Directing stem cell differentiation towards a neuronal fate using nicotinamide

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Abstract

Neuronal cell loss and damage in the central nervous system are characteristics of debilitating brain related-degenerative disorders such as Parkinson's disease. The last four decades of research have focused on the promise of cell replacement therapy to replace lost cells, repair the damage and provide functional recovery in affected neural circuits. A thorough understanding of the signals implicated in the development of neurons will greatly facilitate the use of cell replacement therapy.

This project aimed to investigate the possibility of using nicotinamide, the amide form of vitamin B_3, to promote the development of mature neuronal subtypes from mouse embryonic stem cells, and whether these could form a dopaminergic phenotype, to progress research in stem cell-derived therapies for Parkinson's disease.

Treatment of mouse embryonic stem cell monolayer cultures (46C *Sox1*GFP reporter cell line) with nicotinamide at the early onset of development not only increased the efficiency of neuronal generation but also enriched the ratio of purified neurons to non-neuronal cells. Nicotinamide acted at the initial stages of differentiation to promote accelerated neural lineage entry by embryonic stem cells in adherent monolayer cultures. The pluripotent stem cell and neural progenitor cell populations could be reduced by treating cells with nicotinamide, which also facilitated accelerated neuronal differentiation.

Nicotinamide selectively enhanced the production of catecholaminergic, serotonergic and GABAergic neurons and, moreover, accelerated neuronal maturation. A reduction in the proportion of proliferating cells in nicotinamide-treated cultures was

demonstrated– that is, nicotinamide enhanced cell-cycle exit, thereby promoting neuronal differentiation.

The potential of nicotinamide was introduced to a novel, small-molecule-based strategy using pluripotent stem cell sources. Nicotinamide was shown to function synergistically with signalling molecules known to enhance a dopaminergic phenotype, to direct differentiating cells to adopt a dopaminergic cell fate.

Thus, novel findings suggest that nicotinamide is a key signalling factor in brain development, and is required in a definable dosage range and times for the normal formation of dopamine neurons. This study supports previous evidence that vitamins and their metabolites play a fundamental role in neuronal development.

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Abbreviations

5-HT	5-hydroxytryptamine
6-OHDA	6-hydroxydopamine
AADC	Aromatic L-amino acid decarboxylase
AD	Alzheimer's disease
ANOVA	one way analysis of variance
BBB	blood brain barrier
BDNF	Brain-derived Nerve Factor
bFGF	basic fibroblast growth factor
BG	basal ganglia
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CBP	CREB binding protein
CNS	central nervous system
CRT	cell replacement therapy
DA	dopamine
DAergic	dopaminergic
DAPI	4', 6-diamidino-2-phenylindole
DARPP-32	dopamine and cAMP-regulated phosphoprotein
DAT	dopamine transporter
DBS	Deep brain stimulation
DDC	dopa decarboxylase
dH20	distilled H_2O
DIV	days *in vitro*
DMSO	dimethyl sulfoxide
DYN	dynorphin
EB	embryoid body
EdU	5-ethynl-2'-deoxyuridine

En1	Engrailed-1
ENK	enkephalin
EpiSC	epiblast stem cell
ERK	extracellular-signal-regulated kinase
FCS	foetal calf serum
FI	fluorescence intensity
FSIs	fast-spiking interneurons
Fugene	FuGENE® HD
GABA	γ-amino-butyric acid
Gbx2	gastrulation brain homeobox 2
GDF5	growth/differentiation factor 5
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorestem cellent protein
GIDs	graft-induced dyskinesias
GIRK2	G protein-activated inward rectifier potassium channel 2
GP	globus pallidus
GPe	globus pallidus external segment
GPi	globus pallidus internal division
h	1 hour
H_2O_2	hydrogen peroxide
HD	Huntington's disease
HEK	human embryonic kidney
hfVM	human fetal ventral mesencephalic
iPSC	induced pluripotent stem cell
LB	Lewy body
L-DOPA	L-3, 4-dihydroxyphenylalanine
L-Dopa	Levodopa
LGE	lateral ganglionic eminence

LIDs	l-dopa induced dyskinesias
LIF	leukaemia inhibitory factor
mDA	midbrain DA
mDA	midbrain DA neurons
mEpiSC	mouse epiblast stem cell
mESC	mouse embryonic stem cell
MHB	midbrain hindbrain boundary
min	minute
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSCs	mesenchymal stem cells
MSN	medium spiny neuron
Na2HPO4	disodium hydrogen phosphate
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase diaphorase
NaOH	sodium hydroxide
N-CoR	nuclear receptor co-repressor
NG2	Neuron-glial antigen 2
NGS	normal goat serum
NNMT	nicotinamide *N*-methyltransferase
NSCs	neural stem cells
NTN	neurturin
OPC	oligodendroglial progenitor cell
Otx2	orthodentical homolog 2
PARP	poly ADP ribose polymerase
Pax2	paired box gene
PBS	phosphate buffered saline
PCG-1α	proliferator-activated receptor gamma coactivator 1-alpha

PD	Parkinson's disease
PFA	paraformaldehyde
Pitx3	paired-like homeodomain transcription factor
PLL	poly-L-lysine
PPN	pedunculopontine nucleus
RA	Retinoic acid
RALDH1	retinaldehyde dehydrogenase family 1 gene
RAR	retinoic acid receptor (RAR)
RARE	retinoic acid response element
RF	retinol-free
RPE	retinal pigmented epithelium
RT	room temperature
RXR	retinoid X receptor
SEM	standard error of the mean
Shh	Sonic hedgehog
SIRT1	silencing information regulator 1
SN	substantia nigra
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulata
SP	Substance P
STN	subthalamic nucleus
TANs	tonically active neurons
TBS	tris buffered saline
TdT	terminal deoxynucleotidyl transferase
TGFβs	transforming growth factor βs
TH	tyrosine hydroxylase
VDR	vitamin D receptor
VGlut2	vesicular glutamate transporter protein
VM	ventral mesencephalon

VTA	ventral tegmental area
X-tremeGENE	X-tremeGENE HP DNA Transfection Reagent
α	alpha

Chapter 1: General Introduction

1.0 Introduction

This project aimed to investigate the possibility of using nicotinamide, the amide form of vitamin B_3, to promote the development of mature neuronal subtypes from mouse embryonic stem cells (mESCs) and whether these could form a dopaminergic (DAergic) phenotype. ESCs are considered to be an invaluable *in vitro* biological tool not only for examining the mechanisms of pluripotency, but also to study effects of different factors for directing cell fate decisions throughout early development.

In order to gain an in-depth comprehension of the whole project and for experimental design purposes, it is essential to have an understanding of the basal ganglia (BG) circuitry [the functional brain structure affected by Parkinson's disease (PD)]; PD; transcriptional control of midbrain DAergic neuronal development; how stem cells give rise to DAergic progenitors/neurons; and the role of vitamins and their active metabolites in influencing neural development in the early embryo and the adult central nervous system.

1.1 The anatomy of the basal ganglia

Our understanding of the anatomical and functional organisation of the BG circuitry began more than three decades ago[1,2]. Research from the last decade accomplished additional refinement of pathophysiological BG circuitry models[1,3–5], thus advancing our scientific knowledge regarding the normal and diseased BG; a major cause of impairment in psychomotor disorders. Most importantly, clinical requirements promoted the drive for the development of new therapies in PD. The BG circuitry plays a fundamental role in the control of movement, in addition to associative

learning, planning, working memory and emotion. This chapter will focus on the "motor circuit".

1.1.1 Cells and neurotransmitters of the basal ganglia

The BG is comprised of five interconnected brain nuclei with the majority sending their projections exclusively to other nuclei located within the BG circuitry, establishing a vast subcortical network throughout the forebrain and midbrain. The major nuclei of BG circuitry are the striatum, globus pallidus (GP), subthalamic nucleus (STN) and substantia nigra (SN) (Figure 1.1). The striatum, derived from the telencephalon, is made up of two functionally distinct nuclei; the caudate nucleus and the putamen, composed of neuronal cells sharing similar morphologies. These brain nuclei are partly divided by corticofugal and corticopetal fibers. In rodents, the striatum is shown to exist as an anatomically homogeneous formation. The GP can be subdivided into the globus pallidus external segment (GPe) and globus pallidus internal division (GPi). The SN is located in the mesencephalon (midbrain) and is comprised of an area containing a diffuse portion of neurons called substantia nigra pars reticulata (SNpr) which is comparable to the GP cytologically, and a densely pigmented area called the substantia nigra pars compacta (SNpc), the latter containing DAergic neurons[6].
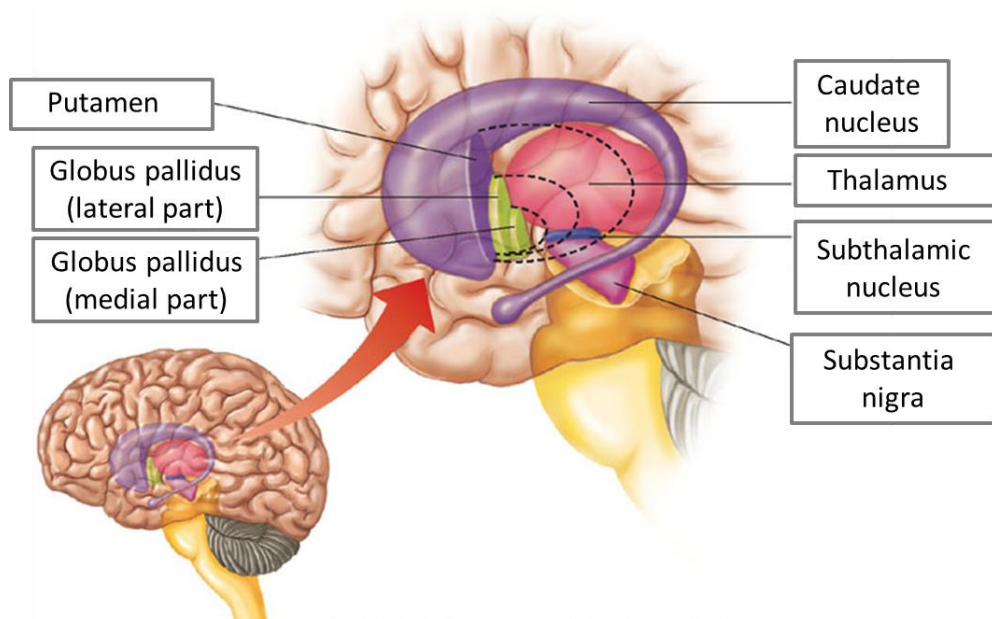
Figure 1.1 The topography of the cortical regions of the brain (left image) and a cross section of the human brain identifying associated structures of the BG circuitry (right image) are illustrated. (Diagram adapted from https://syntaptogaming.files.wordpress.com/2012/01/basalganglia_image1.jpg).

1.1.1.1 Striatal neurons

The striatum is composed mainly of projection neuron types with interspersed populations of interneurons[7]. Neurons located in the striatum are represented mainly by medium spiny neurons (MSNs), i.e. neurons whose dendrites are characteristically enveloped in dense dendritic spines, which account for 90-95% of total striatal neurons[8]. The output nuclei of the BG, i.e. internal and lateral segments of the GP and the SNpr, receive a major input from spiny projection neurons[1,2]. Spiny neurons appear to have a spherical arborisation and utilise γ-amino-butyric acid (GABA) as their predominant neurotransmitter[9], which can be co-localised with substance P/dynorphin (DYN) or enkephalin (ENK)[10].

Five subtypes of dopamine (DA) receptors exist in the striatum. Striatal projection neurons express two main types of DA receptors; D_1Rs and D_2Rs which are G-

protein coupled receptors responsible for modulating the MSNs' response to DA released from nigrostriatal terminals. The inhibitory or excitatory effect carried out by DA is reliant on the type of receptor activated in the postsynaptic element. An enriched level of D_1R mRNA is found on striatonigral substance-P-containing neurons in the direct pathway (see below), whilst D_2Rs are linked to striato-pallidal ENK-immunoreactive neurons in the indirect pathway[11].

The striatum is the only BG nucleus containing various types of interneurons; small/parvalbumin GABAergic medium aspiny neurons[8,9], large cholinergic interneurons [8] and aspiny interneurons containing somatostatin, neuropeptide Y, and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) diaphorase[12]. Cholinergic, tonically active neurons (TANs) containing choline acetyltransferase and GABAergic fast-spiking interneurons (FSIs) are the most abundant types of striatal interneurons. TANs pre-synaptically inhibit glutamatergic input, therefore regulating the excitability of MSNs. The main role of GABAergic interneurons is to synapse on the dendritic shaft of nearby MSNs, thus enabling local collateral arborisation and inhibition to be achieved[13].

1.1.1.2 Pallidal and nigral neurons

Pallidal and nigral neurons receive innervation from the striatum. Neurons from both pallidal segments use GABA as a neurotransmitter, co-localised with ENK, resulting in an inhibitory effect on their target neurons in the thalamus or sub-thalamic nucleus respectively[14]. SNpc neuronal cell types contain neuromelanin and utilse DA as a neurotransmitter. SNpr neurons are GABAergic[15]. Synaptic boutons cover pallidal and nigral neurons.

1.1.1.3 Subthalamic neurons

The STN is the only nucleus of the BG circuitry which contains glutamatergic neurons, causing an excitatory effect on its targets: the GPi and SNpr[16].

1.1.2 The nigrostriatal pathway

Pioneering research carried out by Carlsson, Falck and Hillarp in the 1960s highlighted the existence of two major types of catecholamine neurons in the brain: noradrenaline and DA, identified by applying the formaldehyde histofluorescence method[17]. This anatomical study outlined three dominant trajectories of midbrain DA (mDA) neurons; dorsal striatum, ventral striatum (including nucleus accumbens) and the prefrontal cortex[18].

In the 1970s, immunohistochemical studies employing the rate-limiting enzyme of DA synthesis, tyrosine hydroxylase (TH) enabled the positioning and expression pattern of DA neurons in the central nervous system (CNS) to be revealed. There are nine distinct DAergic cell groups present in the mammalian brain, extending from the mesencephalon to the olfactory bulb (Figure 1.2.)[19].

The use of more refined tract-tracing techniques illustrated the anatomical organisation and projection arrangement of TH$^+$ DA neurons arising from the ventral part of the brain which was much more complex than was originally conceived. Ventral midbrain DA (mDA) cell sources can be classified into three main groups according to their location; A8 (retrorubral area), A9 (SN) and A10 (ventral tegmental area, VTA) (Figure 1.2). Recent research discovered that DA neurons located in the SN not only innervate the striatum, but also project to the STN, GPe and GPi[20]. Furthermore, cells located in the A8 region produce a dorsal and caudal

continuation of the A9 DA cell group, extending their projections to striatal, limbic and cortical areas[21]. These projections have been termed as the nigro-extrastriatal pathway and it has been proposed that they play a fundamental role in BG pathophysiologic disorders and/or perhaps influence the compensatory mechanisms predominant at the early stages of PD.
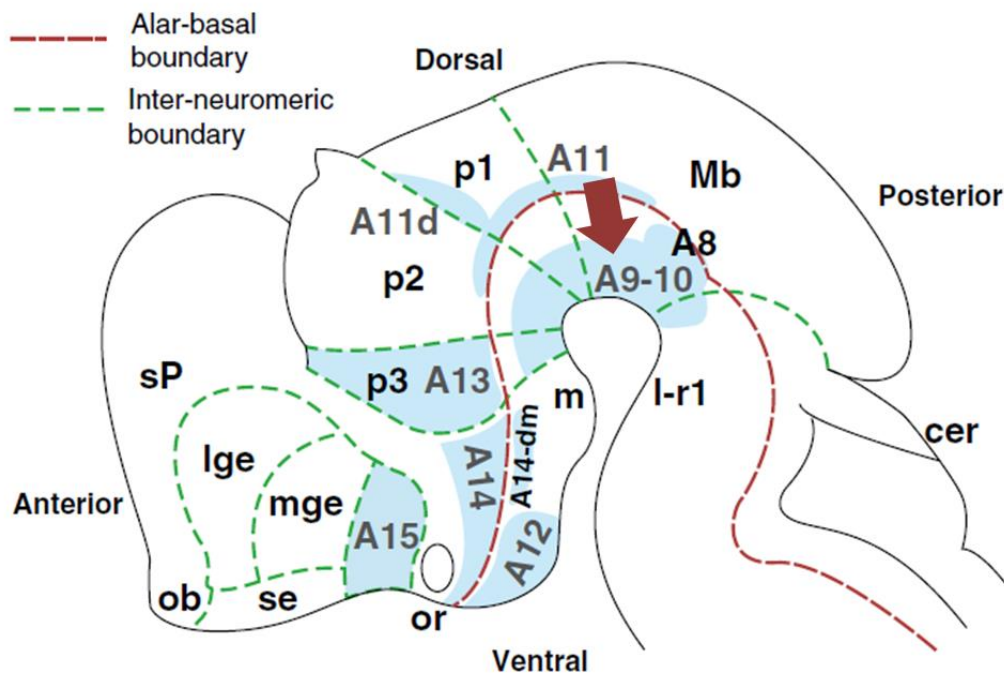
Figure 1.2 DA neuronal subtypes located in the CNS.
This diagram illustrates the location of TH$^+$ DA neurons corresponding to A8-A15 groups in the developing mammalian brain. mDA neurons emerge from the ventral mesencephalic flexure comprising of A9 SNpc and A10 VTA sets of neurons, shown by the red arrow. (Diagram adapted from Siew-Lan Ang, 2006)[22].

The nigrostriatal DA pathway (Figure 1.3) is derived from neurons in both the dorsal and ventral tier of the SNpc, a chief source of DA innervations of the sensorimotor striatum. The nigrostriatal projection is conveyed in a topographical manner, segregated into medial-lateral and dorso-lateral highly convergent projections. The

nigrostriatal system is mainly comprised of neurons from the A9 region in the mesencephalon[21].
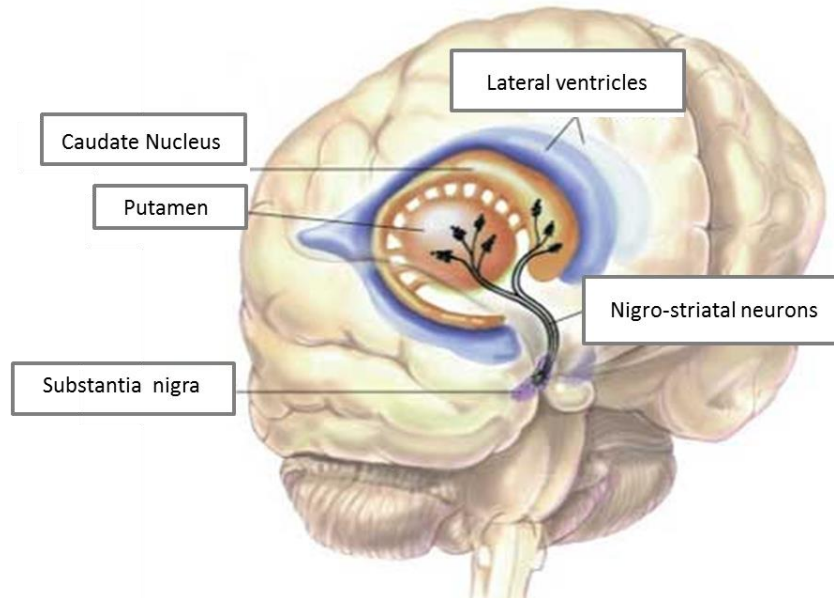
Figure 1.3 Nigrostriatal Pathway. In the normal brain, DAergic neurons localised in the SN region project their axons to the striatum; composed of the putamen and caudate nucleus. (Diagram adapted from © 2001 Terese Winslow, Lydia Kibiuk)

DA neuronal cell types can be characterised by their medium size and non-spiny morphology. Dendritic branches of DA neurons are exceptionally arborised and it has been estimated that a single DA axonal projection can give rise to 1,000,000 terminals[23]. DA axonal projections are thin with a diameter of 0.1-0.8 μm. DA axons projecting exclusively to the dorsal striatum do not project to other areas such as the ventral striatum or neocortex, implying that each subtype of DA neurons has a distinct role within its target area[24].

mDA neurons can be segregated into a *dorsal* and *ventral* tier in relation to their connectivity and morphological properties. Cells dorsally located in the VTA and SN comprise of the *dorsal tier*, in addition to A8 cells projecting to the ventral

striatal, limbic, cortical areas and matrix compartment of the dorsal striatum[21]. *Dorsal tier* cell types can be characterized by their round shape and are calbindin-positive. These cells express low levels of DA transporter (DAT). Dense, angular cells located in the ventral region of the VTA and SN constitutes the *ventral tier*, sending their projections exclusively to the striatum. Compared to dorsal tier cell types, these cells are calbindin-negative and are shown to express relatively high levels of DAT in addition to the G protein-activated inward rectifier potassium channel 2 (GIRK2)[25]. In the SN, ventral tier DA neurons release neurotransmitter DA from a network of dendritic terminals projecting to the striatum where postsynaptic targets are affected. Ventral tier neurons are crucially positioned to influence and directly modulate neurotransmission in the BG. mDA neurons not only play a fundamental role at the level of the caudate and putamen, but also regulate the activity of output pallidal, STN and nigral neurons[26].

The patch and matrix compartmental organisation of the dorsal striatum is critical in regulating the complex, numerous parallel pathways connecting the midbrain, BG, thalamus and cortex responsible for movement[27]. Smaller, patch compartments are embedded in larger matrix compartments, based on the laminar organisation of the cortex. It has been shown that patch neurons preferentially project to ventral tier DAergic neurons in the SNpc and DA cell islands located within the SNr, whereas matrix neurons project to GABAergic neurons found in the SNr[4].

1.1.3 Functional anatomy of the basal ganglia circuitry

The main objective of the BG circuitry is to process signals arising from virtually the whole cerebral cortex. This is accomplished by what is now considered as the classic view of the BG[1,2,28]. Corticostriatal projections carry cortical information to the

striatum where it is processed and integrated with other inputs to the BG, including intralaminar thalamic nuclei, amygdala, hippocampus and dorsal raphe. BG nuclei then generate output signals, which travel through the thalamic target back to the cortex via a *direct* and an *indirect* pathway resulting in the modulation of motor, sensory and cognitive cortical information processing[29].

Researchers employing anatomical and physiological studies illustrated the existence of projections from different cortical areas to specific regions of the caudate and putamen, establishing five parallel information processing circuits. These circuits include a motor, oculomotor, dorsolateral prefrontal, lateral orbitofrontal and an anterior cingulate circuit[30]. The supplementary motor cortex is the major target of the BG motor circuit; however the subcortical 'premotor' areas such as the superior colliculus, the PPN and reticular formation are also influenced.

The striatum (caudate and putamen collectively) is considered to be the main input nucleus to the BG circuitry, whereas the GPi and the SNpr can be classified as the two output nuclei. Like the striatum, it is now accepted that the STN is also recognised as another input nucleus as this structure directly receives afferents from the cerebral cortex, thalamus and brainstem (reviewed in Utter and Basso, 2008)[31].

The direct pathway is innervated by striatal spiny neurons which directly synapse with neurons in the GPi and the SNpr, the majority of which are output neurons (reviewed in Bolam *et al,* 2000)[32]. Neurons in the striatum which innervate the SNpr and GPi are GABAergic and express DYN as a co-transmitter and possess D_1Rs. Under resting conditions, striatal neurons are quiescent. GPi/SNpr neurons are also GABAergic and can be characterised by their high discharge rate which maintains targeted structures in the thalamus (ventral thalamic or subcortical premotor region neurons) and brainstem under tonic inhibitory control.

Firing of corticostriatal glutamatergic neurons activates the circuit, resulting in an initiation of striatal neuron discharge. Consequently, output neurons in the SNpr and GPi are inhibited, resulting in a reduction of their firing. This leads to disinhibition of neurons in the thalamus, resulting in movement. Therefore, in the normal state and functioning of the BG, activation of the direct pathway facilitates movement (Figure 1.4.).

In the indirect pathway, an opposite physiological effect occurs. GABAergic projections exist from the striatum to the GPe, which in turn project to the STN. In turn, glutamatergic outputs in the STN send their axons to the GPi and SNr, which then innervate the thalamus. Upon depletion of DA, striatopallidal neurons inhibit tonically active GPe neurons, thereby freeing STN excitatory neurons from inhibition. Excitation of GABAergic neurons located in the GPi and SNr occurs as a result of the disinhibited STN neurons, causing an inhibitory effect on thalamic output. This leads to reduced excitation on neurons found in the supplementary motor area and movement is arrested (Figure 1.4)[2,27].

In addition to the cortex and thalamus, the DAergic system carries out a fundamental role in each pathway in the BG circuitry. DA modulates glutamatergic synapses from corticostriatal inputs to the BG circuitry. It has an excitatory effect on D_1R-expressing striatal neurons in the direct pathway and an inhibitory effect on D_2R-expressing neurons in the indirect pathway[1]. Therefore, impairment in the control of voluntary movements stems from a loss of DA innervation from the SN, resulting in the deficient release of DA in the striatum.
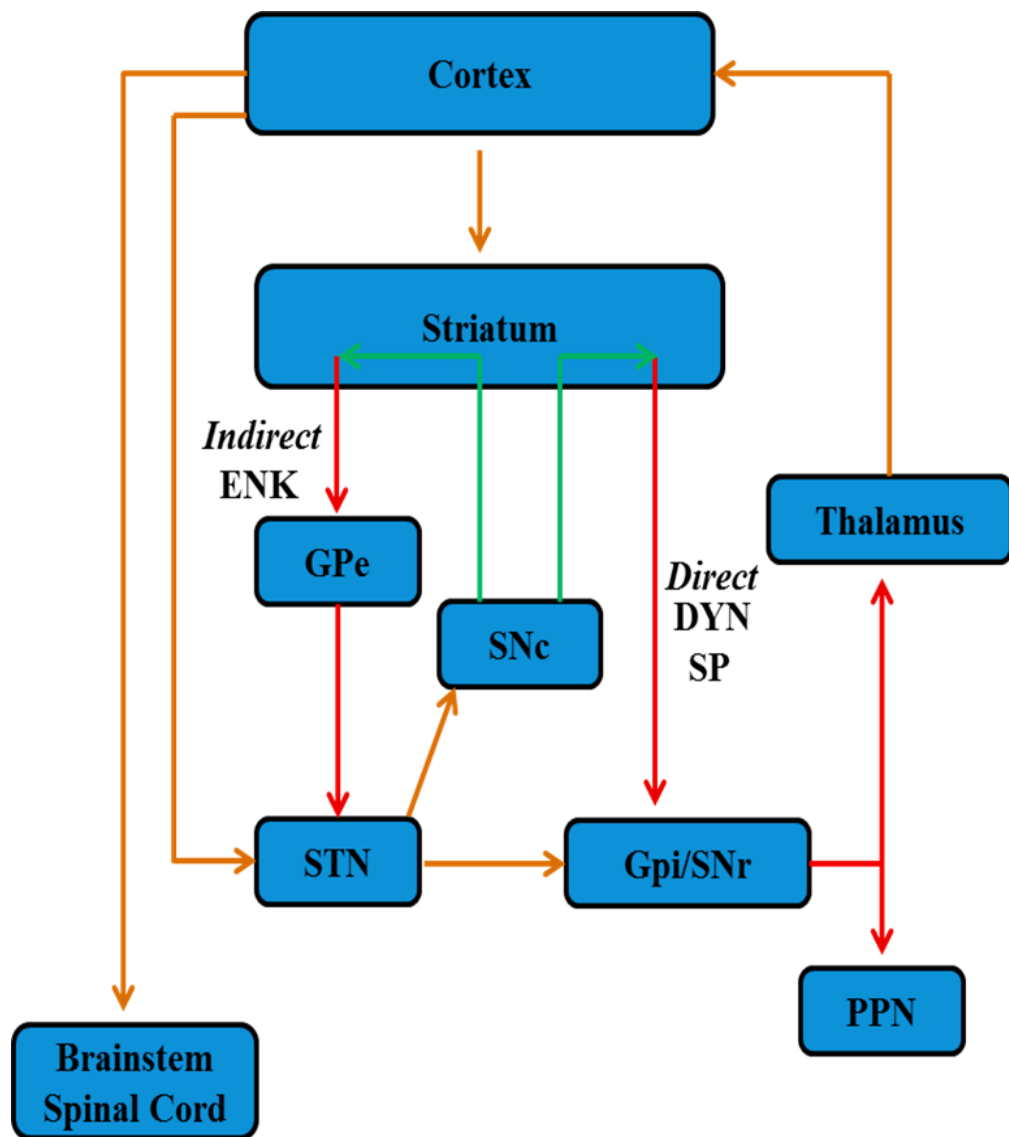
Basal Ganglia circuitry in normal conditions

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Figure 1.4 Schematic diagram of the BG-thalamocortical circuitry in normal conditions. Parallel neuronal networks make up the 'direct' and 'indirect' pathway and are illustrated connecting the striatum (putamen) to the BG output nuclei. GABAergic **inhibitory** neurons are shown as red; glutamatergic **excitatory** neurons are shown in orange; dopaminergic neurons are shown as green.
Abbreviation: SP (Substance P); Enk (enkephalin); GABA (γ-amino-butyric acid); GPe (external globus pallidus); GPi (internal globus pallidus); STN (subthalamic nucleus); SNr (substantia nigra pars reticulata), SNc (substantia nigra pars compacta); PPN (pedunculopontine nucleus) (Diagram modified from Lewis *et al, 2003*).

The highly selective death of DA neurons in the SNpc and insufficient release of

neurotransmitter DA in the *corpus striatum*, results in a decreased inhibitory effect

of the *indirect pathway,* with a reduced excitatory response in the *direct pathway*

(Figure 1.5) The outcome of this activity translates to an excessive activation of the output nuclei of the BG circuitry (GPi and SNr), with a consequential inhibitory affect in the thalamocortical and brainstem motor centres, thus resulting in the development of Parkinsonian motor features[33].
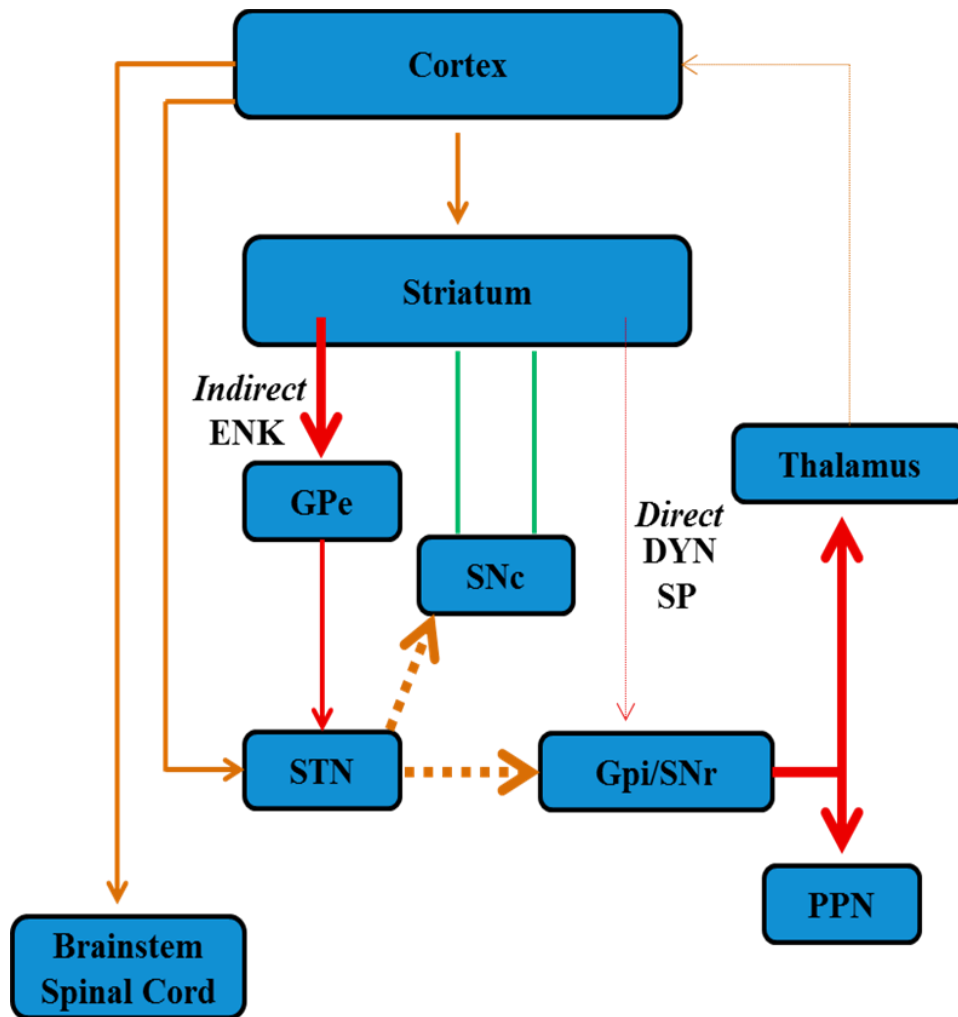
Basal Ganglia circuitry in Parkinson's disease.

Figure1.5 Schematic diagram of the BG-thalamocortical circuitry in Parkinson's disease. GABAergic striatal neurons projecting to the GPi and SNr become depleted, resulting in a reduced inhibitory response from both these BG structures. Therefore, neuronal activity in the direct pathway is reduced, resulting in neurons in the indirect pathway becoming activated. An inhibitory effect on excitatory thalamic neurons is produced from GABAergic neurons localised in the GPi and SNr. This leads to reduced excitation of neurons present in the thalamus, in turn generating a less excitatory response to neurons in the cortex. Attenuated cortical output activity hinders the initiation and execution of voluntary movement, thus resulting in movement arrest and rigidity, which is evident in patients suffering from PD.

GABAergic inhibitory neurons are shown as red; glutamatergic excitatory neurons are shown in orange.
Abbreviation: SP (Substance P); Enk (enkephalin); GABA (γ-amino-butyric acid); GPe (external globus pallidus); GPi (internal globus pallidus); STN (subthalamic nucleus); SNr (substantia nigra pars reticulata), SNc (substantia nigra pars

1.2 Parkinson's disease

"Until we are better informed respecting the nature of the disease, the employment of internal medicine is scarcely warrantable." – J. Parkinson.

Extensive research has been conducted by scientists on the ontogeny of A9 DA neurons, with the aim of gaining a more in-depth knowledge of the chronic human progressive and neurodegenerative disorder, PD. The majority of PD cases are sporadic (approximately 95%), commonly referred to as idiopathic PD.[34] Idiopathic PD is the second most common neurodegenerative disorder worldwide after Alzheimer's disease, affecting approximately 0.5-1% of the population between the ages of 65-69 years, whilst 1-3% of the population are affected over the age of 80 years[35]. In 1817, neurologist James Parkinson first detailed the syndrome and neuropathology of PD in his monograph entitled "An Essay on the Shaking Palsy" or "paralysis agitans". In his work, he described six patients with "involuntary tremulous motion, with lessened muscular power in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from walking to a running pace: the senses and intellects being uninjured"[36]. Despite James Parkinsons' request for a more comprehensive understanding of the underlying causes of this disorder to be achieved for the development of therapies, it was not until 50 years later that any advancement or additional pathophysiological understanding was accomplished[37].

Jean-Martin Charcot, a French physician acknowledged the London apothecary's formal description of PD and established a more elaborated account of the motor abnormities linked to the disease process[38] and also proposed in 1888 that the syndrome should be entitled "maladie de Parkinson" (Parkinson's disease). In the 1950s, Arvid Carlsson and colleagues started to investigate the underlying physiological and anatomical areas of the brain related to the PD process, and found that 80% of DA in the brain is contained within the BG[39]. In 1960, Ehringer and Hornykiewicz observed that the movement abnormalities associated with PD were due to the degeneration of the nigrostriatal circuit originating from the A9 DA cell group. Hornykiewicz noted a significantly higher concentration of DA in the striatum of the normal human brain in comparison to low levels of DA localised in the nucleus caudatus, putamen, nucleus accumbens, SN and GP of PD post mortem samples[40]. Shortly after these discoveries, Levodopa (L-Dopa) was introduced as a treatment for the cardinal motor features of PD. The clinical management of PD symptoms has advanced significantly since the 1960s; however, at present it still remains a challenge to halt the progression of the disease or to restore lost functions in the damaged nigrostriatal pathway.

1.2.1 Clinical and neuropathological characteristics of Parkinson's disease

Deficiencies of nigrostriatal DAergic neurons with a resultant loss of the neurotransmitter DA in the *corpus striatum* (the brain region controlling movement) underlie the pathology of PD. One of the pathological hallmarks of PD is the presence of Lewy bodies, eosinophilic proteinaceous cytoplasmic inclusions containing alpha (α)-synuclein which are observed in prevailing SNpc neurons[41]. Heiko Braak proposed that Lewy body (LB) pathological alterations are not restricted to the SNpc, implying that these protein aggregates follow a distinct

pattern of pathological change with disease progression. Autopsied brains have shown that pathology commences in the olfactory apparatus and caudal brainstem (dorsal motor nucleus of the vagus in the medulla), before extending rostrally up the brainstem where neuronal cell loss is evident in the SNpc[42].

PD can be characterised clinically by the development of three cardinal symptoms namely tremor at rest, rigidity and hypokinesia[34]. Other motor symptoms include bradykinesia, postural abnormalities and a freezing (sometimes referred to as motor block) phenomenon (Table 1.1).

Table 1.1 Glossary of Parkinson's Disease Symptoms	
Akinesia	"Without motion"; inability to initiate movement and is associated with temporary paralysis.
Apathy	State of indifference linked to motivation, personality and cognition.
Ataxia	Loss of balance and coordination.
Bradykinesia	Slowness of movement.
Dyskinesia	Abnormal involuntary movements associated with the onset of striatonigral DAergic degeneration and chronic L-Dopa administration.
Dysphagia	Difficulty in swallowing.
Freezing	Inability to initiate muscular movement in any desired direction.
Masked Facies	Facial muscles become immobilized, resulting in the appearance of a "blank expression"
Micrographia	Tendency for handwriting to become smaller in size.
On-off phenomenon	Alterations between periods of being "on", which represent the patients' ability to control their movements owing to a good responsiveness to medication. "Off" fluctuations develop when the patients experiences symptoms of their underlying parkinsonism, i.e. bradykinesia, rigidity and loss of muscular coordination.
Tremor	Tremor, measuring 4-5 Hz at rest, is the first visible clinical feature recognised in 70% of PD patients, but may be absent in 20%.
Pill-rolling	Tremor can be classified by "pill-rolling", a hand action involving the thumb and forefinger, occurring as a result of involuntary oscillatory movements of agonist and antagonist muscles.
Postural abnormalities	Loss of balance or "unsteady feeling"
Rigidity	Increased muscular tone resulting in stiffness and resistance of bodily movements

Non-motor symptoms occur in over 90% of PD patients and can sometimes be observed as "premotor" phenomena. They include neuropsychiatric symptoms, autonomic disturbances, sensory symptoms such as olfactory impairment, and sleep disorders (Table 1.2)[43]. The appearance of the clinical symptoms of PD occurs with the loss of approximately 60% of the DA neurons in the SNpc and 80% of striatal DA content. This poses a major problem for the treatment of PD, as patient diagnosis occurs only when this neurodegenerative disorder has already advanced to a serious pathological level.

Cardinal features	Tremor at rest, rigidity, bradykinesia/hypokinesia/akinesia, flexed posture/loss of postural reflexes, freezing gait.
Non-motor symptoms Neuropsychiatric	Dementia, depression, anhedonia, apathy, anxiety, slowness of thought, psychosis, sleep disturbances, sexual dysfunction
Craniofacial	Hypomimia, impaired accommodation, sialorrhea and dysphagia, olfactory hypofunction, dysarthria
Autonomic dysfunctions	Bladder dysfunction, erectile dysfunction, impaired gastrointestinal dysfunction, constipation, abnormal thermoregulation and increased sweating.
Sensory symptoms	Reduced sense of smell, pain, numbness and paresthesiae.
Skin	Seborrhoea.
Musculosketal	Scoliosis, peripheral oedema.

Table 1.2. Cardinal and non-motor symptoms linked to the pathology of PD.

1.3 Developmental differentiation of midbrain dopamine neurons

Approximately 400,000-600,000 DAergic neurons (~75 % of all DA neurons) are located within the human ventral mesencephalon (VM), comparable to 20,000-30,000 in the mouse VM[44]. Recent progress, specifically over the last five decades in mouse genetics and knowledge of vertebrate development have aided our understanding of the development of mDA neurons from multipotent progenitors, facilitated through expression of key genetic cascades (activation of internal transcription factors) and cell-extrinsic environmental cues. This information is crucial to further efforts to generate high yields of SNpc DA neurons from stem cell-derived sources (detailed in this chapter), to move us closer to a cell replacement therapy for PD.

In general, during embryonic development, midbrain territory along the anterior-posterior axis and the ventral region of the ventral-dorsal axis is defined by patterning events, closely followed by the induction of mDA neural progenitors in the VM[44]. An important characteristic of these progenitor cells is to maintain a state of pluripotency throughout the development of DA neurons, in addition to responding to key signaling proteins regulating their fate. mDA neurons are then generated from these progenitor cells in the floor plate of the caudal diencephalon and midbrain, producing three distinct clusters of mDA neurons (termed A8; retrorubal field, A9; SNpc and A10; ventral tegmental area). I will discuss the "normal development" of mDA neurogenesis in the SNpc, focusing on cellular and molecular determinants influencing their regional specification, induction, postmitotic differentiation, maturation and maintenance, in order to functionally project and integrate into their desired destinations within the CNS.

1.3.1 Ventral midbrain regionalisation

The commencement of regionalisation occurs during early development when neuralisation causes the lateral edges of the neural plate to scroll upwards to become dorsal. Molecular and cell-fate mapping techniques demonstrated that the temporal and spatial region of neuroepithelial cells (i.e. neurogenic ectoderm) determine the timing and modifications of transcription factors, which in turn influence the specification of DA neurons[45].

The organisation of both A9 and A10 groups is triggered by two important signaling centers located in close proximity: the isthmus, a neuroepithelial signaling center at the midbrain hindbrain boundary (MHB), and the floor plate of the ventral midline of the mesencephalon. This arrangement allows both signaling centers to have an influential effect on both sets of neurons following the secretion of diffusible signals, thus directing the terminal specification of cells within the VM[22].

The axial position of the MHB is established from co-repressive interactions of both orthodentical homolog 2 (*Otx2*) and gastrulation brain homeobox 2 (*Gbx2*) expression domains, and is maintained by FGF8 expression, a fibroblast growth factor8 expressed from the anterior MHB. It should be noted that paired box gene (*Pax2*) is necessary for the induction of FGF8 expression. Additionally, glycoprotein *Wnt* 1 and Engrailed-1 (*En1*) function synergistically with *Otx2* and *Gbx2* to further refine the position of FGF8 expression at the isthmus. Expression of En2 and Pax5 in the MHB commences following the induction and positioning of FGF8 expression, to promote regional specification of the VM[22].

Ventral cells located in the neural plate acquiring regional identity of the midbrain react to the morphogen, Sonic hedgehog (Shh), expression at mouse E8.0 , by obtaining a DA cell fate[46]. Therefore, both the induction and positional location of DA neurons is reliant on overlapping regions of secreted FGF8 and Shh expression (Figure 1.7) emanating from the notochord below the ventral floorplate, as the interplay between these factors will influence where mDA progenitors will arise[47].

1.3.2 Derivation of midbrain dopamine neurons from progenitor cells

Following appropriate early patterning events of the VM, the induction and maintenance of mDA progenitor cells which will ultimately differentiate into mDA neurons is dependent on a number of factors present in the ventral midbrain: Shh, *En1 and En2, Wnt factors, Otx2, Pax2 and Pax5 and Sox* genes (Table 1.3.; Figure 1.6)[22,44]. The interaction of all of these factors present in the ventral midbrain provides a fundamental basis of graded cues enabling neural progenitors to adopt different phenotypes according to their position, (as reviewed by Orme *et al*[48]). Extensive research has been carried out on the specific identity of these progenitor cells due to their potential to be translated as a cell source to replace DAergic neurons lost in PD. Recent research revealed the precise identity of these progenitor cells and showed that floor plate cells residing in the murine VM became neurogenic and later gave rise to DA neurons[49].

1.3.3 Transcription factors: control of midbrain dopamine neuron development

Newly generated progenitor cells are localised within the ventral most proliferative zone of the midbrain neuroepithelium, travelling in a ventral and then lateral manner as they commence the process of differentiation. They can be characterised by their expression of transcription factors which have all been shown to significantly affect

the development of mDA neurons including *Lmx1a, Foxa2, Lmx1b, Msx1/2, Ngn2, Nurr1, Wnt* and (Figure 1.6) at a developmental earlier stage, Shh expression[50].

mDA progenitors have the ability to downregulate *Sox2* expression and are also capable of initiating βIII-tubulin, a neuronal marker expressed in postmitotic immature and mature mDA neurons[51] (Figure 1.6).
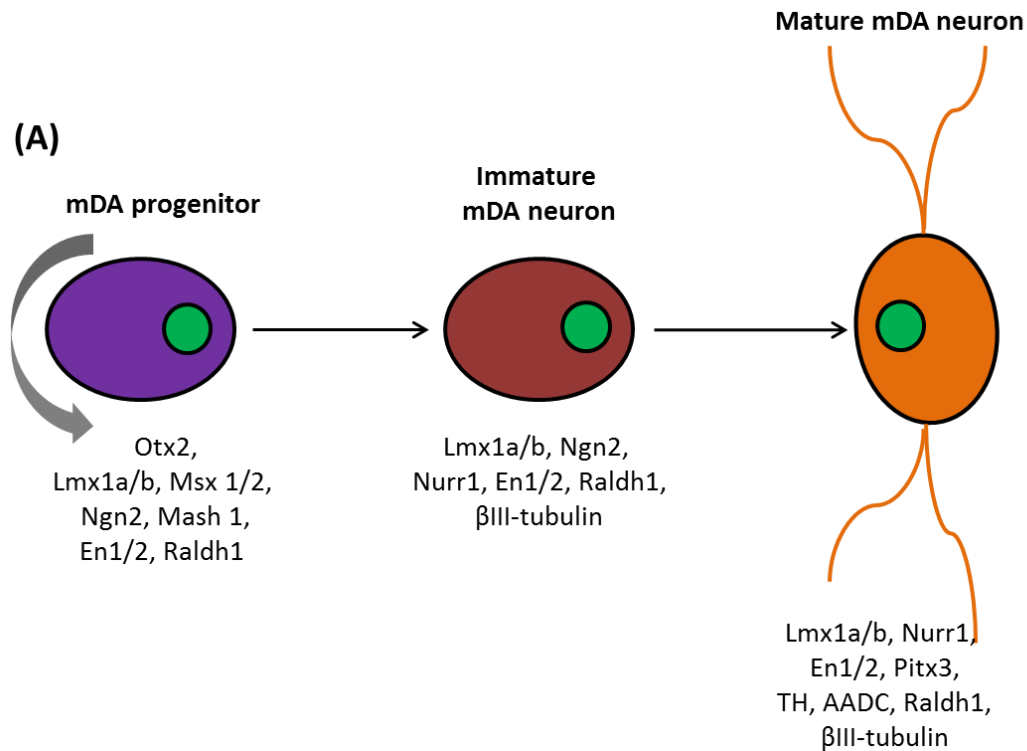
Figure 1.6 Model of mDA neuron generation from early DA progenitors to mature DAergic neurons. This diagram represents the series of stages and the subsequent timing of the activation of transcription factors required for the generation of DA neurons during early embryonic development. (Diagram adapted from Siew-Lan Ang, 2006)[19].

1.3.4 Appearance of terminally differentiated A9 dopamine neurons in the midbrain

In summary, at approximately mouse E10, mDA neural progenitors located in the ventricular zone featuring distinct proliferative capabilities migrate along radial glial fibres until they reach the mantle zone, where they further continue their

differentiation into DA neurons[52]. A number of early specification genes already expressed by these migrating precursors include *En1/2, Lmx1a/b* and *Foxa1/2* and then begin to express aromatic L-amino acid decarboxylase (AADC). AADC functions in converting Dopa into the neurotransmitter DA and experiments have shown that immature DA neurons already express AADC mRNA transcripts[53]. At E10.5, the orphan nuclear receptor for *Nurr1* is expressed and subsequently followed by the exit of precursors from the cell cycle to commence their conversion into postmitotic precursors. This is due to the fact that they have not yet developed into fully differentiated mature DA neurons by this developmental stage. The terminal differentiation step by which cells obtain a DA phenotype is marked by the onset of TH expression, which is present between E9.5 and E11.5 in the ventral midbrain of mice (Figure 1.7). It should be taken into account that this developmental stage is arrested if cells do not express *Nurr*[53]. During this time point at E11.0, the expression of paired-like homeodomain transcription factor, *Pitx3*, indicates the generation of fully differentiated DA neuronal phenotypes, along with *Engrailed and Nurr1* which have functional roles at this terminal stage of DA mature neuronal differentiation (Table 1.3; Figure 1.7).

(A)
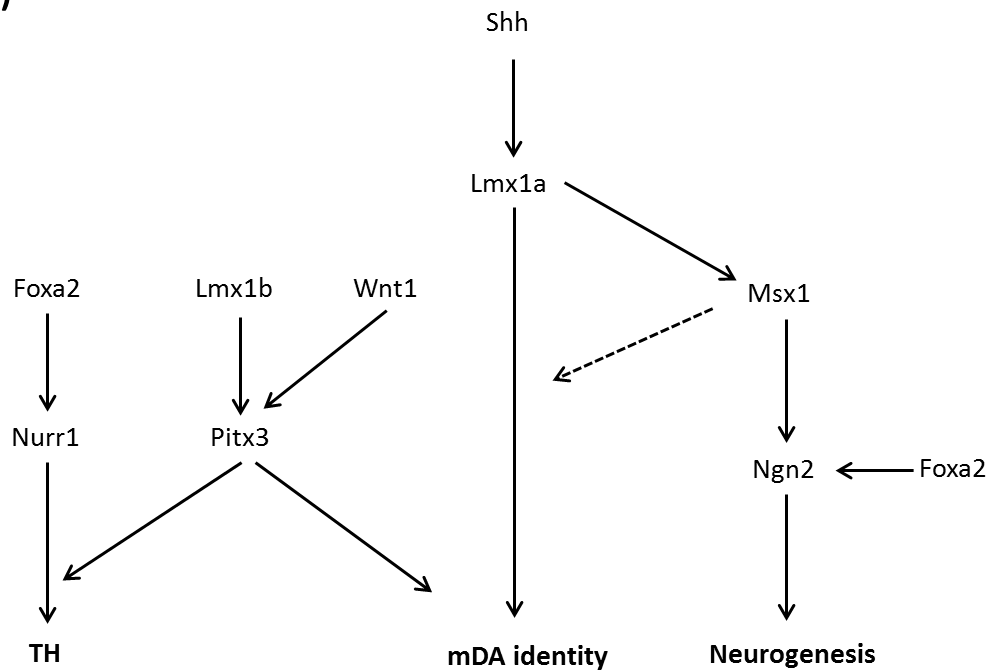
(B)
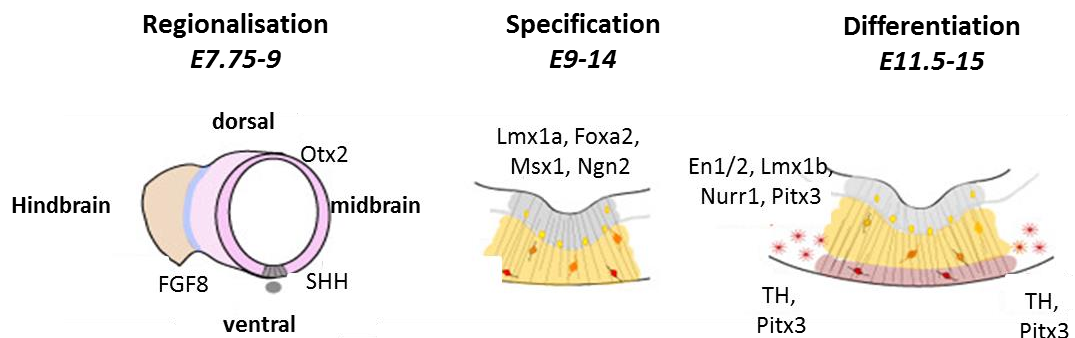
Figure 1.7 Model of mDA neuron generation. (A) This diagram illustrates the signalling cascades involved in the development of mDA neurons. **(B)** Midbrain tissue identity is established during regionalisation of the neural tube. The inductive signals Shh and FGF8 (arising from the notochord (grey circle) and the MHB (blue) respectively), in combination with Otx2 expression play important roles during this stage (E7.75-9). Specification of mDA neuronal identity occurs between E9-14 within the proliferative zone (grey) of the ventral midline. During this developmental stage, Msx1 and Foxa2 drive neurogenesis by regulating Ngn2. Importantly, Lmx1a, in combination with Msx1, specifies mDA neuron cell fate. mDA neuron progenitors enter the intermediate zone (yellow) when they become postmitotic. At this stage, mDA neuron progenitors begin to express the pan neuronal marker, Tuj1, followed by the DA neuron transmitter regulator, Nurr1. Lmx1b and Wnt1 regulate early Pitx3 expression in some Nurr1-expressing cells. The final phase in mDA neuronal differentiation commences as the Pitx3-expressing cells migrate ventrally to the peripheral zone (pink). Eventually, this cell population migrates laterally to

constitute the neural population of the SNpc. At this stage, cells begin to express TH. En1 and En2 are required to promote and maintain survival of the mature mDA neurons localised in the ventral midbrain[50].

1.3.5 Functional maturation of dopaminergic neurite projections from the ventral midbrain

Terminally differentiated mDA neurons undergo functional maturation involving axonal pathfinding and synaptogenesis. Development of mDA axons occurs at E11 in mice (E13 in rat) and by E14.5, the majority of axons have reached their destination. In 2004, Gates and colleagues found that axons initially send their projections dorsally before migrating ventro-rostrally toward the forebrain, by responding to extrinsic instructive cues in the dorsal midbrain and repulsive cues emanating from the caudal brain stem[54]. Chemoattraction then directs migration of these axons toward the telencephalon via the medial forebrain bundle, through the diencephalon where the axons terminate in the striatum and cerebral cortex. This axonal migration and relevant striatal innervation by nigral DA neurons is directed by chemmoattractive cues from the striatal region and chemorepulsion from the cortex. Gates *et al* also showed that contact-dependent inhibitors further assist axonal pathfinding of VM DA neurons by hindering their access through the thalamus. Recently, a study showed how transcription factor *Nurr1* coordinated the expression of the axon genesis gene topoisomerase IIβ (*TopIIβ*), thereby having a regulatory effect on DAergic neuron axonal extensions. A number of molecules implicated in establishing the development of VM DA circuitry and axonal guidance include: Ephrin B2 and its receptor EphB1, netrin receptor DCC and expression patterns of the SLIT family and their receptors robo1/2[44].

1.3.6 Neuroprotective growth factors

Following development of the VM DA circuitry and appropriate innervation of DAergic targets, newly formed axons of these neurons are prone to cell death so therefore contest to develop functional synapses and for survival. Naturally occurring DAergic cell death occurs in the rat near birth, peaking at P2, in addition to a second peak of apoptosis at P14[55]. A family of proteins called neurotrophic factors function to promote growth, survival of newly developing neurons and maintenance of mature neurons. Cell survival at this point is reliant on the limited availability of target derived neurotrophic factors to protect VM DA neurons, namely glial cell line-derived neurotrophic factor (GDNF)[56], neurturin (NTN; member of the GDNF protein family)[57], transforming growth factor βs (TGFβs)[58], brain-derived nerve factor (BDNF)[59] and growth/differentiation factor 5 (GDF5)[60].

Transcription Factor	Expression day in the mDA lineage	Function in mDA cells	References
Otx2	E7.0	Determine forebrain and midbrain territories within developing neural tube. Specification of mDA progenitors	Puelles et al (2003); Vernay et al (2005)
Foxa2	E7.0	Influence mDA neuronal development. Regulate Nurr1 and En1 expression in immature neurons. Also controls TH and AADC expression in differentiated mature DA neurons.	Ferri et al (2007).
Pax2	E7.5	Coordinate precise development of mid/hindbrain area through En2 signalling. Induction of FGF8 expression.	Bouchard et al (2005); Ye et al (2001)
Lmx1b	E7.5	Maintenance of Wnt1 expression and of mature mDA neurons.	Smidt et al (2000)
Foxa1	E7.5	Influence mDA neuronal development. Regulate Nurr1 and En1 expression in immature neurons. Also controls TH and AADC expression in differentiated mature DA neurons.	Ferri et al (2007).
En1/2	E8.0	Generation and survival of mature mDA neurons	Simon et al (2001); Alberi et al (2004)
Wnt1	E8.0	Controls En1/2 expression. Regulates DA progenitor pool and mature DA neuron differentiation.	Danielian and McMahon (1996); Prakash et al (2006); McMahon et al (1992)
Shh	E8.0	Ventral patterning events. DA neuron induction. Govern transcription factor expression in NPCs.	Hynes et al (1995); Briscoe et al (2000)
FGF8	E8.0	Maintenance of MHB. Specification of mesencephalic progenitors.	Ye et al (1998)
Lmx1a	E9.0	Regulates expression of Msx1 for determining DA neuronal cell fate.	Andersson et al (2006a)
Msx1	E9.0	Required for neuronal differentiation. Regulates Ngn2 expression.	Andersson et al (2006b)
Ngn2	E10	Required for neuronal differentiation.	Andersson et al (2006a); Kele et al (2006)
Nurr1	E10.5	Maintenance of mature mDA neurons. Expression of late differentiation markers: TH, AADC, DAT and VMAT.	Smits et al (2003)
Pitx3	E11.0	Essential for TH expression in mature neurons. Survival of SNpc and VTA neurons.	Hwang et al (2003); Nunes et al (2003); van den Munckhof et al (2003); Smidt et al (2004); Maxwell et al (2005).

Table 1.3 Summary of the role of transcription factors required during DAergic neuronal development

1.4 Current approaches to the treatment of PD

At present, no beneficial therapies have been established to potentially prevent the onset of PD or offer support to remaining neurons. Current drug and surgical therapies are capable of reducing PD symptoms but do not prohibit the progressive nature of the disease. Oral preparations of L-3, 4-dihydroxyphenylalanine (L-DOPA) and DA receptor agonists are administered as a PD treatment, with the aim of alleviating motor symptoms. L-DOPA increases DA concentrations in PD patients and unlike DA; it crosses the blood brain barrier (BBB) where it enters the CNS. Here, it is converted to DA by the enzyme aromatic L-amino acid decarboxylase, commonly referred to as dopa decarboxylase (DDC). Continuous L-DOPA delivery via intestinal treatment can also be used, and this continuous release has been attributed to the prevention of L-dopa induced dyskinesias (LIDs). DA agonists function by stimulating DA receptors, thereby aiming to ameliorate motor symptoms associated with PD. In advanced PD cases, administration of apomorphine proves to be an effective non-selective DA agonist, activating D_1Rs and D_2Rs.

A major disadvantage of employing long-term utilisation of DAergic drug therapies is generation of disabling adverse effects, including production of drug-induced motor complications such as on-off fluctuations and LIDs[61]. Deep brain stimulation (DBS) is a surgical treatment used to mitigate PD motor symptoms, involving implantation of a brain pacemaker (electrode) to deliver electrical pulses to two common target structures within the BG; STN and GP. One of the main effects of DBS is to reduce tremor by targeting the STN[62].

1.4.1 Cell replacement therapy

The last four decades of research have brought us closer to turning cell replacement therapy (CRT) into a reality, by aiming to achieve effective repair and functional recovery in brain damage and disease. The premise behind such a therapy would be that identical A9 nigral DA specific neurons transplanted to the DA-depleted striatum could restore DAergic neurotransmission, thereby generating a functionally efficient substitute for the neurons lost/damaged in PD[61–64]. This important attribute of the SNpc component of the striatal grafts enables transplanted neurons to re-innervate and activate the dorsolateral region of the host striatum.

It is evident from previous clinical trials that transplantation of human fetal ventral mesencephalic (hfVM) tissue to the striata of PD patients proved that such grafts were capable of promoting restoration of DAergic function to the striatum[65]. More recent trials provided further proof of concept that transplanted grafts (using hfVM tissue) could functionally reinnervate the denervated striatum, restore DA release and promote significant long-term clinical advancements in a number of PD patients[66].

It should be noted that scientists realised the key to accomplishing effective repair in the adult brain was to transplant specific neuronal subtypes lost to the disease process. Studies showed that it is important for donor neurons to be transplanted at a precise time when their fate is already specified, as reviewed by Dunnett and Rosser, 2011[63]. This is due to the fact that expression of developmental signals in the foetal brain are not present in the adult brain to direct neuronal differentiation. Additionally, donor cells must be within a particular developmental window, i.e. cells too developmentally advanced would be less likely to survive the transplantation procedure.[63] Post transplantation, local signals prompt host axons to

sprout and innervate graft tissue, thereby forming reciprocal connections between graft and host neurons. This was an important finding and turning point in light of the theories surrounding the limited plasticity of the adult mammalian brain, since the time of Cajal[63,67].

1.4.1 .1 Human fetal ventral mesencephalon tissue

The obvious source of cells for a replacement therapy for PD would be from hfVM [68], since this tissue contains postmitotic cells committed to the DAergic lineage. The first cell transplants in PD patients provided proof-of-principle that ectopic engraftments of fetal VM tissue to the striatum not only survived well, as visualised using fluorodopa PET imaging, but also induced functional recovery of motor symptoms, enhanced the functional efficacy of concurrent L-Dopa and moreover augmented the proportion of each day spent in the "on" phase[62,66,68]. DA series of similar translational clinical trial studies in PD patients using intrastriatal hfVM tissue demonstrated long-lasting symptomatic relief in some patients for more than 16 years post-transplantation[62].

Despite some success in the clinic, not all patients experienced positive outcomes and initial clinical trials were impeded by lack of consistent outcomes and graft-induced dyskinesias (GIDs) present in a subgroup of patients[69]. Post-operative GIDs may result from excessive DA release due to continuous neural outgrowths of the grafts[70] or atypical regulatory release of DA originating from the transplanted tissue[71]. Furthermore, the presence of serotonergic neurons (in relation to low yields of appropriate DA nerve cells within the grafts) could potentially lead to false DA release; serotonergic neurons are capable of producing and discharging the

neurotransmitter DA derived from exogenous L-Dopa, but do not have the mechanisms seen in DA neurons to modulate release and re-uptake of DA[72]. Serotonin 1A agonists (buspirone) effectively suppress serotonergic neurons within grafts or alternatively, cell sorting could minimise the prevalence of serotonergic components when generating mDA neurons from SC sources[70,73].

Despite recent suggestions for overcoming these limitations regarding intrastriatal transplantations, it is doubtful that hfVM tissue grafts will be considered as a routine therapeutic procedure for PD, owing to shortcomings with tissue availability and standardisation of the grafts[74]. Furthermore, the harvest of this tissue from termination of pregnancies and requirement of large numbers of foetuses (between three and six foetuses per side of the brain grafted) to produce adequate transplantable neurons further limits the use of this source[62].

1.4.2 Stem cells for dopamine replacement

So, despite success with hfVM transplants and more well-defined understanding of DAergic pathology, there is still no cell replacement therapy (CRT) for PD. Ethical and logistical issues surrounding the use of tissue obtained from aborted human embryos hinder the translation of hfVM to clinic. In this respect, current research is now focusing on generating A9 DA neurons for transplantation from stem cell sources. Research has turned to generating donor cells from stem cell sources, of which human pluripotent stem cells are powerful contenders, with human embryonic stem cells (hESCs) advancing the furthest[74].

However, it remains crucial that a number of prerequisites are met concerning the clinical implementation of pluripotent stem cells toward large-scale clinical

applications to provide a readily available, renewable and bankable source of cells to generate functionally DA neuronal populations[61,63,64,75]. The issues to be considered are outlined as follows:

I. Establishing criteria for directing efficient and robust differentiation and predictive validity of competitive clinical grade protocols.

II. Yields of cells of appropriate neuronal phenotype remain variable. It is fundamental that cells cultured *in vitro* are generated in adequate numbers to achieve a substantial level of engraftment in a clinical setting.

III. It is necessary to ensure that nigral DA neurons are accurately defined. This can be achieved using immunostaining techniques for specific markers typical of these cells, as well as demonstrating gene and transcription factor profiles definitive of distinct brain regions during early embryonic development. Neurophysiological measures and controlled dopamine release must be tested to authenticate neuronal excitability. To eliminate unwanted cells in cultures with respect to ESC and induced pluripotent stem cell (iPSC) sources, cell types must be identified, particularly the number of nonnigral DA neurons, serotonergic neurons, glial cells, undifferentiated stem cells, progenitor cells and nonneural cells.

IV. Post CRT, long term retention of target phenotypes remains poor with limited integration to the host circuitry. Adequate numbers of transplanted DA cells

(i.e. more than 100,000 per human putamen) are required to grow axons and provide reinnervation to the host striatum over distances suitable for the size of the human brain. Stem cell-derived neurons should reverse motor deficits underlying PD over a prolonged period of time to provide symptomatic relief in patients.

V. Finally, it is imperative that risk of adverse effects such as GIDs, secure regulation of proliferation and potential for tumourigenesis encountered using pluripotent and multipotent stem cell sources, neural outgrowths and immune reactions are eliminated from stem cell sources.

1.4.2.1 Embryonic stem cells

Prior to implantation, the inner cell mass (ICM) of the blastocyst generates a source of primitive and highly proliferative cells known as ESCs. ESCs are capable of differentiating into cell types of the three germ layers (pluripotent cell source), including DAergic neurons. Advantageous properties of ESCs include their aptitude to be cultured for extended periods *in vitro* whilst sustaining their pluripotency and their capacity for self-renewal[76].

Transplantation of rodent ESC-derived DAergic neurons to the striatal region of PD rat animal models promoted some degree of functional and behavioural recovery, with axons projecting their extensions beyond the graft core to establish synaptic connections with the host striatum. However, the relatively low survival rate of these neurons is a disadvantage of ESC-derived DAergic neurons[77].

Nevertheless, recent protocols for generating DAergic neurons from ESC sources employing the genetic manipulation of transcription factors to programme the cells

have shown great potential in directing differentiation to high yields of more authentic nigral neurons[78,79]. Despite such encouraging results, further research is required to generate substantial numbers of A9 nigral numbers showing extensive fibre outgrowth and to eradicate the risk of tumourigenesis within transplants[63,74]. Ethical implications inherent with human ESC derivation remain an additional consideration.

1.4.2.1.1 Mouse embryonic stem cells

Early transplantation studies using undifferentiated mESCs showed that this stem cell source could be successfully grafted to the rodent striatum,[80] and were capable of generating DA neurons, and alleviate some of the behavioural deficits in rodent models of PD[81]. However, these studies also demonstrated the formation of teratoma-like tumours.

Specifically, it was first reported that mESCs could be directed to a neural fate by administrating retinoic acid (RA)[82], or bFGF to cultures[83]. Later work focused on the application of developmentally relevant DA specific factors, including Shh, FGF8, BDNF and ascorbic acid (AA), to generate higher yields of DA neuronal populations.[83] This approach increased the number of DA-expressing neurons to ~ 20-30% of the total cell population. Following the discovery that cell-intrinsic transcription factors play a fundamental role during the normal development of DA neurons, scientific studies shifted to a new approach involving the over-expression of these genes in ESCs. In this regard, over-expression of *Nurr1, Pitx3, Lmx1a* and *Lmx1b* lead to the production of large numbers of DA neurons *in vitro*[84–86]. More recently, inhibition of certain pathways has directed the production of large number of neuronal populations in culture from ESCs. For example, disruption of the FGF

signalling pathway has been reported to increase the numbers of DA neurons *in vitro* (this will be discussed in detail in Chapter 6)[87].

1.4.2.1.2 Human embryonic stem cells

The early studies with mESCs lead to the isolation of ESC lines from primate models[88] and humans[89]. However, differences between mESCs and hESCs became apparent; discussed in detail in Chapter 6. Over-expression of transcription factors implicated in DA neuron development was translated to hESCs (via transient transfection), to increase the yield of hESC derived DA neurons in culture, which included *Lmx1a, Nurr1* and *Pitx3*[90,91]. Recent research showed that inhibiting certain molecular pathways, such as the Contraction of Sma and Mad (Mothers against decapentaplegic) pathway (involved in TGFβ signalling), resulted in the rapid conversion of hESCs to a neural fate under *in vitro* culture conditions[92]. Thus, these important studies demonstrated that the ability to reproduce and reliably generate DA neurons *in vitro* from an ESC source was possible.

1.4.2.2 Epiblast stem cells

Recent research found that epiblast stem cells (EpiSCs) provide a more efficient and developmentally-suitable source of pluripotent stem cells for neural induction *in vitro,* and moreover for efficiently directing nigral and striatal neuronal phenotypes *in vitro (reviewed in detail in Chapter 6).* Jaeger and colleagues found that EpiSCs provide a more accelerated DAergic neural differentiation protocol over ESC differentiation methods[87].

1.4.2.3 Patient-specific reprogrammed cells

In light of the limitations associated with hfVM tissue and ESCs, research has also been conducted to investigate patient-specific derived neurons as a potential cell

source which would circumvent some of the key challenges. Studies aimed to reprogramme patients' somatic cells i.e. skin fibroblasts to a stage of pluripotency before directing their conversion to iPSCs and subsequent differentiation to DAergic neurons. In 2006, Yamamaka and associates reprogrammed adult somatic cells to an ESC-like state by applying four defined factors; *Oct 3/ 4, Sox2, c-myc* and *Klf4*. These somatic cells were then converted by retroviral transduction to a pluripotent cell type (i.e. iPSC)[93]. iPSCs are not as restricted by ethical issues to the same extent as human ESCs and hFVM tissue and avoid the risk of immune rejection and subsequent immunosuppressive therapy following autologous transplantation.

However, a number of issues need to be addressed if this stem cell type is to be clinically translated as a PD treatment, owing to the fact that iPSCs derived from PD patients are associated with the prevalence of mutations, polymorphisms or epigenetic marks, making them more susceptible to the occurrence of PD-like features[94]. In addition to this, *c-myc* and *Klf4* linked to oncogenic activity and viral vectors integrated into the genome increase the risk of tumour formation and mutations.

1.4.2.4 Fetal Brain Neural Stem Cells

Fetal brain neural stem cells (NSCs) are classified as multipotent stem cells due to their restricted ability to differentiate only into neural phenotypes, i.e. neurons, astrocytes and oligodendrocytes. This cell type can be obtained directly from an affected PD patient, therefore eradicating risks associated with graft or immune rejection and tumour formation[95]. Early studies using non-differentiated NSCs derived from human tissue transplanted into rat models showed limited

differentiation capabilities *in vivo*. Similarly, translational studies in primate models highlighted the restricted abilities of the NSC progeny to differentiate into DAergic neuronal cell types[62]. However, it should be noted that Shh and Wnt5a signalling did increase DAergic cell yield from differentiated NSCs *in vitro*[96]. Advanced differentiation protocols will need to be devised in order to produce specific cell types from NSCs and establish improved homogeneity between grafts.

1.4.2.5 Bone marrow-derived stromal cells and mesenchymal stem cells

Bone marrow-derived stromal cells and mesenchymal stem cells (MSCs) comprise a small population of cells localised in the bone marrow with the capacity to give rise to adipocytes, chondrocytes and osteoblasts, both *in vitro* and *in vivo*[62]. TH$^+$ neurons generated from non-differentiated murine MSCs were shown to enhance motor performance in mice models[97]. Similar results were observed in MSC-derived DAergic neurons obtained from both rats and human samples post-transplantation in PD animal models[98]. In 2010, an open-labelled clinical trial utilising unilateral autologous MSCs transplanted into the sublateral ventricular zone in PD patients exhibited no adverse effects after 12 months of receiving the transplantation; however only modest clinical improvement was observed in human participants[99].

1.5 Vitamins

A number of key antioxidants, specifically the biologically active metabolites of vitamins including vitamin A (RA), vitamin C (AA) and vitamin D$_3$ (calcitriol) known to play crucial roles in embryonic development, particularly in mDA neuronal development, have successfully been applied *in vitro* to control cell differentiation. This section will summarise unique roles for these vitamins throughout the CNS and most importantly their functioning in stem cell

differentiation and transplantation. Finally, similar possibilities for nicotinamide, the vitamin B$_3$ metabolite will be discussed, with implications for potential neuronal replacement therapies.

1.5.1 Calcitriol

1.5.1.1 The role of calcitriol in the central nervous system

Calcitriol is the hormonally active metabolite of fat-soluble vitamin D. The vitamin D receptor (VDR) protein is widely expressed throughout human and rat brains and specifically in the BG, pontine-midbrain area, cerebellum, thalamus, hypothalamus, hippocampus and olfactory system[100]. VDR is present in most neurons and some glial cells, with robust expression levels observed in SNpc DAergic neurons[101]. This receptor is significantly increased during CNS development, notably during gestation periods (day 12-21) in the rodent brain, with expression levels preferentially localised in areas of cell proliferation, neuroepithleium and differentiating fields[102]. Interestingly, raised VDR expression at E18 in the rat brain corresponds to increasing apoptosis and a reduction in mitosis, suggestive of a role in the maintenance of normal brain functioning[103].

In 2010, Orme and colleagues performed multiplexed quantitative proteomic analysis of the embryonic midbrain to investigate and identify signalling proteins governing neuronal differentiation, maturation and survival in E11-14 tissue. VDR was one of the candidate signalling proteins expressed in developing midbrain tissue at E12 (critical time point in the development of mDA neurons), therefore suggesting a function for vitamin D during DAergic neuron specification.

1.5.1.2 The role of calcitriol during cell differentiation

Orme *et al* investigated the role of the vitamin D_3 metabolite, calcitriol, in primary culture differentiation. Primary E12 VM cultures treated with calcitriol elicited a dose-responsive increase in the number of DAergic neurons. This study showed that the increase in number of DA neurons was directly attributable to a calcitriol-induced increase in GDNF expression[104].

Specifically, the role of the neurotrophic factor GDNF as a neuroprotective agent during both embryonic development and against various forms of neuronal insults has been validated in a number of *in vitro* and *in vivo* studies, and has been long acknowledged to confer neuroprotection to mDA neurons[104–106]. The active metabolite of vitamin D_3 is a potent inducer of GDNF expression[107,108]. Unlike GDNF, vitamin D_3 has the ability to cross the BBB, suggesting a potential neuroprotective function for this compound against neuronal injury.

Wang *et al* found that VM primary rodent cultures pre-treated with vitamin D_3, induced neuroprotection of VM-derived neurons against 6-hydroxydopamine (6-OHDA) or hydrogen peroxide (H_2O_2)-induced cell death *in vitro*. This group also found that administration of vitamin D_3 significantly improved peak locomotor activity and normalised SNpc DA neurons and its metabolites in lesioned 6-OHDA animal *in vivo*, an effect possibly mediated via a GDNF mechanism[109].

1.5.2 Retinoic acid

1.5.2.1 The role of retinoic acid in the central nervous system

RA is a well known endogenous morphogen with important roles in the patterning of the CNS and neuronal differentiation. Specifically, this vitamin metabolite functions in the anteroposterior and dorsoventral patterning of the neural plate and neural tube. Newly formed somites in the dorsoventral axis of the developing neural tube are responsible for generating RA during development, along with other factors, such as

Shh and FGFs, which play fundamental roles in determining cell fate of subsets of sensory neurons, interneurons and motor neurons[110]. With regards to PD research, there is evidence for the functioning of RA at late development, with a suggested role in the establishment of appropriate mDA identity[111].

1.5.2.2 The role of retinoic acid during cell differentiation

RA has successfully been applied to a number of *in vitro* differentiation protocols including neuronal and retinal protocols[112–114]. The binding of RA to the retinoic acid receptor (RAR) and retinoid X receptor (RXR) stimulates the transcription of tissue specific genes resulting in cellular differentiation[115]. RA functions cooperatively with signalling proteins such as Shh and FGFs to improve the regional identity of progenitor cells derived from human ESCs[116].

With respect to PD, high levels of RA signalling functions in the striatum. DAergic neurons localised throughout the nigrostriatal circuit contain high levels of retinaldehyde dehydrogenase family 1 gene (RALDH1), potentially generating RA in axonal terminals which in turns functions in an autocrine manner on neurotransmission or alternatively in a paracrine fashion on striatal cells. The DAergic receptor gene, D2 contains a functional retinoic acid response element (RARE), which is strongly activated by a RARα-RXRγ heterodimer. *Nurr1* is an example of another heterodimeric partner of the RXRs; proving a clear role for RA signalling in the normal functioning of the nigrostriatal circuitry[115].

1.5.3 Ascorbic acid
1.5.3.1 The role of ascorbic acid in the central nervous system

Vitamin C and its metabolite AA are highly concentrated throughout the CNS, and function as antioxidative agents to maintain the normal functioning of brain structures. High levels of vitamin C are necessary in the brain to deal with elevated levels of oxidative stress[117,118]. Along with its neuroprotective role in the CNS, vitamin C is a cofactor in several enzymatic reactions including the synthesis of catecholamines[119], along with a specific role in promoting Schwann cell myelination in the PNS[120].

The foetal rodent brain exhibits high levels of vitamin C expression; and levels have been reported to double between gestation day 15-20 before decreasing significantly prior to birth[121]. Indeed, amongst all organ systems, the highest concentrations of AA are attained in neural tissue, which may be attributable to the existence of an AA-transporter system in neurons, necessary for regulating the steep intra/extracellular concentration gradient[122]. Notably, high concentrations of AA are found in forebrain structures including the neostriatum, a structure abundant in DA terminals with evidence suggesting that the SN plays a crucial role in governing the release of AA in the neostriatum.

1.5.3.2 The role of ascorbic acid during cell differentiation

High levels of SVCT2, the vitamin C transporter are expressed in CNS precursors and embryonic mesencephalic neurons, implying a direct effect on the process of DAergic differentiation[123]. Vitamin C has been used to modulate stem cell transplantation. A number of studies showed that stem cells treated with AA could direct differentiation into increased numbers of DAergic cells[124–126]. Incubation of mouse or human somatic cells with vitamin C generates an increase in the induction of stem cells, mediated in part by regulation of histone demethylase activity, a family

of enzymes necessary for Nanog expression (a master transcription factor for self-renewal of undifferentiated ESCs)[127]. However, AA has been reported to elicit a "double edged sword" effect in a study carried out by Bagga *et al*, where low concentrations of AA had no effect on neuronal numbers and high concentrations were linked to neuronal cytotoxic effects[124].

The anti-oxidative properties of vitamin C have been shown to eliminate free radical accumulation generated by both the endogenous tissue and transplanted exogenous stem cell-derived neurons[122]. Bagga *et al* further demonstrated significantly higher numbers of surviving DAergic neurons in grafts treated with AA, compared to control treatment groups[124]. Similar findings were described by Agrawal and colleagues, who used AA glutathione in combination, to significantly enhance DAergic neuronal survival post transplantation[128]. The potential of this vitamin to significantly reduce apoptosis and moreover, directly favour stem cell generation and differentiation towards a DAergic lineage make ascorbic acid an attractive co-adjuvant to accomplish a CRT for PD.

1.5.4 Nicotinamide

Nicotinamide, the amide form of niacin (vitamin B$_3$), is classified as a water-soluble B complex vitamin. Sources of nicotinamide are obtained from the diet and from the metabolism of endogenous tryptophan, an essential amino acid. Catabolism of the active component of nicotinamide is primarily via *N*-methylation to *N*-methyl nicotinamide (main excretory product) by the xenobiotic enzyme nicotinamide *N*-methyltransferase (NNMT). Remaining nicotinamide produces two important co-enzymes; nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), implicated in over 200 enzymatic reactions.[129] This

compound is integral for the formation of mitochondrial ATP along with other various neuroprotective and anti-oxidant roles[130].

1.5.4.1 The role of nicotinamide in the central nervous system

Nicotinamide can cross the BBB to the CNS via an active transporter. In the CNS, nicotinamide regulates neuronal and vascular cell populations following brain injury. In the early stages of developing cerebral infarction in the ischemic brain, decreased levels of NAD^+ are observed, preceding neuronal apoptosis. Interestingly, administration of nicotinamide 1-2 hours post ischaemia increased brain NAD^+ levels and mitigated necrotic and apoptotic brain injury following focal brain injury[131].

With relevance to PD, NADH is fundamental for the normal functioning of mitochondrial complex 1, which is well established to be defective in MPTP Parkinsonism[132] and idiopathic PD[133,134]. NADH is also integral to the production of tetrahydrobiopterin[135], a co-factor necessary for TH, the rate limiting enzyme in catecholamine biosynthesis, also deficient in PD. NADH is also linked to reduced glutathione, an important anti-oxidant shown to be insufficient at the early stages of PD[129,136].

1.5.4.2 The role of nicotinamide during cell differentiation

Historically, experimental research dating from the 1970s depicted a role for nicotinamide, the active form of vitamin B_3 to function as a morphogen during the process of differentiation to influence cell fate specification. A morphogen is defined as a signalling molecule which directly influences specific cellular responses based on its local concentration. In this context, experiments conducted by Caplan and Ordahl, showed that intracellular NAD played a fundamental role in directing the

choice of cell fate between muscle or cartilage phenotypes. Interestingly, they found that internal pool sizes of NAD localised within the limb mesenchymal cell nucleus were directly influenced by extracellular concentrations of its precursor nicotinamide. The choice mechanism regulating the differentiation of cartilage versus muscle lineages was linked to distinct high internal concentrations of NAD promoting muscle differentiation and low internal NAD levels determining a bone-like phenotype characterised by chondrogenic expression[137].

Consistent with the functioning of other vitamins and their metabolites to effectively direct stem cell differentiation to appropiate cell fates, nicotinamide has also been shown to promote efficient differentiation of pluripotent stem cells toward specific cell populations (detailed account provided in Chapter 3). In addition, Maiese *et al* found that nicotinamide increased cell survival and functions as a neuroprotective agent *in vitro*[138]. Nicotinamide is an inhibitor of Poly-ADP-Ribose (PARP), an enzyme implicated in DNA repair and cell death leading to depletion of both NAD^+ and ATP. Inhibition of PARP by nicotinamide can protect cells from oxidative stress, in addition to apoptosis and necrotic forms of cell death, and influences DNA degradation via a number of cell pathways encompassing PARP, Akt, forkhead transcription factor, mitochondrial membrane polarization, cytochrome C and is an inhibitor of caspase-1, caspase-3 and caspase-8[136].

In animal models of PD, nicotinamide demonstrated neuroprotective properties by attentuating striatal DA depletion and nigral cell loss in acute MPTP-treated animals[139], and significantly enhanced locomotor activity and protected DAergic neurons against MPTP induced neurodegeneration[140]. Thus nicotinamide may potentially offer new therapeutic strategies, as a neuroprotective agent for neurodegenerative disorders of the CNS[141]. In this regard, there is an active uptake

mechanism for nicotinamide in the brain and following systemic administration, this vitamin metabolite is evenly distributed throught brain tissue.

1.6 Aims and strategies of the project.

During neural development, the specification of neurons, their maintenance, axonal elongation and connectivity with other neurons requires complex signalling cascades that are both spatially and temporarily restricted. These signalling cascades can be either internal to developing neurons or exogenously triggered by small molecules. Such exogenous signals include vitamins and their active metabolites. Vitamins are small organic molecules required throughout development and life. Until recently very little research has been undertaken into nicotinamides role in neural development or idiopathic Parkinson's disease. This project aims to investigate the influence of nicotinamide on the development and function of neurons during early embryonic development. The research aims can be described in detail as follows:

i.   Maintain mESCs in an undifferentiated state using LIF (made in house).

ii.   To determine whether the biologically active vitamin B_3 metabolite, nicotinamide, could direct the differentiation of mouse embryonic stem cells, cultured as monolayers, into neurons at either early or late stages of development. If yes, what is the mechanism responsible for nicotinamide-mediated change in cell fate?

iii.   Does nicotinamide treatment influence the proportion of undifferentiated and/or neural progenitor cells in culture?

iv.   Does nicotinamide preferentially influence specific cell types?

v.   To generate epiblast stem cells.

vi.   To investigate whether nicotinamide administration to these epiblast cells potentiates the generation of dopaminergic neurons.

Detailed descriptions of the chapter objectives are stated at the end of the introductions to each experimental chapter.

Chapter 2: Materials and Methods

2.1 Materials

All reagents used throughout this study were purchased from Sigma, UK, unless specified otherwise (media and solution components are detailed in Appendix 2).

2.1.1 Maintenance of undifferentiated mESCs with Leukaemia Inhibitory Factor

2.1.1.1 Maintenance of HEK293 Cell Line

The human embryonic kidney (HEK) 293 cell line (a kind gift from Dr. Mark Pickard, School of Life Sciences, Keele University, UK) was used for the production of leukaemia inhibitory factor (LIF), required to maintain undifferentiated mESCs *in vitro*. The HEK cell line has been reported to provide a robust and reliable platform for gene delivery in *in vitro* biological studies[142]. HEK cells were cultured on a T25 culture flask in RPMI-HEK medium comprising RPMI-1640 medium supplemented with 10% foetal calf serum (FCS) (Biosera, UK) and 4 mM $_L$-glutamine. HEK cells were passaged once they reached a confluency of approximately 90-100% (Figure 2.1). RPMI-HEK medium, 0.025% trypsin solution and phosphate buffered saline (PBS) were pre-warmed to 37°C in a water bath for 1 hour (h). RPMI-HEK medium was aspirated and cells were washed briefly with 5 ml PBS. Trypsin (0.025%; 1 ml) was evenly distributed over the surface of the culture flask and detached cells were centrifuged, and then re-suspended in fresh medium. Cells were split 1:5 every 2 days in 5 ml RPMI-HEK medium and maintained at a temperature of 37°C and CO_2 level of 5.0%. All cell cultures described in this chapter were grown under these conditions.
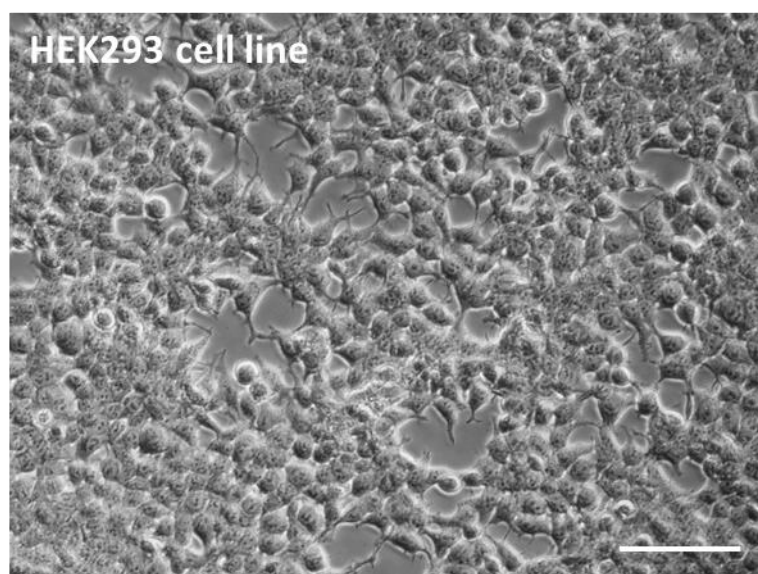
Figure 2.1 Representative image of HEK293 cell line. HEK cells were passaged once they reached a confluency of approximately 90-100%, indicated in the brightfield image. Scale bar = 100 μm

2.1.1.2 Transfection reagent optimisation study

A transfection optimisation experiment was conducted using FuGENE® HD (Fugene) (Promega, Southampton, UK) and X-tremeGENE HP DNA Transfection Reagent (X-tremeGENE) (Roche, West Sussex, UK), to accomplish the highest transfection efficiency for gene delivery into HEK adherent cells. For this comparative study, the plasmid pmaxGFP coding for green fluorescent protein (GFP) (size 3.5kb; Amaxa Biosciences, Cologne, Germany) was used to determine transfection efficiency (Figure 2.2).

One day prior to transfection, HEK cells were passaged and plated at a density of 1.6 x 10^5 cells per well of a 24-well plate, in 500 μl RPMI-HEK medium, in accordance with the transfection reagent manufacturers' guidelines. It is important to point out that experiments were conducted in the absence of antibiotics in medium, due to their adverse effects on transfection[143]. After overnight culture, different ratios

(volume) of Fugene and X-tremeGENE to pmaxGFP plasmid were tested in accordance with the manufactures' guidelines and recommendations (Table 2.1 and 2.2).

Table 2.1 Transfection optimisation: Fugene

pmaxGFP Plasmid (0.55 µg)	Sterile deionized water	Ratio	FuGENE® HD (µl)
1.1 µl	25.75 µl	3:1	1.65 µl
1.1 µl	25.75 µl	2:1	1.1 µl
1.1 µl	25.75 µl	4:1	2.2 µl

Table 2.2 Transfection optimisation: X-tremeGENE

pmaxGFP Plasmid (0.55 µg)	Sterile deionized water	Ratio	X-tremeGENE (µl)
1 µl	49 µl	1:1	0.5 µl
1 µl	49 µl	2:1	1 µl
1 µl	49 µl	3:1	1.5 µl
1 µl	49 µl	4:1	2 µl

Table 2.1 and Table 2.2 Different ratios of Fugene and X-tremeGENE to pmaxGFP plasmid were tested for the transfection reagent optimisation study, in line with the manufactures' guidelines and recommendations.

The following day, transfection levels were determined by examining cultures using fluorescence microscopy and brightfield microscopy. Brightfield microscopy demonstrated that both reagents tested were not toxic to HEK cell cultures (Figure 2.2). The highest level of transfection was achieved in the presence of 1.65 µl Fugene in combination with 0.55 µg pmaxGFP, in comparison to X-tremeGENE which was not as potent for gene delivery [Figure 2.2 (A-B)]. In conclusion, these findings suggested that Fugene was an appropriate reagent to transfect HEK cells for LIF production, to maintain undifferentiated mESC in a pluripotent state.

| | Brightfield | PMAX-GFP | Merged |

A. FuGene HD

B. X-tremeGENE
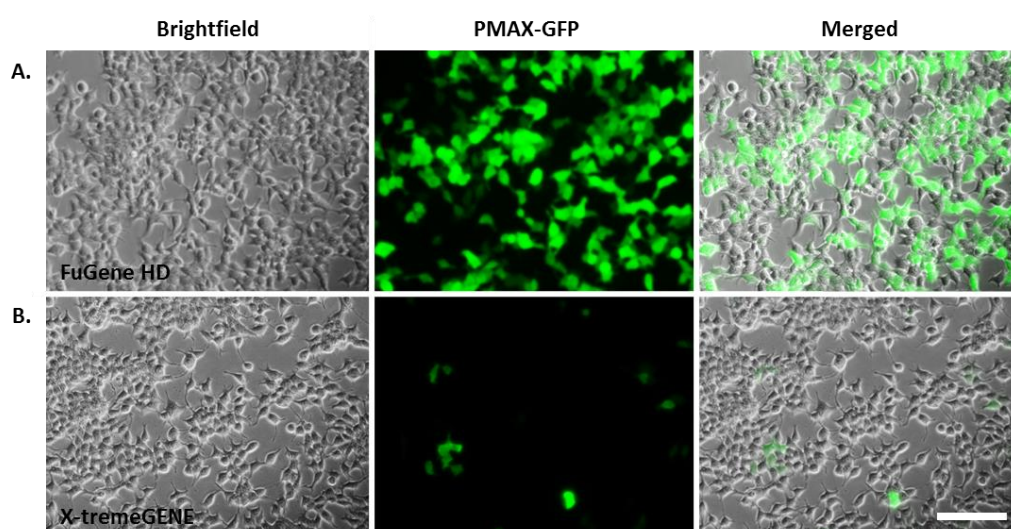
Figure 2.2 Transfection reagents were optimised using HEK 293 cells with pmaxGFP, prior to LIF production (A&B) Representative images showing brightfield, GFP fluorescence and merged images. Scale bars = 100 μm. **(A)** The highest level of transfection was achieved in the presence of 1.65 µl Fugene in combination with 0.55 µg pmaxGFP, in comparison to X-tremeGENE, which was not as potent for gene delivery **(B)**. **(A&B)** Healthy HEK 293 cells maintained their morphology once transfected.

2.1.1.3 Transfection of HEK Cells for LIF Production

HEK cells were plated onto 6-well plates (1.6 x 10^5 cells/well; 2.5 ml RPMI-HEK medium). The following day, human LIF cDNA (made in house by Dr. Eunju Shin, School of Life Sciences, Keele University, UK) and Fugene were used to transiently transfect HEK cells for LIF production. For transfection complex preparation, 772.5 µl sterile deionized water and 49.5 µl Fugene were mixed gently. After 5 min, 12 µg (33 µl) Human LIF cDNA was carefully pipetted directly into the mixture and incubated at room temperature (RT) for 10 minutes (min). Transfection complexes were then added in a dropwise manner to HEK cells per well, gently swirling the 6-well plate to ensure even distribution over the entire well surface and cultured overnight.

The following day, medium was replaced in each well with 2.5 ml RPMI-HEK medium. Following a 4 day culture period, RPMI-HEK medium was collected and centrifuged at 1200 rpm for 5 min and the supernatant collected. The supernatant was centrifuged again and filter-sterilised using a 0.22 µm filter (Millipore, Watford, UK).

2.1.1.4 Titration of LIF

A titration assay was used to determine the concentration of LIF required to maintain pluripotent 46C *Sox1*GFP mESCs (a kind gift from Professor Meng Li, Neuroscience and Mental Health Research Institute, Cardiff University, UK) in an undifferentiated state. 46C mESCs were plated in a 24-well plate at a density of 10^4 per well in 1 ml FCS medium. Cells were allowed to settle in an incubator for approximately 4 h. Medium was replaced with fresh LIF medium, which was serially diluted across the plate (Table 2.3). For control wells, FCS medium supplemented with stock LIF (made in house by Dr. Eunju Shin) or no LIF was added to the last 4 wells of the plate. mESCs were incubated for 4 days.

Table 2.3 Serial diluted LIF medium					
1/40	1/80	1/160	1/320	1/640	1/1280
1/2560	1/5120	1/10240	1/20480	1/40960	1/81920
1/163840	1/327680	1/655360	1/1310720	1/2621440	1/5242880
1/10485760	1/20971520	No LIF	No LIF	Stock LIF	Stock LIF

Table 2.3 Serial diluted LIF medium. The first well had 1500 µl of 1/40 diluted new LIF/FCS medium. FCS medium (750 µl) was placed in the rest of the wells. Medium from the first well (750 µl) was then transferred to the second well and mixed well. This was repeated until the 5th well from last.

To determine the titrated LIF concentration, medium was aspirated and 0.5 ml Leishman's solution was added to each well for a 5 min incubation. Each well was rinsed with sterile deionized water for 1 min. Water was aspirated from each well and plates air-dried. mESC morphology was analysed by holding the plates over white paper for observation. Distinct morphological differences exist between undifferentiated and differentiated mESC. Undifferentiated pluripotent 46C cells form intensely stained small compact colonies which are tightly packed. Cells undergoing differentiation in the absence of LIF appear as larger and faintly stained colonies (Figure 2.3).

Positive and negative LIF controls were observed to specify the limiting dilution of LIF. The highest level of inhibition of differentiation was considered as 1 U/ml. The stock concentration of LIF was determined at 100,000 U/ml, since the final concentration of LIF required is 100 U/ml (allows for dilution between stock and final of 1:1000). Aliquots containing the relevant amount of LIF concentration were stored at 20°C, until required.
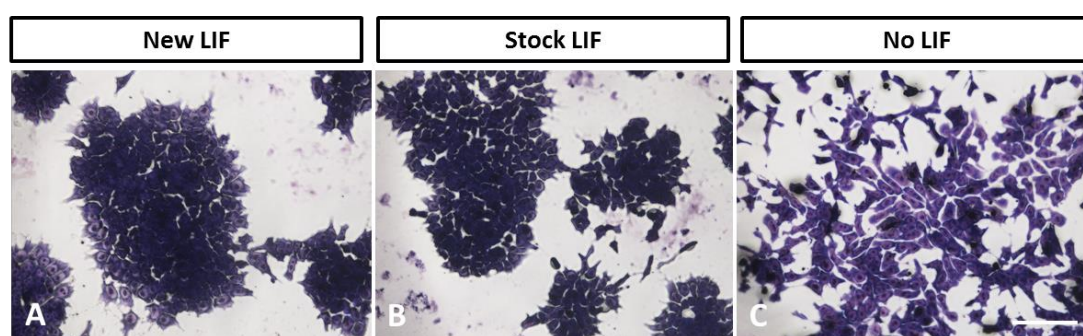
Figure 2.3 Leishman stained mESC colonies under various culture conditions. **(A&B)** 46C mESCs incubated with LIF maintained cells in an undifferentiated pluripotent state. Cells appeared as small rounded intensely stained colonies. **(C)** The absence of LIF induced cell differentiation, indicated by the appearance of larger, spread-out and weaker stained colonies. Scale bar = 100 μm.

2.1.2 Embryonic Stem Cell Culture

ESCs were cultured in Glasgow Modified Eagles Medium (Invitrogen, UK) with the addition of 10% FCS, 0.1 M β-mercaptoethanol, 1 mM $_L$-glutamine, 10 mM non-essential amino acids, 100 mM sodium pyruvate and 100 U/ml LIF (detailed in appendix 2).

2.1.2.1 Thawing

A cryotube of frozen 46C-derived mESCs was rapidly thawed in a water bath heated to 37°C, until a small amount of ice remained. Pre-warmed FCS/LIF medium was added to thaw the remainder of the vial. Cells were centrifuged at 1200 rpm for 3 min. The supernatant was aspirated and cell pellets were re-suspended in warm 10 ml FCS/LIF in a 0.1% gelatin-coated T25 flask.

2.1.2.2 Passage

The 46C mESC line was routinely cultured at a constant plating density of 1×10^6 cell/cm^2 and passaged every 2 days, when cells reached approximately 80% confluency. Prior to passaging, FCS/LIF medium, 0.025% trypsin solution and PBS were pre-warmed to 37°C in a water bath for 1 h. Gelatin solution (0.1%) was used to coat a new T25 culture flask. FCS/LIF medium was aspirated from a confluent T25 flask and the culture was washed twice with 5 ml PBS. Trypsin (1 ml) was evenly dispersed in the culture flask for 1 min at 37°C. Once undifferentiated cells exhibited round morphologies, observed using phase contrast microscopy, the flask was tapped gently by hand to detach the cells. FCS/LIF (5 ml) medium was added to the flask and transferred to a sterile universal centrifuge tube. Cells were centrifuged at 1200 rpm for 3 min. The supernatant was aspirated and cells were re-suspended in

2 ml FCS/LIF. A 1 ml blue pipette tip was used to carefully dissociate cell clumps into a single cell suspension. Cell viability and counts were performed using trypan blue (Invitrogen, Paisley, UK) and a haemocytometer. Undifferentiated mESCs (10^6) were plated in 10 ml FCS/LIF and stored in a 5.0% CO_2 incubator at 37°C.

2.1.2.3 Freezing

The same steps were followed as in 2.1.2.2, as far as the cell viability check. Instead of plating cells in flasks, the supernatant was aspirated and cell pellets were diluted in 10% dimethyl sulfoxide (DMSO) in FCS/LIF medium. 2 x 10^6 cells in 800 µl were aliquoted into cryotubes and placed in a cryochamber at -80°C overnight, before transfer to liquid nitrogen for long-term storage.

2.1.3 *In vitro* Neural Differentiation of mESCs

2.1.3.1 Monolayer Differentiation

For neural induction, undifferentiated mESCs were plated at a density of 9 x 10^4 cells/cm^2 per well of a 0.1% gelatin-coated 6-well dish (day 0). N2B27 serum-free medium (5 ml; appendix 2), was added to each well. Medium was refreshed every alternate day.

Cells differentiated up to a total of 14 days of the monolayer culture period, were washed twice with 5 ml pre-warmed PBS. Trypsin solution (2 ml)was added to each well of the 6-well dish to detach the cells and incubated at 37°C for approximately 1 min. Cells were re-suspended in 10 ml FCS/LIF and centrifuged at 1200 rpm for 3 min. The supernatant was removed and N2B27 medium was added to the pellet and thoroughly pipetted to dissociate cells. Cell viability was observed and cell counts were calculated. Single cells ($3x10^4$) were replated in 30 µl N2B27 medium on 13

mm glass coverslips pre-treated with poly-$_L$-lysine (PLL) (10 µg/ml) and laminin (2 µg/ml) in 24-well plates. Laminin was aspirated from wells prior to cell plating. Following an overnight incubation, wells were flooded with 500 µl N2B27 medium and medium was refreshed every other day until day 14.

2.1.4 Epiblast Stem Cell Culture

2.1.4.1 Generation of epiblast stem cells from 46C-derived mESCs

Mouse Epiblast stem cells (mEpiSCs) were generated from 46C *Sox1*GFP cell line. To begin establishing EpiSC cultures from mESCs, the same initial steps were followed described in section 2.1.2.2.

Briefly, once undifferentiated 46C cells reached 80% confluency in a T25 culture flask, medium was aspirated and cells were rinsed with pre-warmed PBS. Trypsin solution was added to mESCs, and the enzymatic action was quenched with feeder media. mESCs were transferred to a sterile universal centrifuge tube and centrifuged at 1200 rpm for 3 min. The supernatant was aspirated and cells were re-suspended in 2 ml PBS solution. Cell clumps were gently dissociated into a single cell solution using a 1 ml blue pipette tip and counts were manually performed using trypan blue and a haemocytometer.

Undifferentiated mESCs (500,000 cells) were plated in 3 ml FCS/LIF per well of a 0.1% gelatin coated 6-well culture plate. mESCs were incubated overnight at 37°C. The following day once cells reached 60-70% confluency, mESC medium was aspirated from each well and cultures rinsed twice with pre-warmed PBS solution. EpiSC medium (2 ml; appendix 2) was added to each well and cells incubated

overnight at 37°C. The following day, cells reached approximately 90-100% confluency.

A 6-well culture plate were coated with FCS for 30 min at 37°C. Wells were washed with PBS and 3 ml EpiSC culture medium was added to each well. EpiSCs are typically split 1:1, 1:2 and 1:4 every 2 days (the density at which EpiSCs are generated is very important, detailed in section 6.3.4). One well was chosen to be passaged by observing morphology of the cells in the optimal dilution. EpiSCs were rinsed briefly with 1 ml PBS. The rinse was repeated once more with 1 ml PBS and incubated for 2 min at 37°C. The PBS wash was aspirated and replaced with 1 ml pre-warmed EpiSC medium. Cell colonies were scratched and triturated using a 2 ml sterile plastic pipette and cells were plated at three different densities in 2.5 ml EpiSC culture medium per well of a 6-well plate and incubated at 37°C.

2.1.4.2 *In vitro* Neural Differentiation of Epiblast Stem Cells

At day-1, a 6-well plate was coated with fibronectin (30 μg/mL − stock solution 2 mg/mL), for 30 min at 37°C. Cells were passaged and allowed to reach approximately (~) 50-60% confluency at day 0. EpiSC medium was aspirated from each well and cultures were rinsed twice with PBS. EpiSC retinol-free N2B27 medium (2 ml; appendix 2) was added to each well. At day 2, cells were passaged (detailed in 2.1.4.1) and transferred to a 12-well culture plate. Medium was changed every alternate day, until day 14 of differentiation.

2.1.5 Immunocytochemistry

2.1.5.1 Immunocytochemistry using fluorescent antibodies

Differentiated cells were fixed with 4% paraformaldehyde (PFA) for 20 min at 4°C. Fixed cells were washed three times with Tris buffered saline (TBS; appendix 2) for 5 min. Cells were blocked against non-specific binding and permeabilised with 0.02% Triton X-100 and 5% normal goat serum (NGS) (PAA, The Cell Culture Company, Somerset, UK), for 1 h at RT. Primary antibodies (detailed in appendix 3) diluted in 1% NGS blocking buffer were added to cultured cells overnight at 4°C. Negative primary controls consisted of cells treated with blocking buffer without the addition of primary antibodies.

The next day, 3 TBS washes were applied to the cells for 5 min followed by incubation with fluorescent dye-conjugated secondary antibodies (appendix 3), diluted to 1:300 in 1% NGS blocking buffer, for 2 h at RT. Cultures were washed three times with TBS for 5 min. Coverslips were mounted onto the slides using Vectashield hardset mounting medium with 4', 6-diamidino-2-phenylindole (DAPI) (Vector Labs, Peterborough, UK) to counterstain cell nuclei before visualisation the following day.

2.1.5.2 Primary antibody optimisation

Experimental procedures involving animals were conducted with strict adherence to guidelines outlined by the UK Animals, Scientific Act, 1986. At E15, dissection, tissue collection and cell viability counts of striatal and lateral ganglionic eminence (LGE) tissue were carried out by Munyaradzi Kamudzandu (School of Life Sciences, Keele University).

With respect to culturing of dissected tissue, sterile 13 mm glass coverslips were placed in individual wells of 24-well plates and treated with 410 µl PLL (10 µg/ml) and laminin (5 µg/ml) solution. Dissociated cells ($3x10^4$) were plated in 30 µl primary culture medium (appendix 2) as drop cultures for 4-6 h to facilitate cell adherence. Wells were flooded with 500 µl medium, and following 72 h, an additional 500 µl was added to the wells. Medium was refreshed every 3 days and primary cells were maintained in a 5.0% CO_2 incubator at 37°C. The same steps were followed in section 2.1.5.1. for antibody optimisation using immunocytochemistry with fluorescent antibodies.

2.1.6 Cell proliferation assay

To determine the proliferative effect of nicotinamide on ESC-derived cells, a Click-iT® EdU (5-ethynl-2'-deoxyuridine) cell proliferation assay (Invitrogen, Paisley, UK) was used, in accordance with the manufacturer's instructions.

Briefly, adherent monolayer cells were pulsed with EdU (10 µM) for 1 h prior to cell fixation. It is important to point out that the incubation time with EdU is dependent on the cell line's population rate of cell cycle entry and progression (~12 h; personal discussion with Professor Meng Li[144]).

Standard PFA fixation (4% formaldehyde) and detergent permeabilisation (0.5% Triton X-100) were required to facilitate access of the detection reagent to DNA. For EdU detection, a Click-iT® reaction cocktail (appendix 2) was prepared and applied to cells for 30 min. Cells were rinsed with 3% bovine serum albumin (BSA) in PBS for 2 min, followed by PBS for 2 min. To perform dual labelling of cultured cells with EdU and primary antibodies (appendix 3), cells were blocked and permeabilised

with 0.02% Triton X-100 and 5% NGS for 1 h. Immunocytochemsitry with fluorescent antibodies was then performed as detailed in section 2.1.5.1.

2.1.7 Apoptotic assay

The Click-iT® TUNEL Alexa Fluor® imaging assay (Invitrogen, Paisley, UK) was employed to assay nuclear DNA fragmentation; an important biochemical indicator of apoptotic cell death. Fragmented DNA characteristic of apoptotic cells was measured by catalytically integrating fluorescein-12-dUTP at 3´-OH DNA ends, where a polymeric tail was formed. This reaction was catalyzed by the enzyme Terminal Deoxynucleotidyl Transferase (TdT), generating TdT-mediated dUTP Nick-End Labelling.

Monolayer adherent cells were fixed in 4% PFA for 15 min at 4°C. Cells were permeabilised with 0.2% Triton X-100 in PBS for 5 min. Cells were rinsed twice with deionized water. TdT reaction buffer was added to each coverslip for 10 min at RT. Following this, the TdT reaction buffer was removed, and 100 µl TdT reaction cocktail (appendix 2) was added to each coverslip. Coverslips were incubated for 60 min at 37°C, avoiding exposure to light sources. Cells were rinsed with 3% BSA in PBS for 2 min. A Click-iT® reaction cocktail (100 µl) was added to each coverslip for 30 min at RT, protected from light. Cells were rinsed with 3% BSA in PBS for 5 min. To perform dual labelling, cells were blocked and permeabilised with 0.02% Triton X-100 and 5% NGS for 1 h. Immunocytochemsitry with fluorescent antibodies was then performed as detailed in section 2.1.5.1.

2.1.8 Cell Sample Analysis Methods

2.1.8.1 Fluorescence Intensity Measures

Fluorescence Intensity (FI) measures were obtained to determine levels of protein expression of specific neuronal populations derived from *Sox1*GFP mESCs, using ImageJ image analysis software (version 1.45s; NIH). Cell samples were captured at fixed exposure settings using a Hamamatsu ORCA camera with NIS Elements imaging software. Undifferentiated and neural cells located in cell clusters were excluded from FI analyses. FI values were evaluated by converting each colour image to grayscale, calibrated using an FI step tablet and FI readings were corrected for the background.

2.1.8.2 Morphometric Analysis of Neurite Outgrowth

Neuronal cells were photographed from eight to ten random fields per coverslip from three independent experiments using a Hamamatsu ORCA camera equipped with NIS-Elements imaging software. Neuronal cells within cell clusters were omitted from morphometric analysis. Neuritic branching per cell, length of the longest primary neurite and total extent of neurite were analysed to evaluate the effect of nicotinamide on neuritic morphology. Neuronal processes greater than two cell diameters in length were considered as true neurites and neurites of labelled cells were manually traced using the ImageJ plug-in NeuronJ (version 1.4.2; NIF). Neurite length was defined as the distance from the soma to the tip of the longest primary branch and the combined lengths of all neurites per cell were defined as total neurite length. Data obtained represent an average of approximately 50 neurons in each treatment group.

2.1.8.3 Automated Cell Counter

A Countess™ automated cell counter was used to automate cell counting in order to determine total cell counts and viability; taking into account live, dead and total cells differentiated from *Sox1*GFP reporter mESCs. The automated cell counter used the standard trypan blue technique, used for manual cell counting with a haemocytometer. For a typical cell count, 10 μl cell suspensions comprising of both live and dead cells was added to 10 μl 0.4% trypan blue stain. The cell sample mixture (10 μl) was added into one chamber of the Countess™ cell counting chamber slide. The image was optimised using a focusing knob to alter the image for analysis. Live cells appeared to have a bright centre surrounded by a dark edge, whilst dead cells displayed a homogenous blue colour without bright centres.

2.1.8.4 Manual cell counting and statistical analysis

Cell samples were analysed using fluorescence microscopy (Nikon Eclipse 80i microscope) and images acquired using a Hamamatsu ORCA camera. NIS-Elements imaging software, version Br 3.2 was used to manually quantify positive staining in DAPI-stained cultures. Independent experiments were replicated three times and three to four coverslips per group were counted within an experiment. Specific cell populations were analysed by capturing six to eight random fields per coverslip (detailed within each experimental chapter). Data obtained represent an average of approximately 50 DAPI-labelled cells per field of view.

Statistical analysis was performed using GraphPad Prism, version 5.00 (GraphPad Software Inc.) and data were expressed as mean ± standard error of the mean (SEM). Unpaired two-tailed t test was used when comparing two groups. Differences between more than two different groups were analysed using one way analysis of

variance (ANOVA), and post hoc analysis was carried out using Tukey's multiple comparison test. Differences in p-values less than ($\leq$) 0.05 were considered significant.

Chapter 3: Effect of Nicotinamide on Neuronal Differentiation from Mouse Embryonic Stem Cells *In Vitro*

3.1 Introduction

Currently, a major challenge in advancing stem cell therapies to treat a myriad of debilitating brain-related degenerative disorders is to rapidly and efficiently direct pluripotent stem cells to differentiate into pure, high yield populations of desired and mature neuronal phenotypes[145]. To date, protocols for directing *in vitro* differentiation of ESCs have been established; however, additional purification methods to collect homogeneous cell populations are frequently required[146–148]. One main reason for such heterogeneity of stem cell-derived populations is that the majority of ESCs differentiate asynchronously into lineage-biased subsets, thereby generating a chaotic mixture of both undifferentiated and differentiated cells with mixed phenotypes within differentiated progenies[149]. Moreover, for a regenerative cell population, their high capacity for self-renewal predispose them to tumour formation, mostly teratomas or overproliferation of NPCs[150]. In this regard, this potential for tumourigenesis may limit future clinical applications of ESC-derived transplant populations.

Methods for replicating the multistep signalling cascade regulating embryonic development *in vivo* have demonstrated great promise *in vitro* for differentiating neurons from ESCs. Protocols for directing lineage-specific differentiation routinely add or remove inductive signalling molecules to cell cultures. Vitamins are frequently included in differentiation strategies to enhance the *in vitro* derivation of desirable postmitotic cells. The biologically active form of the vitamin is most commonly used, as many cells lack the required metabolic enzymes to convert the inactive form of the vitamin. For example, the active forms of vitamin A (retinoic acid)[116,151–153] and vitamin C (ascorbic acid)[78,79,87,124,126,154,155] are commonly

included in current studies to drive stem cell differentiation toward desired neuronal cell fates.

Vitamins are well-studied small organic molecules known to be vital in the adult, and during growth to support and maintain a healthy brain. They are also well known to play crucial roles during early neuronal development in embryogenesis[104,124,126,152,156]. Experiments dating from the 1970s depict an important role for nicotinamide as a regulator of morphogenesis and cell differentiation (i.e. myogenic and chrondrogenic differentiation)[137]. Nicotinamide, the amide form of vitamin B_3, has since been applied as a differentiation agent to a number of protocols to promote differentiation to specific cell populations from mESCs[157–159] and hESCs[160–163]. Interestingly, existing literature suggests a paucity of information on the potential of nicotinamide to drive ESC differentiation into neural and neuronal populations. Therefore, this experimental chapter sought to determine if nicotinamide could influence the conversion of *Sox1*GFP knock-in mESCs undergoing neural differentiation to mature neurons, based on an adherent monolayer differentiation protocol developed by Ying and co-workers (Figure 3.1)[164].

46C mESCs harbour a knock-in of GFP to one allele of the neural specification marker gene *Sox1,* which is expressed concomitant with the formation of the neural plate in mice[165]. *Sox1* is expressed in neuroepithelial cells. Therefore, the 46C ESC cells express GFP under the control of *Sox1* upon becoming NPCs, and detection of green fluorescence enabled a convenient and facile experimental system to monitor and visualise the process of neural specification. This property makes the 46C ESCs ideal cells to study in the monolayer culture and differentiation system, particularly to evaluate the distinct effects of nicotinamide on self-renewal and terminal

differentiation into mature neurons, astrocytes and oligodendrocytes. Furthermore, this *in vitro* culture system also facilitates visual analysis of the morphological changes of the ESCs during the course of their differentiation and the effect of nicotinamide on this process. A simple and fast protocol was established to:

I. *Maintain 46C mESCs in an undifferentiated state using LIF (made in house).*

II. *Investigate whether nicotinamide facilitates the generation of higher yields of homogenous populations of neurons suitable for transplantation.*

III. *Systematically investigate whether nicotinamide influences the transition through stem cell, neural induction and neuronal differentiation of mESCs, by evaluating the expression of the pluripotency protein, Oct4, associated with tumour forming cells and/or Sox1GFP$^+$ NPC populations.*
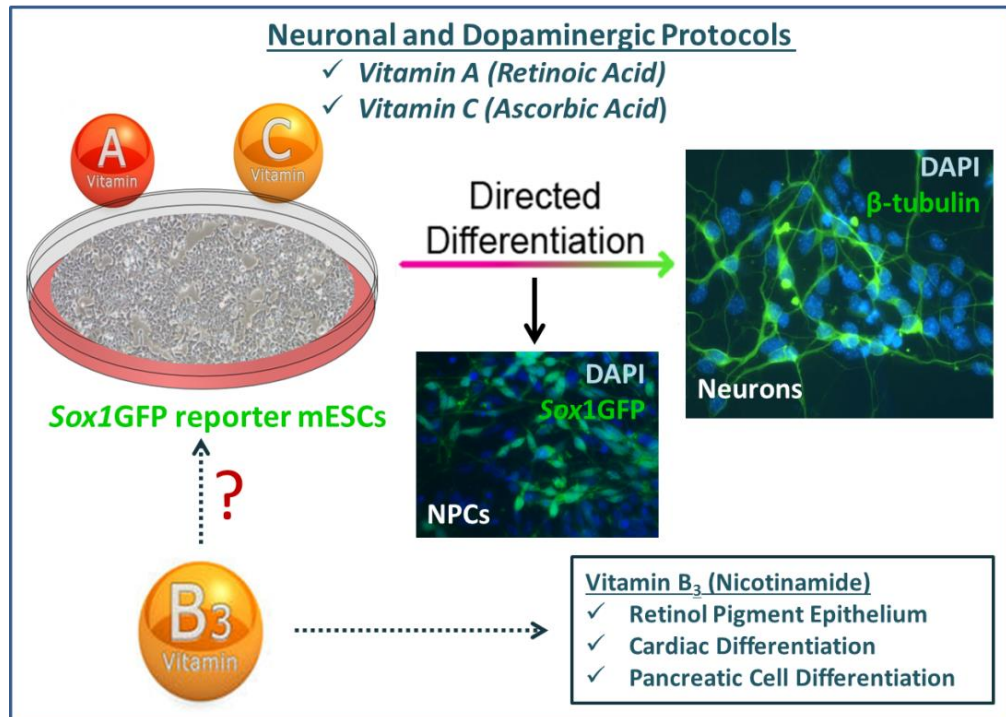
Figure 3.1 Schematic diagram illustrating the overall hypothesis and aim of this study. Developing strategies to channel the promise of stem-cell-derived neurons for future therapies is crucial. Clinical translations of stem cell candidates, such as ESCs, can only commence once all the important challenges have been adequately resolved and protocols are improved to restrict proliferation linked to tumour formation, and to promote differentiation of ESCs to higher and purer yields of desired cell phenotypes[145,166]. Current methods for driving stem cell differentiation towards clinically-relevant lineages routinely apply small molecules, including vitamins A (retinoic acid) and vitamin C (ascorbic acid)[78,79,87]. A number of reports also demonstrate that the biologically active metabolite of vitamin B_3, nicotinamide, efficiently potentiates stem cell differentiation as highlighted in various retinal pigmented epithelium (RPE)[161,162], cardiac[167] and pancreatic[158] stem cell differentiation studies. The present study, for the first time, thoroughly investigates whether nicotinamide could direct neuronal differentiation derived from *Sox1*GFP reporter mESCs, specifically examining its potential across a spectrum of developmental stages toward cell-based therapeutics.

3.2 Experimental Procedure

Taking advantage of GFP expression in the *Sox1*-GFP knock-in 46C cell line, the effect of nicotinamide on neural differentiation was investigated over a 14 day differentiation period. GFP is undetectable in undifferentiated mESCs, but only becomes evident following withdrawal of the cytokine LIF to induce neural differentiation [self-renewal capacity of mESC cells is maintained through LIF-activated signalling[168]; Figure 3.3 (C)].

3.2.1 Cell culture and nicotinamide treatment

It is well documented that undifferentiated ESCs commit to a neural lineage when cultured in feeder-free adherent monolayers, using serum-free N2B27 medium (appendix 2)[164,169]. The latter medium is used because serum acts as a potent antagonist of neural differentiation[149]. To induce monolayer neural and neuronal differentiation until day 14 in culture, undifferentiated ESCs [approximately 80% confluent; Figure 3.3 (B)] were plated on gelatin-coated dishes with N2B27 medium for 7 days. On day 7, cells were replated onto coverslips coated with PLL and laminin until day 14. Medium was changed every alternate day. To determine an appropriate nicotinamide concentration and culture period to potentiate neuronal differentiation, nicotinamide was added to the base medium at varying concentrations (0.5-20 mM; appendix 2) and cells were treated between days 0 and 7 or days 7 and 14 (Figure 3.2, Table 3.1). Control groups were not treated with nicotinamide.

Monolayer differentiation studies using the parental 46C-cell line have shown that GFP expression driven by *Sox1* becomes detectable in cultures at 48 h to 72 h[149,164,170]. GFP expression appears in approximately 60% of cells after the fourth

day of culture[164]; reaching a maximum by the fifth day and remaining at this plateau for several days[149]. A slow decline in GFP becomes evident in monolayer cultures at day 7 and, by day 8, down-regulation of *Sox1* is accompanied by differentiation of precursor cells into neurons and glia. Considering this, in further experiments, the effect of nicotinamide on the process of neural induction and its influence on NPC populations was also investigated. Therefore, two additional culture periods with nicotinamide were introduced: day 0 to 2 and day 2 to 7 (Figure 3.2, Table 3.1).

3.2.2 Immunocytochemistry

To perform a comprehensive time-course analysis of the effect of nicotinamide on the progression through stem cell, neural induction and neuronal differentiation of mESCs, immunocytochemistry using fluorescent antibodies, FI measures and pluripotent colony area measurements were carried out. Immunocytochemistry was performed using primary antibodies against Oct4 (nuclear maker for undifferentiated stem cells and determinant of the pluripotent state) and βIII-tubulin (neuron-specific cytoskeletal marker); with fluorescence detection of native GFP expression to identify the *Sox1* expressing cells/progenitor cells (Figure 3.2). Importantly, it should be noted that images acquired for FI measurements of GFP$^+$ populations were captured using equivalent exposure settings across all conditions.

3.2.3 Image and statistical analysis

For cell quantification and statistical analysis, cell populations were analysed by capturing six to eight random fields per coverslip. Clusters containing mixed populations of neural progenitor and neuronal cells were excluded from data collection, since these cell networks were not countable. Cell count data were averaged across three coverslips per culture and experiments were repeated three to

five times (i.e. n = 3–5 independent experiments). All experiments in this chapter were statistically interpreted using one-way ANOVA followed by a post-hoc Tukey multiple comparison test when required, where more than two independent variables were present, and unpaired t test for two conditions. A level of $p<0.05$ was used as a limit for statistical significance. All data are expressed as mean ± SEM. Detailed protocols are provided in the Material and Methods chapter.
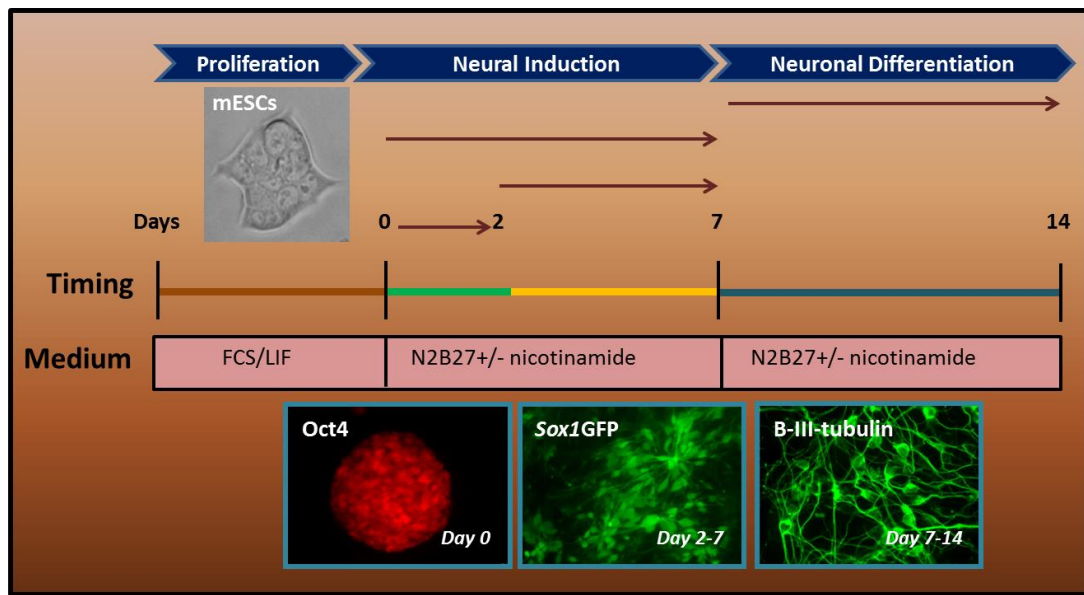
Figure 3.2 Schematic diagram of time-course analysis of neural and neuronal differentiation in the presence of nicotinamide. Exponentially proliferating undifferentiated pluripotent stem cells were maintained in FSC/LIF medium from passage number 24 to 40 [Figure 3.3 (A)]. For ESC differentiation in adherent monoculture, neural commitment and neuronal differentiation is triggered by the absence of serum and withdrawal of self-renewal stimulus (i.e. LIF) at day 0. Defined culture conditions were established to direct the differentiation of mESCs into neuronal cultures. To examine the differentiation potential of the vitamin B_3 metabolite, undifferentiated cells were cultured as monolayers in N2B27 medium in the presence of nicotinamide from days 0 to 7 (i.e. early development) or days 7 to 14 (i.e. late development). The monolayer protocol established by Ying *et al* generates *Sox1* expressing cells (neural progenitors) from around day 2 to day 7[149,164,170]. Therefore, taking advantage of native GFP expression, further studies were conducted to investigate whether nicotinamide would influence the conversion of Oct4^{+} undifferentiated stem cells to *Sox1*GFP neural progenitors and thus drive differentiation toward βIII-tubulin^{+} neurons. The potential of nicotinamide was assessed using immunocytochemistry at different monolayer culture periods until day 14 (Table 3.1).

Experimental Aim	Nicotinamide Culture Period	Fixation Time
Effect on Early Neuronal Development (i.e. Day 0-7)	Day 0-7	Day 14
Effect on Late Neuronal Development (i.e. Day 7-14)	Day 7-14	Day 14
Effect on Neural Induction	Day 0-2 Day 2-7	Day 2 Day 7

Table 3.1 In vitro culture periods and fixation time-points.

3.3 Results

Prior to commencing experimental plating under directed differentiation conditions, it was confirmed that ESCs behaved in a normal manner, with respect to their proliferation [Figure 3.3 (A)].
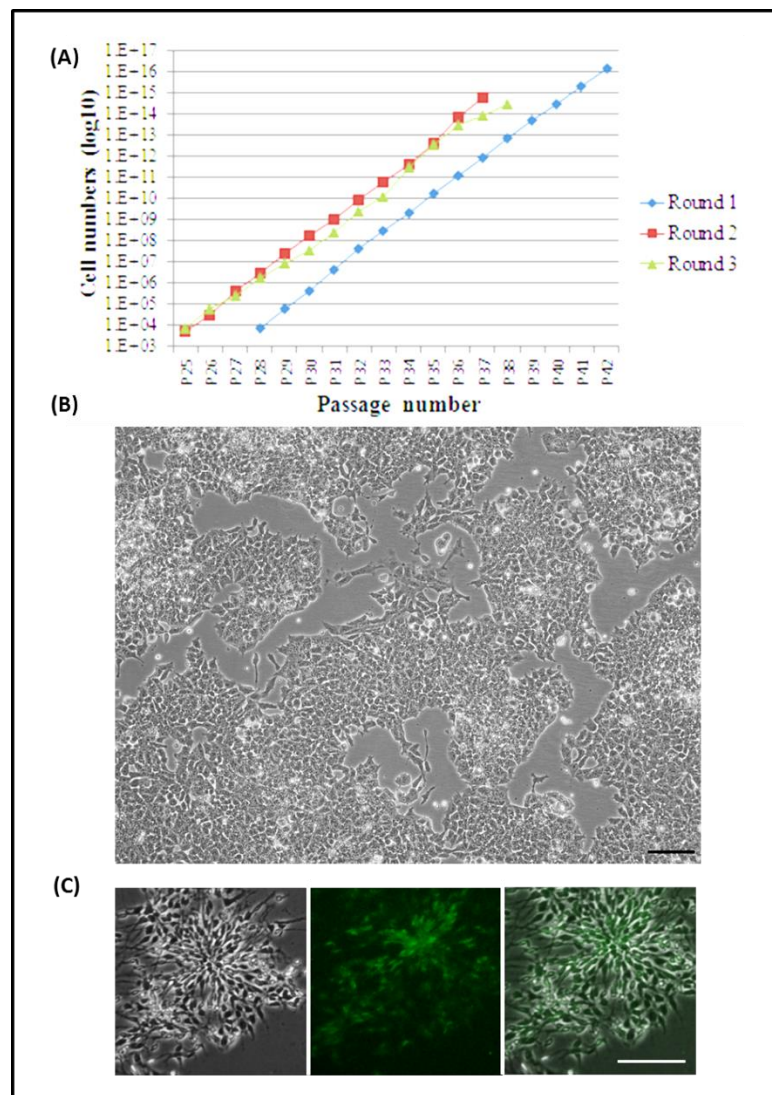
Figure 3.3 (A) Exponential growth curve of *Sox1*GFP mESC line. Exponentially proliferating 46C ESC cultures, represented by the straight line graph were maintained on 0.1% gelatin-coated T25 flasks from passage 25 to passage 42. The normal behaviour of cells, as observed in this study, is crucial to obtaining reliable and accurate results. **(B)** Proliferating mouse embryonic stem cells maintained on gelatin-coated plastic with LIF (~ 80% confluent). **(C)** GFP expression observed in a neural rosette of 46C derived-progenitor cells in differentiated cultures at day 7, plated in N2B27 medium. [(Brightfield (left image), GFP fluorescence (middle image) and *Sox1*GFP 46C cells (right images)] B-C Scale bars = 100 μm.

3.3.1. Nicotinamide increases the percentage of mESC-derived neurons when present at an early developmental stage.

To determine the differentiation potential of the vitamin B_3 metabolite in terms of the percentage of neurons generated from mESCs, the effect of nicotinamide was investigated on differentiation at an early stage of neuronal development (i.e. day 0-7) or late developmental stage (i.e. day 7-14).

Upon exposure of undifferentiated cells to nicotinamide from day 0-7, the percentage of βIII-tubulin expressing neurons measured at 14 days of culture was increased in a concentration dependent manner, peaking at a dose of 10 mM nicotinamide [ANOVA $F_{(4, 268)}$ = 30.1; $p<0.001$; (Figure 3.4 (A) (B)]. Nicotinamide (10 mM) supplementation more than doubled the percentage of βIII-tubulin$^+$ neurons of the control value (29.8 ± 1.6% vs. 12.7 ± 0.9% in control cultures). Addition of 5 mM nicotinamide also significantly enhanced the proportion of neurons (20.7 ± 2.1%; $p<0.01$), whereas 0.5 and 1 mM were ineffective. Thus, the effect of nicotinamide on neuronal differentiation is dose-dependent. With higher levels of nicotinamide (20 mM), cytotoxicity and widespread cell death were evident at 3-4 days of monolayer differentiation, thereby preventing establishment of cell cultures in this condition [Figure 3.5 (A)].

In contrast, nicotinamide did not induce significant differences in the percentage of βIII-tubulin$^+$ neurons between control and 5 or 10 mM nicotinamide conditions, when added between days 7 and 14 [ANOVA $F_{4, 268} = 1.9$; n.s.; Figure 3.4 (C) (D)], indicating that its action is culture stage-dependent.
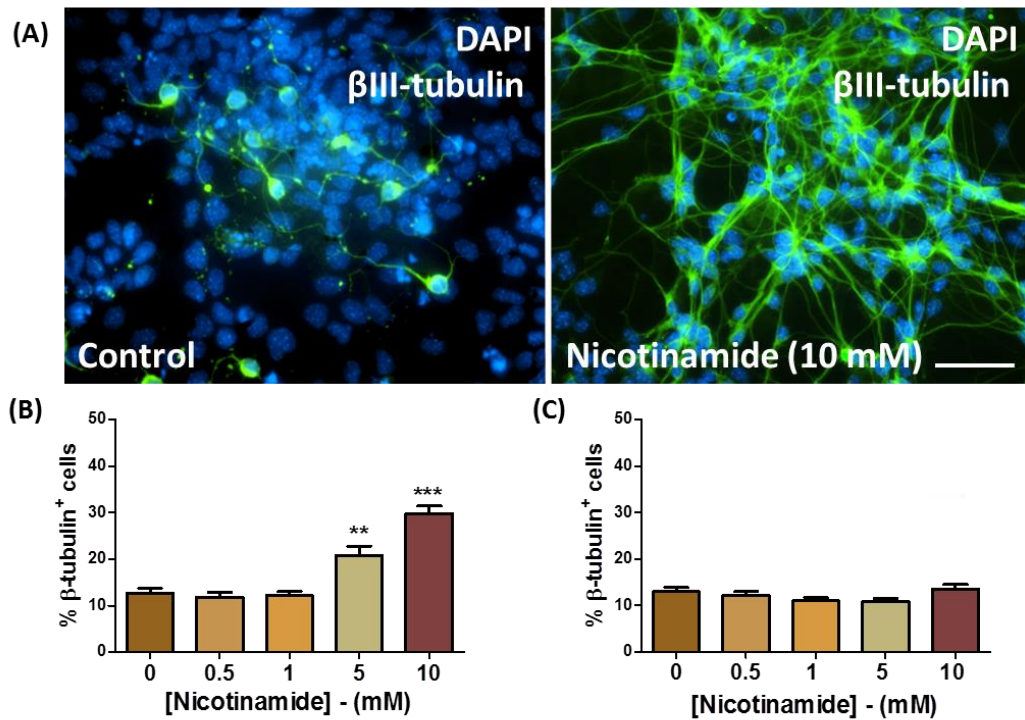
Figure 3.4 Effect of nicotinamide addition to culture media on neuronal differentiation. (A) Immunocytochemical analysis of immature neuronal marker βIII-tubulin (green)-expressing cells and total cells in cultures labelled with DAPI (blue). Scale bar = 50 μm. **(A)** Exposure of undifferentiated cells to 10 mM nicotinamide between day 0 and day 7 increases the percentage of βIII-tubulin-expressing neurons at day 14, compared to untreated cells at 14 days of monolayer culture. **(B)** Bars represent percentage βIII-tubulin-expressing neurons as a percentage of total cells. Nicotinamide (5 and 10 mM) generated an increase in the percentage of βIII-tubulin-expressing neurons, compared to untreated cells, when added to cultures between day 0 and day 7. **(D)** Bars represent percentage βIII-tubulin-expressing neurons as a percentage of total cells. Nicotinamide added from days 7 to 14 had no significant effect on the percentage of βIII-tubulin-expressing neurons.
***$p<0.001$, **$p<0.01$

Nicotinamide-induced cell death (20 mM) was also observed at later stages (i.e. day 7 to day 14) of neuronal development, indicating that this cytotoxic effect was

culture-stage independent, unlike its pro-neuronal differentiation action [Figure 3.5 (A)].
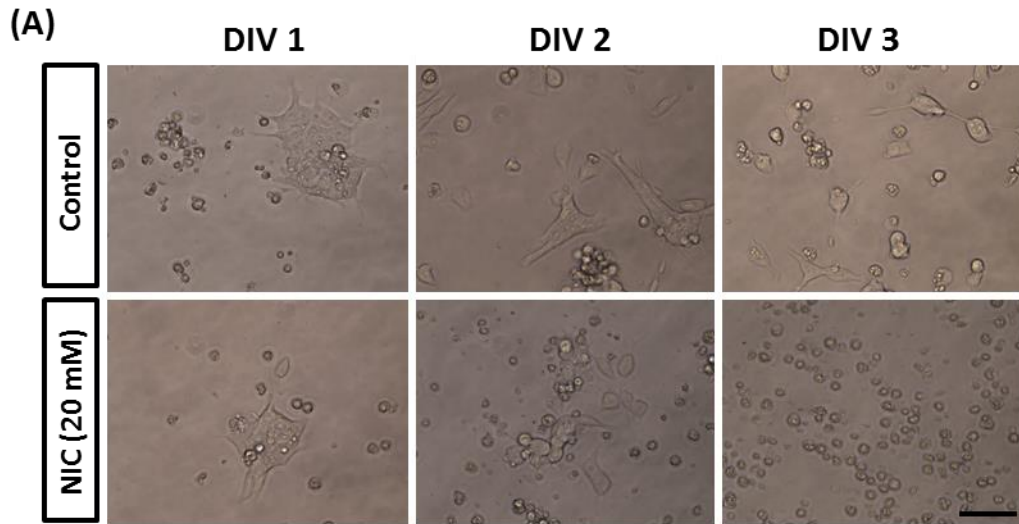
Figure 3.5 (A) Cytotoxic effects of high levels of nicotinamide on neuronal differentiation. Intact cultures from 24 h to 72 h of monolayer differentiation, shown in phase contrast. Higher doses of nicotinamide (20 mM) elicited an obvious cytotoxic effect on monolayer cell survival, at 3 days *in vitro* (DIV). Scale bar = 50 µm.

In summary, nicotinamide administration to undifferentiated cells at early stages of the monolayer protocol exhibited a concentration-dependent enhancement in the conversion of mESCs into βIII-tubulin$^+$ neuronal populations, but elicited no effect on this parameter at the late stages of ESC differentiation. These data suggest that addition of nicotinamide to early differentiation of ESCs may enhance the production of homogenous neuronal populations. Importantly, higher levels of nicotinamide (20 mM) induced cytoxicity and cell death.

3.3.2 The total numbers of cells in nicotinamide-treated cultures were reduced at both early and late developmental stages.

Quantification of DAPI-stained nuclei highlighted a significant reduction in the total number of cells treated with nicotinamide [Figure 3.6 (A) (B)]. At day 14, fewer cells were counted following nicotinamide administration from day 0-7 [ANOVA $F_{4, 268} = 19.5$; Figure 3.6 (C)] and from day 7-14 [ANOVA $F_{4, 268} = 32.7$; Figure 3.6 (D)]. Specifically, cultures supplemented with 5 mM and 10 mM nicotinamide showed a significant decrease in the total numbers of DAPI-labelled cells per mm^2, compared to control conditions at both day 0-7 [2047 ± 69.8 with 5 mM nicotinamide, $p<0.01$; 1754 ± 56.8 with 10 mM nicotinamide, $p<0.001$ vs. 2430 ± 71.0 in control cultures] and day 7-14 [1893 ± 101.1 with 5 mM nicotinamide, $p<0.001$; 1290 ± 12.8 with 10 mM nicotinamide, $p<0.001$ vs. 2556 ± 95.1 in control conditions].

These data suggest that nicotinamide may be functioning to decrease the pluripotent stem cell population (i.e. dividing cells); an important finding which could be translated to current ESC differentiation protocols to reduce the potential risk of tumourigenesis.
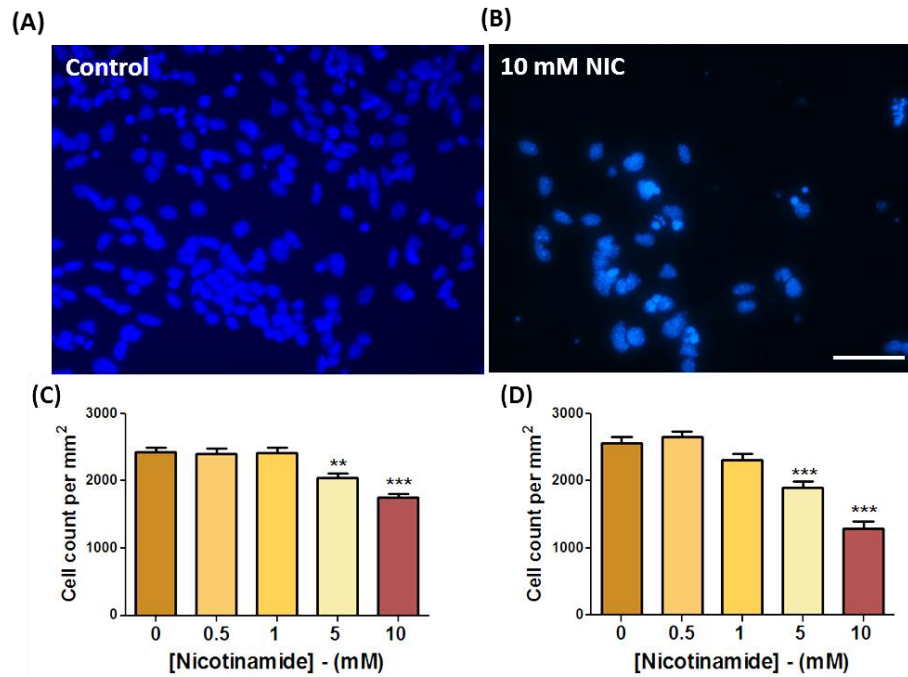
Figure 3.6 Nicotinamide treatment reduced the total cell population. (A-B) Nicotinamide-treated cultures displayed significantly decreased total cell numbers per mm^2. Total cells in cultures labelled with DAPI (blue). Scale bar = 50 μm. **(C-D)** At day 14, fewer cells counterstained with DAPI were observed following nicotinamide treatment from days 0 to 7 and days 7 to 14.
***$p<0.001$, **$p<0.01$

3.3.3 Nicotinamide treatment at early development yielded an enriched population of neuronal cells.

To determine the total number of βIII-tubulin$^+$ neurons per mm^2 across all treatment groups, values for the percentage of βIII-tubulin$^+$ neurons and the total number of DAPI cells per culture were used to calculate the total number of neurons per culture. Findings showed that addition of 10 mM nicotinamide to undifferentiated cells between days 0 and 7 generated significantly more neurons per unit area at 14 days, compared to control conditions (ANOVA $F_{4, 274} = 7.6$; $p<0.01$) [466.8 ± 32.9 vs. 311.7 ± 27.0 with control conditions; Figure 3.7 (A) (B)].

Conversely, addition of nicotinamide to cells at day 7-14 significantly decreased neuronal numbers per mm^2 in the 5 and 10 mM conditions (ANOVA $F_{4,\ 268} = 8.2$; $p<0.01$) [200.9 ± 17.9 with 5 mM nicotinamide, 176.5 ± 20.9 with 10 mM nicotinamide, $p<0.001$ vs. 320.4 ± 28.2 in control conditions; Figure 3.7 (A) (C)].

These crucial findings reveal that addition of nicotinamide at the initial stages of ESC differentiation promoted an enrichment in the number of neurons per unit area by day 14 of monolayer culture. This occurs partly through directed cell differentiation and partly through a reduction in the total number of cells in culture, elicited by nicotinamide treatment.
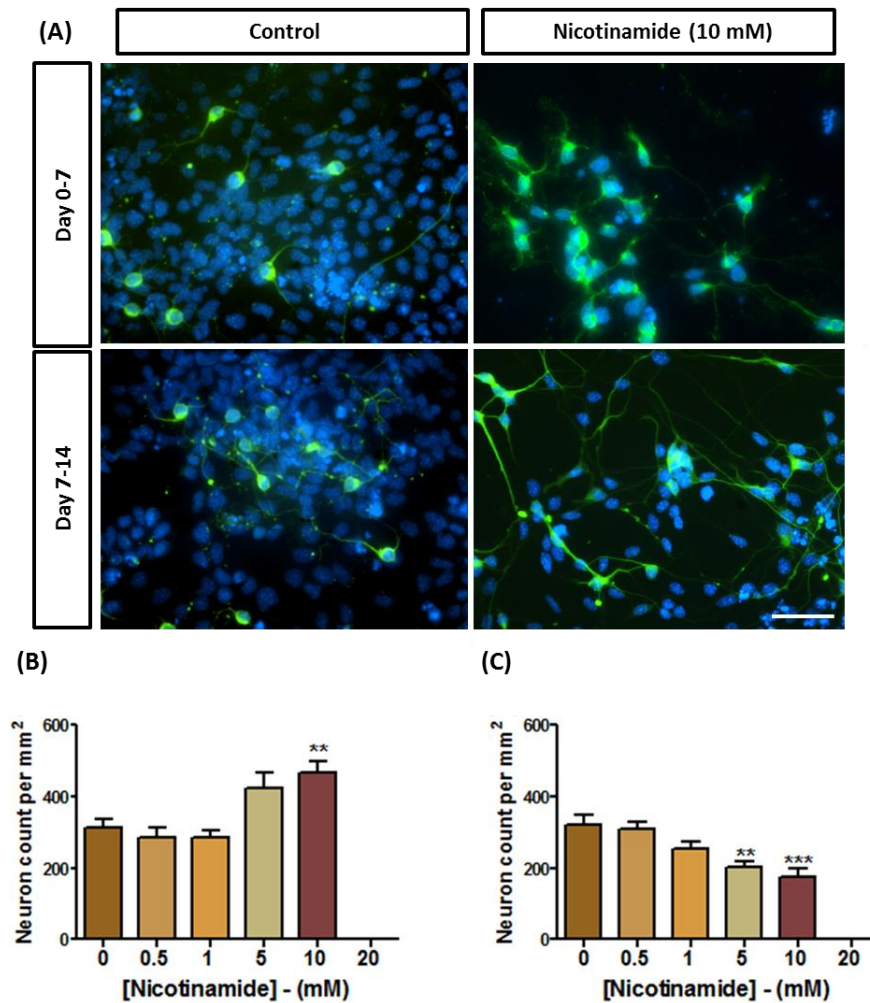
Figure 3.7 Nicotinamide acts at the initial stages of neural differentiation to promote neuronal enrichment. (A) Immunocytochemical analysis of immature

neuronal marker βIII-tubulin (green)-expressing cells and total cells in cultures labelled with DAPI (blue). Representative images demonstrating that nicotinamide treatment between days 0-7 causes a reduction in the total number of cells, concomitant with an increase in the generation of more neurons per unit area at 14 days, therefore promoting neuronal enrichment in monolayer cultures. However, nicotinamide-treated cultures from days 7 to 14 exhibited significantly reduced total cell and neuronal numbers per mm^2. Scale bar = 50 μm. **(B)** Addition of 10 mM nicotinamide to undifferentiated cells from day 0 to 7 significantly increased the number of neurons per unit area compared to control cultures, thereby enriching the ratio of neurons to non-neuronal cells at day 14. **(C)** Nicotinamide (5 and 10 mM) caused a significant reduction in neuronal numbers per mm^2, when added to cultures at late differentiation stages.
***$p<0.001$, **$p<0.01$

3.3.4 Nicotinamide promotes the loss of both pluripotent stem cells and neural progenitor cells.

Having shown that nicotinamide reduced the total number of cells (section 3.3.2), its effects on the heterogeneous population of undifferentiated mESCs and GFP-expressing NPCs in culture at day 14 were more thoroughly investigated. In particular, the differentiation-inducing abilities of 5 and 10 mM nicotinamide were selected to specifically evaluate their effects on stem cell potency and NPCs.

3.3.4.1 The number of colonies expressing Oct4 was dramatically reduced.

The number of undifferentiated stem cell colonies per field, as determined by colonies expressing the stem cell marker Oct4, was reduced by half when 10 mM nicotinamide was added to cultures at the early phases (i.e. days 0-7) of neural differentiation (ANOVA $F_{2, 108} = 17.0$; $p<0.001$) [number of Oct4 colonies per field: 3.2 ± 0.5 vs. 6.8 ± 0.5 in controls; Figure 3.8. (A) (B)]. Cells differentiated with 10 mM nicotinamide from day 7-14 showed a similar effect, where the number of stem cell colonies is reduced to approximately one third of the control conditions

(ANOVA $F_{2,103} = 38.4$; $p<0.001$) [2.7 ± 0.5 vs. 8.1 ± 0.5 in control groups; Figure 3.8. (C) (D)].

Similarly, addition of 5 mM nicotinamide to cultures at early differentiation significantly reduced the number of Oct4-expressing colonies (4.4 ± 0.3; $p<0.001$). This effect of the 5 mM concentration was specific to early stages of differentiation (i.e. days 0-7) and was not replicated at later differentiation stages. These findings implicate a beneficial role for nicotinamide to reduce the numbers of dividing cells in adherent monolayer cultures by day 14.
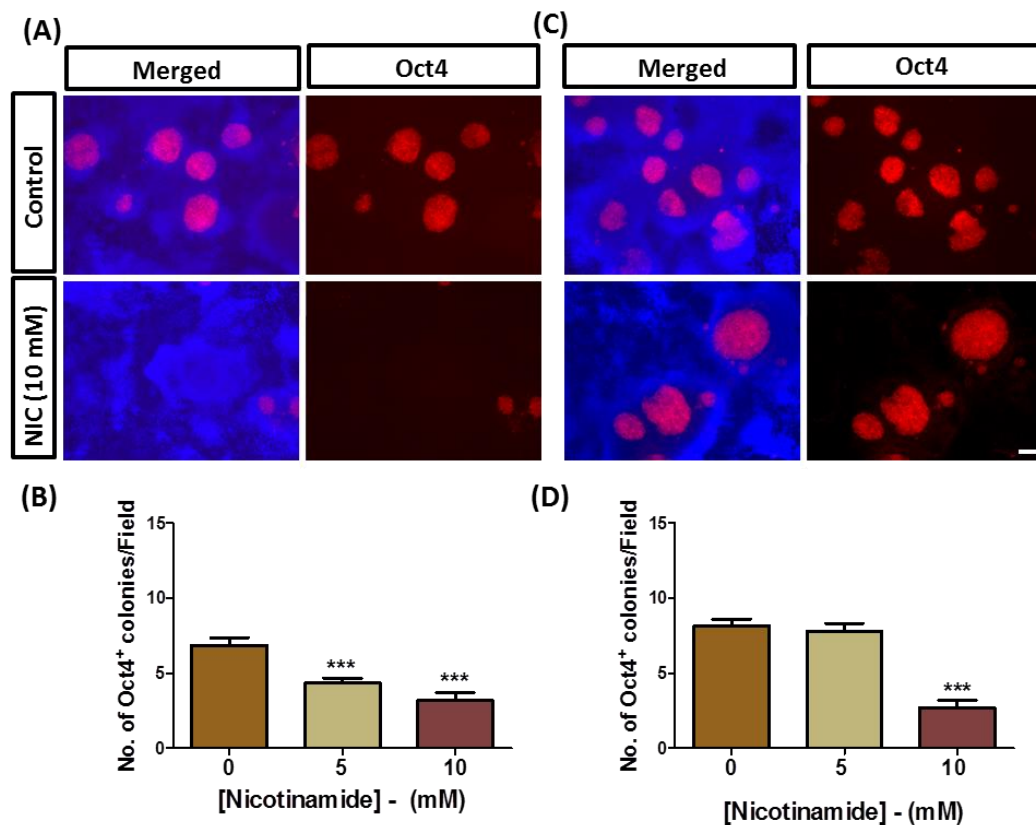
Figure 3.8 Treatment of adherent cultures with nicotinamide decreased the number of undifferentiated colonies expressing Oct4. (A&C) Immunocytochemical analysis of pluripotent stem cell marker Oct4 (red)-expressing colonies and total cells in cultures labelled with DAPI (blue) at day 14. Irrespective of the nicotinamide treatment period, numbers of undifferentiated colonies were lower at day 14, compared to controls. Scale bar = 100 μm. **(B)** Monolayer cultures exposed to both 5 and 10 mM nicotinamide between days 0 and 7 significantly decreased the number of pluripotent colonies per field. **(D)** Nicotinamide-treated

cultures (10 mM) exhibited significantly lower numbers of Oct4$^+$ colonies at day 14 (added from day 7-14).
***$p<0.001$

3.3.4.2 Stem cell pluripotency was down-regulated in nicotinamide conditions.

This experiment also aimed to investigate if nicotinamide would impact on the self-renewing capacity of prevailing undifferentiated colonies remaining in culture at day 14. Round and compact Oct4-expressing colonies are a standard feature of ESC culture conditions. Therefore, the loss of the pluripotency regulator Oct4 was examined by measuring colony area size (μm^2) using Image-J software. Pluripotency was assessed within individual DAPI-labelled colonies as the proportion of cells expressing the Oct4 protein. Therefore, a ratio of the region of red fluorescence (Oct4$^+$) compared to blue fluorescence (DAPI$^+$) within a fixed area was obtained.

Interestingly, nicotinamide significantly decreased the size of Oct4-labelled colonies in cultures treated between days 0 and 7. The percentage of Oct4-labelled areas within DAPI$^+$ colonies was significantly smaller in 10 mM nicotinamide cultures only [17.5 ± 1.7% vs. 37.7 ± 4.2% in control conditions; $p<0.01$; Figure 3.9 (A) (B)]. Cells treated between days 7 and 14 did not have any significant differences in Oct4 expression [Figure 3.9 (C)].

The mean area of colonies (μm^2) was plotted against nicotinamide concentrations, obtained from cultures treated at the early stages of development only. DAPI-labelled colonies remained constant in size across all treatment conditions [Figure 3.9 (D)]. In contrast, 10 mM nicotinamide induced a rapid loss of pluripotency as evidenced by a significant reduction in Oct4-positive colony size [2877 ± 625.2 vs.

7862 ± 317.1 in controls; *p*<0.01; Figure 3.9 (E). This effect was not observed at lower concentrations of nicotinamide (i.e. 5 mM).

In summary, nicotinamide (10 mM) promoted earlier differentiation of mESCs, as evidenced by the pattern of Oct4 expression, which showed a rapid reduction following early nicotinamide exposure. This important finding forms a mutually supportive body of data compatible with the reduction of the total cell population, an effect elicited by nicotinamide at the early stages of neural differentiation. This suggests that the decrease in the pluripotent cell population underlies the significantly lower numbers of total cells in adherent monolayer culture by day 14.
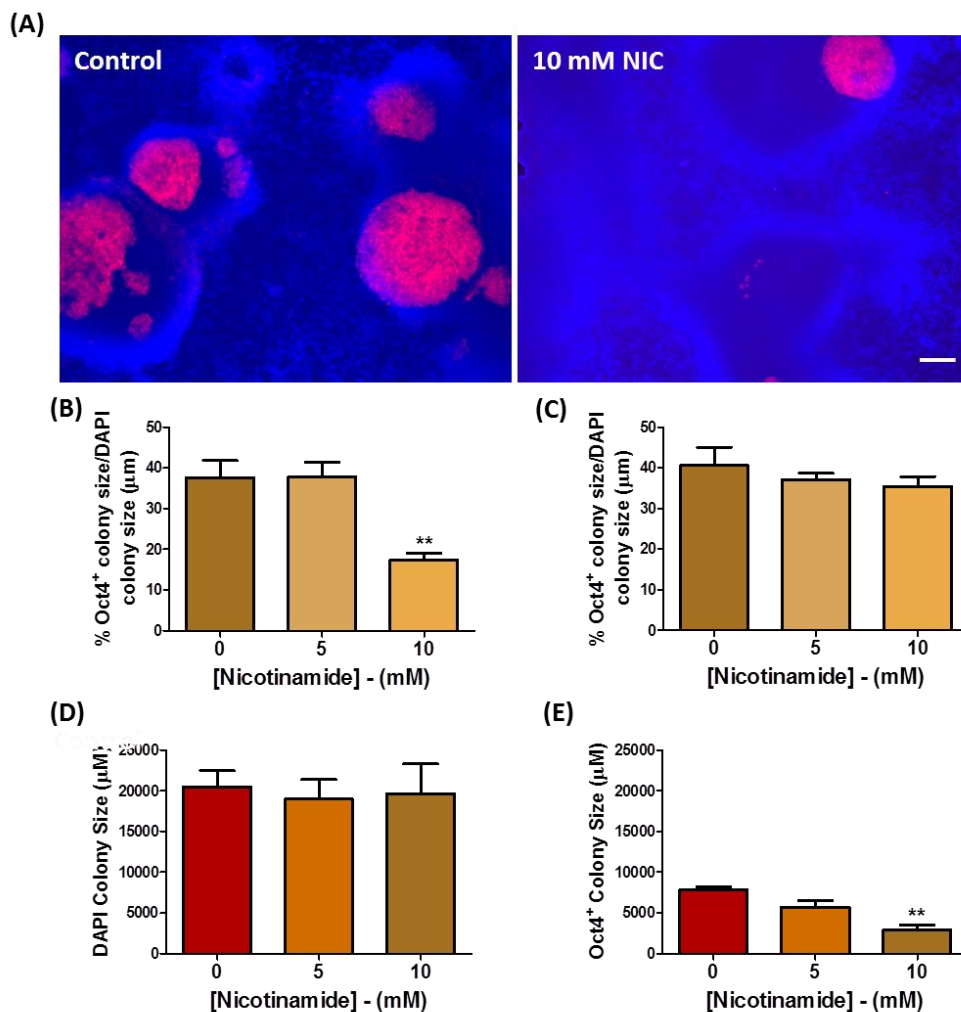
Figure 3.9 Early nicotinamide treatment accelerates stem cell differentiation (A) Semi-quantitative measurements of area size (μm^2) for the pluripotency marker Oct4

(red)-expressing colonies and total cell colonies labelled with DAPI (blue) at day 14. Levels of Oct4-expressing cells within DAPI colonies indicated a more striking difference in 10 mM nicotinamide conditions, compared to controls (treatment period: day 0-7 and differentiated until day 14). Image sets of nicotinamide cultures show DAPI-expressing colonies exhibit reduced number of cells expressing high levels of Oct4. Regions of low pluripotency are also visible in partially pluripotent colonies. Scale bar = 100 μm. **(B)** Percentage of areas containing cells expressing the Oct4 gene within the DAPI-labelled colonies was plotted. Oct4 was observed in less areas within DAPI$^+$ colonies when treated with nicotinamide. **(C)** Late treatment of nicotinamide at day 7 to 14 had no effect on the size of the proportion of DAPI$^+$ colonies that were Oct4$^+$. **(D&E)** Mean area values for DAPI and Oct4 colony size were plotted. DAPI colonies were similar in area (μm^2) across all treatment conditions. Regions of Oct4 expression were markedly reduced in cultures treated with 10 mM nicotinamide from days 0 to 7, suggestive of accelerated stem cell differentiation. ****$p<0.01$**

3.3.4.3 Sox1GFP$^+$ progenitor populations were decreased in cultures treated with nicotinamide.

Further to examining the undifferentiated Oct4 state, the effect of nicotinamide on monolayer-derived *Sox1*GFP-expressing neural progenitors was also assessed by quantifying GFP$^+$ cells. Application of 10 mM nicotinamide between days 0 and 7 significantly reduced the percentage of GFP-expressing NPCs by day 14 of differentiation (ANOVA $F_{2, 195} = 16.3$; $p<0.001$) [$12.8 \pm 1.8\%$ vs. $30.5 \pm 2.6\%$ in controls; Figure 3.10 (A) (B)]. No significant differences in the percentage of GFP$^+$ cells were found between control and treatment conditions when nicotinamide was added at late differentiation stages [Figure 3.10 (C) (D)].
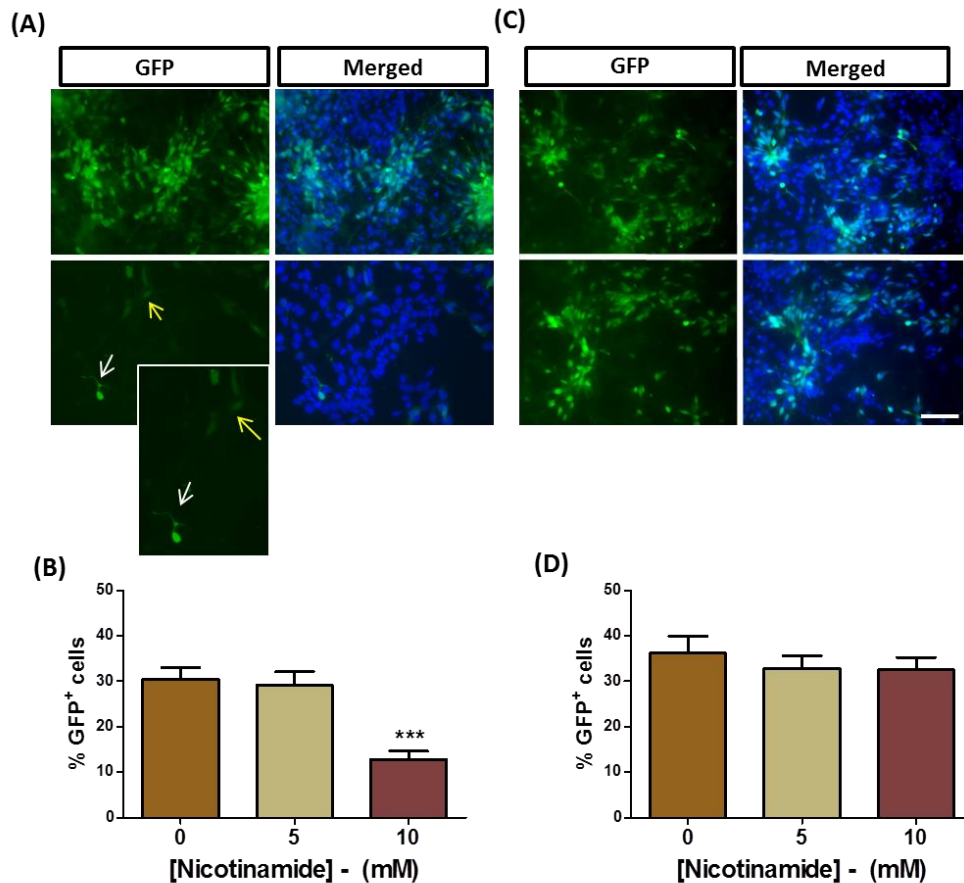
Figure 3.10 Nicotinamide treatment decreases the proliferating *Sox1*GFP⁺ neural precursor population. Cells were cultured for 14 days *in vitro* and analysed with fluorescence microscopy for GFP expression. **(A&C)** Images show native GFP (green)-expressing cells and total cells in cultures (blue) at day 14. Differences in the variability of GFP intensity was noted between cells. Some cells showed intense GFP expression (white arrow) and some less strong GFP expression (yellow arrow). Scale bars = 100 μm. **(A&B)** A significant decrease in the *Sox1*GFP⁺ population was observed in 10 mM nicotinamide-treated cultures, in comparison to untreated cells (day 0-7 conditions). **(C&D)** Nicotinamide added between days 7 and 14 did not affect the population of *Sox1*GFP-expressing cells.
***p<0.001

It should be noted that the well-established neuro-progenitor marker nestin was not investigated in this study. A number of reports found that nestin, an intermediate filament protein, is not an exclusive neural marker and should be used with caution for attributing neural progenitor identity[149,171]. For this purpose, nestin staining was tested in the monolayer culture system using an immunofluorescence experiment. It was noted that many of the strongly GFP-expressing progenitor cells did not show

co-labelling for nestin (Figure 3.11). This observation confirmed that nestin is not a reliable neural progenitor marker, so was therefore excluded from this study.
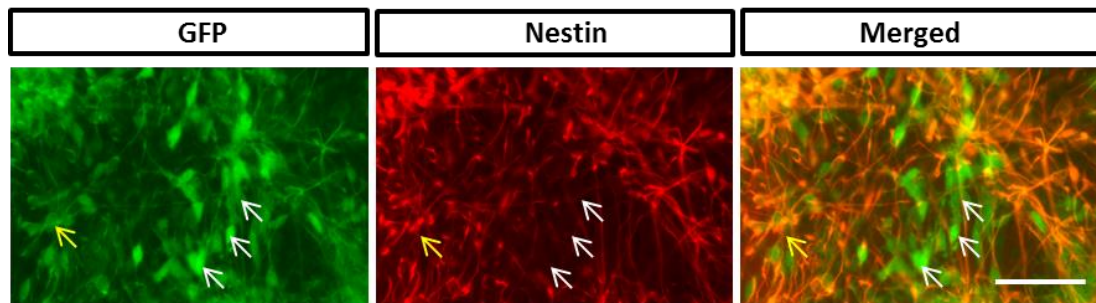
Figure 3.11 Immunocytochemistry of differentiated mESCs to identify neural progenitor cells. Co-localisation of native GFP expression and the neuro-progenitor marker Nestin is evident in some cells (yellow arrows). However, many of the GFP-expressing cells do not co-localise with nestin-positive cells (white arrows). Scale bar = 50 μm

3.3.4.4 Early nicotinamide administration accelerates the loss of neural progenitor populations.

Within monolayer cultures, variations in the intensity of GFP expression was evident, resulting in a spectrum from bright to low intensity GFP$^+$ cells [Figure 3.10 (A)-inset]. It was hypothesised that cells expressing higher levels of GFP represented true *Sox1*$^+$ NPCs, and weaker GFP$^+$ cells were immature neuronal and glial cells containing remnants of GFP protein, despite down-regulation of their GFP gene. This is likely due to the longer half-life of the GFP protein (~ 26 h), in contrast to endogenous *Sox1*[172].

Therefore, to investigate the effect of nicotinamide on intense and weak *Sox1*GFP$^+$ neural progenitor populations, GFP$^+$ cells were scored for levels of reporter protein using FI measures, described previously in chapter 2 (section 2.1.8). Briefly, GFP-expressing cells were categorised as 'weak' (≤ 0.25 FI units), 'moderate' (0.25–0.5 FI units) and 'strong' (≥ 0.5 FI units).

Data for mean FI values highlighted a marked reduction in the relative levels of GFP expression in cells treated with 10 mM nicotinamide between days 0 and 7, compared to all other conditions [ANOVA $F_{5,4410} = 92.5$; $p<0.0001$; Figure 3.12 (A)]. Further, 10 mM nicotinamide applied for the first 7 days of differentiation almost completely eradicated "strong" *Sox1*GFP-expressing cells (i.e. true *Sox1*$^+$ NPCs) from cultures ($0.5 \pm 0.5\%$ vs. $10.1 \pm 0.9\%$ in control conditions; $p<0.001$), concomitantly decreasing the proportion of "moderate" GFP-expressing cells per culture ($11.2 \pm 5.0\%$ vs. $38.6 \pm 0.8\%$ in control conditions; $p<0.01$). Consequently, nicotinamide treatment increased the proportion of weaker expressing GFP$^+$ cells which accounted for $88.3 \pm 5.51\%$ of the GFP$^+$ progenitor population [$p<0.001$; Figure 3.12 (B)], in contrast to control cultures which accounted for $50.8 \pm 4.3\%$ of the GFP$^+$ progenitor population.

Altogether, these important data indicate that early supplementation of differentiating ESC cultures with nicotinamide not only aids the elimination of potential tumorigenic cells from cultures, but also promotes an accelerated loss of pluripotency, thereby speeding up cell-cycle exit from the ESC state. Further, nicotinamide administration at the initial stages of mESC differentiation rapidly decreased the *Sox1*GFP$^+$ NPC population.
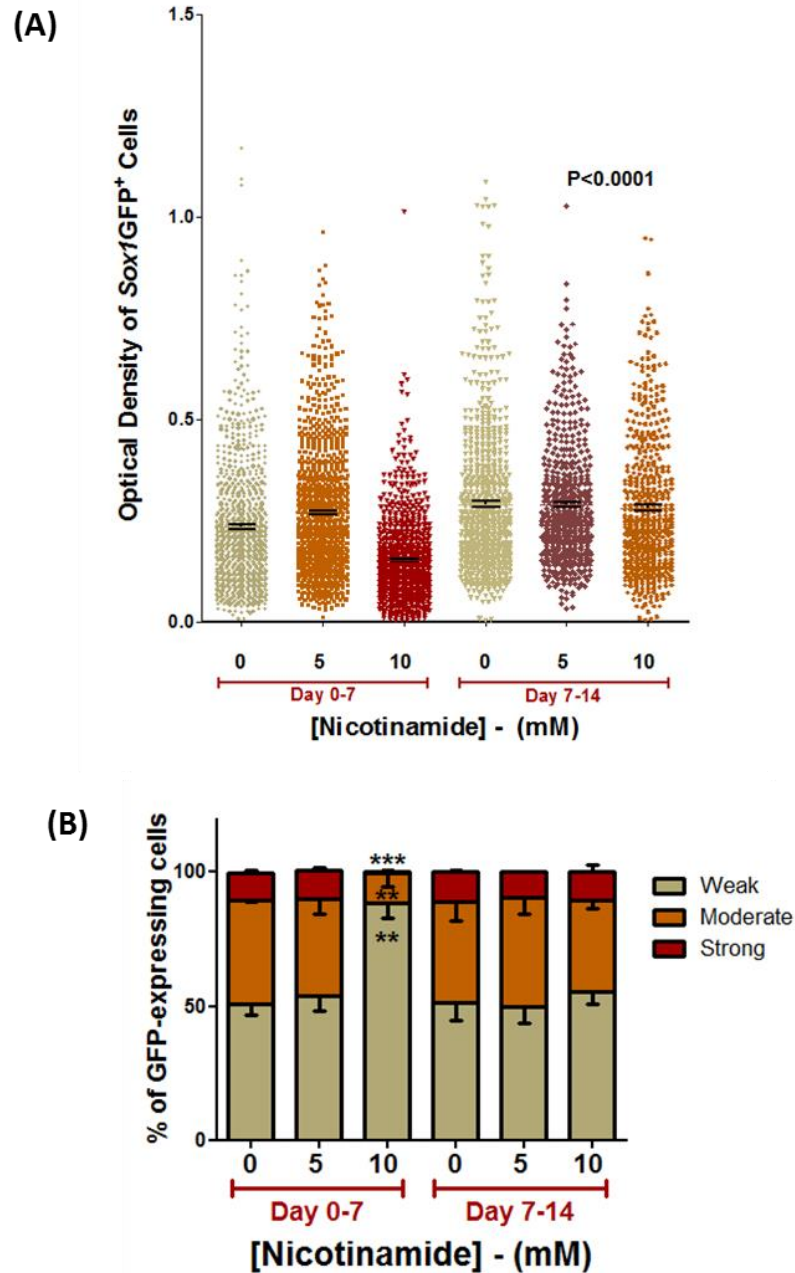
Figure 3.12 Effect of nicotinamide on GFP expression in 46C-derived mESCs
(A) The scatter graph depicts individual optical densities (arbitrary units) of GFP-expressing cells at day 14. FI values are plotted on the y-axis against nicotinamide concentrations on the x-axis. This scatter plot indicates a significant reduction in GFP fluorescence in 10 mM nicotinamide-treated cultures, compared to all other conditions. **(B)** Bar chart showing the proportion of cells with weak (green bars), moderate (orange bars) and strong (red bars) levels of GFP expression. The proportion of 46C-derived cells displaying "strong" and "moderate" levels of GFP expression was significantly decreased in cultures exposed to 10 mM nicotinamide between days 0 and 7, concomitant with an increase in "weaker"-expressing GFP^{+} cells, compared with controls and all other conditions.
***$p<0.001$, **$p<0.01$

3.3.5 Nicotinamide accelerates ESC-derived neural specification

Given the direct effect of 10 mM nicotinamide at the early developmental stages of ESC differentiation (i.e. day 0-7), this section reports experiments which were conducted to further investigate this vitamin concentration on the key developmental steps of neural commitment *in vitro,* in order to gain insight into the mechanism of nicotinamide's action in promoting neuronal differentiation. Adherent monolayer cultures supplemented with nicotinamide at day 0 to day 2 and day 2 to day 7 were analysed for the expression of markers of undifferentiated ESCs, early neural differentiation and early neuronal development in a similar manner to that performed previously in this chapter.

3.3.5.1 Nicotinamide promoted earlier down-regulation of Oct4 in ESCs

To investigate whether nicotinamide could promote mESC entry to the neural lineage in this monolayer culture system, the Oct4 protein was assessed, which is known to be down-regulated early during somatic differentiation[149]. Nicotinamide added to undifferentiated *Sox1*GFP knock-in reporter mESCs at the onset of differentiation (day 0) caused a dramatic reduction in the number of Oct4-expressing colonies per field by day 2 (unpaired t test, t = 6.0; $p<0.001$) [3.3 ± 0.5 vs. 10.2 ± 0.9 in controls; Figure 3.13 (A), Figure 3.16 (A)]. A less marked reduction was observed in treatment conditions from day 2 to day 7 (unpaired t test, t = 2.5; $p<0.05$) [3.8 ± 0.5 vs. 5.3 ± 0.4 in control cultures; Figure 3.13 (B), Figure 3.16 (B)]
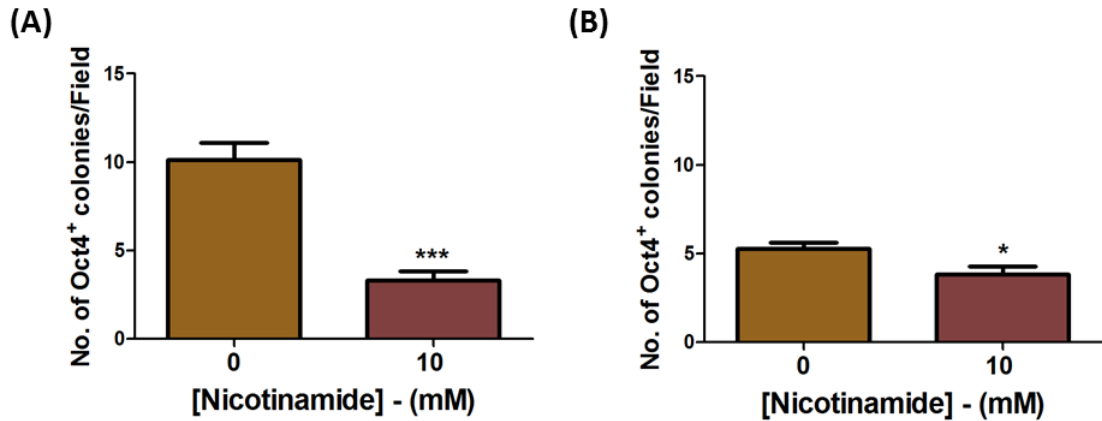
Figure 3.13 Early nicotinamide treatment reduced the number of colonies expressing Oct4. (A&B) The effect of nicotinamide on the undifferentiated mESC population was investigated during monolayer differentiation between days 0-2 **(A)** and days 2-7 **(B).** The number of Oct4-expressing colonies per field was plotted. Cells were counterstained with DAPI.
***$p<0.001$, *$p<0.05$

3.3.5.2 Nicotinamide accelerated neural induction

In accordance with other reports, analysis from this study showed a highly reproducible up-regulation of native GFP expression in control cultures, driven by expression of the *Sox1* gene by day 3-4 of monolayer differentiation. Colonies containing various intensities of GFP expressing cells initially appeared interspersed with GFP⁻ colonies [Figure 3.16 (A)]. Therefore, the effect of nicotinamide on the *Sox1*⁺ progenitor population was assessed at day 4 (treatment: day 0-2) rather than day 2, in addition to the day 2-7 culture period. The extent of GFP expression in colonies was analysed using FI measurements. An area cover of green fluorescence (μm^2) was expressed as a percentage of the total cell/DAPI⁺ area.

At day 4, FI values for the nicotinamide-treated group revealed a slight higher trend in the extent of green fluorescence in monolayer colonies, albeit these differences were not statistically significant [n.s.; Figure 3.14 (A)]. The percentage of native GFP-labelled regions within DAPI⁺ colonies was dramatically enhanced in treatment cultures by day 4, compared to lower distributions of GFP⁺ expression in controls

(unpaired t test, t = 5.6; *p*<0.01) [38.1 ± 1.4% vs. 7.6 ± 5.2% in control groups; Figure 3.14 (B), Figure 3.16 (A)].

Monolayer cultures treated with nicotinamide between days 2 and 7 revealed a slight downward trend in both GFP$^+$ intensity levels and the area coverage of green fluorescence co-localised with DAPI$^+$ colonies, albeit these differences were not statistically significant [n.s.; Figure 3.14 (C) (D), Figure 3.16 (B)]. In summary, these data indicate that nicotinamide added from day 0 of the monolayer protocol enhanced the processes of neural specification.
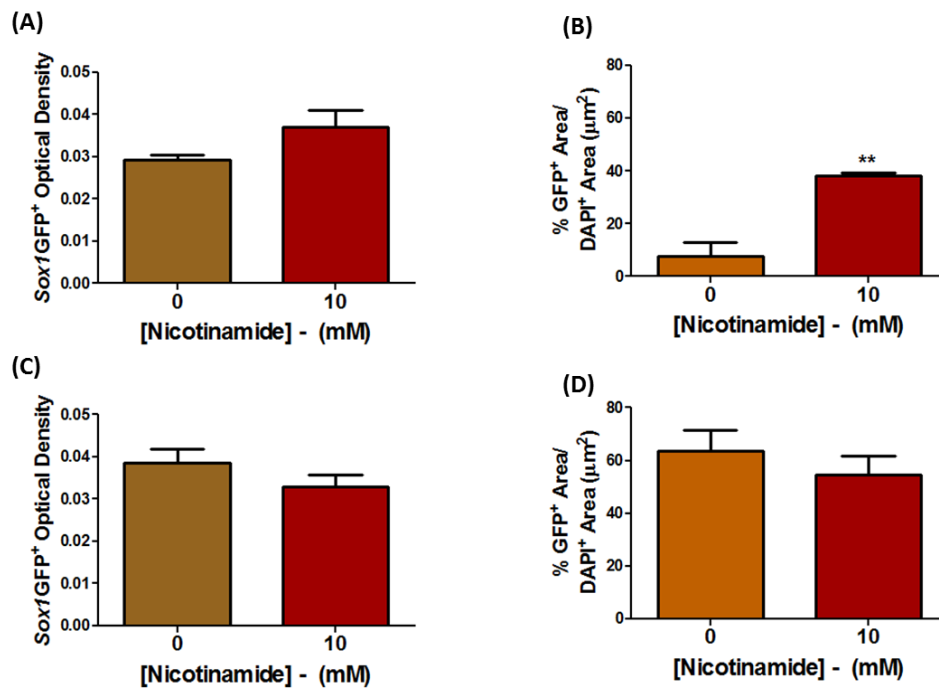
Figure 3.14 Early nicotinamide treatment accelerated neural induction. (A-D) The effect of nicotinamide on neural specification was investigated during monolayer culture periods: day 0-4 **(A&B)** and day 2-7 **(C&D).** FI measurements of GFP-expressing colonies were plotted **(A&C)**. Native GFP-labelled areas were expressed as a percentage of total cell area **(B&D).**
***p*<0.01

3.3.5.3 Nicotinamide treatment directed earlier expression of young βIII-tubulin$^+$ neurons

Consistent with results highlighting a rapid decline in the ESC state and accelerated emergence of GFP$^+$ progenitor cells, the expression of βIII-tubulin was also up-regulated in nicotinamide-treated cultures as early as the neural induction stage in this monolayer system. Although βIII-tubulin$^+$ neurons were evident in cultures as early as day 2, nicotinamide significantly promoted the generation of immature neuronal populations (unpaired t test, t = 2.9; $p<0.05$) [6.8 ± 1.4% vs. 2.63 ± 0.28% in untreated conditions; Figure 3.15 (A), Figure 3.16 (A)]. Surprisingly, a marked increase in the percentage of βIII-tubulin$^+$ neurons was also demonstrated in cultures treated between days 2 and 7, compared to control conditions (unpaired t test, t = 3.6; $p<0.001$;) [7.3 ± 0.9% vs. 3.3 ± 0.6% in control groups; Figure 3.15 (B), Figure 3.16 (B)].

In summary, these data indicate the processes of neural specification and neuronal differentiation were accelerated, under the driving force of nicotinamide acting at the initial stages of ESC differentiation.
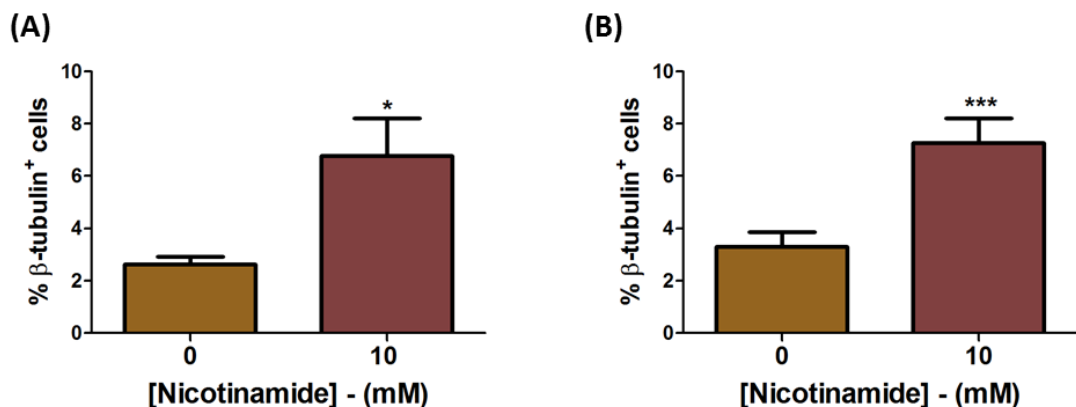
Figure 3.15 Early nicotinamide treatment up-regulated the expression of βIII-tubulin$^+$ neurons. (A&B) The effect of nicotinamide on immature neuronal expression was accessed during monolayer culture periods; day 0-2 **(A)** and day 2-7

(B). Percentage of βIII-tubulin$^+$ cells was plotted over total cells, counterstained with DAPI.
***$p<0.001$, *$p<0.05$
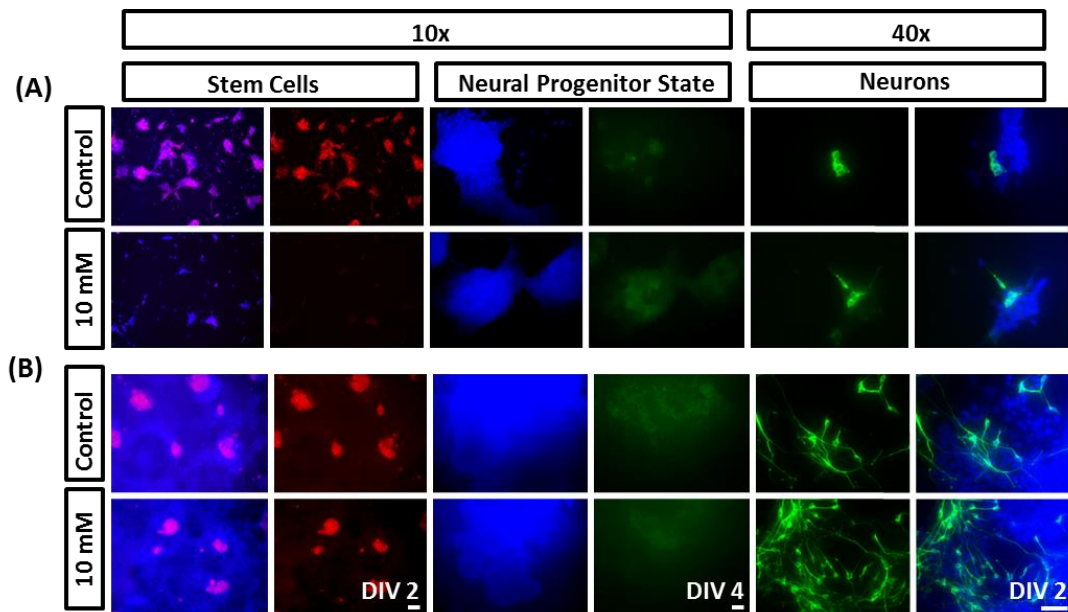
Figure 3.16 Addition of nicotinamide from day 0 of the monolayer protocol accelerated neural induction and neuronal differentiation. Immunofluorescence images of pluripotency (Oct4), early neural (native Sox1GFP expression) and neuronal (βIII-tubulin) proteins. Monolayer cultures were treated from days 0-2 or days 2-7. Nuclei counterstained with DAPI (blue). (A) Nicotinamide acted at the initial stages (days 0-2) of monolayer differentiation to markedly down-regulate the specific marker for ESCs, Oct4, thereby promoting earlier neuroectodermal cell fate commitment, and accelerated the emergence of immature βIII-tubulin+ neurons. (B) Nicotinamide added between days 2 and 7 decreased Oct4-expressing colonies and slowly down-regulated GFP expression, concomitant with enhanced neuronal differentiation.
Scale bar = 100 μm (Stem Cell and Neural Progenitor State panels).
Scale bar = 50 μm (Neuron panels).

3.4 Discussion

To my knowledge, this is the first report which investigated whether nicotinamide, the metabolic product of vitamin B$_3$, could direct 46C *Sox1*GFP mESCs toward neural and further toward neuronal cells *in vitro*. This experimental chapter reports a protocol that renders nicotinamide sufficient to induce accelerated neural specification from mESCs which further progressed to enriched neuronal populations with enhanced efficiency, within 14 days of monolayer differentiation.

A number of *in vitro* protocols have been established to trigger the process of neural induction to generate regionally specified neuroepthelial progenitors and/or terminally differentiated neuronal and glial cells, thereby recapitulating the multistep cascade controlling embryonic development *in vivo*. An initial approach to reconstitute neurogenesis *in vitro* was accomplished through embryoid body (EB) formation or by co-culture with stromal cells or conditioned medium. However, it is often challenging to manipulate and study differentiation within three-dimensional EB aggregates formed in suspension[173]. A more efficient method to achieve neural commitment *in vitro*, avoiding confounding complexities associated with multicellular aggregation co-culture systems relies upon an adherent monolayer (two-dimensional) culture system using ESCs, established by Ying and co-workers[164]. This protocol facilitates the rapid generation of neural progenitors capable of differentiating into neuronal/glial subtypes, with minimum perturbation of the culture or use of uncharacterised media constituents and expensive culture reagents[149,164,170,174]. Therefore, this efficient monolayer culture system was ideal for this study, to investigate the effects of nicotinamide on directing neuronal differentiation in factor-free chemically defined media, without the matrix effects associated with EBs.

The hypothesis under investigation was that nicotinamide could promote enhanced differentiation of *Sox1*GFP reporter mESCs into neuronal populations, to obtain higher yields of enriched neuronal cultures toward transplantation therapies for neurodegenerative disorders, cell biological investigation, neurological disease modelling and pharmaceutical screening.

3.4.1 Differentiation to neurons is highly efficient in nicotinamide-treated cultures.

This study showed that early nicotinamide supplementation to undifferentiated 46C cells increased the percentage of neurons in mESC-derived cultures, concomitant with a decrease in the total number of cells in culture after 14 days. By adding nicotinamide to cells at the onset of differentiation, the efficiency of neuronal generation was significantly increased. The timing of nicotinamide supplementation to cultures during the early steps of monolayer differentiation is consistent with similar protocols reported in the literature, which applied nicotinamide to direct differentiation of hESCs into RPE[161,162], and into human cardiac precursors and cardiomyocytes[163].

Crucially, accompanying the increase in the proportion of neurons, nicotinamide also promoted an enrichment of the neuronal population vs. non-neuronal cells, by reducing the total number of cells, as quantified by DAPI staining. Importantly, this key finding is in agreement with a previous study which controlled the differentiation of human pluripotent stem cells by applying defined factors, including nicotinamide at specific time points to generate approximately 80% of cells characteristic of RPE phenotype, within 14 days of differentiation[162].

Overall, the differentiation-promoting effects of nicotinamide were dose-dependent, being significant at 5 mM and 10 mM doses, with 10 mM nicotinamide having the most pronounced effects, which was in agreement with previous stem cell differentiation studies[159,161,162]. Notably, 10 mM nicotinamide represents a considerably higher concentration than the physiological level *in vivo*. Work carried out by Qin *et al* provided values for nicotinamide concentrations in the brain, which ranged from 100 μM to 300 μM[176]. However, it is important to consider that the intended application of the findings in this study would be in monolayers *in vitro* for cell transplantation, and vitamin metabolites are classically added to cultures

undergoing differentiation at higher concentrations than the physiological level.[104] One reason for this, may be that the functioning of nicotinamide may have synergistic effects *in vivo* and those interacting factors may not be present *in vitro*. Another important point of note is that the level of nicotinamide added to cell cultures *in vitro* may not precisely represent the bioavailable nicotinamide bound to serum proteins *in vivo*. Therefore, the culture concentrations of nicotinamide cannot be directly compared to the physiological levels.

Overall, when added at appropriate concentrations at appropriate developmental stages, nicotinamide has an important role to play in directing mESC differentiation specifically towards a neuronal lineage, which could prove a useful addition to current protocols for directing stem cell differentiation.

3.4.2 Nicotinamide accelerated the differentiation of both stem and progenitor cells towards a neuronal fate.

Cell-to-cell phenotypic heterogeneity is a common and fundamental characteristic of ESCs. However, compounding complexities often arise during *in vitro* ESC differentiation due to this highly heterogeneous nature of *in vitro* differentiated ESC preparations. Asynchronous ESCs often differentiate into lineage-biased subsets[149] and contribute to the significant risk of tumourigenesis due to the presence of undifferentiated ESCs amongst differentiated progenies in grafted transplants[172]. Considering this, it is crucial that tumour-forming cells are eliminated from ESC-derived preparations.

By day 14 of monolayer differentiation, nicotinamide treatment (from day 0-7) reduced both Oct4 and GFP expression of cells in culture, concomitant with the elevated expression of neuron-specific βIII-tubulin, indicative of accelerated

neuronal differentiation. Two quantitative methods were applied to provide an assessment of the effect of nicotinamide on pluripotency: quantification of Oct4 immunoreactive colonies; and measurement of Oct4-labelled areas within DAPI-stained colonies. Addition of nicotinamide at both early and late stages of monolayer differentiation significantly reduced the Oct4$^+$ pluripotent stem cell population from cultures at day 14. Indeed, 10 mM nicotinamide promoted earlier differentiation of mESCs, as indicated by the pattern of Oct4 expression, which declined more rapidly following early nicotinamide exposure.

In line with these findings, *Sox1*GFP$^+$ NPC numbers were rapidly reduced under the driving force of early nicotinamide supplementation, when examined at day 14 of the monolayer protocol. It is therefore reasonable to conclude that more NPCs differentiated earlier into neurons in the presence of nicotinamide. Specifically, semi-quantitative analysis indicated that the proportion of *Sox1*GFP$^+$ cells expressing intense GFP was almost eliminated from cultures. Interestingly, the majority of cells treated with nicotinamide showed uniformly weaker GFP expression. This detailed microscopic analysis of relative GFP intensity levels was used to give a more precise measure of the heterogeneity within cell populations, as observed in 46C ESC-derived cultures, compared with just a cell count of GFP$^+$ cells. Furthermore, this technique using fluorescent microscopy and Image-J software eliminates complexities associated with fluorescent plate readers, which would quantify the total GFP fluorescence present in a culture. Additionally, with respect to experimental design in this study, an anti-nestin antibody was not selected to examine the effects of nicotinamide on the neural progenitor population, since it has been reported that nestin is not an exclusive neural marker,[149,177] which was confirmed in this study. Therefore, analysis of GFP alone in the presence and

absence of nicotinamide was investigated, where GFP has been reported to faithfully mimic endogenous *Sox1* expression during early development in the neuroepithelium and in NPCs *in vitro*[149,164,178].

Overall, findings in this experimental section support previous evidence where nicotinamide was shown to eliminate Oct4$^+$ dividing cells from pluripotent stem cell cultures, thereby speeding up differentiation into clinically relevant lineages[161,162,167].

3.4.3 Nicotinamide accelerated neural specification of Sox1GFP mESCs.

Previous studies have linked nicotinamide with a switch in neural progenitors towards a neuronal fate in the conversion of human ESCs to other neuronal types[179]. Findings from this study determined that nicotinamide is most effective in influencing neuronal differentiation at an early developmental time window (day 0-7 of the 14 day monolayer protocol). A number of neural differentiation studies using the mouse knock-in ESC line 46C cultured in monolayers showed that this method generates neural progenitors from around day 2-7; therefore the results presented in this thesis suggest that nicotinamide may be acting at the initial stages of ESC differentiation to influence the transition of mESCs into neuroectoderm or indeed driving a switch from neural progenitors to neurons in the differentiating cells. This hypothesis is supported by previous data in this experimental chapter showing that after the neural progenitor stage [late born *Sox1*GFP NPCs (day 7)[178]], administration of nicotinamide can no longer direct the differentiation of neurons (i.e. day 7 to 14).

Within 2 days of withdrawing LIF and serum from cultures, the number of Oct4-expressing colonies was dramatically reduced in nicotinamide-treated cultures. Interestingly, adherent monolayer cultures exhibited brighter green fluorescence

within significantly increased GFP-labelled regions following nicotinamide treatment, thereby suggesting that nicotinamide is enhancing neural lineage specification, i.e. accelerating the conversion to neuroectoderm cell fate commitment. Accompanying the accelerated emergence of progenitor cells, the percentage of immature neurons expressing βIII-tubulin was also up-regulated in the presence of nicotinamide, i.e. nicotinamide is facilitating rapid neuronal differentiation. These data are consistent with similar observations in RA-treated *Sox1*-GFP 46C cultures[174].

In this study, it is therefore reasonable to hypothesise that the initial higher frequency of *Sox1*GFP$^+$ cells in monolayer cultures may be due to enhanced viability or conversion of progenitor cells during the early stages of development. In this regard, one study showed that prevention of apoptosis was one of the mechanisms mediating the promotion of neural specification by nicotinamide. Nicotinamide was shown to reduce cell death of human ESC-derived NPCs upon neural induction, via inhibition of PARP, thereby enhancing neutralization[141]. However, it is important to note that higher doses of nicotinamide (20 mM) induced cytotoxic effects on cells within 3 days of application. This is coincident with other differentiation studies which also found that high doses of the active metabolites of vitamin A (retinoic acid: 10-6 μM) and vitamin C (ascorbic acid: 500-5000 μM) produced obvious noxious effects on cell survival[124,174]. In this context, review papers published by Williams *et al* propose a hypothetical link between a hypervitaminosis B_3 state and modern disease phenomena, resulting from a modern Western diet abundant in meat and vitamin supplements[129,180]. Specifically, excess nicotinamide has been linked with neurodegeneration, particularly PD[130], implying that levels of this vitamin require tight regulation in order to sustain normal neuronal functioning. Further to this point,

this report highlighted the narrow range of concentrations at which nicotinamide is active.

Nicotinamide induced a less marked effect on neural induction between days 2 and 7, implying that the inductive role of nicotinamide may function during a narrow time window prior to or at the stage of *Sox1* expression during mESC differentiation. A slight decrease in the number of pluripotent colonies per field was observed at the end of the culture period. Treatment of monolayer cultures with nicotinamide at the initial stages of *Sox1*GFP expression (day 2) elicited a slight down-regulation of GFP expression at day 7. These data show that, in the monolayer culture system, the speed at which nicotinamide promoted ESC differentiation into *Sox1*GFP$^+$ NPCs was faster than the rate of loss of *Sox1*GFP$^+$ cells, thereby suggesting an important role for nicotinamide from day 0 of monolayer differentiation. Surprisingly, the expression levels of βIII-tubulin were dramatically elevated in cultures treated with nicotinamide from day 2 to 7. It is therefore clear that nicotinamide can potentiate pluripotent stem cell differentiation and promote enhanced derivation of mESC-derived neurons across a spectrum of developmental stages during ESC differentiation. This work is highly relevant to future neural-based protocols as it opens up the possibility of applying nicotinamide as a differentiation agent to obtain increased yields of neurons.

3.4.4 Conclusion and Future Studies.

Results obtained in this study strongly support an important role for nicotinamide at the early induction stage during ESC in vitro differentiation, rather than at later stages of neuronal differentiation. Importantly, findings outlined in this study are exciting, as nicotinamide not only increased and accelerated the differentiation of

developing neurons from ESCs, but also generated more enriched neuronal populations, and without the support of expensive exogenous factors, therefore highlighting potential implications for the cost efficient scale-up of future neural cell-based therapies.

The precise mode of action of nicotinamide in mechanisms underlying the promotion of enhanced and accelerated neuronal differentiation remains to be elucidated. A number of studies found that some of its effects may be mediated through inhibition of PARP or silencing information regulator 2 (SIRT1) (this will be discussed in detail in Chapter 5). In future work, it will be of interest to determine if nicotinamide mediates neural specification by enhancing cell-cycle exit by reducing the proportion of proliferating cells and/or selective apoptosis in non-neural populations. In this context, Vaca et al[158] found that nicotinamide could cause an 80% reduction in ESC proliferation, supporting the former mechanism of action, whilst Idelson et al[162] found that prevention of apoptosis in neural populations, rather than augmentation of their proliferation resulted in enhanced RPE differentiation. Whichever mechanism is at work, nicotinamide's influence in reducing ESC numbers and promoting the loss of pluripotency in undifferentiated ESCs, along with reducing the number of proliferating progenitor cells could be translated to current ESC differentiation protocols, thereby reducing the risk of tumorigenicity in stem cell-derived transplants as we advance towards clinical stem cell-based therapies.

It has been reported that neuronal fate specification and progenitor formation are two closely linked processes occurring during ESC differentiation, which may be instructed by the same signals[178]. Therefore, findings outlined in this chapter strongly suggest that nicotinamide could be applied to current neuronal protocols during the early stages of neural differentiation, in combination with factors known

to enhance neuronal phenotypes, to promote subtype specification. The next experimental chapter builds on these concepts, further investigating whether nicotinamide could influence the enrichment and maturity of desired neuronal subtypes, pertinent to common neurological disorders, such as PD, HD and neuropsychiatric disorders.

Chapter 4: Effect of Nicotinamide on Neuronal Subtype Differentiation and Maturation from Mouse Embryonic Stem Cells *In Vitro*

4.1 Introduction

Methods for obtaining specific neural subtypes to substitute cells lost to the disease process routinely apply intrinsic and extrinsic factors to direct cell fate choice. Therefore, identifying factors that regulate specification, differentiation, maturation and survival of developing neurons is important. Recently, increased attention has also focused on stem cell-derived neuronal subtypes as a valuable platform for disease modelling, neurological drug screening and neurotoxicity evaluation, to further develop therapeutic strategies to treat a range of neurological illnesses[181]. However, like CRT, improved methods are required to convert stem cells to highly purified phenotypic neuronal populations in high yields amenable to large screening campaigns, and which share similar morphologies to primary neurons *in vitro*[182,183].

Specific neurotransmitters of the BG neurocircuitries have important functions in the etiology and treatment of neurological disorders. For example, DAergic and serotonergic neurons play important roles in the etiology and treatment of neurodegenerative disorders, including PD[78] and neuropsychiatric diseases[184,185]. The neurotransmitters DA and serotonin or 5-hydroxytryptamine (5-HT) are synthesised by neurons generated from the boundary between the midbrain and hindbrain, and are therefore influenced by similar developmental signals[83]. Midbrain DAergic neurons are of clinical importance due to their selective degeneration in PD, leading to deficits in the control of movement[186]. Therefore, understanding and identifying key signals that increase the development of DAergic neurons may advance the promise of CRT for PD. Serotonergic neurons send their projections to cortical brain regions, thereby regulating behaviours such as cognitive function and mood. Disturbed 5-HT signalling and homeostasis are associated with a myriad of neuropsychiatric disorders, including obsessive compulsive disorder, hyperorexia

(i.e. a compulsive overeating disorder) and impulsivity[185]. Thus, improved protocols to induce *in vitro* serotonergic differentiation from stem cell sources may prove beneficial for developing and testing the safety of therapeutic drug candidates, to progress the treatment of psychiatric disorders[185]. Similarly, it is crucial to fully control the differentiation of pluripotent stem cells into excitatory glutamatergic neurons in a robust and homogenous manner to provide a model system for future mechanistic investigation of early disease pathology and neurotoxicity testing, pertinent to psychiatric conditions and Alzheimer's disease (AD)[182,183,187]. Degeneration of inhibitory GABAergic MSNs in the neostriatum underlies the neuropathology of Huntington's disease (HD), a late-onset inherited neurodegenerative disorder leading to motor disturbances (i.e. chorea movements), together with cognitive and neuropsychiatric impairments[188]. Significant advancements have been made in directing pluripotent human stem cells to generate both authentic nigral and striatal phenotypes *in vitro*, required for PD and HD cell-based therapies respectively[78,79,87,189,190], however, like PD, further research into the signalling pathways governing GABAergic specification and differentiation is necessary to convert stem cells to enriched and high yield populations of GABAergic MSNs[191].

Recently, researchers have reported the significance of nicotinamide to protocols for a range of neurological disorders due to its capacity to ameliorate PD[192,193], HD[194,195], AD[196] and stroke[131] in animal models relevant to these diseases. Specifically, nicotinamide was shown to improve motor deficits in a Drosophila model of PD[193] and promote neuroprotective effects towards DAergic neurons in different PD mouse MPTP models[192]. Nicotinamide has also been reported to alleviate motor deficits and upregulate peroxisome proliferator-activated receptor

gamma coactivator 1-alpha (PCG-1α; a key regulator of mitochondrial biogenesis and respiration) and the neurotrophic factor BDNF in a mouse model of HD[195]; decrease neuronal loss and motor impairment in the Drosophila model of HD[194]; reduce cognitive deficits in AD transgenic mice[196], and function as a robust neuroprotective agent to enhance neurological outcome and decrease brain infarction volume in animal models of stroke[131]. Furthermore, there is growing evidence to suggest an important role for nicotinamide during early development, to promote and enhance various aspects of cellular morphology and maturation[141,197,198].

Building on previous data outlined in Chapter 3, nicotinamide induced neuron-enriched cultures when added at appropriate concentrations at appropriate developmental time-points of monolayer ESC differentiation; however it is not clear whether this vitamin B_3 metabolite could enhance the generation of neurons enriched for particular subtypes implicated in a diverse range of neurological disorders. Therefore, based on existing literature linking nicotinamide to neurological protocols and methods to enhance cellular development, the hypothesis under investigation in this chapter was that nicotinamide could promote the differentiation and/or enrichment of specific neurons exhibiting distinct neurotransmitter phenotypes. 46C mESC-derived neurons widely express neuronal subtype markers, relevant to a range of neurological disorders including PD, HD and neuropsychiatric conditions[170,199], thereby making it an appealing differentiation system to address this hypothesis. This experimental chapter aimed to:

I. *Investigate whether nicotinamide promotes the generation of higher yields of enriched neuronal subtype populations (i.e. catecholaminergic, serotonergic, glutamatergic and GABAergic neurons), to progress stem cell protocols for replacing degenerated neurons for transplantation therapies in BG*

disorders, biological investigation, neurological disease modelling and pharmaceutical screening.

II.   *To study the effect of nicotinamide on neuronal morphogenesis during early development in GABAergic and catecholaminergic populations, since there is a major need to generate functional and mature neuronal cell types for HD and PD, respectively.*

4.2 Experimental Procedure

This chapter aimed to investigate whether the vitamin B_3 metabolite, nicotinamide, could promote either the specification and/or maturation of developing neuronal subtypes characteristic of the BG neurocircuitries, from the 46C *Sox1*GFP reporter cell line.

4.2.1 Cell culture and nicotinamide treatment

To test whether nicotinamide treatment induced neural subtype specification, the effects of different nicotinamide concentrations were selected based on their previous differentiation-inducing effects, outlined in Chapter 3. Thus, monolayer cultures were treated with 5 mM and 10 mM nicotinamide at the initial stages of differentiation (i.e. day 0-7), and the cells were further differentiated up to a total of 14 days (detailed account provided in section 3.2.1).

4.2.2 Immunocytochemistry

Immunocytochemistry and fluorescence microscopy were performed to evaluate the effect of nicotinamide on different classical neurotransmitters phenotypes of the BG

circuitry, which included GABA, glutamate, catecholamine and serotonin. To achieve optimum staining results, newly introduced antibodies to the laboratory were tested in accordance with the manufacturers' recommended dilution ranges, to establish appropriate working dilutions. Primary neuronal cells known to express a particular antigen were selected, to serve as positive controls for the immunocytochemistry. Double-label immunofluorescence was used to detect co-localisation of the neuron specific βIII-tubulin marker with antibodies against the inhibitory neurotransmitter GABA, TH (the rate-limiting enzyme in catecholamine biosynthesis), vesicular protein VGlut2 (marker for excitatory glutamatergic synapses) and 5-HT (immunomarker for serotonin). All cell counts were expressed as a percentage of total neurons counterstained with βIII-tubulin, and as a percentage of total cells counterstained with DAPI.

4.2.3 Morphometric analysis

Morphometric analyses were used to assess the extent of neuronal differentiation in the presence of nicotinamide. Fluorescent images were acquired using a high power objective lens (i.e. x 40 lens) and importantly, exposure settings were kept constant across all conditions. Four morphological parameters were investigated: (1) measurement of neurotransmitter content within neuronal cell bodies (soma); (2) number of primary neurite branches; (3) length of longest neurite (μm), and (4) total neurite extent (μm). Measurements of FI were used to quantify marker expression levels of neurotransmitter phenotypes within individual neuronal cell bodies, using Image-J software (detailed in section 2.1.8.1), and measures of neurite outgrowth were assessed using the ImageJ plug-in NeuronJ (described previously in section 2.1.8.2).

4.2.4 Image and statistical analysis

Analysis of neuronal morphology and maturation were performed on neurons randomly selected from three independent experiments. For all experiments in this chapter, statistical analysis was performed by one-way ANOVA followed by a post-hoc Tukey multiple comparison test when required, using a level of $p < 0.05$ as a limit for statistical significance. Data are expressed as mean ± SEM.

4.3 Results

4.3.1 Primary antibody optimisation for immunocytochemistry

Positive staining controls were used to establish optimum working dilutions and support the validity of newly introduced antibodies to the laboratory, to ensure an accurate interpretation of immunocytochemistry results in this experimental study.
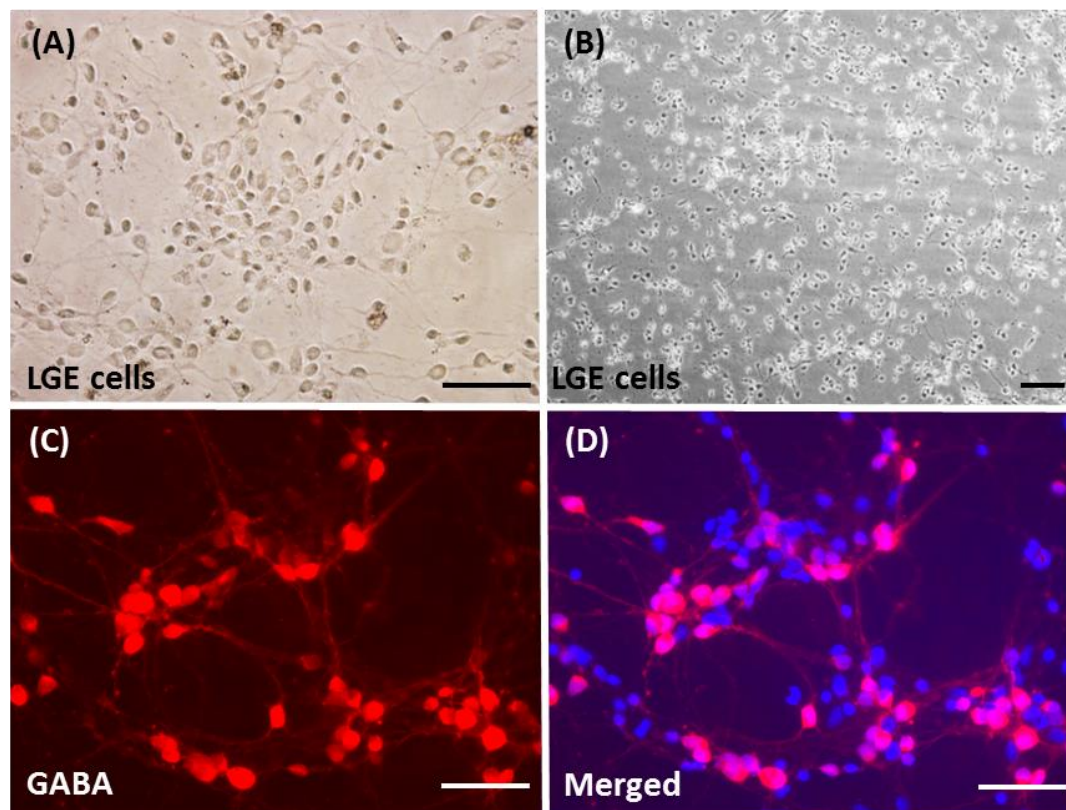
Figure 4.1 Antibody optimisation for immunocytochemistry. Rabbit polyclonal GABA antibody was optimised using LGE-derived primary neuronal cultures, obtained from E15 rat embryos. Striatal GABAergic neurons are derived from the

germinal zones of the LGE located in the subventricular zone of the developing telencephalon.[191] Since the peak period of MSN neurogenesis occurs in the whole ganglionic eminence at approximately E14 in the rat,[63] cultured E15 LGE cells should express high levels of relevant antigen expression required for optimising the anti-GABA antibody. **(A&B)** Bright-field images of E15 LGE-derived primary neurons at 7 DIV **(A)** Scale bar = 50 µm; **(B)** Scale bar = 100 µm. **(C&D)** LGE cultures give rise to the principal source of GABAergic (red)-expressing (projection) neurons. The nuclei of all cells in cultures were labelled with DAPI (blue). **(C&D)** Scale bar = 50 µm.
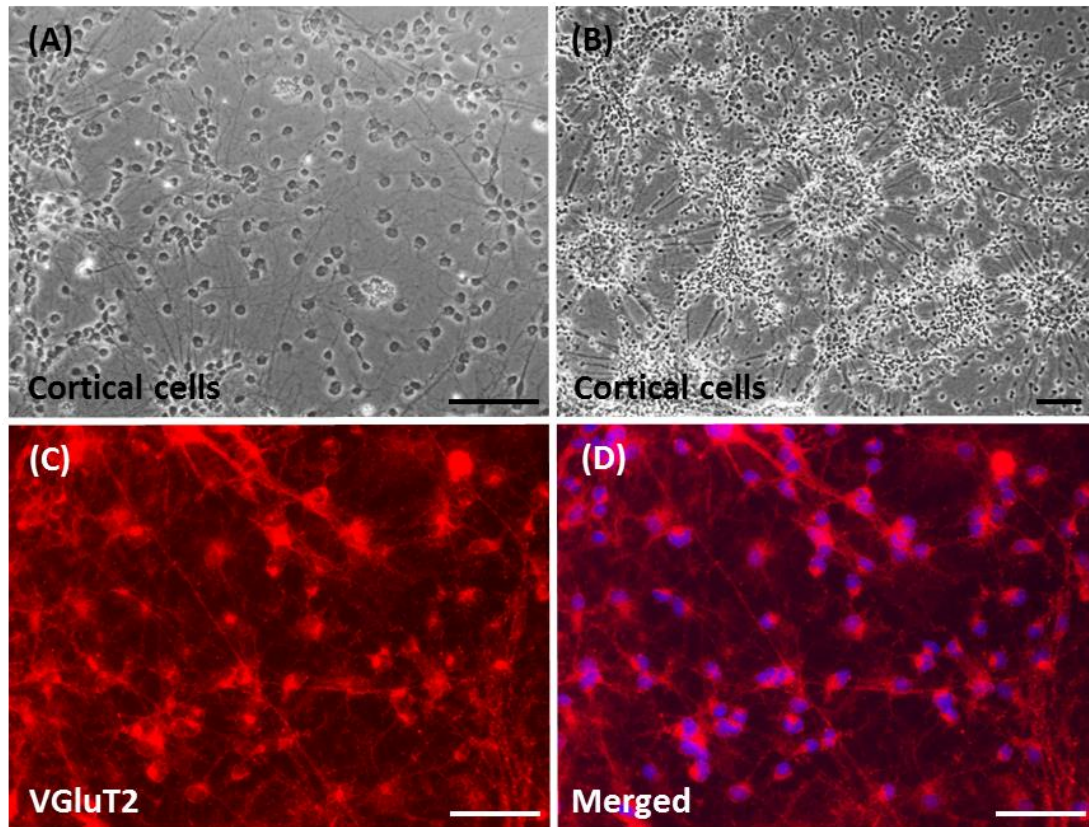
Figure 4.2 Antibody optimisation for immunocytochemistry. Rabbit polyclonal VGlut2 antibody was optimised using developing cortical-derived primary tissue harvested from E15 rat embryos. **(A&B)** Bright-field images of E15 cortical-derived primary neurons **(A)** Scale bar = 50 µm; **(B)** Scale bar = 100 µm. **(C&D)** VGlut2 is a glutamate transporter expressed in the membrane of synaptic vesicles. Representative images of primary cells dissected from the cerebral cortex showing cultures enriched in glutamatergic (red)-expressing neurons. The nuclei of all cells in cultures were labelled with DAPI (blue). **(C&D)** Scale bar = 50 µm.

4.3.2 Nicotinamide selectively enhances the production and enrichment of neurotransmitter phenotypes from mESCs.

To determine the potential of the vitamin B_3 metabolite, in terms of the percentage of specific neurotransmitter subtypes generated from the monolayer-derived *Sox1*GFP knock-in 46C cell line, the effects of nicotinamide on neuronal enrichment and differentiation were investigated. In all experiments, undifferentiated cells were treated with 5 mM and 10 mM nicotinamide at an early stage of development, i.e. between days 0 and 7, and the cells were further differentiated up to a total of 14 days of the monolayer culture period.

4.3.2.1 Nicotinamide treatment enhanced the production and enrichment of catecholaminergic neurons from mESCs.

In agreement with previous evidence, factor-free neural differentiation (i.e. N2B27 differentiation medium without inductive signalling molecules or exogenous growth factors) of *Sox1*GFP mESCs produced low numbers of catecholaminergic-expressing neurons per culture, as examined by TH immunocytofluorescence.[164] However, 10 mM nicotinamide treatment induced almost a two-fold increase in the percentage of cells double-labelled for βIII-tubulin and TH [ANOVA $F_{(2,8)} = 132.5$; $p<0.001$] [20.0 ± 0.8% vs. 7.5 ± 0.2% in control conditions; Figure 4.3 (A-C)], thereby generating significantly purer TH^+ neuronal populations. Additionally, in cultures exposed to 5 mM nicotinamide, the percentage of catecholaminergic cells per total neurons was also significantly higher [13.9 ± 0.3%; $p<0.001$; Figure 4.3 (C)], albeit this effect was not so marked as for 10 mM nicotinamide.

Consistent with data in Chapter 3, outlining the ability of nicotinamide to promote a concentration-dependent increase in the percentage of βIII-tubulin-expressing

neurons, nicotinamide treatment also elicited a significant increase in the percentage of TH$^+$ immunoreactive neurons of the whole cell population, in a concentration dependent manner, peaking at a dose of 10 mM [ANOVA $F_{(2,11)}$ = 17.8; $p<0.001$]. At 10 mM nicotinamide concentration, the percentage of TH$^+$ neurons per total cells was significantly increased, in comparison to all other treatment groups [$4.2 \pm 0.5\%$ vs. $0.6 \pm 0.3\%$ in controls; Figure 4.3 (A-B) (D)].

These results show that mESCs differentiated in serum-free medium with nicotinamide alone, devoid of other exogenous proteins or signalling molecules associated with their development *in vivo*, showed enhanced catecholaminergic differentiation and more importantly, enrichment for TH-expressing neurons.
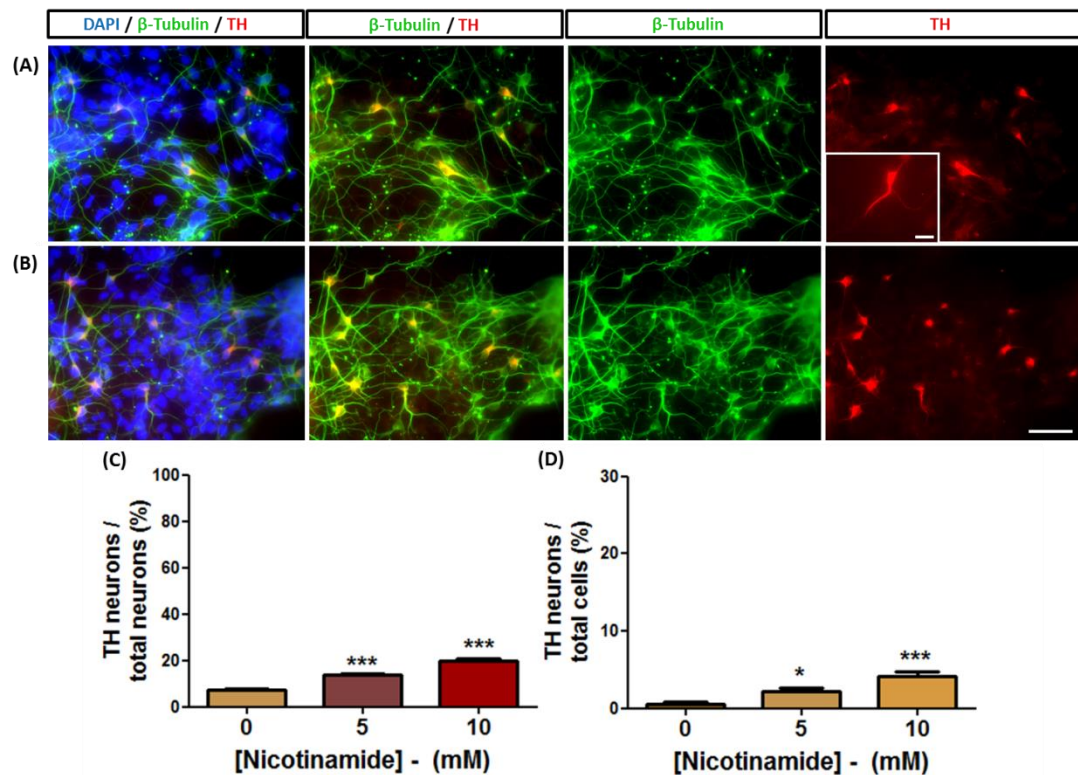
Figure 4.3 Addition of nicotinamide increased and enriched the population of catecholaminergic neurons from mESCs. (A&B) Immunocytochemical analysis of monolayer cultures supplemented with N2B27 medium without nicotinamide **(A)** and with the addition of 10 mM nicotinamide **(B)**. Representative images show immunostaining with antibodies specific to TH (red) and the immature neuronal

marker βIII-tubulin (green). Total cells in cultures counterstained with DAPI (blue). Scale bar = 50 μm.
(C) Bars represent percentage TH immunoreactive neurons as a proportion of total βIII-tubulin-expressing neurons. Monolayer cultures exposed to both 5 mM and 10 mM nicotinamide between days 0 and 7 generated significantly enriched yields of TH neurons at day 14, in a dose-dependent manner. **(D)** Bars represent percentage TH immunoreactive neurons as a proportion of total cells. Differentiating cultures treated with 10 mM nicotinamide show a significant increase in the percentage of TH-positive neurons per culture, compared to untreated conditions. ***$p < 0.001$

To investigate whether nicotinamide influenced another DAergic marker, co-expression of TH⁺ neurons with the DAT was investigated. Immunocytofluorescence analysis showed that TH⁺ neurons co-labelled with DAT were not detected at day 14, in monolayer cultures [Figure 4.4 (A)]. This observation might signify that neurons are still at an early stage of terminal differentiation, since DAT is a well-established late-stage marker of mature DAergic phenotypes[200]. Equivalently, immunofluoresence for the vesicular glutamate transporter protein, VGlut2 expressed in synaptic vesicles in glutamatergic neurons, was not detected in neurons differentiated from mESCs [Figure 4.4 (B)], suggesting that formation of functional synapses is still ongoing at this time point of differentiation in adherent cultures.
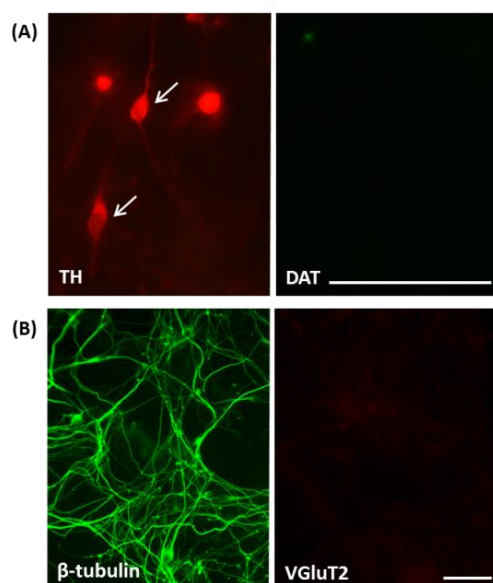
Figure 4.4 (A) Double-staining techniques indicated that DAT (a later marker of mature DAergic neurons) was not co-localised with TH-expressing neurons at day 14 of monolayer differentiation. **(B)** Expression of VGlut2 immunoreactivity, a glutamate transporter found in the membrane of synaptic vesicles was not detected in differentiated neuronal cells. Scale bars = 50 μm

4.3.2.2 Nicotinamide treatment enhanced the production and enrichment of serotonergic neurons from mESCs.

Monolayer cultures treated with early administration of nicotinamide also revealed a concentration-dependent effect on the differentiation of 5-HT-expressing neurons. Cells exposed to 10 mM nicotinamide gave rise to a significantly higher percentage of enriched neurons double-labelled for βIII-tubulin and 5-HT [ANOVA $F_{(2,185)}$ = 11.8; $p<0.001$] [36.1 ± 3.6% vs. 9.2 ± 3.1% in control cultures; Figure 4.5 (A-C)]. In addition, 5 mM nicotinamide significantly enriched the yield of serotonergic neurons expressed as a percentage of all βIII-tubulin$^+$ neurons [28.5 ± 4.3% with 5 mM nicotinamide; $p<0.01$; Figure 4.5 (C)]. Consequently, nicotinamide (5 mM and 10 mM) induced a larger proportion of serotonergic neurons within the total DAPI$^+$ cell population [ANOVA $F_{(2,210)}$ = 14.80] [3.1 ± 0.5% with 5 mM nicotinamide; $p<0.01$; 4.9 ± 0.6% with 10 mM nicotinamide; $p<0.001$ vs. 0.8 ± 0.3% in control groups; Figure 4.5 (D)].

Taken together, these experiments indicate that cultures induced with a period of early exposure to nicotinamide enhanced the yield and purity of TH-positive and 5-HT-positive neurons from mESCs *in vitro*. Therefore, it is reasonable to speculate that nicotinamide treatment in conjunction with appropriate inductive signalling factors during neural specification could be important for directing and optimising yields of stem cell-derived cells adopting a ventral midbrain/hindbrain fate.
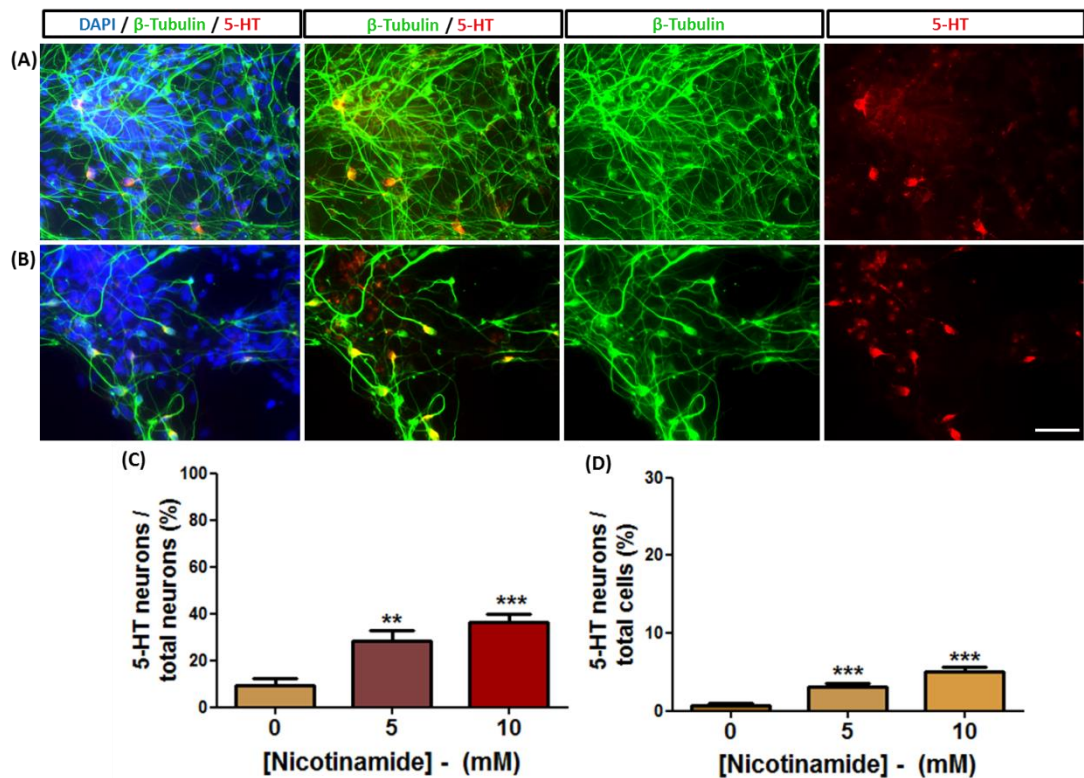
Figure 4.5 Addition of nicotinamide increased and enriched the population of serotonergic neurons from mESCs. (A&B) Immunocytochemical analysis of monolayer cultures supplemented with N2B27 without nicotinamide **(A)** and with the addition of 10 mM nicotinamide **(B)**. Representative images show immunostaining with antibodies specific to serotonin (red) and immature neuronal marker βIII-tubulin (green). Total cells in cultures counterstained with DAPI (blue). Scale bar = 50 μm.

(C) Bars represent percentage serotonergic immunoreactive neurons as a proportion of total βIII-tubulin-expressing neurons. Dose responsive increase in the percentage of serotonergic immunoreactive neurons was obtained with nicotinamide, thus enriching the yield of serotonin-expressing neurons. **(D)** Bars represent percentage serotonergic immunoreactive neurons as a proportion of total cells. The percentage of serotonergic neurons obtained from mESCs increased when nicotinamide was added to media up to a concentration of 10 mM.

***$p<0.001$, **$p<0.01$.

4.3.2.3 Nicotinamide treatment enhanced the production of GABAergic neurons from mESCs.

Double immunofluorescence with anti-GABA and neuron-specific βIII-tubulin demonstrated that the majority of *Sox1*GFP-derived neurons were immunopositive for GABA, in line with other studies using the *Sox1*GFP knock-in 46C cell line[164,172].

Interestingly, in undifferentiated cultures exposed to 5 mM and 10 mM nicotinamide concentrations, the percentage of GABA-expressing neurons co-labelled with βIII-tubulin was not significantly different from levels obtained under control conditions [Figure 4.6 (A-C)]. Therefore, nicotinamide did not enrich the proportion of neurons expressing the inhibitory neurotransmitter, GABA.

However, consistent with the higher proportion of βIII-tubulin neurons observed in nicotinamide groups, 10 mM nicotinamide-stimulated conditions doubled the percentage of GABAergic neurons of the total cell population labelled with DAPI, compared to untreated cells [ANOVA $F_{(2,153)} = 35.4$; $p<0.001$] [22.8 ± 1.2% vs. 10.7 ± 0.8%; Figure 4.6 (A-B) (D)]. No significant differences in the percentage of GABA-expressing neurons were observed between control and 5 mM nicotinamide treatment conditions.
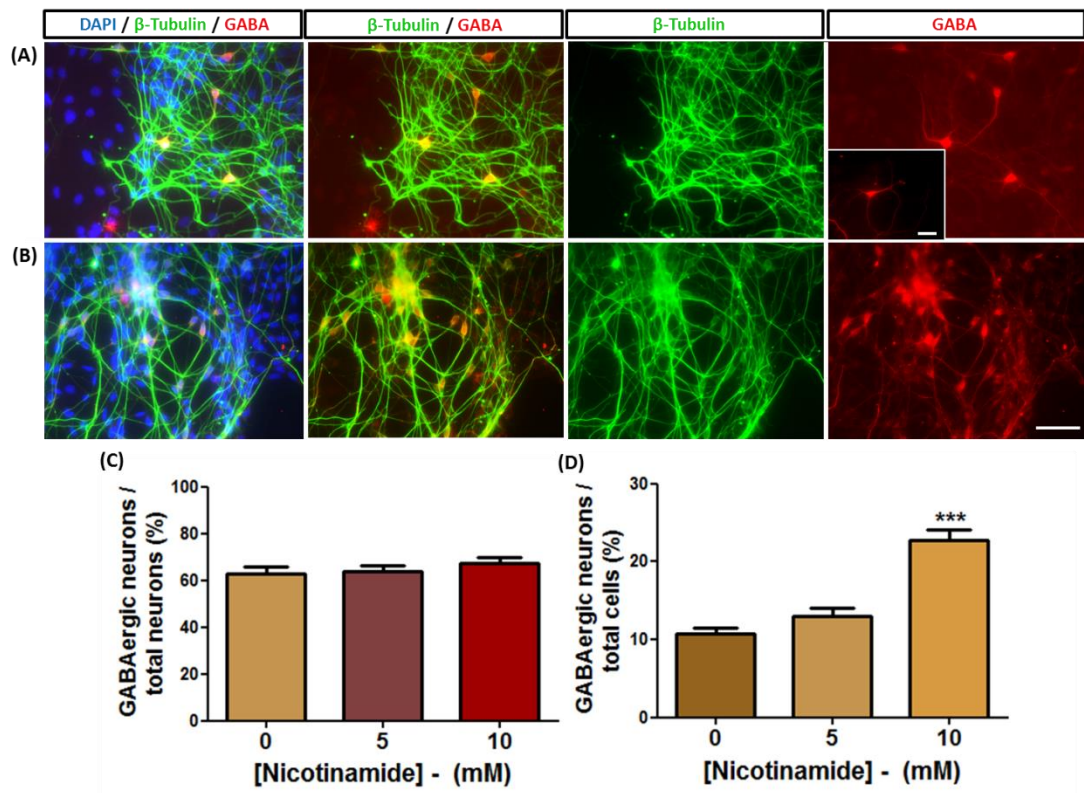
Figure 4.6 Addition of nicotinamide increased the population of GABAergic neurons from mESCs. (A&B) Immunocytochemical analysis of monolayer cultures supplemented with N2B27 without nicotinamide **(A)** and with the addition of 10 mM nicotinamide **(B)**. Representative images show immunostaining with antibodies

specific to the inhibitory neurotransmitter GABA (red) and immature neuronal marker βIII-tubulin (green). Total cells in cultures counterstained with DAPI (blue). Inhibitory GABAergic neurons represented the most prevalent neuronal subtype in *Sox1*GFP mESC-derived monolayer cultures. Scale bar = 50 µm.

(C) Bars represent percentage GABAergic immunoreactive neurons as a proportion of total βIII-tubulin-expressing neurons. Nicotinamide had no effect on this parameter. **(D)** Bars represent percentage GABAergic immunoreactive neurons as a proportion of total cells. Nicotinamide (10 mM) added to media generated a significant increase in the percentage of the total GABAergic cell population, compared against control groups.

***p<0.001

To assess whether nicotinamide influenced the population of mature striatal GABAergic MSNs, cultures were processed immunocytochemically with antibodies against GABA and the striatal specific marker, Dopamine and cAMP-regulated Phosphoprotein (DARPP-32). Immunocytofluorescence analysis showed that mature DARPP-32 expressing GABAergic neurons were undetectable under the differentiation conditions used in this monolayer culture system (Figure 4.7), and which may also further support the hypothesis that 46C-derived neurons are at an early stage of maturation at day 14 of monolayer differentiation.
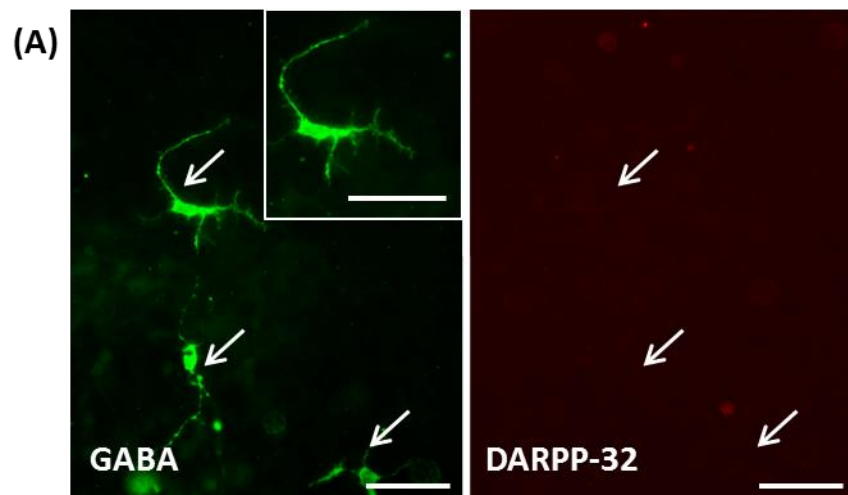
Figure 4.7 Double-staining techniques indicated that DARPP-32 (a marker of striatal projection neurons) was not co-localised with GABA-expressing neurons at day 14 of monolayer differentiation. Scale bar = 50 µm

In summary, these data demonstrate that *Sox1*GFP derived-mESCs differentiated with an early exposure to nicotinamide exhibit higher yields of catecholaminergic, serotonergic and GABAergic neurons per culture. Importantly, differentiated cell populations induced with nicotinamide were selectively enriched for catecholaminergic and serotonergic neurons, but not for GABAergic cells. These important findings suggest that nicotinamide added at appropriate concentrations at the onset of the ESC differentiation process, may function as an important signalling factor to determine neural commitment/subtype specification to certain neurotransmitter phenotypes *in vitro.*

4.3.3 Nicotinamide differentiation accelerates the early stages of neuronal development in catecholaminergic and GABAergic populations.

The effect of the vitamin B_3 metabolite on neurodevelopment and maturation was next investigated in *Sox1*GFP mESC-derived catecholaminergic and GABAergic neuronal populations. Early neuronal development commences with elongation of neurite processes followed by axon differentiation, dendritic branching and synaptic formation[201]. Therefore, the effect of nicotinamide on a series of distinct maturation stages during differentiation was studied to assess primary neurite outgrowth (i.e. length of the longest neurite), total neurite outgrowth and the number of primary neurite branches per neuron.

In addition, similar to GFP immunoreactivity (outlined in Chapter 3, section 3.3.4.4.), catecholaminergic and GABAergic neurons exhibit variations in the intensity of TH and GABA expression respectively, resulting in a spectrum from weak to strong fluorescence. It was hypothesised that cells expressing higher levels

of immunostaining represented more mature neuronal subtypes, since strong staining reflects more neurotransmitter content. Therefore, to investigate the effect of nicotinamide on weak and intense-expressing neuronal subtypes, individual cell bodies were scored for levels of protein using FI measures, described previously in Chapter 2 (section 2.1.8.1).   Briefly, GABA and TH-expressing cells were categorised as 'weak' (FI ≤ 0.1 units) and 'strong' (FI ≥ 0.2 units).

4.3.31 Nicotinamide promotes various aspects of TH+ neuronal morphology

Image analysis indicated that for TH+ cells, the average primary neurite length was significantly higher in 10 mM nicotinamide conditions than those of control and 5 mM nicotinamide conditions [123.6μm ± 6.5 for 10 mM groups; $p<0.001$; 97.9μm ± 7.0 for 5 mM groups; n.s., vs. 74.5μm ± 6.1 in controls; Figure 4.8 (A-B) (D)].

Further, binning the data into the proportion of TH+ neurons with short (≤ 60μm), medium (60-120μm) and long (≥ 120μm) primary neurite processes, confirmed that 10 mM nicotinamide treatment increased neurite extensions in cells derived from *Sox1*GFP mESCs. Specifically, the proportion of 46C-derived TH+ cells displaying "long" primary neurite outgrowths was significantly increased in cultures exposed to 10 mM nicotinamide only [0.4 ± 0.1 vs. 0.1 ± 0.1 in control conditions; $p<0.05$; Figure 4.8 (E)], concomitant with a significant decrease in the proportion of "shorter" TH+ primary neurites [0.1 ± 0.0 vs. 0.4 ± 0.0 in control groups; $p<0.05$; Figure 4.8 (E)].

Monolayer cultures treated with 10 mM nicotinamide also resulted in TH+ neurons with significantly increased total neurite extent [197.9μm ± 11.5 vs. 118.8μm ± 7.0 in controls groups; $p<0.05$; Figure 4.8 (F)]. Although the numbers of neurites per neuron were not changed [Figure 4.8 (G)], nicotinamide promotes neurite elongation

in TH^{+} cells, thus suggesting an important role at the early maturation stages of neuronal development.

In agreement with the rapid maturation effect elicited by 10 mM nicotinamide, a substantial increase in the proportion of catecholaminergic cells displaying more neurotransmitter content was observed. Cultures treated with 10 mM nicotinamide revealed an upward trend in the percentage of neurons per culture displaying "strong" TH immunofluorescence [0.9 ± 0.0 vs. 0.4 ± 0.2 in untreated conditions; Figure 4.9 (C) (H)], concomitant with a marked downward trend in the percentage of "weak" TH-expressing cells in differentiated cultures (0.1 ± 0.0 vs. 0.6 ± 0.2 in controls). Therefore, following brief exposure to nicotinamide between day 0 and 7, mESC-derived TH^{+} neurons appeared to more mature since they possessed extended primary neurite outgrowths and increased total neurites, correlating with a pronounced upward trend in the percentage of TH^{+} neurons expressing intense immunoreactivity.
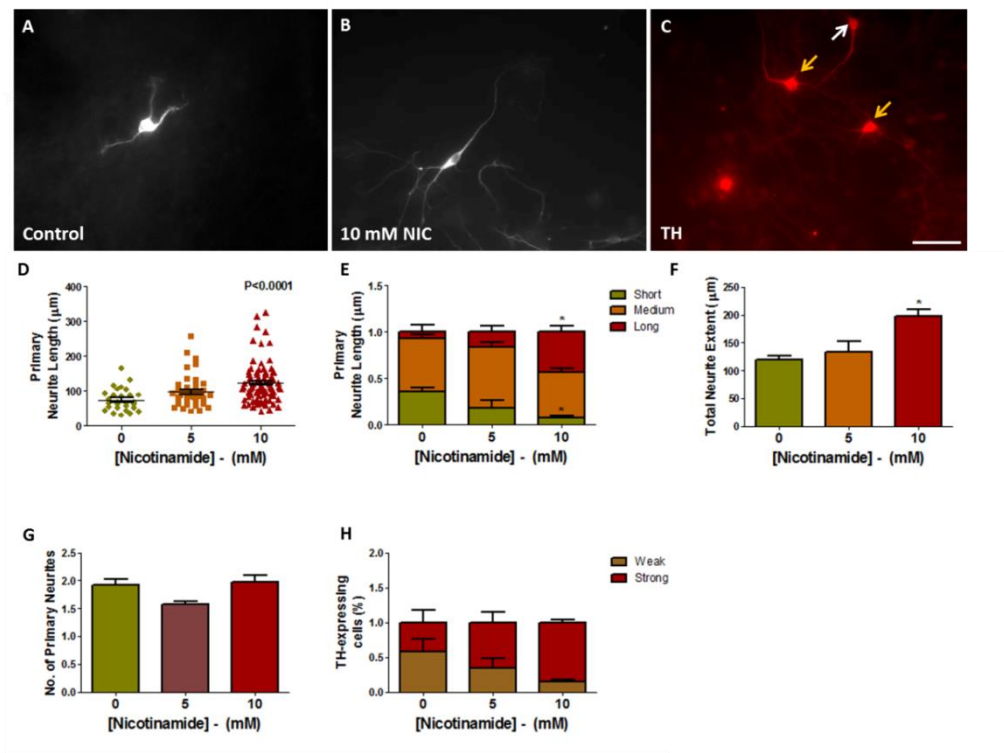
Figure 4.8 Nicotinamide enhanced neuronal maturation in DAergic populations. (A-B) Representative images of TH$^+$ neurons in **(A)** monolayer control conditions and **(B)** cultures with 10 mM nicotinamide. Morphometric analyses were performed on fluorescent images inverted to black and white for clarity. **(C)** The levels of protein expression in TH-expressing neuronal cell bodies were assessed to establish the degree of maturation of neurons with and without nicotinamide treatment. Some cells showed intense TH immunoreactivity (yellow arrows) and some less strong TH expression (white arrow). Scale bars = 50 μm. **(D)** The scatter graph depicts individual readings for primary neurite length in TH-expressing cells at day 14. Average neurite length was significantly increased in 10 mM nicotinamide-treated cultures, compared to all other conditions. **(E)** Bar chart showing the proportion of cells with short (green bars), medium (orange bars) and long (red bars) values of primary neurite outgrowth (μm). The proportion of 46C-derived cells displaying "short" primary processes was significantly decreased in cultures exposed to 10 mM nicotinamide between days 0 and 7, concomitant with an increase in "longer" primary neurites, compared with controls. **(F)** In addition to increased primary neurite extension in TH-expressing cells, increases in total neuritic extent (μm) were highly significant in 10 mM nicotinamide cultures versus controls. **(G)** The number of primary neurites per neurons was not significantly different across all conditions. **(H)** Bar chart showing the proportion of cells with weak (brown bars) and strong (red bars) levels of TH expression (arbitrary units), indicative of neurotransmitter content within cell soma. A marked upward trend showing the proportion of 46C-derived cells displaying "strong" levels of TH expression was demonstrated in cultures exposed to 10 mM nicotinamide, concomitant with a downward trend in "weaker"-expressing TH$^+$ cells, compared with controls and 5 mM nicotinamide conditions.

*$p<0.05$

4.3.3.2 Nicotinamide promotes various aspects of GABAergic neuronal morphology.

Similar to morphometric analysis performed in TH-expressing neuronal populations, several types of GABAergic neuronal morphologies were also observed in monolayer cultures at day 14 of differentiation, i.e. GABAergic neurons with short, medium and long neurite processes, and these neurons exhibited differences in fluorescence intensities.

Data for mean primary neurite outgrowth values highlighted a significant increase for neurite length in postmitotic GABA neurons treated with 10 mM nicotinamide [116.2μm ± 6.2 vs. 80.9μm ± 4.4 in controls; $p<0.001$; Figure 4.9 (A-B) (D)]. Similarly, 5 mM nicotinamide treatment promoted a significant increase in neurite length [102.1μm ± 5.1; $p<0.05$; Figure 4.9 (D)].

Specifically, in 10 mM nicotinamide conditions, an upward trend in the proportion of cells exhibiting "medium" (51.5 ± 1.3 vs. 47.8 ± 4.6 in controls; n.s.) and "long" (34.5 ± 2.9 vs. 15.2 ± 1.2 in controls; n.s.) neurite outgrowths were shown, subsequently resulting in a significant reduction of the proportion of GABAergic neurons displaying "short" primary neurite branches [14.0 ± 2.4 vs. 36.9 ± 4.2 in control conditions; $p<0.001$; Figure 4.9 (E)]. Similarly, cells exposed to 5 mM nicotinamide revealed a comparable trend, resulting in a significant reduction in the proportion of neurons displaying "shorter" neurite lengths compared to untreated cultures [19.9 ± 0.4 vs. 36.9 ± 4.2 in control conditions; $p<0.05$.; Figure 4.9 (E)].

Further, total neurite extent was significantly increased in 10 mM nicotinamide cultures, compared to controls [146.9μm ± 6.7 vs. 104.3μm ± 5.1 in control groups; $p<0.001$; Figure 4.9 (F)]. Nicotinamide treatment induced no significant changes in the number of primary neurites per neuron, compared to untreated conditions [Figure 4.9 (G)].

In cultures treated with 10 mM nicotinamide, enhanced maturation of GABAergic neurons at the early stages of neurodevelopment was further evidenced by elevated levels of neurotransmitter content within neuronal cell bodies. Binning analysis showing the percentage of 46C-derived neurons exhibiting "weak" and "strong" levels of GABA immunoreactivity indicated an upward trend in the percentage of

neurons expressing intense GABAergic staining, observed in cultures differentiated with 10 mM nicotinamide [0.6 ± 0.0 vs. 0.5 ± 0.1 in untreated conditions; Figure 4.9 (C) (H)]. Correspondingly, the percentage of weak-expressing GABAergic neurons was reduced in 10 mM nicotinamide-treated cultures [0.4 ± 0.0 vs. 0.5 ± 0.1 in control conditions; Figure 4.9 (C) (H)].
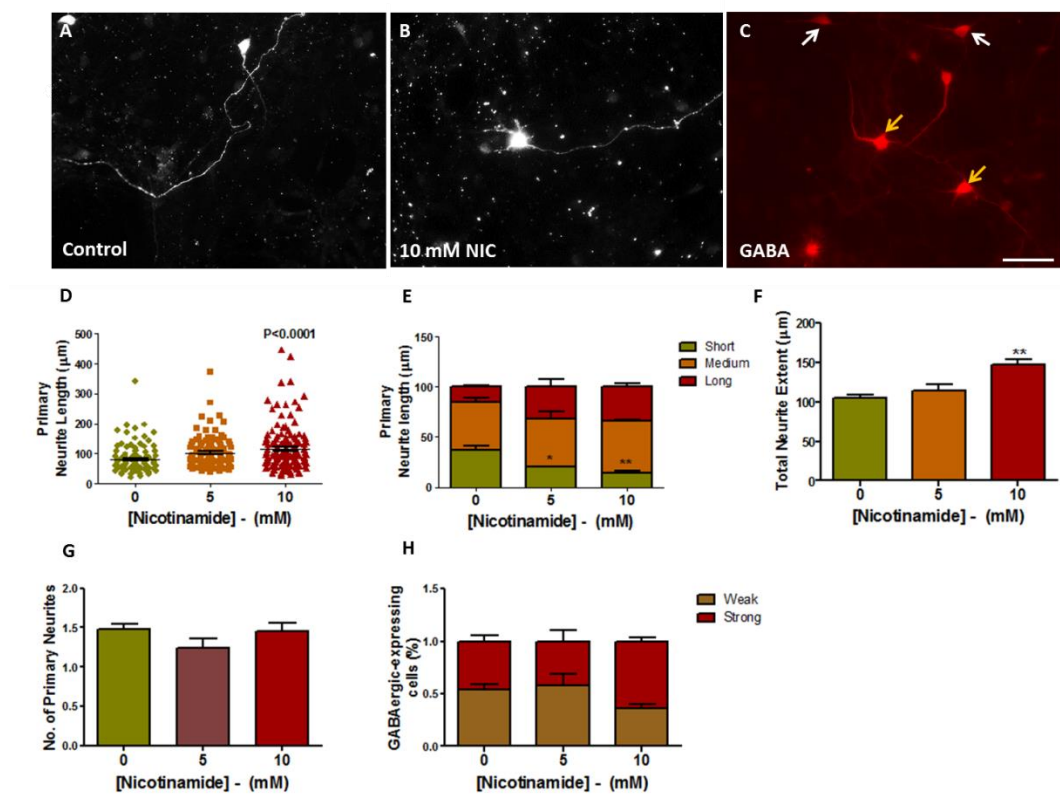
Figure 4.9   Nicotinamide enhanced neuronal maturation in GABAergic populations. (A-B) Representative images of GABAergic neurons in **(A)** monolayer control conditions and **(B)** cultures with 10 mM nicotinamide. **(C)** Some cells showed intense GABA immunoreactivity (yellow arrows) and some less strong GABA expression (white arrows). Scale bars = 50 μm. **(D)** The scatter graph depicts individual readings of primary neurite length (μm) in GABA-expressing cells at day 14. Average primary neurite length was significantly increased in 5 mM and 10 mM nicotinamide-treated cultures, compared to control conditions. **(E)** Bar chart showing the proportion of cells with short (green bars), medium (orange bars) and long (red bars) values of primary neurite outgrowth (μm). The proportion of 46C-derived cells

displaying "short" primary processes was significantly decreased in cultures exposed to 5 mM and 10 mM nicotinamide between days 0 and 7, concomitant with an obvious increased trend in "longer" neuritic processes, compared with controls. **(F)** Greater neurite extents were shown in 10 mM nicotinamide cultures versus controls. **(G)** There was no difference in the number of primary neurites per neurons between controls and nicotinamide-stimulated conditions. **(H)** Bar chart showing the percentage of cells with weak (brown bars) and strong (red bars) levels of GABA immunoreactivity(arbitrary units), correlating to the levels of neurotransmitter content within cell soma. An upward trend showing the proportion of 46C-derived cells displaying "strong" levels of GABA expression was demonstrated in cultures exposed to 10 mM nicotinamide, which correlated with a downward trend in "weaker"-expressing GABAergic cells, compared with controls and 5 mM nicotinamide conditions.

***p<0.001, **p<0.01, *p<0.05

In summary, differentiation with nicotinamide (10 mM) rapidly enhanced early morphological features in TH^+ and $GABAergic^+$ cells. These data suggest that nicotinamide may exert a neurite promoting action on young neuronal subtypes. Moreover, upregulated levels of the expression of selected neuronal subtype proteins further confirmed the accelerative effect of nicotinamide on neuron maturation. These findings strongly support previous data outlined in Chapter 3, highlighting the importance of nicotinamide-promoted differentiation to rapidly induce the loss of stem cell identity, accelerate neural fate and in turn accelerate neuronal maturation during early development.

4.4 Discussion

Findings demonstrated in this experimental chapter show that the biologically active vitamin B_3 metabolite, nicotinamide, not only functions as a neuroprotective agent in several neurodegenerative diseases, as reported in the literature but also has an important role to play during cell differentiation. The ESC monolayer model system was chosen for this study since DAergic, serotonergic and GABAergic neurons can

be efficiently obtained from *Sox1*GFP[+] populations[172,202], thus making it an ideal system to fully evaluate the effects of nicotinamide on neuronal subtype lineage analysis and neurodifferentiation. In order to address the question whether nicotinamide stimulates DAergic, serotonergic and GABAergic differentiation, mESCs carrying a neuroepithelial specific vital reporter (*Sox1*GFP knock-in 46C cell line) were exploited in combination with N2B27 neural differentiation medium, independent of exogenous and inductive signalling molecules and without the requirement of feeder cells.

In this study, a novel function for nicotinamide during neural subtype differentiation was shown: namely altering the proportion of neurons expressing specific phenotypic properties in a concentration-dependent manner, thus generating selectively enhanced and/or purer neuronal subtype yields pertinent to a range of BG-associated disorders. In addition, its function was also associated with enhanced neurite elongation and increased levels of neurotransmitter content within the cell bodies of distinct neuronal subpopulations, therefore indicative of an accelerative effect on the maturation of *Sox1*GFP-derived neurons. These important findings provide crucial evidence that under serum-free and factor-free differentiation conditions, enhanced neural subtype commitment and neuronal morphogenesis were predominantly due to the independent effect of nicotinamide, applied during distinct stages of SC neural fate (i.e. day 0-7).

4.4.1 Nicotinamide promotes neural phenotype differentiation in the absence of exogenous inductive molecules from ESCs.

With relevance for future treatment of PD, ongoing research into stem cell sources such as ESCs, adult stem cells and iPSCs are being considered for DA neuronal

replacement therapies[74]. However since the most advanced work to date is being undertaken with ESCs[78,79,155], this chapter investigated whether nicotinamide may be a useful signalling molecule to generate DAergic neurons from mESCs *in vitro*. To my knowledge, this current experimental study presents the first report investigating the effect of nicotinamide on the production of catecholaminergic cells in a monolayer differentiation paradigm, and showed that administration of this compound at the initial stages of ESC differentiation enhances the yield of TH-expressing neurons.

In control cultures, no appreciable numbers of TH^+ cells were observed, which is also in line with other studies where few DAergic cells are generated in the absence of the ventralising signals; Shh and FGF8[199]. However, 10 mM nicotinamide treatment directly increased the percentage of TH^+ neurons of the total cell population. In addition, nicotinamide increased the percentage of TH^+ neurons per total neurons, producing purer DAergic neuronal yields and highlighting the relevance of nicotinamide for catecholaminergic differentiation. A study conducted under similar monolayer differentiation conditions (a total of 14 days of differentiation) reported that the number of TH^+ neurons (expressed as a percentage of the total $\beta III\text{-tubulin}^+$ population) derived from *Sox1*GFP$^+$ populations increased when Shh and FGF8 were applied at peak neurogenesis (i.e. *Sox1*$^+$ NPC production), corresponding to ~28% of the percentage of TH-expressing neurons versus ~9% in control cultures (comparable to $7.5 \pm 0.2\%$ in control conditions reported in this experiment)[178]. These important observations suggest that nicotinamide supplementation alone during ESC differentiation displayed almost a similar potency to these well-established inductive signalling factors, thus suggesting a strong

applicative potential for nicotinamide in current restorative cell-based protocols for PD.

The effect of nicotinamide was also focused on 5-HT differentiation from mESCs *in vitro*. The 5-HT-synthesising neurons represent another ventral cell fate generated *in vivo*, originating from the anterior and posterior of the isthmic organiser, which provides a source of signalling factors regulating midbrain and hindbrain patterning[83]. Therefore, methods developed for generating DAergic neurons from stem cell sources share similar differentiation pathways with serotonergic neurons *in vivo*[203]. Interestingly, in addition to enhanced and enriched DAergic differentiation with nicotinamide, comparable effects were also observed with respect to serotonergic differentiation from the 46C *Sox1*GFP cell line, suggesting that nicotinamide is also important for directing serotonergic neuronal fates. Specifically, treatment of monolayer cultures with 10 mM nicotinamide in particular had a distinct activity in promoting differentiation of ESCs into significantly higher and purer serotonergic neuronal yields. In light of these findings, future work should investigate the effect of nicotinamide with Shh, FGF8 and noggin (a known agonist of BMP), shown to also be important during hindbrain serotonergic neuronal differentiation[185,203], which may further increase and enrich yields of serotonergic neurons in ESC culture systems, to open new therapeutic perspectives for the treatment of neuropsychiatric disorders. With relevance to serotonin and PD, postmortem studies show that the pathology in PD extends past the striatal region[204] and the DAergic system[205]. Specifically, PD animal model studies highlighted a link between dysfunction of the norepinephrine and serotonergic neuronal systems with the occurrence of non-motor symptoms[206,207], which was further confirmed by several positron emission tomography reports in PD patients[208,209]. In this context,

Politis *et al* recently reported a major loss of 5-HT expressing neurons in PD patients, particularly in raphe nuclei and regions receiving serotonergic projections involved in the regulation of functions such as sleep, mood and emotion.[205] Interestingly, this phenomenon was observed in patients who had previously been treated with DAergic fVM tissue grafts 13-16 years earlier. Politis and colleagues therefore propose that additional grafts of 5-HT expressing neurons may be required in future PD cell-based therapies to ameliorate non-motor symptoms by restoring serotonergic neurotransmission in affected cerebral targets[205]. In this context, nicotinamide may prove beneficial in future protocols for producing high and enriched yields of 5-HT neurons from stem cells *in vitro*.

MSN GABAergic neuronal differentiation derived from pluripotent sources such as hESCs or hiPSCs has been explored, however the differentiation efficiency was low for striatal GABAergic neurons[210,211]. In this study, under control conditions, the vast majority of neuronal cells produced by monolayer culture were $GABA^+$, reflecting similar findings in other studies using *Sox1*-GFP-derived cultures[164,172,212]. Research conducted by Chung *et al* showed that FACS-purified GFP^+-derived GABAergic neurons represented $51.1 \pm 4.9\%$ of the total neuronal population at day 10 of differentiation; Shin and colleagues showed that the percentage of GABAergic neurons obtained under physiological oxygen levels at day 17 of monolayer differentiation corresponded to 70-75 % of the βIII-tubulin population[213], in line with the cell-counting analysis performed in this experiment after 14 days of monolayer culture. Results reveal that nicotinamide (10 mM) treatment of mESCs could stimulate a significant increase in the production of GABAergic neurons of the total cell population; however this compound did not selectively enrich the proportion of GABA-expressing neurons generated from *Sox1*GFP^+ cells. Nevertheless, this does

not negate the potential of nicotinamide as a useful candidate in future protocols for accelerating maturation in GABAergic populations.

GABA^{+} neurons were checked for striatal lineage by immunostaining with DARPP-32, which is expressed in all MSN neurons in the striatum[214]. Expression of DARPP-32 was not detected in terminally differentiated cultures at day 14, however, most studies report that stem cell-derived cultures generate 10-20 % DARPP-32^{+} MSNs[215]. Thus, to fully determine the effects of nicotinamide on MSN-differentiation, future work should focus on the effects of nicotinamide in conjunction with ventralising factors using Shh or an agonist, purmorphamine, which are necessary to promote the emergence of a forebrain phenotype from ESCs[215], or indeed with RA which has been shown to function a key GABAergic differentiation signal in the BG circuitry[191].

Finally, the effect of nicotinamide was also investigated on glutamatergic differentiation. Challenges arise in glutamate immunocytochemistry due to the difficulty involved in accurately distinguishing immunocytochemical staining, resulting from metabolic glutamate, from labelling caused by transmitter glutamate[216]. Therefore, with respect to experimental design in this study, an anti-glutamate antibody was not selected, which was previously employed in the report conducted by Chung and colleagues[172]. Instead, βIII-tubulin^{+} neurons were analysed by immunocytochemistry for co-expression with the glutamate transporter marker, VGlut2. Glutamatergic neurons were not detected in this experimental system at day 14 leading to the hypothesis that monolayer-derived cells are still at the early phases of termination undergoing the formation of functional synapses, since VGlut2 is expressed in the membrane of synaptic vesicles. Collectively, the absence of VGlut2

and DAT under the differentiation conditions established in this study are consistent with similar observations in *Sox1*-GFP 46C cultures[170].

4.4.2 Nicotinamide accelerates the formation of neuronal processes.

Several reports support a role for nicotinamide in cellular maturation and differentiation processes[141,198,217]. Therefore, the effects of nicotinamide on cell morphology in TH-expressing and GABAergic-expressing populations were next addressed. During neurogenesis, following cell-cycle exit, immature neurons initiate the formation and elongation of neurites, which ultimately develop into axons and dendrites. In this context, this study reports a positive role for nicotinamide at the initial phases of neuronal maturation in young subpopulations. Specifically, increased primary neurite outgrowth and total neurite output were observed in GABAergic and DAergic neuronal populations. Thus, *Sox1*GFP-derived neurons differentiated with nicotinamide between days 0 and 7 undergo neuronal morphogenesis earlier than cells induced with N2B27 medium alone. These findings are consistent with the faster differentiation of ESCs, earlier emergence of *Sox1*GFP$^+$ NPCs and, as a consequence, βIII-tubulin$^+$ neuronal cells in nicotinamide-treated monolayer cultures; findings outlined in Chapter 3. This rapid maturing effect elicited by the vitamin B$_3$ metabolite was further confirmed by stronger GABA and TH immunofluorescence, indicative of increased levels of neurotransmitter content within cell bodies. These data obtained from both transmitter phenotypic populations signify an important role for nicotinamide at the initial stages of neuronal maturation.

4.4.3 Conclusion and Further Studies

Nicotinamide selectively enhanced and enriched the production of catecholaminergic and serotonergic neurons and, moreover, enhanced various aspects of neuronal morphology in catecholaminergic and GABAergic populations. In accordance with findings in Chapter 3, these data suggest that nicotinamide may function as a critical instructive molecule at the initial stages of embryonic stem cell differentiation, to rapidly and efficiently direct lineage specific differentiation /neural commitment to highly enriched neuronal lineages by eliminating non-neural cells.

With respect to PD, it is important to point out that the majority of TH$^+$-expressing DA neurons generated under these monolayer differentiation conditions in the absence of patterning factors are heterogeneous with regard to regional identities and may not represent midbrain specific DAergic phenotypes[178], since their differentiation is dependent on Shh signalling[218]. Considering this, in future work, it will be of interest to investigate the combined effect of nicotinamide with the sequential treatment of factors functioning during normal development to promote midbrain specific DAergic neurons. In addition, to investigate the effects of nicotinamide on glutamatergic differentiation, it will be necessary to differentiate neurons for a longer culture period and then investigate whether nicotinamide influences glutamatergic differentiation at a later time point of the monolayer protocol. To determine an appropriate later time point, the extent of differentiation could be checked for the presence of the synaptic proteins Syn1 and PSD 95, which are pre- and post-synaptic markers respectively. Their expression is localised in a "punctate pattern" along neurites, similar to the pattern of expression for VGlut2[170].

Future work will also be required to elucidate the mechanisms underlying the promotion of accelerated neuronal maturation, which would lead to a greater understanding of neuronal differentiation. A possible explanation for this additional effect of nicotinamide on enhanced neuronal development may be that this signalling molecule directly induces genes linked to neuronal maturation or with the mature state.

The next chapter will focus on elucidating the mechanism(s) mediating neural specification by nicotinamide - that is, induction of cell-cycle exit and/or selective apoptosis in non-neural populations, and will also address the possible role of sirtuins (silent information regulator 2 (Sir2) family of proteins) during mESC differentiation in the *Sox1*GFP model system.

Chapter 5: Investigation of the Mechanism(s) Underlying the Enrichment and Enhancement of Neuronal Differentiation by Nicotinamide.

5.1 Introduction

A major challenge for advancement of clinical neuronal replacement therapies is the production of high yields of purified neuronal populations of appropriate phenotype with control over proliferation to prevent tumourigenesis. Previous findings demonstrated that treatment of mESC (46C *Sox1*GFP reporter cell line) monolayer cultures with the vitamin B$_3$ metabolite, nicotinamide, at the early onset of development decreased the total number of cells in cultures, resulting in a greater ratio of neurons to non-neuronal cells. Specifically, by day 14, nicotinamide treatment (from day 0-7) reduced the populations of undifferentiated cells and *Sox1*GFP$^+$ progenitors, in addition to reducing Oct4 and GFP expression in the cultures concomitant with enhanced expression of βIII-tubulin, indicative of accelerated neuronal differentiation. Thus, this marked reduction in distinct cell populations forms a mutually supportive body of data compatible with the previous data showing reduction of the total cell population at this time point. The cellular mechanisms of nicotinamide in this regard are currently unknown. Therefore, the hypotheses under investigation in this study were to determine whether nicotinamide promoted inhibition of proliferation, and/or whether nicotinamide induced cell death in specific cell populations.

Further, nicotinamide has been extensively characterised as a potent inhibitor of sirtuins, which were identified as NAD+-dependent Class III histone deacetylases. Sirtuin proteins are known to play important roles in cellular processes including aging, inflammation, and stress resistance[219]. Specifically, the cellular [NAD$^+$]/[NADH] ratio controls the deacetylase activity of sirtuins, where NAD$^+$ functions as an activator and nicotinamide and NADH work as inhibitors[220].

There are seven known sirtuins (SIRT1-7) in mammals which are linked with varied subcellular localisation and enzymatic activity. The most extensively studied sirtuin, SIRT1, deacetylates a wide range of histones and non-histone proteins[221], and is ubiquitously expressed throughout the brain[222]. Initially SIRT1 was described as a nuclear protein shown to shuttle to the cytoplasm during neuronal differentiation and neurite outgrowth[219,223]. SIRT2 and SIRT3 exhibit NAD-dependent deacetylase activity[219]. SIRT2 represents a cytoplasmic protein known to deacetylate tubulin, and has also been described in the nucleus during cell-cycle progression[219]. SIRT3, SIRT4 and SIRT5 represent mitochondrial sirtuin proteins; however they are each implicated in different enzymatic activities. SIRT4 functions as an ADP-ribosyltransferase, whereas SIRT5 is involved in distinct activities such as NAD-dependent demalonylase, desuccincylase, and deacetylase activities[219]. SIRT6 displays deacetylase and ADP-ribosyltransferase activities and is associated with chromatin in the nucleus[219]. SIRT7 is a NAD-dependent deacetylase which functions as a nucleolar protein[219].

Interestingly, SIRT1 plays an important role in mESC differentiation[224], particularly during differentiation into neural progenitors[223,225]. In this regard, there is strong evidence linking SIRT1 with a cell-fate decision switch in progenitor cells towards neurons or astrocytes [Figure 5.1 (A-C)]. Inhibition of SIRT1 has been shown to determine neural cell fate by manipulating the differentiation of neural precursor cells towards a neuronal lineage, at the expense of a glial fate[225]. Since the 46C *Sox1*GFP cell line has also been reported to generate glial cell types[169,170], this study sought to determine whether this mechanism is operative, i.e. does the SIRT1 inhibitor, nicotinamide, reduce glial commitment at the expense of neurons in the monolayer protocol?

SIRT1 has also been shown to be important for DA neuronal differentiation, and its spatial regulation may prove crucial in TH gene regulation[226]. In this context, during neurogenesis, SIRT1 undergoes a transient nuclear translocation to promote neuronal differentiation [Figure 5.1(A)][223]. Given that nicotinamide promoted the differentiation of TH^{+} neurons in Chapter 4 (section 4.3.2.1), immunocytochemistry was used to assess the localisation of SIRT1 within distinct neural populations across a spectrum of developmental stages. Furthermore, a reduction in SIRT1 expression is conducive to neural fate determination and induction of neuronal differentiation[227]. Therefore, to investigate whether SIRT1 is down-regulated during differentiation, the expression of SIRT1 at the protein level was assessed at different stages of the monolayer protocol.

To thoroughly investigate these hypotheses, experiments were designed to assess the influence of nicotinamide on cell viability, apoptosis, proliferation and SIRT1 expression during monolayer differentiation. This experimental chapter aimed to:

I. *Measure the effect of nicotinamide on cell viability in differentiating monolayer cultures.*

II. *Investigate the causes of the cell number reduction in nicotinamide-treated cultures – e.g., induction of cell-cycle exit and/or induction of cell death in specific cell populations.*

III. *Perform a preliminary study to evaluate SIRT1 expression during mESC differentiation in the Sox1GFP model system.*

Figure 5.1 The role of SIRT1 during neurogenesis. (A) It has been reported that SIRT1 is predominantly localised in the cytoplasm in NPCs. Under differentiation conditions, SIRT1 translocates to the nucleus to interact with the nuclear receptor co-repressor (N-CoR). This complex (i.e. N-CoR/SIRT1 complex) binds to the *Hes1* gene (downstream target of Notch, which promotes neural lineage entry[149]), thereby directing a switch in NPCs towards neuronal differentiation. Representative image shows immunostaining with antibodies specific to SIRT1 (red) and the neuronal marker βIII-tubulin (green). SIRT1 staining is shown to be primarily nuclear within the soma, under differentiation conditions (DIV 7). **(B)** Inhibition of SIRT1 promotes neural progenitors toward neuron differentiation. Under reducing conditions, the transcription factor *Hes1* recruits the CREB binding protein (CBP), which activates *Mash1* transcription (implicated during neuronal differentiation) and directs NPCs to assume a neuronal fate. Representative image show immunostaining with antibodies specific to SIRT1 (red) and the neuronal marker βIII-tubulin (green). **(C)** In an oxidising environment, SIRT1 binds with Hes1 on the *Mash1* promoter, resulting in histone deacetylation. The transcription of *Mash1* is repressed, thereby directing NPC differentiation in favour of astrogenesis. Representative image shows immunolabeling with anti-glial fibrillary acidic protein (GFAP; a principal

intermediate filament protein of astrocytes). Diagram adapted from Rodriguez *et al*[228]. **(A-C)** Scale bars = 50 µm.

5.2 Experimental Procedure

Cells were treated for 7 days with 10 mM nicotinamide, and then assays performed at 7 and 14 days, which consisted of: viability, apoptosis, proliferation and SIRT1 expression.

5.2.1 Cell culture and nicotinamide treatment

*Sox1*GFP reporter mESCs were cultured in serum-free medium supplemented with N2 and B27, as previously described.

5.2.2 Immunocytochemistry

Cell immunofluorescence was carried out in accordance with the protocol outlined in section 2.1.5.1. In this experimental chapter, the antibodies used for immunocytochemistry were against Oct4, βIII-tubulin, GFAP, NG2 [Neuron-glial antigen 2; chondroitin sulphate proteoglycan 4; a chondroitin sulfate proteoglycan surface marker of oligodendroglial progenitor cells (OPCs)] and SIRT1. In addition, fluorescence detection of native GFP expression was used to identify the *Sox1* expressing cells/progenitor cells in adherent monolayer cultures.

5.2.3 Cell viability assay

The viability of 46C-derived cells was measured using the Countess[TM] Automated Cell Counter, which was based on the trypan blue exclusion method to distinguish viable from nonviable cells. Viable and non-viable cell counts were counted using the automated cell counting system, following manufacturer guidelines. Three counts per sample were averaged to obtain one value per replicate (i.e. n = 3). Cell viability

was calculated as follows: number of viable cells/total number of cells (viable + non-viable). Detailed protocols are provided in the Material and Methods chapter.

5.2.4 Apoptotic assay

The effect of nicotinamide on apoptosis was investigated at day 7 and day 14 in adherent *Sox1*GFP-derived cultures. Apoptosis was determined using an *in situ* TUNEL assay, to preferentially label DNA strand breaks generated during apoptosis. This method was combined with examination of cell nuclei integrity, and microscopy assessments of cellular morphology were performed using fluorescence and phase contrast microscopy. Thus, cells undergoing apoptosis were quantified only when they stained positively in the TUNEL assay and exhibited a pyknotic nucleus (caused by chromatin condensation). Healthy and apoptotic cells were plotted as a percentage of the total numbers of DAPI-stained nuclei per microscopic field. A detailed protocol for this experiment is provided in section 2.1.7.

5.2.5 Cell proliferation assays

The goal of this study was to assess the influence of nicotinamide on cell proliferation, by utilising an EdU cell proliferation assay approach *in situ* in *Sox1*GFP-dervied cultures. To investigate whether nicotinamide elicited an effect on the proliferation of undifferentiated mESCs, progenitor cells and neurons, the number of cells that incorporated EdU following exposure to nicotinamide (from day 0-7) within these distinct cell populations was calculated at days 7 and 14. This was achieved using double-staining techniques to identify EdU/Oct4 and EdU/βIII-tubulin double-labelling in control and treated cultures. Co-labelling of EdU with native *Sox1*GFP-expression was used to identify progenitor cells which had undergone mitosis. To test for differences in proliferation between the two

conditions, the percentage of proliferating cells was determined by calculating the number of proliferating cells/total cells and the number of proliferating cells/specific cell types. A detailed protocol for this EdU incorporation study is provided in section 2.1.6.

5.2.6 Image Analysis

All cell counts were produced from a minimum of six random fields per coverslip. Cell clusters containing mixed populations of neural progenitor, neuronal and glial cells were excluded from data analyses, since individual cells within these dense networks could not be reliably identified.

5.2.7 Statistical Analysis

In each experiment, data from three coverslips per culture were averaged, with all experiments bring repeated 3-5 times (i.e. n = 3–5 independent experiments). Data replicates were analysed using an unpaired Student t test. Results were considered statistically significant for p values less than 0.05. Data are expressed as mean ± SEM.

5.3 Results

5.3.1 Early nicotinamide treatment reduced total live cell numbers, but did not affect cell viability in monolayer cultures.

In section 3.3.2, manual quantification of DAPI-stained nuclei at day 14 showed that 10 mM nicotinamide during the first 7 days of differentiation reduced cell number. In the present study, a Countess™ automated cell counter was used to validate this analysis by measuring the number of viable cells in control and nicotinamide-treated cultures at 7 and 14 days respectively. Further, to rule out the possibility that

widespread cell death underlies the reduction in cell number; the influence of nicotinamide (10 mM) on cell viability in differentiating monolayer cultures was assessed at different developmental stages. In the present study, viability was assessed in terms of both total and percentage viable cells in 0.5 ml N2B27 media.

Administration of 10 mM nicotinamide from day 0 to 7 of monolayer differentiation generated a significant reduction in the total number of live cells by day 7, in contrast to control conditions (unpaired t test, t = 3.6) [433,333 ± 350,000 vs. 2800000 ± 476,638 in control conditions; $p<0.05$; Figure 5.2 (A-B)]. However, nicotinamide did not affect culture viability at this time-point (unpaired t test, t = 1.3; n.s.) [81.5 ± 0.5% vs. 87.0 ± 3.2% in control conditions; Figure 5.2 (C)].

These parameters were also addressed in cultures treated with nicotinamide from day 0-7 and further differentiated up to a total of 14 days. This also revealed a decrease in absolute live cell numbers in nicotinamide-treated cultures (unpaired t test, t = 1.6; n.s.) [94,444 ± 27,981 vs. 219,722 ± 75,195 in control conditions; Figure 5.2 (D)], in agreement with the data collected previously by manual counting. As at day 7, early nicotinamide administration did not alter the viability of cultures at day 14 (unpaired t test, t = 0.4; n.s.) [76.3 ± 3.8% vs. 72.0 ± 9.8% in control conditions; Figure 5.2 (E)].

To conclude, data generated from live cultures using the automated cell counting system strongly suggests that the reduction in cell number in nicotinamide conditions does not result from an induction of cell death, since the cell viability did not significantly differ from control culture.
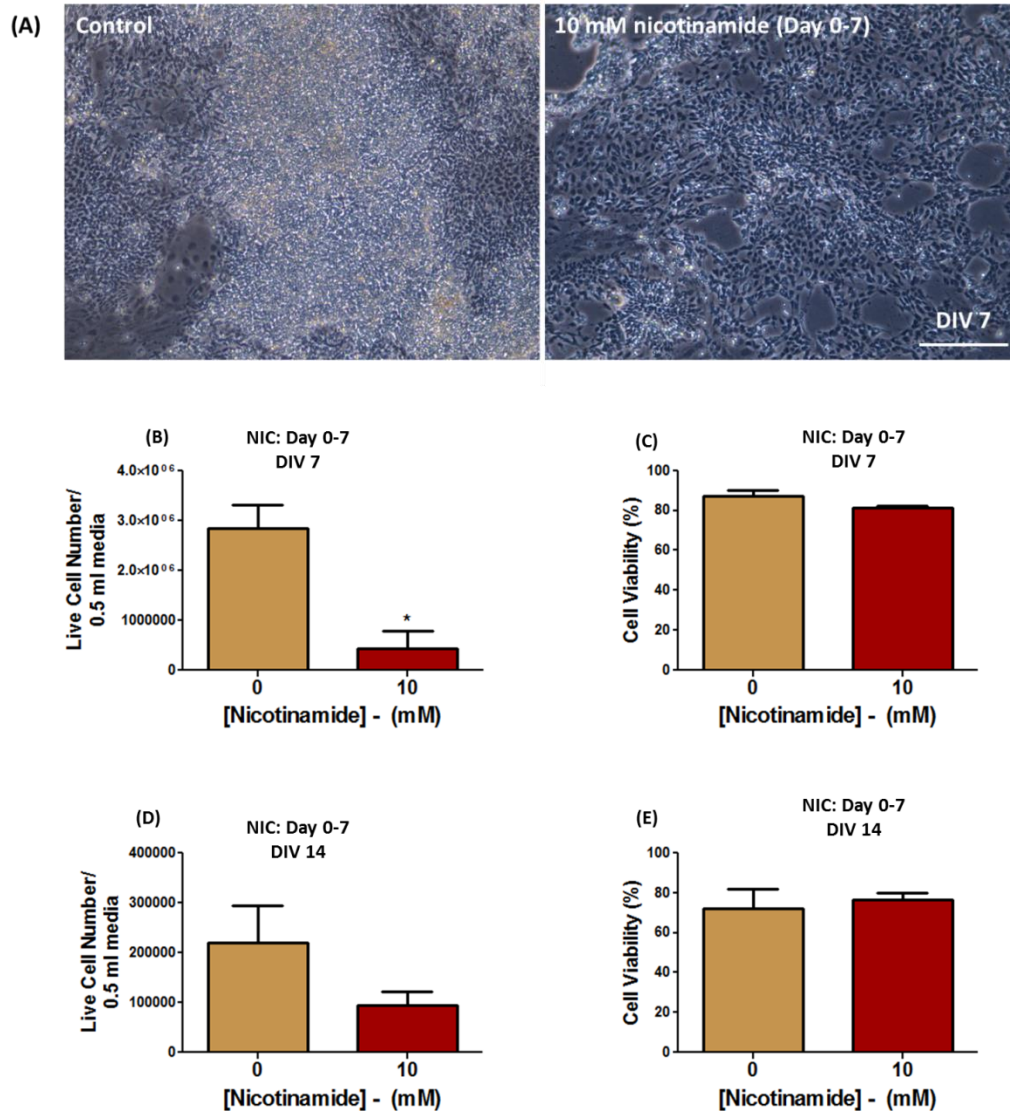
Figure 5.2 Nicotinamide reduced the number of live cells in cultures derived from *Sox1*GFP mESCs, without altering cell viability. Monolayer cultures were exposed to nicotinamide between days 0 and 7, and viability assessed in terms of both total and percentage viable cells at days 7 and 14 of differentiation. **(A)** Phase contrast images of mESC monolayer cultures after 7 days in differentiation medium, without and with nicotinamide treatment. A marked reduction in cell density in nicotinamide-treated cultures was evident at day 7 *in vitro*, compared to untreated cultures. Scale bar = 100 μm. **(B)** Administration of 10 mM nicotinamide between days 0-7 significantly reduced the number of viable cells at 7 DIV. **(C)** Nicotinamide did not elicit any significant differences in the percentage of viable cells remaining in the cultures. **(D&E)** Nicotinamide administered during the first 7 days of differentiation induced a reduction in live cell numbers at 14 DIV, without evidence of toxicity. $*p<0.05$

5.3.2 Addition of nicotinamide at the initial stages of embryonic stem cell differentiation did not induce apoptosis in Sox1GFP-derived cultures.

To verify the previous results in section 5.3.1, showing that nicotinamide (10 mM) treatment did not alter cell viability in mESC-derived cultures; the effect of this small molecule on apoptosis was further investigated by means of the TUNEL technique, in combination with morphological analysis of cell nuclei (Figure 5.4). This effect was evaluated using fluorescence and phase contrast microscopy to quantify the pyknotic (condensed or fragmenting morphologies; Figure 5.3) and total (healthy plus pyknotic) nuclei per microscopic field at 7 and 14 days, respectively.

Figure 5.3 During the early process of apoptosis, cell shrinkage and pyknosis are visible in cell nuclei counterstained with DAPI, using fluorescent microscopy. **(A)** Representative image shows monolayer cells stained with DAPI at 4 DIV, exhibiting condensed nuclei (indicated by the yellow arrow), or pyknotic or fragmented nuclei (indicated by the white arrows), which are characteristic of apoptotic cells. Nuclear integrity was also studied via phase contrast microscopy (inset). White arrows indicate pyknotic nuclei. Scale bar = 50 μm.

Nicotinamide addition during the early stages of differentiation did not significantly alter the percentage of apoptotic cells at day 7, relative to untreated groups (unpaired t test, t = 0.5, n.s.) [15.8 ± 2.1% in nicotinamide treated vs. 14.4 ± 1.4% in control conditions; Figure 5.4 (B)]. Similarly, by day 14, the percentage of nuclei exhibiting pyknosis and TUNEL staining did not significantly differ from control cultures (unpaired t test, t = 0.1, n.s.) [11.3 ± 1.4% in nicotinamide treated vs. 11.5 ± 0.4% in control conditions; Figure 5.4 (C)].

Together, these findings strongly suggest that the reduced total cell population in nicotinamide-treated cultures was not due to induction of apoptosis. Instead, these data suggest a reduction in the proportion of proliferating cells - that is, nicotinamide enhances cell-cycle exit to promote neuronal differentiation.
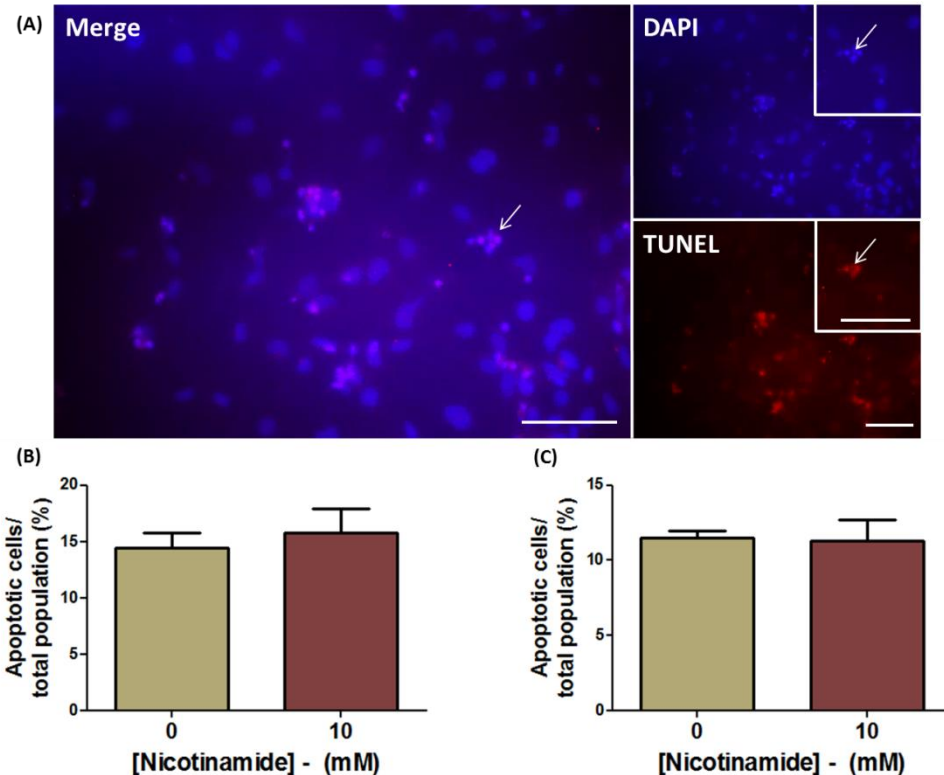
Figure 5.4 Addition of nicotinamide during the early stages of neural differentiation does not induce apoptosis in adherent monolayer cultures. (A)

Apoptosis was determined by evaluation of nuclear integrity and by TUNEL assay at days 7 and 14. Representative images show immunocytochemical labelling of TUNEL-positive cells (red) and total cells in culture counterstained with DAPI (blue). Apoptotic cells were quantified when they stained positively in the TUNEL assay and exhibited a pyknotic nucleus (Merge). Scale bars = 50 µm. **(B&C)** The percentages of apoptotic cell populations were not significantly altered by early nicotinamide treatment in adherent monolayer cultures at days 7 **(B)** and 14 **(C)**, respectively.

5.3.3. Nicotinamide treatment reduced the proportion of proliferating cells in monolayer cultures by promoting the exit of neural progenitors from the cell cycle.

With respect to the decline in the total cell number at day 7 and day 14, the next hypothesis under investigation was to determine whether nicotinamide was acting to direct cells to exit the cell cycle and become postmitotic, in line with the accelerative effect on neuronal differentiation and maturation reported in the previous chapters 3 and 4.

5.3.3.1 BrdU incorporation assay was not a reliable technique to measure proliferation activity in monolayer cultures.

5-bromo-2'-deoxyuridine (BrdU) is incorporated into *de novo* synthesised DNA as a substitute for thymidine. This incorporation assay permanently labels cells undergoing proliferation, including daughter cells until it is diluted out through multiple rounds of cell division[229].

Initially, mESC-derived control and treatment cultures were incubated with BrdU (10 µM) for the first 7 days of differentiation to label all dividing cells. The rationale behind this experimental design was to reveal the proportion of cells that were proliferating during a defined time period (i.e. in the presence of nicotinamide during days 0-7), rather than performing a pulse-labelling experiment prior to cell fixation

which would only reveal the cells that were proliferating at the time of sample collection. However, BrdU treatment for 7 days demonstrated cytotoxic effects, evidenced by widespread monolayer cell death during this process *(data not shown).*

Therefore, the experimental design in this study was altered in accordance with other cell proliferation methods published in the literature.[230] Monolayer cells were pulsed for 1 h with BrdU (10 µM) at day 7, prior to fixation, to label proliferative cells. Detection of BrdU is determined by immunostaining with specific anti-BrdU antibodies (appendix 3)[229]. In this context, a major disadvantage of this proliferation assay is that the complementary base pairing in double-stranded DNA hinders access of the antibody (i.e. anti-BrdU) to BrdU subunits[231]. Therefore, harsh denaturing conditions involving HCl are required to facilitate antibody access to BrdU inserted into genomic DNA, often resulting in tissue damage including degradation of the specimen structure[229]. Furthermore, it has been reported that BrdU labelling is rarely compatible with other staining assays[229].

The present study showed that BrdU-labelling generated an ambiguous staining pattern, where BrdU immunostaining was undetectable within DAPI-stained cell nuclei. Instead, immunofluorescent labelling was shown to be localised within apparent artefacts/dead cell fragments (Figure 5.5). Thus, given these drawbacks, an alternative method to measure cell proliferation during monolayer differentiation was required.
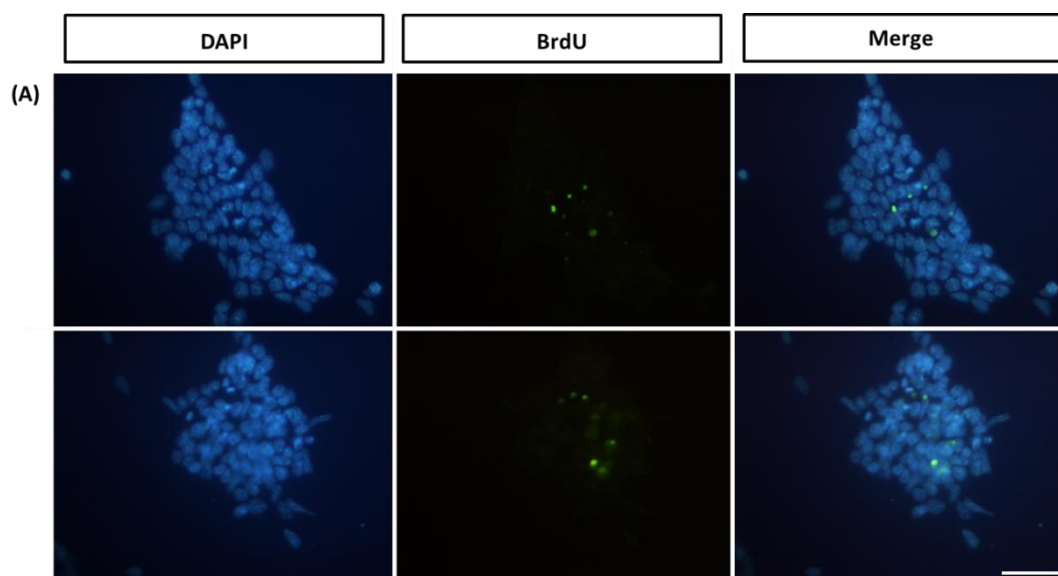
Figure 5.5 BrdU assay proved an unreliable technique to measure cellular proliferation in the monolayer protocol. (A) Immunocytochemical labelling of BrdU (green) and total cells in culture (blue). Representative images show punctate green staining, which was not specific to cell nuclei, and was characteristic of non-specific immunostaining picked up by dead cell fragments. Thus, evidence of cells undergoing proliferation could not be observed using the BrdU incorporation assay in monolayer cultures at day 7. Scale bar = 50 µm

5.3.3.2 Detection of the EdU cell proliferation assay was accomplished in the monolayer protocol.

An alternative assay was established to investigate the effect of nicotinamide on cell proliferation during monolayer differentiation. This approach was based on the incorporation of another thymidine analog, EdU into *de novo* DNA during monolayer differentiation. EdU-labelling is detected by click chemistry (i.e. covalent cross-linking of its ethynyl group with a fluorescent azide). Due to the small size and chemical nature of the fluorescent azide, effective diffusion is facilitated through fixed samples to readily access incorporated EdU nucleosides in genomic DNA[229]. Therefore, unlike the BrdU incorporation assay, no harsh treatments with HCl are necessary, and the physical integrity of specimens is preserved. Further, it has been

demonstrated that the EdU proliferation assay can be reliably performed with other immunostaining techniques. In this study, EdU was shown to provide an easy and sensitive alternative to the BrdU assay, evidenced by its incorporation into the DNA of DAPI-stained cell nuclei at day 7 of differentiation [Figure 5.6 (A)].

The first goal of this experiment was to measure the percentage of proliferating cells in cultures treated with N2B27 alone or with addition of nicotinamide from day 0-day 7. Monolayer cultures received EdU for 1 h prior to cell fixation at the end of the 7 day culture period. This pulse-labelling experiment revealed that 61% of cells in cultures that had been differentiated without nicotinamide showed positive EdU signal, whereas only 29% of cells in the cultures containing nicotinamide were labelled with EdU (unpaired t test, t = 10.6, $p<0.001$) [$28.7 \pm 2.9\%$ vs. $60.7 \pm 0.9\%$ in control conditions; Figure 5.6 (A-B)]. Collectively, these data suggest that N2B27 medium alone supports a higher level of continued proliferation in monolayer cultures at day 7 of differentiation, whereas exposure to 10 mM nicotinamide during days 0-7 promotes the exit of dividing cells from the cell cycle.

Nicotinamide was previously found to induce accelerated neural fate commitment and in turn neuronal maturation during early development. Therefore, the second aim of this study was to determine whether this vitamin B_3 derivative was directing cells to exit the cell cycle at an earlier time point than cell-cycle exit in untreated cultures. To achieve this, EdU-labelled cells were classified as having either strong or weak incorporation of EdU in their cell nuclei [Figure 5.6 (A-inset)]. Using the NIS-image software, strongly labelled cells were characterised as exhibiting expression levels usually within the intensity range of 240-260, and were interpreted as those that had exited the cell cycle immediately after incorporating EdU in S-phase (i.e. the synthesis phase that represents the stage of the cell cycle in which DNA replication

occurs; between G1 phase and G2 phase). Weak EdU expression was more commonly observed at levels of 140-160, and was interpreted as resulting from either late S-phase incorporation prior to cell fixation resulting in limited uptake of EdU, or from EdU dilution following one or more cycles of cell division. Thus, since weakly-expressing EdU labelled cells are open to several interpretations, the effect of nicotinamide on cells displaying "strong" EdU labelling was quantified.

The percentage of EdU-labelled cells in nicotinamide-treated cultures exhibiting "strong" expression levels were slightly reduced, however did not significantly differ from control cultures (unpaired t test, t = 1.4, n.s.) [11.3 ± 3.6% in nicotinamide treated cultures vs. 17.4 ± 2.2% in control conditions; Figure 5.6 (C)].
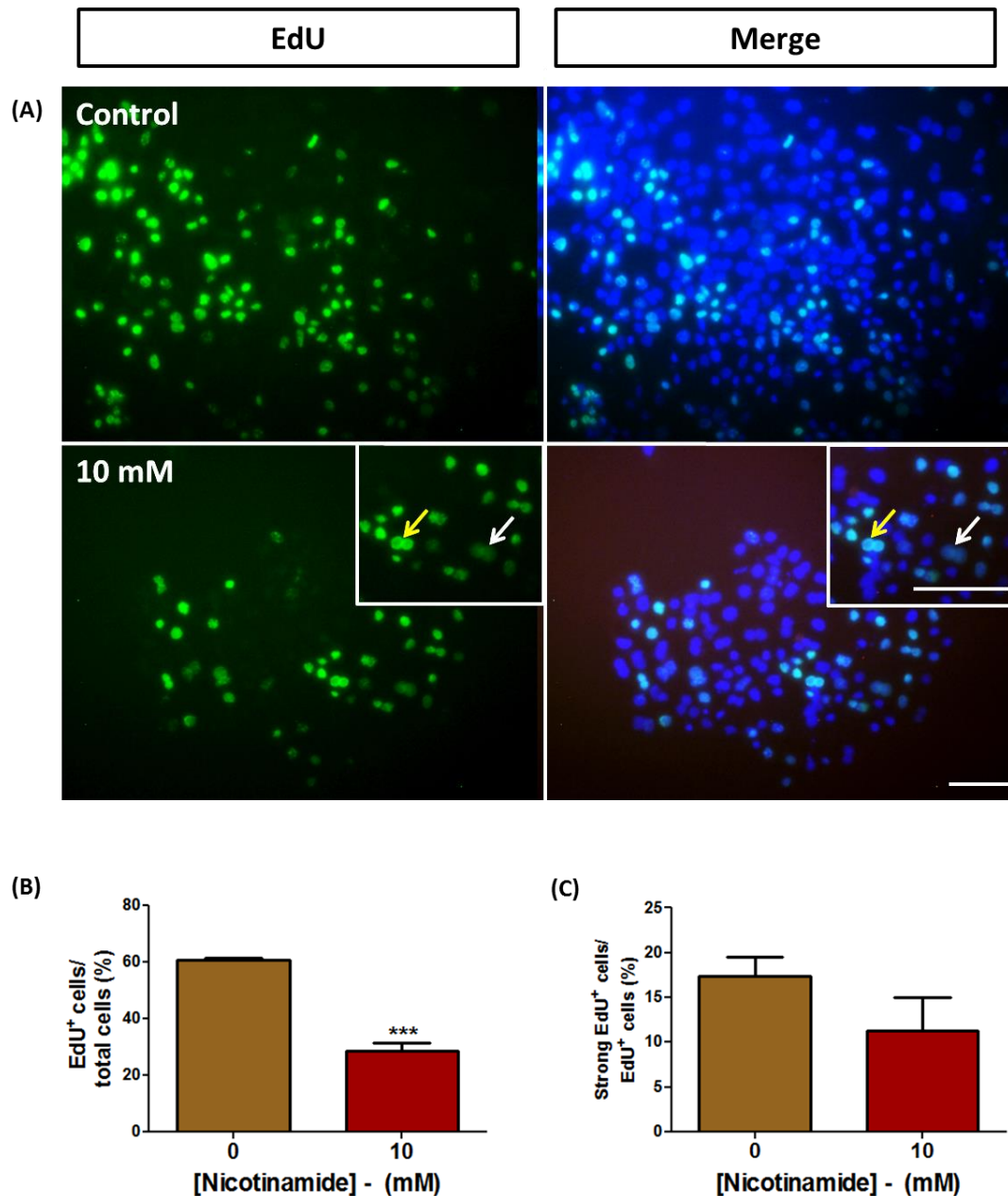
Figure 5.6 Inhibition of *Sox1*GFP-derived cell proliferation by nicotinamide. **(A)** Assessment of EdU immunoreactivity was made using standard fluorescence microscopy. Fluorescent images show EdU$^+$ cells (green), co-labelled with DAPI nuclear staining (blue). Scale bar = 50 μm. Arrows indicate cells expressing strong EdU labelling (yellow) and weaker EdU immunostaining (white). Some EdU-labelled cells demonstrated the formation of doublets following mitosis (indicated by white and yellow arrows). A reduction in cell proliferation was observed in 10 mM nicotinamide-treated cultures, in contrast to control groups. Scale bar = 50 μm **(B)** The data provided by EdU incorporation indicated that mESC-derived cells differentiated with 10 mM nicotinamide resulted in a reduction in cell proliferation, compared to control conditions. **(C)** The percentage of strongly labelled EdU-positive cells indicated a downward trend in cultures treated with nicotinamide from day 0-7, compared to untreated groups.

***p<0.001

5.3.3.3 Early nicotinamide administration reduced proliferation of the Sox1GFP⁺ cell population.

To gain further insight into the effects of nicotinamide on the cell cycle, the uptake of EdU was measured within specific cell populations in control and treatment cultures. In this study, the percentage of Oct4, *Sox1*GFP$^+$ and βIII-tubulin-expressing cells double-positive for EdU were analysed. Here, it should be noted that images were acquired using a high power objective lens (i.e. x40 magnification), to accurately identify individual cells co-labelled with EdU.

At day 7, the percentage of undifferentiated stem cells per field was determined by quantifying individual cells expressing the stem cell marker, Oct4. Findings showed that addition of 10 mM nicotinamide between days 0 and 7 caused a slight decrease in the proportion of Oct4$^+$ cells, expressed as a percentage of total cells (unpaired t test, t = 1.0, n.s.) [16.9 ± 5.9% in nicotinamide treated cultures vs. 23.6 ± 3.3% in control conditions; Figure 5.7 (A-B)]. However, the percent of EdU labelled cells within the Oct4$^+$ population was comparable to controls (unpaired t test, t = 0.5, n.s.) [9.2 ± 4.0% in nicotinamide treated cultures vs. 11.5 ± 1.8% in control conditions; Figure 5.7 (A-B)].
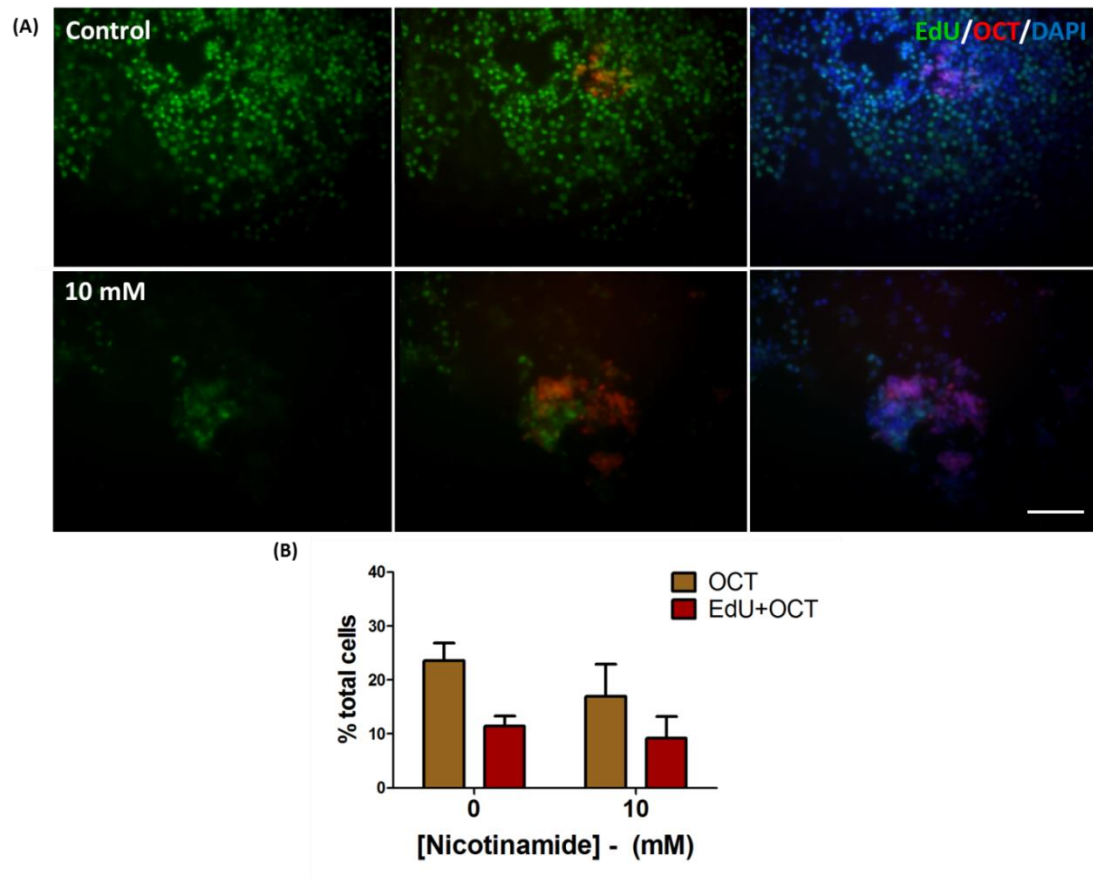
Figure 5.7 Colocalisation of EdU and the undifferentiated cell marker, Oct4.
(A) Immunofluorescent images of EdU-labelled (green) and double-labelled undifferentiated cells (Oct4, red) in monolayer cultures at day 7. The nuclei of all cells in cultures were labelled with DAPI (blue). Scale bar = 50 μm. **(B)** Nicotinamide treatment generated a slight reduction in the percent of Oct4-expressing cells by day 7, but did not alter the percent of EdU-labelled cells within this population.

In contrast, proliferation was significantly reduced in progenitor cells co-localising *Sox1*GFP$^+$ expression and EdU-labelling (unpaired t test, t = 4.8, p<0.01) [3.0 ± 2.0% in nicotinamide treated cultures vs. 13.2 ± 0.6% in control conditions; Figure 5.8 (A-B)], and the percentage of GFP$^+$ cells was also significantly decreased by day 7 (unpaired t test, t = 3.8; p<0.05) [9.7 ± 6.4% in nicotinamide treated cultures vs. 33.6 ± 0.3% in control conditions; Figure 5.8 (A-B)].

This important finding shows that 10 mM nicotinamide is functioning during early development to restrict $Sox1^+$ precursor proliferation.
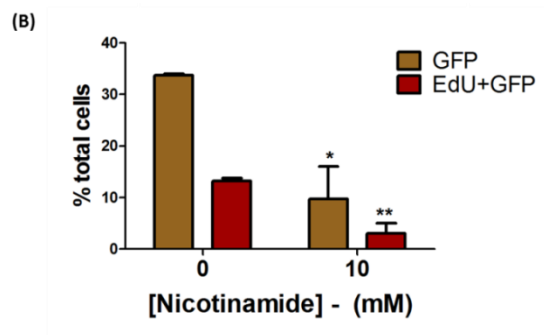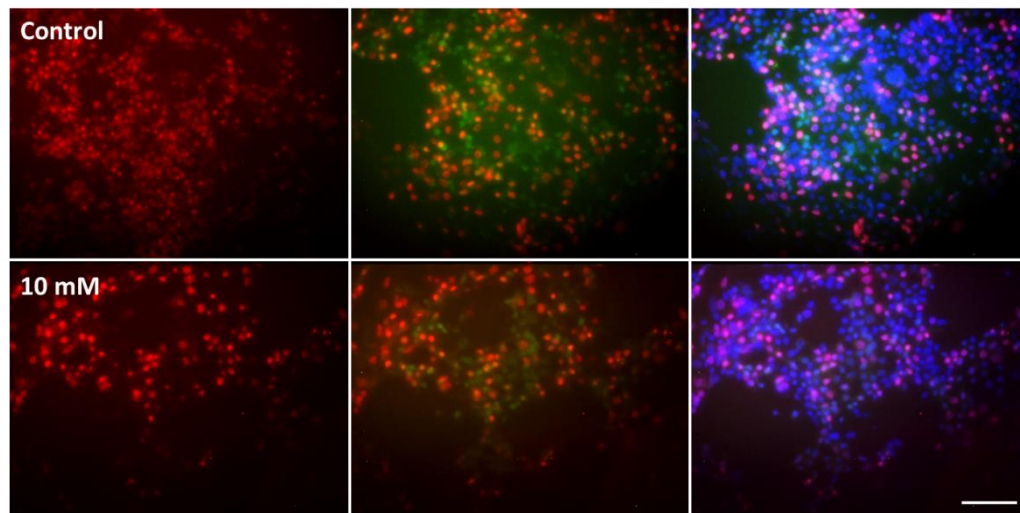
Figure 5.8 Colocalisation of EdU and native GFP-expressing progenitor cells. **(A)** Immunofluorescent images of EdU-labelled (red) and double-labelled native GFP (green)-expressing cells in monolayer cultures at day 7. The nuclei of all cells in cultures were labelled with DAPI (blue). Scale bar = 50 μm. **(B)** Nicotinamide treatment significantly reduced the percentage of GFP-expressing cells, concomitant with a decrease in the percent of EdU-labelled cells in the *Sox1*GFP-postitive population.
**p<0.01; *p<0.05

Subsequently, 10 mM nicotinamide had a significant effect on neuronal differentiation, concomitantly increasing the βIII-tubulin-positive neuronal population (unpaired t test, t = 10.8 $p<0.001$) [15.2 ± 0.9% in nicotinamide treated cultures vs. 3.6 ± 0.4% in control conditions; Figure 5.9 (A-B)]. Colocalisation of EdU and βIII-tubulin demonstrated no detectable difference in the levels of

incorporation between treated and untreated cultures (unpaired t test, t = 0.2, n.s.) [0.8 ± 0.1% in nicotinamide treated cultures vs. 0.9 ± 0.1% in control conditions; Figure 5.9 (A-B)]. Since the vast majority of βIII-tubulin-expressing neurons were rarely double-labelled for EdU, it is reasonable to conclude that many of the differentiated neurons were postmitotic at the stage of differentiation when EdU was applied to the cultures.
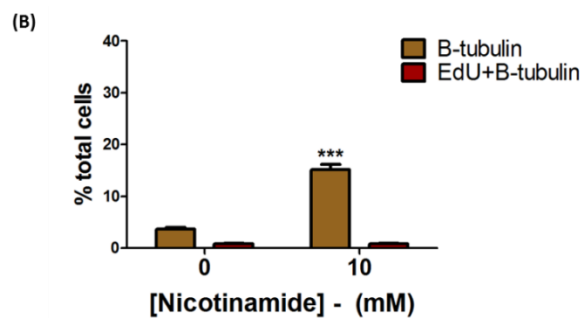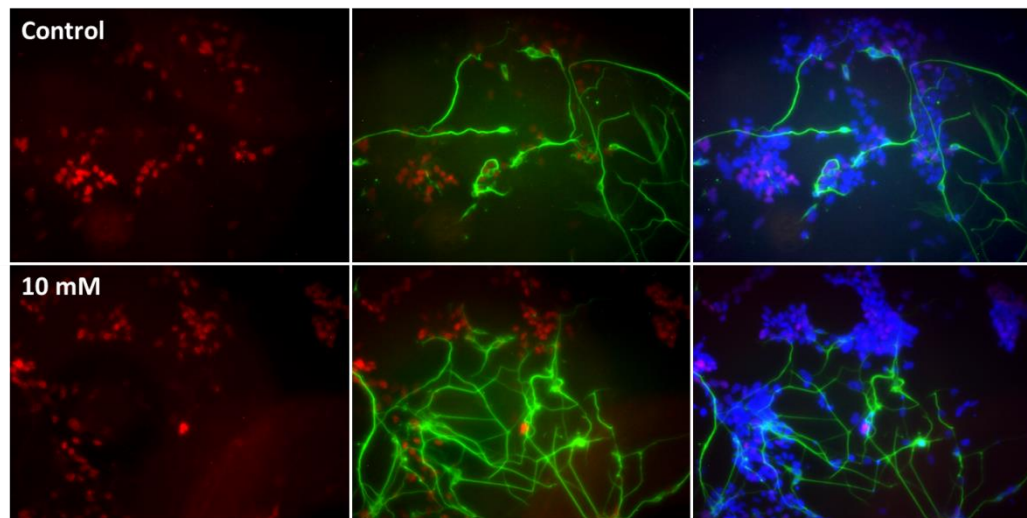
Figure 5.9 Colocalisation of EdU and the immature neuronal marker βIII-tubulin.
(A) Immunofluorescent images of EdU-labelled (red) and double-labelled βIII-tubulin (green)-expressing cells in monolayer cultures at day 7. The nuclei of all cells in cultures were labelled with DAPI (blue). Scale bar = 50 μm. **(B)** Nicotinamide treatment significantly enhanced expression of neuron-specific βIII-tubulin, without altering the percentage of EdU-labelled cells within the neuronal population.
 ***$p<0.001$

Collectively, important and novel data generated in this study indicate that nicotinamide is involved in the switch between proliferation and differentiation during early development. Specifically, addition of this small molecule during the initial stages of embryonic stem cell differentiation enhanced cell cycle exit to halt proliferation of neural progenitors, thereby driving a switch from neuroectodermal precursors to neurons within differentiating cultures. This result is in line with the effect of nicotinamide (between days 0-7) on accelerated neuronal differentiation and maturation by day 14. Furthermore, this section provides further evidence that nicotinamide is most effective in influencing neuronal differentiation at an early developmental time window.

5.3.3.4 EdU incorporation was not detected at day 14 of monolayer differentiation.

To test for differences in proliferation in undifferentiated ESCs, $Sox1$GFP$^+$ progenitor cells, and βIII-tubulin$^+$ cells at day 14, cultures were maintained as feeder-free adherent monolayers from day 0-7, using serum-free N2B27 medium in the presence/absence of nicotinamide treatment. At day 7, cultures were incubated with 10 μM EdU for 1 h to label all diving cells, and cells were then replated onto coverslips coated with PLL and laminin until day 14. At this point, cell samples were processed for immunocytochemistry.

It was observed that EdU incorporation was not detectable in monolayer cultures. This finding suggests that EdU may have been incorporated into *de novo* synthesized DNA at day 7, but diluted out through multiple rounds of cell division by day 14 of differentiation or cell death (Figure 5.10).
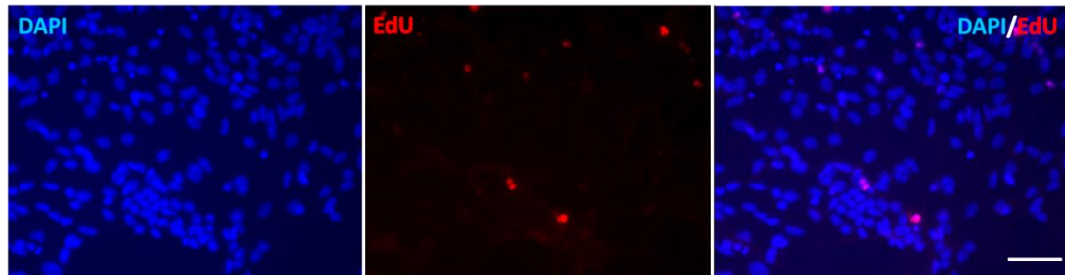
Figure 5.10 Immunocytochemical labelling of EdU (red) and total cells in culture (blue). EdU-labelling was undetectable at day 14 of the monolayer protocol following an EdU pulse-labelling experiment at day 7. This observation suggests a dilution of EdU label caused by cell division. Representative images show punctate red staining, which was not specific to cell nuclei, possibly characteristic of non-specific immunostaining picked up by dead cell fragments. Scale bar = 50 μm.

5.3.4 Under monolayer differentiation conditions, nuclear SIRT1 staining in neural progenitor and neuronal cell populations is down-regulated during monolayer differentiation.

It has been reported that SIRT1, an NAD$^+$-dependent histone deacetylase is highly expressed in mESCs, in contrast to differentiated tissues[224]. Therefore, *Sox1*GFP-derived undifferentiated pluripotent mESCs, maintained in FSC/LIF medium (i.e. culture media for mESC proliferation; appendix 2) were selected to serve as a positive control to establish optimum working dilutions of anti-SIRT1 antibody (Figure 5.11; appendix 3).
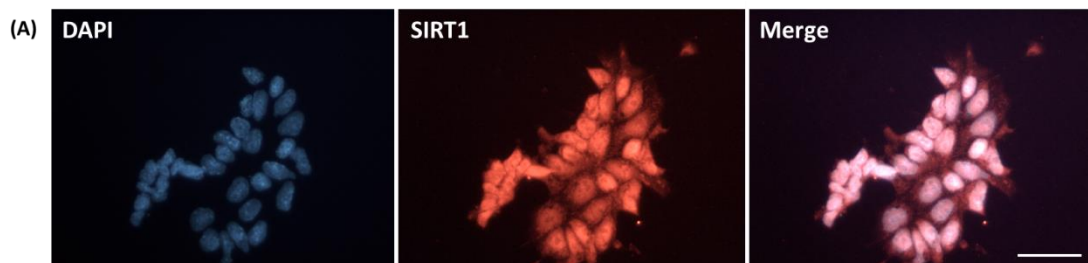
Figure 5.11. Anti-SIRT1 optimisation for immunocytochemistry. Rabbit polyclonal SIRT1 antibody was optimised using undifferentiated mESC cultures as a positive control. **(A)** Immunostaining of mESC cultures with anti-SIRT1 (red) and DAPI staining (blue), which was used to visualise cell nuclei. 46C-derived mESC cultures strongly expressed SIRT1 in their cytoplasm (yellow arrow) and cell nuclei

(white arrow); visualised by fluorescent and phase contrast microscopy. Scale bar = 50 µm.

5.3.4.1 SIRT1 is predominantly localised in the nucleus during monolayer differentiation.

It has previously been reported that SIRT1 plays a modulatory role during neuronal differentiation[232]. A study carried out by Hisahara *et al*, found SIRT1 to be localised within the cytoplasm of embryonic and adult NPCs, but was transiently localised to cell nuclei in response to differentiation stimulus (Figure 5.1)[223]. Therefore, in this study, defined culture conditions were established to direct the differentiation of 46C-derived mESCs into neuronal cultures. Immuno-stained differentiated cells were visualised using a 40x lens to accurately visualise subcellular structures. Immunocytochemisty showed that SIRT1 is predominalty localised within the nucleus of monolayer-derived cells at 14 DIV (Figure 5.12).
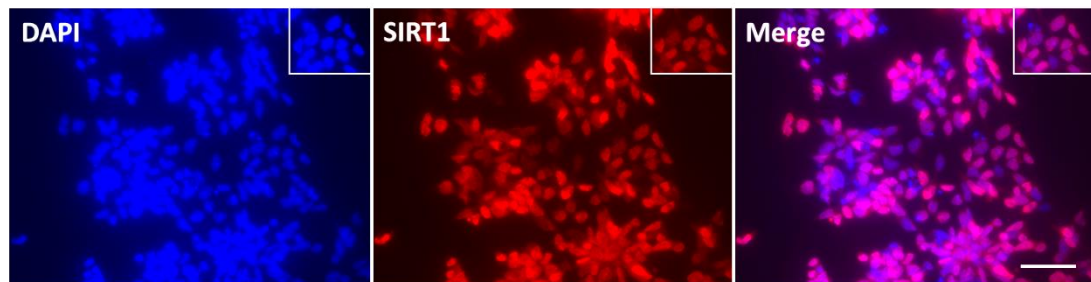
Figure 5.12. SIRT1 localisation under adherent monolayer differentiation conditions. Immunofluorescence detection of *Sox1*GFP-derived cultures labelled with anti-SIRT1 (red). DAPI staining (blue) was used to stain the total cell population and to visualise cell nuclei. Merge image shows that SIRT1 is highly expressed in differentiated cells at day 14 of the monolayer protocol, and revealed that SIRT1 staining is primarily nuclear. Scale bar = 50 µm

5.3.4.2 A progressive and substantial decrease in SIRT1 expression was observed during Sox1GFP-derived monolayer differentiation.

SIRT1 expression has been reported to be down-regulated during differentiation of hESCs and mNSCs[233,234]. Therefore, in this study, expression levels of SIRT1 were monitored in neural and neuronal cell populations, as they matured from day 7 to day 14 of adherent monolayer differentiation.

During the first 7 days of differentiation, SIRT1 was shown to be strongly expressed in monolayer cultures, in contrast to day 14 [Figure 5.13 (A-B)]. Notably, higher levels of SIRT1 staining were shown within GFP⁻ populations at day 7; these could potentially be undifferentiated cell colonies [i.e. Oct4⁺ colonies; Figure 5.13 (A)]. Immunofluorescence staining of SIRT1 at day 7 revlealed that SIRT1 expression was predomiantly nuclear in βIII-tubulin-expressing cells.

Interestingly, by day 14, decreases in the overall expression levels of SIRT1 were observed in cultures. Specifically, SIRT1 nuclear staining was shown to be reduced in distinct neural populations, i.e. GFP⁺ and βIII-tubulin⁺ cells at this time point [Figure 5.13 (B)].

These preliminary observations demonstrate that SIRT1 expression progressively decreases in ESC-derived cultures as time *in vitro* increases, in line with previous findings reported in the literature[234,235]. Based on this evidence, a decrease in SIRT1 expression may play a role in supporting neuronal differentiation[235]. Furthermore, it has been reported that the spatial regulation of SIRT1 is critical for inducing the neuron-specific transcription programme (Figure 5.1)[228]. In this context, immunostaining results suggest that differential SIRT1 localisation exists between undifferentiated mESCs (detected in the nucleus and cytoplasm), in contrast to

differentiated neural and neuronal cell populations which showed SIRT1 staining to be primarily nuclear.
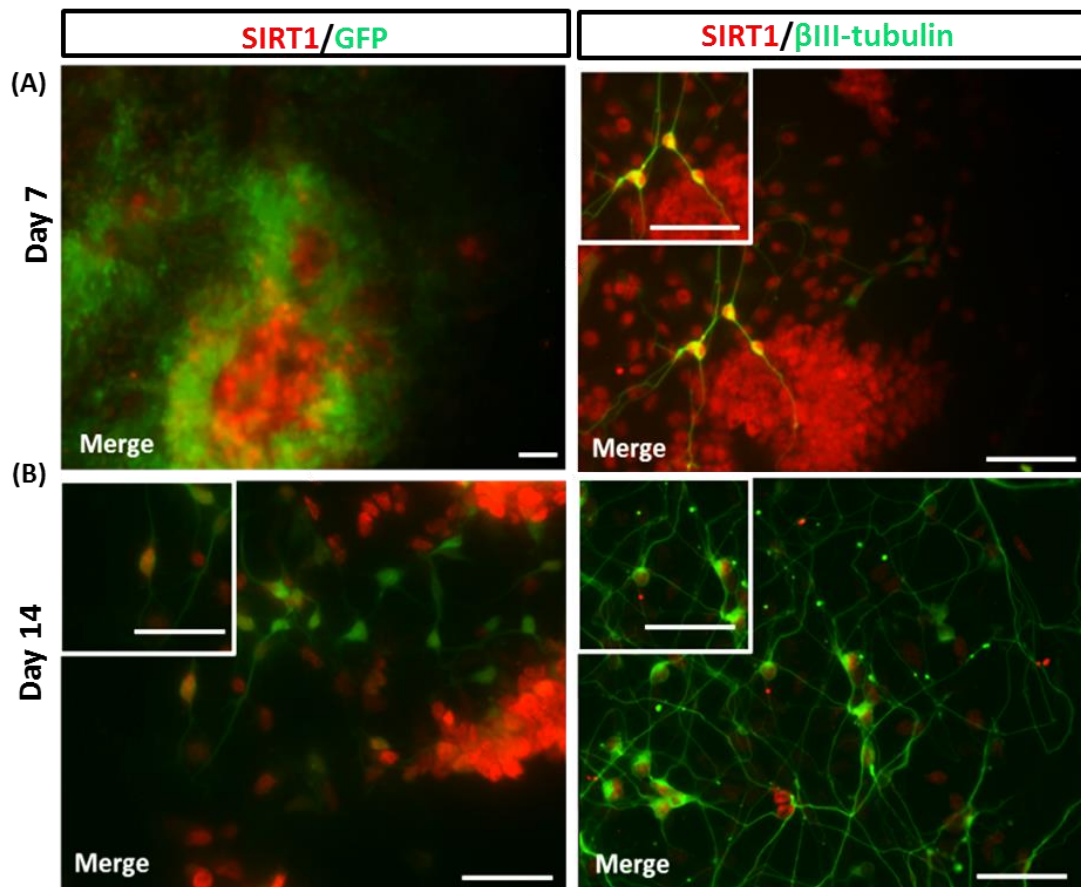
Figure 5.13 Expression of SIRT1 in *Sox1*GFP-derived monolayer cultures. Immunocytochemical analysis of cells expressing the immature neuronal marker βIII-tubulin (green) or native GFP (green), co-labelled with anti-SIRT1 (red). Scale bar = 50 μm. **(A)** Levels of SIRT1 were initially high in cultures assessed at day 7 of the monolayer protocol. In particular, SIRT1 was strongly expressed within GFP-negative populations, possibly indicative of pluripotent stem cell colonies. Double-staining analysis revealed that SIRT1 expression was mostly located within the cell nucleus of βIII-tubulin^{+} cells at seven days of differentiation. **(B)** As time in culture increases, SIRT1 expression was shown to decrease. Colocalisation of nuclear SIRT1 expression was observed in GFP^{+} progenitor cells and βIII-tubulin-expressing cells at day 14, albeit in decreased levels compared to day 7.

5.3.4.3 Addition of nicotinamide to monolayer cultures elicited no effect on glial differentiation.

SIRT1 inhibition has been linked to alterations in NPC cell-fate decisions *in vitro*, causing increased differentiation of NPCs into neurons, at the expense of astrocytes (Figure 5.1)[236]. Further, previous results in this experimental chapter showed that

nicotinamide, a SIRT1 inhibitor, functioned during early development to direct a switch from neural progenitors to neurons, by inhibiting proliferation. For this purpose, this study aimed to test whether nicotinamide could also direct glial differentiation from *Sox1*GFP mESC-derived monolayer cultures. Immunocytofluorescence analysis was performed to investigate the expression of the astrocyte marker, GFAP and the early OPC marker, NG2, in differentiated cultures at day 14. Total numbers of cells expressing GFAP and NG2 were presented as a percentage of the total DAPI cell count.

GFAP-expressing cells exhibiting long cellular projections[169,237], similar to those of radial glial cells, were detected at day 14, albeit in small numbers [Figure 5.14 (A)], in striking contrast with the number of βIII-tubulin –expressing neurons generated at this time point (section 3.3.1). There was no significant effect on the percentage of GFAP$^+$ cells at day 14 in cultures of cells incubated with nicotinamide between days 0 and 7 compared to control cultures, (unpaired t test, t = 1.0, n.s.) [0.3 ± 0.1% in nicotinamide treated cultures vs. 0.5 ± 0.2% in control conditions; Figure 5.14 (A-C)]. Similarly, there was no difference in NG2 expression when comparing differentiated cells treated with or without nicotinamide (unpaired t test, t = 1.2, n.s.) [3.2 ± 0.5% in nicotinamide treated cultures vs. 2.3 ± 0.6% in control conditions; Figure 5.14 (B-C)].

Taken together, these data reveal that the GFAP protein and the early OPC marker, NG2 were expressed in very few cells of the *Sox1*GFP-derived cultures at day 14; constituting less than 5% of the total cell population. In addition, mature GFAP$^+$ cells displaying characteristic large flattened cell bodies [Figure 5.1 (C)] were absent from monolayer cultures at this time-point. Thus, it is reasonable to conclude that

day 14 of monolayer differentiation might be too early to assess for the effect of nicotinamide on glial fate, in line with previous reports in the literature[169,170,238].
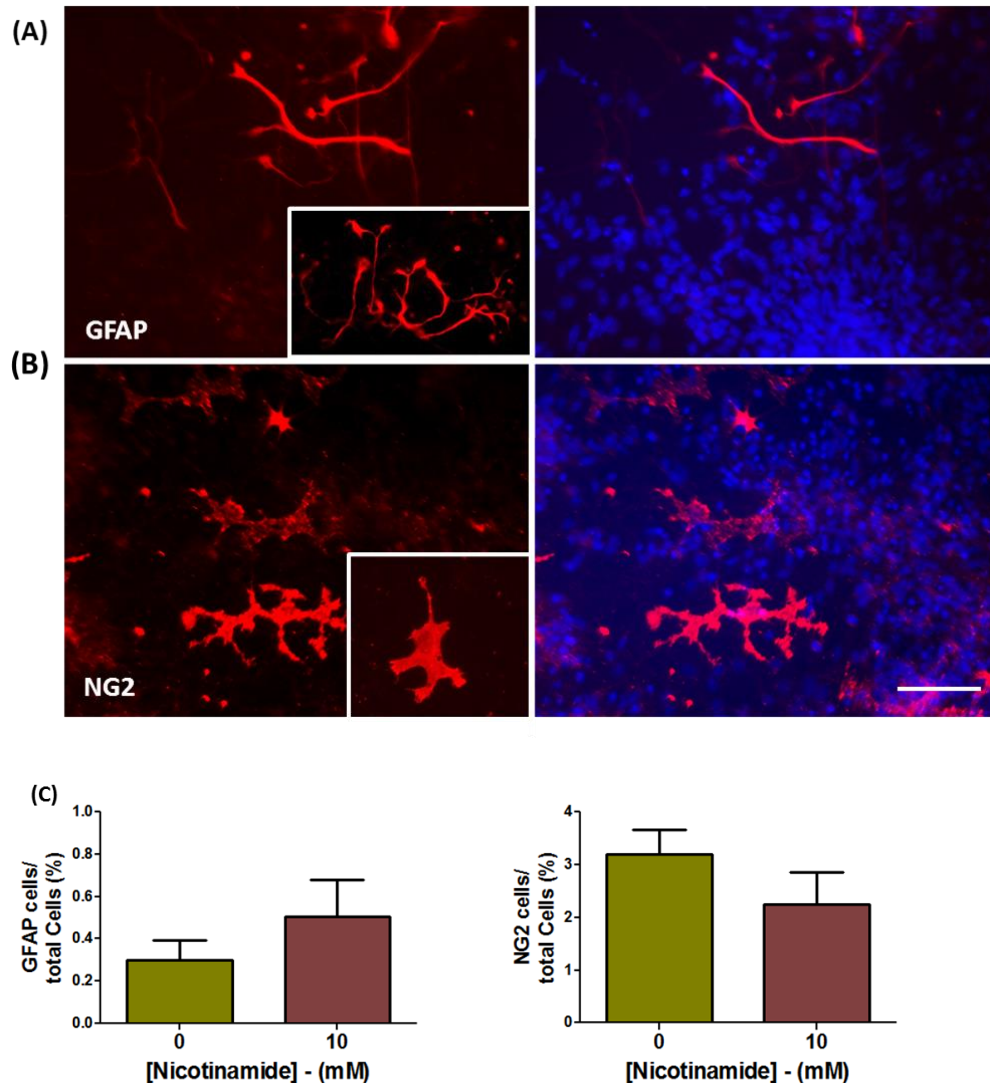
Figure 5.14 Treatment of adherent cultures with nicotinamide elicited no effect on glial differentiation. Immunofluorescence images of glial populations generated from mESC-derived monolayer cultures, showing immunostaining with antibodies specific to the astrocyte marker, GFAP **(A)** and the OPC marker, NG2 **(B)**. **(A)** GFAP-expressing cells with leading processes and elongated cell bodies (inset) were observed at day 14 of monolayer differentiation. **(B)** Higher magnification image of monolayer cells immunostained for NG2 (inset), displaying a prominent cell with multiple processes, representative of their morphology *in vivo*. Cell nuclei counterstained with DAPI (blue). Scale bar = 50 μm. **(C)** GFAP[+] and NG2^{+} cells were detected in cultures at a very low percentage. No significant effects on glial

expression (i.e. GFAP or NG2 production) were demonstrated following induction with 10 mM nicotinamide treatment between days 0-7.

5.4 Discussion

5.4.1 Nicotinamide did not induce apoptosis or alter cell viability in monolayer cultures.

For nicotinamide-induced neuronal differentiation to be of benefit to future neural-cell based therapies, it is crucial that this compound does not decrease the total cell population by inducing cell death or reducing overall cell viability in differentiating cultures. In this context, since higher concentrations of nicotinamide (i.e. 20 mM) elicited cytotoxic effects on monolayer cell survival (section 3.3.1), one of the hypotheses under investigation in this study was to determine whether 10 mM nicotinamide caused a reduction in cell number (section 3.3.2) by inducing cell death.

By adding nicotinamide to cells at an early stage of the differentiation, a decrease in total live cell numbers was observed at 7 and 14 days; verified using an automated cell counting system, based on trypan blue viability analyses. Crucially, undifferentiated ESCs cultured in the presence of 10 mM nicotinamide (from day 0-7) showed no differences in cell viability of the remaining cells at days 7 or 14, when compared with control cultures. These results were further confirmed by analysis of pyknosis and TUNEL staining of apoptotic cells, demonstrating that nicotinamide did not increase apoptosis levels in monolayer cultures at these time points. Collectively, this evidence strongly suggests that of the addition of nicotinamide to future neural cell-based therapies offers a safe method to direct differentiation. As importantly, these findings strongly indicate that nicotinamide may be functioning

instead to cause a reduction in the proportion of proliferating cells, to cause a decrease in total cell numbers.

5.4.2 Nicotinamide regulates the cell cycle to halt proliferation of Sox1GFP-derived neural progenitor cells.

Neural differentiation requires cells to exit the cell cycle and become postmitotic, during *in vitro* and *in vivo* development[239]. Thus, regulating the transition between proliferation and differentiation is fundamental to neurogenesis. In this respect, control of cell cycle is crucial in the differentiation of ESCs, as we advance towards clinical stem cell-based therapies.

Vitamins have been reported to influence cell cycle progression in cancer cell lines[240–242]. Further, a reduction in proliferation has previously been reported upon exposure of ESC-derived cultures to 10 mM nicotinamide[158]. The ability of nicotinamide to regulate the number of neurons produced and function as an inducer of neural maturation during monolayer differentiation, reported previously in chapters 3 and 4, served as early evidence that nicotinamide may be driving this neural proliferation-to-differentiation switch during development.

Based on this evidence, the present study therefore sought to investigate the influence of nicotinamide on DNA synthesis in proliferating monolayer-derived cells. This hypothesis was addressed using an EdU incorporation assay, which was demonstrated to be a much more sensitive method than BrdU detection, in agreement with findings in the literature[231]. Initial findings generated in this study reported that control cultures induced with N2B27 differentiation medium alone contained more proliferating cells, in contrast to cultures exposed to nicotinamide, where the population of proliferating cells was reduced by half at day 7 of the monolayer

protocol. This early and robust effect suggests that nicotinamide may be acting at the initial stages of ESC differentiation to influence the transition of mESCs into neuroectoderm or indeed driving a switch from neural progenitors to neurons in the differentiating cells. Similarly, the vitamin A derivative, RA has been shown to regulate the proliferation-to-differentiation switch[242]. Specifically, RA has been reported to promote differentiation and inhibit cell proliferation by directing cell cycle arrest in ESCs[239,243]. With particular relevance to the findings discussed in this thesis, early administration of RA to differentiating adherent mono-cultures was found to restrict the proliferation of $Sox1^+$ neuroectodermal precursors, resulting in enhanced neurite outgrowth and βIII-tubulin expression[243]. In the present study, nicotinamide significantly reduced the percentage of GFP$^+$ cells, concomitant with a decrease in the number of actively proliferating $Sox1$GFP$^+$ NPC cells. As far as I am aware, this is the first report to demonstrate that the vitamin B$_3$ metabolite is influencing a similar switch to RA - that is a neural proliferation-to-differentiation switch from neural progenitors to neurons.

5.4.3. Analysis of SIRT1 expression during ESC differentiation

The present study showed that, in mESCs, SIRT1 expression levels were initially high but were down-regulated during neuronal differentiation, in agreement with similar expression patterns reported in the literature[228,234,235]. In this context, inhibition of SIRT1 by nicotinamide has been shown to direct NPCs toward motor neuron differentiation[160], suggesting a further role for SIRT1 down-regulation during neural differentiation. In addition, a reduction in SIRT1 expression may be a prerequisite for successful differentiation[234], since it has been reported that over-expression of SIRT1 inhibits neural differentiation[235]. In this study, SIRT1 staining was shown to be expressed in βIII-tubulin$^+$ cell nuclei as early as day 7 of

differentiation, implying that SIRT1 is required at the early stages of the differentiation process.

5.4.4 The role of SIRT1 during neuronal development.

SIRT1 also plays an important role during neural development[221], regulating brain structure and neuronal morphology through axon elongation[244], neurite outgrowth and branching[201,227,245,246]. With particular relevance to the findings outlined in the previous experimental chapter 4, SIRT1 knockdown with small hairpin RNA has been reported to promote neurite extension[227], and increase neurite number and total neurite output[246]. Nicotinamide also inhibits another member of the sirtuin family, SIRT2, which deacetylates βIII-tubulin, and also plays a relevant role in regulating the extension of cell processes[198]. Similarly, SIRT2 knockdown was also shown to increase hippocampal neurite outgrowth[247] and the arborisation complexity observed in primary oligodendrocyte precursors[248]. These findings indicate that the specific effect of nicotinamide on the appearance of postmitotic neurons, observed in sections 4.3.3.1 and 4.3.3.2 may be mediated by SIRT1/SIRT2 inhibition. Therefore, in future work, to test whether nicotinamide primarily enhances neuronal maturation in *Sox1*GFP-derived neural subtypes by mechanisms that involve SIRT1/2 downregulation, treatment of mESC cultures with small molecule compounds such as the SIRT1/2 inhibitor cambinol or a SIRT1 pharmacologic activator, resveratrol between days 0-7 of monolayer differentiation should address this hypothesis.

It should be noted that other publications report that SIRT1 overexpression, an effect mimicked by resveratrol and NAD, regulates dendritic development in hippocampal neurons, while interfering with the catalytic deacetylase activity of SIRT1 by nicotinamide treatment decreased the number of primary and secondary dendrites[201]. These findings are consistent with similar observations in neurons differentiated in

the presence of SIRT inhibitors, which exhibited fewer and shorter neurites than control conditions[223]. In this regard, SIRT1 is likely to have a complex and perhaps context-specific effect in neuron biology. It can be hypothesised that nicotinamide may function as an activator or inhibitor of SIRT1, based on cellular requirements. Further, it cannot be excluded that differences associated with specific properties of distinct cellular models may explain the inconsistent nature of SIRT1 findings in the literature.

5.4.5 Conclusion and Further Studies

In conclusion, data generated in this study strongly supports a role for nicotinamide in neural commitment of *Sox1*GFP-derived ESCs and in down-regulating neural progenitor proliferation. In addition, the influence of nicotinamide on SIRT1 may be necessary for the correct establishment of specific differentiation programmes during ESC development.

Future studies into how nicotinamide regulates cell-cycle exit would lead to a greater understanding of its role during neuronal differentiation. In this regard, cell proliferation and differentiation are tightly coordinated by cell cycle controls, implicating the formation of complexes between cyclins and cyclin-dependent kinases (CDKs), a process inhibited by cyclin-dependent kinase inhibitors (CKIs)[239]. Kim *et al* hypothesised that RA functions to supress cyclin genes leading to an induction of CKI genes, thereby accelerating cell-cycle exit and promoting ESC neural differentiation[239]. Therefore, it will be of interest to determine whether nicotinamide acts in a similar manner to regulate cell cycle genes during ESC differentiation.

Since targets of SIRT1 include master transcription factors and cell cycle regulators[249], it will also be important to thoroughly explore the functioning of SIRT1 during ESC differentiation. Based on preliminary observations investigating SIRT1 protein expression at different stages of adherent monolayer differentiation, it will be necessary to ascertain whether SIRT1 activity is down-regulated following nicotinamide treatment in neural and neuronal populations. This could be assessed using a SIRT1 activity assay, employed by Zhang and colleagues[160]. In this study, the SIRT1 inhibitor, nicotinamide, did not influence glial commitment, in accordance with a number of reports[179,234,236]. However, in future work, it will be necessary to differentiate *Sox1*GFP-derived cells for a longer culture period and then investigate whether nicotinamide influences glial differentiation at a later time point of the monolayer protocol, since a neurogenic switch to gliogenic NPCs has been reported in later monolayer cultures, at approximately day 16[169].

Chapter 6: Effect of Nicotinamide on Midbrain Dopaminergic Neuron Production from Mouse Pluripotent Stem Cells

6.1 Introduction

Efficient induction of ventral mDA neurons from pluripotent stem cell sources is critical for advancing their potential in neuronal replacement therapies relevant to PD[166]. In this regard, the therapeutic use of pluripotent stem cells will require robust and safe methods for generating mDAergic neurons *in vitro.* Thus, understanding the molecular signalling cascades governing cell fate choice during DA neuron development is fundamental. To date, a successful method for promoting the correct specification and development of mDA neurons *in vitro* relies upon the addition of key developmental morphogens to the differentiation media, to induce a cascade of gene transcription resembling normal development *in vivo,* and therefore steer midbrain-specific DA cell fate from pluripotent stem cells[50]. Regulatory factors implicated in mDA neuron development are discussed in detail in the introductory Chapter 1.

Findings reported in Chapter 4 (section 4.3.2.1) highlighted an essential role for nicotinamide during catecholaminergic development. Specifically, a monolayer differentiation procedure completely devoid of exogenous proteins and signalling molecules associated with DA neuron development *in vivo* was devised, to fully elucidate if nicotinamide functions as an instructive small molecule for catecholaminergic differentiation *in vitro.* This study presented evidence for the first time that administration of this vitamin metabolite during an early developmental time-window of ESC monolayer differentiation significantly enhanced and enriched the generation of *Sox1*GFP mESC-derived TH$^+$ neurons by day 14. However, there are a number of limitations associated with this ESC differentiation model. It is clear that neural progenitors and neurons in general could be derived from mESCs with higher frequency (detailed in section 3.3.1 and 3.3.4.3) than the production of TH$^+$

neurons (section 4.3.2.1), under the experimental conditions applied. In Chapter 3, the heterogeneous nature of ESC *in vitro* differentiation was demonstrated using the *Sox1*GFP model system; highlighting the presence of undifferentiated mESCs and GFP-expressing NPCs within differentiated progenies in monolayer cultures at day 14 (section 3.3.4.1 and 3.3.4.3). Heterogeneity is a common feature across ESC differentiation paradigms, resulting in the presence of intermediate progenitor cells of distinct stages of development and/or regional identity in differentiating cultures, thereby generating heterogeneous responses to specific inductive signalling molecules[87]. Further, it has been reported that the acquisition of a midbrain specific DAergic cell fate is not supported under *Sox1*GFP mESC monolayer differentiation conditions, evidenced by the absence of key midbrain markers such as Foxa2, Lmx1a and Pitx3 in the study carried out by Parmer and Li (2007)[250].

To address some of these issues, the current study aimed to translate earlier work with nicotinamide to a recent novel method of mDA neuron differentiation[87], to potentiate the robust production of authentic midbrain-specific DAergic neurons and therefore explore the full potential of this vitamin B_3 metabolite in its ability to drive pluripotent stem cells towards a DA neuron cell fate. This protocol is based on modifications to the original adherent monolayer differentiation protocol established by Ying *et al* (2003)[199] and offers an ideal and small-molecule-based paradigm[87]. Moreover, this novel differentiation method offers several advantages over current protocols of producing midbrain-specific DA neurons, due to its ability to direct the highly efficient generation of functional, "authentic" mDAergic neurons via generation of intermediate epiblast cells. Populations of this intermediate epiblast cell, derived from post-implantation blastocysts (E5.75–E6.5) are more homogenous than the original stem cell populations (i.e. ESCs; derived from the ICM of an

embryo at the earlier blastocyst stage; Figure 6.1)[251], and share defining features associated with human ESCs[252] used in current protocols for PD[78,79,155]. Furthermore, pluripotent epiblast cells are more synchronised[87] and should therefore respond much more consistently to external signals for neural induction *in vitro.*

Based on these findings, one of the primary aims of this study was to determine whether nicotinamide could direct a more developmentally primed stem cell population to midbrain specific DAergic neurons, based on monolayer differentiation system developed by Jaeger and colleagues[87]. Briefly, Jaeger *et al* (2011) explored the temporal actions of FGF signalling in mDA neuron fate specification of mouse pluripotent stem cells and reported that a brief inactivation of FGF/extracellular-signal-regulated kinase (ERK) signalling upon neural induction induced mDA neural progenitor characteristics. Successive activation of FGF signalling resulted in the maintenance of DAergic competence in differentiating monolayer cultures. Furthermore, FGF inhibition directed a midbrain specific fate in the derived DA neurons at the expense of other regional identities such as forebrain and caudal neural fates. Subsequent combinatorial stimulation with Shh, FGF8 and an array of survival-promoting factors applied in a stepwise manner directed the robust differentiation of "authentic" mDA neuronal yields from both human and mouse pluripotent stem cells. The authors demonstrated that it was possible to obtain TH expression in over 52% of the neurons, which efficiently co-expressed midbrain specific markers including Foxa2, Lmx1a and Pitx3.
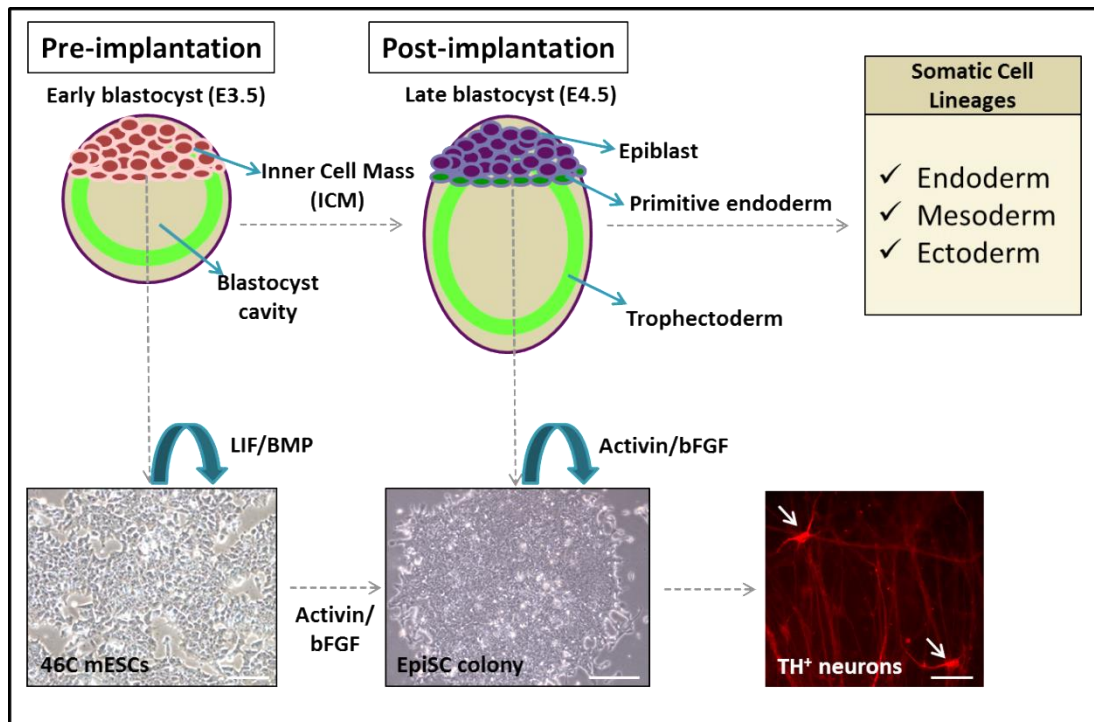
Figure 6.1 Schematic diagram representing the conversion of mESCs to novel epiblast cells during embryonic development *in vivo* and *in vitro*. Pluripotent mESCs are derived from the ICM at the early blastocyst stage[253], and are dependent on LIF and BMP signalling to maintain their pluripotency. Following implantation, occurring at approximately E4.5 in mouse embryos, pluripotent ICM cells constitute an epithelial layer, known as the epiblast, which differentiates during gastrulation to generate the three primary germ layers (i.e. endoderm, mesoderm and ectoderm), giving rise to all adult tissues. For almost 25 years, mESCs represented the only pluripotent cell type derived from mouse embryos, prior to implantation in the uterus[251]. However, the discovery that novel epiblast cells could be isolated from the late epiblast layer of rodent post-implantation embryos (E5.75-6.5) indicated that additional stages of pluripotency could be stabilised *in vitro*[252,254]. Thus, conversion of "naïve" mESCs (pre-implantation embryos) into somatic cell lineages occurs via the primitive ectoderm/epiblast stage (post-implantation embryos), when pluripotent EpiSC lines dependent on activin/nodal and FGF signalling to sustain their pluripotent state are derived[252,254]. Therefore, generation of EpiSCs from mESCs *in vitro* can be achieved using chemically defined activin and basic fibroblast growth factor (bFGF)-containing medium. "Developmentally primed" EpiSCs robustly differentiate into the major somatic cell lineages, including DAergic neurons.

To my knowledge, the combinatorial/synergistic effects of nicotinamide with signalling factors implicated in the induction and maintenance of mDAergic neurogenesis has not been reported before. Therefore, the hypothesis under

investigation in this experimental chapter was that nicotinamide combined with appropriate protein growth factors and small molecules, applied at appropriate concentrations and time points previously determined to direct DAergic specification[87] could further augment mouse pluripotent stem cell differentiation into enriched midbrain specific DAergic neuronal populations. To thoroughly explore the full potential of nicotinamide, *Sox1*GFP mESCs were induced to convert into specialist mEpiSCs of the late epiblast, and the effects of this vitamin metabolite on DAergic differentiation were then examined in both mouse pluripotent stem cell populations representative of different developmental states. This experimental chapter aimed to:

I.   *Investigate whether nicotinamide combined with mDAergic related signalling molecules promotes the generation of higher yields of enriched mDA containing neurons from mESCs.*

II. *Generate EpiSCs from the Sox1GFP mESC knock-in ESC line.*

III. *Investigate if early nicotinamide treatment of these epiblast cells potentiates the generation of mDAergic neurons.*

6.2 Experimental Procedure

Given the direct stimulatory effect of early nicotinamide (10 mM) supplementation on catecholaminergic differentiation (section 4.3.2.1), this experimental chapter sought to further evaluate the potential of this vitamin concentration in directing mDA fate from mouse pluripotent stem cell sources: i.e. *Sox1*GFP reporter mESCs and mEpiSCs. Therefore, nicotinamide treatment was introduced to an adherent,

small-molecule-based neural differentiation paradigm, developed by Jaeger *et al*, which employed the temporally controlled exposure of mouse pluripotent stem cells to an FGF/ERK-deficient environment, together with inductive signalling molecules pertinent to mDA neuron development[87].

6.2.1 mESC differentiation with midbrain DAergic neural-inducing factors and nicotinamide treatment.

*Sox1*GFP reporter mESCs were maintained feeder-free, as previously outlined (Chapter 2, section 2.1.3.1)[87,199,250]. Briefly, 46C-derived monolayer cultures were treated with 10 mM nicotinamide at the early stages of neural differentiation (i.e. day 0-7), and the cells were further differentiated up to a total of 14 days. Where indicated (Figure 6.2), N2B27 medium was supplemented with PD0325901 (1 µM; to temporally block FGF/ERK activity; Axonmedchem, Groningen, The Netherlands), ventralising signals Shh (200 ng/ml; R&D systems, Abingdon, UK) and FGF8b (100 ng/ml; PeproTech, London, UK), and pro-survival factors GDNF (10 ng/ml; PeproTech), BDNF (10 ng/ml; PeproTech) and AA (0.2 mM; Sigma, Aldrich, UK).
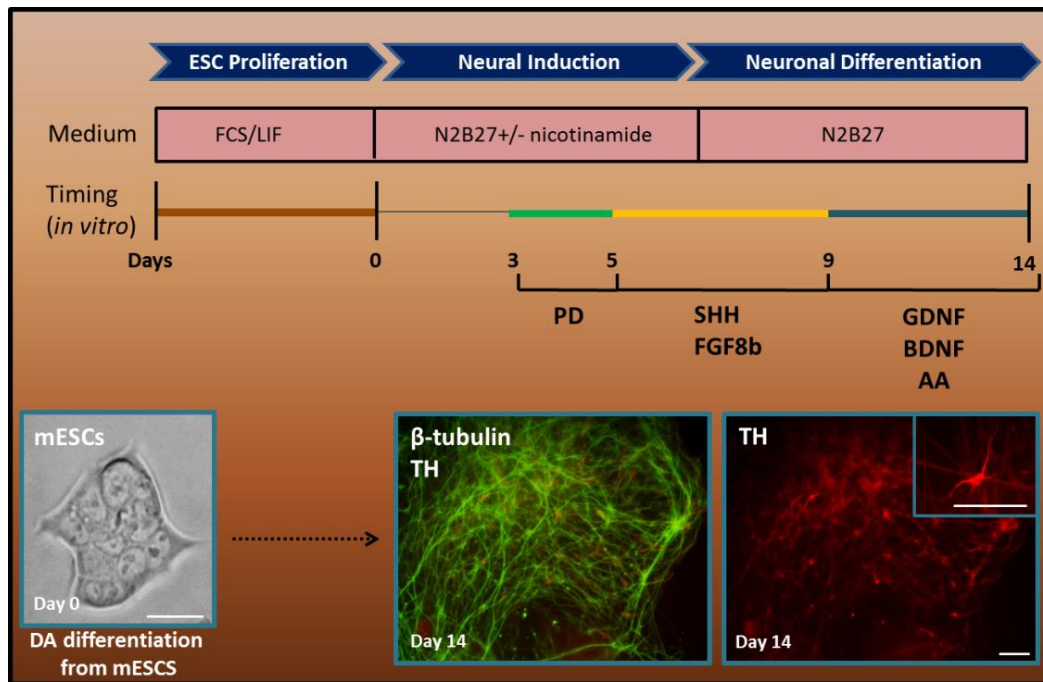
Figure 6.2 Schematic procedure to investigate the influence of nicotinamide on the induction of midbrain specific DA neuron differentiation from *Sox1*GFP mESCs. Exponentially proliferating undifferentiated pluripotent mESCs were maintained in FCS/LIF medium. Withdrawal of self-renewal stimulus (i.e. LIF), where the cells are switched to N2B27 is designated as day 0 of monolayer differentiation. Defined culture conditions[87] were established to direct the differentiation of 46C mESCs into mDAergic neuronal cultures. To examine the differentiation potential of the vitamin B_3 metabolite on mDA neuron production, undifferentiated cells were cultured as monolayers in N2B27 medium in the presence of nicotinamide during the early stages of ESC differentiation, combined with the stepwise addition of key inductive and maturation signalling factors at distinct time points of mDA neuron differentiation, until day 14.

Brief pharmacological inhibition of FGF/ERK signalling upon neural induction, reported to induce midbrain-specific traits in differentiating cultures[87], was achieved using a PD compound (potent MEK blocker: PD0325901) between days 3 and 5. Shh and FGF8b were added between days 5-9, which spans the peak of neurogenesis (i.e. *Sox1*^{+} NPC production is at its peak). A panel of survival-promoting factors; i.e. GDNF, BDNF and AA were added to cultures between days 9-14, to promote the survival and maturation of 46C mESC-derived TH-expressing neurons. The potential of nicotinamide in this experimental paradigm was assessed for immunocytochemistry at day 14 of monolayer differentiation, with antibodies specific to the dopaminergic neuron marker, TH (red) and the immature neuronal marker, βIII-tubulin (green).

6.2.2 Conversion of naïve mESCs to the primed mEpiSC pluripotent state, via activin and bFGF treatment.

mEpiSCs were generated from the 46C *Sox1*GFP reporter cell line and maintained in a pluripotent and self-renewing state by bFGF and Activin/Nodal signalling (appendix 2)[252]. This protocol is detailed in the Material and Methods' chapter (section 2.1.4.1).

6.2.3 EpiSC differentiation with mDAergic neural-inducing factors and nicotinamide treatment.

Monolayer neural differentiation of mEpiSCs was established based on the protocol developed by Jaeger *et al* (2011)[87]. One modification to this differentiation procedure was devised in the present study; i.e. presence/absence of 10 mM nicotinamide between days 0 and 7. Briefly, mEpiSCs were plated on fibronectin-coated plastics and cultured in EpiSC media, until approximately 50-60% confluency. Cultures were rinsed twice with PBS and switched to retinol-free (RF) N2B27 differentiation medium (appendix 2); designated as day 0 of monolayer differentiation. Similar to 46C mESC differentiation, medium with factors was replenished every alternate day. Where indicated (Figure 6.3), the concentration of added small molecule factors was: presence/absence of nicotinamide (10 mM), PD0325901 (1 µM), Shh (200 ng/ml), FGF8b (100 ng/ml), GDNF (10 ng/ml), BDNF (10 ng/ml) and AA (0.2 mM), which were added to differentiating monolayer cultures every alternate date until day 14, after which the cells were processed for immunocytochemistry. A detailed protocol of EpiSC differentiation is provided in the Material and Methods' chapter (section 2.1.4.2).
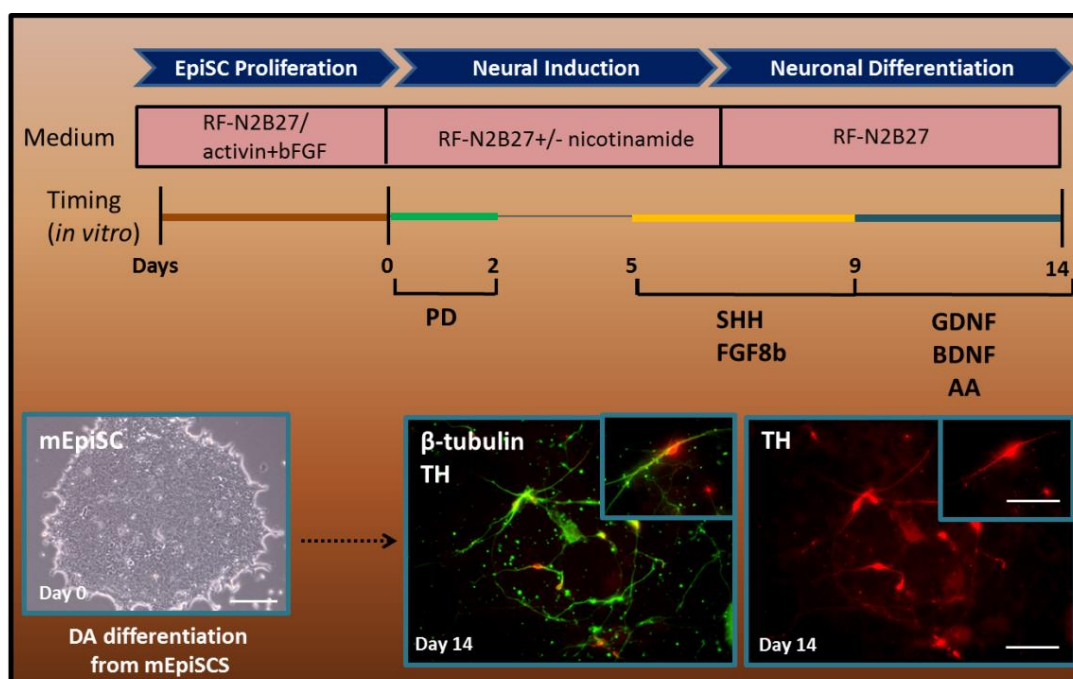
Figure 6.3 Schematic procedure for inducing DA neuron differentiation from mEpiSCs, using a small-molecule-based strategy with nicotinamide treatment. Proliferating undifferentiated pluripotent EpiSCs were maintained in EpiSC medium, supplemented with activin and bFGF, until approximately 50-60% confluency. To induce monolayer neural differentiation of EpiSCs, self-renewal stimuli (i.e. activin/bFGF) were removed and the cells were switched to RF-N2B27 (i.e. day 0 of monolayer differentiation). To examine the differentiation potential of the vitamin B_3 metabolite on mDAergic neuronal production from mEpiSCs, undifferentiated cells were cultured as monolayers in RF-N2B27 medium in the presence of nicotinamide from days 0 to 7, and combined with the stepwise addition of inductive and maturation signalling factors at distinct stages of mDA neuron differentiation, until day 14. A brief inhibition of FGF/ERK signalling on exit of the lineage-primed epiblast pluripotent state was achieved using a PD compound (potent MEK blocker: PD0325901) between days 0 and 2. PD0325901 was administered at an earlier time point since neural induction was accelerated in the EpiSC differentiation system (occurs during the first 2-3 days of EpiSC differentiation). Shh and FGF8b were applied between days 5-9 and an array of survival-promoting factors were added to cultures during neuronal differentiation between days 9-14. The potential of nicotinamide in this experimental paradigm was assessed by immunocytochemistry at day 14 of monolayer differentiation.

6.2.4 Immunocytochemistry

To study the effect of nicotinamide on mDAergic generation from pluripotent mouse stem cell sources, immunocytochemical staining was performed as previously described (Material and Methods' chapter, section 2.1.5.1), using primary antibodies

against the neuron-specific postmitotic βIII-tubulin marker and TH, the early marker of DA neurons. Cell nuclei were counterstained with DAPI.

6.2.5   Image and statistical analysis

For preliminary assessment of the effects of nicotinamide treatment on mDA neuronal generation from 46C-derived mESCs, cell quantification of TH^+ neurons (expressed as a percentage of the total cell population) was performed manually on 6-8 random fields of view. Cell count data were averaged across three coverslips per culture and experiments were repeated three to five times (i.e. n = 3–5 independent experiments). To determine the preliminary effects of nicotinamide on mDA neuron differentiation of mEpiSCs, the average number of TH-expressing neurons per field was quantified manually from 6-8 random fields of view, in each well of a 12-well tissue culture plate (i.e. n = 1).

In this experimental chapter, where multiple data replicates were collected, treatment groups were compared using an unpaired t test. Results were considered statistically significant for p values less than 0.05. Where specified, data plotted on graphs are expressed as mean ± SEM.

6.3 Results

6.3.1 Enhanced generation of TH-expressing cells from mESCs via adherent monolayer differentiation.

Following a total of 14 days of differentiation, using inductive signalling molecules but without nicotinamide, a substantial number of βIII-tubulin$^+$ neurons, corresponding to enhanced yields of TH$^+$ cells was evident under defined culture conditions, following the stepwise treatment of 46C-derived ESC cultures with small molecule signalling factors implicated during normal DAergic development *in vivo*. High-density βIII-tubulin$^+$ and TH$^+$ neuronal cell clusters were frequently observed under this culture paradigm (Figure 6.4 and 6.5).
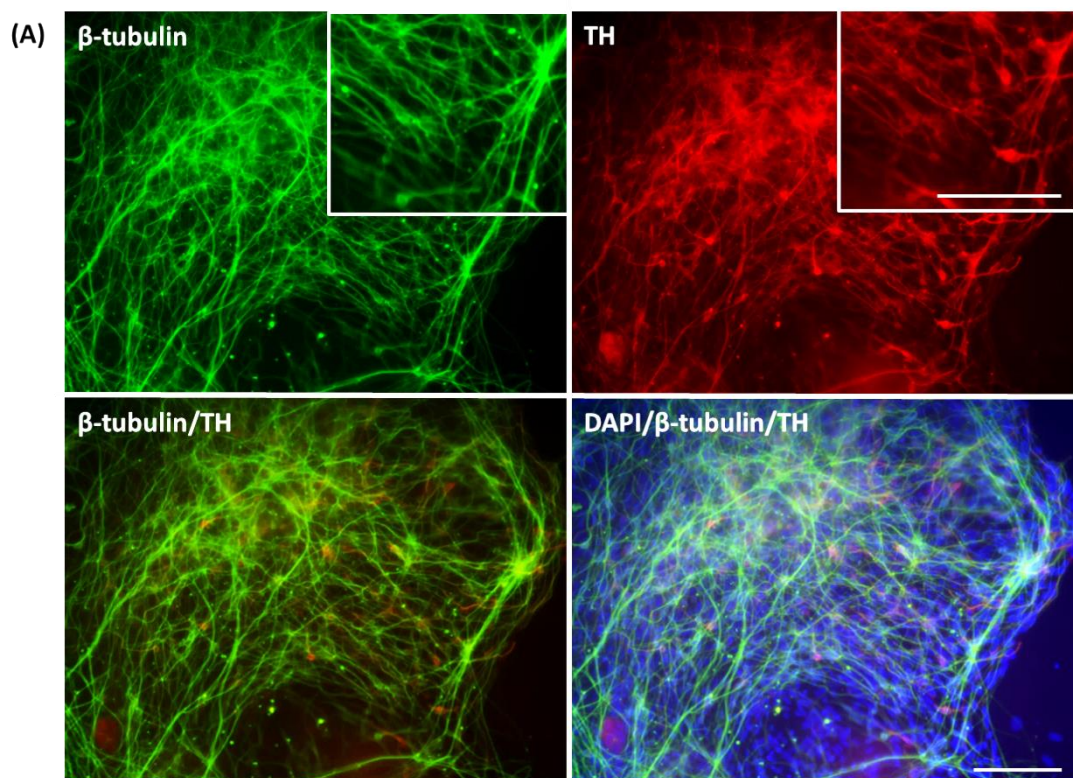
Figure 6.4 Enhanced generation of TH-expressing neurons, derived from 46C mESCs via monolayer differentiation. (A) Immunofluorescence images of TH$^+$ neuronal populations generated from mESC-derived monolayer cultures, showing immunostaining with antibodies specific to the DA neuronal marker, TH (red) and the immature neuronal marker, βIII-tubulin (green). Double-staining techniques indicated co-localisation of TH-expressing neurons with βIII-tubulin$^+$ neurons, at day

14 of monolayer differentiation. The nuclei of all cells in cultures were labelled with DAPI (blue). Under defined differentiation conditions,[87] higher frequencies of TH immunoreactive cells were robustly produced, compared to monolayer cultures without exogenous inductive and pro-survival signalling factors, in which no appreciable numbers of TH$^+$ cells were detected (detailed in section 4.3.2.1). Scale bar = 100 μm.
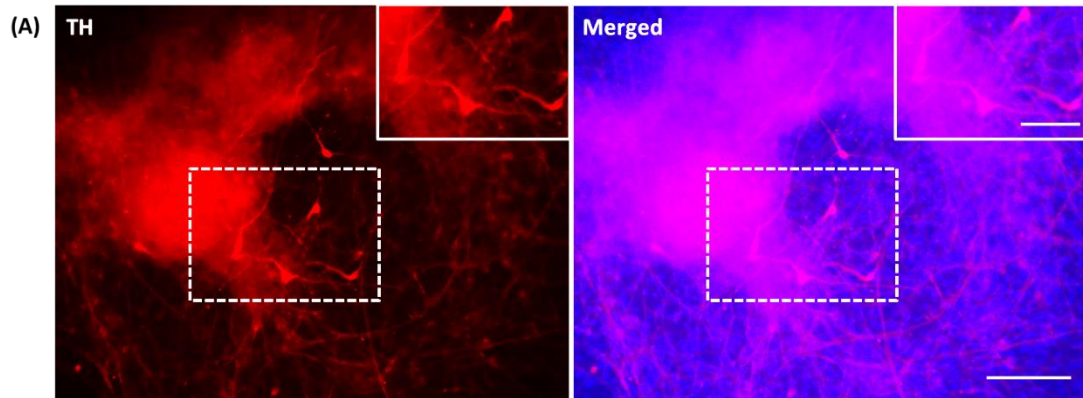
Figure 6.5 (A) TH-expressing cells were frequently observed in cell clusters, counterstained with DAPI, which displayed obvious neuronal morphologies. Scale bar = 100 μm.

6.3.2 Stepwise directed differentiation of 46C-derived mESCs to mature neuronal cultures.

Treatment of mESCs during the course of their differentiation with well-established inductive molecules, which have been successfully applied in a number of *in vitro* differentiation protocols, particularly to direct mDA neuron differentiation (Figure 6.2)[78,87] results in the development of neuronal morphologies and formation of TH$^+$ cell networks, observed in a more mature neuronal culture system (Figure 6.6).
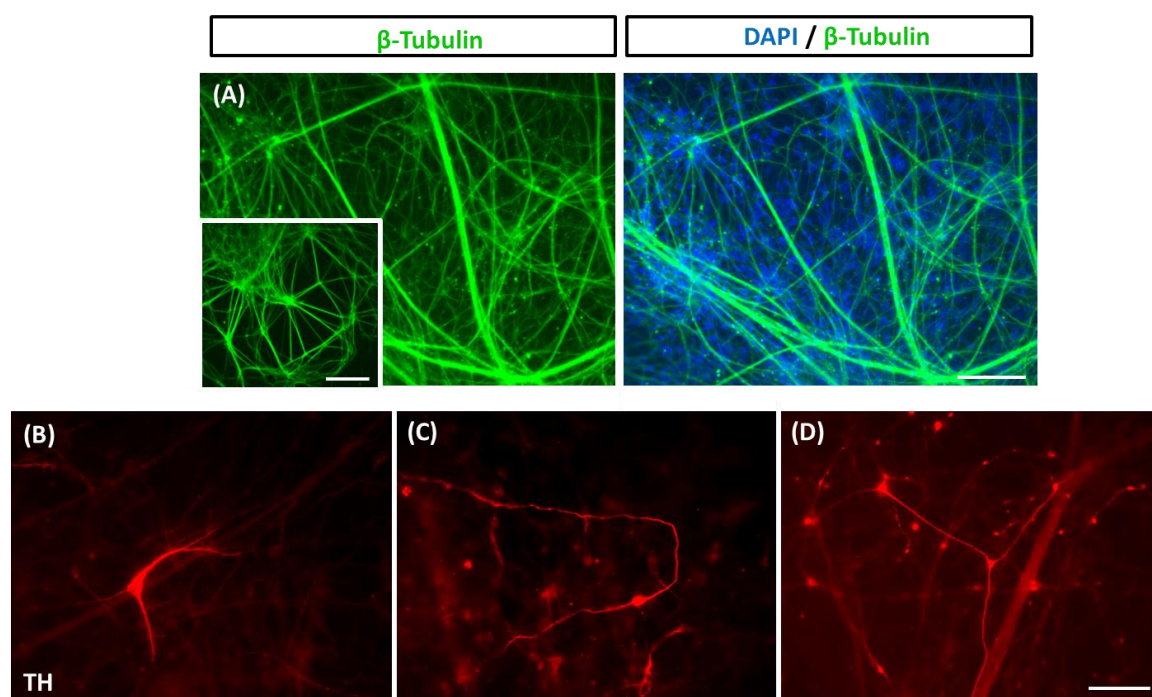
Figure 6.6 Enhanced neuronal maturation in mESC-derived cultures. (A) Extensive axonal branching and elaborate neuronal networks (inset) in βIII-tubulin$^+$ neuronal populations (green) were observed; a possible contribution of the combined effects of GDNF, BDNF and the vitamin C metabolite, AA, during the late stages of neuronal development. The nuclei of all cells in cultures were counterstained with DAPI (blue). **(B-D)** TH immunoreactive cells displayed enhanced neuronal morphologies, increased neurite outgrowth, and formed TH$^+$ neuronal networks, following 14 days of differentiation. **(A-D)** Scale Bars = 100 μm.

6.3.3 Nicotinamide treatment enhances the production of TH-expressing cells from mESCs.

To determine the potential of the vitamin B$_3$ metabolite, in terms of the percentage of TH$^+$ cells generated from the monolayer-derived *Sox1*GFP knock-in 46C cell line, nicotinamide treatment was included in the Jaeger protocol, outlined in section 6.2.1. *Sox1*GFP mESCs were differentiated up to a total of 14 days of the monolayer culture period (Figure 6.2).

Under this differentiation method, early supplementation of 10 mM nicotinamide (i.e. day 0-7) elicited a significant increase in the percentage of TH$^+$ immunoreactive

neurons of the whole cell population (unpaired t test, t = 11; $p<0.001$) [12.7 ± 0.4% vs. 6.2 ± 0.4% in control conditions; Figure 6.7 (A-C)].

With respect to cell quantification in this experiment, individual βIII-tubulin-expressing neurons were not quantified manually for data analyses due to the high density of βIII-tubulin$^+$ neuronal networks. Therefore, it was not possible to express the number of TH$^+$ cells as a proportion of total neurons, to determine if this effect is associated with an enrichment of TH$^+$ versus total neurons.

The current study further confirms an important role for nicotinamide during the early stages of ESC differentiation, possibly at the level of NPC formation, to direct the production of ESC-derived TH$^+$ neurons during monolayer differentiation. It is reasonable to conclude that nicotinamide may function synergistically with protein signalling molecules such as Shh and FGF8 during neural specification to direct differentiating neural progenitors adopting a DAergic cell fate.
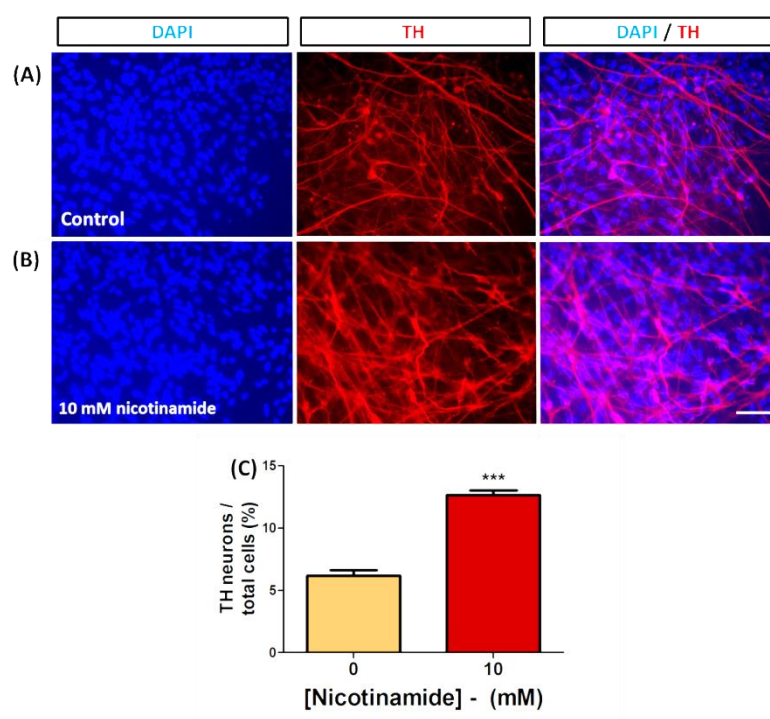
Figure 6.7 Enhanced production of TH$^+$ neurons, derived from 46C mESCs via monolayer differentiation with nicotinamide treatment. Immunocytochemical

analysis of monolayer cultures supplemented with N2B27 medium without nicotinamide **(A)** and with the addition of 10 mM nicotinamide **(B)**. Representative images show immunostaining with the antibody specific to TH (red). Total cells in cultures counterstained with DAPI (blue). Scale bar = 50 μm. The percentage of TH$^+$ neurons obtained from mESCs increased when 10 mM nicotinamide was added to media. **(C)** Bars represent percentage TH immunoreactive neurons as a proportion of total cells. Differentiating cultures treated with 10 mM nicotinamide show a significant increase in the percentage of TH-positive neurons per culture, compared to control conditions.
***$p<0.001$

6.3.4 Small molecule induced induction of mESCs into a primed epiblast state.

In the next part of the study, pluripotent mEpiSCs were successfully established from *Sox1*GFP mESCs under directed EpiSC culture conditions without LIF but including activin and bFGF induction, to sustain the self-renewal capacity and pluripotency in EpiSC cultures (Figure 6.8).

Cells were routinely passaged every other day, and 10-15 passages were required to purify epiblast cultures from mESCs. Unlike mESC maintenance, EpiSCs are not trypsinised to a single cell suspension. Thus, cell cultures are not counted for passaging, therefore passaging densities must be determined empirically. The plating density of EpiSCs is important to maintain their undifferentiated state, and also to ensure that cells are not plated sparsely/at low densities, as this would result in extra population doublings to reach confluency.
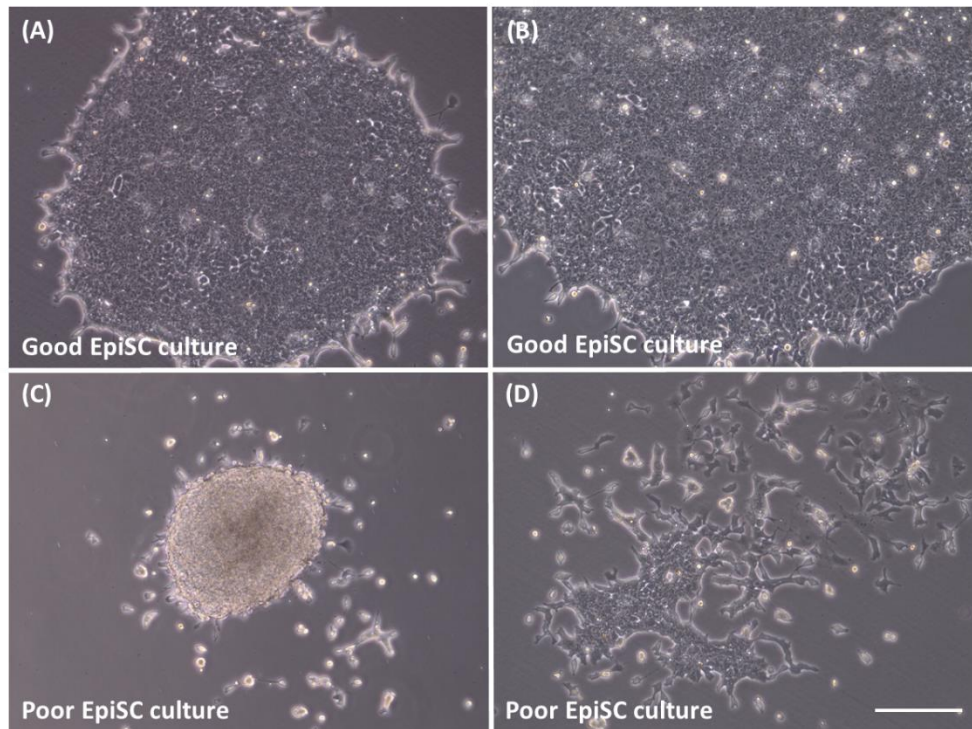
Figure 6.8 Derivation of EpiSCs from *Sox1*GFP knock-in cell line. Representative brightfield cell images of EpiSC cultures. **(A&B)** EpiSCs should form flat, compact and rounded colonies with an epithelial morphology. Prominent nucleoli and a high nuclear/cytoplasmic ratio are both characteristic of EpiSC cultures. **(B)** It is important that colonies show sharp, defined borders, with no evidence of differentiation. **(C&D)** When converting mESCs to EpiSC cultures, it is important to observe the morphology of the cells and ensure that they form tightly packed colonies without forming 3D structures (indicative of differentiation) **(C)** or dissociating into single cells **(D)**. Scale bar = 100 μm.

6.3.5 Differentiation of mEpiSCs with nicotinamide supplementation into DAergic neuronal populations.

Nicotinamide treatment was introduced to a novel method of mDA neuron differentiation, to investigate its effect on the differentiation of pluripotent epiblast cells, derived from the *Sox1*GFP mESC line. EpiSCs were subjected to a monolayer differentiation protocol (Figure 6.3), previously established in ESCs[87]. In this study, further efforts were made to enhance the population of TH-expressing neurons by

addition of nicotinamide to the early stages of EpiSC differentiation, combined with the sequential application of inductive signalling molecules to potentiate DAergic differentiation from EpiSCs.

TH^+ neuronal cells located in dense $DAPI^+$ and βIII-tubulin$^+$ cell clusters were not included in data collection, since these neural networks were unquantifiable and therefore not expressed as a percentage of total neurons or as a percentage of total cells counterstained with DAPI (Figure 6.9). Further, due to the high density of cells in clusters, it was not possible to provide a quantitative assessment in terms of the number of TH neurons independent of treatment. Thus, preliminary findings in this EpiSC differentiation study are presented as the mean TH^+ neuronal cell count per field, observed in low density areas.

Preliminary data suggest that nicotinamide did not induce significant differences in the mean number of TH^+ neurons per field of view, when added between days 0 and 7 of EpiSC neural differentiation. However, it is important to point out that the results of this study are preliminary and were obtained from one experiment (i.e. n = 1); therefore further work to optimise the EpiSC model system is necessary.

Although further optimisation is required using both the mESC and mEpiSC differentiation models to establish appropriate plating densities, important preliminary findings generated under the mESC experimental paradigm strongly suggest that nicotinamide may function as a key factor to stimulate a higher extent of differentiation toward a DAergic cell fate in ESCs.
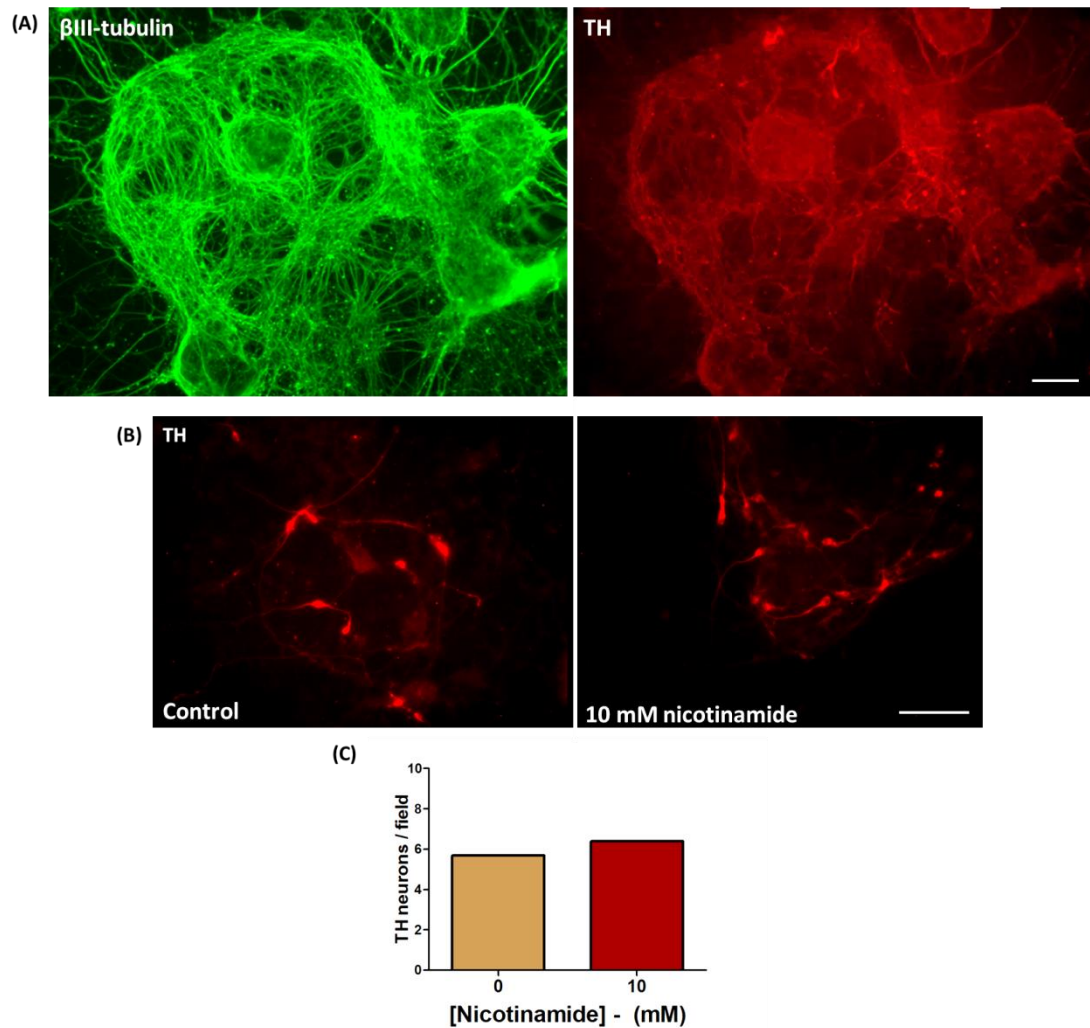
Figure 6.9 Effect of nicotinamide on the generation of TH-expressing neurons, from mEpiSCs via monolayer differentiation. (**A**) Representative images of neural differentiation of mEpiSCs by inactivating the FGF signalling pathway. Immunocytofluorescence images show total cells in cultures labelled with DAPI (blue), and immunostaining with antibodies specific to immature neuronal marker βIII-tubulin (green-expressing cells) and TH (red). Scale bar = 100 µm. (**B**) Immunocytochemical analysis of monolayer EpiSC-derived cultures supplemented with RF-N2B27 medium without nicotinamide and with the addition of 10 mM nicotinamide. Representative images show immunostaining with the antibody specific to TH (red). Nicotinamide added during the early stages of EpiSC differentiation elicited no effect on the average number of TH-expressing neurons per field. Scale bar = 100 µm. (**C**) Bars represent the mean TH$^+$ neuronal cell count per field. Preliminary results show that nicotinamide, when added between days 0 and 7, did not induce differences in the number of TH$^+$ neurons per field between control and 10 mM nicotinamide conditions.

6.4 Discussion

Identification of differentiation conditions to direct efficient commitment to a DAergic cell fate is crucial to maximise the potential of pluripotent stem cells towards a CRT for PD. The current study utilised pluripotent mESCs and mEpiSCs to identify the effects of nicotinamide on DAergic neural differentiation. This experimental chapter provides further crucial evidence that the biologically active vitamin B_3 metabolite, nicotinamide, functions as a key factor to direct the development of TH^+ neurons from mESCs. Thus, this important preliminary finding strongly suggests that our understanding of the genetic cues governing the induction of mDA NPCs and the specification of the mDA neuronal cell fate is far from complete, despite rapid progress over the past few years.

6.4.1 Nicotinamide significantly enhances TH^+ neuronal differentiation from Sox1GFP mESCs.

To my knowledge, the current study presents the first report demonstrating significantly enhanced neural conversion of mESCs to TH^+ neurons, using nicotinamide supplementation during the early stages of ESC *in vitro* development. Specifically, nicotinamide added to monolayer cultures, at an effective concentration (i.e. 10 mM) previously determined to efficiently promote catecholaminergic differentiation, stimulated a significant increase in the production of TH-expressing neurons of the total cell population. This finding highlights the reproducible nature of the effect of nicotinamide on catecholaminergic differentiation, using two distinct experimental paradigms[87,201]. It should also be noted that attempts to increase the number of DAergic neurons by nicotinamide induction should focus on the early developmental time window of ESC development (i.e. day 0-7). In this regard,

nicotinamide does not have a further instructive role during neuronal differentiation of NPCs (i.e. day 7-14; section 3.3.1).

Another important point of note from this study established that 10 mM nicotinamide supplementation alone during mESC-derived TH$^+$ neuronal differentiation (generated 4.2% of TH$^+$ cells per culture; section 4.3.2.1;), displayed almost a similar potency to the yield of TH-expressing cells per culture produced in control conditions (6.2%), under the experimental paradigm devised by Jaeger *et al* Thus, this vitamin B$_3$ metabolite functioned as an important signalling molecule to direct the production of new TH-expressing cells from ESCs, sharing a similar potency to well-established extracellular factors known to induce mDA specification. In other studies, the vitamin A metabolite, RA has been reported to function synergistically with Shh, FGF and BMP to direct the patterning of differentiating progenitor cells, by directing differentiating rostral-like cells to develop caudal characteristics[257].

Due to the highly-dense βIII-tubulin$^+$ neuronal networks observed under this mESC differentiation paradigm, it was not possible to quantify individual βIII-tubulin-expressing neurons localised within elaborate networks of axonal processes; a possible attribution of the neuroprotective factors administered between days 9 and 14 of differentiation. Therefore, it was not feasible to determine whether TH$^+$ neuronal enrichment was further enhanced by nicotinamide in the current monolayer differentiation system. Nevertheless, preliminary data do not negate the potential of nicotinamide as a useful candidate in future protocols for increasing TH$^+$ cell differentiation. In future work, neuronal cultures plated at various cell densities should be investigated, to optimise mESC seeding densities in order to reduce the number of high-density neuronal networks by day 14 of monolayer differentiation.

Alternatively, confocal microscopy may prove advantageous to quantify TH$^+$/βIII-tubulin$^+$–cells to confirm colabeling for both markers, and therefore determine whether nicotinamide further enhances cultures enriched for DAergic neurons, toward generating homogenous populations of DAergic neurons suitable for a transplantation therapy for PD.

DAergic neurons are localised throughout the vertebrate CNS, with the largest assembly in the midbrain. However, approximately 25 % of DA containing neurons are present in other brain regions[178] Many *in vitro* differentiation studies determine mDA neuronal phenotype by assessing for TH expression in differentiated cultures.[203] However, TH expression is observed in all catecholaminergic neuronal subtypes, such as adrenergic and noradrenergic neurons[258]. In this regard, the Parmer and Li study reported that TH immunoreactive neurons co-expressed Nurr1, a gene characteristic of DAergic markers but excluded from other catecholaminergic neurons, therefore indicating that *Sox1*GFP-derived TH$^+$ neurons are likely DAergic[178]. Since the protocol established in this study included appropriate instructive patterning molecules necessary for directing DA cell fate, it is reasonable to speculate that the majority of TH-containing neurons reflect DAergic populations. However, it was also reported that under the *Sox1*GFP model system, monolayer-derived TH expressing neurons do not express additional mesencephalic phenotypic markers[178]. Thus, the extent to which 46C mESCs generated DA neurons co-expressing midbrain markers in the presence of nicotinamide remains to be elucidated in an alternative pluripotent stem cell model.

6.4.2 Neural differentiation of epiblast-derived stem cells.

Studies performed in mice models highlighted two distinct pluripotent states *in vivo*, which can be mimicked *in vitro*[252,254]. Specifically, classic mESC cultures are obtained from preimplantation embryos, whilst epiblast cells are derived from postimplantation embryos. However, significant differences exist between mESCs and mEpiSCs. mEpiSCs share many defining features with both pluripotent hiPSC and hESC sources, such as signalling pathways to maintain their pluripotent state; stability of their epigenetic status; cellular morphologies, and their ability to differentiate similarly under defined culture conditions[251]. During early embryogenesis, the epiblast contains more progressed pluripotent cells derived from the ICM, thereby generating the most immediate precursors of the early somatic lineages[259]. Thus, the EpiSC differentiation system serves as an advantageous model to investigate germ-layer specification, since these developmentally primed cells epitomise the last pluripotent state prior to gastrulation. Furthermore, the broad similarities that exist between epiblast cells and hESCs derived from the late epiblast indicate that cells of human origin more closely reflect characteristics of mEpiSCs than mESCs[252]. Thus, the potential of nicotinamide was examined in a more efficient protocol using intermediate epiblast cells[87].

Neural differentiation using mEpiSCs was based on a strategy which offered several advantages over current strategies for directing DAergic differentiation[87]. Firstly, this protocol has demonstrated a propensity to direct midbrain regional identity in the generated DA neurons more readily from pluripotent cell sources including mEpiSCs, miPSCs and two hESC lines. Furthermore, previous protocols using ESCs to generate TH^+ neuronal populations have often proved unreliable and highly variable between experiments, which may be due to the temporal and spatial heterogeneity observed in ESC-derived NPCs[87]. However, Jaeger and colleagues

reported a simple strategy to enhance the generation of TH$^+$ neurons co-expressing Foxa2, Lmx1a and Pitx3, likely due to the more synchronous conversion of EpiSCs to the neuroepithelial fate, thereby facilitating efficient capture of mDA-competent progenitors. Further, previous findings outlined in Chapter 4 demonstrated a bias toward the generation of high yields of GABAergic neurons from mESCs (61%), with a maximum of 7.5% TH$^+$ neurons reported in control conditions, without any exogenous stimuli. Interestingly, the Jaeger study found that neural progenitors exposed to sequential treatment with the small inhibitory PD molecule and ventralising signals (i.e. Shh and FGF8) displayed reduced expression of non-DA immunomarkers, therefore indicating the necessity of appropriate signals required to direct efficient mDA fate specification and differentiation[87].

However, it is clear from this study that extensive optimisation of the EpiSC model is required to improve culture conditions and plating densities for differentiation of EpiSCs, to fully evaluate the effects of nicotinamide on mDA specification and differentiation. It is important to note that the midbrain DA neuron protocol established by Jaeger and colleagues is suboptimal for inducing TH immunoreactive cells, co-expressing FOXA2, Pitx3 and Lmx1a expression[87]. Therefore, in future work is will be of interest to determine whether nicotinamide further augments mDA neuron differentiation.

6.4.3 Conclusion and Further Studies

This study provides an important foundation for the role of nicotinamide in differentiation of mESCs, however further studies are essential. Although a number of optimisation issues including plating densities need to be overcome with respect to the mESC and mEpiSC differentiation protocol, it is encouraging to note that

combinatorial use of nicotinamide with appropriate inductive and pro-survival signalling molecules generates significantly increased TH$^+$ neuronal populations from *Sox1*GFP mESCs. However, complications can arise when a protocol established in a mouse model system is adapted for use with human cells. Therefore, translation of the potential of the small molecule nicotinamide to a more efficient protocol using the EpiSC model, comparable to the human differentiation system is crucial. Further optimisation of this novel method will provide new information on the importance of vitamin B$_3$ in the production of new mDA nerve cells. Moreover, access to a large and enriched population of midbrain dopaminergic cell types, using the EpiSC differentiation protocol, should yield a scalable source of neurons for evaluation in preclinical models to test the potential of nicotinamide, and its ability to promote neural differentiation, towards development of future neural cell-based therapies for patients. In these contexts, other areas worth investigating in future studies include the influence of nicotinamide on SIRT1 during DA neurogenesis[226,232,260].

Chapter 7: Summary and General Discussion

7.1 Summary of thesis findings

Knowledge of the effects and timing of inductive molecules is fundamental for advancing prospective therapies to generate neuronal populations from ESCs. In this regard, a monolayer system, comprising a cell line engineered to express a reporter of neural specification, *Sox1*GFP, was employed here, to facilitate direct visualisation of the neural progenitor state in living cells with fluorescence microscopy. This advantageous feature of the 46C line allowed, for the first time, a detailed investigation of the effects of the vitamin B_3 metabolite, nicotinamide, on ESC-derived cell populations at any point during monolayer differentiation. It was demonstrated in this thesis, for the first time, that nicotinamide plays an important role in directing mESC differentiation specifically towards a neuronal lineage.

The findings presented in this thesis can be summarised as follows:

- By adding nicotinamide to cells at an early stage of the monolayer protocol, differentiation of *Sox1*GFP mESCs was directed rapidly into the neural lineage. Nicotinamide added at later stages of differentiation had no effect on neuronal induction.

- In nicotinamide-treated cultures, the marker for pluripotent ESCs, Oct4, was down-regulated rapidly, concomitantly increasing GFP^+ neural precursor cells and immature neurons at day 4. By day 14, nicotinamide treatment (from day 0-7) reduced both $Oct4^+$ and GFP expression concomitant with enhanced expression of neuron-specific βIII-tubulin, indicative of accelerated neuronal differentiation.

- Treatment with nicotinamide at the initial stages of ESC differentiation not only promoted neuronal differentiation, but also reduced the total number of cells in adherent monolayer cultures by day 14 of differentiation. This important finding showed that this vitamin derivative fucntions to promote the enrichment of neurons versus non-neuronal cells.

- Nicotinamide selectively enhanced the production of specific neuronal phenotypes, i.e. catecholaminergic and serotonergic neuronal populations.

- The differentiation-promoting effects of nicotinamide were dose dependent, being significant for 5 mM and especially 10 mM doses. High doses of nicotinamide (20 mM) showed an obvious noxious effect on cell suvival. Crucially, cultures treated with 10 mM nicotinamide doses demonstrated no adverse effects on cell viability or cell death.

- The mechanims by which nicotinamide reduced total cell numbers in cultures and by which cells alter their fate were addressed by investigating cell cycle exit and apoptotic mechanisms in specific cell populations. For the first time, nicotinamide was demonstrated to act at the initial stages of ESC differentiation to suppress *Sox1* precursor proliferation, thereby promoting the transition from neural progenitors to neurons. This important finding is in agreement with the ability of nicotinamide to efficiently commit cells to a neuronal fate, and accelerate maturation and neurite formation in catecholaminergic and GABAergic populations.

- In a preliminary study to address the mechanism of action of nicotinamide on neuronal differentiation, the expression of a potential target for its action, SIRT1, was shown to progressively decrease in monolayer cultures with time

in vitro, in agreement with reports showing that down-regulated SIRT1 expression is fundamental to support neuronal differentiation.

- Immunocytochemical analyses further indicated that the sub-cellular localisation of SIRT1 is predominantly nuclear in neural progenitor and neuronal cell populations. Given that SIRT1 has been reported to modulate chromatin structure by deacetylating specific lysine residues in histones[261], these observations support function of the target protein within the neuron.

- The potential of nicotinamide was combined with external signalling factors known to enhance a DAergic phenotype. Specifically, nicotinamide functioned synergistically with signalling molecules implicated in the induction and maintenance of mDAergic neurogenesis to enhance TH-expressing neuronal populations from *Sox1*GFP mESCs.

- Pluripotent mEpiSCs were successfully established from *Sox1*GFP mESCs under directed EpiSC culture conditions. In future work, optimisation of this differentiation model should enable access to a large and enriched population of mDAergic cell types to test the potential of nicotinamide on their survival and maturation.

The immediate implications of these findings towards the potential of nicotinamide, a derivative of vitamin B_3, in future neuronal replacement therapies have been discussed in detail within each experimental chapter. ESCs are considered to be an invaluable in vitro biological tool not only for examining the mechanisms of pluripotency, but also to study effects of different factors for directing cell fate decisions throughout early development. Further to this, ESCs facilitate disease

modelling, pharmacological screening and most importantly, serve as an unlimited source of pluripotent cells toward regenerative therapies.

Currently, the challenge in ESC biology to advance the potential of pluripotential stem cell sources is to establish criteria for determining effective differentiation[61−63]. In this regard, significant issues need to be resolved before cells are translated to the clinic, including the production of high yields of purified neuronal populations of appropriate phenotype with control over proliferation to prevent tumorigenesis[63]. Novel findings presented in this thesis suggest that nicotinamide may be a key external signalling factor in helping to achieve this goal, to translate effective laboratory cell-based protocols to patients in clinical trials. The discussion here will consider future development of this PhD project.

7.2 General discussion

Results presented in this thesis establish that nicotinamide functions as a regulator of neuronal differentiation and neurite outgrowth and maturation, thus supporting previous evidence of the fundamental role of vitamins and their metabolites during early development[104,115,126,128,161−163,191,239,242,243,262].

The importance of vitamin signalling in the development of mDA neurons was recently reported in a proteomics study[263], which identified key signalling molecules linked to vitamin B_3 and vitamin D_3 specifically. Protein expression in the rat embryo during mDA neuronal development was studied using an iTRAQ proteomics approach, and successfully identified signalling proteins including: nicotinamide nucleotide dehydrogenase, vitamin D receptor and vitamin D binding prepeptide[263]. Therefore, expression of key signalling proteins for these vitamins is present at the correct time and place to direct mDA neurogenesis. Further, the final experimental

chapter presented in this thesis (Chapter 6), reported that the combinatorial use of nicotinamide with appropriate inductive and pro-survival signalling molecules generated significantly increased TH$^+$ neuronal populations from *Sox1*GFP mESCs. This critical finding, shows for the first time, that the vitamin B$_3$ metabolite functions as a key factor in directing ESCs adopting a DAergic fate.

7.2.1. Future directions to identify distinct mechanisms underlying nicotinamide-induced DAergic neural lineage-specification of Sox1GFP mESCs.

Detailed knowledge of the exact timing of action of small molecules during neuronal differentiation is not only critical for establishing effective differentiation protocols to direct clinically relevant lineages for regenerative therapies, but also to uncover mechanisms underlying small molecule induced lineage-specification of pluripotent stem cells. Therefore, with respect to future mechanistic investigations related to this project, it will be necessary to establish the exact time-window of ESC differentiation where nicotinamide predominantly acts on cells to increase TH$^+$ neuronal production (i.e. between days 0-7).

Findings presented in this thesis provide critical insight into the effects of nicotinamide during the initial phases of ESC development. It is interesting to note that addition of nicotinamide from day 0 of monolayer differentiation elicited a more potent effect on neuronal differentiation by day 7, which promoted significantly enhanced yields of βIII-tubulin$^+$ neurons [15.2% vs. 3.4% in control groups; section 5.3.3.3, Chapter 5], in contrast to nicotinamide treatment between days 2-7 [7.3% vs. 3.3% in control groups; section 3.3.5.3, Chapter 4], which showed less effect.

Sox1 expression is detectable in all NPCs during early time points of embryonic development, but is not detectable in the VM at the stage when DAergic neurons are produced during embryonic development *in vivo*[178]. Since, the time-window for DAergic specification is quite narrow (i.e. pre-*Sox1* expression stage, tightly linked with the acquisition of a pan-neuroectodermal fate), it will be of interest to determine whether nicotinamide exclusively promotes DAergic differentiation in progenitor cells prior to *Sox1* expression (i.e. day 0-2), or during the stage of *Sox1*GFP$^+$ NPC formation (early born progenitor cells observed between days 3-4, and late born progenitors detected at day 7). Further to this point, in experimental chapter 4, a plausible explanation for the early differentiation-inducing effects of nicotinamide on catecholaminergic and serotonergic populations may be that this early time window (i.e. day 0-2) precedes the generation of TH and 5-HT NPCs, thereby suggesting that nicotinamide may drive the ontogenetically early processes for DAergic and serotonergic specification, analogous to those which occur at the midbrain-hindbrain organiser and the primitive streak during normal development *in vivo*.

Thus in future work to uncover the key developmental stage where nicotinamide acts during neuronal development, further experiments will be required, to determine the effects of nicotinamide on the process of neural induction (days 0-2), or between days 2-7 to investigate its influence NPC populations (day 2-7). Of particular interest to this study, it will be necessary to further differentiate the cells for a total of 14 days rather than examining differentiated cultures for TH expression at days 2 and 7 respectively, since Parmer and Li reported the presence of very few TH-expressing neurons at day 10 of differentiation, using the *Sox1*GFP model system[178]. Alternatively, the use of other reporter cell lines, such as the *Pitx3*GFP knock-in cell

line to track midbrain-specific DA differentiation from stem cells *in vitro*, would provide further insight into the effects of nicotinamide on DAergic fate specification.

Currently, the precise mechanism by which nicotinamide exerts its actions on developing neurons remains to be elucidated. One interesting hypothesis for the precise mode of action of nicotinamide in mechanisms underlying the promotion of enhanced TH[+] neuronal differentiation, is a potential for SIRT1, a critical factor reported for stem cell function, that acts by controlling cell fate decision[232,233]. Specifically, it was previously reported that nicotinamide directed cardiac lineage specification from the pluripotent state of hESCs[163]. A follow-up study reported that nicotinamide promoted nuclear translocation of NAD dependent histone deacetylase SIRT1 and global chromatin silencing, thereby suggesting that a predominant epigenetic mechanism via SIRT1 underlies the nicotinamide-induced hESC cardiac fate determination[259]. Similarly, with respect to DAergic differentiation, SIRT1 has been reported to play a role in *Nurr1* control of TH expression in human NSCs, during DA neurogenesis[226].

For future development of this project, to investigate the specific elements of the SIRT1 signalling mechanism during neuronal development, it will be necessary to block this pathway to assess how it affects neuronal differentiation. This hypothesis could be addressed using a blocking experiment with a small molecule inhibitor of downstream targets of their known signalling pathway, or by introducing short interfering RNA to inhibit expression of downstream targets.

7.2.2 Future directions to address whether there is a synergistic effect of the active forms of vitamin B₃ and vitamin D₃ on the development of DA neurons?

A number of reports show that neuronal development in the CNS is dependent on a complex array of multiple signalling factors[44,263]. Currently, it is not known whether vitamin metabolites act independently or in concert with each other, through converging pathways to influence neuronal development during ESC differentiation. In this context, nicotinamide has been reported to cooperate with RA or vitamin D_3 to regulate cell differentiation and cell cycle arrest[264]. The effects of calcitriol (active metabolite of vitamin D_3) are mediated through the binding of this ligand to its nuclear receptor and heterodimer formation with RXR and RAR[104]. In turn, these retinoid receptors interact with other coactivator and corepressor nuclear receptors, thereby initiating the expression of more than 500 genes[265]. Endogenous RXR ligands found in the CNS are capable of activating heterodimers formed between RXR and the orphan nuclear receptor[266], Nurr1 *in vivo*; an essential transcription factor required for the development and maintenance of mDA neurons[267]. Thus, it is likely that vitamin metabolites function in defined locations, at appropriate developmental stages, and that different combinations function synergistically to direct neuronal development.

In future studies, it will be of interest to investigate vitamin metabolites, such as nicotinamide and calcitriol in combination, at the stage of differentiation where they were reported to predominantly function. Orme *et al* (2013) reported that calcitriol acts at a late stage of differentiation to promote neuroprotection *in vitro* to midbrain DA neurons by up-regulating GDNF expression[104]. Therefore, to investigate this hypothesis, nicotinamide applied at the initial stages of ESC differentiation could be critical for rapidly and efficiently promoting neural commitment to highly enriched neuronal lineages, before specifically defining a DAergic phenotype with calcitriol and other molecules, detailed in section 6.2.1. It is important to consider that

application of nicotinamide throughout the 14 days of monolayer differentiation (i.e. day 0 to day 14) may prove advantageous to keep pluripotent cells to a minimum, as discussed in Chapter 3.

7.2.3 Future directions to translate the potential of nicotinamide to a functional protocol of mDA differentiation.

To develop cells for PD therapy, cells must possess the capacity to generate mDA neurons, ideally those of SNpc character. In this regard, mESCs demonstrate a lack of ability to produce TH$^+$ neurons possessing mesencephalic markers when differentiated under monolayer conditions[178]. Efficient monolayer neural differentiation requires low cell density, suggesting that close cell-cell interaction is essential for induction of an mDA neuronal phenotype[50]. Thus, in this thesis, it was not possible to evaluate the effects of nicotinamide on midbrain specific DA differentiation using the *Sox1*GFP mESC model system. Further optimisation of mEpiSC neural differentiation model would overcome this obstacle, which is known to potentiate the generation of midbrain specific DAergic neurons[87].

Another important concern for directed differentiation protocols is the functional evaluation of neuronal populations generated, to determine whether cells display appropriate physiological behaviours to those of the same phenotype *in vivo*[268]. Based on the previous effects of nicotinamide on enhanced neuronal maturation of derived catecholaminergic cells from mESCs (section 4.3.3), it will be interest to examine whether this vitamin B$_3$ metabolite also functions as an inducer of neural maturation in midbrain specific DAergic populations, derived from developmentally primed epiblast cells.

Further to this point, clinical motor features appear in PD patients, when over 60% of DAergic neurons have been lost, causing an 80% depletion of striatal DA levels. It has been hypothesised that the lag between the appearance of motor symptoms and DAergic neuronal loss may be due to a compensatory mechanism, where remaining DAergic neurons sprout to maintain striatal innervation[269]. Thus, small molecule factors with the ability to promote DAergic neurite outgrowth hold much promise to significantly prolong the pre-symptomatic stage of PD.

Thus, this thesis describes the pivotal functioning of nicotinamide as a regulator of neuronal differentiation and maturation, thus supporting previous evidence of the fundamental role of vitamins and their metabolites during early development. The implications for this project are wide. In particular, novel findings generated from this project are very exciting, as nicotinamide uniquely appears to drive dopamine neuron differentiation as effectively as known cocktails of signalling factors routinely applied in current published methods. Thus, nicotinamide may become a valuable component of cell culture solutions to aid the conversion of stem cells to dopamine neurons, helping to increase the efficiency of this process, the safety of the cells produced, and reduce costs, as we progress towards a patient-specific therapy for PD.

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Appendices

Appendix 1: Medium and solution components

FCS/LIF medium, 10% for 550 ml:

Media and solution components	Quantity	Supplier
Glasgow Modified Eagles Medium	500 ml	Invitrogen, Paisley, UK
Foetal bovine serum	51 ml	Biosera, East Sussex, UK
Non-essential amino acids	5.5 ml (10 mM)	Invitrogen, Paisley, UK
L-glutamine	5.5 ml (1 mM)	Sigma, Aldrich, UK
Sodium pyruvate	5.5 ml (100 mM)	Invitrogen, Paisley, UK
β-mercaptoethanol	550 µl (0.1 M)	Sigma, Aldrich, UK
Leukaemia inhibitory factor	100 U/ml	Made in house

N2B27 medium, for 50 ml:

Media and solution components	Quantity	Supplier
DMEM/F12	24.5 ml	Invitrogen, Paisley, UK
Neurobasal medium	24.75 ml	Invitrogen, Paisley, UK
N2	250 µl	Fisher Stem cellientific, Loughborough, UK
B27	500 µl	Fisher Stem cellientific, Loughborough, UK
L-glutamine	500 µl (1 mM)	Sigma, Aldrich, UK
β-mercaptoethanol	50 µl (0.1 M)	Sigma, Aldrich, UK

EpiSC Retinol-free N2B27 medium, for 50 ml:

Media and solution components	Quantity	Supplier
DMEM/F12	24.5 ml	Invitrogen, Paisley, UK
Neurobasal medium	24.75 ml	Invitrogen, Paisley, UK
N2	250 µl	Fisher Stem cellientific, Loughborough, UK
B27 (without vitamin A)	500 µl	Fisher Stem cellientific, Loughborough, UK
L-glutamine	500 µl (1 mM)	Sigma, Aldrich, UK
β-mercaptoethanol	50 µl (0.1 M)	Sigma, Aldrich, UK

EpiSC proliferation medium (Added to retinol-free N2B27 medium), for 50 ml:

Media and solution components	Quantity	Supplier
Human FGF-2 (bFGF)	60 µl	PeproTech, London, UK
Activin	40 µl	R&D Systems, Abingdon, UK

Nicotinamide (Dose-response assay), for 15 ml N2B27 medium:

1 mM stock solution; base medium = Neurobasal medium

Nicotinamide Molarity	Stock Solution
0.5 mM	7.5 µl
1 mM	15 µl
5 mM	75 µl
10 mM	150 µl
20 mM	300 µl

EdU Click-iT® reaction cocktail

EdU Click-iT® reaction cocktail	Quantity: for 1 coverslip
1X Click-iT® EdU reaction buffer	430 µl
CuSO4	20 µl
Alexa Fluor® azide	1.2 µl
1X Click-iT® EdU buffer additive	50 µl
Total Volume	500 µl

TdT reaction cocktail

TdT reaction cocktail	Quantity: for 1 coverslip
TdT reaction buffer	94 µl
EdUTP	2 µl
TdT	4 µl
Total Volume	100 µl

TUNEL Click-iT® reaction cocktail

TUNEL Click-iT® reaction cocktail	Quantity: for 1 coverslip
Click-iT® reaction buffer	**97.5 μl**
Click-iT® reaction buffer additive	**2.5 μl**
Total Volume	**100 μl**

112 solution: 10 ml penicillin-streptomycin-amphotericin B (5000 U/ml), 12 g glucose dissolved in 28 ml sterile water, and 10 ml 200 mM L-glutamine

Gelatin solution (0.1%): 1% gelatin solution was made up in distilled H_2O (dH_2O), autoclaved to sterilise and stored at 4ºC. Stock solution was further diluted in sterilised water for a working solution.

PFA, 4%: 18 g Disodium hydrogen phosphate (Na2HPO4), 9 g Sodium Chloride (NaCl), and 40 g PFA were added to 700 ml dH_2O overnight. The pH was adjusted to 7.3 with orthophosphoric acid or sodium hydroxide (NaOH), and dH_2O was added to make the volume up to 1 L.

Primary culture medium, for 50 ml: 42.75 ml Neurobasal medium, 5 ml foetal bovine serum, 1.25 ml 112 solution (see appendix), 500 μl B27 and 500 μl penicillin-streptomycin-amphotericin B (5000 U/ml).

TBS: 12 g trizma base and 9 g NaCl were made up in 1 L dH_2O. The pH was adjusted to 7.4 using 32% HCl.

Trypsin solution for ESTEM CELL (0.025%): 490 ml PBS, 5 ml chick serum, 0.186 g EDTA and 5 ml 2.5% trypsin were mixed together. The solution was filter-sterilised (0.2 μm) and aliquoted. Aliquots were stored at -20°C.

TXTBS: 250 ml TBS and 500 μl triton X-100 were mixed together. The pH was adjusted to 7.4 using 32% HCl.

Appendix 2: Antibody Lists

Primary Antibody	Dilution	Supplier
	Cell	
Mouse anti-Oct4	1:100	Santa Cruz
Mouse anti-Nestin	1:200	BD Sciences
Mouse anti-βIII-tubulin	1:500	Covance
Rabbit anti-βIII-tubulin	1:500	Covance
Rabbit anti-GABA	1:750	Sigma
Rabbit anti-VGlut2	1:150	Synaptic Systems
Rabbit anti-Tyrosine hydroxylase	1:1000	Chemicon
Mouse anti-Serotonin	1:500	Abcam
Rabbit anti-DARPP-32	1:1000	Chemicon
Rat anti-BrdU	1:1000	Oxford Biotechnology
Rabbit anti-SIRT1	1:250	Millipore
Rabbit anti-GFAP	1:500	DakoCytomation
Mouse anti-NG2	1:150	Millipore

Secondary Antibody	Dilution	Supplier
Goat anti-mouse Alexa Fluor 547	1:300	Cheshire Sciences
Goat anti-mouse Alexa Fluor 490	1:300	Cheshire Sciences
Goat anti-rabbit Alexa Fluor 488	1:300	Cheshire Sciences
Goat anti-rabbit Alexa Fluor 547	1:300	Cheshire Sciences
Goat anti-rat Alexa Fluor 488	1:300	Cheshire Sciences

Appendix 3: Manuscript Articles

Published article:

1. **Griffin, S. M.**, Pickard, M. R., Orme, R. P., Hawkins, C. P. & Fricker, R. A. Nicotinamide promotes neuronal differentiation of mouse embryonic stem cells *in vitro*. *Neuroreport*, 24, 1041–6 (2013).

*Some of the data included in Chapter 3 was accepted for publication by Neuroreport on 25 September 2013.

Conference article publications (Special edition journal issues):

Conference: 3-6/9/2013, International Symposia on Neural Transplantation (INTR12), Cardiff, UK.

1. **Griffin, S. M.**, Pickard, M. R., Orme, R. P., Hawkins, C. P. & Fricker, R. A. Nicotinamide promotes neuronal differentiation of mouse embryonic stem cells in vitro. *Neuroreport* 25, 139–163 (2014).

Conference: 7/5/2014, Association of British Neurologists Meeting (ABN), Cardiff, UK.

2. **Griffin S.** *et al* The influence of nicotinamide on the development of neurons. *J Neurol Neurosurg Psychiatry* (2014); 85:e4 doi:10.1136/jnnp-2014-309236.199

Manuscripts in preparation:

1. **Griffin SM**, Pickard MR, Orme RP, Hawkins CP, Williams AC, Fricker RA. Accelerated and efficient neuronal differentiation of *Sox1*GFP mouse embryonic stem cell *in vitro*. (2016)

2. **Griffin SM**, Pickard MR, Orme RP, Hawkins CP, Williams AC, Fricker RA. Nicotinamide enhances the development of dopamine and serotonin neurons from mouse embryonic stem cells – a key player in the etiology of Parkinson's disease? (2016)

3. **Griffin SM**, Williams AC, Fricker RA. Vitamins and the brain: A review of nicotinamide's key functions in early embryonic development. (2016)

4. **Griffin SM**, Williams AC, Fricker RA. Nicotinamide: therapeutic potential in Parkinson's disease. (2016)

Appendix 4: Lists of talks in conferences

1. 26/11/2014: Careers in Neuroscience Symposium, Galway, Ireland

2. 27-28/11/2014: Network for European CNS Transplantation & Restoration (NECTAR) Data Blitz Presentation, Galway, Ireland.

3. 9-11/12/2015: Network for European CNS Transplantation & Restoration (NECTAR) Data Blitz Presentation, Lund, Sweden.

Appendix 5: Awards

1. Poster presentation: 26/4/2012, Keele Postgraduate Symposium 2012, Keele, UK - won the first prize.

2. Poster presentation: 12/7/2012, Vitae Midlands Hub Research Exhibition, 2012, Coventry, UK - won the second prize.

3. Travel award to attend International Symposia on Neural Transplantation (INTR12), Cardiff, UK, 2013.

4. Travel award to attend Network for European CNS Transplantation & Restoration (NECTAR) Meeting, Galway, Ireland, 2014.

5. Travel award to attend Network for European CNS Transplantation & Restoration (NECTAR) Meeting, Lund, Sweden, 2015.