Figure 6.12). There was no significant difference in the proportion of DARPP-32 positive neurons as a percentage of  $\beta$ -III-tubulin neurons when cultures were plated on PLL/laminin substrate, after 7 days differentiation with respect to purmorphamine concentration (purmorphamine,  $F_{1,12} = 0.28$ ,  $p = \text{n.s.}$ ) or differentiation treatment (BDNF,  $F_{1,12} = 2.13$ ,  $p = \text{n.s.}$ ); untreated precursors without BDNF ( $4.16 \pm 1.77\%$ ), purmorphamine treated precursors without BDNF ( $3.82 \pm 0.89\%$ ), untreated precursors with BDNF ( $6.91 \pm 2.04\%$ ) and purmorphamine treated precursors with BDNF ( $5.60 \pm 1.24\%$ ) (Figure 6.10; C). There was also no significant difference in the proportion of DARPP-32 positive neurons when cultures were plated on PLL/laminin substrate and allowed to differentiate for 14 days, with respect to addition of purmorphamine (purmorphamine,  $F_{1,12} = 0.65$ ,  $p = \text{n.s.}$ ) or differentiation treatment (BDNF,  $F_{1,12} = 1.50$ ,  $p = \text{n.s.}$ ); untreated precursors without BDNF ( $2.26 \pm 0.54\%$ ), purmorphamine treated precursors without BDNF ( $2.38 \pm 0.49\%$ ), untreated precursors with BDNF ( $6.42 \pm 2.76\%$ ) and purmorphamine treated precursors with BDNF ( $3.58 \pm 1.85\%$ ) (Figure 6.10; F).



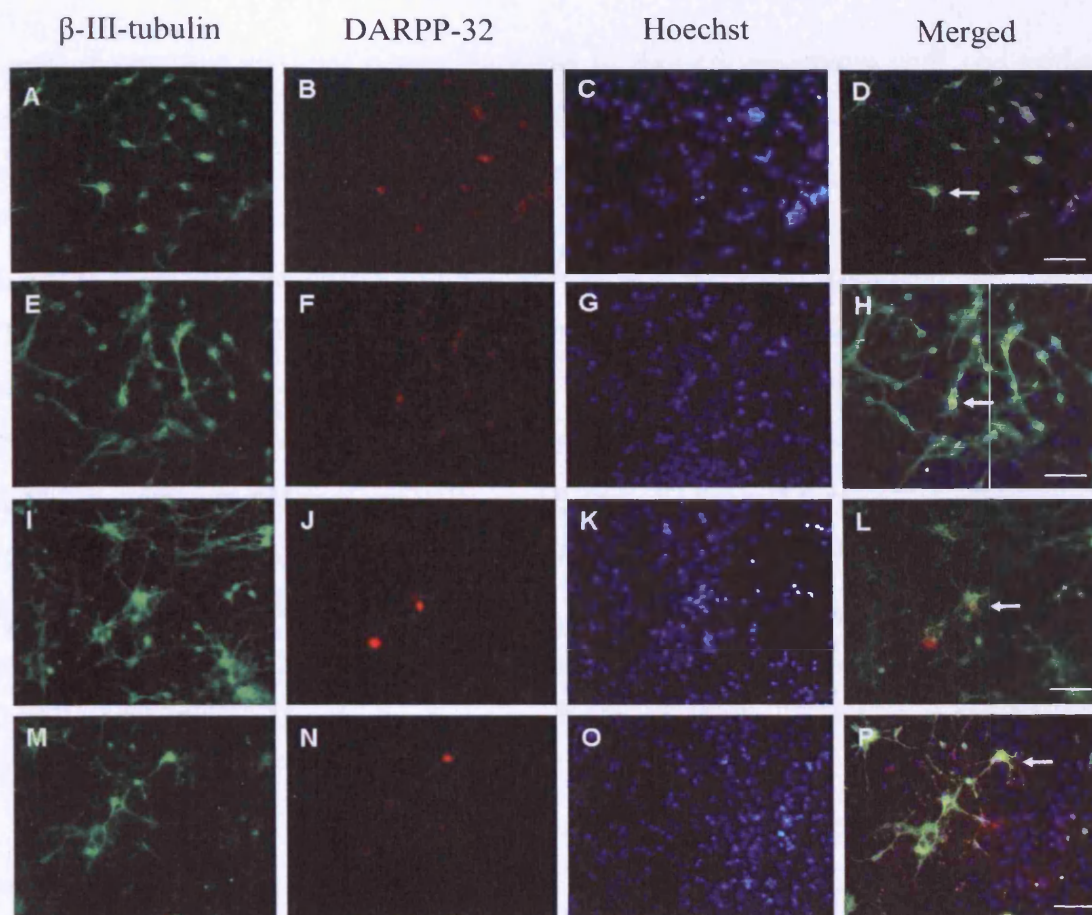


**Figure 6.10.  $\beta$ -III-tubulin, GABA and DARPP-32 yield from day 16 NEBs.** NEBs following 16 days in neural induction medium were harvested, dissociated and plated onto PLL/laminin substrate and allowed to differentiate for 7 days (A, B and C) or 14 days (D, E and F). Neural induction cultures were untreated or treated with 0.5  $\mu$ M purmorphamine, and subsequent differentiation was in neuronal differentiation medium either untreated or with addition of 50 ng/ml BDNF. Cultures were fixed and triple-labelled for  $\beta$ -III-tubulin, GABA and Hoechst nuclear stain, or  $\beta$ -III-tubulin, DARPP-32 and Hoechst nuclear stain.  $\beta$ -III-tubulin positive neurons are represented as a percentage of total Hoechst cells (A and D), GABA positive neurons are represented as a percentage of  $\beta$ -III-tubulin positive neurons (B and E) and DARPP-32 positive neurons are represented as a percentage of  $\beta$ -III-tubulin positive neurons (C and F). Each bar on the graphs represents a mean of 3 different neural induction cultures and error bars represent SEM.



**Figure 6.11. Photomicrographs showing expression of  $\beta$ -III-tubulin and GABA from day 16 NEBs following differentiation, using immuno-fluorescence.** Since there was no significant difference in  $\beta$ -III-tubulin and GABA yield between cultures of the various treatments, this figure shows a representative selection of photomicrographs from some of the different culture conditions. NEBs following 16 days in neural induction medium were harvested, dissociated and plated onto PLL/laminin substrate and allowed to differentiate for 7 days (A-H) or 14 days (I-P). Neural induction cultures were untreated or treated with  $0.5\mu\text{M}$  purmorphamine, and subsequent differentiation was in neuronal differentiation medium either untreated or with addition of  $50\text{ng/ml}$  BDNF. Cultures were fixed and triple-labelled for  $\beta$ -III-tubulin (red), GABA (green) and Hoechst nuclear stain (blue). Untreated precursors without BDNF for 7 days (A-D); untreated precursors with addition of BDNF for 7 days (E-H); untreated precursors without BDNF for 14 days (I-L);  $0.5\mu\text{M}$  purmorphamine treated precursors with addition of BDNF for 14 days (M-P). Arrows (D, H, L and P) indicate cells co-expressing  $\beta$ -III-tubulin and GABA. Scale bars =  $50\mu\text{m}$ .



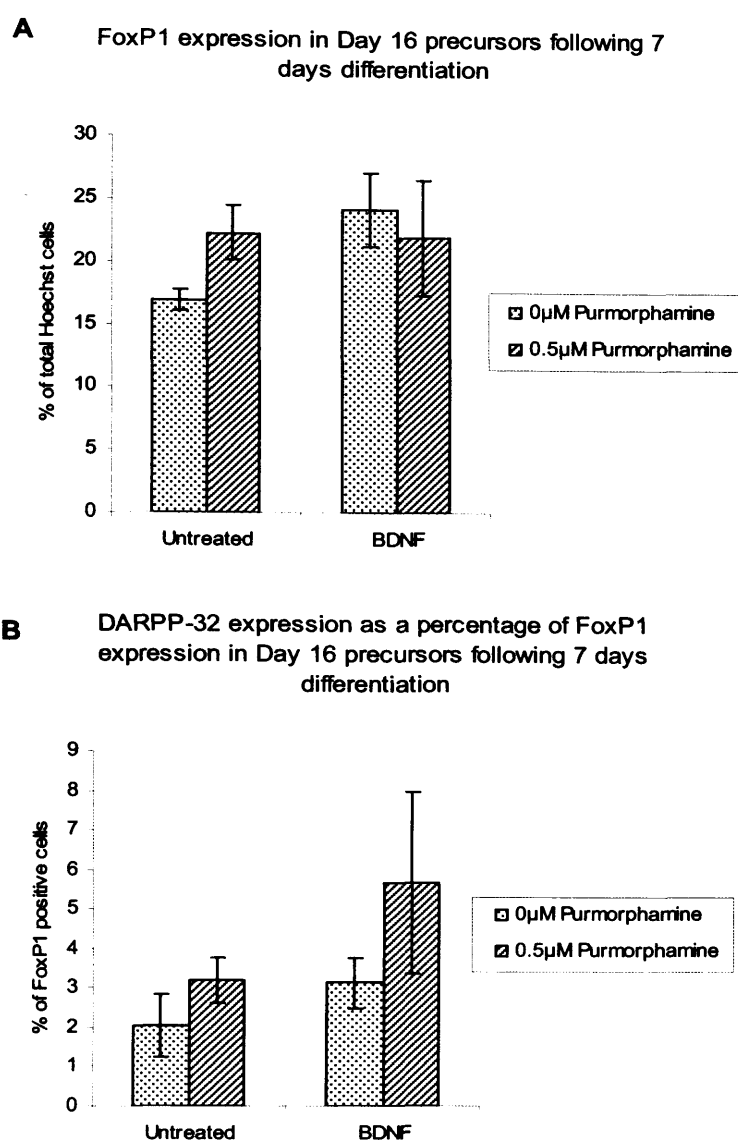


**Figure 6.12.** Photomicrographs showing expression of  $\beta$ -III-tubulin and DARPP-32 from day 16 NEBs following differentiation, using immuno-fluorescence. Since there was no significant difference in  $\beta$ -III-tubulin and DARPP-32 yield between cultures of the various treatments, this figure shows a representative selection of photomicrographs from some of the different culture conditions. NEBs following 16 days in neural induction medium were harvested, dissociated and plated onto PLL/laminin substrate and allowed to differentiate for 7 days (A-H) or 14 days (I-P). Neural induction cultures were untreated or treated with  $0.5\mu\text{M}$  purmorphamine, and subsequent differentiation was in neuronal differentiation medium either untreated or with addition of  $50\text{ng/ml}$  BDNF. Cultures were fixed and triple-labelled for  $\beta$ -III-tubulin (green), DARPP-32 (red) and Hoechst nuclear stain (blue). Untreated precursors without BDNF for 7 days (A-D);  $0.5\mu\text{M}$  purmorphamine treated precursors with addition of BDNF for 7 days (E-H); untreated precursors without BDNF for 14 days (I-L);  $0.5\mu\text{M}$  purmorphamine treated precursors with addition of BDNF for 14 days (M-P). Arrows (D, H, L and P) indicate cells co-expressing  $\beta$ -III-tubulin and DARPP-32. Scale bars =  $50\mu\text{m}$ .

Expression of FoxP1 was analysed in day 16 precursors with and without purmorphamine addition to neural induction medium, and with and without BDNF addition to subsequent neuronal differentiation cultures (Figure 6.13 and 6.14). Fluorescent immunocytochemistry demonstrated co-expression of FoxP1 and DARPP-32 in day 16 NEBs following 7 days in neuronal differentiation medium (Figure 6.14). There was no significant difference in the proportion of FoxP1 positive cells yielded when day 16 precursors, with or without purmorphamine, were plated on PLL/laminin substrate and differentiated for 7 days in neuronal differentiation medium with or without BDNF, with respect to addition of purmorphamine (purmorphamine,  $F_{1,12} = 0.29$ ,  $p = \text{n.s.}$ ) or addition of BDNF (BDNF,  $F_{1,12} = 1.34$ ,  $p = \text{n.s.}$ ); untreated precursors without BDNF ( $16.89 \pm 0.81\%$ ), purmorphamine treated precursors without BDNF ( $22.25 \pm 2.14\%$ ), untreated precursors with BDNF ( $24.10 \pm 2.91\%$ ) and purmorphamine treated precursors with BDNF ( $21.92 \pm 4.65\%$ ) (Figure 6.13; A).

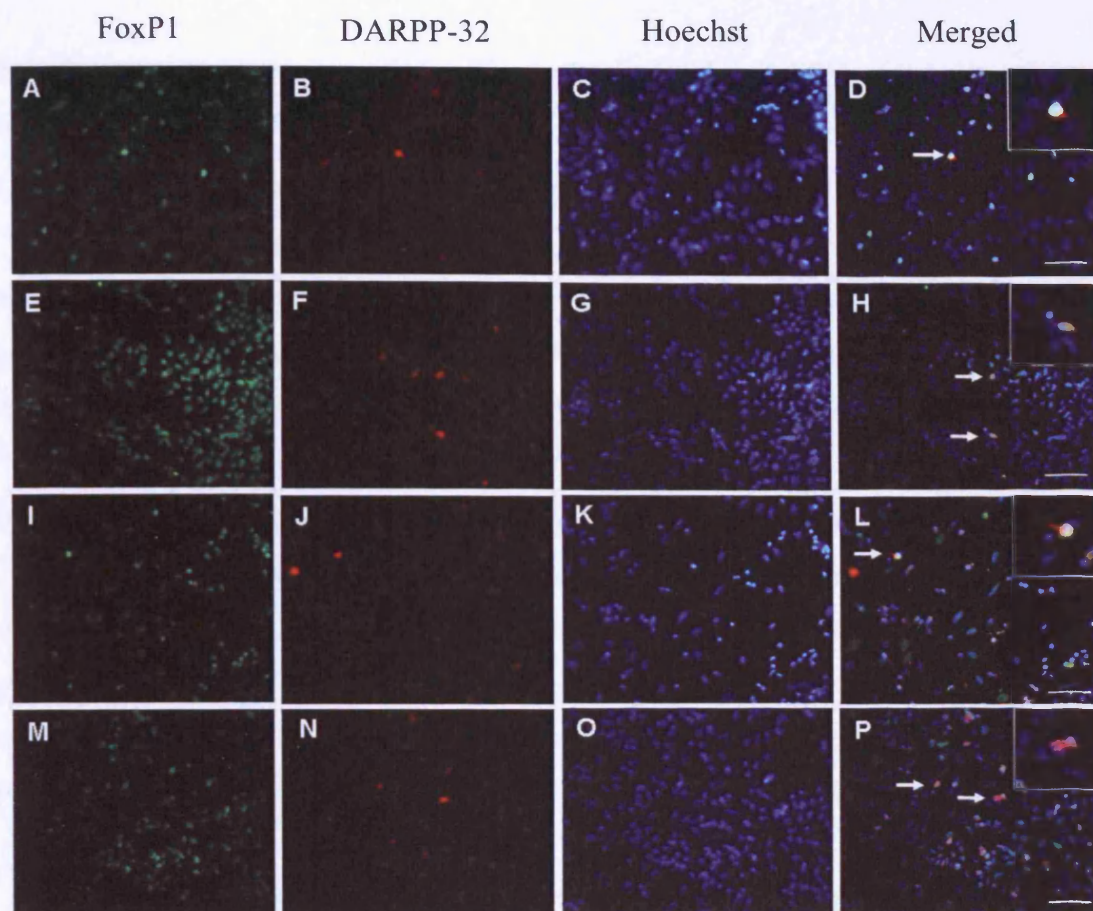
There was no significant difference in the proportion of DARPP-32 positive neurons yielded, as a percentage of FoxP1 positive cells, when day 16 precursors, with or without addition of purmorphamine, were plated on PLL/laminin substrate and differentiated for a further 7 days in neuronal differentiation medium with or without BDNF, with respect to addition of purmorphamine (purmorphamine,  $F_{1,12} = 2.94$ ,  $p = \text{n.s.}$ ) or addition of BDNF (BDNF,  $F_{1,12} = 2.64$ ,  $p = \text{n.s.}$ ); untreated precursors without BDNF ( $2.04 \pm 0.81\%$ ), untreated precursors with BDNF ( $3.14 \pm 0.65\%$ ), purmorphamine treated precursors without BDNF ( $3.19 \pm 0.58\%$ ) and purmorphamine treated precursors with BDNF ( $5.67 \pm 2.29\%$ ) (Figure 6.13; B).

A summary of the neuronal expression in LGE-derived neurons and day 16 NEBs following neuronal differentiation under the various culture conditions is shown in Appendix 7.



**Figure 6.13. FoxP1 and DARPP-32 expression in day 16 NEBs.** NEBs following 16 days in neural induction medium were harvested, dissociated and plated onto PLL/laminin substrate and allowed to differentiate for 7 days (A and B). Neural induction cultures were untreated or treated with 0.5µM purmorphamine, and subsequent differentiation was in neuronal differentiation medium either untreated or with addition of 50ng/ml BDNF. Cultures were fixed and triple-labelled for FoxP1, DARPP-32 and Hoechst nuclear stain. FoxP1 positive cells are represented as a percentage of total Hoechst cells (A) and DARPP-32 positive neurons are represented as a percentage of FoxP1 positive cells (B). Each bar on the graphs represents a mean of 3 different neural induction cultures and error bars represent SEM.





**Figure 6.14. Photomicrographs showing expression of FoxP1 and DARPP-32 from day 16 NEBs following differentiation, using immuno-fluorescence.** Since there was no significant difference in FoxP1 and DARPP-32 yield between cultures of the various treatments, this figure shows a representative selection of photomicrographs from some of the different culture conditions. NEBs following 16 days in neural induction medium were harvested, dissociated and plated onto PLL/laminin substrate and allowed to differentiate for 7 days (A-H) or 14 days (I-P). Neural induction cultures were untreated or treated with 0.5  $\mu$ M purlmorphamine, and subsequent differentiation was in neuronal differentiation medium either untreated or with addition of 50ng/ml BDNF. Cultures were fixed and triple-labelled for FoxP1 (green), DARPP-32 (red) and Hoechst nuclear stain (blue). Untreated precursors without BDNF for 7 days (A-D); 0.5  $\mu$ M purlmorphamine treated precursors with addition of BDNF for 7 days (E-H); untreated precursors without BDNF for 14 days (I-L); 0.5  $\mu$ M purlmorphamine treated precursors without BDNF for 14 days (M-P). Arrows (D, H, L and P) indicate cells co-expressing FoxP1 and DARPP-32, and insets (D, H, L and P) show higher magnifications of double-labelled cells. Scale bars = 50  $\mu$ m.

## 6.5 Discussion

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### *DARPP-32 expression in LGE-derived neurons*

The commitment of striatal projection neurons to the patch or matrix compartment of the striatum is related to the embryonic stage at which neurons are generated (van der Kooy and Fishell, 1987). The early-born neurons (by E12 in the mouse) are destined to the patch compartment whereas the mid to late-born neurons (by E15-16 in the mouse) populate the matrix compartment (van der Kooy and Fishell, 1987). The two compartments emerge clearly *in vivo* at P0-2 (Passante et al., 2008). MSNs are found in both the patch and matrix striatal compartments and therefore include a combination of early and mid to late-born neurons. Peak production of MSNs is at E15 (Marchand et al., 1986), but DARPP-32 is not expressed until later. DARPP-32 expression in the mouse is detected at low levels at P0, with an increase to adult levels over the first four post-natal weeks (Ehrlich et al., 1990).

The LGE is the origin of the DARPP-32 positive MSNs found in the adult striatum. When mouse E14 LGE was differentiated *in vitro* for 7 days, in the presence of FCS and B27, the proportion of neurons that expressed DARPP-32 was shown to increase by nearly 3 times with the addition of 50ng/ml BDNF when compared with untreated differentiation cultures. Since there was no increase in the total neuron number, as determined by  $\beta$ -III-tubulin expression, the BDNF-induced increase in DARPP-32 positive neurons appears to be specific and not just due to an overall enrichment of neuronal differentiation. BDNF has previously been reported to exert a dose-dependent survival effect on DARPP-32-positive neurons derived from embryonic rat striatum (Nakao et al., 1995), and in mouse primary striatal cultures, addition of 100ng/ml BDNF to serum-free culture medium resulted in a 4-fold increase in DARPP-32 expression after 24 hours (Ivkovic et al., 1997).

In the neuronal differentiation culture system applied here, addition of RA to LGE differentiation cultures did not result in an increase in DARPP-32 expression. When both BDNF and RA were added concomitantly, there was a slight increase in DARPP-32 expression when compared with untreated cultures, however, this was only half that seen in cultures treated with BDNF alone. This was not consistent with

a previous study, also using mouse LGE at E13.5, which reported that addition of exogenous RA (10nm and 1 $\mu$ M) to LGE-derived neuronal cultures resulted in an increase in DARPP-32 expression which was twice that of untreated control cultures (Toresson et al., 1999). In contrast to the neuronal differentiation medium employed in this study, Toresson et al used serum-free differentiation medium, in order to exclude the effects of undefined growth factors present at varying levels in FCS, including retinol. This might be one explanation for the different responses of LGE cultures following addition of exogenous RA. Retinyl acetate is also a component of the growth supplement B-27, which is added to neuronal differentiation medium in this study and aids survival of neurons in culture, but is absent from the other differentiation protocols mentioned (Nakao et al., 1995; Ivkovic et al., 1997; Toresson et al., 1999). In our study we were interested in optimising the DARPP-32 output from the neuronal differentiation culture system. Since the presence of FCS and B-27 encourages neuronal differentiation, we used this as our baseline system for expression of striatal markers.

The effect of BDNF on DARPP-32 expression in E14 mouse LGE neuronal cultures might be to induce neurons to become DARPP-32 positive, since we, and others (Nakao et al., 1995; Ivkovic et al., 1997) demonstrate an increase in DARPP-32 positive neurons following BDNF addition to LGE neuronal cultures. Nakao et al also reported that BDNF acted as a survival factor for DARPP-32 positive neurons (Nakao et al., 1995). Another possibility is that BDNF might decrease the time taken for DARPP-32 to be 'switched on' in those neurons whose fates are already specified to become DARPP-32 positive neurons. Our results would favour the latter hypothesis, since longer-term neuronal differentiation of LGE cultures, up to 14 days, in untreated neuronal differentiation medium resulted in 3 times more DARPP-32-expressing neurons than LGE cultures that were differentiated for 7 days. DARPP-32 expression yielded from untreated LGE cultures differentiated for 14 days, was only slightly higher than BDNF treated LGE cultures differentiated for 7 days. When BDNF was added to LGE cultures that were differentiated for 14 days, there was no difference in DARPP-32 between untreated cultures differentiated for the same time. This suggests that in this neuronal differentiation system, BDNF reduces the differentiation time of DARPP-32 positive neurons in LGE cultures.

Differentiation of LGE-derived neurons in homotopic co-culture on LGE-derived glial cells resulted in increased DARPP-32 expression following 7 days



differentiation when compared with LGE-derived neurons differentiated on PLL/laminin substrate for the same period of time. When LGE-derived neurons were differentiated in LGE-derived glia co-culture for 14 days, there was no difference in DARPP-32 expression compared with 7 days differentiation in co-culture. In our study, there was a two-fold increase in DARPP-32 positive neurons following 7 days differentiation in co-culture (up to 8%) compared with differentiation on substrate. Skogh and Campbell reported 10-15% DARPP-32 positive neurons following 7 days in co-culture and noted a decrease in DARPP-32 yield with higher passage numbers of LGE-derived glia prior to co-culture (Skogh and Campbell, 2003). This suggests that glial cells are releasing different factors or different levels of factors following different periods of time in culture. Although differentiation of LGE cultures on PLL/laminin substrate for 14 days yielded significantly more DARPP-32 positive neurons than those differentiated for 7 days, the differentiation cultures at day 14 were not consistently healthy in appearance and therefore not reliable for subsequent immuno-fluorescent analysis. This might suggest that the *in vitro* neuronal differentiation culture system used here is not (always) favourable for long-term differentiation of mouse striatal cultures.

In the CNS, glial cells release soluble factors and signal to neurons regulating neuronal activity. *In vitro*, glia-derived factors have been shown to be important for the development of synapses (Pfrieger and Barres, 1997; Vicario-Abejon et al., 1998; Blondel et al., 2000). Factors such as BDNF and neurotrophin-3 (NT-3) have been shown to regulate synaptogenesis in hippocampal cultures, and these factors do not require the presence of glia to act on neurons (Song et al., 1997).

*In vitro* studies using organotypic cultures looking at development of striatal neurons and the formation of the patch compartment of the striatum have suggested that the cortex provides signals to the developing striatum, and the corticostriatal afferents might be necessary for correct development of the early-born striatal neurons (Snyder-Keller et al., 2001; Snyder-Keller, 2004; Snyder-Keller et al., 2008). Furthermore, it has been demonstrated that the stage of development of the cortical neurons is important for the maturation of striatal neurons (Snyder-Keller, 2004; Snyder-Keller et al., 2008). Thus a co-culture system of LGE-derived neurons or NEBs, with neurons from the developing cortex or ventral mesencephalon might prove beneficial in increasing the proportion of striatal projections neurons within cultures.

Since DARPP-32 is a marker of mature striatal MSNs, it would be useful to identify a reliable marker of MSN precursor cells. FoxP1 was identified as a potential candidate following a screen to analyse up-regulation of striatal genes during embryonic development (Kelly et al., in preparation). Further analysis using RT-PCR, *in situ* hybridization and fluorescent immunocytochemistry on developing mouse striatum, revealed that FoxP1 is expressed in the striatum and is upregulated from E12 through to E16 by 6 fold, with expression maintained through to adulthood (Kelly et al., in preparation; Ferland et al., 2003). Immunofluorescence shown here demonstrates that FoxP1 and DARPP-32 are co-expressed in mature striatal neurons. Neuronal differentiation of LGE cultures showed no significant difference in FoxP1 expression when cultures were untreated or treated with BDNF, but expression of DARPP-32 positive neurons, as a percentage of FoxP1 cells, was increased with addition of BDNF. Expression of FoxP1 in MSNs, both prior to their expression of DARPP-32 and then concomitantly with their expression of DARPP-32, suggests that in the 7 day differentiation period of LGE-derived neurons, BDNF does not induce cells to become striatal precursors, but simply pushes the striatal precursors which are already present to express DARPP-32.

#### *Neuronal differentiation of day 16 NEBs*

ES cell-derived neural precursors subjected to 16 days in neural induction medium followed by a further period in neuronal differentiation medium as a monolayer culture, are shown to generate neurons, among other cell types. To date there is no literature in mouse which assesses the neuronal analysis of these neural precursors with the aim to generate DARPP-32 positive MSNs *in vitro*. When day 16 neural precursor cultures are allowed to differentiate on PLL/laminin substrate for 7 and 14 days they generate  $\beta$ -III-tubulin positive neurons along with GFAP positive glia. There was significantly more GFAP expression at day 14 than at day 7, but the proportion of  $\beta$ -III-tubulin positive neurons did not differ significantly between the two time points. Terminal differentiation of earlier stage neural precursors has been reported to generate  $\beta$ -III-tubulin positive neurons (Bouhon et al., 2005; Dihne et al., 2006) and, with longer-term differentiation up to 14 days, GFAP positive glia (Bouhon et al., 2005). Later stage neural precursors at day 20 have also been reported to generate  $\beta$ -III-tubulin positive neurons as well as GFAP-positive cells and oligodendrocytes (Bouhon et al., 2006). It has been proposed that ES cell-derived

neural precursors undergo a transition period from early stage 'neurogenic' to late stage 'gliogenic' precursors (Bouhon et al., 2006).

The neuronal yield of day 16 neural precursors did not change when cultures were differentiated on PLL/laminin for 7 and 14 days with and without the addition of BDNF. There was also no difference in neuronal yield when cultures were differentiated on LGE-derived glia for 7 and 14 days with and without the addition of BDNF. As with LGE cultures, there was variable consistency in generation of healthy cultures that could be taken forward for further analysis, following long-term differentiation of neural precursors for 14 days.

The previous chapter looked at generating a greater proportion of ventral telencephalic precursors within the neural precursor population, as a way to attempt to increase mature striatal neurons following subsequent terminal differentiation. Addition of low concentrations of the SHH agonist purmorphamine resulted in a marginal increase in *Nkx2.1* and *Dlx1/2* expression of day 16 neural precursors. These purmorphamine treated neural precursors, along with untreated neural precursors, have been subjected to the neuronal differentiation protocol that was applied to the LGE-derived neurons to assess whether or not DARPP-32 expression can be generated from the neuronal population.

Neuronal yield was not changed between the different conditions of neural induction or neuronal differentiation, and within the neuronal population, there was no effect on the expression of DARPP-32 or GABA. The percentage of  $\beta$ -III-tubulin positive neurons that co-expressed GABA was about 60%, which is similar to that previously reported following 7 days differentiation of day 8 neural precursors, where 55% of  $\beta$ -III-tubulin positive neurons expressed GABA (Bouhon et al., 2006). Terminal differentiation of human ES cell-derived neural precursors resulted in the generation of neuronal cells, 36% of which co-expressed GABA and 53% of which co-expressed DARPP-32 (Aubry et al., 2008). Assessment of the novel striatal marker FoxP1 revealed that there was no difference in expression between the different culture conditions and there was also no difference in the proportion of cells that co-expressed FoxP1 and DARPP-32 in the day 16 neural precursors.

It appears that the day 16 neural precursors are not responsive to BDNF in the same way as the LGE-derived neurons. The heterogeneous nature of the ES cell-derived neural precursor cultures might make it difficult to see an effect on a specific cell type, and there might be an effect on other cell lineages that are present. The

LGE-derived neurons have been pre-specified in the developing brain to become striatal cells whereas the ES cell-derived neural precursors have undergone only limited specification. It is possible that the neural precursors are not at the optimal stage required for responsiveness to certain inductive cues. Results presented in this chapter also indicate the differences between cultures, where cell counts show wide variation, due to heterogeneity and differences in the proportions of cell lineages within cultures, which results in large error bars and in turn influences the statistical significance of findings.

In this study the immature neuronal marker  $\beta$ -III-tubulin was used. The use of more mature neuronal markers such as MAP2A, NeuN and neurofilament, in conjunction with  $\beta$ -III-tubulin would allow assessment of the maturity of the cultures. One explanation for the differences between LGE-derived neurons and day 16 NEB-derived neurons in their response to factors such as BDNF, other than the heterogeneity of cells present in the NEB cultures, might be the degree of maturity of the neural cells. Data in Chapter 5 demonstrating the lower expression of dorso-ventral markers in NEBs compared with LGE or MGE, suggested that NEBs were not at the same developmental stage as the developing striatum. The data in this chapter revealing lower FoxP1 expression in NEBs and also lower ratio of DARPP-32:FoxP1 double-labelled cells suggest that NEBs are immature in comparison to the cells of the developing striatum.

The next step will be to explore the effects of the conditions applied here within a population of forebrain or, more specifically, ventral telencephalic-like cells derived from ES cells. Generation of a pure population of cells using cell-sorting techniques would enable determination of direct responses of these specific cells to the different conditions.

### *Conclusions*

Work presented in this chapter demonstrates the potential of ES cell-derived neural precursors to differentiate into various striatal phenotypes. The expression of DARPP-32 positive neurons, from day 16 NEBs following neuronal differentiation, albeit at a low percentage of total neurons and therefore an even lower percentage of the total cell population, demonstrates the potential of these cells to generate the desired mature phenotype, MSN, which is encouraging. However, the presence of true, mature MSNs needs to be confirmed with other markers, including markers

which demonstrate the presence of more mature neurons. Differentiation of the LGE-derived neurons suggests that if an LGE-like population of precursors can be generated from ES cells, there is the potential to increase the DARPP-32 yield from NEBs, however this likely requires further directed differentiation of NEBs in the neural induction culture prior to neuronal differentiation both *in vitro* and *in vivo*.

Since DARPP-32 positive neurons were generated from NEBs *in vitro*, the next chapter will analyse NEBs produced using the same protocol, following transplantation into the adult mouse lesioned striatum. In the *in vivo* host environment NEBs will have access to additional diffusible and contact factors that may prove important in further specifying and directing cells towards the mature neuronal phenotype.

# Chapter 7

## *in vivo characterisation of NEBs (allograft model)*

### 7.1 Summary

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Neural transplantation of NEBs into the QA lesioned striatum was reported in Chapter 4, using a xenograft paradigm (mouse-to-rat). In Chapter 4, NEBs derived using standard neuralising conditions were insufficient to produce striatal-like grafts following *in vivo* differentiation. Subsequent chapters attempted to further direct cells and resulted in some expression of DARPP-32 *in vitro*. This chapter applies information gained from previous chapters, comparing transplantation of NEBs (day 16 untreated or purmorphamine treated) and E14 mouse striatum (LGE, MGE and WGE) into the mouse host brain. Allografts offer the potential for donor cells to develop in a same-species environment, which might also enhance their responsiveness to host signals by providing species-specific signals. Six and twelve weeks post-transplantation no teratomas were detected in the NEB grafts and there was no significant difference in graft volume between any group. Grafts were small with a notable presence of dead cells. NEB donor cells contained the *LacZ* reporter gene to allow for post-transplantation identification of Foxg1 positive cells (as used in Chapter 4). Neither the antibody against  $\beta$ -galactosidase nor the X-gal reaction detected any positive cells in the NEB-derived grafts. All analysis was carried out using Nissl staining to mark the graft area, and without being able to positively identify cells as graft or host-derived, counts represent cell numbers present within this area. Grafts were analysed for neuronal phenotype, in particular striatal neurons. There was no significant difference in NeuN positive cells within the graft area of NEB-derived or E14-derived grafts at both 6 and 12 weeks post-transplantation. DARPP-32 expression was not significantly different between groups at 6 weeks, but at 12 weeks there were significantly more DARPP-32 positive neurons in WGE-derived grafts compared with untreated day 16 NEB grafts. There was no significant difference in FoxP1, parvalbumin and calbindin expression between transplant groups at both survival time points, suggesting no difference between NEB-derived and E14-derived grafts. Assessment of the mouse striatum as a viable host for transplantation needs to be addressed, and identification of all donor cells in a systematic study would help to give a clearer indication of what is happening to donor cells post-transplantation.

## 7.2 Introduction

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In Chapter 4 of this thesis, mouse ES cell-derived neural precursors following 8 and 16 days in neural induction medium, were grafted into the QA-lesioned rat striatum. Following transplantation the day 8 NEB grafts developed teratomas and analysis for this group was performed earlier than anticipated, at 2 weeks post-transplantation. The day 16 NEB grafts, at 6 weeks post-transplantation, generated grafts which comprised a heterogeneous mix of cells including cells of neuronal lineage, some of which expressed calbindin and parvalbumin, however there was no DARPP-32 expression in Foxg1-positive donor-derived cells, as identified using an antibody against  $\beta$ -galactosidase to detect the *LacZ* reporter gene. Chapter 5 attempted further directed differentiation of NEBs looking at the effect of SHH on expression of markers of the ventral telencephalon. Addition of the SHH agonist purmorphamine at low concentrations (0.5 $\mu$ M) resulted in increased expression of the ventral markers *Nkx2.1* and *Dlx2*. In this chapter, day 16 NEBs were transplanted with or without the addition of 0.5 $\mu$ M purmorphamine.

Since mouse-to-rat grafts are a xenograft paradigm there was the requirement for immunosuppression in the form of daily administration of CsA to prevent graft rejection. Allograft studies bypass the need for immunosuppression, allowing for longer-term survival post-transplantation and eliminating confounding factors such as slow, ongoing immune rejection. There is also the potential for donor cells to be receptive to species-specific signals and thus develop more thoroughly.

Currently, the assessment of allograft studies prior to the translation of human ES cells to clinical trials can only be performed in the mouse or monkey paradigm, since rat ES cell lines, which have only recently been established, are still undergoing development (Buehr et al., 2008; Li et al., 2008). The mouse-to mouse system is more readily accessible to researchers than the monkey-to-monkey system, with the potential to use larger transplant cohorts, the development of various mouse models of HD, and for functional analysis, there are numerous behavioural tests which have been optimised in mice, including those that reveal deficits in HD mice genetic models, and would therefore be useful when attempting to demonstrate improvements following transplantation.

Transplantation of mouse ES cell-derived neural precursors into mouse models of HD has not been widely reported. Dihne et al (2006) transplanted mouse

ES cell-derived neural precursors at different stages during neural precursor induction, into the QA-lesioned mouse striatum. Differences in the expression of nestin (neural precursor marker), BrdU (marker of dividing cells) and  $\beta$ -III-tubulin (immature neuronal marker) prior to transplantation, resulted in generation of macroscopically different grafts. In precursor populations with relatively high expression of nestin (>95%) and BrdU (42%), and low  $\beta$ -III-tubulin expression (<3%), there was a much greater risk of tumour formation, with graft volume up to 26mm<sup>3</sup>. When nestin and BrdU expression were lower (14% and 6%, respectively), and  $\beta$ -III-tubulin expression was higher (76%) there was no tumour formation at 16 weeks post-transplantation (Dihne et al., 2006).

Transplantation of E13-14 mouse LGE into the striatum of the R6/2 transgenic mouse model of HD resulted in graft survival following 6 weeks post-transplantation, with limited integration and only marginal improvements in the behavioural phenotype (Dunnett et al., 1998). The authors reported that grafts in the transgenic striatum behaved similarly to those in the control striatum, suggesting that the transgenic host environment is able to accommodate the introduction of donor cells.

Work in our group looking at striatal graft projections has shown that grafts derived from expanded mouse neural precursors generate more axonal projections than those grafts derived from E14 mouse primary foetal striatum (Kelly et al., 2007). This study reported 94% graft survival in mouse-to-mouse grafts in the unilateral QA-lesion model. Graft volumes were not significantly different between mouse foetal primary cells and mouse expanded neural precursors, ( $0.80 \pm 0.09\text{mm}^3$  and  $0.88 \pm 0.87\text{mm}^3$ , respectively). However, long-term expanded neural precursors, of both mouse and human origin, exhibit reduced neurogenic potential compared to short-term expanded neural precursors (Anderson et al., 2007) and reduced survival following long-term post-transplantation time points (Zietlow et al., 2005).

In this chapter mice with unilateral striatal QA lesions received transplants of (i) day 16 NEBs untreated, (ii) day 16 NEBs purmorphamine treated, (iii) E14 mouse LGE, (iv) E14 mouse MGE or (v) E14 mouse WGE, and were allowed to survive post-transplantation for 6 or 12 weeks. Morphological analysis was carried out, including assessment of graft volume, and more detailed analysis of phenotype looking in particular at striatal cell type was performed and compared between transplant groups.



## 7.3 Experimental Procedures

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### *ES cell culture*

The mouse ES cell line *Foxg1-lacZ* was used in this study. NEBs were derived from *Foxg1Z* mouse ES cells as described in Chapter 2. Briefly, ES cells were transferred from ES cell culture medium to ADF+ medium at a density of 50,000 cells per ml, medium was replaced every 2 days and 20ng/ml FGF2 was added from day 4. Neural induction cultures were untreated (ADF+ plus 20ng/ml FGF2 from day 4) or treated with the SHH agonist purmorphamine (ADF+ plus 20ng/ml FGF2 and 0.5 $\mu$ M purmorphamine from day 4) prior to transplantation. On day 16 NEBs were harvested, incubated in accutase and a single-cell suspension was generated (see section 2.1.4). Cells were counted using a haemocytometer and viability was determined using trypan blue exclusion.

### *Dissection and dissociation of mouse E14 LGE, MGE and WGE*

The three striatal dissections were used because of the differences in gene expression of the LGE and MGE regions, and the subsequent differences in neuronal phenotypes which originate in the two regions.

LGE, MGE and WGE were dissected from E14 C57Bl6/J mouse forebrain and dissociated to generate a single-cell suspension, as described in Chapter 2. Briefly, tissue was incubated in trypsin and DNase for 20 minutes at 37°C followed by a further 5 minute incubation in trypsin inhibitor. Tissue was then washed in DMEM/F-12, centrifuged at 1000rpm for 3 minutes, the remaining pellet was resuspended in 200 $\mu$ l DMEM/F-12 and triturated 10-15 times to generate a single-cell suspension which was counted, and viability was determined using trypan blue exclusion.

### *Neural transplantation*

Adult female C57Bl6/J mice received a unilateral QA lesion of the right striatum. Two weeks after lesioning mice received transplants of: (i) day 16 *Foxg1Z* NEBs untreated (FGF2 alone) (n = 8), (ii) day 16 *Foxg1Z* NEBs purmorphamine treated (FGF2 and purmorphamine) (n = 8), (iii) LGE (n = 8), (iv) MGE (n = 8) and (v) WGE (n = 8), into the lesioned striatum. Lesions and grafts were carried out as described previously in Chapter 2.

### *Histology and Immunohistochemistry*

At 6 and 12 weeks post-transplantation animals were transcardially perfused and brains were taken for histological analysis. Brains were sectioned coronally on the freezing-stage microtome at 40 $\mu$ m. 1:12 series' were processed for Nissl staining using cresyl violet, histochemical staining for AChE, and further 1:12 series' were processed for immunohistochemistry with anti- $\beta$ -galactosidase (1:1000), anti-NeuN (1:4000), anti-DARPP-32 (1:10,000) (a gift from Paul Greengard), anti-FoxP1 (1:500), anti-calbindin (1:20,000) and anti-parvalbumin (1:4000).

The immunohistochemical staining protocol was the same for each antibody, as described in Chapter 2. Antibody staining was visualised using the Vector SG kit, and to determine graft area in stained sections, a Nissl stain was performed using cresyl violet, since neither  $\beta$ -galactosidase nor X-gal were detected.

Controls were carried out in parallel, where the primary antibody was omitted from the procedure.

### *Quantification and Statistical analysis*

Cells expressing markers of interest were counted using a Leitz light microscope and stereology software. Graft volume was determined as described in Chapter 2.

Statistical analyses were performed using two-sample t-tests and one-way ANOVA with Tukey-Kramer *post-hoc* comparisons when appropriate, using Minitab 15 software.

## 7.4 Results

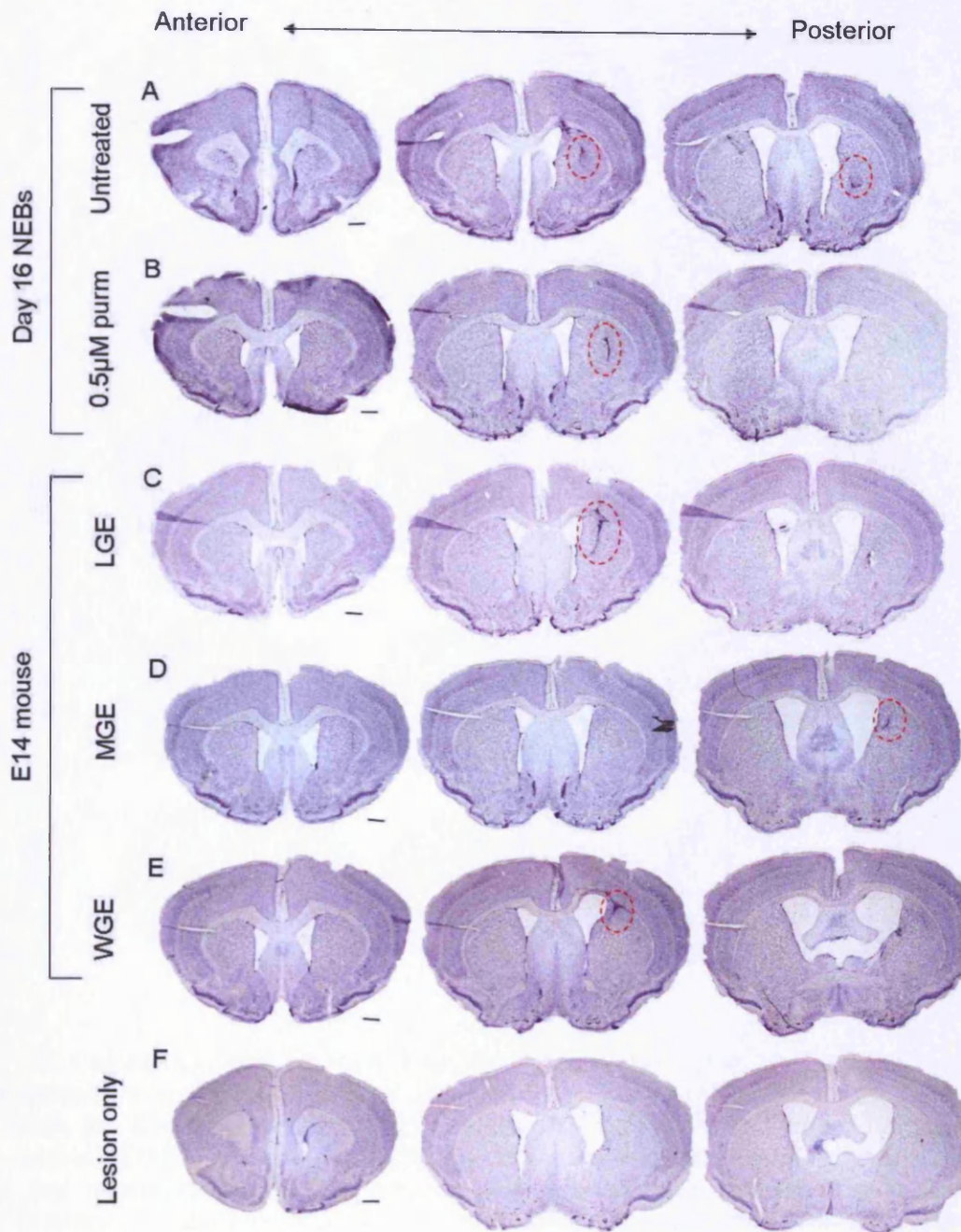
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### *Graft survival and morphology*

NEBs were cultured in neural induction medium and at day 4 addition of either 20ng/ml FGF2 alone (day 16 NEBs untreated), or 20ng/ml FGF2 and 0.5 $\mu$ M purmorphamine (day 16 NEBs purmorphamine treated) (see Chapter 5), which was then maintained to day 16 when NEBs were harvested for transplantation. E14 mouse LGE, MGE and WGE were dissected, dissociated and transplanted as primary tissue, without expansion. Adult mice received unilateral QA lesions to the right striatum and two weeks later day 16 NEBs or E14 mouse striatal cells were transplanted into the lesion site. Prior to transplantation cell viability was determined using the trypan blue exclusion method: (i) day 16 NEBs untreated 80.99%; (ii) day 16 NEBs purmorphamine treated 78.85%; (iii) E14 mouse LGE 91.05%; (iv) E14 mouse MGE 92.19% and (v) E14 mouse WGE 91.18%. Grafts were allowed to survive for 6 and 12 weeks post-transplantation.

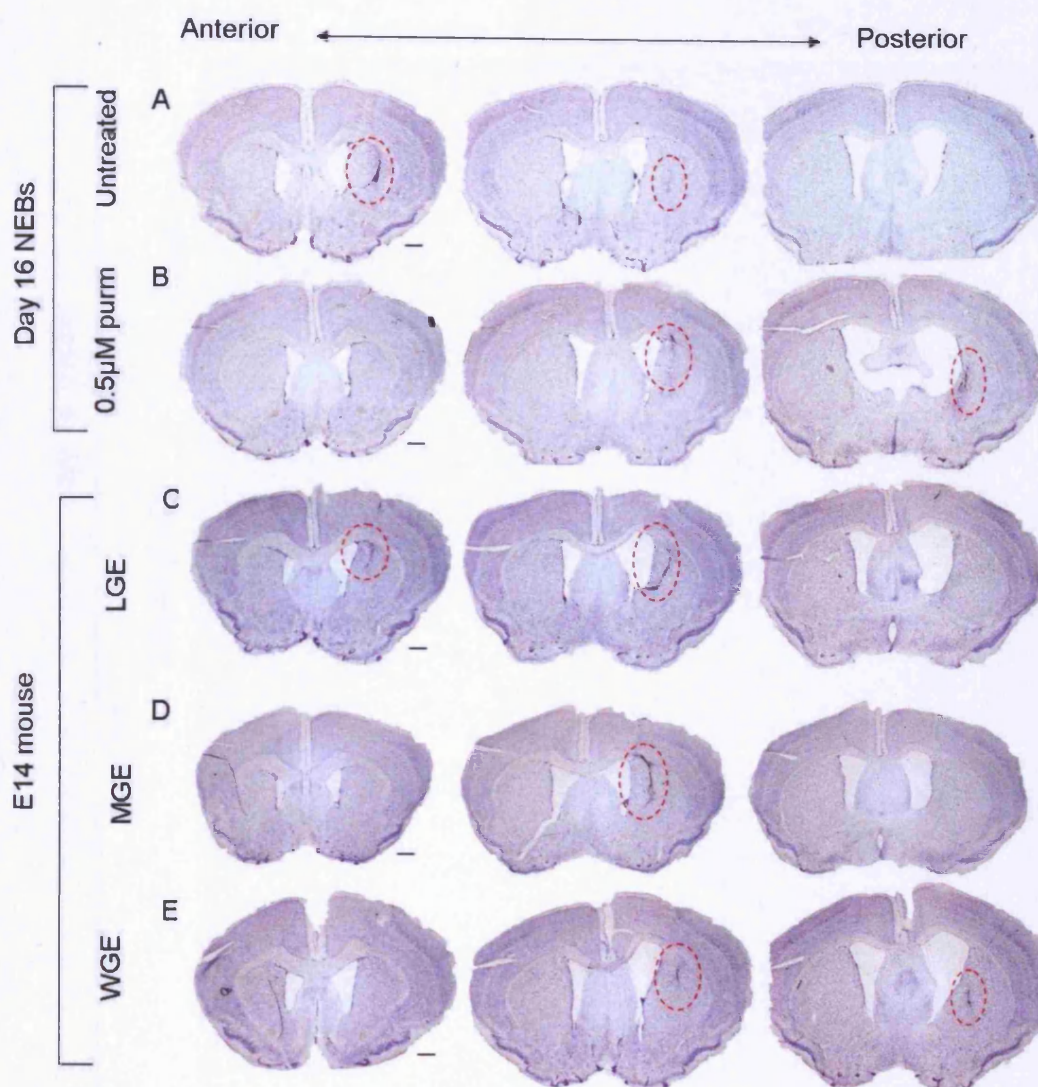
Nissl staining using cresyl violet revealed the presence of very small, pencil-like grafts in all transplant groups at 6 weeks (Figure 7.1) and 12 weeks (Figure 7.2) post-transplantation. Grafted tissue can be identified by increased density in Nissl staining in the right striatum. The QA lesion resulted in enlargement of the right ventricle caused by cell death in the lesioned striatum. Higher power images of Nissl staining further demonstrated the small grafts and revealed the notable presence of dead cells within the graft areas (Figure 7.3). Graft volume was estimated using graft area as determined by Nissl staining (Figure 7.4), but there was no significant difference between groups at 6 weeks ( $F_{4,20} = 0.68$ ,  $p = \text{n.s.}$ ) or 12 weeks ( $F_{4,19} = 1.79$ ,  $p = \text{n.s.}$ ). There were also no significant differences between the two survival time points for each group; day 16 NEBs untreated ( $t_7 = 0.12$ ,  $p = \text{n.s.}$ ); day 16 NEBs purmorphamine treated ( $t_7 = 0.71$ ,  $p = \text{n.s.}$ ); E14 mouse LGE ( $t_7 = 1.08$ ,  $p = \text{n.s.}$ ); E14 mouse MGE ( $t_7 = 0.18$ ,  $p = \text{n.s.}$ ) and E14 mouse WGE ( $t_6 = 0.57$ ,  $p = \text{n.s.}$ ).

Interestingly, however, combining the transplant groups to make two groups (day 16 NEBs versus E14 mouse) for analysis of graft volume revealed a significant difference, with E14 mouse derived grafts ( $1.00 \pm 0.15\text{mm}^3$ ) generating significantly bigger grafts than day 16 NEB derived grafts ( $0.52 \pm 0.08\text{mm}^3$ ) ( $t_{39} = 2.41$ ,  $p < 0.05$ ) (Figure 7.5).



**Figure 7.1.** Coronal sections of Nissl staining, using cresyl violet, at 6 weeks post-transplantation of transplant groups: Day 16 untreated NEBs (A), Day 16 purmorphamine treated NEBs (B), E14 mouse LGE (C), E14 mouse MGE (D), E14 mouse WGE (E) and QA lesion only (F), in mouse striatum. Grafted tissue is identified by increased density in staining in the right striatum. One representative brain per group is shown in each row, and left to right shows anterior to posterior positioning in the brain. Red dotted lines outline graft area. Scale bars = 500µm. Abbreviations: purm – purmorphamine; LGE – lateral ganglionic eminence; MGE – medial ganglionic eminence; WGE – whole ganglionic eminence.

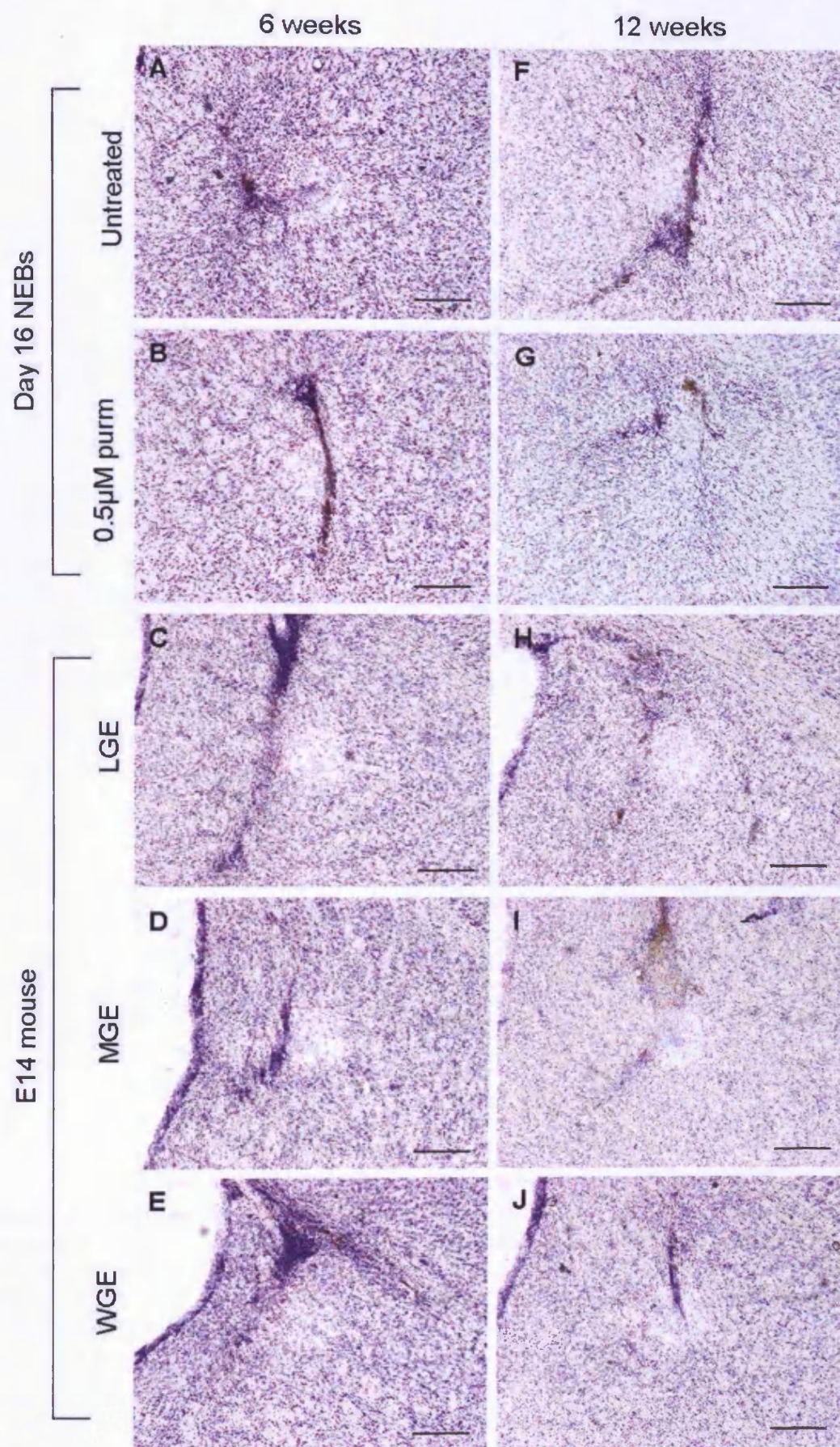


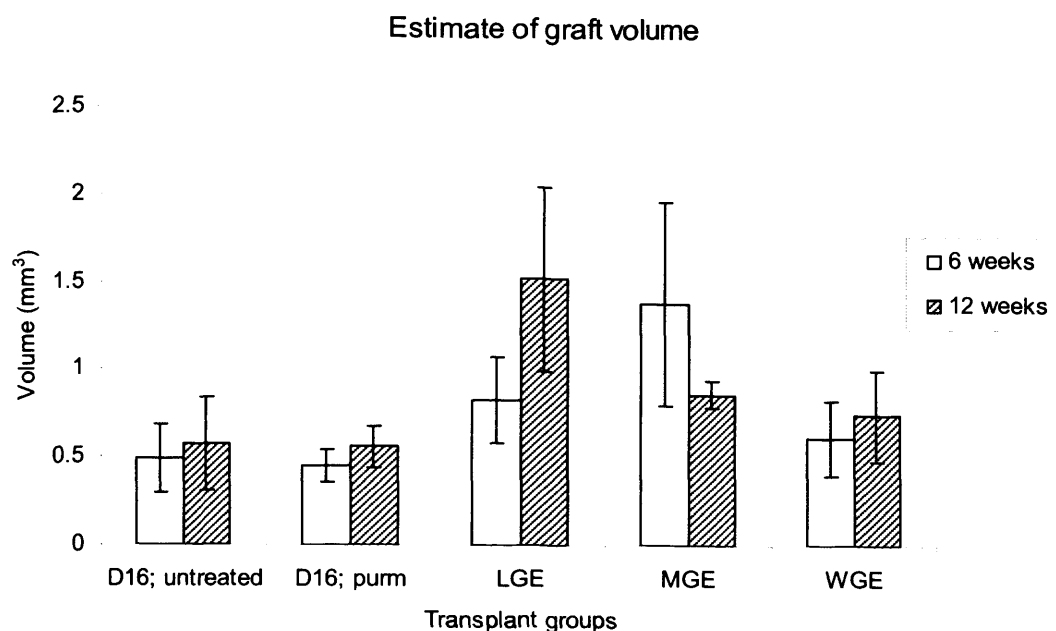


**Figure 7.2.** Coronal sections of Nissl staining, using cresyl violet, at 12 weeks post-transplantation of transplant groups: Day 16 untreated NEBs (A), Day 16 purmorphamine treated NEBs (B), E14 mouse LGE (C), E14 mouse MGE (D) and E14 mouse WGE (E), in mouse striatum. Grafted tissue is identified by increased density in staining in the right striatum. One representative brain per group is shown in each row, and left to right shows anterior to posterior positioning in the brain. Red dotted lines outline graft area. Scale bars = 500 $\mu$ m. Abbreviations: purm – purmorphamine; LGE – lateral ganglionic eminence; MGE – medial ganglionic eminence; WGE – whole ganglionic eminence.

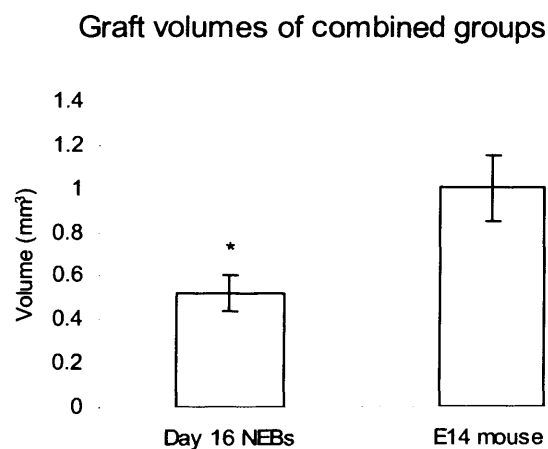
**Figure 7.3 (overleaf).** Photomicrographs showing higher power images of Nissl staining at 6 weeks (A-E) and 12 week (F-J) post-transplantation following transplants of day 16 NEBs or E14 mouse tissue, into the QA-lesioned mouse striatum. There was a notable presence of dead cells within the grafts (seen as brown cells). Scale bars = 200 $\mu$ m. Abbreviations: purm – purmorphamine; LGE – lateral ganglionic eminence; MGE – medial ganglionic eminence; WGE – whole ganglionic eminence.







**Figure 7.4.** Graft volumes were estimated using areas determined with Nissl staining. Each bar on the graph represents a mean of 4 different animals and error bars represent SEM. Abbreviations: purm – purmorphamine; LGE – lateral ganglionic eminence; MGE – medial ganglionic eminence; WGE – whole ganglionic eminence.



**Figure 7.5.** Estimation of graft volumes of combined transplant groups. Day 16 NEBs includes day 16 NEBs untreated and day 16 NEBs purmorphamine treated grafts at 6 and 12 weeks survival ( $n = 16$ ); E14 mouse includes LGE, MGE and WGE grafts at 6 and 12 weeks survival ( $n = 23$ ). Bars on the graph represent mean graft volumes for the combined groups and error bars represent SEM. (\* $p < 0.05$ ).

*Neuronal analysis of grafts*

The day 16 NEB donor cells were derived from the Foxg1Z mouse ES cell line and therefore contained the *LacZ* reporter gene, which would enable post-transplantation identification of Foxg1 positive cells (as in Chapter 4). However, in this instance, neither the antibody against  $\beta$ -galactosidase nor the X-gal histochemical reaction resulted in detection of any positive cells in the NEB-derived grafts. All analysis was therefore carried out using Nissl staining to mark the graft area, and without being able to positively identify cells as graft or host derived, counts represent cell numbers present within this area.

Grafts were analysed for expression of the neuronal marker NeuN (Figure 7.6; A-F and Figure 7.7). NeuN positive cells were seen within the graft areas of those grafts derived from day 16 NEBs and E14 mouse striatum (Figure 7.6; A-F). There was no significant difference in NeuN expression within grafts between transplant groups at 6 weeks ( $F_{4,20} = 1.24$ ,  $p = \text{n.s.}$ ) or 12 weeks ( $F_{4,20} = 0.47$ ,  $p = \text{n.s.}$ ) post-transplantation.

Grafts were further analysed for striatal markers DARPP-32, FoxP1, parvalbumin and calbindin (Figure 7.6 and 7.7). DARPP-32 positive neurons were rarely seen within the graft areas of day 16 NEB-derived grafts, with immuno-positive cells detected at the graft periphery (Figure 7.6; G-L). E14 mouse striatum-derived grafts exhibited more frequent DARPP-32 positive neurons within the grafted area than the day 16 NEB-derived grafts, and there were almost no DARPP-32 positive cells detected in the lesion area of the QA-lesion only brains. Expression of DARPP-32 was not significantly different between transplant groups at 6 weeks post-transplantation ( $F_{4,20} = 0.21$ ,  $p = \text{n.s.}$ ), but there was a significant difference between transplant groups at 12 weeks post-transplantation ( $F_{4,19} = 4.05$ ,  $p < 0.05$ ) (Figure 7.8; A). *Post-hoc* comparisons revealed a significant difference between grafts from day 16 untreated NEBs and grafts from E14 mouse WGE.

FoxP1 expression was detected in grafts derived from both day 16 NEBs and E14 mouse striatum (Figure 7.6; M-R). FoxP1 immuno-positive cells were occasionally seen within the lesion only site, and the presence of large numbers of FoxP1 positive cells clearly marked the boundary of the QA lesion. Expression of FoxP1 was not significantly different between transplant groups at 6 weeks ( $F_{4,20} = 0.17$ ,  $p = \text{n.s.}$ ) or 12 weeks ( $F_{4,19} = 0.54$ ,  $p = \text{n.s.}$ ) post-transplantation (Figure 7.8; B).



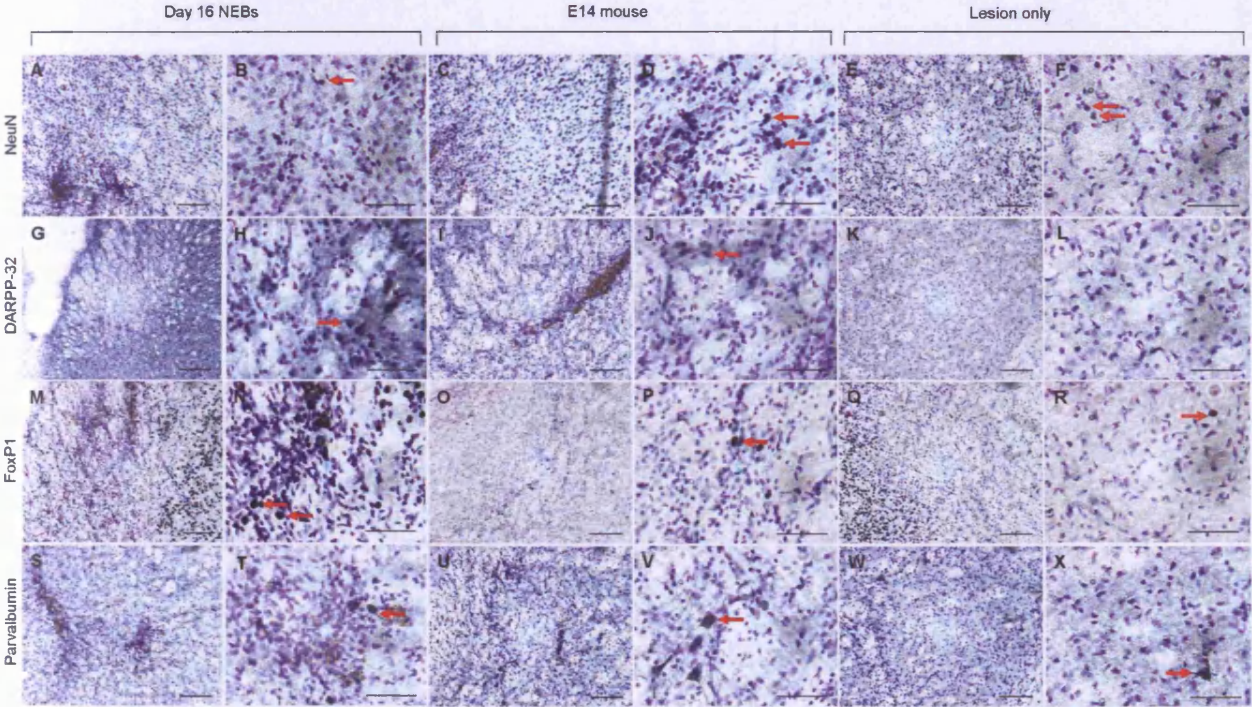
Parvalbumin positive cells were detected within the grafted area of all transplant groups, and were occasionally seen within the lesion area of the QA lesion only brains (Figure 7.6; S-X). Expression of parvalbumin was not significantly different between transplant groups at 6 weeks ( $F_{4,20} = 0.08$ ,  $p = \text{n.s.}$ ) or 12 weeks ( $F_{4,19} = 0.80$ ,  $p = \text{n.s.}$ ) post-transplantation (Figure 7.8; C); and the same was true for calbindin expression; 6 weeks ( $F_{4,20} = 0.21$ ,  $p = \text{n.s.}$ ) and 12 weeks ( $F_{4,19} = 0.33$ ,  $p = \text{n.s.}$ ) (Figure 7.8; D).

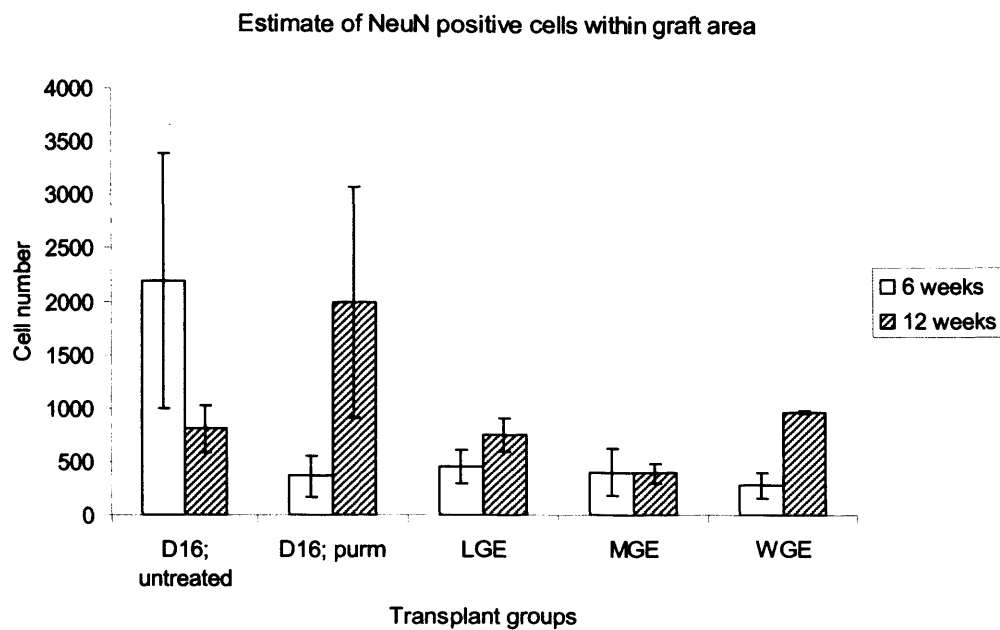
Since the graft sizes were so small AChE staining failed to show any patch-matrix structure and did not demonstrate any differences between transplant groups (Figure 7.10).

When transplant groups were combined (day 16 NEBs versus E14 mouse), there was no significant difference in expression of NeuN positive cells, due to high standard deviation within the day 16 NEB graft group ( $t_{34} = 0.90$ ,  $p = \text{n.s.}$ ) (Figure 7.10). There was also no significant difference in expression of FoxP1 ( $t_{37} = 0.51$ ,  $p = \text{n.s.}$ ), parvalbumin ( $t_{38} = 0.53$ ,  $p = \text{n.s.}$ ) and calbindin ( $t_{37} = 0.67$ ,  $p = \text{n.s.}$ ) between the two groups when data were combined (Figure 7.10). DARPP-32 expression was significantly different between the two groups, with more DARPP-32 present in E14 mouse grafts than Day 16 NEB graft ( $t_{37} = 2.96$ ,  $p < 0.05$ ).

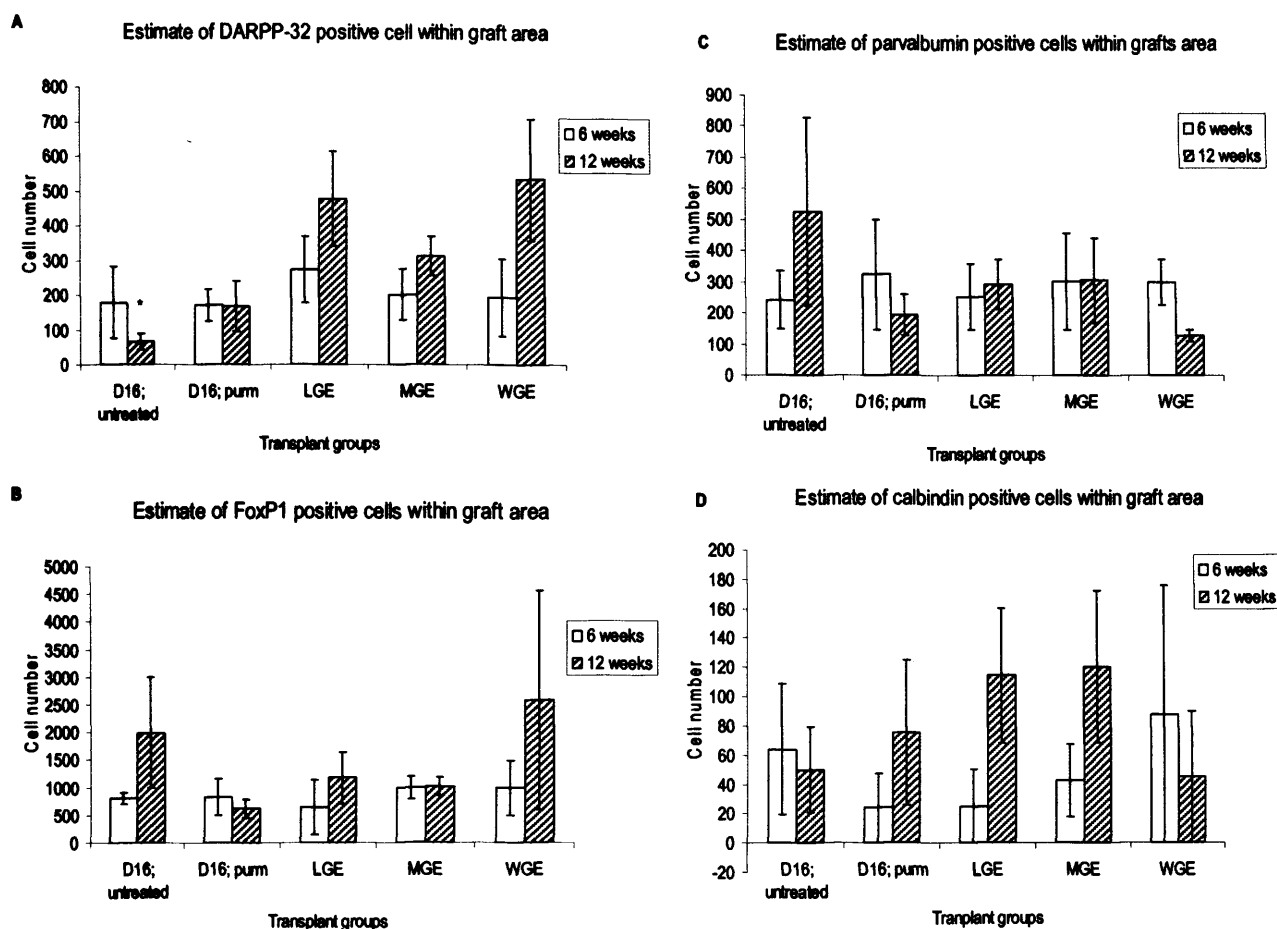
A summary of graft volumes and cell numbers of the various phenotypes found within graft areas is shown in Appendix 8.

**Figure 7.6. (overleaf).** Photomicrographs showing NeuN (A-F), DARPP-32 (G-L), FoxP1 (M-R) and parvalbumin (S-X) staining (grey), double-labelled with cresyl violet (purple). Red arrows indicate cells positive for the marker of interest. Scale bars = 100 $\mu\text{m}$  for lower power images and 50 $\mu\text{m}$  for higher power images.



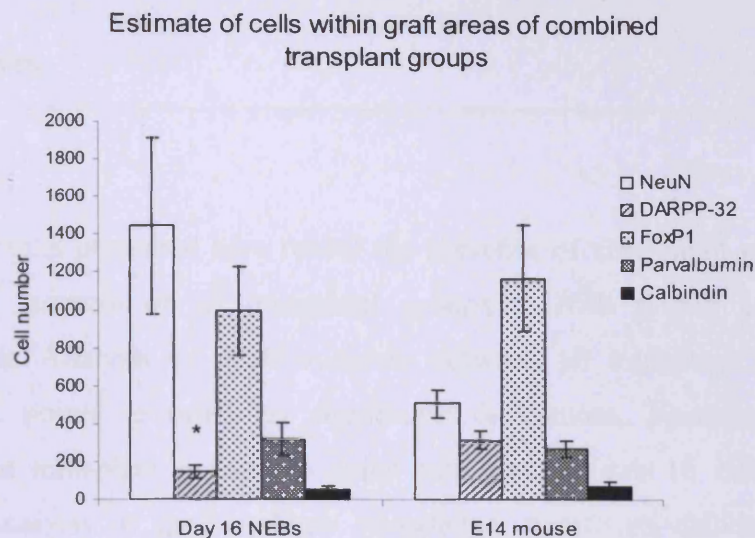


**Figure 7.7.** NeuN expression within graft areas. Each bar on the graph represents a mean of 4 different animals and error bars represent SEM. Abbreviations: purm – purmorphamine; LGE – lateral ganglionic eminence; MGE – medial ganglionic eminence; WGE – whole ganglionic eminence.

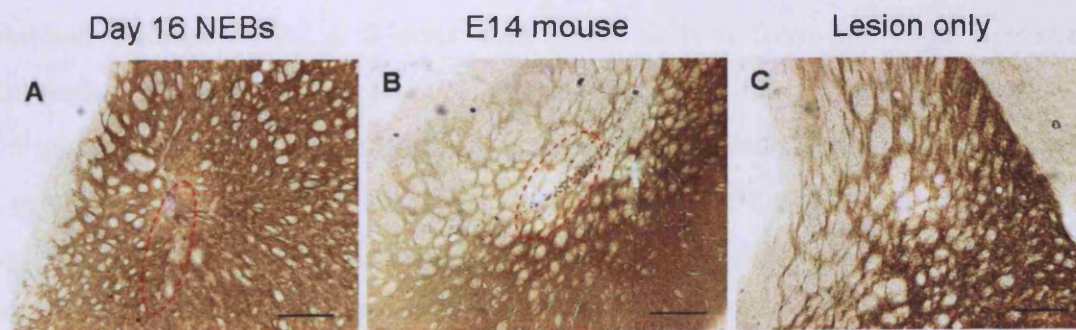


**Figure 7.8.** Expression of DARPP-32 (A), FoxP1 (B), parvalbumin (C) and calbindin (D) within graft areas. Each bar on the graph represents a mean of 4 different animals and error bars represent SEM. (\* $p < 0.05$ ). Abbreviations: pum – pumorphamine; LGE – lateral ganglionic eminence; MGE – medial ganglionic eminence; WGE – whole ganglionic eminence.





**Figure 7.9.** Estimation of cell numbers of markers of interest within graft areas, of combined transplant groups. Day 16 NEBs includes day 16 NEBs untreated and day 16 NEBs purmorphamine treated grafts at 6 and 12 weeks survival ( $n = 16$ ); E14 mouse includes LGE, MGE and WGE grafts at 6 and 12 weeks survival ( $n = 23$ ). Bars on the graph represent mean graft volumes for the combined groups and error bars represent SEM. (\* $p < 0.05$ ).



**Figure 7.10.** Photomicrographs of AChE staining in the transplant groups (A and B) and the QA lesion only brain (C). Scale bars = 200µm.

## 7.5 Discussion

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### *Graft volume*

The results presented here reveal the presence of very small grafts within the QA lesioned striatum of all transplant groups at both 6 and 12 weeks post-transplantation. Analysis of graft volumes between all transplant groups at both survival time points revealed no significant differences. Interestingly, however, combining the transplant groups to make two groups (day 16 NEBs versus E14 mouse) for analysis of graft volume revealed a significant difference, with E14 mouse-derived grafts generating significantly bigger grafts than day 16 NEB-derived grafts. It is, however, encouraging to note the absence of any teratoma formation in the day 16 NEB-derived grafts. This supports previous findings reported in Chapter 4 where we demonstrated no teratoma formation following transplantation of day 16 NEBs into the rat QA lesioned striatum.

Dihne et al (2006) reported graft volumes of up to  $26\text{mm}^3$  in grafts derived from early stage neural precursors, with high expression of nestin and BrdU, that generated tumours. Early stage precursor grafts that did not generate tumours exhibited volumes of  $2.2 \pm 0.3\text{mm}^3$  and grafts derived from late stage precursors exhibited volumes of  $1.6 \pm 0.3\text{mm}^3$  (Dihne et al., 2006). Striatal grafts derived from E14 mouse WGE generated volumes of  $0.80 \pm 0.09\text{mm}^3$  when cells were transplanted as primary tissue, and  $0.88 \pm 0.87\text{mm}^3$  when cells were expanded in culture for 10 days prior to transplantation (Kelly et al., 2007). The graft volumes that we report here in all transplant groups are comparable with those reported in these studies and overall suggest a potential caveat regarding graft survival and development of mouse-to-mouse transplantation. Thus these small graft volumes are not necessarily an indicator of failed grafts, since the 'gold standard' of primary tissue has produced equally small grafts.

Immuno-suppression was not employed in our study because of the assumption that it is not required in allograft studies. The Foxg1Z mouse ES cell line used in our study was derived using mice of the C57Bl6/J (*Black6*) background strain. To bypass potential graft rejection issues due to strain differences, we used C57Bl6/J

mice as our transplant hosts and the E14 striatal tissue was taken from embryos of C57Bl6/J time-mated mice.

One study which appears to contradict this problem of small-volume mouse-to-mouse grafts looked at transplantation of E13-14 mouse LGE into the striatum of the transgenic HD mouse model R6/2 (Dunnett et al., 1998). Photomicrographs representing Nissl staining of selected grafts, demonstrated the presence of large grafts in both the wild-type control striatum and the transgenic striatum following 6 weeks survival post-transplantation. However, no graft volumes were reported in this study, which would indicate whether the grafts presented were representative of the whole cohort. The small, pencil-like grafts reported in this chapter are not unique to this study and have been demonstrated by other colleagues using the mouse-to-mouse system within our group, indicating a major limitation for further studies using this paradigm.

#### *Identification of donor cells post-transplantation*

The Foxg1Z mouse ES cell line used in this study has the *LacZ* reporter gene incorporated so that it is under the control of the Foxg1 promoter and therefore when cells express Foxg1, expression of *LacZ* is switched on. *LacZ* can be detected using antibodies against  $\beta$ -galactosidase, or the histochemical X-gal assay. Following the demonstration of this as a marker of cells post-transplantation in Chapter 4, the aim was to apply this strategy here and again identify the Foxg1-expressing cells and analyse them for co-expression of markers of interest. However, in this instance neither method proved successful, and no positive cells could be identified in the day 16 NEB-derived grafts. One possibility is that there are no Foxg1-expressing cells in the grafts which could be due to poor graft survival and development, as indicated by the Nissl staining. Another possible explanation is that Foxg1 might have been switched off, as cells have developed beyond the stage at which they would express Foxg1. However, in Chapter 4 Foxg1 was widely expressed throughout the graft areas at 6 weeks post-transplantation and expression was demonstrated following differentiation of cells both *in vitro* and *in vivo* (see Chapter 4).

Dihne et al (2006) used a transgenic mouse ES cell line that expressed enhanced green fluorescent protein (eGFP) under the control of the  $\beta$ -actin promoter, which yields ubiquitous expression of GFP, and were able to detect GFP-expressing cells at 16 weeks post-transplantation. The use of the GFP transgene reporter for post-

transplantation analysis of primary mouse striatal grafts has demonstrated down-regulation of the GFP between 2 and 4 weeks post-transplantation (Eriksson et al., 2003), with further down-regulation observed up to 12 weeks (Kelly et al., 2007).

Visualisation of human neural progenitor cells and human ES cell-derived neural precursors post-transplantation has been demonstrated using pre-labelling incubation with the DNA-specific fluorochrome bisbenzimidazole (Hoechst) (Aleksandrova et al., 2001; Song et al., 2007). However, in both of these reports only short-term survival post-transplantation was assessed (up to 3 weeks) and therefore more long-term analyses are required to determine if this is a viable option for long-term visualisation of cells post-transplantation. Prior to transplantation cells can be incubated with viral vectors, such as the LacZ virus, which will label a proportion of the cells and these can later be visualised using antibodies against  $\beta$ -galactosidase. This has previously been demonstrated for both mouse and human foetal precursor cells (Kelly et al., 2007).

### *Neuronal analysis of grafts*

As a result of not being able to positively identify any graft-derived cells, all analysis was carried out using the Nissl stain to mark the area of the graft and cell counts are estimated as cell numbers present within graft area. There was no significant difference in NeuN positive cells between the transplant groups and even when transplant groups were combined (day 16 NEBs versus E14 mouse) there was no significant difference (Figure 7.10). There was also no significant difference in expression of FoxP1, parvalbumin and calbindin between all transplant groups and when data were combined (Figure 7.10). The high degree of deviation of the cell numbers of the various phenotypes within the transplant groups, and the small numbers of animals within each group, reduce the likelihood of a statistically significant outcome.

DARPP-32 expression was not significantly different at 6 weeks post-transplantation between any of the transplant groups. However at 12 weeks post-transplantation, there were significantly more DARPP-32 positive neurons within the graft area of E14 mouse WGE-derived grafts than day 16 untreated NEB-derived grafts. To date DARPP-32 expression has not been reported in mouse ES cell-derived neural precursors following transplantation, although graft-derived neurons expressing  $\beta$ -III-tubulin, neurofilament and GAD65/67 have been detected at 16



weeks post-transplantation (Dihne et al., 2006) and mouse ES cell-derived neural precursors transplanted into the rat QA lesioned striatum generated neurons expressing GABA and AChE following 6 weeks survival *in vivo* (Dinsmore et al., 1996). The demonstration of no significant difference in neuronal yield between all transplant groups is a somewhat remarkable and encouraging result for the potential of ES cells in cell-replacement therapies. Although we cannot positively distinguish between graft and host-derived cells, it is possible that at least a proportion of the DARPP-32 positive neurons observed within the graft area are graft-derived, or host-derived but as a result of the grafted cells, since there are very few DARPP-32 positive neurons present in the lesion only striatum.

Transplantation studies looking at developing mouse primary striatum grafted into the adult rat QA-lesioned striatum show DARPP-32 expression at 2 weeks post-transplantation (Olsson et al., 1995). Olsson et al (1995) looked at grafts derived from E14 mouse MGE versus E14 mouse LGE, and at 2 weeks post-transplantation there was no significant difference in graft volume, but whereas in the MGE-derived grafts only 5% DARPP-32 positive cells were observed, in the LGE-derived grafts this increased to about 50% of the cross-sectional area.

Striatal tissue grafts are made up of two distinct regions or zones. The P-zones comprise DARPP-32 and AChE positive neurons and these areas 'intermingle' with regions called NP-zones that don't stain for striatal markers (Graybiel et al., 1989). In WGE grafts, P-zones are usually about one third of the total graft, whereas in LGE-alone grafts, P-zones can go up to 80-90% of graft (Pakzaban et al., 1993). LGE allografts of rat tissue at two to three months post-transplantation comprise AChE-positive regions, where DARPP-32 positive cells are also detected, interspersed with few AChE-poor regions (Deacon et al., 1994). Regarding behavioural effects of striatal grafts, there is correlation between the proportion of 'striatal-like' cells within grafts and the extent of recovery observed in paw-reaching tasks (Nakao et al., 1996).

In our study, transplantation of E14 mouse LGE, MGE and WGE into the QA-lesioned mouse striatum, did not result in a significant difference in DARPP-32 expression between any of the striatal graft-derived regions. Also AChE staining shown in our study did not reveal any clear differences between transplant groups as was expected from the reports discussed previously.

### *Conclusions*

This chapter was an exploratory study which aimed to compare day 16 NEB-derived grafts with E14 mouse striatum-derived grafts over two survival time points. The proportion of neurons in day 16 NEB-derived grafts was comparable to E14 striatum-derived grafts. At 12 weeks post-transplantation there were significantly more DARPP-32 positive neurons in WGE-derived grafts than untreated day 16 NEB-derived grafts. However, DARPP-32 expression was not significantly different between purmorphamine-treated day 16 NEB-derived grafts and striatal-derived grafts, indicating the potential for ES cell-derived NEBs in cell-replacement strategies.

However, not only were the graft sizes small, but there was also no detection of the Foxg1-LacZ positive cells post-transplantation. Full confidence that cells were derived from the day 16 NEB grafts was therefore not possible, and cell counts could only be represented as number of cells present in the graft area, as determined by Nissl staining.

Work presented in this chapter highlights a potential caveat of the mouse-to-mouse allograft paradigm and suggests the need for a systematic assessment of the mouse striatum as a viable host for transplantation. In addition, reliable methods for identification of all donor-derived cells in order to distinguish them from host-derived cells must be applied in order to determine what is happening to donor cells post-transplantation.

## Chapter 8

### General Discussion

*Neural transplantation in HD aims to replace the neurons that are lost, predominantly the MSNs of the striatum; reconstruct the affected circuitry; alleviate the associated symptoms; and possibly slow the disease progression. Primary foetal striatal tissue is currently the 'standard' cell source for transplantation and is the origin of pre-programmed MSN precursors. However, tissue from this source is limited and raises ethical and logistical constraints suggesting the need for an alternative supply of donor cells. The requirement of any alternative donor cell source is to have the capability to generate the specific mature MSN phenotype, which could be utilised in cell-replacement strategies and for screening potential drug candidates.*

*This thesis looked at mouse ES cells as a model system for neural induction and directed differentiation of neurally induced ES cells to enrich for a population of ventral telencephalon-like cells which could subsequently, under optimal terminal differentiation conditions, generate striatal projection neurons, both in vitro and in vivo.*

#### 8.1 In vitro neural induction of mouse ES cells

Mouse ES cells offer a potential alternative source of donor cells for transplantation strategies in neurodegenerative diseases and have been shown to generate neural precursors *in vitro* when cultured under defined serum-free conditions, with the formation of NEBs that express markers of the neuroectoderm including Sox1 and Pax6 (Ying et al., 2003; Bouhon et al., 2005; Watanabe et al., 2005). Not only are mouse ES cells a robust model for assessing development, but they can also be easily manipulated allowing incorporation of reporter genes which can readily permit visualisation of markers of interest. Work in this thesis utilised a mouse ES cell line, Foxg1Z, where the reporter gene *LacZ* was integrated into the genome replacing most of the *Foxg1* coding sequence so that expression of  $\beta$ -galactosidase is under the control of the *Foxg1* promoter (Xuan et al., 1995). *Foxg1* is

the earliest known marker of the telencephalon (Tao and Lai, 1992) and the focus of Chapter 3 was to generate optimal numbers of *Foxg1*-expressing cells in the neural induction culture system, which would then be taken forward in subsequent chapters for further directed differentiation and terminal neuronal differentiation *in vitro* and *in vivo*. In Chapter 3, it was demonstrated that addition of FGF2 to neural induction cultures of mouse ES cells at day 4 results in an increase in *Foxg1* positive cells when analysed at day 8, which was consistent with data from a previous study (Bouhon et al., 2006). Addition of the Wnt inhibitor DKK-1 did not result in an increase in the *Foxg1* population which was surprising when compared with other reports (Aubert et al., 2002; Watanabe et al., 2005; ten Berge et al., 2008). However, interpretation of the results reported in Chapter 3 indicated some fundamental discrepancies between studies including culture systems and cell lines utilised, as well as composition of defined medium. As a result of the heterogeneity of the neural induction protocols applied, drawing comparisons between the findings in each study is not always straight forward. Differences in protocols can result in different outputs of expression. The presence of different components endogenously in culture medium alters the conditions, which might have a knock-on effect on expression of genes, since specific conditions might be required for induction of certain markers. Ideally, in order to achieve a more reliable and reproducible method for the neural induction of ES cells, standardisation of protocols is required, which is not a realistic option at this stage since protocols are still being developed. It is therefore important to approach similarities and differences with caution and with the mind-set that reported findings are restricted to each individual culture system. A comparison of different protocols in carefully controlled conditions would give a reliable indication and understanding of the differences between the various groups.

A recent comparison of gene expression profiles in three different mouse ES cell lines, revealed that undifferentiated ES cells in their pluripotent state, derived from different mouse strains (i.e. different genetic backgrounds) and carrying different genetic modifications exhibit almost invariant patterns of gene expression (Mansergh et al., 2009). However, it is possible that even slight deviations in gene expression early on in development might be responsible for more significant differences that occur later in development. Later analysis of gene expression using an EB-based method of ES cell differentiation, demonstrated a high degree of variability between EBs, even when the starting number of cells was controlled using the

'hanging drop' protocol, and even in those EBs generated within the same culture (Mansergh et al., 2009). Application of a similar strategy to analyse NEB development in defined suspension conditions would enable the gain of more fundamental understanding of this culture system.

Another potential cause of the differences in gene expression observed between neural induction cultures derived from mouse ES cells, both within different studies and within experiments, is the stage of ES cell growth at which cells are transferred from ES cell culture conditions to neural induction conditions. In general, for maintenance of mouse ES cells, cells are cultured on gelatin and initially plated as a crude single cell suspension. Then, as cells divide and proliferate, they form colonies of daughter cells that gradually increase in size over time in culture, and are typically passaged and replated every two to three days, depending on the cell line. Observations made while culturing mouse ES cells indicate that relatively early passage, rather than later in this maintenance period, helps to maintain 'neat' ES cell colonies with few undifferentiated ES cells. It would be useful to determine if initiation of neural induction (or indeed induction of any other lineage) at different phases of ES cell growth alters subsequent differentiation of cells towards that lineage. This information might prove valuable in the generation of more reproducible protocols for lineage induction.

## *8.2 Transplantation of mouse ES cells*

In mouse ES cell cultures with defined neural induction conditions, neural precursor markers, such as nestin, are initially shown to up-regulate over time through to day 8, and then decrease with continued culture (Bouhon et al., 2005), while the ES cell marker, Oct-3/4, down-regulates over time in culture (Bouhon et al., 2005; Bouhon et al., 2006). Although this was also demonstrated in Chapter 4, Oct-3/4 was seen to persist beyond day 8 with very low expression present even at day 12, and it was switched off by day 16. The persistence of undifferentiated ES cells in the cell population presents problems when considering cells for transplantation and one stigma attached to transplantation of ES cells is their potential for uncontrolled proliferation. Alongside encouraging observations of graft-derived differentiation to neurons and glia (Benninger et al., 2000) and region-specific neural markers (Wernig et al., 2004), development of non-neural tissue clusters and teratomas has been

reported following transplantation of EBs and NEBs derived from mouse ES cells (Brustle et al., 1997).

The work presented in Chapter 4 demonstrates the problem of transplanting ES cells prematurely, when a proportion of cells still express ES-like and pluripotent markers. Transplants of early-stage NEBs (day 8) generated teratoma-like masses, whereas later-stage NEBs (day 16) generated large grafts that expressed heterogeneous cell types including neurons, but no teratomas.

One way to overcome this might be longer-term culture prior to terminal differentiation and transplantation. However, although this might result in further down-regulation (abolishment) of ES cells, other important markers of interest might also down-regulate over time, since we show that the neural precursor marker nestin down-regulates between day 8 and day 16, and also beyond day 16, at day 24, expression of markers of the ventral telencephalon were decreased to levels below those seen at day 8 (data not shown).

Other strategies involve purification of cell populations using techniques such as FACS and MACS, where cells can be sorted according to expression markers of interest, either by labelling with cell surface antibodies or integrating reporter genes tagged to the specific cells of interest. An advantage of integrating a reporter gene into a cell line, as well as aiding visualisation of a specific gene of interest, enables purification of a population of cells that expresses the gene of interest and is a homogeneous population. The cleavage product of X-gal is non-fluorescent and toxic to viable cells, which deems it unsuitable for FACS analysis. For detection of  $\beta$ -galactosidase activity in living cells, the  $\beta$ -galactosidase substrate, 5-chloromethylfluorescein di- $\beta$ -D-galactopyranoside (CMFDG) (*Molecular Probes, Invitrogen*) that yields a green fluorescent product, has been developed. Using an 'Influx pinocytic cell-loading' reagent, CMFDG can be introduced into living cells without significantly altering normal cell function and will therefore allow FACS analysis of Foxg1Z NEBs, using unstained cells to set the background and auto-fluorescence controls, which should enable a pure population of Foxg1 cells to be obtained. Unfortunately though, when we attempted to apply these techniques to NEBs, recovery of cells following enzymatic and mechanical dissociation, CMFDG incubation and hypotonic shock, followed by FACS collection was not sufficient for continued analysis; single cells did not re-form spheres or aggregates following FACS and viability dramatically decreased so that cells were no longer useable.

Some studies have demonstrated success with FACS purification of cell populations both with mouse and human ES cells, using genetically labelled ES cell lines and cell surface antigens (Pruszek et al., 2007; Hedlund et al., 2008). However, both these studies utilised 'refined' and 'gentle' FACS conditions compared with 'standard' FACS conditions, and for further *in vitro* analysis of cells post-FACS purification, co-culture with primary astrocytes was essential for their survival (Donaldson et al., 2005; Hedlund et al., 2008).

Another approach is to treat cultures in order to eliminate the proliferating cells. Mitomycin C is a chemotherapeutic agent that arrests cell proliferation and has previously been used to treat neurally induced human ES cells prior to transplantation (Sanchez-Pernaute et al., 2005). Although this could prove beneficial for prevention of tumours, it would not however prevent the heterogeneity of lineages that exists in cultures.

### 8.3 Directed differentiation

During neural development, induction and specification of specific cell types within the neural tube requires the presence of certain factors at precise levels in order to generate the full complement of cell types. The practicality of mimicking this *in vitro* is not straight forward, since concentrations and length of exposure time need to be determined. Refinement of factors involved and the windows of exposure for their optimal addition will require systematic studies with sequential application to determine the specific effects and requirements of each factor.

In Chapter 5, we attempted to direct the differentiation of NEBs using the SHH agonist purmorphamine and then the SHH antagonist cyclopamine, since SHH is important in ventralising the telencephalon. The gradient effect of SHH in the developing neural tube suggests that different concentrations of SHH will give rise to expression of various dorso-ventral markers at different levels. Following addition of these two factors to neurally induced mouse ES cells we demonstrated that NEBs are responsive to these factors, but did not observe a distinct pattern of dorso-ventral expression. Dessaud et al, (2007) demonstrated that cells in the neural tube can become desensitised to SHH following long periods of exposure, and this affects the way in which they respond. The desensitisation results in a precise duration and concentration of the SHH signal, which is responsible for the subsequent levels of

gene expression (Dessaud et al., 2007). The challenge *in vitro* will be to analyse different exposure times of various SHH concentrations in the culture system, and determine the optimum of each in order to generate increased expression of ventral telencephalic precursors.

Generation of reporter cell lines with reporter genes tagged to ventral telencephalic markers should make for an efficient method of visualising cells of interest. In the lab we have initiated the generation of such cell lines, in particular, the *Dlx1/2* gene tagged to the GFP reporter. This cell line will allow for fluorescent analysis of *Dlx*-expressing cells, which enable simple demonstration of the effects of factors on the *Dlx* population.

It is important to note that even if we generate a population of ventral telencephalic-like precursors, this is not the overall solution, since differentiation of primary striatal precursors both *in vitro* and *in vivo*, does not generate vast quantities of DARPP-32 expressing MSNs, and will likely require some further signalling input to increase this.

#### 8.4 Is generation of MSNs from ES cells achievable?

In Chapter 6 the *in vitro* neuronal differentiation of mouse ES cells was addressed along with differentiation of striatal-derived neurons. The origin of MSNs, the LGE, used as a standard for DARPP-32 differentiation, did not generate vast numbers of DARPP-32 positive neurons. One explanation for this might be that MSN precursors are particularly susceptible to cell death following dissection and dissociation procedures. Another possibility is that, although the precursors are already specified to become MSNs, they still require specific signals to enable differentiation into the mature neuronal phenotype. Further work to identify and apply such signalling factors to striatal differentiation cultures is needed with subsequent application to neuronal differentiation cultures of ES cells to determine if, under the right conditions, these cells have the capacity to generate DARPP-32 positive neurons.

Since we demonstrated an increase in the proportion of DARPP-32 positive neurons following addition of BDNF to LGE-derived neurons, cells could benefit from transplantation with the addition of BDNF to the cell suspension. Delivery of neurotrophic factors, such as GDNF, prior to transplantation in models of PD, has



proved beneficial both by increasing the yield of dopaminergic neurons and protecting the brain from lesion-induced impairments (Torres et al., 2005; Dowd et al., 2005).

If DARPP-32 can be achieved *in vitro*, one strategy might be to pre-differentiate NEBs in neuronal differentiation medium (with inclusion of appropriate signalling factors) prior to transplantation. The degree of pre-differentiation would require optimisation and manipulation of cells might prove challenging, since differentiated neurons exhibiting projections, might be more susceptible to damage.

Presently, DARPP-32 is the standard mature MSN marker employed. To date limited expression of DARPP-32 from ES cells has been reported, and even then only from human ES cell differentiation (Aubry et al., 2008; Nasonkin et al., 2009). The 'Aubry' DARPP-32 positive neurons were derived from cells subjected to a complex sequential addition of factors that were then transplanted into the rat QA lesioned striatum and allowed to differentiate for 4-6 weeks or 13-21 weeks (Aubry et al., 2008). In contrast, the 'Nasonkin' DARPP-32 positive neurons were derived from cells that were subjected to limited factors in culture prior to transplantation and were allowed to differentiate *in vivo* in the unlesioned rat striatum for up to 6 months (Nasonkin et al., 2009). On the one hand, Aubry et al suggest the need for a complex 'cocktail' of factors with specific exposure times in culture followed by a relatively short *in vivo* period, whilst, conversely, Nasonkin et al suggest that a simple culture system but long term differentiation *in vivo* is required for DARPP-32 generation. A combination of the two protocols, with more defined directed differentiation *in vitro* followed by long term differentiation *in vivo* would be an obvious option, although with the longer-term graft survival in the 'Aubry' protocol, massive graft overgrowth was reported, along with the DARPP-32 positive neurons, suggesting that the *in vitro* differentiation phase needs to be refined.

Analysis of differentiation of DARPP-32, and other striatal markers, post-transplantation is necessary to ascertain if the signals from the CNS environment are adequate to generate markers of the desired phenotype. The presence of DARPP-32 in graft-derived cells is encouraging but it does not necessarily mean these neurons will be functional and produce a beneficial behavioural effect. It will be important to determine if neurons within the graft are functional, using electrophysiology, and to assess behavioural recovery of graft recipients using a selection of tests known to be sensitive to the QA lesion model.

### 8.5 Mouse-to-mouse allografts

In this thesis mouse ES cells were utilised, and in Chapter 7 were applied in the mouse-to-mouse allograft paradigm in the QA lesioned striatum model of HD. However, in this study we indicated a potential problem with this system, since only small, pencil-like grafts were identified exhibiting poor development. This finding was not remarkable, and it was later recognised as a recurring problem within our group and others. However, it has not been seen in the rat-to-rat allograft system. Addressing this issue will require a thorough assessment of different combinations of donor and host tissue, taking into account the developing brain region for dissection and donor cells, the species and strain of both donor and host, the state of the host brain (lesioned or non-lesioned), and the administration of immunosuppression.

With the recent emergence of rat ES cell lines, there is the potential for the allograft paradigm to be assessed in the rat system. However, although expression of the immature neuronal marker  $\beta$ -III-tubulin has been demonstrated following differentiation of rat ES cells (Li et al., 2008; Buehr et al., 2008), to date there have been no reports demonstrating specific neural induction of rat ES cells and subsequent directed differentiation. It is possible that induction and differentiation conditions required for rat ES cells will differ from those required for mouse and human ES cells.

### 8.6 Where next?

The aims of this thesis were to attempt to generate DARPP-32 positive neurons from mouse ES cells. However, this was not achieved here, either *in vitro* or *in vivo*. Firstly, re-addressing the initial neural induction stage of mouse ES cell cultures is necessary to attempt to generate a homogeneous population of cells of the neural lineage. The subsequent directed differentiation protocol will then need to be refined, with systematic studies looking at the effects on gene expression of exposure times of the factors added. Effects of SHH in this study were only analysed following chronic administration, and it will be important to dissect the response of NEBs at different SHH concentrations and different exposure times, to try to mimic the actions of SHH in the developing neural tube and generate a range of precursor cell types.

Since foetal striatal tissue is currently the standard used for transplantation in the HD paradigm and this has not been shown to generate vast quantities of DARPP-32 *in vitro* or *in vivo*, it is clear that optimisation of the culture of this source of cells is required. In this study, foetal striatal tissue was used for differentiation only. Expansion of these cells prior to terminal differentiation is an option that has been investigated in attempts to increase cell numbers and therefore reduce the amount of starting material required. However, over time in culture, although overall cell number is increased, the neuronal yield is decreased (Kelly et al., 2003). Addition of factors, such as SHH, might enhance the induction and survival of striatal neurons and therefore increase the yield of DARPP-32 positive neurons following differentiation. If DARPP-32 yield can be optimised from foetal striatal cells by manipulating culture conditions for expansion and differentiation, these conditions can be transferred to ES cells for differentiation.

There are limited reports on the transplantation of ES cells into models of HD and before these cells can be seriously considered for clinical application they need to be assessed in terms of the functional benefit they can offer within the HD brain. It will be important to determine the degree of benefit this cell source can provide with respect to both motor and cognitive tasks in animal models of HD.

### *Concluding remarks*

This thesis highlights some important issues relating to neural induction of ES cells and their capacity to be directed towards more specific precursor types. Achieving a 100% neural population is an absolute requirement before these cells can be seriously considered for clinical application for cell-replacement in HD.

Generation of ventral telencephalic-like precursors with more stringent directed differentiation regimes will take us a step closer, but assessment of neuronal function using electrophysiological techniques, and graft function following transplantation using an array of behavioural tests will be required in order to further this work.

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## Appendices

### *Appendix 1: Reagents and Suppliers*

Reagent	Supplier	Address
Accutase	<i>PAA Labs</i>	<i>Farnborough, Hampshire, UK</i>
Advanced DMEM/F12	<i>Gibco</i>	<i>Paisley, Scotland, UK</i>
B-27	<i>Gibco</i>	
BDNF	<i>Peprotech</i>	<i>New Jersey, USA</i>
B-mercaptoethanol	<i>Sigma</i>	<i>Gillingham, Dorset, UK</i>
BSA	<i>Sigma</i>	
Cyclopamine	<i>Calbiochem</i>	<i>Hull, UK</i>
DKK1	<i>R&amp;D Systems</i>	<i>Abingdon, Oxfordshire, UK</i>
DMEM/F12	<i>Gibco</i>	
DMSO	<i>Sigma</i>	
DNAse	<i>Sigma</i>	
EGF	<i>Sigma</i>	
FCS	<i>Gibco</i>	
FGF2	<i>R&amp;D Systems</i>	
FuGENE 6	<i>Roche</i>	<i>West Sussex, UK</i>
Gelatin	<i>Chemicon</i>	<i>Chandlers Ford, Southampton, UK</i>
HBSS	<i>Gibco</i>	
Hoechst	<i>Fisher</i>	<i>Loughborough, Leicestershire, UK</i>
IMDM	<i>Gibco</i>	
Insulin	<i>Sigma</i>	
KSR	<i>Gibco</i>	
Laminin	<i>Sigma</i>	
L-Glutamine	<i>Gibco</i>	
Lipid concentrate	<i>Gibco</i>	
Lipofectamine	<i>Gibco</i>	
Non-Essential Amino acids (MEM)	<i>Gibco</i>	
Opti-MEM	<i>Gibco</i>	
PBS	<i>Gibco</i>	
Penicillin Streptomycin	<i>Gibco</i>	
PLL	<i>Sigma</i>	
Purmorphamine	<i>Calbiochem</i>	
RA	<i>Sigma</i>	
SYBR Green	<i>Bioline</i>	<i>London, UK</i>
Transferrin	<i>Sigma</i>	
Trypan blue	<i>Sigma</i>	
Trypsin	<i>Worthington</i>	<i>Freehold, New Jersey, USA</i>
Trypsin inhibitor	<i>Sigma</i>	
Trypsin/EDTA	<i>Gibco</i>	



## Appendix 2: Recipes

### 2a) Cell culture

<b>Mouse ES cell culture medium:</b> IMDM 15% KSR 1% PS Non-essential amino acids (1mM) L-glutamine (2mM) $\beta$ -mercaptoethanol LIF	<b>Neuralising medium (ADF+):</b> Advanced DMEM/F-12 1% PS L-Glutamine (2mM) Lipid concentrate Insulin Transferrin $\beta$ -mercaptoethanol
<b>Mouse ES cell freezing medium:</b> 10% DMSO Mouse ES cell culture medium	<b>Neuronal differentiation medium:</b> DMEM/F-12 1% PS 2% B-27 1% FCS
<b>Glial expansion medium:</b> DMEM/F-12 1% PS 10% FCS L-Glutamine (2mM) EGF (20ng/ml)	<b>Glial differentiation medium:</b> DMEM/F-12 1% PS 10% FCS L-glutamine (2mM)
<b>COS cell culture medium</b> DMEM 10% FCS L-Glutamine (2mM) 1% PS	

## 2b) Immunohistochemistry

<b>Prewash buffer</b> 18g di-sodium hydrogen phosphate 9g sodium chloride 1L distilled water pH 7.3	<b>Fixative (4% paraformaldehyde)</b> 40g PFA 1L prewash buffer Heat with addition of NaOH to dissolve pH 7.3
<b>Sucrose</b> 250g sucrose 1L prewash buffer pH 7.3	<b>X-Gal Assay</b> <b>X-Gal solution:</b> 0.5g X-gal 5ml N,N-Dimethylformamide <b>Ferro/Ferri-cyanide solution:</b> 1.050g Ferrocyanide 0.825g Ferricyanide 0.2g MgCl <sub>2</sub> 495ml PBS
<b>4X TBS</b> 48g Trizma base 36g sodium chloride 1L distilled water pH 7.4	} Store at 4°C, dark. Add 1ml X-Gal solution to 49ml Ferro/Ferri-cyanide solution. Fix cells in 1% formalin, 5 minutes. Wash 3x PBS. Add X-gal staining solution, leave at 37°C for 2-4 hours, then transfer to 4°C overnight.
<b>TXTB</b> 0.2% Triton-X100 in 1X TBS pH 7.4	
<b>TBZ</b> 0.02% sodium azide in 1X TBS	
<b>TNS</b> 6g Trizma base 1L distilled water pH 7.4 with HCl	

**Appendix 3: Antibodies for immunohistochemistry and fluorescent immunocytochemistry**

PRIMARY, SECONDARY and BLOCKING SERA									
Primary Antibody	Species	Supplier	Dilution	Normal Serum	Blocking dilution	Normal dilution	Secondary Antibody	Dilution	Comments
Calbindin	Mouse	Sigma	1:20,000	Horse	3%	1%	Horse anti mouse	1:200	2ary rat adsorbed
DARPP-32	Mouse	Cornell Univ	1:10,000	Goat	3%	3%	Goat anti-mouse $\alpha$ 594	1:200	Already diluted 1:50, needs 1:200 dilution
				Horse	3%	1%	Horse anti mouse	1:200	
Doublecortin	Guinea pig	Abcam	1:500	Goat	3%	1%	Goat anti-guinea pig	1:200	Discontinued
FoxP1	Rabbit	Abcam	1:500	Goat	3%	3%	Goat anti-rabbit $\alpha$ 488	1:200	Antigen retrieval required
				Goat	3%	1%	Goat anti-rabbit	1:200	
GABA	Rabbit	Sigma	1:500	Goat	3%	3%	Goat anti-rabbit $\alpha$ 488	1:200	Fix with glutaraldehyde
GFAP	Rabbit	DAKO	1:2000	Goat	3%	3%	Goat anti-rabbit $\alpha$ 488	1:200	
				Goat	3%	1%	Goat anti-rabbit	1:200	
Mash1	Mouse	BD Pharm	1:200	Goat	3%	1%	Goat anti-mouse $\alpha$ 594	1:200	
Nestin	Mouse	BD Pharm	1:400	Goat	3%	1%	Goat anti-mouse $\alpha$ 594	1:200	
NeuN	Mouse	Chemicon	1:4000	Horse	3%	1%	Horse anti mouse	1:200	2ary rat adsorbed
Nkx2.1	Mouse	DAKO	1:100	Goat	3%	1%	Goat anti-mouse $\alpha$ 594	1:200	
Oct-3/4	Mouse	Santa Cruz	1:100	Goat	3%	1%	Goat anti-mouse $\alpha$ 594	1:200	
Otx2	Rabbit	Millipore	1:200	Goat	3%	1%	Goat anti-rabbit $\alpha$ 488	1:200	
Parvalbumin	Mouse	Sigma	1:4000	Horse	3%	1%	Horse anti mouse	1:200	2ary rat adsorbed
Pax6	Mouse	DSHB	1:50	Goat	3%	1%	Goat anti-mouse $\alpha$ 594	1:200	
$\beta$ -galactosidase	Mouse	Promega	1:1000	Goat	3%	3%	Goat anti-mouse $\alpha$ 594	1:200	2ary rat adsorbed
				Horse	3%	1%	Horse anti mouse	1:200	
$\beta$ -III tubulin	Mouse	Sigma	1:1000	Goat	3%	1%	Goat anti-mouse $\alpha$ 594	1:200	
$\beta$ -III tubulin	Rabbit	Sigma	1:500	Goat	3%	1%	Goat anti-rabbit $\alpha$ 488	1:200	

**Appendix 4: QPCR primer sequences**

Primer pairs were generated using:

*Mouse Genome Informatics* (<http://www.informatics.jax.org/>)

*Ensembl* (<http://www.ensembl.org/index.html>)

*Primer 3 Input* ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi))

QPCR primer sequences (designed for melting at 60°C)

Primer	Forward Sequence	Reverse Sequence
Axin2	CCATGACGGACAGTAGCGTA	CTGCGATGCATCTCTCTCTG
Dlx2	GTCTCCGGTCAACAACGAG	CAGGGCCAGATACTGGGT
Emx2	TCTGGGTCATCGCTTCCAAG	CTAGCCTTAAAAGCTGGGACG
Gli3	ATTCCCGTAGCAGCTCTTCA	GGGTAGGTGAAGCTCAATGC
Gsx2	TGGGAGGCTCCGATACCAG	CGGGACAGGTACATATTGGAAGA
Mash1	CTGGGAATGGACTTTGGAAG	TTTTTCTGCCTCCCCATTT
Ngn2	AACTCCACGTCCCCATACAG	GAGGCGCATAACGATGCTTCT
Nkx2.1	GTCTTCGGAAAGACAGCATC	GTGCTTTGGACTCATCGACA
Otx2	CCCGGTACCCAGACATCTT	CGGCACTTAGCTCTTCGATT
Patched1	TCTTGCTCTTGGTGTGTTGGTG	CCCAGTCCTGTCCTCAAATG
Pax6	GTGGCGACTCCAGAAGTTGT	AGAAAGACCCCCTCGGATAA
Shh	CCAATTACAACCCCGACATC	AGAGATGGCCAAGGCATTTA
$\beta$ -actin	GCTCTTTTCCAGCCTTCCTT	CGGATGTCAACGTCACACTT

## ***Appendix 5: Production of Leukaemia Inhibitory Factor (LIF)***

Leukaemia Inhibitory Factor (LIF) is necessary for the culture of mouse embryonic stem (ES) cell lines in order to maintain pluripotency and to prevent differentiation of cells. ES cell lines may be maintained in an undifferentiated state by addition of serum and LIF to the culture medium (Ishiwata et al., 2001). Removal of LIF has been shown to induce ES cell differentiation, as observed by a reduction in Oct 4 expression (Faherty et al., 2005). Addition of LIF to ES cell medium has little effect on growth rate of ES cells, but it does influence the decision of cells to self-renew or differentiate (Zandstra et al., 2000). The aim here was to produce LIF suitable for addition to ES cell medium for maintenance and culture of mouse ES cell lines.

### ***(i) Preparation of LIF plasmid DNA and Transfection***

LIF expression vector was kindly donated by Dr Vladimir Buchman. The expression vector (Ampicillin resistant) was transformed into TOP 10 competent cells, as described in section 2.2.6. The bacterial suspension was inoculated in LB media with ampicillin (150µg/ml). A midi prep was performed following instructions from the Qiagen Midi prep kit (section 2.2.7). The DNA was stored at -20°C in TE at 1µg/µl until use.

For transfection of LIF plasmid DNA, COS cells were used and transfection reagent FuGENE 6 was used according to instructions in the kit. After 24 hours, the transfection mix was discarded and ES cell culture medium without LIF or PS was added and incubated at 37°C overnight. After a further 24 hours, the medium was collected into a 50 ml falcon tube, stored at 4°C and another volume of ES cell culture medium without LIF or penicillin/streptomycin was added and left at 37°C overnight. After 24 hours, the medium was collected, pooled with that collected 24 hours earlier, 'LIF-conditioned media' aliquots were stored at -20°C and COS cells were discarded.

### ***(ii) Testing LIF conditioned medium***

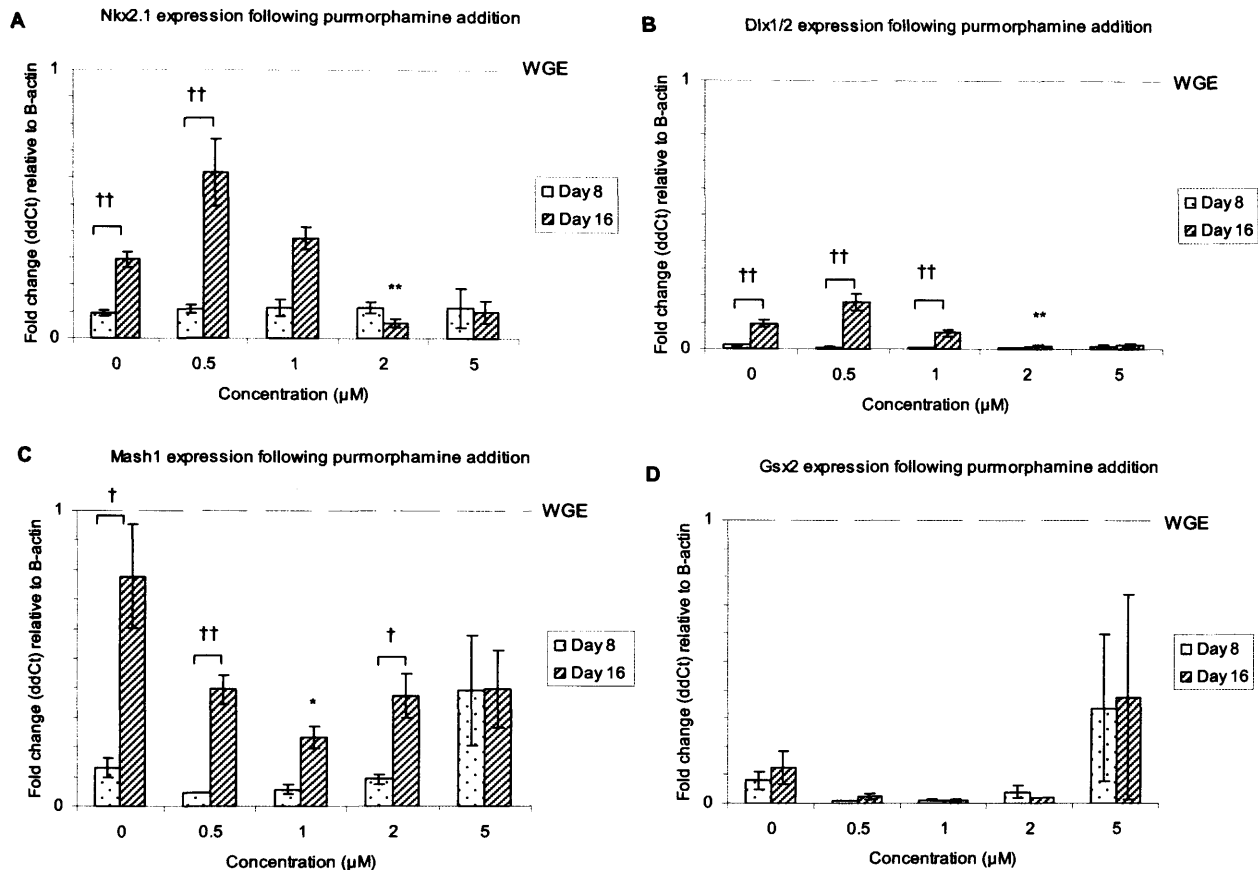
For testing of LIF conditioned medium, the mouse ES cell line CGR8.8 (129Ola derived) was used. ES cells were plated in ES cell culture medium without LIF, onto gelatin-coated 6-well plates (*Costar*) at various densities. The cell densities

tested were 1000 cells/well, 2000cells/well and 5000cells/well. Various dilutions of LIF conditioned media were added (1:2000, 1:1000, 3:2000 and 1:500), and for controls, 10ng/ml commercial LIF was added to one well and another well was left without any LIF.

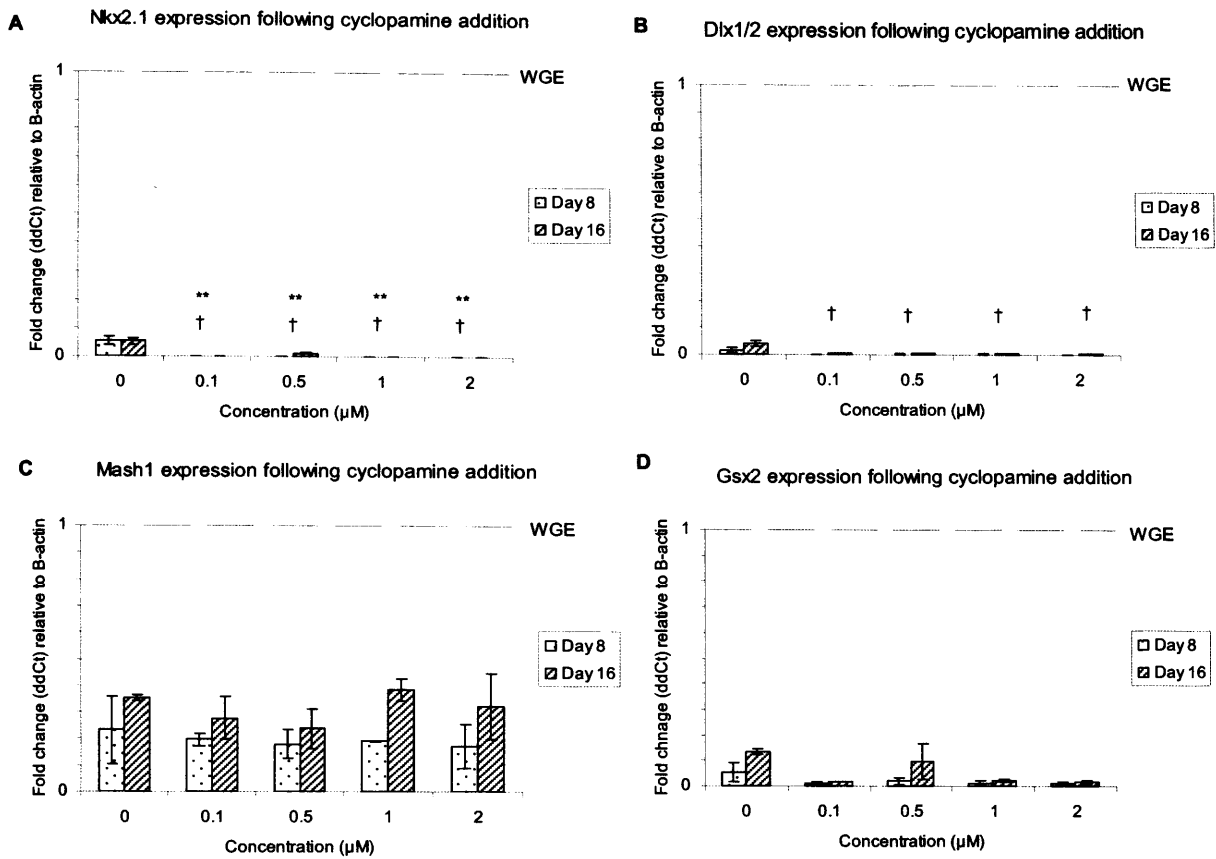
Medium was changed daily with replacement of the appropriate concentration of 'LIF conditioned medium'. Each day the cultures were checked microscopically for differences in morphology in order to determine the optimum concentration of LIF conditioned media required for maintenance of the ES cell cultures.

## Appendix 6: Dorso-ventral gene expression relative to WGE

Gene expression analysis of NEBs following 8 and 16 days in ADF+ neural induction culture and treated with the SHH agonist purmorphamine or the SHH antagonist cyclopamine is shown relative to expression in E14 mouse WGE.



**Figure 1.** Expression of the ventral markers *Nkx2.1* (A), *Dlx1/2* (B), *Mash1* (C) and *Gsx2* (D) were analysed using semi-quantitative RT-PCR on cDNAs prepared from ES cell-derived NEBs at day 8 and day 16 of neural induction in ADF+ with 20ng/ml FGF2 and different concentrations of the SHH agonist purmorphamine present from day 4. Gene expression is shown relative to  $\beta$ -actin. The broken line crossing the y-axis at 1 represents relative expression in the E14 mouse WGE. Each bar on the graphs represents a mean of 3 different neural induction cultures and error bars represent SEM. Brackets indicate significance between the day 8 and day 16 NEBs at a particular concentration. \* = significant difference from WGE control. † = significant difference between NEB groups. (\* $p$ <0.05, \*\* $p$ <0.01, † $p$ <0.05, †† $p$ <0.01).



**Figure 2.** Expression of the ventral markers *Nkx2.1* (A), *Dlx1/2* (B), *Mash1* (C) and *Gsx2* (D) were analysed using semi-quantitative RT-PCR on cDNAs prepared from ES cell-derived NEBs at day 8 and day 16 of neural induction in ADF+ with 20ng/ml FGF2 and different concentrations of the SHH antagonist cyclopamine present from day 4. Gene expression is shown relative to  $\beta$ -actin. The broken line crossing the y-axis at 1 represents relative expression in the E14 mouse WGE. Each bar on the graphs represents a mean of 3 different neural induction cultures and error bars represent SEM. Brackets indicate significance between the day 8 and day 16 NEBs at a particular concentration. \* = significant difference to untreated NEBs at day 8. † = significant difference to untreated NEBs at day 16. (\*\*p<0.01, †p<0.05).



**Appendix 7:** A summary of the neuronal expression in LGE-derived neurons and day 16 NEBs following in vitro neuronal differentiation.

Neuronal differentiation conditions		LGE-derived neurons		Day 16 NEBs; untreated		Day 16 NEBs; purmorphamine	
		7 days	14 days	7 days	14 days	7 days	14 days
<b>β-III-tubulin</b> as a % of total Hoechst cells	PLL/laminin; untreated	71.56 ± 2.56%	67.06 ± 6.40%	30.71 ± 4.89%	15.11 ± 2.05%	25.43 ± 1.03%	22.30 ± 1.60%
	PLL/laminin; BDNF	70.15 ± 0.41%	-	30.58 ± 0.69%	23.13 ± 3.39%	27.01 ± 3.97%	21.56 ± 1.79%
	PLL/laminin; RA	71.17 ± 4.05%	-	-	-	-	-
	PLL/laminin; BDNF & RA	70.32 ± 3.02%	-	-	-	-	-
	LGE glia; untreated	-	-	25.08 ± 7.67%	15.18 ± 0.31%	-	-
	LGE glia; BDNF	-	-	28.12 ± 3.56%	20.03 ± 2.24%	-	-
<b>DARPP-32</b> as a % of β-III-tubulin positive neurons	PLL/laminin; untreated	3.74 ± 0.11%	13.38 ± 2.37%	4.16 ± 1.77%	2.26 ± 0.54%	3.82 ± 0.89%	2.38 ± 0.49%
	PLL/laminin; BDNF	10.93 ± 0.88%	11.68 ± 2.30%	3.82 ± 0.89%	6.42 ± 2.76%	5.60 ± 1.24%	3.58 ± 1.85%
	PLL/laminin; RA	2.29 ± 0.47%	-	-	-	-	-
	PLL/laminin; BDNF & RA	5.27 ± 0.68%	-	-	-	-	-
	LGE glia; untreated	7.77 ± 0.96%	4.42 ± 2.82%	-	-	-	-
<b>FoxP1</b> as % of Hoechst	PLL/laminin; untreated	39.59 ± 3.80%	-	16.89 ± 0.81%	21.20 ± 2.01%	22.55 ± 2.14%	22.03 ± 1.60%
	PLL/laminin; BDNF	35.10 ± 3.15%	-	24.10 ± 2.91%	15.94 ± 3.42%	21.92 ± 4.65%	21.56 ± 1.79%
<b>DARPP-32</b> as % of FoxP1	PLL/laminin; untreated	9.51 ± 3.16%	-	2.04 ± 0.80%	-	3.19 ± 0.58%	-
	PLL/laminin; BDNF	26.36 ± 3.23%	-	3.14 ± 0.65%	-	5.67 ± 2.29%	-
<b>GABA</b> as a % of neurons	PLL/laminin; untreated	-	-	62.75 ± 7.28%	57.42 ± 6.73%	60.18 ± 6.95%	54.88 ± 7.05%
	PLL/laminin; BDNF	-	-	68.52 ± 3.82%	67.24 ± 4.51%	67.26 ± 7.10%	62.53 ± 5.92%

**Appendix 8:** *A summary of graft volumes and cell numbers of various phenotypes in graft areas following transplantation into the mouse QA-lesioned striatum.*

	Transplant group	Survival (weeks)	Estimate of graft volume (mm <sup>3</sup> )	Estimate of number of cells of each phenotype within graft area				
				NeuN	DARPP-32	FoxP1	Parvalbumin	Calbindin
NEBs	Day 16; untreated	6	0.49 ± 0.19	2193.06 ± 1191.22	178.59 ± 102.30	800.64 ± 105.21	240.98 ± 92.51	64.01 ± 44.81
		12	0.58 ± 0.27	805.61 ± 217.30	65.12 ± 21.93	1983.90 ± 1001.94	524.60 ± 303.99	50.08 ± 29.22
	Day 16; purmorphamine	6	0.45 ± 0.09	364.34 ± 192.59	171.23 ± 45.25	822.85 ± 326.57	322.69 ± 175.93	23.82 ± 23.81
		12	0.56 ± 0.12	1996.60 ± 1082.13	166.93 ± 73.05	623.63 ± 170.76	193.47 ± 66.84	75.13 ± 49.53
E14 mouse primary foetal tissue	LGE	6	0.83 ± 0.25	451.38 ± 158.03	273.13 ± 95.17	639.08 ± 493.68	252.12 ± 105.09	25.08 ± 25.08
		12	1.52 ± 0.53	755.50 ± 154.32	476.16 ± 135.70	1171.25 ± 459.05	291.80 ± 81.48	114.47 ± 46.12
	MGE	6	1.38 ± 0.59	403.97 ± 225.36	200.93 ± 73.33	990.63 ± 207.82	301.56 ± 155.02	42.52 ± 24.76
		12	0.86 ± 0.07	393.49 ± 92.90	312.08 ± 54.73	1011.47 ± 160.23	304.89 ± 137.57	120.12 ± 52.12
	WGE	6	0.62 ± 0.22	278.17 ± 123.35	192.22 ± 111.85	993.57 ± 499.86	298.81 ± 72.34	88.05 ± 88.04
		12	0.74 ± 0.26	968.40 ± 19.17	856.93 ± 496.43	2585.64 ± 1987.24	127.46 ± 18.87	45.07 ± 45.06

***Appendices reference list***

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