Nicotinamide, neural cells, and Parkinson's disease: exploring nicotinamide's effects on proliferation, neuronal differentiation, and neuroprotection within *in vitro* models

Emma Louise Green

A thesis submitted for the degree of

Doctor of Philosophy in Neuroscience

March 2021

Keele University

Abstract

Parkinson's disease (PD) is characterised by motor symptoms caused by degeneration of dopaminergic neurons in the substantia nigra. The cause remains elusive and current treatments cannot halt or reverse progression. This thesis explores the effects of nicotinamide on cells associated with PD; exploring its roles in the development and protection of dopaminergic neurons, and its influence on microglia.

Nicotinamide was tested on 46C mouse embryonic stem cells. Neural differentiation, indicated by *Sox1-GFP* expression, was identified by flow cytometry and immunocytochemistry. Neuroprotective effects of nicotinamide were assessed using a 6-hydroxydopamine primary ventral mesencephalon (VM) cell model of PD, and tyrosine hydroxylase (TH) immunocytochemistry to identify dopaminergic neurons. Effects of nicotinamide on $Iba1^+$ microglia were assessed in VM cultures and in isolated rat cortex microglial cultures.

Nicotinamide limited proliferation of 46C cells over 7 days of differentiation. *Sox1-GFP* expression was lower in nicotinamide-treated cultures compared to control, but peak expression occurred earlier. β-III-$tubulin^+$ neuron numbers remained unchanged at day 7. In VM cultures, TH^+ neuron numbers were higher in nicotinamide-treated cultures at days 1, 4, and 7. However, nicotinamide did not protect TH^+ neurons against 6-hydroxydopamine. Nicotinamide treatment showed reversible impairment of $Iba1^+$ microglial proliferation in VM cultures. Lower numbers of $Iba1^+$ microglia were observed in isolated microglial cultures with 10 mM nicotinamide; however, nicotinamide did not prevent granulocyte-macrophage colony-stimulating factor-induced proliferation.

Thus, nicotinamide could be utilised for novel PD treatments including promoting neuronal differentiation and restricting unwanted progenitor cell proliferation in stem cell

replacement therapy. Nicotinamide may also enhance dopaminergic differentiation or survival in primary VM cultures. Finally, nicotinamide's ability to reduce microglial proliferation could be utilised as a novel therapy to slow progression at PD onset or may offer protection from immune rejection in cell replacement therapies.

Contents

List of tables and figures

Abbreviations

ΔΨm – mitochondrial membrane potential

2D – 2-dimensional

3D – 3-dimenstional

6-OHDA – 6-Hydroxydopamine

AA – ascorbic acid

AD – Alzheimer's disease

ADPR – adenosine diphosphate-ribose

ANOVA – analysis of variance

APOE4 – apolipoprotein E type 4

ATP – adenosine triphosphate

BBB – blood brain barrier

BDNF – brain derived neurotrophic factor

BG – basal ganglia

BH4 – tetrahydrobiopterin

Calbindin – calcium binding protein

cAMP – cyclic adenosine monophosphate

CNS – central nervous system

COMT – catechol-O-methyltransferase

CRT – cell replacement therapy

CSF-1 – colony-stimulating factor-1

DAPI – 4',6-diamidino-2-phenylindole

DAT – dopamine transporter

DBS – deep brain stimulation

DMSO – dimethyl sulfoxide

dH_2O – distilled water

DIV – days *in vitro*

E# – embryonic day

ERK – extracellular signal-regulated kinase

ESC – embryonic stem cell

FOI – field of interest

GABA – gamma-aminobutyric acid

GDNF – glial cell line-derived neurotrophic factor

GFAP – glial fibrillary acidic protein

GFP – green fluorescent protein

GID – graft induced dyskinesia

GIRK2 – G protein-activated inward rectifier potassium channel 2

GM-CSF – granulocyte-macrophage colony-stimulating factor

GMP – good manufacturing practice

GSH – Glutathione

H_2O_2 – hydrogen peroxide

hESC – human embryonic stem cell

HVA – homovanillic acid

Iba1 – ionized calcium binding adaptor molecule 1

IL – interleukin

iNOS – inducible nitric oxide synthase

iPSC – induced pluripotent stem cell

LB – Lewy body

LPS – lipopolysaccharide

MAO – monoamine oxidase

MAP – mitogen-activated protein

mESC – mouse embryonic stem cell

MPP^+ – 1-methyl-4-phenylpyridinium

MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NA – nicotinic acid

NAD – nicotinamide adenine dinucleotide

NADP – nicotinamide adenine dinucleotide phosphate

NaMN – nicotinic acid mononucleotide

NAMPT – nicotinamide phosphoribosyl-transferase

NCM – neural cell media

Nestin – neuroectodermal stem cell protein

NMN – nicotinamide mononucleotide

NMNAT – nicotinamide mononucleotide adenylyltransferase

MMP-9 – matrix metalloproteinase 9

NNMT – nicotinamide N-methyltransferase

NR – nicotinamide riboside

NSC – neural stem cell

NSM – nicotinamide supplemented NCM

O_2^- – superoxide

•OH – hydroxyl radical

OD – optical density

P# – postnatal day

PARP – poly (ADP-ribose) polymerase

PBS – phosphate buffered saline

PD – Parkinson’s disease

PDL – poly-D-lysine

PEI – polyethyleneimine

PFA – paraformaldehyde

PI – propidium iodide

RNS – reactive nitrogen species

ROS – reactive oxygen species

SEM – standard error mean

Shh – sonic hedgehog

SN – substantia nigra

SNpc – substantia nigra pars compacta

SNpr – substantia nigra pars reticulata

STAT – signal transducer and activator of transcription

TH – tyrosine hydroxylase

TNF-α – tumour necrosis factor alpha

Trk B – tropomyosin receptor kinase B

VEGF – vascular endothelial growth factor

VM – ventral mesencephalon

VMAT – vesicular monoamine transporter

VTA – ventral tegmental area

WGE – whole ganglionic eminence

Acknowledgments

I would like to say how extremely grateful I am for the opportunity to complete this PhD candidacy. I have learnt so much over the past four years, and a lot more than I was expecting to. There have many ups and downs and I know that this journey would not have been possible without the tremendous support I have received from so many wonderful people.

Firstly, I would like to thank my supervisors Dr Stuart Jenkins and Professor Rosemary Fricker. I think it goes without saying that this would not have been possible without you both. Please know how appreciative I am for all of your help and understanding over this time, thank you for all of the knowledge and skills that you have helped me to develop; and for your enthusiasm that has allowed me to have a wonderful time during my PhD both within and beyond the laboratory.

I also need to thank all of the wonderful people who work in the Huxley building including the admin staff, cleaners, technicians, PhDs, post-docs, and lecturers who have provided a truly lovely environment to have worked in over this time. Special mentions go to Dr Monte Gates for your wisdom, help, and advice over these years. Dr Florian Noulin, you have taught me so much and helped me on so many occasions, thank you for all the advice, coffee, and badminton games. Dr George Joseph, you have saved me from insanity with your technical assistance, especially with the operation of the microscope and your image J macro coding skills. Dr Nana Efua Andoh, your advice, friendship, and know-how have been an amazing asset to me through these years. I wish you the best of luck in your future and thank you for all of your kindness and advice, you are an inspiration. And also, Dr Yolanda Gómez Gálvez, your strength, passion, and wisdom has pushed me not only to be a better scientist but a better person. Thank you for everything; you have reignited my love for cycling, shown

me how beautiful Stoke-on-Trent is, and helped me get involved with so many outreach activities.

I especially need to thank the lovely ladies with whom I shared my office space. Aina, Farhana, Jess, and Catia, you're all beautiful, strong, inspirational women. Thank you for all the laughter, you have kept me sane through these crazy times, my experience would not have been half as fun without you.

I also need to thank the students that have been involved in my project and the lab including Elizabeth Attia, Natasha Hill, and Amy Brown-Lyons. Thank you for your assistance in the lab and with data analysis, and for helping me to develop my skills and explore options for developing my research.

A big thank you also goes to all of my friends especially Katy, Hannah, Yasemin, Emma, Emily, Alison, Chin, Sarah, Clare and Kristel. You have provided tremendous emotional support throughout this roller-coaster ride and I am eternally grateful for your friendship. You are all such wonderful people and each of you unique; with you I know that I can take on anything.

To my partner Blessing, thank you for sticking with me through this. I know it has not always been easy, but your support and love has helped me through. You help me to see things in a different light, question preconceptions, and always make me smile. Thank you for keeping me smiling through this.

And last but far from least, thank you to my wonderful family. Mum, you are so strong and independent, you have given me the perseverance I have needed for this journey. Dad, your kindness and work ethic have helped me through this. Grandad, your generosity is inspiring, I would not be where I am today without you. To my brothers, thank you for your support, I know you are always there for me. You all have made me who I am, taught me to be kind, independent, and pragmatic, without you I wouldn't be where I am today. Thank you to you all.

Publication during PhD

Whilst undertaking my PhD candidacy I co-authored a published review article titled "The Influence of Nicotinamide on Health and Disease in the Central Nervous System".

Fricker, R. A., Green, E. L, Jenkins, S. I, Griffin, S. M. (2018) 'The Influence of Nicotinamide on Health and Disease in the Central Nervous System', *International Journal of Tryptophan Research*. SAGE Publications Ltd, 11, pp. 1–11. doi: 10.1177/1178646918776658.

This is presented in Appendix A.

1 General introduction

1.1 Introduction to Parkinson's disease

1.1.1 Prevalence of Parkinson's disease is expected to continue rising

Neurodegenerative disorders describe a group of diseases associated with the progressive loss of neurons from the central nervous system (CNS) resulting in a wide range of symptoms from cognitive decline to movement impairment. Common neurodegenerative disorders include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease and multiple sclerosis and although all fall under this general term, they have vastly different presentations, aetiologies, and treatments. Much research is focused on understanding these diseases, their causes and developing new therapies and treatments.

An estimated 10 million people are diagnosed with PD worldwide and this number is only expected to increase in future years (Fredericks *et al.*, 2017) placing a heavy socioeconomic strain on societies. Between 1994-2013 in the UK, caring for PD patients was estimated to cost the NHS £5022 ± 4058 per year compared to age-matched controls £2001 ± 2000, with costs including inpatient hospital stays, prescribed medication, and accident and emergency visits. However, these authors suggest that this estimate is conservative and that costs arising from nursing home, social services, lost productivity, and costs covered by patients/carers, or private healthcare, are not included (Weir *et al.*, 2018). Therefore, PD places a heavy financial burden on government, and personal funds, and these costs are expected to rise.

Due to the nature of PD, incidence rates (the number of diagnoses per 100,000 people) and prevalence (the number of people affected) are difficult to determine (Stern and

Siderowf, 2010). Some trends in PD diagnosis are consistently found across meta-analysis studies including age and gender which can contribute to predictive factors. The prevalence of PD is increased with age, with studies indicating highest prevalence between the ages of 70-90 years. Significantly increased prevalence is also found in males compared to females (Horsfall *et al.*, 2013; Pringsheim *et al.*, 2014; Hirsch *et al.*, 2016; Savica *et al.*, 2016).

Early onset PD (< 50 years of age) can often be misdiagnosed, as PD is typically considered an age-related condition, and so may not be considered as a diagnosis in younger people (Schrag, Ben-Shlomo and Quinn, 2002). High symptomatic variability (Postuma *et al.*, 2016) and the presence of a prodromal symptomatic phase (typically non-motor or subtle motor changes; Mahlknecht, Seppi and Poewe, 2015) preceding motor symptoms could also delay disease diagnosis (Berg *et al.*, 2015); diagnosis is mostly dependent on the presence of bradykinesia, rigidity and resting tremor, although recent guidelines now suggest the inclusion of prodromal features (Postuma *et al.*, 2016). In this regard, studies can fail to show a clear trend in incidence rates over time, with both increases and decreases predicted across different studies due to differences in the data collection and analysis, changes in definitions of PD, and geographical location. For instance a decrease in incidence rates of either 6% or 1% was observed in the UK in meta-analysis data dependent on the inclusion criteria (Horsfall *et al.*, 2013); whilst another study found a significant increase in incidence rate in a single county in the U.S.A. (Savica *et al.*, 2016). Overall, prevalence of PD is expected to rise over the coming years due to increases in population size (Dorsey *et al.*, 2007; Rossi *et al.*, 2018), the increasing average age of the population, and increases in life expectancy, with projections expecting a doubling in PD cases by 2050 (Bach *et al.*, 2011). With no effective long-term treatments available, efforts to slow, halt, and reverse disease progression are greatly needed.

1.1.2 Parkinson's disease can be difficult to diagnose

PD is now one of the most common movement disorders (Oertel and Schulz, 2016) displaying a range of symptoms (Table 1.1). It is often characterised by the motor symptoms bradykinesia (a slowness in movement), resting tremor and rigidity, although non-motor symptoms including dementia, sleep disturbances, and autonomic dysfunction may also be present. Although PD is well characterised, the gold standard for diagnosis is expert opinion (Postuma *et al.*, 2016), with diagnostic accuracy estimated to be at 82% (Rizzo *et al.*, 2016), highlighting the need for an accurate and objective diagnostic test. Many diagnoses are based on the motor symptoms, requiring the presence of bradykinesia alongside either rigidity, resting tremor, or postural instability. Diagnosis is further strengthened by exclusion criteria (for instance exposure to a neurotoxin) and supportive criteria (such as unilateral onset and positive response to levodopa medication). Non-motor symptoms are not currently included as part of the diagnosis described by the Parkinson's Disease Society Brain Bank which is followed by National Institute for Health and Care Excellence (NICE) guidance (Rogers *et al.*, 2017).

Further subtype classification for PD has been suggested, with the presence of motor symptoms classed as clinical PD, the presence of prodromal motor and non-motor symptoms classed as prodromal PD, and the occurrence of neurodegeneration without symptoms classed as preclinical PD (Postuma *et al.*, 2016). However, although prodromal features can provide a critical tool in the early diagnosis and treatment of PD, research into these symptoms is still relatively new. Their variability and coincidence with other diseases mean that they cannot currently be used as definitive diagnostic criteria (Berg *et al.*, 2015). This emerging field could provide beneficial insights into disease progression and treatment.

Table 1.1 Table showing some the motor and non-motor symptoms that patients with PD may experience. Many of the non-motor symptoms manifest during the prodromal phase. *Denotes symptom associated with medication (Mahlknecht, Seppi and Poewe, 2015).

Motor Features	**Non-motor Features**			
	Neuropsychiatric	**Sleep**	**Autonomic dysfunction**	**Sensory**
Tremor Rigidity Bradykinesia Postural instability Motor fluctuations Dyskinesia*	Cognitive impairment Depression Psychosis Dementia Impulse control disorder*	Insomnia Daytime sleepiness REM sleep disturbance	Gastro-intestinal Genito-urinary Circulatory	Pain Hyposmia Visual changes Hallucinations*

The symptoms of PD, and treatment regimen, place a heavy burden on both people with PD and their carers. A decrease in mortality (PD mentioned on the death certificate) was observed between 1993-2006, possibly due to improvements in PD treatments and general medicine (Mylne *et al.*, 2009); although, despite decades of research, the underlying mechanisms of PD, and an effective long-term treatment, remain elusive.

1.1.3 Motor symptoms of Parkinson's disease are caused by striatal dopaminergic denervation

Originally described by James Parkinson in 1817 (Parkinson, 2002), the cause of the motor symptoms of PD was only discovered in 1960 when striatal dopamine deficiency was described by Ehringer and Hornykiewicz (Tolosa *et al.*, 1998). The pathology has since been more accurately described. Braak et al. (2006) proposed 6 stages of Lewy body (LB) formation that progress within PD. LBs are abnormal aggregates of the presynaptic protein alpha-

synuclein which is involved in neurotransmitter release, synaptic plasticity, and possibly the regulation of dopamine storage and synaptic vesicle recycling (Johns, 2014). In the disease pathology suggested by Braak et al. (2006), stages 1 and 2 include the appearance of LBs in the medulla oblongata/pontine tegmentum and anterior olfactory structures. During stages 3 and 4 initially subtle, but later severe, changes occur in the substantia nigra pars compacta (SNpc) and other areas of the basal midbrain and forebrain resulting in a reduction in dopamine neurons leading to the motor symptoms of PD. In the final stages, 5 and 6, lesions appear in the neocortex and can account for the non-motor symptoms. Although this staging can account for a number of the prodromal features, there is debate about the validity and accuracy of this proposed staging process across subtypes of PD (for instance early onset vs. late onset; Burke, Dauer and Vonsattel, 2008; Dickson *et al.*, 2010; Jellinger, 2019).

Despite the significance of non-motor symptoms in patient quality of life, research has typically focussed on the motor-symptoms of PD, the cause of which has long been established as the loss of dopaminergic neurons within the SNpc. Cheng, Ulane, & Burke (2010) reviewed estimates of the extent of SNpc neuron loss and whilst some studies observed losses of 50-70% of dopaminergic neurons at motor symptom onset, more recent estimates support a more modest decrease in SNpc dopaminergic neurons of ~30%, compared to age-matched controls, at symptom onset.

The substantia nigra (SN) is part of a collection of nuclei, termed the basal ganglia (BG), and is composed of 2 distinct regions, the pars reticulata (SNpr) and pars compacta. The SNpr is composed of gamma-aminobutyric acid (GABA) neurons projecting to the thalamus and is degenerated to a lesser extent by PD pathology, not contributing to the motor symptoms discussed here. The dopaminergic neurons of the SNpc project to the striatum (another structure of the BG) and are almost exclusively degenerated in PD. The BG, located in the midbrain (Figure 1.1), are a collection of nuclei involved in a number of circuits including eye

movement control, executive functions, emotional regulation, and the control of movement (Fazl and Fleisher, 2018), which will be the focus of this thesis. Some of the brain regions involved in motor control are shown in Figure 1.2. Signals from the cortex feed into the nuclei of the BG, and pons and cerebellum, motor loops which feed to the thalamus and back to the cortex. This simplified model of the BG (Figure 1.3 A) has both excitatory and inhibitory connections (yellow and red respectively) and is often used to illustrate this brain circuit. Despite some pitfalls and its simplicity, numerous animal models have confirmed the functionality of the BG in line with this model (Fazl and Fleisher, 2018), although more complex and detailed anatomy of the connections within the BG and dopaminergic signalling pathways are still being uncovered (Haber, 2014; Ogawa and Watabe-Uchida, 2018).

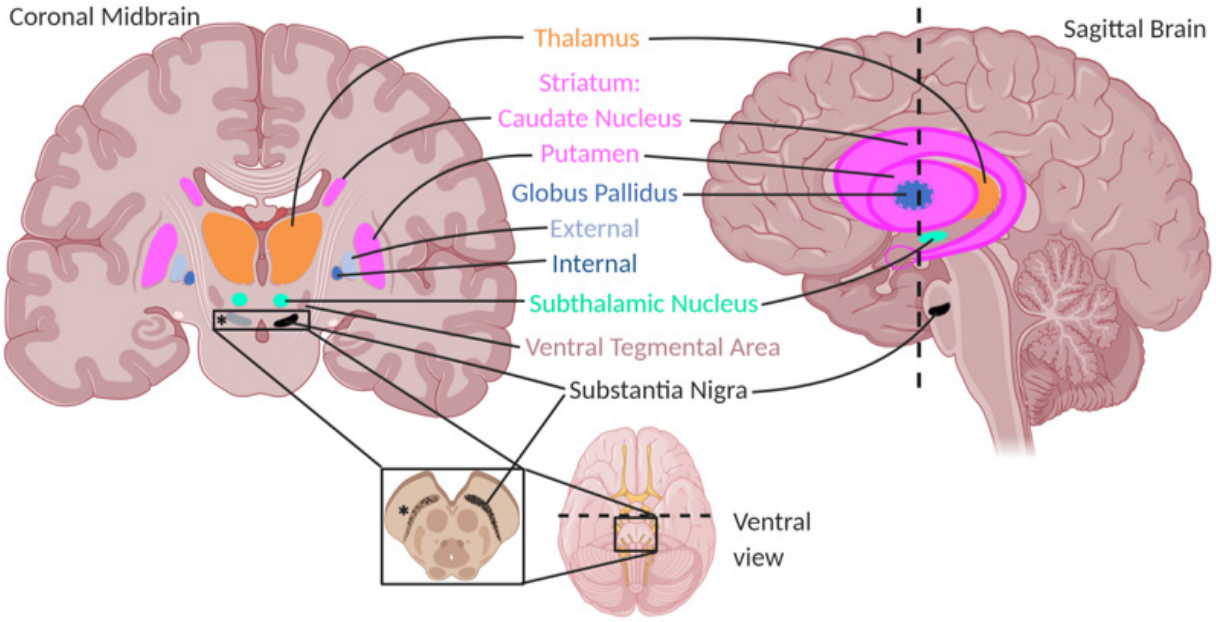


Figure 1.1 Diagrams representing the nuclei of the basal ganglia and related structures (thalamus, ventral tegmental area) within the human brain. Although the size and positioning are not precise, these views give a general idea of the location and relational positioning of nuclei commented on in this PhD thesis. The sagittal brain diagram shows the approximate positioning of the basal ganglia within the brain, however, the thalamus and globus pallidus are positioned behind the putamen and cannot be accurately represented from this view. The coronal midbrain section (position indicated by the dashed line) has the substantia nigra super-imposed to show relative vertical positioning but is actually more caudal in relation to the other bodies of the basal ganglia. *Denotes loss of substantia nigra dopaminergic neurons and subsequent neuromelanin pigmentation in PD. Created with Biorender.com.

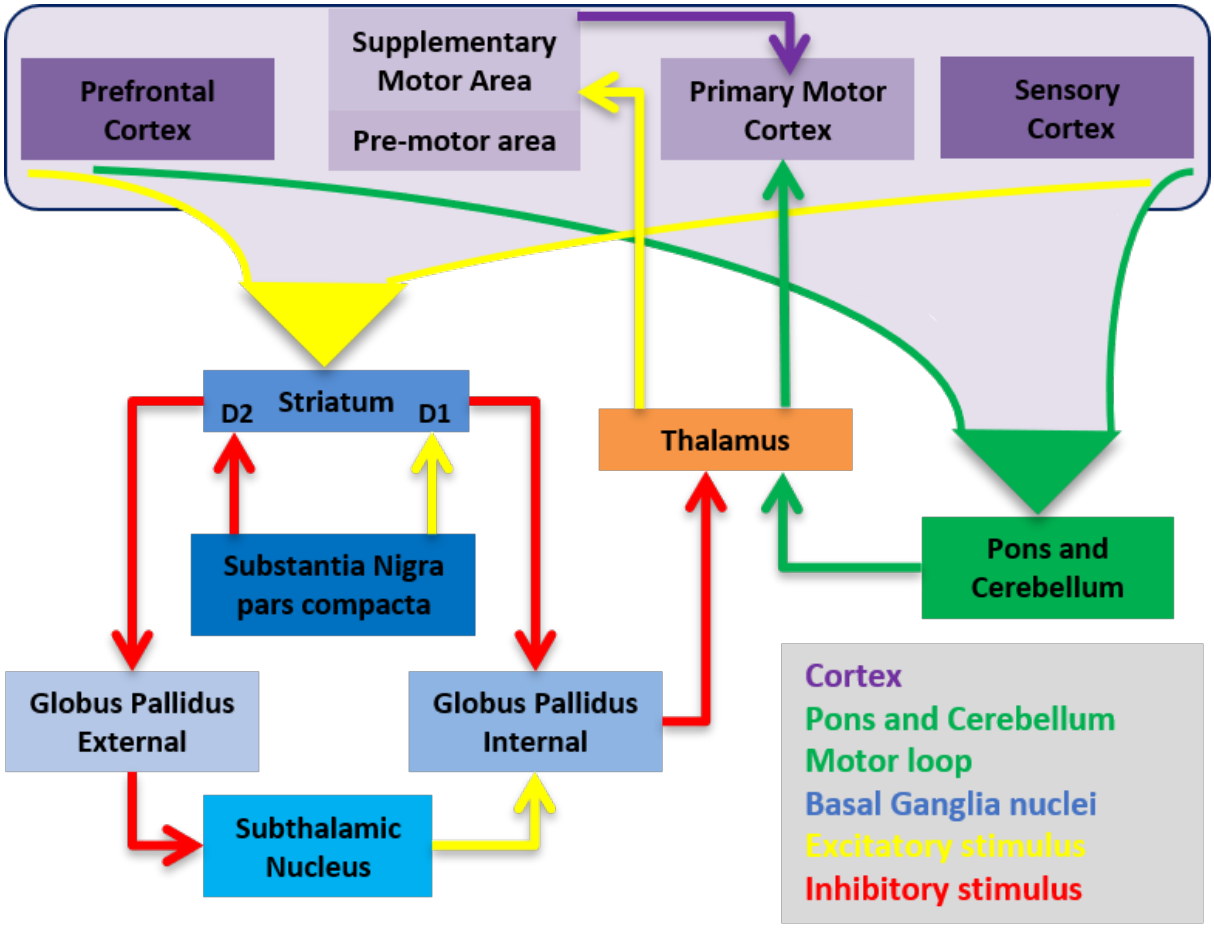


Figure 1.2 Schematic illustrating some of the areas, connections and pathways involved in movement. Purple areas are in the cortex and are all interconnected with a particularly strong connection between the supplementary motor area and the primary motor cortex being highlighted here. They feed into loops within the basal ganglia and cerebellum, which both feed back through the thalamus. Green corresponds to the pathway that involves the cerebellum and pons which are involved in precision and timing of movement. Blue encompasses the basal ganglia inputs involved in control and initiation of movement with excitatory connections in yellow and inhibitory connections in red. Neurons in the striatum contain either the D1 or D2 dopamine receptor which correspond to the direct and indirect pathways respectively.

Simply, dopaminergic neurons from the SNpc project to the striatum where dopamine can act as either an excitatory or inhibitory neurotransmitter dependent on the type of receptor that it binds to (D1 or D2 respectively). This gives rise to the direct (via the globus pallidus internal segment) and indirect (via the globus pallidus external segment) pathways (Figure 1.3 A). In PD, the loss of dopaminergic neurons in the SNpc, and subsequent depletion of dopamine in the striatum, disrupts the signalling pathway through the basal ganglia restricting the output from the thalamus to the cortex (Figure 1.3 B). Although analysis of dopamine levels in the striatum can be difficult to measure, extrapolate, and compare across

studies (either due to degradation post mortem, or the different *in vivo* techniques), it is estimated that dopamine levels in the striatum are ~50% lower, compared to healthy individuals, when PD motor-symptoms arise (Cheng, Ulane and Burke, 2010). Whilst the decrease in dopaminergic SN neurons is substantial, it is the more profound loss of striatal dopaminergic innervation which is responsible for the motor symptoms of PD.

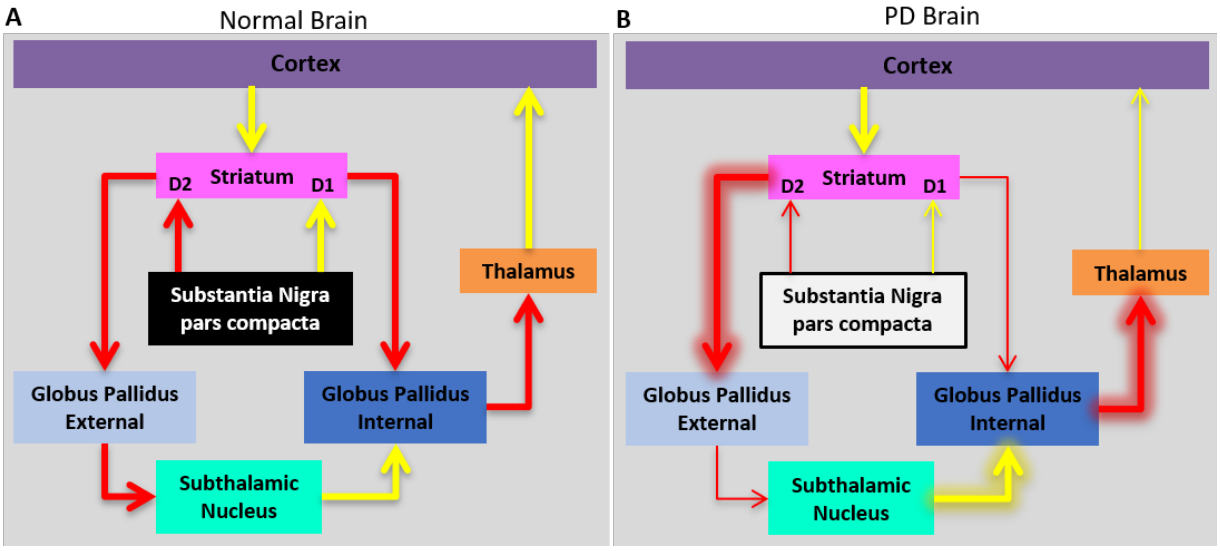


Figure 1.3 Diagram showing simplified circuitry of the basal ganglia and associated structures (cortex and thalamus).
A) Normal brain circuity through the BG. Signalling for movement is sent from the cortex (purple), filtered through the nuclei of the BG and then sent back to the cortex via the thalamus (orange). Two pathways, the direct (D1) and indirect (D2), work through inhibitory (red) and excitatory (yellow) connections which culminate in disinhibition of the thalamus and therefore initiate movement. The direct pathway receives an excitatory signal from the SNpc which, when combined with the signal from the cortex, activates an inhibitory signal to the GPi, this reduces the inhibitory signal to the thalamus, thus initiating movement. The indirect pathway, however, receives an inhibitory signal from the SNpc. This overrides the excitatory signal received from the cortex and inhibits the inhibitory signal going to the GPe. This means that the GPe is able to inhibit the STN turning off the excitatory signal going to the GPi and also releasing the thalamus from inhibition.
B) In PD, dopaminergic signalling from neurons in the SNpc is lost meaning that signalling through the BG is disrupted. Increased thickness of arrow indicates increased strength, decreased thickness represents diminished strength. The reduced signals from the SNpc mean that the D1 pathway does not have sufficient activation to inhibit the GPi and the D2 pathway is excited by the striatum without being inhibited by the SNpc culminating in over-inhibition of the thalamus. BG: basal ganglia, GPe: globus pallidus external, Gpi: globus pallidus internal, SNpc: substantia nigra pars compacta, STN: subthalamic nucleus

The substantial decrease in striatal dopamine levels and dopaminergic neurons in the SNpc that is necessary before clinical symptoms are evident is thought to be due to compensatory mechanisms. There are a number of theories for compensatory mechanisms,

although this phenomenon has proven difficult to study due to the progressive, asymmetric, and prodromal/preclinical nature of PD; and a lack of animal models that accurately recapitulate these characteristics. Three stages of compensatory mechanisms have been proposed, from initial dopamine homeostatic mechanisms occurring in the striatum, to changes in BG activity adjusting for striatal dopamine loss, and finally compensatory mechanisms outside of the BG accommodating for BG signalling disruption (Bezard, Gross and Brotchie, 2003). These exact mechanisms are debated and there is also suggestion of contralateral mechanisms whereby the still functioning BG can compensate for abnormal thalamocortical signalling, with clinical manifestation apparent only after dopamine depletion on the secondarily affected side (Obeso *et al.*, 2004). The presence of compensatory mechanisms explains the dissociation between the initial decrease in striatal dopamine levels and later appearance of motor symptoms arising from the significant loss of dopaminergic neurons from the SNpc.

It is clear that striatal dopamine denervation and subsequent dopaminergic cell death are responsible for the motor symptoms of PD, however, the exact pathology of this cell death remains undefined. A number of factors are thought to be involved including α-synuclein aggregation/LB formation, mitochondrial dysfunction, and oxidative stress.

1.1.4 Lewy body formation is a pathological hallmark of Parkinson's disease

One of the pathological hallmarks of PD is the presence of LBs which are caused by the progressive accumulation of α-synuclein (Lashuel et al. 2013). This protein is expressed in the brain and is involved in synaptic processes and neurotransmitter release, however, its precise function is unknown (Mehra, Sahay and Maji, 2019). Toxicity occurs from intracellular

accumulation of α-synuclein, thought to be caused by an imbalance between synthesis, aggregation, and removal. Unfolded α-synuclein monomers can interact, forming dimers which propagate to generate oligomers. Oligomers form fibrils by further addition of monomers, and the accumulation of fibrils leads to the formation of LBs (Lashuel *et al.*, 2013). These intermediary stages, oligomers and fibrils, are suggested to be toxic through aberrant interactions with cytosolic proteins and organelles (Lashuel *et al.*, 2013), and it is thought that LB formation is a protective mechanism to contain and limit damage from oligomers and fibrils. However, a recent LB formation *in vitro* model suggests that toxicity within neurons does not occur during fibrillization, but appears after LB formation (Mahul-Mellier *et al.*, 2020).

It is suggested that α-synuclein fibrils, initially formed in the pre-synaptic terminals (Kramer and Schulz-Schaeffer, 2007), are transported to the perinuclear region over time where they form LB inclusions. The formation of LBs also involves the sequestration of other proteins, vesicles, lipids, microtubule complexes, and organelles including mitochondria and endoplasmic reticulum, all of which have been found within LB plaques (Mahul-Mellier *et al.*, 2020). This sequestration is thought to contribute to pathology through disruption of normal cellular function.

Although the mechanisms behind aberrant α-synuclein appearance and subsequent toxicity in PD are not well understood, it is thought that α-synuclein filaments could impede axonal transport of essential proteins travelling from the soma to the dendrite (Galvin, Lee and Trojanowski, 2001). This results in a loss of structural and molecular integrity in the axon and dendrite which could cause axons to 'die back' and disconnect the SNpc from the striatum. The accumulation of α-synuclein could also lead to neurodegeneration through other detrimental mechanisms such as mitochondrial dysfunction, oxidative stress, and neuroinflammation.

1.1.5 Mitochondrial dysfunction and oxidative stress are involved in dopaminergic cell death

Abnormal functioning of mitochondria can have a deleterious effect on a number of cellular pathways, culminating in cell death (Henchcliffe and Beal, 2008). Studies have found abnormal function and structure of mitochondria in the brains of some, but not all, PD patients (Moon and Paek, 2015). Post mortem analysis of the SNpc has found that although mitochondrial mass is unaffected in PD, activity of complex I (also known as NADH-ubiquinone reductase; Schapira *et al.*, 1990) and complex II (succinate dehydrogenase; Grünewald *et al.*, 2016) is reduced in the SN of PD patients.

The cause of the mitochondrial dysfunction observed in SN dopaminergic neurons of PD brains is unknown. However, α-synuclein aggregation and LB formation (Mahul-Mellier *et al.*, 2020), genetic mutations (Zeng *et al.*, 2018), and calcium dysregulation (Surmeier *et al.*, 2011) may contribute. The mitochondrial dysfunction and mitochondrial DNA defects observed in PD are linked to decreased mitochondrial membrane potential, reduced adenosine triphosphate (ATP) synthesis, and increased levels of reactive oxygen species (ROS) leading to oxidative stress (Moon and Paek, 2015; Chen, Turnbull and Reeve, 2019).

Cellular oxidative stress refers to the over-production of ROS such as peroxides, hydroxyl radicals and superoxide, over cellular neutralisation mechanisms. ROS formation is part of normal cell functioning, however, uncontrolled ROS production results in oxidative stress and widespread cellular disruption through lipid peroxidation, mitochondrial dysfunction, and damage to nuclear DNA and cytosolic RNA (Montine *et al.*, 2002; Guo *et al.*, 2013; Sanders and Greenamyren, 2013). Indicators of ROS are present in the brain areas affected by PD (Andersen, 2004), and serum from PD patients show higher levels of oxidative stress markers and lower levels of antioxidants (Medeiros *et al.*, 2016).

Dopaminergic neurons of the SNpc are also more susceptible to oxidative damage than other neurons, which may explain their selective degeneration in PD. This is thought to be due to higher endogenous ROS production as a result of increased levels of iron, increased monoamine oxidase (MAO; an enzyme involved in the metabolism of dopamine) activity (Wang and Michaelis, 2010), and the reaction of dopamine with oxygen, producing superoxide radicals (Halliwell, 2006). The SNpc of PD patients also show reduced levels of the antioxidant glutathione (Pearce *et al.*, 1997) suggesting a redox imbalance and increased oxidative stress. The presence of neuromelanin, the black pigment in dopaminergic neurons of the SNpc, has also been implicated as a contributor to the selective vulnerability of these neurons, however, its involvement in neurodegeneration/neuroprotection is debated (Vatassery, 1992; Zecca *et al.*, 2001, 2008; Dias, Junn and Mouradian, 2013; Knörle, 2018).

Other potential reasons for susceptibility of SNpc neurons is their relatively large size and protracted axonal transportation which increases neurofilament content, energy requirements, mitochondrial activity, and ATP production (Wang and Michaelis, 2010). It appears that throughout disease progression, it is the axons and their terminals in the striatum, rather than the cell soma in the SNpc, that is the initial site of degeneration (Cheng, Ulane and Burke, 2010). Although the cause of the motor symptoms of PD is very well described, the mechanisms behind the loss of these dopamine neurons are still being elucidated, and the initial cause for this pathology is still unknown for the majority of PD cases.

1.1.6 The cause of Parkinson's disease remains elusive

Despite the well described pathology of PD, the cause of dopaminergic cell death is still mostly unknown. Having a family member with PD is one of the highest risk factors (Noyce *et al.*, 2012), with first-degree relatives having a two- to three-fold increased likelihood of developing PD (Balestrino and Schapira, 2020). Gentic or 'familial' forms of PD have been

identified, and these are thought to account for approximately 10% of cases (Thomas and Beal, 2007), with several genes linked to the disease. These forms are often associated with early-onset cases (diagnosis before 50 years of age); with genetic forms accounting for approximately 50% of early-onset cases and only 2-3% of late-onset cases (Zeng *et al.*, 2018). The first identified genetic link associated with PD was a mutation observed in the *SNCA* gene which encodes for α-synuclein (Polymeropoulos *et al.*, 1997). A number of other genetic mutations are linked to the disease, typically associated with abnormal α-synuclein aggregation and mitochondrial dysfunction (Simón-Sánchez *et al.*, 2009; Singleton, Farrer and Bonifati, 2013; Zeng *et al.*, 2018), these are shown in Table 1.2 including identified mutations and a brief description of how these mutations are thought to contribute to disease progression.

Table 1.2 Table displaying some of the genes (commonly used abbreviations and names) and mutations linked to PD. An associated role of each gene and some of the downstream effects linked to mutations are also described. Although this is not an exhaustive list of genetic mutations and their interactions, these are used to study genetic causes of PD and possible pathology. Adapted from: Polymeropoulos *et al.* (1997); Singleton, Farrer and Bonifati (2013); O'Regan *et al.* (2017); Zeng *et al.* (2018); Chen, Turnbull and Reeve (2019).

Gene	Mutation	Role	Downstream effects of mutation	Onset association
SNCA (synuclein alpha)	A53T, A30P, E46K, H50Q	α-synuclein protein	Mis-folding and reduced autophagy	Associated with early-onset and rapid progression
LRRK2 (Leucine-rich repeat kinase 2)/PARK8	G2019S, R1441C, I2020T, G2385R	Kinase enzyme	Enhanced α-synuclein mobility and aggregation, and mitochondrial dysfunction	Associated with late onset, associated with 4% familial PD cases
PINK1 (PTEN-induced putative kinase 1)	G411S, I368N, Q456X, A168P, H271Q, L347P, G309D	Mitochondrial protein kinase	Mitochondrial dysfunction and calcium dysregulation, reduced Parkin recruitment	Associated with early-onset, 1-8% of familial cases
PARK2 (Parkin)	R42P, R46P, K211N, C212Y, C253Y, C289G, C441R, R275W	E3 ubiquitin ligase	Inhibits mitochondrial Parkin recruitment and subsequent mitophagy	Associated with early-onset and 10-25% of cases
PARK7 (Protein deglycase DJ-1)	L166P, M26I, L10P, P158Δ, D149A, *DJ1*$^{-/-}$, L172Q	Redox sensitive chaperone protein	Attenuation of antioxidant response, mitochondrial dysfunction	Associated with early-onset, 1-2% familial cases
VPS35 (Vacuolar protein sorting 35)	D620N, *VPS35*$^{-/-}$, R524W, P316S	Regulates transmembrane protein sorting	Mitochondrial dysfunction, decrease in complex I and II activity, α-synuclein aggregation	Associated with late-onset and accounts for 1% familial PD cases
GBA (glucocerebrocid-ase)	*GBA*$^{-/-}$, N370S, L444P	Lysosomal enzyme	Reduced enzyme activity, accumulation of α-synuclein, mitochondrial dysfunction, macroautophagy dysfunction, endoplasmic reticulum stress	5-25% of 'idiopathic' PD cases carry a GBA mutation

The remaining 90% of PD cases, classed as idiopathic, are thought to be due to environmental factors including age, toxins, and diet. These contributing factors may lead to cell death through accumulation of α-synuclein/LB formation, mitochondrial dysfunction, oxidative stress, and/or inflammatory activation.

With just 1.2% of idiopathic cases classed as early-onset (Parkinson's UK, 2019), age seems to be the main contributing risk factor for PD. Aging is associated with a number of brain changes including reduced brain weight (Elobeid *et al.*, 2016), mitochondrial dysfunction (Chen, Turnbull and Reeve, 2019), decreased striatal dopamine and death of SNpc neurons (Carlsson, 1987; Huot, Lévesque and Parent, 2007), and presence of LBs (Elobeid *et al.*, 2016), which are not associated with any symptoms of disease. Although these individuals may be classed as pre-clinical, very few aged brains completely lack any characteristics associated with neurodegenerative pathology (Wyss-Coray, 2016). Disruption of neurotrophic factors, such as brain derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), may also contribute to disease pathology with concentration changes observed in the ageing brain (Huot, Lévesque and Parent, 2007).

Levels of BDNF, and the ratio of tyrosine hydroxylase (TH; an enzyme involved in the production of dopamine and present in SNpc dopaminergic neurons) to BDNF mRNA decreased more substantially with age in the SNpc compared to ventral tegmental area (VTA; dopaminergic nuclei situated laterally to the SN) of mice (Shaerzadeh *et al.*, 2020). However, no significant difference in plasma BDNF was observed between PD and control groups (Costa *et al.*, 2019). Levels of GDNF have been found to be decreased in the hippocampus of PD brains compared to control, however, levels of cerebral dopamine neurotrophic factor and basic fibroblast growth factor were increased (Virachit *et al.*, 2019). This suggests that although increasing age is a risk factor for PD, other factors also influence the specific and increased degeneration observed in the SNpc PD brains compared to age-matched controls.

Results from meta-analysis studies should be interpreted with caution due to heterogeneity across studies and within study biases, however, this data can give clues to factors with potential causal links to PD surrounding environment and lifestyle. A number of positive and negative external factors have been identified as associated with PD.

The development of severe parkinsonism in a group of young drug users after self-administration of a synthetic heroin revealed 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine (MPTP) as a selective toxin of dopaminergic SN neurons (Langston *et al.*, 1999). Parallels in cellular pathology between cell death in MPTP-poisoning and PD highlights the potential that PD could be caused by environmental toxins (Schapira *et al.*, 1990). In humans, SN neurons are closely aligned to blood vessels suggesting that the SN may be more susceptible to blood-borne agents (Webster, 2001). Farming, rural living, and well water drinking have been linked to a higher risk of PD and this is often thought to be associated with pesticide exposure, which also holds an increased risk of PD (Noyce *et al.*, 2012). However, another study has shown 12% higher incidence rates in urban compared to rural areas (Horsfall *et al.*, 2013). Differences in the collection of this data may account for the discrepancy observed here in incidence rates between rural and urban living. Rural and urban living may both lead to exposure to toxins but from different sources. These studies suggest that environmental toxins may contribute to an increased risk of PD, however, no specific toxin or route of exposure has been reliably associated with cases of PD.

Certain lifestyle and diet choices may also contribute to the risk of developing PD. Smoking, alcohol intake, drinking coffee, and physical activity are reported to reduce the risk of PD (Noyce *et al.*, 2012; Bellou *et al.*, 2016). Data from meta-analysis studies can have both the benefit of encompassing many factors, and the downfall of being nonspecific. For instance, whilst increased dairy consumption is associated with an increased risk of developing PD, body mass index, fat, and cholesterol intake (which can also be associated with dairy products) do

not appear to influence the risk (Jiang *et al.*, 2014; Bellou *et al.*, 2016). Other dietary factors including vitamin intake/deficiency have also been implicated to varying degrees (Agim and Cannon, 2015; Bellou *et al.*, 2016; Yang *et al.*, 2017). A rise in PD in the UK between 1850 and 1960 correlates with increases in meat intake and this trend is seen in populations across the world (Hill and Williams, 2017). It has also been suggested that a vegan diet reduces the risk of PD (McCarty, 2001). However, dietary factors and influences arising from these can be difficult to measure due to the range of nutrients found within foods and the requirement of participants to accurately record their dietary habits.

Other correlating factors such as comorbidity with depression, anxiety, and constipation are associated with PD, however, it is not known whether these are preclinical symptoms, rather than causative risk factors (Bellou *et al.*, 2016; Yang *et al.*, 2017). As described above, mitochondrial dysfunction and oxidative stress are pathological features of PD, although it is unclear whether these represent a causal feature or are in response to damaging stimuli. Aside from chemical toxins that can cause the increased oxidative stress observed in PD, inflammation may also play a role in the cascade of events leading to neuronal cell death.

Inflammation in the brain is part of a protective mechanism to maintain cellular health and homeostasis by removal of detrimental material. However, neuroinflammation is associated with the release of ROS (Wang *et al.*, 2015) and an uncontrolled/unwarranted pro-inflammatory response can cause cell and tissue damage, and has been linked to the loss of dopaminergic neurons in PD (Guo *et al.*, 2018). Upregulation of the neuroinflammation signalling pathway has been observed in an α-synuclein *in vitro* LB mouse model (Mahul-Mellier *et al.*, 2020) suggesting a link between α-synuclein/LBs with an inflammatory response. However, it is not certain what may trigger an uncontrolled inflammatory response that could lead to specific SNpc cell death in humans.

The contributing factors to neurodegeneration in PD are well described, however, the initial factor in the cascade of events leading to specific SNpc cell death remains elusive. Despite the presence of this disease worldwide, significantly lower prevalence is reported in Asia compared to North America, Europe, and Australia, and authors suggest that genetic or environmental factors could explain these differences (Pringsheim *et al.*, 2014). The wide array of risk factors associated with this disease also point to gene-environment interactions and it is probable that no single factor is responsible for PD (Horsfall *et al.*, 2013). This range of potential contributing factors, the difficulty in studying the disease *in vitro*, and difficulty studying disease onset due to a preclinical phase mean that understanding features of dopaminergic SN neurons in health and disease may assist in understanding this specific neurodegeneration and treatments to prevent it.

1.2 Substantia nigra dopamine neurons are more susceptible to degeneration

Dopamine (synthesis and breakdown shown in Figure 1.4) is a neurotransmitter used in six dopaminergic pathways in the human brain (mesolimbic, mesocortical, nigrostriatal, tuberoinfundibular, hypothalamospinal projection, and incertohypothalamic) and are associated with dopaminergic subtypes classified as A8-A16 (Table 1.3) numbered based on caudal to rostral location (Goridis and Rohrer, 2002). These different pathways also correspond to differing levels of dopamine in specific brain regions with lowest levels in the cortex (0.1 μg/g) and highest in the striatum (10 μg/g; Webster, 2001). Dopamine has widespread activity, being linked to movement, motivation, reward and pleasure, memory/learning, lactation, sexual behaviour, and nausea (Klein *et al.*, 2019).

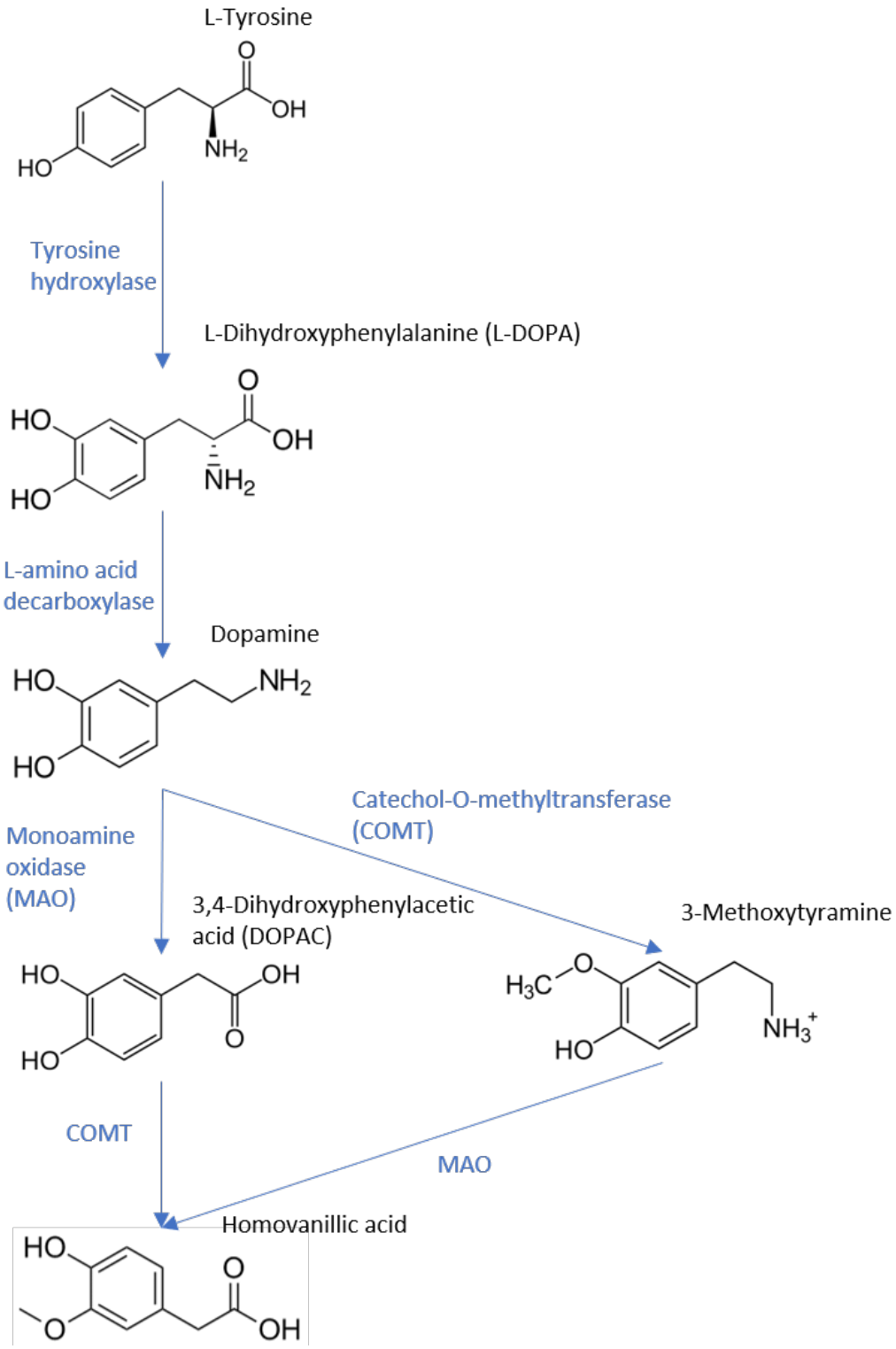


Figure 1.4 Schematic showing the synthesis and breakdown of dopamine. Molecules are shown in black, enzymes are shown in blue. Dopamine synthesis in neurons of the substantia nigra pars compacta starts with the conversion of L-tyrosine to L-Dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH; a commonly used marker of dopaminergic neurons). L-DOPA is then converted into dopamine by L-amino acid decarboxylase. Dopamine is then stored in vesicles within the cell by vesicular monoamine transporter (VMAT). After release into the synaptic cleft, and subsequent post-synaptic stimulation, dopamine is either taken up by the pre-synaptic neuron via the dopamine transporter (DAT) or metabolised by monoamine oxidase (MAO) and catechol O-methyl transferase (COMT) resulting in the final production of homovanillic acid via the intermediate compounds 3,4-Dihydroxyphenylacetic acid and/or 3-Methoxytyramine.

Table 1.3 Table showing the 9 subtypes of dopaminergic neurons, their associated pathways, location and some prominent efferent connections, associated behaviour/homeostatic activities, and diseases associate with disruption/loss of these neurons. *Associated hormones include gonadotrophin releasing hormone, thyroid stimulating hormone, and growth hormone. SNpc: substantia nigra pars compacta; VTA: ventral tegmental area; ADHD: attention deficit hyperactive disorder. Contributing papers: Björklund, Lindvall and Nobin (1975); Gasbarri *et al.* (1996); Le *et al.* (1999); Webster (2001); Blum *et al.* (2008); Haber (2014); Subramaniam and Roeper (2016); Cooper, Robison and Mazei-Robison (2017); Howes *et al.* (2017); Klein *et al.* (2019).

Associated Pathway		**A#**	**Location in Brain/Areas involved**	**Associated activity**	**Associated diseases**
		A8	Retrorubral field to mesolimbic projection and hippocampus	Motor coordination	
Nigrostriatal pathway		A9	SNpc to dorsal striatum	Motor control, Pain modulation	Parkinson's disease, Huntington's disease
Mesocorticolimbic projection	Mesolimbic pathway	A10	VTA to nucleus accumbens and olfactory tubercle	Reward based learning	Addiction, Schizophrenia, ADHD
	Mesocortical pathway		VTA to prefrontal and perirhinal cortex	Executive functions	Schizophrenia, ADHD
Hypothalamospinal projection		A11	Dorsal posterior hypothalamus to neocortex, dorsal horn, intermediolateral nucleus	Motor functions and muscular sensations	Restless leg syndrome
Tuberoinfundibular pathway		A12	Arcuate nucleus to pituitary gland	Prolactin release	Hyperprolactinaemia
Incertohypothalamic pathway		A13	Zona incerta to hypothalamus	Hormone release*	
		A14	Periventricular nucleus		
		A15	Rostral hypothalamic and periventricular nucleus		
		A16	Olfactory bulb	Smell	

Once dopamine has been synthesised, it is stored in vesicles by vesicular monoamine transporter (VMAT) 1 and 2 until it is released into the synaptic cleft (Lopes *et al.*, 2017). Dopamine can elicit either inhibitory or excitatory responses dependent on the dopamine receptor it binds to. These receptors are often referred to as D1-D5, their binding effect and brain regions associated with highest expression are shown in Table 1.4. The D1 receptor, associated with an excitatory response, is the most abundantly expressed in the brain followed by the D2 receptor, associated with an inhibitory response, and the two are very rarely co-expressed in the same neuron (Klein *et al.*, 2019). Differences in neuronal dopamine receptor expression appears to be a feature explored in the study of schizophrenia (Howes *et al.*, 2017) but does not appear to be related to PD. To prevent continuous stimulation of the post-synaptic neuron, dopamine is removed from the synaptic cleft by either reuptake into the presynaptic neuron by the dopamine transporter (DAT), or it is deaminated by monoamine oxidase (MAO) or catechol O-methyl transferase (COMT), which are produced by both neurons and glia (Lopes *et al.*, 2017).

Table 1.4 Table showing the different dopamine receptors, their binding effect, and brain areas with abundant expression (Klein *et al.*, 2019).

Receptor	Binding effect	Brain regions
D1	Excitatory	Caudate putamen, nucleus accumbens, substantia nigra pars reticular, olfactory bulb, amygdala, frontal cortex
D5	Excitatory	
D2	Inhibitory	Striatum, globus pallidus external, nucleus accumbens (core), ventral tegmental area, hypothalamus, amygdala, cortical areas, hippocampus, pituitary
D3	Inhibitory	
D4	Inhibitory	

Dopaminergic subtypes based on location, gene/protein expression, and electrophysiological patterns exist within the mammalian brain. The A9 dopaminergic neurons of the nigrostriatal pathway within the SNpc and A10 dopaminergic neurons of the mesolimbic and mesocortical projections from the VTA, are the most abundant and well-studied dopamine neurons in the brain (Webster, 2001).

Dopaminergic neurons of the VTA receive inputs from the lateral orbital cortex, ventral striatum and pallidum, and lateral amygdala; whilst dopaminergic neurons of the SNpc receive inputs from sensory cortex, globus pallidus, entopeduncular nucleus, SNpr, subthalamic nucleus and zona incerta (Ogawa and Watabe-Uchida, 2018). Recent studies have further subtyped these based on location within these specific nuclei, and afferent and efferent connections (Subramaniam and Roeper, 2016; Poulin *et al.*, 2018).

Despite their regional proximity, dopaminergic neurons of the SNpc and VTA show distinct characteristics (Table 1.5), including efferent connections to the striatum (dorsal and ventral respectively), electrophysiology (Krashia *et al.*, 2017), and expression of ion channels (review: Gantz *et al.*, 2018). Marker expression differences have also been observed in mature dopaminergic neurons, with all SNpc neurons expressing GIRK2 (G protein-coupled inwardly-rectifying potassium channel 2) but only ~55% of VTA neuron expressing GIRK2 (Reyes *et al.*, 2012). Marker expression, morphology and projections even vary within the ventral/dorsal subpopulations of SN and VTA, highlighting the complexity of these midbrain dopaminergic neurons (Björklund and Dunnett, 2007).

Table 1.5 Table showing differences in marker expression across A8, A9 and A10 subtype dopaminergic neurons. Neurons of the A8 retrorubral field appear to be less studied, - indicates information for this marker was not commented on.

Marker/ Characteristic	**A8**	**A9**	**A10**	**Reference**
Nurr1	Not essential	Essential	Essential	Le *et al.* (1999)
Calbindin	Low	Low	High	Liang, Sinton and German (1996)
GIRK2	-	High	Low	Reyes *et al.* (2012)
Pacemaking mechanism	-	Calcium ion channels	Sodium ion channels	Khaliq and Bean (2010)
Cytosolic dopamine	-	> A10	< A9	Mosharov *et al.* (2009)
DAT	-	High	Low	Björklund and Dunnett (2007)
Neuromelanin	-	High	Low	Kastner *et al.*, (1992)

Midbrain dopamine neurons in retrorubral field (A8) and VTA (A10) also degenerate in PD but to a lesser extent than those in the SNpc (A9; German *et al.*, 1992; Alberico, Cassell and Narayanan, 2015). Calcium binding protein (Calbindin) is differentially expressed across the dopaminergic nuclei of the midbrain and Calbindin^{+} neurons are selectively spared in both PD and MPTP-induced degeneration (German *et al.*, 1992; Haber, 2014). Calbindin^{+} neurons also express lower levels of DAT (Björklund and Dunnett, 2007) which may contribute to their decreased vulnerability.

Dopaminergic neurons of the SNpc are large with long, unmyelinated axons and high numbers of synapses (Bolam and Pissadaki, 2012). A dopaminergic neuron of the VTA is estimated to have approximately 12,000-30,000 synapses, whilst an SNpc dopaminergic neuron is estimated to give rise to approximately 102,000-245,000 synapses. These features of SNpc neurons result in a high energy demand and could make them more susceptible to perturbations in energy supply (Bolam and Pissadaki, 2012) leading to increased vulnerability.

Neuromelanin is a dark, melanin pigment present in the SN and locus coeruleus of the human brain (and some other mammals including primates, horses, and sheep), appearing after 2-3 years of age in humans (Zecca *et al.*, 2001) and progressively increasing with age (Knörle, 2018). Higher content of neuromelanin is observed in SNpc neurons compared to VTA, and the number of pigmented neurons is significantly lower in PD brains suggesting increased vulnerability of pigmented neurons to degeneration (Kastner *et al.*, 1992). The involvement of neuromelanin in dopamine neuron degeneration in PD and its cytotoxic/cytoprotectant properties are debated. Neuromelanin has been found to be involved in cellular iron accumulation, oxidative stress, and calcium binding, although its role in these dopaminergic neurons and their pathology in PD remains unknown (review: Knörle, 2018).

The differences highlighted here are observed between mature dopaminergic neurons of the SNpc and VTA and may contribute to the differing vulnerability of these neurons to degeneration in PD. However, specification of these subsets of neurons within the midbrain and differential expression of specific markers is also observed in their embryonic development.

1.3 Midbrain development and homeostasis

The development and maintenance of A9 dopaminergic neurons requires a complex combination of transcription factors and diffusible signals, shown in a simplified diagram in Figure 1.5 (Castelo-Branco *et al.*, 2003; Arenas, Denham and Villaescusa, 2015). Whilst Nurr1, Pitx3, and Lmx1b expression are essential in the maintenance of A9 and A10 midbrain dopaminergic neurons, these post-mitotic transcription factors are not essential for other dopaminergic cell subtypes (Goridis and Rohrer, 2002; Bissonette and Roesch, 2016). Understanding the development of these A9 neurons can contribute to the development of treatments and also an understanding of potential causes of neurodegeneration.

However, neurons only constitute a minority of the cellular composition in the human brain, with the majority broadly classed as glia (Jessen, 2004). Glia can be further subtyped into microglia (discussed in Chapter 1.4) and macroglia, which account for astrocytes, oligodendrocytes, and oligodendrocyte precursors (also termed NG2-glia) of the CNS. Diffusible factors termed netrins (extracellular proteins related to laminin), arising from the floor plate of the embryonic neural tube, are involved in directing both neuron and macroglia development. Neuroepithelial progenitor cells transform into radial glia which initially generate neurons before astrocyte and oligodendrocyte precursor generation through factors including *Sox9*, *Olig1*, and *Olig2* (Losada-Perez, 2018).

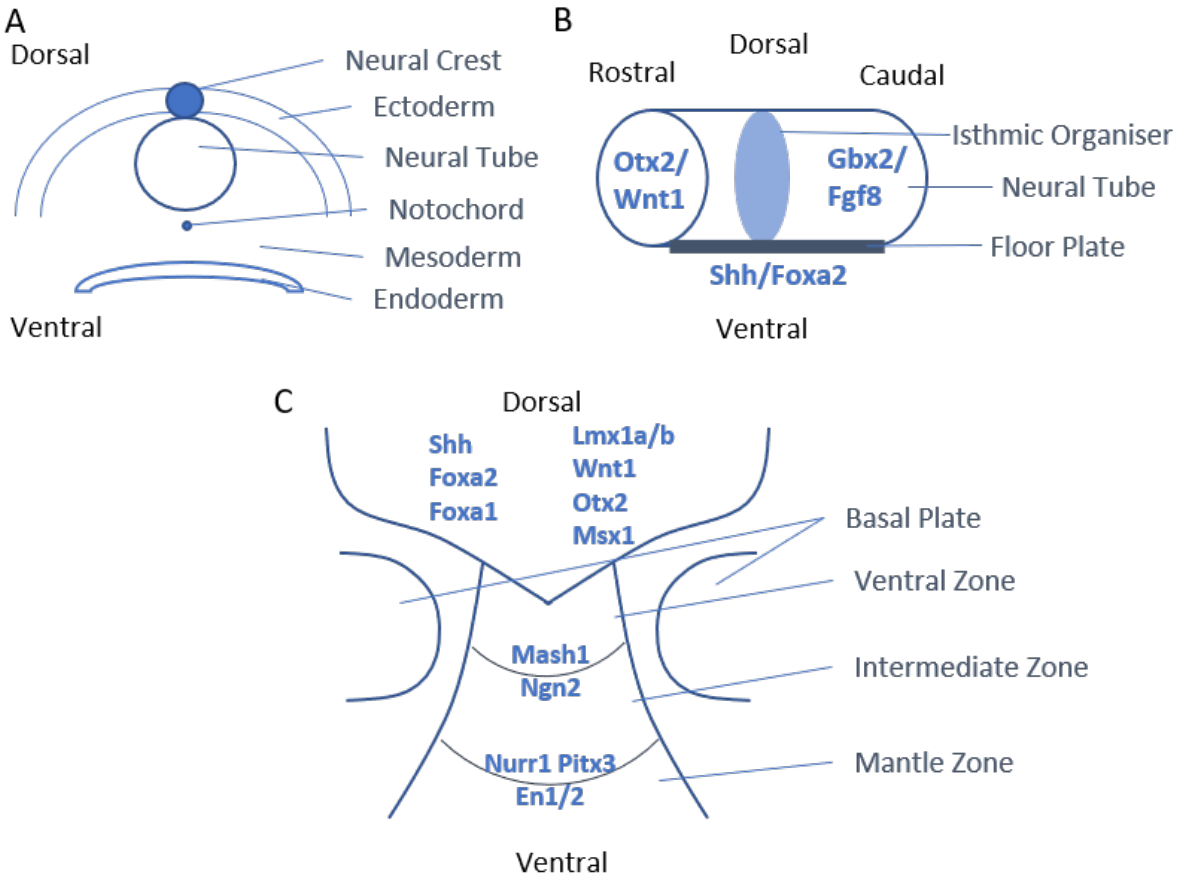


Figure 1.5 Diagram illustrating signalling patterns in the development of A9 dopaminergic neurons *in vivo*.
A (coronal view of embryo):
After the development of the three germ layers at around 3 weeks in human development (Pansky, 1982) and around embryonic day 7 in mouse (Gray and Ross, 2011), signalling at the rostral end of the embryo leads to the formation of the neural tube.
B (lateral view of neural tube):
Coordinated expression and repression of Otx2 and Gbx2 (respectively) forms the midbrain-hindbrain boundary where the isthmic organiser (IsO) is generated. Sonic hedgehog (Shh), secreted by the notochord, induces Foxa2 which signals the formation of the floor plate (FP), from which, midbrain dopaminergic neurons are generated (Ono *et al.*, 2007). Cells rostral to the IsO express Otx2 which regulates Wnt1, whilst cells caudal to the IsO, express Gbx2 which regulates Fgf8 (through Pax2). This gradient induces caudal/rostral patterning.
C (coronal view of floor plate):
During this time, the FP expresses Shh, creating a gradient regulating ventral/dorsal specification. Wnt1 is also expressed in two medial bands within the FP. High expression of Shh upregulates Foxa2 which, alongside Foxa1 and Otx2, regulates expression of Lmx1a and Lmx1b. Lmx1b is required for the differentiation of dopamine progenitors; whilst Lmx1a is required for the specification of dopaminergic neurons in the FP, it also induces expression of Msx1 which supresses basal plate fate. An auto-regulatory loop is formed with β-catenin which upregulates Lmx1a and Otx2. Lmx1a/b upregulate each other as well as Wnt1, Msx1, Nurr1 and Pitx3. The Shh-Foxa2 and Lmx1a/b-Wnt1-Otx2 networks regulate expression of Mash1 and Ngn2 in the ventral zone of the FP. Dopaminergic neurogenesis takes place in the ventral zone, as neuroblasts descend through the intermediate zone to the mantle zone, they differentiate into dopamine neurons and express transcriptions factors Nurr1, Pitx3, En1 and En2 which are essential for maturation and maintenance. From here, A9 neurons migrate towards the SNpc with maturation typically occurring through weeks 6-11 in humans, and starting at E10.5 in mice (Arenas, Denham and Villaescusa, 2015).

The importance of glia is apparent in their roles in migration and axon guidance, neuron survival and regeneration, synapse maintenance, signal transduction, and immune response (Jessen, 2004; Losada-Perez, 2018). Astrocytes are the most abundant glial subtype in the CNS (Jessen, 2004) and are essential to neuron survival, with dysfunction related to a number of neurodegenerative disorders including PD (Phatnani and Maniatis, 2015). One of the important roles of glial cells is the breakdown of neurotransmitters. As described in Figure 1.4, dopamine is degraded by MAO and COMT. MAO has two isoforms, A and B; both isoforms are expressed in astrocytes, whilst only MAO-A is expressed in dopaminergic neurons (Youdim and Bakhle, 2006). COMT is expressed by both astrocytes and microglia, with CNS expression highest in microglia, and it is not present in neurons of the SNpc (Meiser, Weindl and Hiller, 2013). Although glia possess the ability to metabolise dopamine, their role in PD is often overlooked. However, their roles in dopamine transport are becoming an area of study (Meiser, Weindl and Hiller, 2013; Winner *et al.*, 2017; Petrelli *et al.*, 2020).

Glia can be difficult to study *in vitro*, often displaying non-physiological phenotypes and expression profiles when isolated and expanded in culture (Ransohoff, 2016). However, their importance in development and homeostatic maintenance of the brain is clear and research into this field could help to find novel neurodegenerative/neuroprotective mechanisms relevant to many CNS diseases including PD.

1.4 Microglia

1.4.1 Microglia are the immune cells of the central nervous system

Present in similar numbers to neurons, microglia represent 10-20% of the glial population (Soulet and Rivest, 2008). Unlike other CNS cell types which develop from the neural plate (Bear, Connors and Paradiso, 2007), microglia originate from yolk sac macrophages and migrate into the embryonic brain (Ginhoux *et al.*, 2013) maintaining their capacity for self-renewal into adulthood (Bruttger *et al.*, 2015). This migration is thought to take place very early in development, before formation of the blood brain barrier (BBB). Microglial cultures have been harvested from as early as embryonic day 10 (E10) rat brains (Ghosh and Ghosh, 2016). *In vivo*, their three-dimensional organisation is considered homogeneous and territorial with microglia keeping consistent cell to cell distances (Nimmerjahn, Kirchhoff and Helmchen, 2005).

Whilst they are present throughout the CNS, their population density can change depending upon their location (Akiyama and McGeer, 1990). Microglia make up 5% of the cortex cell population increasing to 12% of the SN, with the basal ganglia being one of the most densely populated areas in mice (Lawson *et al.*, 1990). Microglial density also varies across the BG of mice, with the VTA showing the lowest density and the SN showing the highest (De Biase *et al.*, 2017). Alongside changes in population densities, microglia also exhibit morphological variation and unique expression profiles based on their location and organism age, this indicates how adaptive they are to their micro-environment (Lawson *et al.*, 1990; de Haas, Boddeke and Biber, 2008; Boche, Perry and Nicoll, 2013; Crain, Nikodemova and Watters, 2013; Ginhoux *et al.*, 2013; Hayley *et al.*, 2015; Ghosh and Ghosh, 2016; Tan, Yuan and Tian, 2020).

Microglia play important roles in brain development (Bilimoria and Stevens, 2015; Anderson and Vetter, 2018; Menassa and Gomez-Nicola, 2018) and homeostasis (Paolicelli *et al.*, 2011; Schafer *et al.*, 2012), with altered function associated with neurodegenerative conditions, including PD. Microglia are heavily involved in tissue remodelling, axon guidance, and synaptic pruning in brain development, with ongoing supportive and adverse interactions affecting adult neurogenesis in both the healthy and injured brain (Ekdahl, Kokaia and Lindvall, 2009) highlighting their importance and influence throughout life.

Although microglia share many features of circulating monocytes and tissue macrophages (Perry, Hume and Gordon, 1985; Akiyama and McGeer, 1990; Boche, Perry and Nicoll, 2013), not all that applies to circulating monocytes can be applied to microglia (Pepe *et al.*, 2017; Haage *et al.*, 2019). Just as in the peripheral immune system, microglia display pro-inflammatory and anti-inflammatory states that have been classed to reflect those of the peripheral immune system, although the states of microglia are less well-defined and are still being uncovered.

1.4.2 Microglia activation involves switching between phenotypes classed as M0, M1, and M2

In healthy tissue, microglia have a highly ramified shape with active and dynamic processes (Soulet and Rivest, 2008). Typically, the soma shows few signs of migration, however, the processes are continuously forming and withdrawing, displaying highly motile filopodia-like projections (Nimmerjahn, Kirchhoff and Helmchen, 2005). This state is typically referred to as the 'resting/surveillance', or M0 state in which microglia are surveying the environment. However, microglia change their morphology, gene expression, function, motility and proliferation capacity in response to stimuli indicating immediate or potential

disruption of brain homeostasis (Nimmerjahn, Kirchhoff and Helmchen, 2005; Kettenmann *et al.*, 2011). These changes result in microglia displaying M1 or M2 phenotypes, typically related with pro-inflammatory and anti-inflammatory functions respectively (Figure 1.6). These states are thought to be transitory, and, as individual cells can express both M1 and M2 features simultaneously, there is continuing debate about which markers are genuinely indicative of phenotype (Donat *et al.*, 2017), with markers and morphology also varying across brain regions in healthy tissue (Lawson *et al.*, 1990; De Biase *et al.*, 2017).

Microglia are constantly sensing their surroundings and are equipped with a range of receptors including toll-like receptors, nucleotide-binding oligomerisation domains, and scavenger receptors to recognise potentially harmful stimuli (Orihuela, Mcpherson and Harry, 2016). In response to pro-inflammatory signals, microglia become "classically activated", displaying an M1 phenotype with larger soma and shorter processes, typically described as amoeboid, or rod-like in shape (Davis, Foster and Thomas, 1994), and increased motility (Figure 1.6). This stage is often related to urgent responses and defence, attempting to limit damage and combat infection (Abd-El-Basset and Fedoroff, 1995; Nimmerjahn, Kirchhoff and Helmchen, 2005; Cho *et al.*, 2006). During this stage microglia express a variety of cytokines including tumour necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-12, and free radicals such as reactive oxygen and nitrogen species (ROS; RNS) meaning that this state is often related to neuronal damage induced by inflammation (Wang *et al.*, 2015).

"Alternatively activated" or M2 phenotypes include anti-inflammatory, repair-promoting, and phagocytic behaviours (Smith *et al.*, 2012). This polarisation has been further classified into M2a, M2b, and M2c subtypes with M2a associated with repair and regeneration, M2b associated with immune modulation (Chhor *et al.*, 2013), and M2c associated with immune suppression (Gensel and Zhang, 2015). However, these subtypes remain controversial, being mainly described from *in vitro* studies.

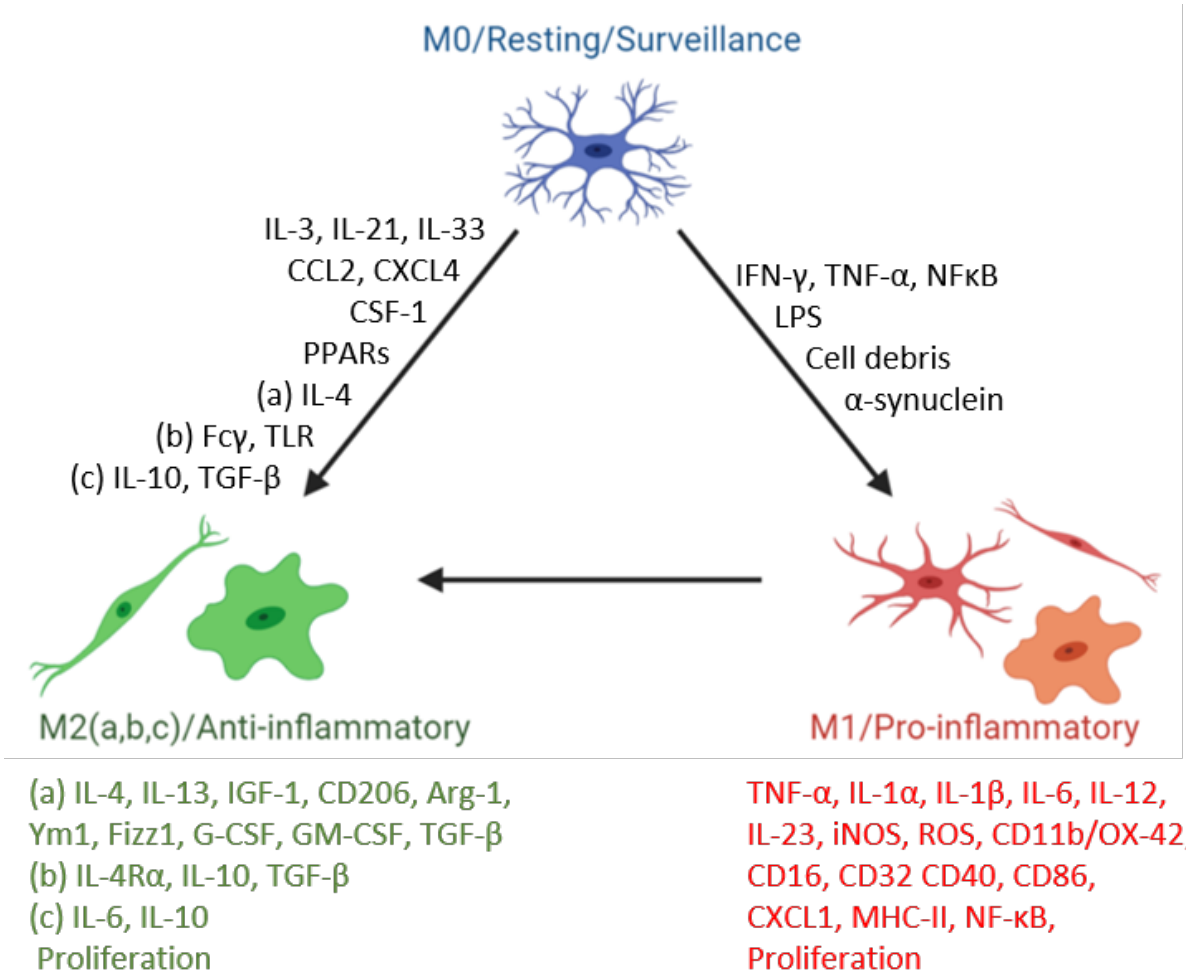


Figure 1.6 Diagram illustrating some of the features of microglia. Morphology, expression profiles, and released factors change from M0 to either M1 (red) or M2 (green) with different activation stimuli shown in black. Red text indicates some of the characteristics associated with M1 activation, green text indicates characteristics of M2 activation, which can be further subtyped into a, b, and c associated factors.
Arg: arginase; CCL2: monocyte chemoattractant protein; CD: cluster of differentiation molecule; CSF – colony stimulating factor; CXCL: chemokine ligand; Fcγ: fragment crystallisable gamma; Fizz1: Resistin-like molecule alpha/found in inflammatory zone protein; G-CSF: granulocyte colony-stimulating factor; GM-CSF: Granulocyte-macrophage colony-stimulating factor; IFN-γ: interferon gamma; IL: interleukin; iNOS: nitric oxide synthase; LPS: lipopolysaccharide; MHC-II: major histocompatibility complex class II; NF-κB: nuclear factor-κB; PPARs: peroxisome proliferator-activated receptors; ROS: reactive oxygen species; TGF-β: transforming growth factor beta; TNF-α: tumour necrosis factor alpha; Ym1: eosinophil chemotactic factor-lymphocyte/chitinase 3-like 3. Zhang *et al.* (2005); Chhor *et al.* (2013); Tam and Ma (2014); Gensel and Zhang (2015); Orihuela, Mcpherson and Harry (2016); Pepe *et al.* (2017). Created using BioRender.com

1.4.3 Microglia have been implicated in neurodegeneration

The activation state of microglia has been implicated in a number of brain diseases, including epilepsy and dementia (Devinsky *et al.*, 2013; Clayton *et al.*, 2017), with microglia observed at sites of neurodegeneration, both post-mortem and *in vivo*, in a variety of diseases including AD, PD (McGeer *et al.*, 1988; Fan *et al.*, 2015), multiple sclerosis, and amyotrophic lateral sclerosis (Liu and J.-S. Hong, 2003). Therefore, the inflammatory response of microglia has become a potential target for treatment for a number of neurodegenerative conditions (Liu and J.-S. Hong, 2003).

Their exact role in neurodegeneration is unclear but microglia are thought to exacerbate the degeneration of neurons. Fan et al. (2015) found significant increases in microglial activation and decreases in glucose metabolism (which reflects neuronal synaptic function) in patients with AD, amnestic mild cognitive impairment (the transitional stage between healthy and AD), and PD dementia. Although this highlights a relationship between microglia activation and neuronal death, it does not necessarily show that microglia are the cause of neuronal death in AD and PD.

Hypotheses for the microglial role in neurodegeneration include a sustained pro-inflammatory state resulting in the generation of ROS and RNS causing neuronal damage; and an inability of microglia to clear aberrant or aggregated proteins leading to neurotoxicity and further stimulating a pro-inflammatory environment (Orihuela, Mcpherson and Harry, 2016). A potential cause for unwarranted microglial activation leading to degeneration is a process known as priming. It is possible that age or disease “primes” microglia, resulting in an exaggerated immune response (heightened or prolonged) to subsequent stimuli (whether infection, trauma, or something previously innocuous), potentially causing damage to otherwise healthy tissue (Perry and Teeling, 2013).

The strongest genetic risk factor for late onset AD is apolipoprotein E type 4 (APOE4) (Corder *et al.*, 1993; Farrer, 1997; Liu *et al.*, 2013; Belloy, Napolioni and Greicius, 2019). Expression has been found in some, but not all, astrocytes, <10% of microglia, and injured neurons (Uchihara *et al.*, 1995; Grehan, Tse and Taylor, 2001; Xu *et al.*, 2006). Although the mode of action is not clear, it is thought that isoforms of APOE4 are differentially involved in amyloid beta deposition and clearance (Kim, Basak and Holtzman, 2009; Liu *et al.*, 2013). Furthermore, APOE has been linked to increased inflammatory microglial phenotype (Krasemann *et al.*, 2017; Ulrich *et al.*, 2018), however, microglial depletion in the P301S tau transgenic AD mouse model which is homozygous for human APOE4 prevented neurodegeneration (Shi *et al.*, 2019) suggesting that it is the reaction of microglia to amyloid beta deposition driving neuronal degeneration.

Proliferation is an indicator of microglial activation (Gensel and Zhang, 2015) and increased microglial numbers at sites of neurodegeneration have been associated with disease progression (Olmos-Alonso *et al.*, 2016; Clayton *et al.*, 2017; Mathys *et al.*, 2017). Increasing microglia numbers and pro-inflammatory factors IL-1β and TNFα have been observed in a mouse model of frontotemporal dementia, corresponding with disease symptoms (Clayton *et al.*, 2017). Whilst proliferative markers for microglia in this frontotemporal dementia model were low initially, Mathys *et al.* (2017) observed high proliferation early on in a mouse AD model. Inhibition of microglial proliferation (by the tyrosine kinase inhibitor GW2580) showed improvements in behaviour deficits and a shift from pro-inflammatory to anti-inflammatory phenotype, without affecting amyloid plaques (protein aggregates associated with AD pathology) in a mouse model of AD (Olmos-Alonso *et al.*, 2016). However, depletion of microglia has been shown to suppress the propagation of abnormal tau protein aggregation through brain regions in another AD mouse model (Asai *et al.*, 2015). Despite discrepancies in these studies, they indicate that microglia change with

disease progression, and that the route of pathogenesis differs across brain regions and disease models.

A common risk factor in a number of neurodegenerative diseases, including PD, is ageing (Johnson, 2015; Collier, Kanaan and Kordower, 2017). Alongside a variety of other biological changes, microglial expression patterns change throughout life, suggesting a potential for different microglial responses to stimuli at different ages (Crain, Nikodemova and Watters, 2013). Ageing has been associated with changes in the neuroinflammatory environment, and microglia dysfunction could be a contributing factor to age associated neurodegeneration (Grabert *et al.*, 2016). Contrary to over-active inflammation of microglia, it is also hypothesised that dystrophic, dysfunctional, or senescent microglia may result in reduced microglial neuroprotection (Streit and Xue, 2009; Askew *et al.*, 2017). Ageing of microglia may be more problematic than for peripheral macrophages, as the latter are replenished from bone marrow stem cells, whereas microglia self-renew, perhaps with increased senescence. Although it is not clear how senescent microglia may contribute to neurodegeneration, aged microglia show compromised surveying activity in the M0 state, with fewer branches and slower process motility. Response to pro-inflammatory stimuli showed decreased migration and a pro-longed inflammatory response (Damani *et al.*, 2011). A mouse model of tau-dependent degeneration also found a positive correlation between senescent cells and neuronal loss, with removal of these senescent glia preventing gliosis, tau deposition and neuronal degeneration (Bussian *et al.*, 2018).

Long term use of nonsteroidal anti-inflammatory drugs during mid-life has been shown to reduce the risk of AD, but not other forms of dementia (in ’T Veld *et al.*, 2001). Therefore, further exploration of the roles of microglia in neurodegeneration, and perhaps a more targeted approach to microglia, are required to fully assess potential benefits and development of improved treatments.

1.4.4 Roles of microglia in Parkinson's disease

As previously discussed, there are a number of ways that microglia could be implicated in neurodegenerative disease including PD, and a number of studies have shown the possibility of microglia involvement specific to PD, including increased inflammatory markers present in the serum of PD patients (Medeiros *et al.*, 2016).

A number of studies have shown that activated microglia can destroy otherwise healthy neurons. Injection of lipopolysaccharide (LPS; molecules found on many bacterial species which elicit an inflammatory response) into the nigrostriatal pathway of rats resulted in microglia activation and subsequent destruction of dopaminergic neurons, despite a lack of toxic effect of LPS to dopamine neurons alone (Bronstein *et al.*, 1995; Herrera *et al.*, 2000). Microglia have also been shown to destroy dopaminergic neurons after transection of the medial forebrain bundle (Cho *et al.*, 2006), suggesting that microglia may destroy damaged neurons.

Injection of neuromelanin into the SN of rats resulted in strong microglia activation and significant dopamine neuron loss (Zecca *et al.*, 2008) suggesting that the presence of neuromelanin may be a contributing factor in the selective vulnerability of SNpc dopaminergic neurons. Neuromelanin is phagocytosed and degraded by microglia, however it also elicits a pro-inflammatory response through release of detrimental factors including ROS (W. Zhang *et al.*, 2011). Although neuromelanin is also present in healthy individuals, it is possible that cell death triggered by external factors could release neuromelanin which in turn exacerbates dopaminergic neuronal degeneration of the SNpc, with a feed forward cycle of neuronal damage and uncontrolled inflammation amplifying and potentiating neurotoxicity (Gao and Hong, 2008). This idea is supported by Langston *et al.* (1999) with the observation of continued degeneration in individuals with MPTP-induced parkinsonism displaying ongoing

degeneration of dopaminergic neurons years after the initial insult with microglial clusters around neurons and extracellular neuromelanin in the SNpc.

Dopaminergic neurons of the SNpc may also be inherently more vulnerable to degeneration exacerbated by microglia due to their higher iron content. Iron was able to potentiate pro-inflammatory microglial activation and neurotoxicity in rat neuron-astrocyte-microglia cultures leading to selective and progressive dopamine neuron degeneration (Zhang *et al.*, 2014). Both of these mechanisms, an adverse response to iron or neuromelanin, are based on neurons dying and releasing their contents, heightening the microglial inflammatory response, rather than instigating the inflammation. It remains unclear whether inflammation may be associated with the onset of PD pathology, or whether it simply exacerbates the process.

Microglia are closely involved in synapse maintenance and constantly receive signals from neurons. Dopamine receptors 1-4 are expressed on human microglia (Mastroeni *et al.*, 2009) and dopamine has been found to be involved in inflammatory regulation. It is suggested that decreased dopamine signalling induces a pro-inflammatory response through a change in dopamine receptor stimulation from low-affinity receptors (in response to high homeostatic conditions) to high-affinity receptors (in response to reduced dopamine levels; Broome *et al.*, 2020). Further suggesting a role for microglia in the propagation of dopaminergic neuron death.

Microglia may also be involved in the spread and toxicity of α-synuclein. PD derived exosomes containing α-synuclein were phagocytosed by microglia, inducing an inflammatory response with the release of pro-inflammatory cytokines, nitric oxide production, and increased proliferation. Microglia were also shown to transmit this α-synuclein from human exosomes to mouse striatal neurons. Exosomes also suppressed autophagy, increasing the accumulation of α-synuclein (Xia *et al.*, 2019). Microglia senescence with age, observed by a

decrease in branching complexity, soma size increases and 'surveillance area' decreases, in SNpc and VTA of aged mice (1, 6, 9, 18, 24 months; Shaerzadeh *et al.*, 2020), may also be a factor in α-synuclein pathology in PD.

Microglia show a clear role in the pathogenesis of PD, although the exact relationship, and series of degenerative events, is difficult to assess. Pro-inflammatory microglia likely contribute to disease progression, therefore, the switching of microglia to an anti-inflammatory M2 state could be critical to ending inflammation and supporting repair/regeneration. There is reason to believe that neuro-immunomodulatory treatments targeting microglia could be therapeutically beneficial in PD (Liu and J.-S. Hong, 2003; Choi, Koppula and Suk, 2011). However, progression in this area of research relies on the use of models of PD.

1.5 Models of Parkinson's disease

The inaccessible nature of the brain in general, and specifically the SNpc, alongside a dissociation between PD pathology and motor-symptom onset, means that studying the molecular changes occurring in PD is often not possible until post-mortem. Whilst this captures the end stages of the disease, early pathology is not able to be elucidated. Not only does this limit our understanding of possible causes, but also disease onset, the ideal point in time when most interventions would be applied. Therefore, PD models that reproduce key features of the disease and its progression are often used to study both potential factors that could cause the disease as well as possible treatments.

The variety of pathologies, including progressive degeneration, α-synuclein aggregation, mitochondrial dysfunction, and inflammation; coupled with a natural susceptibility that is restricted to humans; make it difficult to study complete PD pathology in one model.

1.5.1 *In vivo* models of Parkinson's disease

Animals are not naturally susceptible to PD, the symptoms must be induced and so models do not capture the full spectrum of PD, how it begins in idiopathic cases, and how it progresses. Full characteristics of SNpc dopamine neurons are also not present in rodent models, for example, the presence of neuromelanin is restricted to humans, primates, and some other mammals (Webster, 2001). However, models do allow for study of some of the motor and behavioural deficits experienced by people with PD. A number of animal models of PD have been developed, typically dependent on the administration of a toxin that targets dopaminergic neurons. The use of 6-hydroxydopamine (6-OHDA) and MPTP are perhaps the most popular models, although the use of rotenone and lactacystin also produce parkinsonian

symptoms and pathological hallmarks of PD. Some of the animal models of PD are described below, although this list is not exhaustive.

Since 6-OHDA cannot cross the BBB (Simola, Morelli and Carta, 2007) administration of 6-OHDA is applied by direct injection, typically unilaterally, into the SN or medial forebrain bundle, inducing the loss of the nigrostriatal dopaminergic pathway. Injection of desipramine (a noradrenaline transporter inhibitor) 30-60 mins prior to 6-OHDA improves specificity of dopaminergic toxicity. This method is commonly chosen for the rat model of PD, with administration of amphetamine used to study the unilateral motor deficits induced by destruction of the nigrostriatal pathway through rotation tests (Duty and Jenner, 2011). Toxicity occurs through accumulation of 6-OHDA into dopaminergic neurons which subsequently results in neuronal dysfunction through oxidative stress (via auto-oxidation of 6-OHDA) and direct inhibition of mitochondrial activity (Simola, Morelli and Carta, 2007). Microglial activation (Cicchetti *et al.*, 2002) and the pro-inflammatory marker TNF-α (Mogi *et al.*, 2000) are also increased in the 6-OHDA rat model which corresponds to the neuroinflammation response observed in PD brains.

The toxin MPTP was later discovered as a selective toxin for SN dopaminergic neurons with the effects also observed in the primate brain, generating a primate model of PD (Duty and Jenner, 2011). MPTP is able to cross the BBB, after which it is taken up and metabolised by astrocytes through MAO activity producing the toxin 1-methyl-4-phenylpyridinium (MPP^+) which is then selectively taken up by dopaminergic neurons where it inhibits mitochondrial activity (Dias, Junn and Mouradian, 2013; Chen, Turnbull and Reeve, 2019). However, rodents are less sensitive than primates to MPTP toxicity due to a more rapid clearance of MPTP and its metabolites (Johannessen *et al.*, 1985), although some specific mouse breeds are more susceptible (Duty and Jenner, 2011). One of the proposed mechanisms for MPTP-induced

toxicity is through poly(ADP-ribose) polymerase (PARP) activation, with *PARP*$^{-/-}$ mice showing resistance to MPTP mediated toxicity (Mandir *et al.*, 1999).

The organic pesticide rotenone is also used to generate animal models of PD. This toxin is able to easily cross the BBB and diffuse into neurons where it accumulates within mitochondria inhibiting complex I (Duty and Jenner, 2011). Degeneration starts in the axon terminals retrogradely progressing towards to the soma, and also involves the aggregation of α-synuclein and appearance of LBs (Greenamyre, Betarbet and Sherer, 2003). Other hallmarks of PD degeneration are also observed including oxidative stress, and microglial inflammation, however, toxicity is not limited to the CNS and it can produce mortality through cardiovascular toxicity (Duty and Jenner, 2011).

Another method for the *in vivo* modelling of PD is through proteasome inhibition. Proteasome dysfunction is a possible pathogenic mechanism for the degeneration of dopamine neurons in PD. A proteasome inhibitor commonly used is lactacystin, which is typically, and most reliably, used to produce *in vivo* models generating PD-like symptoms from injection into the SN or medial forebrain bundle of rodents. The mechanisms for toxicity are suggested to include the production of ROS, mitochondrial dysfunction, and accumulation of iron and α-synuclein (Bentea, Verbruggen and Massie, 2017).

Genetic based models have also been developed, typically using mutations linked to the hereditary forms of PD such as *LRRK2*, *PINK1*, *GBA*, and *DJ-1* (Dawson, Ko and Dawson, 2010). These models can provide insights into pathogenesis and molecular mechanisms of the disease as well as providing a testbed for potential new treatments, although their application may be restricted to genetic, rather than idiopathic, PD.

These *in vivo* models all provide methods to study the mechanisms behind, and potential treatments for, PD. Each have advantages and disadvantages with reviews in the literature both across and within these model systems (Greenamyre, Betarbet and Sherer,

2003; Dawson, Ko and Dawson, 2010; Duty and Jenner, 2011; Bentea, Verbruggen and Massie, 2017). The most commonly used and well-characterised toxin for *in vivo* models appears to be MPTP, although 6-OHDA is also widely used.

1.5.2 *In vitro* models of Parkinson's disease

In vitro models provide a more controlled environment to study the cellular and molecular pathophysiological mechanisms that may take place in PD degeneration, with advantages and disadvantages to their simplicity. A variety of cell lines and primary derived cultures, alongside the use of genetic manipulations and the toxins described above, are typically used to generate *in vitro* PD models. To accurately recapitulate the dopaminergic neuron degeneration that occurs in PD, ideally, cell models should have the same features as SNpc dopaminergic neurons (described in Table 1.5). Some of the available cell lines and culture techniques used to model PD are described below, however, these examples are not exhaustive.

The PC12 cell line is derived from rat pheochromocytoma (Zou *et al.*, 2016) and has the ability to synthesise, store, release, and uptake catecholamines, including dopamine, as well differentiate into neuron-like cells in response to nerve growth factor. Advantages of the use of these cells as an *in vitro* model include that they are well-studied and characterised, can be genetically modified, and have characteristics of dopaminergic neurons (review: Malagelada and Greene, 2008). However, this cell line is derived from cancerous rat adrenal tissue, severely limiting its application to human dopaminergic neurons. Nevertheless, research can be extrapolated to other PD models and this cell line can provide a method for the quick processing of theories surrounding PD.

The RCSN-3 cell line is derived from adult rat SN. In their proliferative state, RCSN-3 cells express enzymes involved in catecholaminergic synthesis, including TH, synthesise and

release dopamine, and express dopamine, noradrenaline, and serotonin transporter (review: Paris *et al.*, 2008). Whilst these cells hold the advantage of derivation from the SN, they are not truly representative of dopamine neurons.

One of the most commonly used cell lines described as an *in vitro* PD model is the human neuroblastoma cell line SH-SY5Y (Kermer *et al.*, 2015; Anis *et al.*, 2018; Elyasi *et al.*, 2018; Morroni *et al.*, 2018; Rafiepour *et al.*, 2018; Wang *et al.*, 2018). These cells are easy to maintain and proliferate in culture and upon application/withdrawal of specific factors can differentiate into catecholaminergic-like neurons expressing markers involved in the synthesis of dopamine and noradrenaline. This cell line also holds the advantage of being human. However, whilst this cell line has proven valuable in the understanding of PD pathology, its derivation from a neuroblastoma means that it cannot accurately recapitulate A9 dopaminergic neurons (review: Xicoy, Wieringa and Martens, 2017), and do not display their specific characteristics as outlined in Chapter 1.2.

Human embryonic stem cell (hESC) lines are typically used to study the development of dopaminergic neurons with the aim of reliably generating large numbers of dopaminergic neurons (this is discussed in more detail in Chapter 1.7). However, much of this research is focussed on the generation of neurons as a therapeutic tool (discussed in Chapter 1.6.3) rather than modelling disease; this may be due to the difficulty in generating large numbers of specific A9 dopaminergic neurons (Arenas, Denham and Villaescusa, 2015) to then model degeneration. Neural stem cell (NSC) lines such the *v-myc* immortalised hVM1 cell line derived from human ventral mesencephalon provide a source of cells that more readily differentiate into midbrain dopaminergic neurons. NSCs allow for the study of factors involved in later specification of midbrain dopamine neurons and can also provide models for screening of pharmacological therapies for PD (Villa *et al.*, 2009). The use of stem cells for modelling PD can be difficult due to large variation in dopamine neuron yield across cell lines and

differentiation protocols. However, these cell lines can provide a valuable tool in studying development and vulnerabilities of dopaminergic neurons through genetic manipulation of these cells (Simmnacher *et al.*, 2020).

Induced pluripotent stem cells (iPSCs) can also provide a valuable tool for understanding the pathology of PD. A number of iPSC cell lines derived from PD patients have been developed and differentiated towards dopaminergic phenotypes allowing for the study of the molecular mechanisms involved in hereditary forms of PD. These cells have been critical in the study of PD, especially surrounding genetic risk factors, although there can be great variability across cell lines dependent on reprogramming methods and differentiation protocols. Whilst improvements are still needed, iPSCs provide great potential for PD modelling *in vitro* and further exploration of disease pathogenesis and treatment options (review: Hu *et al.*, 2020).

Primary cultures derived from the developing ventral mesencephalon (VM) have been considered the gold standard for *in vitro* modelling of PD (Pruszak *et al.*, 2009). This method allows for generation of genuine SNpc dopamine neurons, typically from rat, and as well as providing a testbed for protection from toxic insult in mature cultures, it can also be used to assess developing midbrain dopaminergic neurons. These cultures hold the advantage of containing other cell types present in the SN, providing an *in vitro* environment that more closely mimics the brain and allowing for the study of cell-cell interactions (Pruszak *et al.*, 2009). Also related to this technique is the use of VM organotypic slice cultures, typically derived from postnatal rat. These hold the advantage of maintaining cellular interactions and 3-dimensional structure, as well as identification of the A8, A9, and A10 midbrain dopaminergic regions. However, they are more labour intensive, sensitive, and vulnerable to degradation than embryonic VM cultures and can present variation within, and across experimental repeats (Stahl, Skare and Torp, 2009).

These cell cultures represent the initial set-up, however, to truly model PD, dopaminergic cell death needs to be induced. As described in Chapter 1.5.1, toxins are typically used to target dopaminergic neurons. Due to the need for conversion of MPTP to MPP^+ by astrocytes, it is not often used in *in vitro* models and 6-OHDA tends to be the most popular toxin to induce cell death in these cultures. MPTP also holds a higher risk of toxicity to humans than 6-OHDA, making it a more dangerous toxin for researchers to handle. Typically, the choice of toxin is dependent on the cell death pathway that is under investigation.

Despite decades of research and the development of a myriad of *in vivo* and *in vitro* PD models, the pathogenesis of idiopathic PD is not yet fully understood, and effective long-term treatments are not yet available for the motor symptoms or non-motor symptoms of the disease.

1.6 Treatments for the motor symptoms of Parkinson's disease

1.6.1 Current and future therapies for Parkinson's disease

There are a variety of methods currently used to treat PD throughout disease progression, with focus relating to the motor (Fox *et al.*, 2011) and non-motor symptoms (Seppi *et al.*, 2011) of the disease, with short-term effectiveness of pharmacological and surgical treatments (Goetz *et al.*, 2005). The main treatment for the symptoms of PD is pharmacological, aiming to restore dopamine levels, with neurosurgery (deep brain stimulation; DBS) to restore the disrupted signalling through the BG, typically reserved for more advanced disease stages. Other therapies such as physiotherapy and speech therapy are also used alongside these (Oertel and Schulz, 2016). Although current treatments can alleviate symptoms, no long-term, or restorative therapies are available.

Some of the current treatments, and their mode of action, for the motor symptoms of PD are displayed in Figure 1.7. Levodopa was the first and remains the most effective drug for treating the motor symptoms of PD (Thanvi, Lo and Robinson, 2007) by crossing the BBB and replenishing dopamine levels. However, long term use leads to further complications such as dyskinesias and on-off fluctuations of dystonia which are hard to counteract (Fabbrini *et al.*, 2007).

Dopamine agonists mimic dopamine and act directly on D1 and D2 receptors (Brooks, 2000). However, they are typically ineffective without levodopa supplementation (Pilleri and Antonini, 2015), can induce hallucinations (Poewe, 2008), and decrease in effectiveness over a 4 year period (Borgemeester, Lees and van Laar, 2016).

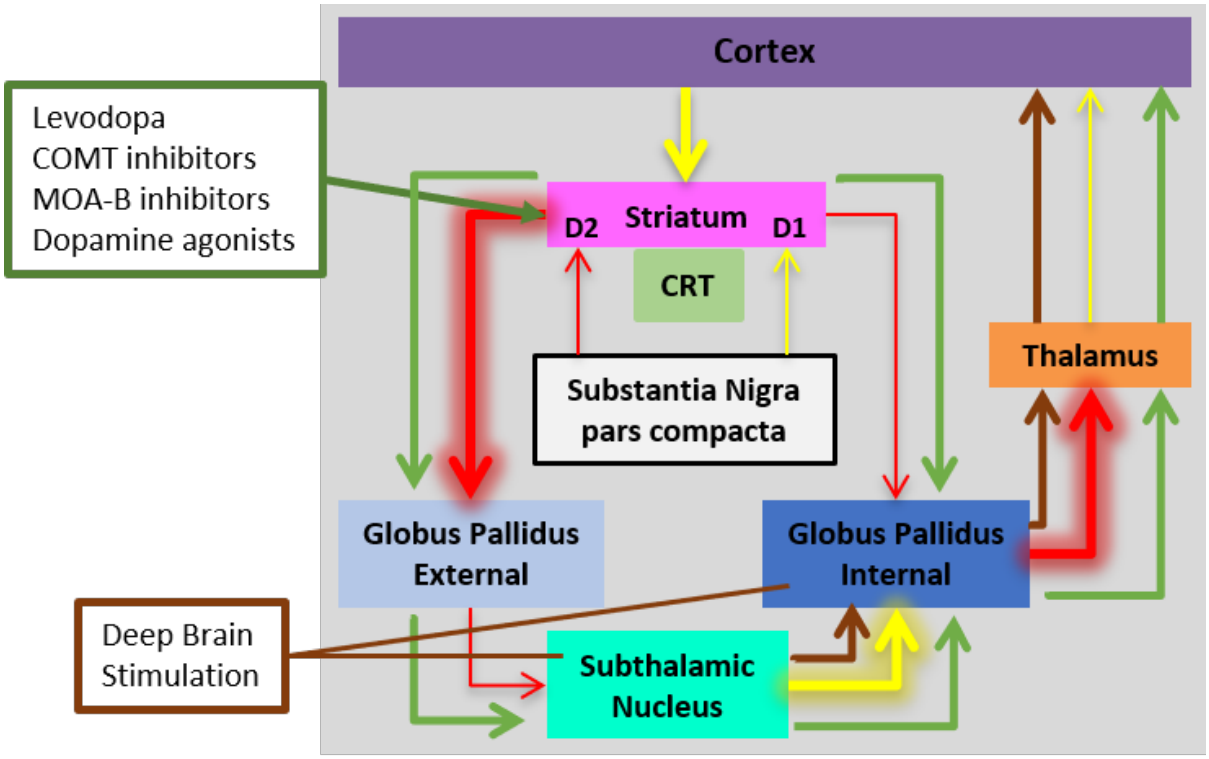


Figure 1.7 Schematic showing how current treatments ease the motor symptoms of PD. Building on Figure 1.3 B, loss of SNpc dopaminergic neurons disrupts signalling through the nuclei of the BG (red inhibitory, yellow excitatory; increased/decreased thickness represents increased/decreased strength of signal respectively). Pharmacological intervention through administration of Levodopa (the precursor to dopamine which can cross the BBB), COMT inhibitors (reduce the breakdown of dopamine), MOA-B inhibitors (limit the breakdown of dopamine), and dopamine agonists (compounds which activate D1 and D2 receptors) increase the level of available dopamine and dopamine signalling in the striatum. CRT also aims to increase levels of dopamine by placement of dopaminergic neurons into the striatum. These interventions work by restoring the lost dopaminergic signalling in the striatum which in turn restores the direct (D1) and indirect (D2) signalling pathways through the nuclei of the BG (green arrows) releasing the thalamus from inhibition.
Deep brain stimulation is a neurosurgical approach to counter the motor symptoms of PD. Electrodes are typically placed within the STN or GPi, although different targets can produce different effects meaning that DBS can be tailored to each individual patient (Pizzolato & Mandat 2012). Continuous, high frequency stimulation is used to inactivate the target area and ease signalling disruption (brown arrows) to the thalamus, restoring signalling to the cortex. BBB: blood brain barrier, BG: basal ganglia, COMT: catechol-O-methyltransferase, CRT: cell replacement therapy, GPe: globus pallidus external, GPi: globus pallidus internal, MOA-B: monoamine oxidase B, SNpc: substantia nigra pars compacta, STN: subthalamic nucleus.

COMT inhibitors can be used alongside levodopa, prolonging its pharmacological effect by blocking degradation (Kiss and Soares-Da-Silva, 2014). They can provide a neuroprotective effect from levodopa-induced neuropathy (Cossu *et al.*, 2016), however, they can also be toxic to the liver (Kaakkola, 2010). Similarly to COMT, the administration of MAO-B inhibitors prevent the breakdown of dopamine (Schapira, 2011). However, these can also cause

hallucinations and headaches, and can result in the production of neurotoxic metabolites (Park *et al.*, 2013).

These pharmacological treatments increase levels of dopamine to restore BG signalling, however, this increase is not restricted to the striatum and can affect other dopamine signalling pathways. Intelligent dosing of medications significantly improves the lives of PD patients, however, combinations and timings can become complex and need tailoring to each individual. They also do not represent a long-term treatment solution as they only restore dopamine levels temporarily (i.e. immediately after administration), lose effectiveness over time due to non-physiological activation of dopamine receptors and continued neurodegeneration (Cenci and Konradi, 2010), and can produce debilitating side effects that outweigh initial benefits (Foster and Hoffer, 2004).

DBS is a surgical technique that places electrodes into specific sites within the brain (Figure 1.7) utilising continuous high frequency stimulation to inactivate the target area, thereby blocking the signals that cause the motor symptoms of PD, notably the resting tremor. DBS has shown significant improvements on test scores (UPDRS-III and PDQ-39; Deuschl, 2006) lasting at least 5 years (Krack *et al.*, 2003), and has also been found to partially alleviate some non-motor symptoms of PD (Wolz *et al.*, 2012).

Although the symptoms of PD can be fairly well managed at the start of the disease, there is nothing to slow, halt or reverse disease progression, and current treatments lose effectiveness over time and can produce debilitating side effects (Oertel & Schulz 2016). Therefore, there is a major unmet need for therapies that offer neuroprotection for dopamine neurons, and/or can restore/replace the dopaminergic signalling system.

1.6.2 Future therapies may serve to protect dopamine neurons from degeneration

As discussed in Chapter 1.1.6, the cause of dopaminergic degeneration in idiopathic PD remains unknown despite an in-depth understanding of the pathological features involved in their demise (Chapters 1.1.3, 1.1.4, 1.1.5). The dissociation between commencement of striatal dopamine loss and appearance of clinical features reaching the threshold for a diagnosis make it difficult to detect the disease at onset, although serum markers and risk factors may assist in overcoming this difficulty (Chapter 1.1.6). With this potential development it may be possible to protect dopamine neurons from the pathological process before substantial cell death and induction of symptoms by providing the optimum environment for their survival.

Preventing the pathological process may be possible through administration of neuroprotective compounds. Administration of the growth factors neurturin and GDNF directly into the brain of PD patients found improvements in motor symptoms in some studies, however, placebo-controlled trials failed to show growth factor-induced improvements over placebo administration. There are a number of possible reasons for the outcomes of these trials, with one being suboptimal patient selection where degeneration of the nigrostriatal pathway was too advanced (Kirkeby and Barker, 2019). Application of this type of neuroprotective treatment may only be beneficial to less clinically advanced patients by slowing disease progression rather than restoring dopamine signalling.

Inflammation and microglia have also been implicated in PD (Chapter 1.4.4), therefore, prevention of disease progression may also be possible through modulation of the brain's immune system. The influence of anti-inflammatory medication on the risk of developing PD is not clear. Exposure to some non-steroidal anti-inflammatory drugs, such as ibuprofen, but

not aspirin, can reduce the risk of developing PD (Rees *et al.*, 2011). Two specific immunosuppressants, corticosteroids and inosine monophosphate dehydrogenase inhibitors, have also been strongly associated with a lower risk of PD (Racette *et al.*, 2018). This suggests that perhaps a more targeted approach to immune modulation is required for PD.

Microglia may also be involved in a potential future therapy for PD, (in association with cell replacement therapy [CRT, described below]). Thus, through microglial interaction with grafted cells and/or their roles in embryonic brain development, microglia may one day be utilised to support the restoration of the nigrostriatal pathway.

1.6.3 Cell transplantation is a promising therapy to restore dopamine signalling

The aim of CRT is to restore the lost motor functions in PD by grafting dopaminergic neurons into the striatum to restore dopamine signalling. Ideally graft placement would be into the SN, to allow the rebuilding of complex topographical efferent and afferent connections (Haber, 2014), however, restoration of efferent connections from SN to striatum is not currently possible, due to inhibitory signals located within the nigrostriatal pathway in the adult brain. However, some progress has been made which shows potential for the reformation of full circuitry (Toledo-Aral *et al.*, 2003; Shen, Hoffer and Wang, 2009; Thompson *et al.*, 2009; Ghosh *et al.*, 2019).

CRT for PD has been tested in a number of clinical trials, with tissue from aborted human foetuses, containing SN progenitors, transplanted into the striatum of patients. A number of studies have shown reductions in motor symptoms, with some patients able to discontinue medication, and effects reaching 18 years post-transplant (Lindvall *et al.*, 1990; Freed *et al.*, 1992; Kefalopoulou *et al.*, 2014), considerably higher than other treatments.

However, results have been varied and some patients received little to no benefit from the procedure, or developed graft induced dyskinesia (GID; Freed *et al.*, 2001; Olanow *et al.*, 2003; Barker *et al.*, 2013).

The development of GID as a side effect of CRT is poorly understood yet it is a potential barrier to the development of CRT as a therapy for PD. The replication of GID in animal models has proven difficult, however, Kordower *et al.* (2016) found that a higher dopamine deficit prior to grafting increased vulnerability to GID in parkinsonian monkeys. Although no relationship was found between the magnitude of dopamine reinnervation (measured by positron emission tomography) and the development of GID in grafted patients (Hagell *et al.*, 2002), Ma *et al.* (2002) suggest that their development could be due to unbalanced and uneven increases of dopamine within the graft. Another suggestion is that GIDs arise from the presence of serotonergic neurons within grafts (Politis *et al.*, 2010; Shin *et al.*, 2012) highlighting the need for well-characterised and defined graft sources.

The discrepancies between early CRT trials are often accredited to the large variations regarding tissue source and preparation, aftercare, disease progression prior to transplantation, and assessment methods. A recent, ongoing clinical trial, TRANSEURO, is attempting to address the inconsistencies prevalent in CRT research for PD. Collaborators have drawn from raw data to develop a standardised technique designed to provide the best clinical outcome, with robust assessment measures for data collection pre- and post-transplantation. Conclusions drawn from this trial will help to determine the future of CRT for PD (Barker *et al.*, 2019).

Grafts have been shown to survive and integrate, with surviving transplanted dopamine neurons observed post mortem (Hagell and Brundin, 2001) and fluorodopa positron-emission tomography showing an increase in the synthesis and storage of dopamine in the striatum post-transplantation (Lindvall *et al.*, 1990; Freed *et al.*, 1992).

Currently, the gold standard graft for CRT is considered to be human foetal VM tissue, although it is recognised that this is an unsustainable source (Barker *et al.*, 2019; Parmar, Grealish and Henchcliffe, 2020). Therefore, protocols to generate suitable supplies of dopaminergic neurons from other sources are still required for CRT to be a sustainable, standardised therapy for PD. Hagell and Brundin (2001) suggest that, in order to achieve clinical benefit, the grafted cells must express phenotypic and genetic markers of A9 dopaminergic neurons, be excitable and release dopamine, contain no proliferating cells, and survive transplantation in numbers of at least 100,000.

One of the consistent problems with CRT is the poor survival rate of dopamine neurons, with numbers depleted to 1-20% of the total originally grafted. This cell death occurs through both necrosis and apoptosis arising from factors including sample preparation and a non-supportive host environment (inducing oxidative stress or lacking in suitable growth factors; review: Castilho, Hansson, & Brundin, 2000). The use of immunosuppression in CRT is varied and the effects are not well understood, although the consensus seems to be that immune suppression is beneficial to graft survival (Moriarty, Parish and Dowd, 2018). The recruitment of microglia to the graft site may be beneficial, or at least not detrimental, to grafted dopamine neurons (Duan *et al.*, 1998; Zietlow, Dunnett and Fawcett, 1999). However, the activation state of recruited microglia (M1/M2) and their subsequent roles in graft integration and survival, are not well described. Furthermore, the duration of the immune response to the transplantation process is unknown. These factors contribute to the ambiguity of the role of microglia in CRT, and consequently the need for immunosuppression (review: Tomov, 2020).

Biomaterials which allow for provision of a supportive, cell adhesion environment, or localised and sustained release of growth factors, or protection from host immune cells may

assist in developing CRT as a treatment for PD (Moriarty, Parish and Dowd, 2018) by helping the survival of transplants and also reducing the volume of tissue required.

Stem cells, defined by their ability to both self-renew and to differentiate and produce mature progeny, are a potential source of dopaminergic neurons for CRT. They can be classified by their potential for differentiation as totipotent (able to give rise to all cell types including embryonic and extra-embryonic), pluripotent (able to give rise to all embryonic cell types), multipotent (able to give rise to a more restricted cell lineage such as mesodermal, ectodermal or endodermal), and oligopotent (able to give rise to more restricted cells such as those within an organ; Wagers & Weissman 2004). Their importance in clinical research includes their use as tools for the study of development, disease modelling, drug screening, and gene identification (both as genetic risk factors or as therapeutic targets); and their potential for use in cell therapies (Reubinoff *et al.*, 2001; Shakhova and Sommer, 2010).

Human stem cells can be derived from a number of sources (Figure 1.8), with both human ESCs and iPSCs thought to be the most suitable and sustainable source of dopaminergic neurons for CRT (Parmar, Grealish and Henchcliffe, 2020). Grafts derived from these sources reportedly contain fewer astrocytes and serotonergic neurons (which contribute to graft induced dyskinesia) than VM tissue grafts; and may also promote vascularisation through inclusion of vascular leptomeningeal cells. ESCs or iPSCs from stem cell banks would provide a defined and consistent supply of dopaminergic progenitors, easily assessed for quality control purposes. However, ESC and some iPSC grafts (derived from iPSC banks) do have the limitation of being allogenic, presenting the risk of immune rejection or need for long-term immune suppression. The use of allogenic ESCs without immune suppression showed poor survival and no functional recovery in a monkey MPTP CRT model (Hallett *et al.*, 2014). However, researchers have proposed that the use of homozygous human leukocyte antigen typed iPSCs could match over 90% of a country's population (Nakatsuji, Nakajima and

Tokunaga, 2008; Taylor *et al.*, 2012) reducing the need for immune suppression and risk of rejection.

The use of autologous iPSCs can eliminate the risk of immune rejection and has shown functional motor recovery and survival for up to 2 years in a monkey MPTP PD model (Hallett *et al.*, 2014). This study also showed no recovery with the use of an alternative differentiation protocol, and others have shown differences in the numbers of surviving TH^+ neurons between different iPSC lines (Kikuchi *et al.*, 2017), highlighting the importance of the differentiation protocol and donor cells in the survival of grafted cells. The variability arising from iPSCs raises concerns of reproducibility, lack of quality control checks, and an increased time period for the generation of suitable cells for transplantation. Retaining genetic risk factors of PD also poses another detriment to the use of autologous iPSCs (Fan, Winanto and Ng, 2020), although only 10% of PD cases have a known genetic risk factor. No differences were found between iPSC grafts derived from healthy and PD individuals in an MPTP monkey model (Kikuchi *et al.*, 2017), suggesting that autologous iPSCs may not pose an increased risk. However, this model was only followed up for 24 months, and as the specific cause of PD remains unknown it is suspected that gene-environment interactions play a role (Horsfall *et al.*, 2013). Also, the age-related nature of PD suggests the potential for degeneration in iPSCs may be delayed, although the long-term *in vivo* viability of dopaminergic neurons from PD patient-derived iPSCs is difficult to study.

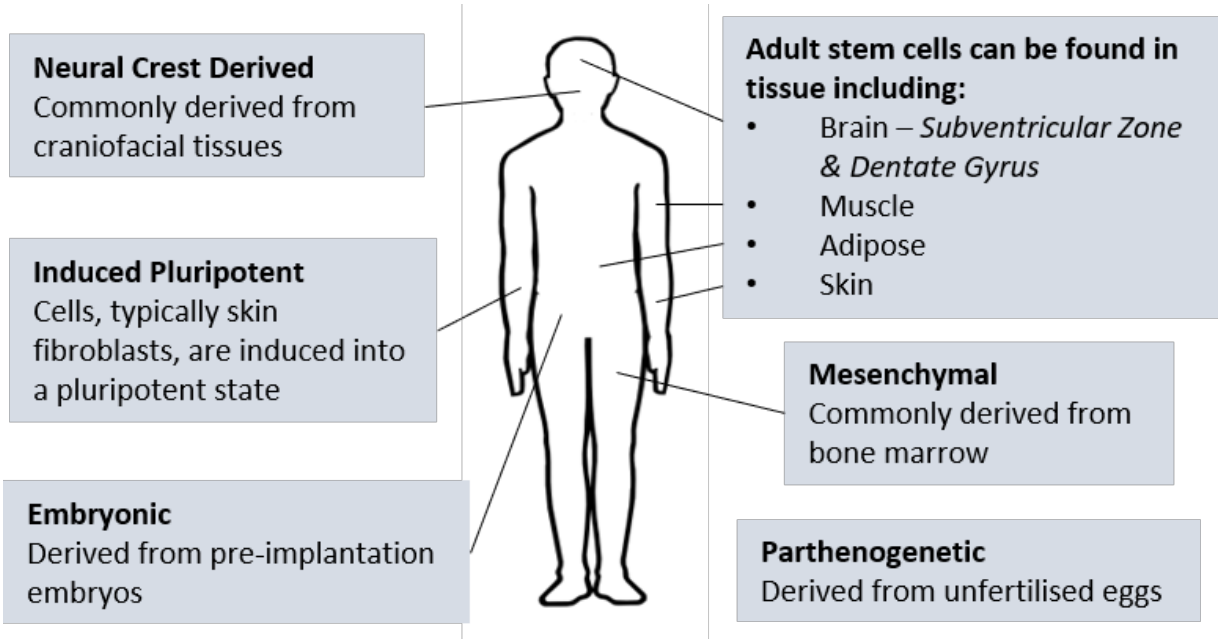


Figure 1.8 Diagram illustrating some of the sources of human stem cells that may be applicable to cell replacement therapies. (Wagers and Weissman, 2004; Revazova *et al.*, 2007; Chen, He and Zhang, 2009; Trounson and McDonald, 2015)

The promising results from the use of stem cell grafts in animal models of PD encourages the potential use of stem cells in CRT for PD. However, *in vitro* differentiation of stem cells into dopaminergic neurons is a complex process requiring a number of factors. Protocols often try to recapitulate the natural development of midbrain dopaminergic neurons.

1.7 Dopaminergic neuron development *in vitro*

As discussed in Figure 1.5, the *in vivo* development and maintenance of midbrain dopaminergic neurons requires a complex signalling pattern throughout embryogenesis. Replication of this signalling pathway may assist in the development of dopaminergic neurons suitable for CRT for PD. It is also important to be able to identify this subset of neurons prior to terminal differentiation as mature neurons show lower viability after transplantation (Barker *et al.*, 1995; Fricker *et al.*, 1996; Brederlau *et al.*, 2006) and therefore neural progenitors or immature neurons may provide a more useful cell population for transplantation.

In vitro generation of midbrain dopaminergic neurons has vastly improved. Early protocols were able to differentiate large numbers of TH^+ neurons, but not with the A9 specificity required for use in CRT (Arenas, Denham and Villaescusa, 2015). Protocols can now generate high percentages of dopaminergic neurons between 60-97% TH^+ from human ESCs and iPSCs. However these protocols utilise co-culture and feeder cell techniques that are not clinically transferable due to the risk of contamination with undesirable cell types or the risks associated with xenogeneic products which are commonly used as feeder cells (Kriks *et al.*, 2011; Gonzalez *et al.*, 2013; Llames *et al.*, 2015). A selection of studies that have shown differentiation and transplantation of TH^+ neurons into PD models are listed in Table 1.6 with survival of TH^+ neurons composing up to 54% of grafted cells (Kirkeby *et al.*, 2012). Arenas, Denham and Villaescusa (2015) provide an in-depth review on current generation of A9 specific neurons from pluripotent stem cell sources. They highlight a number of future challenges and criteria for the generation of neurons suitable for CRT for PD including the need for short and scalable protocols using defined components in line with good manufacturing practice (GMP) guidelines (Bedford *et al.*, 2018) . One class of molecules that

are well defined and essential to life are vitamins, which may hold potential for use in the generation of GMP grade cell products.

Table 1.6 Table showing some studies that have successfully transplanted dopaminergic neurons, derived from human stem cells, in PD models.

Dopamine Neuron Yield	Cell type	Differentiation protocol/added factors	Graft survival	Motor deficits	Dopaminergic markers	Other cell types present	Reference
TH/FOXA2$^+$: ~2.6% of grafted cells in mouse; ~5.6% in rat; monkey not stated	ESCs H9, H1 iPSCs 2C6, SeV6	Dual SMAD inhibition, floor plate induction. Shh, purmorphamine, FGF8, CHIR99021, AA, BDNF, GDNF, transforming growth factor type β3 (TGFβ3), dibutyryl cyclic AMP (db-cAMP)	Yes, 18-20 weeks 6-OHDA rat and mouse; 1 month in MPTP monkey	Improved in rat and mouse, monkey not stated	TH, LMX1A, FOXA2, NURR1, TFF3, PITX3, Dopamine, DOPAC, HVA, GIRK2, DAT	Few GABA$^+$, 5-HT$^+$ neurons	Kriks *et al.* (2011)
54 % TH$^+$ in graft	ESCs H9, SA121	Dual SMAD inhibition and embryoid body formation, activation of Wnt signalling (using CHIR99021), Shh Maintained on mouse embryonic fibroblasts	Yes, 18 weeks in 6-OHDA rat	Improved	EN1, NURR1, LMX1A, FOXA2, TH, AADC, GIRK2	Not stated	Kirkeby *et al.* (2012)
40% TH$^+$ in graft	iPSCs 836B3	Dual SMAD inhibition and floor plate induction, activation of wnt signalling (CHIR99021) Isolation of CORIN$^+$ cells	Yes, 16 weeks in 6-OHDA rat	Improved	MAP2, LMX1A, EN1, NURR1, TH, FOXA2, AADC, PITX3, GIRK2	No Ki67$^+$ proliferation 1.2% 5-HT$^+$ neurons	Doi *et al.* (2014)
TH$^+$: ~3-4% of grafted cells	ESCs H9	Dual SMAD inhibition, Shh, purmorphamine, CHIR99021, AA, BDNF, GDNF, TGFβ3, DAPT, db-cAMP	Yes, 4-6 months in 6-OHDA mouse	Improved	LMX1a, FOXA2, NURR1, TH, Dopamine		Steinbeck *et al.* (2015)
3,716 ± 1,026 TH$^+$ per 100,000 grafted	ECS, RC17, H9	Cells grown on Lam-111, 16 day protocol, basal medium Neurobasal + DMEM/F12 with N2. Additional SB431542, Noggin, Shh-C24II, CHIR99021, FGF8b, B27, BDNF, AA; GMP grade protocol	Yes, 6-OHDA rat	Improved	FOXA2, OTX2, LMX1A, LMX1B, TH, GIRK2	Few CALB$^+$ neurons	Kirkeby *et al.* (2017)
6.4 ± 4.9 x 10^4 per graft	8 iPSC lines, healthy and PD donors	Dual SMAD inhibition and floor plate induction, isolation of CORIN$^+$ cells, db-cAMP, DAPT	Yes, MPTP monkey	Improved	FOXA2, TUJ1, NURR1, TH, GIRK2, DAT	No or few OCT4$^+$, SOX1$^+$, PAX6$^+$, Ki67$^+$ proliferating markers 5-HT$^+$, GABA$^+$, GFAP$^+$, VGLUT$^+$, CHAT$^+$ neurons	Kikuchi *et al.* (2017)

1.8 Vitamins in the central nervous system

Between the early 19th century and mid-20th century, the structure and major functions of each of the 13 key vitamins was elucidated (Table 1.7; Semba, 2012) and it is now common knowledge that vitamins, in small amounts, are essential for maintaining health throughout the body, including the brain.

Table 1.7 Table displaying the 13 key vitamins, other names by which they can be known, and some examples of their physiological functions. RDI: recommended daily intake is given in mg per day for adults (women/men if values differ), data obtained from Public health England. * denotes no recommended intake given, doses below are not considered detrimental. Adapted from Fricker *et al.* (2018).

Vitamin	Other Names	Examples of physiological functions	RDI (mg)
A	Retinol, Retinoic Acid, Retinal, Carotenoid	Growth, maintenance of skin, bone development, maintenance of myelin, maintenance of colour and peripheral vision, steroid hormone synthesis, resistance to infection	0.6/0.7
B_1	Thiamine	Growth, appetite, digestion, nerve activity, energy production, coenzyme in pyruvate metabolism	0.8/1
B_2	Riboflavin	Growth and development of foetus, constituent of flavoproteins, redox systems and respiratory enzymes, maintenance of mucosal, epithelial and eye tissues	1.1/1.3
B_3	Nicotinamide, Niacin, Nicotinic acid	Maintenance of NAD and NADP, coenzyme in lipid catabolism, oxidative deamination	13.2/16.5
B_5	Pantothenic Acid	Lipid metabolism, protein metabolism, acetylcholine production, part of coenzyme A in carbohydrate metabolism	*< 200
B_6	Pyridoxine, Pyridoxol, Adermine	Protein, carbohydrate, and lipid metabolism, coenzyme in amino acid metabolism, growth	1.2/1.4
B_7	Biotin, protective factor X	Growth, maintenance of skin, hair, bone marrow and sex glands, coenzyme for carboxylation reactions, biosynthesis of aspartate and unsaturated fatty acids	*< 0.9
B_9	Folic acid, Folacin, Folinic acid	Synthesis of nucleic acid, differentiation of embryonic nervous system, metabolism of tyrosine and histidine, synthesis of choline, one-carbon transfer mechanisms	0.2
B_{12}	Cobalamin	Coenzyme in nucleic acid, protein and lipid synthesis, maintenance of epithelial cells and nervous system	0.15
C	Ascorbic acid	Absorption of iron, antioxidant, growth, wound healing, formation of cartilage, dentine, bone and teeth, maintenance of capillaries	40
D	Vitamin D_3, Cholecalciferol, Calcitriol	Normal growth, Ca and P absorption, increases citrate blood levels, maintains and activates alkaline phosphatase in bone, maintains serum calcium and phosphorus levels	0.1
E	Tocopherol, Tokopharm, Tocotrienols	Antioxidant, normal growth maintenance, aids absorption of unsaturated fatty acids, maintains normal muscular metabolism and integrity of vascular system and central nervous system	3/4
K	Prothrombin factor, Menaquinones	Blood clotting mechanisms, electron transport mechanisms, growth, prothrombin synthesis in liver	0.1 /kg

Studies have shown the importance of vitamins in the differentiation and survival of neuronal cells (Kasuya, 1975; Bain *et al.*, 1995; Christie *et al.*, 2008; Erceg *et al.*, 2008; Ulatowski and Manor, 2015). They have an antioxidant role in the central and peripheral nervous system (Vatassery, Smith and Quach, 1989; Vatassery, 1992), with imbalance during pregnancy shown to adversely affect antioxidant defence mechanisms (Roy *et al.*, 2014), and to alter adult gene expression of neurotrophins and signalling molecules such as BDNF, nerve growth factor, tropomyosin receptor kinase B (TrkB) and cyclic adenosine monophosphate (cAMP) response element binding protein (CREB; Sable *et al.*, 2014). This highlights their importance in the developing nervous system. Vitamins are commonly used in a number of different *in vitro* differentiation protocols (Griffin *et al.*, 2017) and nicotinamide has been shown to enhance the differentiation potential of calcitriol and retinoic acid in human myeloblastic leukaemia cells (Shen and Yen, 2009). The potential interactions of vitamins A (retinoic acid), B_3 (nicotinamide), C (ascorbic acid; AA), and D_3 (calcitriol) in the development of A9 dopaminergic neurons are displayed in Figure 1.9.

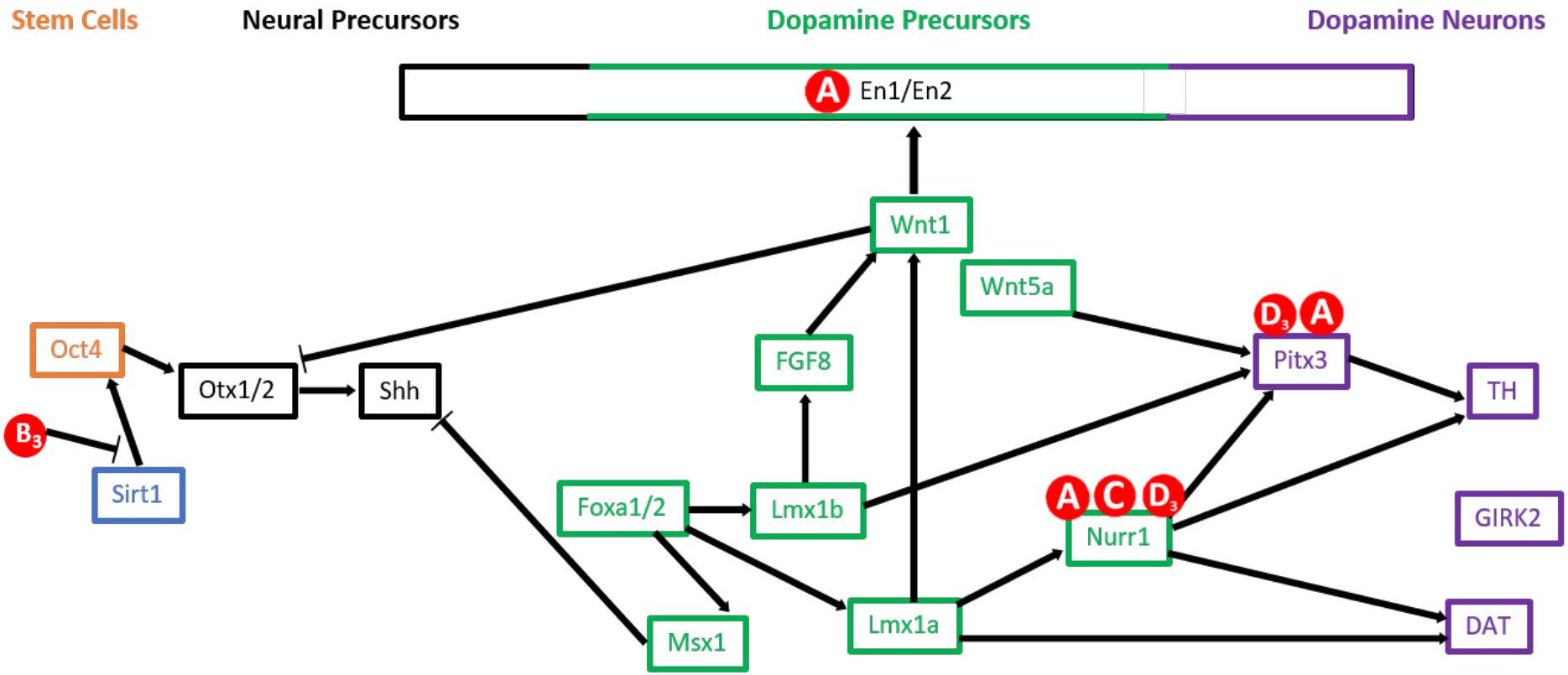


Figure 1.9 Schematic showing genes and proteins involved in dopaminergic differentiation and how vitamins A, B_3, C, and D_3 may interact with these. Orange boxes indicate stem cell expression, black boxes represent expression at the neural precursor stage, green shows expression in dopamine precursors and purple shows expression in mature dopamine neurons. En1/2 are essential for dopaminergic development and maintenance. Arrow-headed lines show upregulation and bar-headed lines downregulation. Vitamin B_3 (nicotinamide) may promote differentiation through inhibition of Sirt1 which is required by Oct4 to maintain self-renewal and inhibit differentiation (Williams *et al.*, 2016). Vitamin A (retinoic acid) increases levels of En1 (Cooper *et al.*, 2010), mediates Nurr1 signalling (Wallen-Mackenzie *et al.*, 2003), and can counteract Pitx3 deficiency (Jacobs *et al.*, 2007). Vitamin C (ascorbic acid) promotes Nurr1 recruitment (He *et al.*, 2015). Vitamin D_3 (calcitriol) upregulates GDNF (Orme, Bhangal and Fricker, 2013) which induces Pitx3 expression (Peng *et al.*, 2011) and maternal deficiency of calcitriol reduces Nurr1 expression (Cui *et al.*, 2010).

1.9 Calcitriol and Parkinson's disease

Calcitriol is an active metabolite of vitamin D_3. Although commonly known for its importance in bone growth, the vitamin D receptor is found in over 36 cell types (Norman, 2008). In developing rats, the vitamin D receptor is found in the superior colliculus which later becomes the dopaminergic midbrain (Veenstra *et al.*, 1998) and its onset at E15 coincides with the establishment of dopaminergic neurons suggesting a link between calcitriol and dopamine neuron development and maintenance (Gates *et al.*, 2006). This timing also corresponds with an increase in neural apoptosis and decrease in mitosis (Burkert, McGrath and Eyles, 2003) and deficiency of vitamin D_3 during development results in reduced levels of Nurr1 (Cui *et al.*, 2010) suggesting a role in dopaminergic maturation.

Vitamin D_3 has also been found to have neuroprotective properties (Wang *et al.*, 2000, 2001; Soilu-Hänninen *et al.*, 2012) and deficiency could be a contributing factor to PD (Newmark and Newmark, 2007). However, a study of genetically reduced vitamin D_3 levels showed no clear causal risk of developing PD (Larsson *et al.*, 2017).

Calcitriol increased levels of dopamine in the striatum and SN of 6-OHDA treated rats, however, this effect was not seen in 'aged' (22 month old) rats suggesting that neuroprotection via calcitriol could be reduced with age (Cass and Peters, 2017). Orme, Bhangal and Fricker (2013) found neuroprotective properties of calcitriol through upregulation of GDNF in cultures derived from E12 rat VM. With peak neurogenesis of TH^+ neurons occurring at E12 in the rat embryo, and almost 80% of dopamine neurons generated between E12 and E14 (Gates *et al.*, 2006), calcitriol may exert therefore neuroprotective effects on dopaminergic neurons at an early time point. Together, this suggests that calcitriol may play an important role in the development, maturation, and maintenance of dopamine neurons, however, calcitriol alone may not be sufficient.

1.10 Nicotinamide may also have roles in Parkinson's disease

Nicotinamide is suggested to have roles in both health and disease (Williams and Ramsden, 2005a), although due to a wide range of derivatives and biological affects, the exact role and mechanisms of nicotinamide's activity is unclear. This PhD has focussed on uncovering the potential effects that nicotinamide has on neural cell types and how this may be utilised in research specific to PD.

1.10.1 Vitamin B_3 metabolism

Vitamin B_3 is the collective term used to describe the water soluble molecules nicotinic acid (NA; a.k.a. niacin) and nicotinamide (a.k.a. niacinamide) which are obtained through diet from foods including meat, fish, eggs, mushrooms and fortified cereals (Bogan and Brenner, 2008). This is the body's chief source of nicotinamide adenine dinucleotide (NAD^+), although the essential amino acid tryptophan can also provide a source of NAD^+ via metabolism in the liver and kidneys (Fukuwatari and Shibata, 2013). More recently, nicotinamide riboside (NR), found in cow's milk, has also been discovered as a precursor to NAD^+ (Bieganowski and Brenner, 2004). The processes involved in the generation of NAD^+ from tryptophan, NA, nicotinamide, and NR, as well as a number of metabolites and pathways surrounding nicotinamide, are shown in Figure 1.10.

Although tryptophan, NA, nicotinamide, and NR produce NAD^+, each precursor has a different route and therefore different molecular intermediates, enzyme requirements, effects, advantages, and disadvantages. Tryptophan requires the kynurenine pathway, NA the Preiss-Handler pathway and nicotinamide and NR the Salvage pathway (Figure 1.10).

Tryptophan metabolism occurs via the kynurenine pathway (Figure 1.10) which has two branches active in different tissues and cell types. These produce a range of biologically and neurologically active intermediates (for review see: Castro-Portuguez & Sutphin, 2020). However, with the 8-step process producing just 1 mg of nicotinamide from 67 mg of tryptophan (Fukuwatari and Shibata, 2013) and low activity of kynurenine pathway enzymes in brain compared to peripheral organs (Schwarcz *et al.*, 2012) it may not be the most productive method for increasing NAD^+ levels in the brain.

NA requires a 3-step process known as the Preiss-Handler pathway for conversion to NAD^+ (Figure 1.10). However, high doses of NA given as a drug results in the adverse effect of flushing and therefore, NA supplementation is not considered a tolerable route for increasing NAD^+ levels in humans.

Nicotinamide is converted to NAD^+ via the 2-step salvage pathway (Figure 1.10) with nicotinamide phosphoribosyl-transferase (NAMPT) producing the intermediate component nicotinamide mononucleotide (NMN), which is converted to NAD^+ by the enzyme nicotinamide mononucleotide adenylyltransferase (NMNAT) of which there are three isoforms (NMNAT1, NMNAT2, NMNAT3). NR enters the salvage pathway producing the NMN intermediate through nicotinamide riboside kinase activity and synthesising NAD^+ in a 2-step process. The use of NR can increase levels of NAD^+ (T. Yang *et al.*, 2007) without increasing levels of nicotinamide. Nicotinamide can be considered as a feedback/signalling molecule as it is produced by and inhibits sirtuin and PARP activity, and, through this mechanism, may be involved in the maintenance of NAD^+ levels (Feldman *et al.*, 2015). NR may be beneficial in increasing NAD^+ levels whilst bypassing the inhibitory action of nicotinamide.

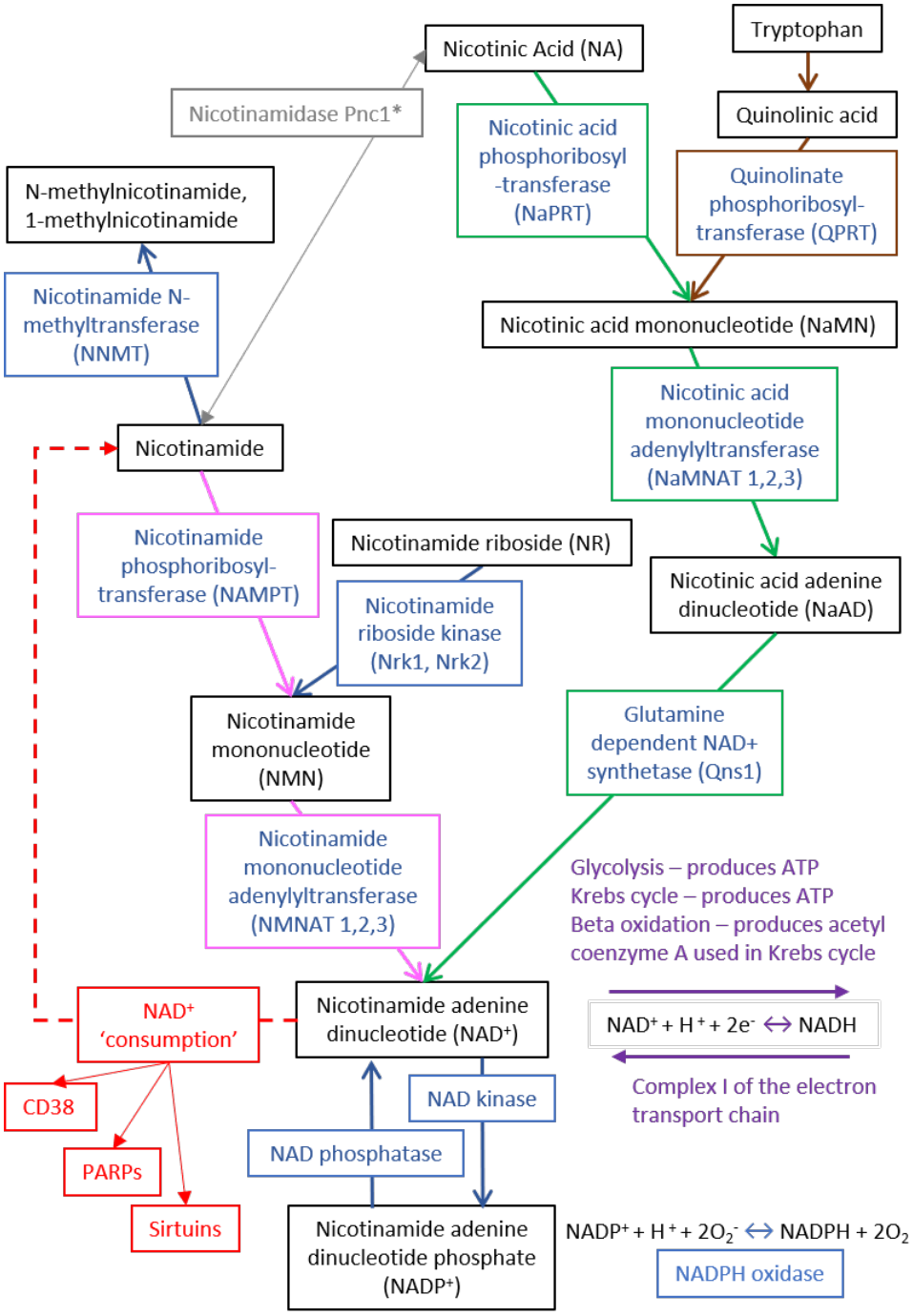


Figure 1.10 Schematic showing the pathways in the production of NAD^+ and compounds associated with nicotinamide. The Kynurenine pathway (highlighted by brown arrows), generating NAD^+ from tryptophan (tryptophan to quinolinic acid not shown in detail), is an 8-step process, producing a range of biologically and neurologically active intermediates. The Preiss-Handler pathway (highlighted by green arrows) utilises a 3-step process to generate NAD^+ from nicotinic acid (NA). The salvage pathway (shown in pink) is a 2-step process converting nicotinamide to NAD^+ which is then recycled back to nicotinamide through NAD^+ 'consumption' (red) by CD38 (cyclic ADP ribose hydrolase), PARPs (poly(ADP-ribose) polymerases), and sirtuins. Nicotinamide riboside also enters the salvage pathway through conversion to nicotinamide mononucleotide. Nicotinamidase, or Pnc1 (grey), bi-directionally converts nicotinamide to NA, although this enzyme is only present in invertebrates which do not possess NAMPT and instead convert nicotinamide to NA for NAD^+ generation via the Preiss-Handler pathway, suggesting that the salvage pathway is more evolutionarily advanced (Vrablik *et al.*, 2009; Imai and Guarente, 2016). Each molecule/compound is written in black, whilst enzymes are written in blue. Purple shows the processes that convert NAD^+ to NADH, and the reverse. Adapted from Fricker *et al.* (2018).

Aside from the salvage pathway, nicotinamide can also be converted to N-methylnicotinamide or 1-methylnicotinamide, metabolites commonly found in urine, and low levels can be considered a marker of niacin deficiency (Bender, Earl and Lees, 1979). This takes place through nicotinamide N-methyltransferase (NNMT) activity, an enzyme that is expressed in the CNS with varying levels across brain regions and is present in dopaminergic neurons, but not glia, of the SN (Parsons *et al.*, 2002).

The plasma concentrations of NA and nicotinamide vary across studies. NA has been suggested at 4-122 nM (Ashourian and Mousdicas, 2006) with concentrations of up to 57,000 nM in children (Adams and Audhya, 2011). Whilst observed serum levels of nicotinamide have included 840 nM (Ito *et al.*, 2020) and approximately 4000 nM (Odum and Wakwe, 2012). The differences observed across these studies could arise from dietary differences across subjects. Oral administration of nicotinamide dose-dependently increases plasma concentrations (between 30 mins to 4 hours correlating with increased dose, with more variation between individual patients at higher doses) with final plasma concentrations of approximately 1.2 mM (156 ± 34 μg/mL) and 1.4 mM (177 ± 23 μg/mL) for 6 g and 10 g doses respectively. Doses of up to 6 g were well tolerated, however, half of patients ingesting 10 g had severe nausea and vomiting (Dragovic *et al.*, 1995). Direct application of 10 mM nicotinamide to human pluripotent stem cells has increased intracellular concentrations from 0.01 mM to 1.5 mM after 1 hour of incubation (Meng *et al.*, 2018). This suggests that *in vitro* studies can be extrapolated to the *in vivo* environment as administration of exogenous nicotinamide can increase concentrations within both *in vitro* and *in vivo* systems.

Nicotinamide and its derivatives have been reported to be at a higher concentration in cerebrospinal fluid (~15 μM) than in serum, with approximately 0.5 mM/kg in the brain. After intravenous injection into rabbit, nicotinamide was more readily taken up into the

cerebrospinal fluid than NA (Spector, 1979); whilst NA was shown to be a better precursor to NAD^+ than nicotinamide in the liver, intestine, and kidney (Bogan and Brenner, 2008).

The potential for nicotinamide to treat a variety of diseases including neurological disorders, skin disorders, and cancer has been discussed across the literature. Hwang and Song (2020) provide a review of nicotinamide, potential uses, and the positive and negative effects experienced with high doses. The effects of nicotinamide on the body may be through direct effects of nicotinamide, through conversion to nicotinamide derivatives, or through enzymes that utilise NAD^+ such as sirtuins, poly(ADP-ribose) polymerases (PARPs), and CD38 (Banasik, Stedeford and Strosznajder, 2012).

1.10.2 Nicotinamide and NAD^+ in the brain

Nicotinamide crosses the BBB (Spector and Johanson, 2007) and can be converted into NAD^+ by neural cells, leading to increased levels of NAD^+, NADH, $NADP^+$, and NADPH, collectively termed the pyridine nucleotides (Banasik, Stedeford and Strosznajder, 2012), and has been shown to be transported into mouse astrocytes through extracellular pH-sensitive mechanisms (Suzuki *et al.*, 2010). Administration of nicotinamide to mice increased levels of NAD^+, NADH, $NADP^+$, and NADPH in the brain. Although increases varied across different brain regions, significant increases were seen in striatum and midbrain regions (Klaidman, Mukherjee and Adams, 2001).

Generation of NAD^+ through the salvage pathway is initiated by the conversion of nicotinamide to NMN by the enzyme NAMPT. Expression of NAMPT has been observed in the rat and human brain (Kitani, Okuno and Fujisawa, 2003), and specifically in neurons (Stein *et al.*, 2014), with higher expression in neurons than astrocytes (Zhang *et al.*, 2010; Wang *et al.*, 2011). Overexpression of *NAMPT*, *in vivo* and *in vitro*, has shown neuroprotective effects

(Wang *et al.*, 2011), and knockout, in mouse forebrain excitatory neurons, has led to cortical atrophy and abnormal dendrite morphology (Stein *et al.*, 2014).

NMN is then converted to NAD^+ by NMNAT, whose expression has been observed in brain tissue (Orsomando *et al.*, 2012). The activity of NMNAT has also been implicated in neuroprotection and is suggested to be specifically involved in axon protection (Sasaki, Araki and Milbrandt, 2006; Coleman and Freeman, 2010; Ljungberg *et al.*, 2012; Lavado-Roldán and Fernández-Chacón, 2016).

Nicotinamide is implicated as an important modulator of CNS health and disease, with involvement in neuronal injury, stroke, Huntington's disease, AD, and PD (Fricker et al., 2018). Overexpression of Pcn1 (also known as nicotinamidase; which bi-directionally converts nicotinamide to NA) extends drosophila lifespan (Balan *et al.*, 2008). Overexpression of NAMPT is protective in *in vivo* and *in vitro* stroke models, whilst blocking NAMPT (using FK866) aggravates neuronal injury in these models, an effect that was prevented by addition of NMN (Wang *et al.*, 2011).Treatment of an AD mouse model with NMN restored respiratory function (Long *et al.*, 2015); and application of nicotinic acid mononucleotide (NaMN), NMN, and NR can delay axonal degeneration after axotomy (Sasaki, Araki and Milbrandt, 2006). If mitochondrial dysfunction and axonal degeneration are features of PD (as discussed in Chapters 1.1.5 and 1.1.6), increasing levels of NMN and NR, which are involved in NAD^+ synthesis, may be beneficial for neuroprotection.

NAD is present in two forms, oxidised (NAD^+) and reduced (NADH), which are vital cofactors in glycolysis, beta-oxidation, Krebs cycle, and the electron transport chain. These redox reactions cycle the reversible conversion of NAD^+ to NADH (Figure 1.10), never depleting the pool of NAD^+. A portion of NAD^+ can be converted into $NADP^+$, with the addition of a phosphate group by NAD kinase, which can also cycle between oxidised ($NADP^+$) and reduced (NADPH) forms, or be converted back to NAD^+ through NAD phosphatase. Anabolic redox

reactions use $NADP^+$/NADPH, whereas NAD^+/NADH is used for catabolic reactions (Bogan and Brenner, 2008; Spaans *et al.*, 2015). Collectively, the pyridine nucleotides are crucial in around 200 enzymatic reactions with functionality in a number of cytoprotectant roles (Williams and Ramsden, 2005b).

Reduced serum levels of NAD^+ and NADH are found in patients with multiple sclerosis (Braidy *et al.*, 2013) and NAD homeostasis is altered with age (Massudi *et al.*, 2012; Braidy *et al.*, 2014; Zhu *et al.*, 2015) suggesting a role in age-related diseases. Overexpression of NAMPT increases NAD^+ levels and protects cells through protection of mitochondrial NAD^+ levels (H. Yang *et al.*, 2007).

NAD^+ is utilised by the mitochondrial respiratory electron transport chain in the synthesis of ATP, a molecule that provides energy in many cellular processes. Depletion of NAD^+ can also lead to depletion of ATP and decreased cell viability (Berger *et al.*, 1987; Stubberfield and Cohen, 1988), suggesting that nicotinamide could provide protection through maintenance of NAD^+ and subsequently ATP levels. Nicotinamide has also been found to maintain mitochondrial membrane potential and prevent membrane depolarisation resulting in the release of cytochrome c (involved in apoptotic signalling), an effect observed in models of oxidative stress (Li, Chong and Maiese, 2006).

There are many cellular pathways and functions affected by NAD^+, and NAD^+ levels, meaning that NAD^+ can be considered a signalling molecule, with increased levels inducing cell survival mechanisms (Schultz and Sinclair, 2016). NAD^+ is used by processes which 'consume' it, leading to the production of nicotinamide which is recycled into the salvage pathway to generate more NAD^+. 'Consumers' of NAD^+ include sirtuins, PARPs and CD38 and may be a potential mode of action through which nicotinamide exerts effects (Li, Chong and Maiese, 2006).

1.10.3 Nicotinamide and sirtuins

Sirtuins are a family of histone deacetylases involved in the regulation of many essential biological processes throughout the body and have been linked to aging and longevity (Imai and Guarente, 2016). There are 7 isoforms, named Sirt1-Sirt7, which mostly function in the deacetylation of proteins resulting in changes to protein surface charge, conformation, and interactions. In this process, the acetylated group is transferred from the protein to ADP-ribose, using NAD^+, and releasing nicotinamide (Wang *et al.*, 2019). The actions of sirtuins are complex and are still being uncovered, although their activity in neurodegenerative disease, including PD, has long been established (Jęśko *et al.*, 2017; Tang, 2017).

Sirtuin mRNA expression is highest in the brain compared to other organs and is often linked to cellular replicative lifespan (Michishita *et al.*, 2005). Sirt1 expression can be considered both neuroprotective and neurotoxic dependent on cell type, stress, and conditions, for example showing neuroprotection in models of axonal degeneration and AD (Sansone *et al.*, 2013).

Nicotinamide is a non-competitive inhibitor of sirtuins and inhibits Sirt1, Sirt2, Sirt3 and Sirt6 to different extents (Bitterman *et al.*, 2002; Avalos, Bever and Wolberger, 2005), and it is proposed that NAD^+ levels regulate sirtuin activity (Feldman *et al.*, 2015).

1.10.4 Nicotinamide and poly(ADP-ribose) polymerases

PARPs are a family of 17 proteins that utilise NAD^+ as a substrate for generating ADP-ribose modification on target proteins and are involved in a number of cellular functions including cell survival, DNA repair, and genomic stability (Herceg and Wang, 2001; Amé, Spenlehauer and De Murcia, 2004; Cseh *et al.*, 2017). PARP-1 is the most abundantly

expressed and best characterised isoform (Cseh *et al.*, 2017). It is activated by DNA strand breaks and involved in DNA repair, with inhibition of PARP-1 increasing the sensitivity of cells to DNA damaging agents (Herceg & Wang, 2001). However, PARP-1 also plays a role in cell death through both apoptosis and necrosis (review: Virág & Szabó, 2002) with severe stress leading to over-activation (Cimadamore *et al.*, 2009); and PARP-1 activation leading to NAD^+ and ATP depletion (Alano *et al.*, 2010), and involvement in the release and translocation of apoptosis-inducing factor (Herceg and Wang, 2001; Amé, Spenlehauer and De Murcia, 2004). PARP-1 has also been implicated in neurite outgrowth (Stoica *et al.*, 2014), proliferation (Hong *et al.*, 2019) and inflammation (Kauppinen and Swanson, 2005). It can be described as a double-edged sword due to involvement in both cell survival and cell death.

Nicotinamide is an inhibitor of PARP-1; although K_i and IC_{50} values vary across the literature ranging from 5.6 – 52 µM and 31 – 210 µM respectively (Banasik, Stedeford and Strosznajder, 2012), this suggests that serum levels, especially with supplemented nicotinamide, may be adequate to inhibit PARP-1 activity. Nicotinamide can rescue primary mouse astrocytes from cell death induced by a DNA alkylating agent (N-methyl-N'-nitro- N-nitrosoguanidine) which activates PARP-1 expression (Suzuki *et al.*, 2010). Since PARP-1 activity cleaves NAD^+ into ADP-ribose and nicotinamide, the release of nicotinamide could be considered as feedback inhibition. PARP inhibition has also shown cytoprotective effects from hydrogen peroxide (Zhang *et al.*, 2000), and genetic disruption of PARP provides neuroprotection in cerebrovascular disease models (Eliasson *et al.*, 1997).

1.10.5 Nicotinamide and cyclic ADP ribose hydrolase

Cyclic ADP ribose hydrolase, also known as CD38, is a transmembrane glycoprotein which converts NAD^+ into nicotinamide, adenosine diphosphate-ribose (ADPR), and cyclic ADPR (Hamblin, 2003). It is expressed in neurons (Mizuguchi *et al.*, 1995), astrocytes (Verderio

et al., 2001), and microglia (Mayo *et al.*, 2008). CD38 has a number of functions including a role in calcium signalling, through the production of cyclic ADPR (Verderio *et al.*, 2001), and roles in neurodegeneration and neuroinflammation, proposed to be through CD38 regulation of NAD^+ levels (for review see: Guerreiro *et al.*, 2020).

In primary cultured mouse microglia, cyclic ADPR promoted pro-inflammatory microglia activation, associated with increased expression and activity of CD38, and increased intracellular calcium concentration, following exposure to the pro-inflammatory stimuli LPS and IFNɣ (Mayo *et al.*, 2008).

Overexpression of CD38 (in the human embryonic kidney 293T cell line) led to a 35% decrease in intracellular NAD^+ levels (Hu *et al.*, 2013). With CD38 expression and activity increasing with age it is thought to be involved in the age-related decline of NAD^+. CD38 is also involved in the degradation of NMN, indicating a further possibility for modulating CD38 in therapies aiming to restore NAD^+ levels (Camacho-Pereira *et al.*, 2016).

1.10.6 Nicotinamide and Parkinson's Disease

Nicotinamide may have a role in PD, however, the exact implication is unknown (Williams and Ramsden, 2005a; Hill and Williams, 2017). Significant reductions in the level of NAD^+, nicotinamide ribonucleotide (an NAD^+ salvage metabolite), and NR were found in PINK1 mutant drosophila. Dietary supplementation of nicotinamide improved mitochondrial function and prevented the loss of dopaminergic neurons (Lehmann, Loh and Martins, 2017). Similarly, NR supplementation protected dopamine neurons and climbing ability in the drosophila GBA-PD model (Schöndorf *et al.*, 2018).

GBA-PD iPSCs show a reduced NAD^+/NADH redox state suggesting a reduction in NAD^+. Treatment with nicotinamide, NMN and NR showed increases in NAD^+ levels (nicotinamide showed the least increase and NR the most). NR also restored mitochondrial cristae

morphology and reduced mitochondrial ROS production in GBA-PD neurons. Therefore increasing NAD^+ levels may rescue mitochondrial dysfunction The effect of NR was abolished by a Sirt1 inhibitor, suggesting a potential mechanism for this effect. (Schöndorf *et al.*, 2018). Nicotinamide has also shown protection in an MPTP mouse model (Anderson, Bradbury and Schneider, 2008), further suggesting the potential for nicotinamide as a neuroprotectant.

Significantly upregulated levels of *NAMPT* mRNA have been observed in blood samples from PD patients (Santiago, Littlefield and Potashkin, 2016). Inhibition of NAMPT in a PC12 6-OHDA model increased the vulnerability of these cells to toxicity, whilst application of NMN (the product of NAMPT activity) protected cells (Zou *et al.*, 2016). This suggests a role of NAMPT, and/or the substrates involved, in PD, although the exact relationship is unclear.

NA has been shown to reduce inflammation in RAW264.7 cells (a mouse cell line with macrophage/monocyte morphology; Giri *et al.*, 2019) and can skew peripheral macrophages from M1 polarisation to M2 polarisation in PD patients (Wakade *et al.*, 2018). This is proposed to be mediated through GPR109A, an anti-inflammatory receptor with a high affinity for NA, which is upregulated in the blood and SN of PD patients (Wakade *et al.*, 2014). However, nicotinamide has virtually no GPR109A activity (Pike, 2005) but may still exert effects on immune cells directly, through increased NAD^+ levels (Haag *et al.*, 2007), or through nicotinamide's interactions with sirtuins and PARPs.

However, it is also possible that high levels of nicotinamide may induce detrimental effects. Levels of nicotinamide are high in meat, particularly beef, and a correlation of increased meat in diets and PD incidence is observed across international populations (Hill and Williams, 2017). Argentina, with high beef consumption has one of the highest incidence rates, whilst poor, rural China and Nigeria, with mostly vegetarian diets, have the lowest incidence rates (Williams and Ramsden, 2005b). Furthermore, a vegan diet is suggested to reduce the risk of PD (McCarty, 2001).

A method for which high levels of nicotinamide may increase the risk of PD is through NNMT activity which converts nicotinamide to N-methylnicotinamide (Figure 1.10) a substrate that is toxic to dopamine neurons (Fukushima *et al.*, 2002), likened to MPP^+ (Parsons *et al.*, 2002), and has been linked to PD pathology (Fukushima, 2005). Higher levels of NNMT activity have been found in brains from PD patients compared to controls (Parsons *et al.*, 2002) and this activity is also reflected in substantially higher levels of N-methylnicotinamide in urine of PD patients (Green *et al.*, 1991).

As discussed in Chapter 1.1.6, intake of alcohol, caffeine and nicotine (through smoking) are associated with a lower risk of developing PD. These compounds may interfere with NNMT activity decreasing the production of N-methylnicotinamide (Williams and Ramsden, 2005b).

It is unclear the exact role that nicotinamide may play in PD, whether it is neurotoxic, neuroprotective, or how this interaction may be conveyed through the variety of mechanisms and derivatives that nicotinamide possesses. Further research into the effects of nicotinamide on neural cells implicated in PD may assist in the development of future therapies or provide insight into the pathology of PD.

1.11 Aims and Objectives

Despite decades of research and an in-depth knowledge of the structural organisation of the nuclei involved in PD neurodegeneration, and the variety of pathological features displayed, a true understanding of the pathological mechanisms remains largely unknown and a curative therapy elusive. However, ongoing research in this field can help to highlight, develop, and improve potential treatments for PD including understanding the roles of non-neuronal cells. Similarly, although vitamins are known to exert a myriad of effects on the CNS, and nicotinamide has been suggested as a possible neuroprotectant, further investigation is required before their full potential can be harnessed.

Therefore, the aims of this PhD thesis are:

I. To assess the potential of nicotinamide in the differentiation of dopaminergic neurons from stem cells to aid the development of CRT for the treatment of the motor symptoms of PD.

II. To assess the potential protective actions of nicotinamide on dopaminergic neurons, which may assist in the development of future therapies for PD.

III. To explore the potential effects of nicotinamide on microglia and whether this could be utilised to understand the mechanisms of immune modulation; both in neurodegeneration generally, and its potential as a future therapy for PD.

2 Materials and Methods

2.1 Reagents, antibodies, and equipment

General reagents used in this PhD project are shown in tables.

- Reagents, their manufacturing company and relevant storage conditions are described in Table 2.1.
- Primary antibodies and their dilutions are supplied in Table 2.2.
- Secondary antibodies and their dilutions are shown in Table 2.3.
- Equipment and software used in this PhD thesis are displayed in Table 2.4.

2.2 Overall experimental design

This PhD project used 2 key analytical techniques, microscopy and flow cytometry, to analyse cell cultures. The experimental design for microscopy analysis is shown in Figure 2.1. Typically, each experiment consisted of at least 3 experimental repeats or 'n' numbers. This term is used to describe each experiment where cells were derived from separate biological sources including different litters, split litters (2 tandem experiments from one litter split from dissection stage), or split cell cultures. Using multiple experimental repeats ensures results are observed across separate biological repeats are not confined to a singular biological anomaly. It also contributes to producing more robust data with smaller error margins. It is generally accepted that scientific experiments should be repeated at least in triplicate to confirm observed results.

Each experimental repeat contained control and treatment conditions. Each treatment condition had 3-4 coverslips termed 'technical replicates'. Each technical replicate consisted of 3-4 micrographs/images; this allowed for observation of an area and quantitative cell

numbers that were deemed representative of the cell population. Cell numbers counted from these images were depictive of treatment conditions and were sufficient to show the observed differences through statistical tests.

For flow cytometry, each condition had 1 or 2 samples, termed ‘technical replicates’, run through the flow cytometer and 4 experimental repeats (n number). The use of technical replicates ensures reliability and consistency within each experimental repeat and considers error that may arise within experimental repeats or from equipment.

Table 2.1 Table of reagents used in this PhD thesis.

Product Name	Manufacturer	Code	Solvent	Storage of solution
6-Hydroxydopamine hydrobromide	Sigma	162957	85 mM ascorbic acid	100 mM, 10 µL aliquots, -80°C, no freeze thaw
B27	Gibco	17504044		
Borate buffer – Sodium tetraborate	Sigma	221732	Water	25 mM, adjust to pH 8, stored at 4°C, sterile filtered
Calcitriol	R&D Systems	2551	DMSO	
CHIR-99021	Sigma	SML1046		
Dnase	Fisher Scientific	10649890		
Dulbecco's Modified Eagle Medium (DMEM) 4.5 g/L w/o L-glutamine w/o sodium pyruvate	Lonza	12-733f		
Dulbecco's Modified Eagle Medium (DMEM)-F12 with Ultraglutamine	Lonza	BE04-687F/U1		
EC23	StemCell Technologies	73102		
Foetal bovine serum	Corning	35-079-CV		
Gelatin 0.1% Embryomax, porcine, type A	Sigma	ES-0006-B		
Glasgow's Minimum Essential Medium (GMEM) high glucose	Gibco	21710025		
Glucose	Sigma	G7021	Water	30% solution (15 g into 50 mL), sterile filtered, stored at 4°C
GM-CSF	PeproTech	400-23	Water	25 µg/mL in 0.1% bovine serum albumin (PBS), -80°C
HEPES	HyClone	SH30237.01		
Laminin, mouse	Merck	CC095	PBS	-20°C
L-Ascorbic acid	Sigma	A4544	Water	Powder kept in dark at -20°C, solution used immediately
MycoAlert Plus Mycoplasma detection kit	Lonza	LT07-701		
N2	Gibco	17502048		
Neurobasal	Gibco	21103049		
Nicotinamide	Sigma	N0636-100G	Neurobasal	1 M -20°C, 200 µL aliquots, 1 month, no freeze-thaw

Normal Donkey Serum	Jackson ImmunoResearch	017-000-121	Water	-20°C
Normal Goat Serum	Jackson ImmunoResearch	005-000-121-JIR	Water	-20°C
Paraformaldehyde	Fisher Scientific	11473704	PBS	4% stored at -20°C
PD0325901	Sigma	PZ0162		
PenStrep: Penicillin Streptomycin	Lonza/SLS	LZDE17-602E		
Poly-D-Lysine	Corning/SLS	354210	PBS	-20°C
Polyethylenimine	Aldrich	408727	25 mM borate buffer	10% stored at 4°C, sterile filtered
Propidium Iodide	Invitrogen	P3566		
Sodium Pyruvate	Lonza	LZBE13-115E		
Triton X-100	Sigma	X100	PBS	
Trypan Blue 0.4%	Fisher Scientific	15250061		
TrypLE	Fisher Scientific	10573283		
Ultraglutamine	Lonza	BE17-605E/U1		
Vectashield mounting medium with DAPI	Vectorlabs	H-1200		
β-mercaptoethanol	Merck	M3148		

Table 2.2 Table of primary antibodies used in this PhD thesis.

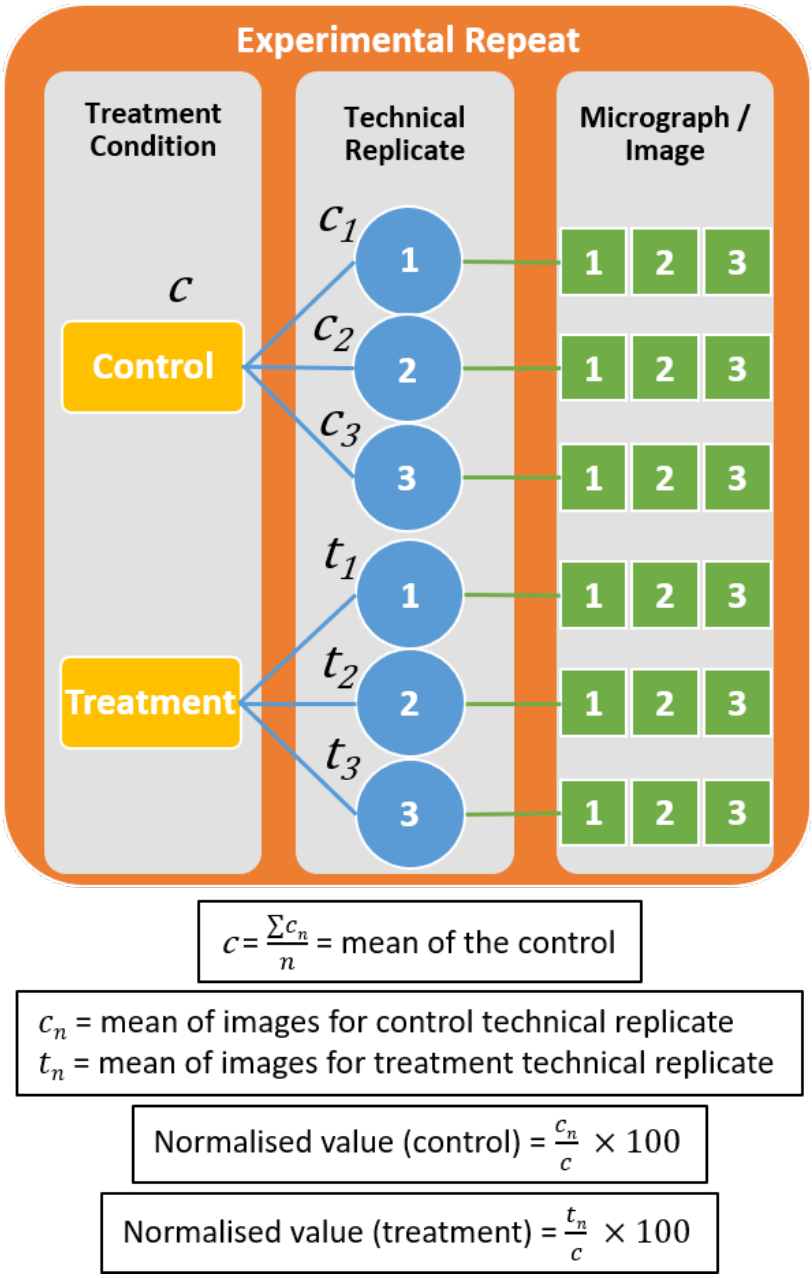
Target	Host Species	Manufacturer	Code	Dilution Ratio
GFAP	Rabbit	DAKO	Z0335	1:1000
GFAP	Mouse	Biologend	644701	1:500
GFP	Rabbit	Novus Biologicals	NB600-308	1:1000
Iba1	Goat	Abcam	ab5076	1:500
Nestin	Mouse	BD Biosciences	611658	1:200
Serotonin	Goat	Abcam	ab66047	1:500
Tyrosine hydroxylase	Rabbit	Merck	AB152	1:1000
β-III-tubulin	Mouse	Biolegend	801201	1:1000
β-III-tubulin	Rabbit	Biolegend	802001	1:1000

Table 2.3 Table of secondary antibodies used in this PhD thesis.

Fluorophore	Target Species	Host Species	Manufacturer	Code	Dilution Ratio
488	Mouse	Goat	Invitrogen	A28175	1:500
488	Mouse	Donkey	Jackson Immuno	715-545-150	1:500
488	Mouse	Donkey	Invitrogen	A21202	1:500
594	Goat	Donkey	Jackson Immuno	705-585-003	1:500
594	Rabbit	Goat	Invitrogen	A-11012	1:500
647	Rabbit	Donkey	Abcam	ab150067	1:500

Table 2.4 Table of equipment used in this PhD thesis.

Equipment/Software	Brand	Model
Dissecting microscope	Leica	S6D
Flow Cytometer	Guava	EasyCyte 6-2L
InCyte	Guava	3.1
FlowJo	BD Biosciences	X.0.7
Microscope	Nikon	Eclipse 80i
Microscope	Nikon	Eclipse Ti
Microscope camera	Hammamatsu	C4742-95
NiS Elements	Nikon	2.32
Image J	National Institutes of Health	1.51j8
Plate reader	Promega	Glomax Multi Detection System
Prism	Graphpad	8



$$c = \frac{\sum c_n}{n} = \text{mean of the control}$$

c_n = mean of images for control technical replicate
t_n = mean of images for treatment technical replicate

$$\text{Normalised value (control)} = \frac{c_n}{c} \times 100$$

$$\text{Normalised value (treatment)} = \frac{t_n}{c} \times 100$$

Figure 2.1 Schematic demonstrating the experimental design and equations used to normalise data. Each experiment consisted of at least 3 experimental repeats or 'n' numbers (orange). Experimental repeats contained control and treatment condition. Each treatment condition had 3-4 coverslips termed 'technical replicates', with 3-4 micrographs/images taken of each technical replicate. Equations explain the calculation used to normalise data. The average quantified value/measurement from each technical replicate was divided by the average number of the control condition of that experimental repeat, then multiplied by 100 to give the percentage, relative to the average control.

2.3 General cell culture:

2.3.1 Maintenance, incubation, and media

Each cell culture system was maintained in a humidified incubator at 37 °C with 5% CO_2. All cell culture was carried out using aseptic techniques with the use of a class II laminar flow hood. Non-sterile reagents such as phosphate buffered saline (PBS) and distilled water dH_2O were autoclaved at 121 °C and 20 psi (20 mins) prior to use. Any reagents that were not able to be autoclaved, such as media components, were sterile filtered through 0.2 µm syringe filters.

Different culture media, outlined in Table 2.5, were used for each culture system. The mouse embryonic stem cell line 46C was maintained in 2i and N2B27 media for proliferation and differentiation respectively. Primary rat embryonic day 14 (E14) cultures were dissected using dissection medium and maintained in neural cell medium (NCM). Pure microglial cultures were maintained in D10 media. All media were stored at 4 °C and kept for no longer than 2 weeks.

When handling cells, all reagents and media were either warm (pre-warmed to 37 °C in a water bath), or cold (kept on ice or at 4 °C) as stated in the text.

2.3.2 Preparation of cell adhesion surfaces

Cell culture flasks, wells and coverslips were coated to promote adherent, monolayer growth. Each culture system required different substrates, the procedure for each is outlined below, and preparation of each substrate was based on protocols previously established in the laboratory.

For 0.1% gelatin coating, the bases of flasks/wells were fully covered with 0.1% gelatin and left for a minimum of 30 mins at room temperature. The gelatin was then aspirated and plasticware was washed with warm PBS, which was removed just prior to cell seeding.

Table 2.5 Table showing the media used for each culture system in this PhD thesis.

Culture System	Media	Reagent	Concentration
46C	N2B27	DMEM-F12 with Ultraglutamine	1:1 Neurobasal
		Neurobasal	1:1 DMEM-F12
		Ultraglutamine	1 mM
		B27	1 X
		N2	0.5 X
		PenStrep	100 U/mL
		β-mercaptoethanol	0.1 mM
46C	2i	N2B27	
		CHIR99021	3 µM
		PD03259010	1 µM
Primary rat E14 VM and WGE	Dissection media	DMEM-F12	
		HEPES	50 mM
		Glucose	0.6 %
Primary rat E14 VM and WGE	NCM (Neural Cell Media)	Neurobasal	
		Glucose	0.45%
		PenStrep	100 U/mL
		Foetal bovine serum	1 %
		B27	1 X
		Ultraglutamine	1 mM
Pure Microglial cultures (and mixed glial starter cultures)	D10	DMEM (4.5 g/mL glucose)	
		Foetal bovine serum	10%
		Ultraglutamine	2 mM
		Sodium Pyruvate	1 mM
		PenStrep	100 U/mL

Prior to coating, coverslips were dipped in 70% ethanol and dried under ultraviolet (UV) light for sterilisation. Coverslips were then coated with either poly-D-lysine (PDL) alone, PDL and laminin, or polyethylenimine (PEI) and PDL, dependent on each culture system.

For PDL coating, coverslips were submerged in PDL (10 μg/mL) for a minimum of 30 mins at room temperature. Coverslips were then washed 3 times with dH_2O and left in the final wash until aspiration just prior to cell seeding.

For PDL and laminin coating, coverslips were submerged in PDL (10 μg/mL) for a minimum of 1 hour at room temperature. PDL was then aspirated and coverslips were washed 3 times with dH_2O. Laminin (2 μg/mL) was then applied, ensuring complete coverage of coverslips, which were placed in the incubator at 37 °C overnight. Laminin was removed and coverslips were washed 3 times with dH_2O, aspirating the final wash just prior to cell seeding.

To coat coverslips with PEI and PDL, coverslips were immersed in PEI (0.05%) for a minimum of 1 hour at room temperature. PEI was then removed and coverslips were washed 4 times with dH_2O, then left in PDL (10 μg/mL) overnight in the incubator. Coverslips were washed 3 times with dH_2O with the final wash aspirated just prior to cell seeding.

2.3.3 Cell counting and seeding

To seed cell cultures at the appropriate density, cell counts were undertaken using a Neubauer chamber. After centrifugation and aspiration of supernatant, cells were resuspended in the appropriate media. A sample of cells was diluted into trypan blue, by a dilution factor of 10, to allow visualisation and exclusion of dead cells (trypan blue positive). 10 μL of trypan blue/cell mixture was transferred to the haemocytometer for counting. The number of trypan blue negative cells were counted in at least 2 of the 4x4 corner grids, including cells touching the top and left lines and excluding those touching the bottom and right lines. The number of cells across grids was averaged, then multiplied by the trypan blue dilution factor, then multiplied by 10 to give the number of cells in 1 μL. Cells were then diluted into media to the appropriate concentration for seeding.

Cells were seeded by either flooding the well (and coverslip) with media containing the correct number of cells per well, or by using micro-drop cultures. Micro-drop cultures were formed by placing 30-50 µL of medium containing the specified density of cells into the centre of a dry coverslip forming a defined drop on the surface. This method allowed for a high-density cell colony in the centre of the coverslip with all viable cells attached to the coverslip. After transferring plates to the incubator for 4 hours allowing cell attachment, wells were then flooded with treatment medium.

2.4 Mouse embryonic stem cell culture

2.4.1 The 46C cell line

The mouse embryonic stem cell (mESC) line 46C (a kind gift from Professor Meng Li, Neuroscience & Mental Health Research Institute, Cardiff University) was used to study the effects of nicotinamide on neuronal differentiation. This cell line contains the green fluorescent protein gene (*GFP*) under the *Sox1* promoter allowing for visual identification of neural precursors expressing Sox1. This cell line was generated from E14Tg2a.IV cells after gene targeting where the open reading frame of *Sox1* was replaced with *GFPiresPac*. This allows for accurate recapitulation of *in vitro Sox1* signalling with undetectable levels in undifferentiated cells, increasing to a significant proportion of cells expressing GFP upon neural induction (Ying *et al.*, 2003). This cell line provides a valuable tool for the identification of neural precursors within differentiating populations.

2.4.2 Thawing and proliferation of the 46C cell line

The 46C cell line had been frozen in 10% dimethyl sulfoxide (DMSO) in medium at a density of 2 x 10^6 cells in 800 µL. Vials were removed from liquid nitrogen storage and rapidly thawed in warm water. Cells were transferred to a 15 mL tube containing 5 mL warm N2B27 medium (Table 2.5) and centrifuged at 1000 rpm for 3 minutes. Supernatant was removed and cells were resuspended in warm 2i medium (Table 2.5) and transferred to a gelatin coated T25 flask (described in Chapter 2.3.2).

Proliferating 46C cells were maintained in 0.1% gelatin coated T25 flasks in 2i media (Table 2.5). Cultures were passaged after reaching 80% confluence. Cells were washed with warm PBS, then enzymatically detached using warm TrypLE (synthetic trypsin; 2 mins). Cells were collected into a 15 mL tube with 10 mL warm N2B27 and centrifuged at 1200 rpm for 3

mins. Supernatant was discarded and the pellet resuspended in warm 2i medium. Live cells were counted (Chapter 2.3.3) and seeded at 5 x 10^5 cells per gelatin coated T25 flask with 2i medium. Medium was changed every 2-3 days. Cultures were used between passage numbers 26-32. Thawed cultures were passaged at least once before use.

2.5 Use of animals for primary tissue cultures

Adult Sprague Dawley rats were maintained in appropriate conditions in line with the Home Office Code of Practice for the housing and care of animals bred, supplied, or used for scientific purposes covered by Keele University site licence X350251A8. Animals were housed in the accredited animal facility at Keele University with ad libitum access to food (Special Diet Services RM1 (E) 801002) and water, and with controlled temperature (21 ± 2 °C) and photoperiod (12 hour light-dark cycle). Each cage was supplied with substrate, hide, chew block and bedding. Females were 12-15 weeks old when paired for mating in a mesh bottom cage with ad libitum access to food and water. The tray underneath the cage was checked daily for the plug ejected by the female once mated. Once the plug was seen, marking Day 0 of gestation, females were removed and housed with other pregnant dams. Animals were killed using licenced Schedule 1 methods in accordance with the Animals (Scientific Procedures) Act of 1986.

2.5.1 Dissection and maintenance of E14 rat ventral mesencephalon and whole ganglionic eminence cultures

Primary ventral mesencephalon (VM) and whole ganglionic eminence (WGE) cultures were obtained from E14 rat embryos. Pregnant Sprague Dawley rats were sacrificed by cervical dislocation at 14 days from the plug date (defined as E0). The abdomen was sprayed with 70% ethanol before laparotomy to remove embryos from the uterus. Embryos were killed humanely using a standard Schedule 1 procedure appropriate for their gestation (decapitation) and the heads collected into dissection media (Table 2.5) kept on ice. The VM and/or WGE was then dissected, as described in Dunnett & Björklund (1992) and shown in Figure 2.2, from each head individually under sterile conditions using a dissection microscope

in a laminar flow hood. The dissected tissue was collected into 1 mL dissection media kept on ice. Once dissections were complete, the collected tissue was washed twice with fresh, cold dissection media, then TrypLE DNase (consisting of DMEM-F12 with 0.5X TrypLE and 50 U/mL DNase) was added and cells were incubated at 37°C for 20 mins. TrypLE DNase was removed and tissue pieces were washed 3 times with cold DMEM DNase (DMEM-F12 with 50 U/mL DNase). Tissue pieces were then resuspended in 200 µL DMEM DNase and were mechanically dissociated using a P200 pipette. Cell density was calculated (Chapter 2.3.3) whilst cells were centrifuged at 1200 rpm for 3 mins at 4 °C. The pellet was then resuspended in room temperature NCM (Table 2.5) and cultures were seeded at 5-6 x 10^4 cells in 50 µL micro-drop cultures onto PEI and PDL coated coverslips (described in Chapter 2.3.2). Micro-drop cultures were allowed to attach for 4 hours before wells were flooded with warm NCM. At this point, cultures were described as 0 days *in vitro* (DIV).

Primary rat E14 VM and WGE cultures were maintained in NCM in an incubator at 37 °C with 5% CO_2 and humidified atmosphere. The initial full media change took place at 3 DIV, with full media changes thereafter every 2-3 days to ensure removal of debris and replenishment of any media components or vitamins in treatment conditions.

2.5.2 *In vitro* PD model using 6-OHDA

To create an *in vitro* PD model, 6-OHDA was used to selectively destroy dopaminergic neurons within VM cultures. To prevent rapid oxidation of 6-OHDA, an ascorbic acid (AA) solution was used as a vehicle. To ensure continuity across experimental repeats, a stock solution of 100 mM 6-OHDA in 85 mM AA (stored at -80 °C) was used. For cell application, 100 mM 6-OHDA (in 85 mM AA) was diluted into 85 mM AA to 2.5 mM 6-OHDA concentration. This solution (2.5 mM 6-OHDA in 85 mM AA vehicle) was then diluted 1:100 into NCM resulting in cells receiving final concentrations of 25 µM 6-OHDA in 850 µM AA.

At 7 DIV, media was aspirated and replaced with NCM containing 25 µM 6-OHDA or vehicle control (850 µM AA) for 4 hours. After treatment, 6-OHDA/AA media was aspirated and cells were washed twice with warm NCM and left to recover in NCM for 48 hours.

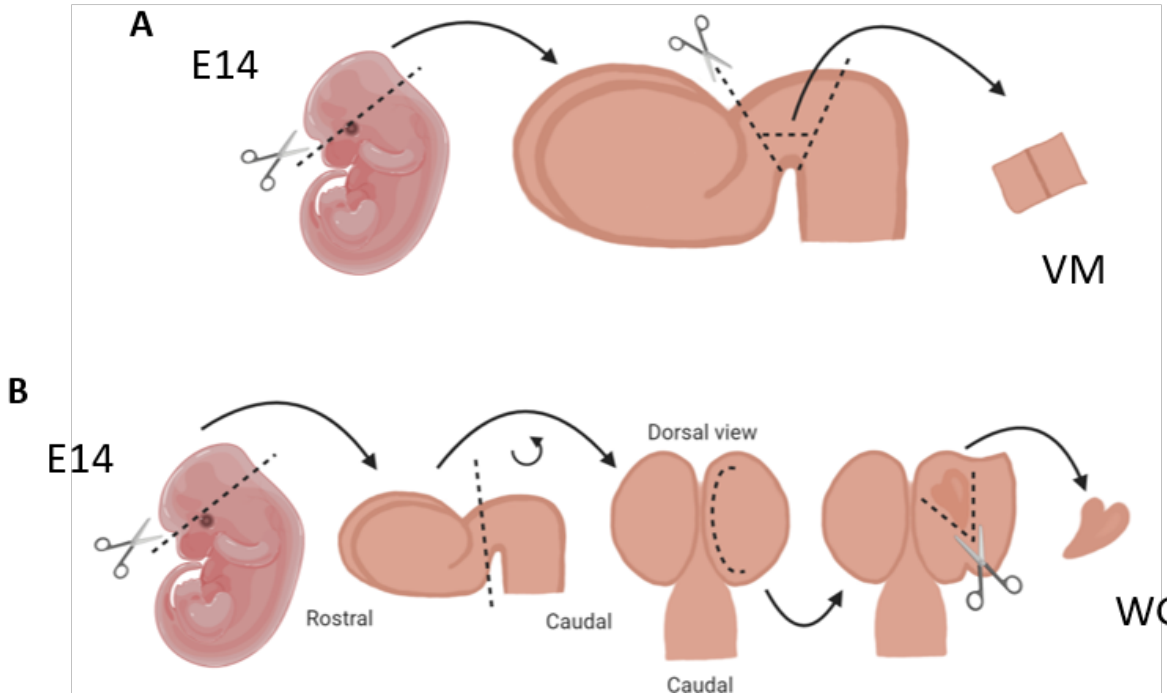


Figure 2.2 Diagram showing the dissection of VM and WGE tissue. A) Diagram showing dissection of VM tissue from E14 rat. An initial cut was made above the eye to remove the brain. A series of cuts, as shown, were then made to dissect the VM B) Diagram showing dissection of WGE from E14 rat. An initial cut above the eye was made to remove the brain and the cortices were removed from the mid- and hindbrain. Cuts were then made along the dorsal, medial cortex and folded aside to reveal the lateral ventricular cavity. The WGE appears as a heart-shaped bulge which can then be removed with a superficial horizontal cut. This was then repeated on the second hemisphere. E: embryonic day; VM: ventral mesencephalon; WGE: whole ganglionic eminence. Created using BioRender.com

2.6 Derivation of pure microglial cultures from mixed glial cultures

Pure microglial cultures were obtained from rat mixed glia cultures. Mixed glial cultures were derived from postnatal day 0-3 (P0-3) Sprague Dawley rats. Animals were killed using a standard Schedule 1 procedure (overdose of pentobarbital) and the brains dissected out. Under sterile conditions, the cerebellum and olfactory bulbs were removed, the cerebral cortices isolated, and gently rolled on autoclaved handtowel to remove meninges. The cerebral cortices were then mechanically dissociated, seeded into PDL coated flasks, and maintained in D10 media (Table 2.5) for 7-10 days allowing cultures to become confluent, and for stratification of the cell layers, such that oligodendrocyte precursor cells and microglia were displaced above a confluent bedlayer of astrocytes.

Shaking was used to separate floating and loosely adherent microglia from mixed glia cultures. Flasks had their headspace sealed to maintain 5% CO_2, then were placed in a 37 °C rotary shaker at 220 rpm for 1.5-2 hours. This process detaches microglia from the monolayer culture keeping them suspended in the media. Media was then collected from the flasks and centrifuged at 1200rpm for 3 mins. The supernatant was discarded and the cell pellet resuspended in warm D10 media. Cell density was calculated (Chapter 2.3.3) and cells were seeded at the required density for each experiment onto PDL coated coverslips or wells and maintained in D10 media with full media changes every 2-3 days.

2.7 Preparation of flow cytometry samples

To prepare cells for flow cytometry analysis, media was removed and cells were washed with warm PBS. TrypLE was applied for 2 mins to enzymatically detach cells which were collected into warm N2B27 in a 15 mL tube and centrifuged at 1200 rpm for 3 mins. The supernatant was discarded and cells were resuspended in 1 mL of cold PBS and transferred to a 1.5 mL Eppendorf tube. Cells were centrifuged again at 1200 rpm for 3 mins, the supernatant was discarded and cells resuspended in 500 µL cold PBS. Once again, cells were centrifuged at 1200 rpm for 3 mins, the supernatant discarded, and cells resuspended in 200 µL of cold PBS. This cycle of washes ensured removal of media. Samples were kept on ice to minimise any enzymatic activity and degradation of cells until use. Cells were passed through the flow cytometer using Incyte software and data was later analysed using FlowJo software (Table 2.4). For excitation of fluorophores the blue (488 nm) laser was used alongside green (525/30 nm) and red (695/50 nm) emission filters for detection of GFP and propidium iodide (PI) respectively. Further details of flow cytometry experiments are given in Chapter 3.2.

2.8 Fixation and Immunocytochemistry

Cells on coverslips or in wells were fixed using 4% paraformaldehyde (PFA) for 15 minutes then washed 3 times with PBS. Samples remained in PBS at 4 °C until staining. Blocking solution (5% normal goat or donkey serum in PBS, 0.3% Triton X-100) was applied for 1 hour to limit non-specific antibody binding. Primary antibodies (Table 2.2) in blocking solution were applied and left at 4 °C overnight. Samples were washed 3 times for 3 mins with PBS and blocking solution was applied again for 30 mins. Corresponding secondary antibodies (Table 2.3) in blocking solution were applied and samples were incubated in the dark at room temperature for 2 hours. Samples were then washed 3 times for 3 mins and coverslips were mounted with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI). The outside of mounted coverslips were sealed with clear nail varnish for preservation of samples.

The primary antibodies and dilution used in this PhD thesis are shown in Table 2.2. β-III-tubulin is a microtubule present almost exclusively in neurons (Lee *et al.*, 1990) with exceptions discussed in Chapter 3.4.1. Here it has been used to identify neuronal populations in both VM and 46C cultures.

Tyrosine Hydroxylase (TH) is the rate-limiting enzyme that catalyses the hydroxylation of tyrosine to L-DOPA (Figure 1.4) and is expressed exclusively in catecholaminergic neurons (Daubner, Le and Wang, 2011). In this PhD thesis TH was used to identify dopamine neurons in VM cultures. Although some serotonergic neurons may be included in cultures, dependent on the positioning of the caudal cut during dissection (Dunnett and Björklund, 1992), later examination for serotonin staining revealed the presence of a couple of serotonin$^+$ neurons within some coverslip cultures (data not shown). This allowed the belief that the majority of TH$^+$ neurons within these cultures were dopaminergic.

Neuroectodermal stem cell protein (Nestin) is a cytoskeletal protein expressed in NSCs and some non-neural cells. Here it is used to identify NSCs in VM cultures, which do not

contain the non-neural cells (e.g. heart, skeletal muscle) also known to express Nestin (Bernal and Arranz, 2018).

GFAP is an intermediate filament present in astrocytes and is involved in pro-inflammatory activation of astrocytes in response to injury or insult (McKeon and Benarroch, 2018). It has been used to identify astrocytes in VM cultures in this PhD thesis.

Ionized calcium binding adaptor molecule 1 (Iba1) is found to be exclusively expressed in microglia (Ito *et al.*, 1998). Involved in cytoskeletal reorganisation, it is uniformly expressed throughout the cytoplasm (Korzhevskii and Kirik, 2016). It was used here to identify microglia in VM cultures, and confirm identity in high purity microglial cultures derived from mixed glia cultures.

2.9 Acquisition of cell images for analysis

Microscopy was performed on either a Nikon Eclipse 80i or Nikon Eclipse Ti microscope. Images were captured using a black and white digital camera (Hammamatsu) and NiS Elements software. The same exposure settings were used for each fluorescence stain within each experimental repeat. Images were exported from NiS Elements software in Tag Image File Format (TIFF). Each TIFF contained a 'stack' of images termed 'slices' for each fluorescence channel used. TIFFs were analysed and transformed (e.g. colour application, scale bar addition etc.) using Image J software (Schneider, Rasband and Eliceiri, 2012). Lookup tables (LUTs) within Image J were used to apply relevant colours to each slice and to merge colour channels to show co-localisation of stains within images.

Images taken at 10X magnification defined a 1.16 mm^2 field of interest (FOI). Magnification at 20X equalled an FOI of 0.29 mm^2. Images taken at 40X magnification had an FOI of 0.073 mm^2. FOIs were taken randomly across the culture moving from left to right and top to bottom with no overlapping fields. To obtain representative images of cells of interest, the edges of VM cultures, which typically contain mostly proliferating cells, were avoided where possible. In order to reliably count cells of interest, large clumps of cells (identified as large numbers of $DAPI^+$ nuclei, visible across multiple focal panes in the z axis), which would be difficult to quantify, were also avoided (FOI was rejected, and new FOI tested). Although all imaging followed the above guidelines, slightly differing techniques of imaging and analysis were utilised dependent on which cell type was to be analysed. Details of the technique used in each instance are described in the methods sections of each results chapter.

2.10 Measuring optical density

Optical density measurements were used to quantify the expression of markers within cultures. This technique was used when counting was either not possible due to crowded/clumped cell distributions, or to provide further quantitative data about expression levels.

To prepare images for optical density analysis, an Image J macro was used to transform TIFF stacks into individual images for each fluorescence channel slice. The slices were then colour inverted (high intensity staining originally shaded as white was changed to black) meaning that higher optical density measurements corresponded to higher levels of expression.

The optical density of images was measured using the Image J Measure function. Measurements were calibrated using the step tablet (which provides steady gradations from white to black) and protocol supplied by the National Institutes of Health (NIH), Image J website (NIH, no date). The mean grey value was measured for each step on the tablet and entered into the 'Calibrate' dialog box alongside calibration values supplied by NIH. "Rodbard" was selected from the "Function" dropdown menu, and O.D. was entered in the "Unit" field. The mean grey value was then measured for each image as a whole resulting in values displayed in OD units.

2.11 Normalisation of results with variation across experimental repeats

Due to the nature of working with the dissection of biological tissue, variation was observed across some experimental repeats. This could arise from biological differences in the tissue used, due to variables such as: exact time between plug formation and dissection (which could easily vary by ~ 12 hours), variations in litter number (which can affect rate of development for individual pups), and variation in embryo/pup size (which may be related to individual rate of development, and could also influence accuracy of dissection and yield of desired tissue). Differences could also arise from the size of the dissected tissue with no way to replicate the exact accuracy of tissue dissection, yield, or purity across experimental repeats.

Data sets were normalised if variations in cell counts were observed across experimental repeats. Trends across treatment conditions were conserved with normalisation. To normalise, the average quantified value/measurement from each technical replicate was divided by the average number of the control condition of that experimental repeat, then multiplied by 100 to give the percentage, relative to the average control. This is shown in Figure 2.1.

2.12 Statistical analysis using GraphPad Prism software

Prism software was utilised for all statistical analysis. One-way and two-way ANOVA, *t*-tests and linear regression were utilised. The statistical analysis used for each set of data is outlined in the relevant chapter. Post-hoc analysis for ANOVA was utilised for more detailed statistical information across treatment groups. Bonferroni's multiple comparisons test was used to compare two selected treatments within an ANOVA. Tukey's multiple comparisons test was used to compare every treatment with every other treatment within an ANOVA. Dunnett's multiple comparisons test was used when comparing each treatment against the control within an ANOVA.

All data described within text and displayed in graphs is mean ± SEM (standard error mean) unless otherwise stated. Statistical significance is displayed as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Graphs were also generated using Prism software.

3 Nicotinamide in directing neuronal differentiation

3.1 Introduction

3.1.1 Nicotinamide has been used in differentiation protocols

Cell replacement therapy has the potential to reverse, and offer long-term relief, from the motor symptoms of PD, through the replacement of lost dopaminergic neurons. However, researchers are still in pursuit of a reliable source of specific midbrain dopaminergic neurons. Stem cells may provide readily available and consistent neurons for grafts, with low immunogenicity. However, a suitable protocol for large scale, reproducible production of dopaminergic neurons, which survive transplantation in required numbers, is yet to be fully established (Chapter 1.6.3). Further enhancement of protocols for the differentiation of dopaminergic neurons and their survival *in vitro* may benefit from the addition of nicotinamide.

Protocols have shown the use of nicotinamide in the differentiation of pluripotent cells (specifically ESCs and iPSCs) into insulin-secreting (Vaca *et al.*, 2008), cardiac (Parsons *et al.*, 2011), motoneuron (Y. Zhang *et al.*, 2011), and retinal pigmented epithelium (Idelson *et al.*, 2009; Buchholz *et al.*, 2013) cells.

Specifically, nicotinamide has shown effectiveness in promoting neural differentiation (Cimadamore *et al.*, 2009; Idelson *et al.*, 2009; Y. Zhang *et al.*, 2011; Buchholz *et al.*, 2013; Griffin *et al.*, 2013, 2017). The application of 10 mM nicotinamide has been shown to promote neuronal differentiation of the mESCs used in this PhD thesis. Both numbers, and the percentage neurons of total $DAPI^+$ cells, were significantly, and dose-dependently increased with the application of 5 and 10 mM nicotinamide (Griffin *et al.*, 2013). Application of 10 mM nicotinamide during early differentiation also increased neuronal maturation of differentiated

mESCs and reduced the number of undifferentiated stem cells determined by Oct4 expression (Griffin *et al.*, 2017). Reduced levels of *NANOG* and *Oct4* mRNA in nicotinamide treated hESCs also suggest that nicotinamide promotes differentiation; whilst inhibition of meso-endoderm marker expression and induction of ectoderm marker expression further suggests that nicotinamide promotes neural differentiation (Meng *et al.*, 2018). Cimadamore et al. (2009) found that addition of 5 mM nicotinamide to hESCs increased neuralisation and cell viability. Alongside differentiation, studies have also suggested that nicotinamide has roles in stem cell pluripotency, survival, and proliferation (Meng *et al.*, 2018).

3.1.2 Nicotinamide inhibits proliferation in a number of different cell types

Nicotinamide dose dependently promoted hESC survival, and concentrations of 10 mM reduced cell growth over 4 days (Meng *et al.*, 2018). Nicotinamide has also been shown to inhibit proliferation in some cancer-related cell lines, although the mode of action is debated (Audrito *et al.*, 2011; Petin *et al.*, 2019; Malesu *et al.*, 2020). The addition of 10 mM nicotinamide to D3-ES cells (an mESC line) reduced proliferation by 70% when directed towards an insulin-secreting cell fate (Vaca *et al.*, 2008). However, concentrations of 1 mM and below have been shown to increase proliferation and maintenance of pluripotency in human ESCs and iPSCs (Son *et al.*, 2013). As discussed in Chapter1.10, nicotinamide could influence a range of mechanisms behind the effects observed in pluripotent cell cultures.

3.1.3 Nicotinamide's mode of action includes Sirt1, PARP-1 and kinase inhibition

Studies exploring the mechanisms of nicotinamide often refer to the inhibitory effects that nicotinamide exerts on Sirt1 and PARP-1 (discussed in Chapters 1.10.4 and 1.10.5).

Inhibition of Sirt1 has been suggested as a mechanism in nicotinamide-induced neural differentiation (Y. Zhang *et al.*, 2011; Liu *et al.*, 2014). Levels of Sirt1 are higher in foetal, compared to adult, brain (Michishita *et al.*, 2005), and Sansone et al. (2013) found reduced levels of Sirt1 in neuronally differentiated NG108-15 cells (a rodent neuroblastoma/glioma hybrid cell line with neuronal growth properties) compared to proliferating cells. Inhibition of Sirt1, through application of sirtinol, induced cell death in proliferating NG108-15 cells; whilst cell death was significantly lower in neuronally differentiated cells. Sirt1 overexpression also resulted in decreased expression of the neuronal marker MAP5, whilst Sirt1 silencing promoted neuronal net formation (Sansone *et al.*, 2013). This suggests that nicotinamide-induced Sirt1 inhibition may contribute to the accelerated neuronal differentiation/maturation described above.

Nicotinamide also acts as a PARP-1 inhibitor and this is often cited as a mechanism for nicotinamide-induced effects. PARP-1 knockout mice show reduced brain weights, and NSCs derived from these mice show decreased proliferation rates and increased apoptosis, showing that PARP-1 is involved in NSC proliferation and survival (Hong *et al.*, 2019). Cimadamore et al. (2009) found that addition of 5 mM nicotinamide to hESCs increased neuralisation and also increased cell viability through nicotinamide-induced PARP-1 inhibition, resulting in decreased cell death. They found that PARP-1 is strongly expressed in human neural progenitor cells, but it's expression in hESCs is significantly lower (Cimadamore *et al.*, 2009) suggesting that inhibition of PARP-1 may help to protect differentiating neural progenitor cells. However,

PARP-1 knockout mouse NSCs showed decreased neuronal, and increased astrocyte ($GFAP^+$) and oligodendrocyte ($Olig2^+$) differentiation (Hong *et al.*, 2019). PARP inhibition supressed the formation of neurospheres in a concentration dependent manner and reduced the viability of neural stem/progenitor cells (Okuda *et al.*, 2017).

Although nicotinamide has been shown to inhibit PARP-1 activity, and this may have a role in the effects observed, complete inhibition may not be responsible for the nicotinamide-induced neurogenesis discussed above. The assessment of 5 different PARP inhibitors (not including nicotinamide) showed variability in their effects on proliferation, differentiation and survival with mouse neural stem/progenitor cells (Okuda *et al.*, 2017), highlighting the complexity of downstream effects caused by PARP inhibition and other regulatory influences that PARP inhibitors may exhibit.

Nicotinamide may also exert neuroprotective effects through inhibition of Rho-associated protein kinase activity and other kinase cascades (Meng *et al.*, 2018). Although this is relatively new research, it provides another potential avenue for therapeutic applications of nicotinamide in neurodegeneration. This research also shows the wide-ranging effects that nicotinamide can have on cellular function and the many mechanisms associated with it.

3.1.4 Aims of Chapter 3

The use of nicotinamide in stem cell cultures could help to advance the development of dopaminergic differentiation protocols for CRT to provide treatment for PD. As previously discussed, nicotinamide has been used in the development of a number of differentiation protocols and has been shown to enhance neuronal differentiation whilst limiting stem cell proliferation. There is potential for CRT in the treatment of neurodegenerative conditions, however, one of the obstacles to CRT for PD is the need for protocols that can reliably and efficiently generate cells suitable for grafting. Therefore, this chapter aims to ascertain how nicotinamide may be used in the differentiation of stem cells for CRT for PD by assessing the effects of nicotinamide on 46C mESCs using immunocytochemistry and flow cytometry. The specific aims of this work were to:

I. Assess the effect of nicotinamide on proliferation of 46C cells during a pro-neuronal differentiation protocol.

II. Assess the effect of nicotinamide on neuralisation and neuronal differentiation.

3.2 Methods

3.2.1 Mycoplasma testing of 46C cell line

Mycoplasma are a type of bacteria, commonly found to infect cells in culture, that can interfere with normal cell functions, such as metabolism, compromising experimental data (review: Drexler & Uphoff, 2002). To confirm that the cells used in these experiments were mycoplasma-free prior to differentiation, the 46C cell cultures were tested for presence of mycoplasma using the MycoAlert PLUS kit (Lonza).

The reagent and substrate were reconstituted as instructed. Supernatant from cells grown in Glasgow's minimum essential medium (GMEM) for at least 24 hours was used to test cultures for mycoplasma. GMEM, rather than complex culture media was used, to simplify the experiment, removing extraneous media components, in order to rule out the cells as a source of contamination. GMEM supernatant was centrifuged at 1200 rpm for 3 minutes to remove cells and debris, then mixed with the reagent. Luminescence was read by a plate reader (Promega Glomax Multi detection system) after 10 minutes of reagent addition. The substrate was then added and the plate was read 10 minutes later. Results were calculated by dividing the second luminescence reading (substrate) by the initial luminescence reading (reagent). If this ratio is < 1, the sample is negative for mycoplasma.

3.2.2 Neuronal differentiation of the 46C cell line

For neuronal differentiation of 46C cells, proliferating cells (Chapter 2.4.2) were washed with warm PBS then enzymatically detached using warm TrypLE for 2 mins. Cells were collected in warm N2B27 media, centrifuged at 1200 rpm for 3 mins. The supernatant was discarded, the pellet resuspended in warm N2B27, and cell density calculated (Figure 3.1).

Cells were seeded into gelatin coated 6 well plates at a density of 1 x 10^5 cells per well, in warm N2B27 media (day 0) with full media changes every 2-3 days.

On day 7, differentiating cells were washed with warm PBS, enzymatically detached using warm TrypLE for 2 mins, collected in 10 mL warm N2B27 and centrifuged at 1000 rpm for 3 mins. The supernatant was discarded and the pellet resuspended in warm N2B27. Cells were counted and seeded on to PDL and laminin coated glass coverslips in 30 µL micro-drop cultures containing 3 x 10^4 cells. Micro-drop cultures were allowed to adhere for 4 hours before flooding the well with warm N2B27 media. Cultures were maintained for a further 7 days with full media changes every 2-3 days, throughout the differentiation protocol.

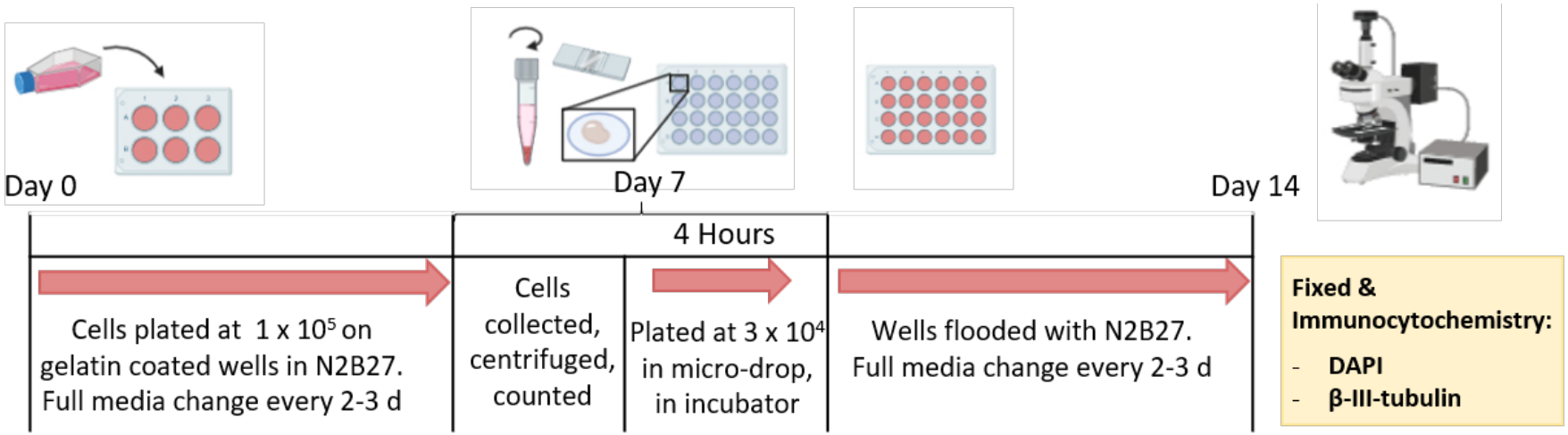


Figure 3.1 Diagram showing the 14 day protocol used for neuronal differentiation and analysis of 46C cultures. Created using BioRender.com

3.2.3 Analysis of cells over the initial 7 days of differentiation

The effects of nicotinamide on the neural differentiation of the 46C cell line was explored. Cells were plated into the differentiation protocol as described in Chapter 3.2.2. Nicotinamide concentrations of 5 and 10 mM were applied to cells in N2B27 media with full media changes every 2-3 days. Cells were fixed each day between day 1-7 and analysed by immunocytochemistry for GFP and β-III-tubulin expression (Chapter 2.8). Micrographs were obtained at 10X magnification using an inverted microscope to allow imaging of the cells in the 6-well plate. Efforts were made to plate the cells on gelatin coated glass coverslips at the same plating density, however, cells did not attach. It was deemed most appropriate to grow and image the cells in the 6 well plates. GFP expression in fixed samples across days 1-7 was analysed using OD (Chapter 2.10), and β-III-tubulin^{+} cells were manually quantified at day 7.

Cells were also analysed by flow cytometry everyday between day 1-7 of the differentiation protocol. Flow cytometry samples were prepared as described in Chapter 2.7.

3.2.4 Gating of cell populations within flow cytometry samples

Flow cytometry is a method in which particles within liquid samples can be processed individually. With cells, this is obtained by forcing the liquid into a thin stream over a detection point in which only single cells can pass. At the detection point, variables including fluorescence, forward scatter (forward deflection of light; indicative of size) and side scatter (indicative of granularity) of each “event” are measured. Due to the nature of cell culture and the flow cytometry sample preparation, cell samples likely contain debris from dead cells which is also detected by the flow cytometer. Therefore, within flow cytometry samples, events need to be gated so that analysis can be focused on purely the intact cell population.

PI and Triton X-100 were used to identify the cell population within these flow cytometry samples, and to distinguish cells from debris. PI binds to DNA, an interaction which enhances the fluorescence of PI (~20-30 fold). However, PI cannot permeate into live cells, meaning that PI fluorescence is an indicator of membrane permeability and, therefore, cell death. This means it is commonly used to detect irreversibly compromised cells within a population. In this experiment PI was used at a concentration of 10 µg/mL to detect dead cells within flow cytometry samples.

Triton X-100 is a detergent commonly used to permeabilise cells (Borner *et al.*, 1994; Ahn *et al.*, 1997). In this experiment 0.01% Triton X-100 was used to permeabilise cells, inducing cell death, within flow cytometry samples. By using PI and Triton X-100 it is possible to identify the cell population within samples.

In this experiment, 46C cells were harvested after 3 days in either proliferation conditions (2i media) or differentiation conditions (N2B27 media). Cells were processed as described in Chapter 2.7. Proliferating and differentiating flow cytometry samples were then split into conditions containing PI alone or PI with 0.01% Triton X-100 (PI + Triton) and were left on ice for 15 mins before flow cytometry analysis.

3.2.5 Gating of GFP$^+$ cells within cell populations

To assess the GFP$^+$ population, gating needed to be set to discern GFP$^-$ and GFP$^+$ populations. Possible gates were placed at $10^{1.2}$, 10^2 and $10^{2.2}$ on the green fluorescence log scale. These gates were chosen based on a search of the literature showing gates placed across these points for differentiating 46C cultures (Aubert *et al.*, 2003; Ying *et al.*, 2003; Diogo, Henrique and Cabral, 2008; Incitti *et al.*, 2014). All three gates showed similar trends in GFP expression in these 46C control cultures over 7 days of differentiation (Figure 3.2). The percentage of GFP$^+$ cells given at each timepoint within these gates was used to determine

the most appropriate gating strategy. Gating GFP^+ populations as those above 10^2 in the green fluorescence log scale was deemed the most accurate gating strategy. This was based on a low GFP^+ population at day 1 (4.32 ± 0.96 SEM) and a plateauing in GFP^+ cells at days 5, 6, and 7 reaching a maximum of 77.49% (± 3.02 SEM) at day 7, similar to results seen by Ying et al. (2003).

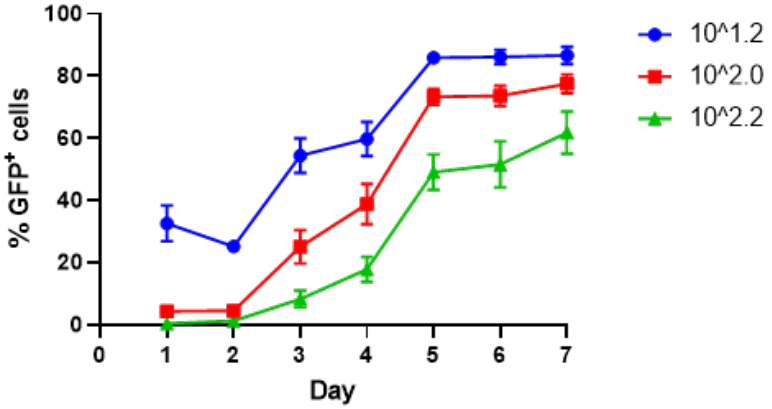


Figure 3.2 Graph showing the percentage of GFP^+ cells in flow cytometry samples (cell population gated as described in 3.2.4). Initially, three gates were set at $10^{1.2}$, 10^2 and $10^{2.2}$ in green fluorescence to discern GFP^- and GFP^+ populations.

3.3 Results

3.3.1 46C cultures tested negative for mycoplasma

As detailed in Chapter 3.2.1, supernatant from 46C cell culture was tested for the presence of mycoplasma prior to culturing of this cell line for this PhD thesis. Results were calculated by dividing the substrate luminescence reading (15383) by the reagent luminescence reading (22799) providing a ratio of 0.67 (Table 3.1). As this ratio is < 1, the sample was classed as negative for mycoplasma.

Another simple test for mycoplasma contamination is the presence of extra-nuclear fluorescence resulting from mycoplasma DNA. Extra-nuclear DAPI fluorescence was not observed in differentiated 46C cultures during immunocytochemical analysis of fixed cultures (Figure 3.3). All cultures were routinely assessed using this method, with no signs of extra-nuclear DAPI observed.

Table 3.1 Table showing mycoplasma kit test results. Luminescence readings taken after addition of reagent and substrate to cell supernatant. The ratio of substrate to reagent is < 1 indicating a negative mycoplasma result.

Reagent luminescence	Substrate luminescence	$\frac{\text{Substrate}}{\text{Reagent}}$
22799	15383	0.67

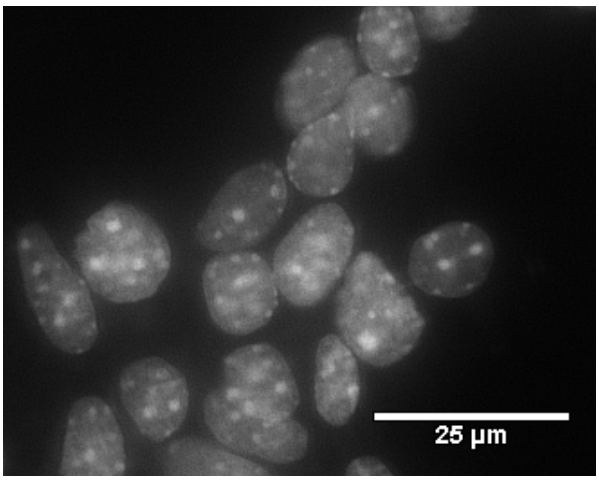


Figure 3.3 Image showing DAPI staining of differentiated 46C cells. No extra-nuclear staining is visible indicating absence of mycoplasma.

3.3.2 Validating the production of β-III-tubulin$^+$ cells in the neuronal differentiation protocol for 46C cells

To determine whether nicotinamide has effects on the differentiation of stem cells to a neuronal fate, a testbed differentiation protocol was established (Chapter 3.2.2). Micrographs taken from 4 experimental repeats, each with 2-3 technical replicates, were counted to ascertain the percentage of β-III-tubulin$^+$ neurons within differentiated cultures. Differentiation of the 46C cell line, using this 14 day protocol, resulted in β-III-tubulin$^+$ cell populations comprising an average of 16.33% ± 4.15 of the total cell population (Figure 3.4, A-C). β-III-tubulin$^+$ cells displayed typical neuronal morphology with small somas and extending neurites (Figure 3.4, D). This indicates that the differentiation protocol is able to produce β-III-tubulin$^+$ neurons from the 46C cell line.

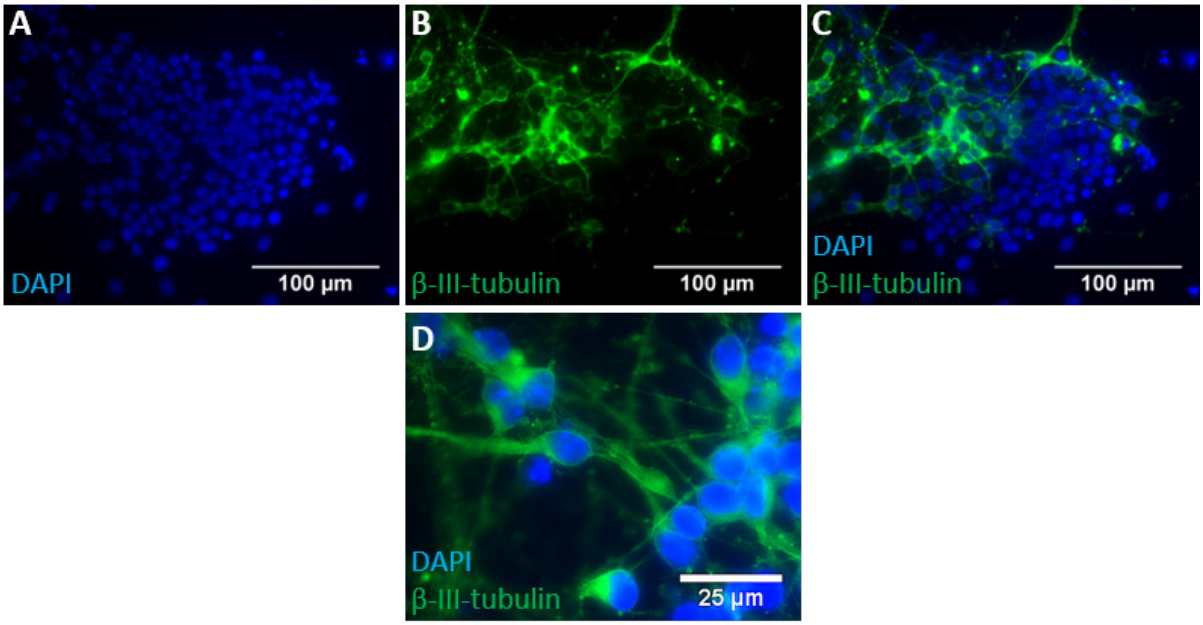


Figure 3.4 Representative images of 46C cultures after the 14 day differentiation protocol. A) DAPI. B) β-III-tubulin. C) Merge of DAPI and β-III-tubulin. D) Magnified image showing typical neuronal morphology of small soma and extending neurites.

3.3.3 Nicotinamide reduced 46C *Sox1*-GFP expression over the initial 7 days of differentiation

Having validated a model of neuronal differentiation, cultures were treated with nicotinamide across the first 7 days, based on previous work showing the effects of nicotinamide occurring within the early days of differentiation (Griffin *et al.*, 2013). Expression of GFP was quantified indicating *Sox1* expression which is indicative of neural precursors. GFP expression was not visible in control cultures at day 1 of differentiation (Figure 3.5 A), however expression was widespread at day 7 (Figure 3.5 B).

Due to the high density of cells observed over increasing days, it was not possible to identify individual cells to perform cell counts to quantify GFP expression. Therefore, optical density (OD) was used to assess GFP expression.

OD measurements provided a simple assessment for immunocytochemistry. The mean grey value of 10X magnification micrographs showing GFP^+ cells was measured. The changes in OD levels with time in culture could be the result of changes in cell numbers, morphology and/or GFP expression levels. Four images were taken from each of 3 technical replicates across 3 experimental repeats. As there was variation between experimental repeats, mean OD values of each technical replicate were normalised to the average day 1 control value of each experimental repeat.

Two-way ANOVA of GFP OD data, normalised to control day 1, showed that GFP expression was affected by time in culture ($F_{(6, 168)} = 16.49$, $p < 0.0001$), nicotinamide treatment ($F_{(2, 168)} = 12.06$, $p < 0.0001$), and showed an interaction effect of these parameters ($F_{(12, 168)} = 9.481$, $p < 0.0001$). Post-hoc analysis using Dunnett's multiple comparison test revealed a significant increase in GFP OD in control cultures at days 5 (103 ± 0.46, $p < 0.01$), 6 (106 ± 0.96, $p < 0.001$) and 7 (110 ± 1.45, $p < 0.001$) of differentiation compared to day 1 (100 ± 0.06; Figure

3.6 A, E). Cultures treated with 5 mM nicotinamide also showed a significant increase in GFP OD at day 5 of differentiation compared to day 1 (104 ± 0.76 vs. 101 ± 0.22, $p < 0.001$; Figure 3.6 B, E). Treatment of cultures with 10 mM nicotinamide did not show any significant difference in GFP OD over 7 days (Figure 3.6 C, E). These results show a significant increase in GFP expression that increases over days 5-7 of differentiation in control cultures. Cultures treated with 5 mM nicotinamide showed a significant increase in GFP OD only at day 5 compared to day 1, whilst cultures treated with 10 mM nicotinamide did not show any significant differences in GFP OD throughout differentiation.

Further post-hoc analysis showed significant differences between control and nicotinamide treated cultures. The addition of 5 mM nicotinamide to differentiating 46C cultures resulted in significantly lower GFP OD measurements at days 6 and 7 compared to control cultures (103 ± 0.27 vs. 106 ± 0.96, 102 ± 0.29 vs. 110 ± 1.5; Tukey's $p < 0.01$; Figure 3.6 D). The addition of 10 mM nicotinamide to differentiating cultures also resulted in lower GFP OD compared to control cultures at days 6 and 7 (102 ± 0.19 vs. 106 ± 0.96, 102 ± 0.5 vs. 110 ± 1.5;Tukey's $p < 0.001$; Figure 3.6 D). These results show that cultures treated with nicotinamide, at both 5 and 10 mM, resulted in lower GFP expression, measured by OD, than control cultures at days 6 and 7 of differentiation.

A significant interaction of time and nicotinamide treatment was also observed. Further post-hoc analysis showed that differentiating cultures treated with 5 mM nicotinamide showed a significant increase in GFP OD at days 5 (Dunnett's $p < 0.001$) and 6 (Dunnett's $p < 0.05$) compared to day 1 control cultures (Figure 3.6 D). A significant increase in GFP OD was also observed in 10 mM nicotinamide treated cultures at day 5 of differentiation compared to day 1 control cultures (Dunnett's $p < 0.05$; Figure 3.6 D). This shows an interaction effect with *Sox1*-GFP expression significantly increased between days 5-

7 in control conditions, at days 5 and 6 with 5 mM nicotinamide treatment, and at day 5 with 10 mM nicotinamide treatment, when compared to day 1 control cultures.

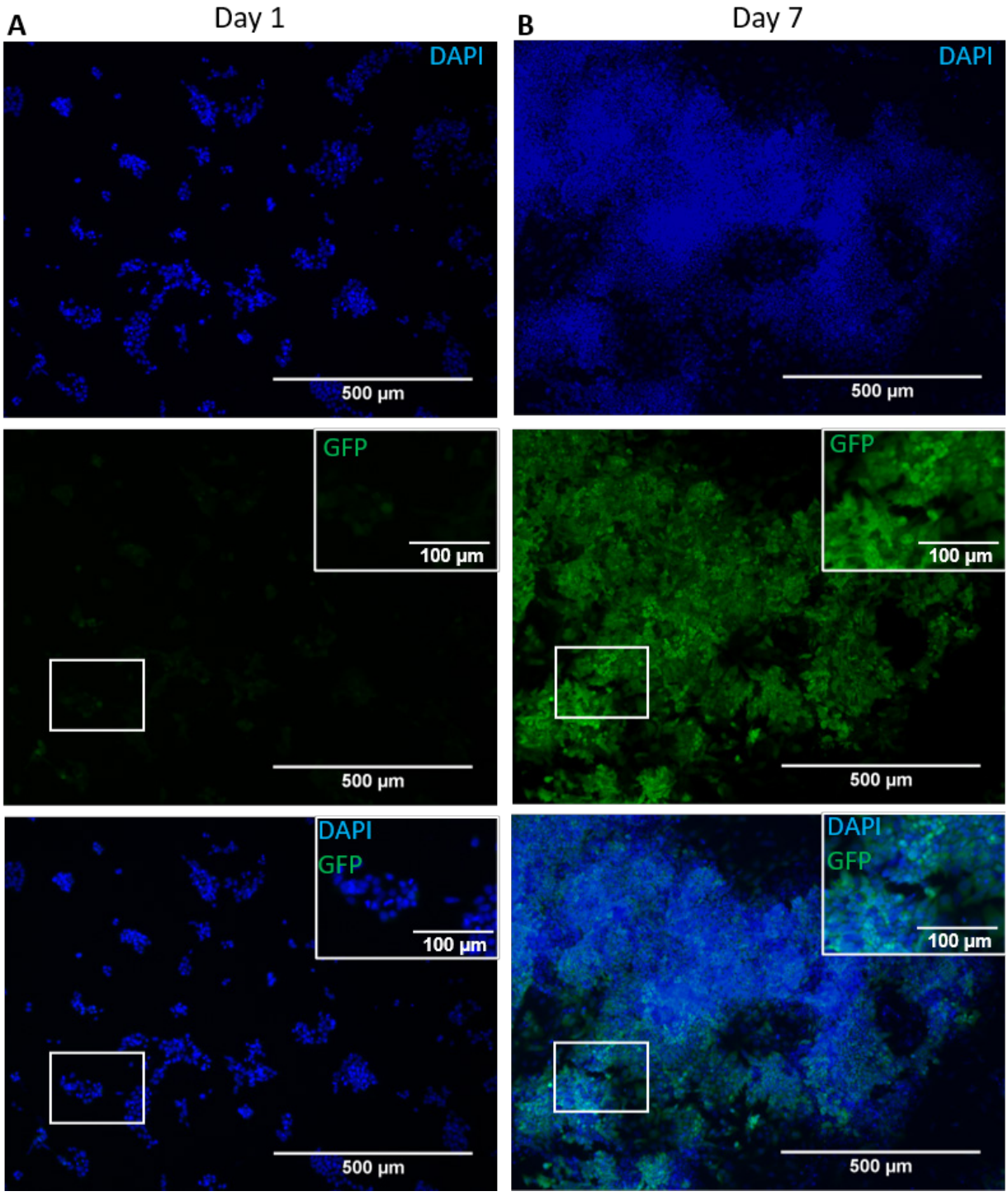


Figure 3.5 Representative images of GFP expression in 46C control cultures.
A) Day 1 of differentiation showing fewer DAPI$^+$ nuclei and few GFP$^+$ cells. Inset shows faint GFP expression associated with nuclei.
B) Day 7 of differentiation showing higher numbers of DAPI$^+$ nuclei and GFP$^+$ cells showing clear GFP expression. Inset shows cytoplasmic GFP expression surrounding nuclei.

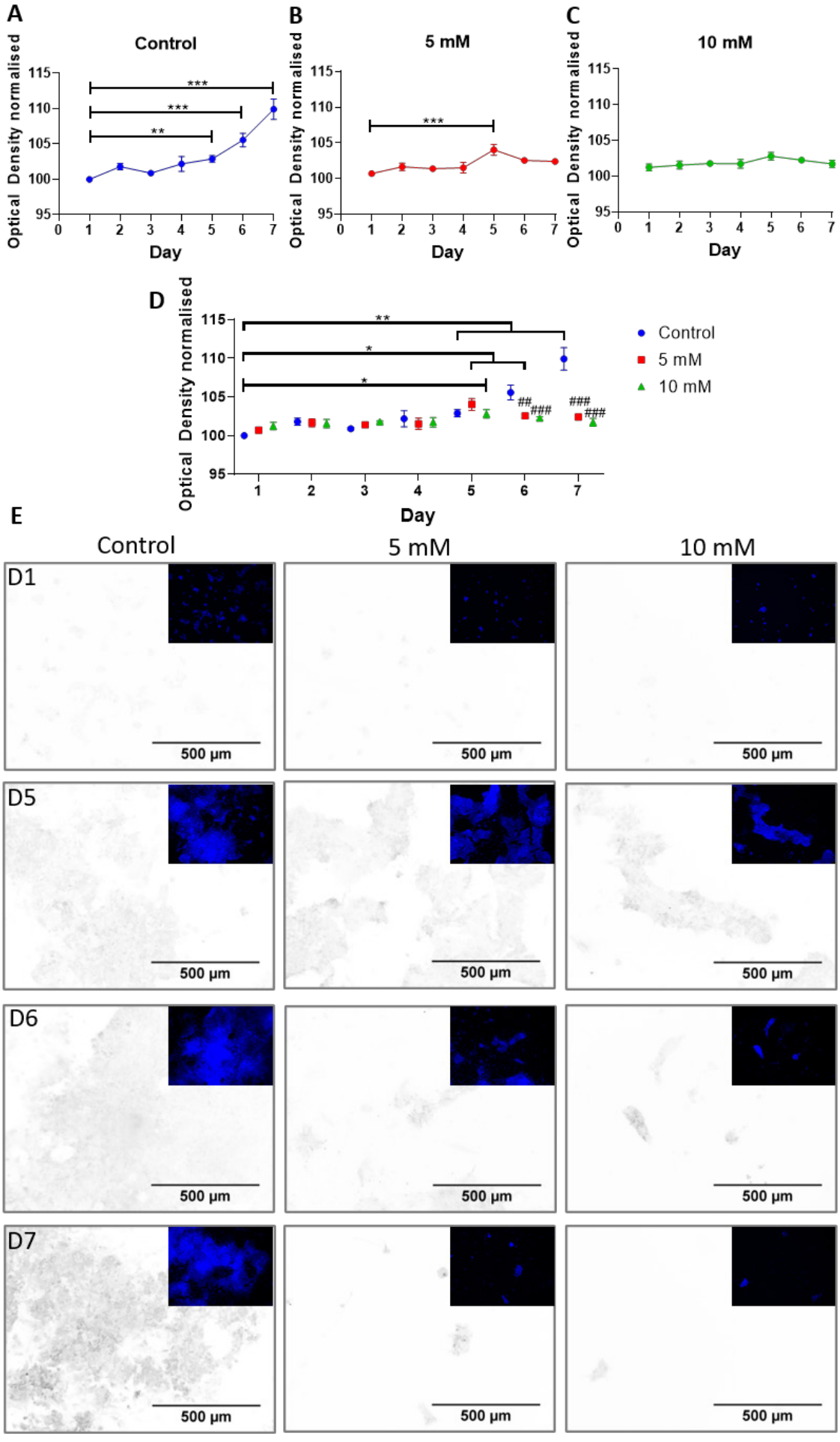

A
Control
B
5 mM
C
10 mM
Optical Density normalised
Day
D
Control
5 mM
10 mM
E
D1
D5
D6
D7
500 µm

Figure 3.6 Nicotinamide affected GFP expression in differentiation 46C cultures.
A) Graphs showing the optical density measurements normalised to control day 1 cultures. A significant increase in GFP expression was observed at days 5, 6, and 7, compared to day 1 of differentiation.
B) Graph showing the optical density measurements normalised to control day 1 in 5 mM nicotinamide treated cultures. A significant increase in GFP expression was observed at day 5 compared to day 1 of differentiation.
C) Graph showing the optical density measurements normalised to control day 1 in 10 mM nicotinamide treated cultures. No significant increase in GFP expression was observed across differentiation. ** $p < 0.01$; *** $p < 0.001$.
D) Graph showing the optical density measurements normalised to control day 1 in all treatment conditions. A significant ANOVA interaction effect was observed in control (blue), 5 mM nicotinamide (red), and 10 mM nicotinamide (green) treated cultures compared to day 1 control cultures; * $p < 0.05$; ** $p < 0.01$. A significant difference in GFP expression in nicotinamide treated cultures compared to control cultures was also observed at days 6 and 7; ## $p < 0.01$; ### $p < 0.001$.
E) Representative inverted, greyscale images (with blue DAPI inserts) used for optical density measurements. Images show how optical density measurements from each treatment condition at days 1, 5, 6 and 7 reflect cell numbers in these cultures.

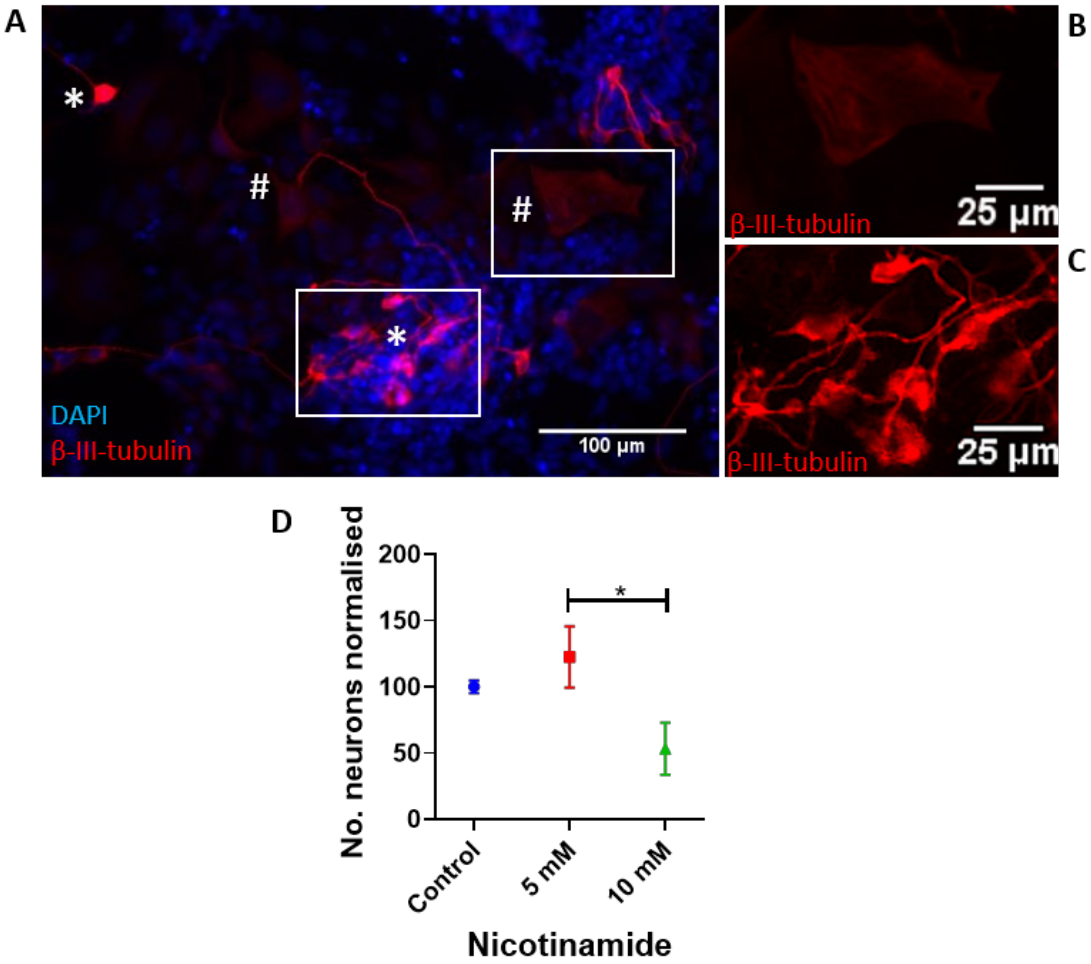
These results show that the addition of nicotinamide at concentrations of 5 and 10 mM significantly altered GFP OD measurements compared to control over 7 days of differentiation. Control cultures showed an increase in GFP OD over 7 days, whilst 5 mM nicotinamide showed a peak at day 5 which decreased and plateaued at days 6-7, and 10 mM nicotinamide showed a slight peak at day 5 although levels remained low compared to control. The use of OD measurements for GFP fluorescence here provides an estimate of GFP expression but cannot consider the percentage of GFP^+ cells as $DAPI^+$ cell counts were not possible due to high density populations.

3.3.4 Numbers of β-III-tubulin$^+$ cells at day 7 of differentiation are affected by nicotinamide concentrations

Next, β-III-tubulin expression was assessed as an indicator of neuronal differentiation. Numbers of β-III-tubulin$^+$ cells were counted from micrographs taken at 10X magnification to assess neuron numbers at 7 days of differentiation. However, not all β-III-tubulin$^+$ cells showed a neuronal morphology of small cell soma and presence of neurites with higher expression

intensity, a minority (observed in some micrographs) displayed a large, flat shape with lower expression intensity (Figure 3.7 A, B). In this experiment, the number of β-III-tubulin$^+$ neurons was of interest, therefore, only β-III-tubulin$^+$ cells with a neuronal morphology (as displayed by * in Figure 3.7 A, C) were counted and analysed.

Numbers of β-III-tubulin$^+$ cells with neuronal morphology were counted within 4 images across 3 technical replicates and 3 experimental repeats. Due to variation in numbers of β-III-tubulin$^+$ cells across experimental repeats (control averages ranging from 14-43), the number of β-III-tubulin$^+$ neurons was normalised to the control as described in Chapter 2.11). One-way ANOVA showed an effect of nicotinamide treatment ($F_{(2, 24)} = 4.49$, $p < 0.05$). Tukey's multiple comparisons test showed no significant difference in the number of β-III-tubulin$^+$ cells (with neuronal morphology) at 7 days of differentiation between control and nicotinamide treated cultures (Figure 3.7 D, E). However, neuron numbers were slightly higher in 5 mM nicotinamide treated cultures compared to controls, and were lower in 10 mM nicotinamide treated cultures compared with controls, at 7 days in culture. Therefore, when nicotinamide treated cultures were compared directly, there were significantly lower numbers of neurons in 10 mM nicotinamide treated cultures compared to 5 mM nicotinamide treated cultures at 7 days in culture (Tukey's $p < 0.05$; Figure 3.7 D, E).


A
B
C
*
#
#
*
DAPI
β-III-tubulin
100 µm
β-III-tubulin
25 µm
β-III-tubulin
25 µm

D
No. neurons normalised
200
150
100
50
0
*
Control
5 mM
10 mM
Nicotinamide

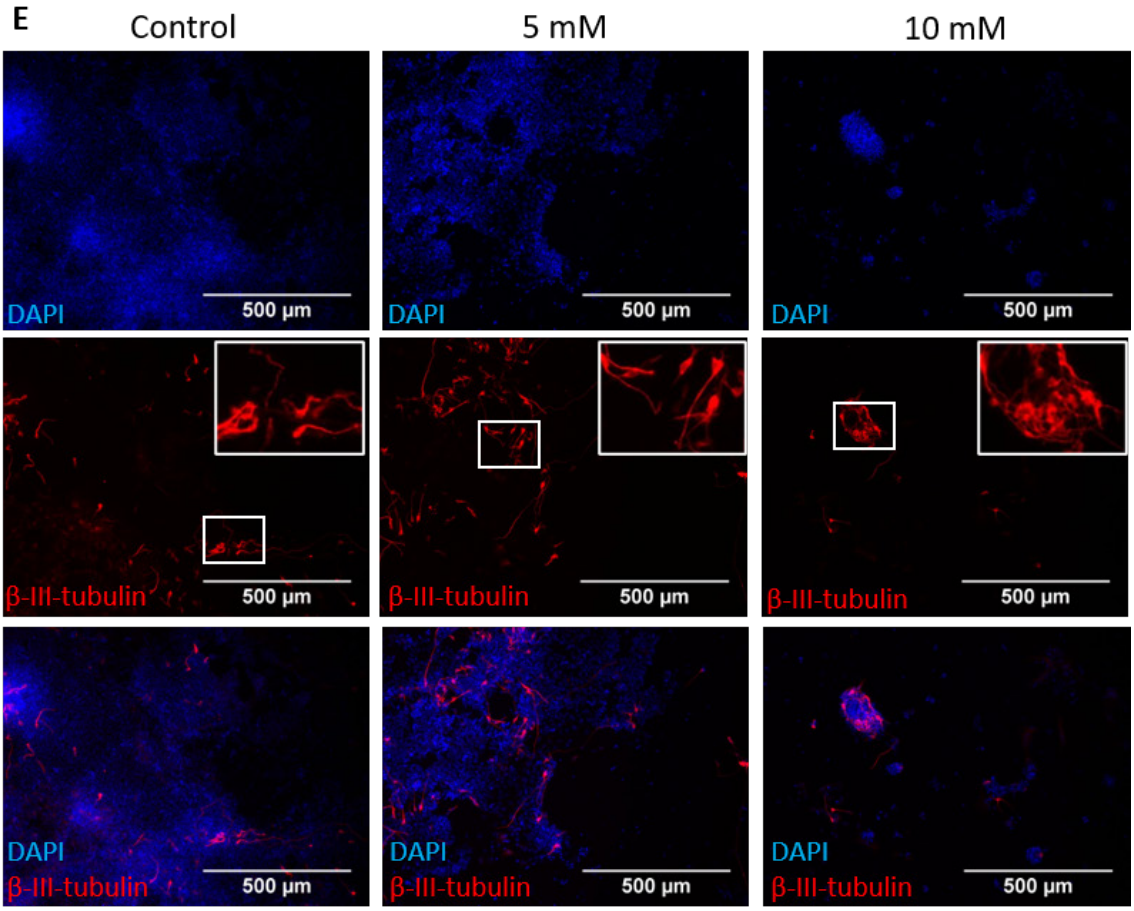

E
Control
5 mM
10 mM
DAPI
500 µm
β-III-tubulin
500 µm

Figure 3.7 The number of β-III-tubulin$^+$ cells did not differ between control and nicotinamide treated cultures.
A) Micrograph showing merge of DAPI and β-III-tubulin staining in 46C cultures at day 7 of differentiation. * Indicates examples of neuronal β-III-tubulin staining with a small cell soma, extending neurites, and high intensity expression. # Indicates non-neuronal β-III-tubulin staining with large, flattened cell soma and low intensity expression.
B) Magnified example of non-neuronal β-III-tubulin staining with large, flattened cell soma and low intensity expression.
C) Magnified example of neuronal β-III-tubulin staining with a small cell soma, extending neurites, and high intensity expression.
D) Graph showing numbers of β-III-tubulin$^+$ neurons at day 7 of differentiation normalised to control cultures. A significant decrease in neuron numbers was observed between 5 mM and 10 mM nicotinamide treatment conditions ($p < 0.05$).
E) Representative micrographs showing DAPI and β-III-tubulin staining across treatment conditions at day 7 of differentiation. Inserts show neuronal morphology.

3.3.5 Gating of cell populations within 46C flow cytometry samples and representation of data

Flow cytometry was used to provide a more detailed analysis of the effects of nicotinamide on differentiating 46C cultures, in particular, addressing numbers/proportion of cells expressing GFP, which was not possible to derive accurately using micrographs of fixed cultures. For accurate analysis, gating parameters needed to be established to distinguish cells from debris within the flow cytometry sample. Propidium iodide (PI) and Triton X-100 were used to identify the cell populations within samples of proliferating and differentiating 46C cell cultures as described in Chapter 3.2.4. Cells grown in both proliferation and differentiation media showed similar trends. As differentiating samples were to be analysed for GFP expression, only results from differentiating cells are described below.

Addition of PI to flow cytometry samples resulted in the formation of two peaks in the red fluorescence channel which were classed as PI$^-$ and PI$^+$, respectively, as separated by a gate placed at 10^1, in between the peaks (Figure 3.8 A, B). In cell samples from differentiation medium, 26% of total events were PI$^+$ (Figure 3.8 A). As a positive control, 0.01% Triton X-100 was added to a parallel differentiating cell sample. This increased PI$^+$ events to 61% of total

events (Figure 3.8 B), consistent with the anticipated effects of Triton X-100: increased plasma membrane permeability of cells within the sample, allowing PI to bind to DNA and subsequently fluoresce. By applying this PI^+/PI^- gate of red fluorescence against forward scatter (which provides an estimate of the size of the event), it was possible to identify the shift in red fluorescence predominantly occurring in events above $10^{2.3}$ in forward scatter in both PI only and PI with Triton X-100 samples (Figure 3.8 C, D). That is, the bottom right quadrant (PI^+, Forward Scatter $<10^{2.3}$) showed an extremely low number of events, consistent with PI staining being associated with cells whose membranes are compromised, rather than fragments/debris. From this it was decided that events above $10^{2.3}$ in forward scatter would be considered as the cell population of the sample (i.e. excluding debris). This data was then plotted as forward scatter vs. side scatter (a general measure of granularity) to show the cell population without the use of fluorescence markers and channels (Figure 3.8 E, F).

When events were gated to limit analysis to the cell population, the percentage of PI^+ cells in differentiating samples without triton was 35.4% (Figure 3.8 G), which increased to 90.7% in samples treated with Triton X-100 (Figure 3.8 H). Fluorescence microscopy confirmed PI staining within cells (Figure 3.8 I, J) showing a larger proportion of PI^+ cells within samples with Triton X-100 compared to samples with PI alone. These images also confirm a greater presence of debris in samples with Triton X-100. This is also observed in graphs E and F with a lower percentage of events classed as whole cells in samples treated with Triton X-100 (66%) compared to samples with no Triton X-100 added (72%; Figure 3.8 E, F).

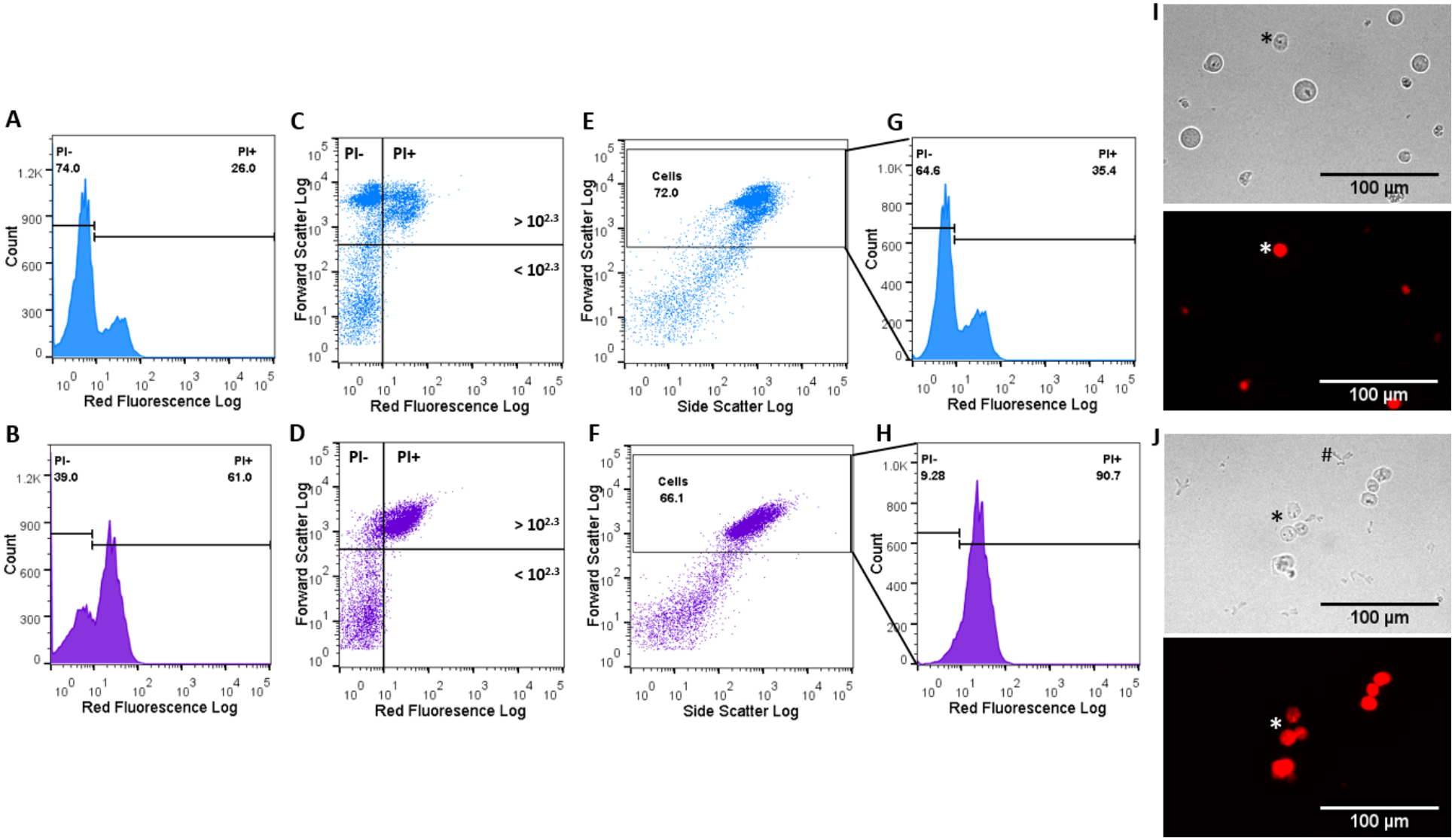

A
PI-
74.0
PI+
26.0
Count
Red Fluorescence Log
B
PI-
39.0
PI+
61.0
Count
Red Fluorescence Log
C
PI-
PI+
> 10^2.3
< 10^2.3
Forward Scatter Log
Red Fluorescence Log
D
PI-
PI+
> 10^2.3
< 10^2.3
Forward Scatter Log
Red Fluoresence Log
E
Cells
72.0
Forward Scatter Log
Side Scatter Log
F
Cells
66.1
Forward Scatter Log
Side Scatter Log
G
PI-
64.6
PI+
35.4
Count
Red Fluoresence Log
H
PI-
9.28
PI+
90.7
Count
Red Fluoresence Log
I
100 µm
100 µm
J
100 µm
100 µm

Figure 3.8 Flow cytometry gating for cell population.
A) Histogram of red fluorescence showing flow cytometry samples of differentiating cells with PI only. A gate separating PI+ and PI- events in red fluorescence was set at 10^1.
B) Histogram of red fluorescence showing flow cytometry samples of differentiating cells with PI and 0.01% Triton X-100. A gate separating PI+ and PI- events in red fluorescence was set at 10^1.
C) Scatter graph showing forward scatter (y; used as a relative measure of size) and red fluorescence (x) for samples with PI only. A shift in red fluorescence is observed mostly in events above $10^{2.3}$. This indicates the cell population.
D) Scatter graph showing forward scatter (y; used as a relative measure of size) and red fluorescence (x) for samples with PI and 0.01% Triton X-100. A shift in red fluorescence is observed mostly in events above $10^{2.3}$. This indicates the cell population.
E) Scatter graph showing forward scatter (y) against side scatter (x; representative of granularity) for differentiating cells with PI only.
F) Scatter graph showing forward scatter (y) against side scatter (x; representative of granularity) for differentiating cells with PI and 0.01% Triton X-100.
G) Histogram of red fluorescence of the "cell" gated population shown in E with gate indicating PI- (below 10^1) and PI+ (above 10^1) staining for PI only differentiating samples.
H) Histogram of red fluorescence of the "cell" gated population shown in F with gate indicating PI- (below 10^1) and PI+ (above 10^1) staining for PI with Triton X-100 differentiating samples.
I) Brightfield and fluorescence microscopy showing PI staining (red) within cells (*) in differentiating PI only conditions.
J) Brightfield and fluorescence microscopy showing PI staining (red) within cells (*) in differentiating PI with Triton X-100. # shows an increase in debris in samples with Triton X-100.

This established the methodology used for the gating of cell populations within flow cytometry samples in the following experiments exploring the effects of nicotinamide on proliferation and GFP expression in 46C cultures.

To assess cell numbers, results, measured in forward scatter, are presented as cells (based on the gate set at log $10^{2.3}$ to include cells and exclude debris) as a percentage of total events (which include both cells and debris). Representing results this way was deemed most appropriate, as absolute numbers of cells could be affected by changes in the processing of the flow cytometry samples (e.g. the amount of sample processed). To check the validity of this measure, absolute cell numbers were checked within each experimental run and data showed the same trends as the percentage of total events measure used when analysing the combined experimental data (data not shown).

3.3.6 The number of cells in cultures over 7 days of differentiation were lower with nicotinamide treatment

Flow cytometry samples were analysed from differentiating 46C cell cultures across days 1-7, treated with either 5 or 10 mM nicotinamide, with a full media change every other day. The results were gated as described in Chapter 3.3.5 allowing for analysis of the cell population within the sample.

Two-way ANOVA revealed significant differences in the percentage of cells out of total events across nicotinamide treatment ($F_{(2, 125)} = 60.68$, $p < 0.0001$) and day of differentiation ($F_{(6, 125)} = 9.36$, $p < 0.0001$) with a significant interaction ($F_{(12, 125)} = 5.302$, $p < 0.0001$).

Nicotinamide treatment significantly reduced the cell population compared to control cultures. Control cultures reached a peak of cells at 95 ± 1.8 percent at day 7 of differentiation, whilst cultures treated with 5 mM nicotinamide peaked at 40 ± 8.5 percent at day 3, and cultures treated with 10 mM nicotinamide peaked at 25 ± 10 at day 3. These results show a significant decrease in cells with nicotinamide treatment compared to control; with the percentage of cells peaking at day 3 in nicotinamide treated cultures compared to day 7 in control cultures.

An increase in the percentage of cells across days in differentiation was observed in control cultures. Post-hoc analysis showed a significant increase in the percentage of cells in control flow cytometry samples between day 1 and days 3, 4, 5, 6, and 7 (Dunnett's $p < 0.001$; Figure 3.9 A). No significant difference in the percentage of cells across days in differentiation was observed for cultures treated with 5 mM or 10 mM nicotinamide (Figure 3.9 B, C respectively). These results indicate that numbers of cells increased across 7 days of differentiation in control 46C cultures, but not in cultures treated with 5 mM or 10 mM nicotinamide.

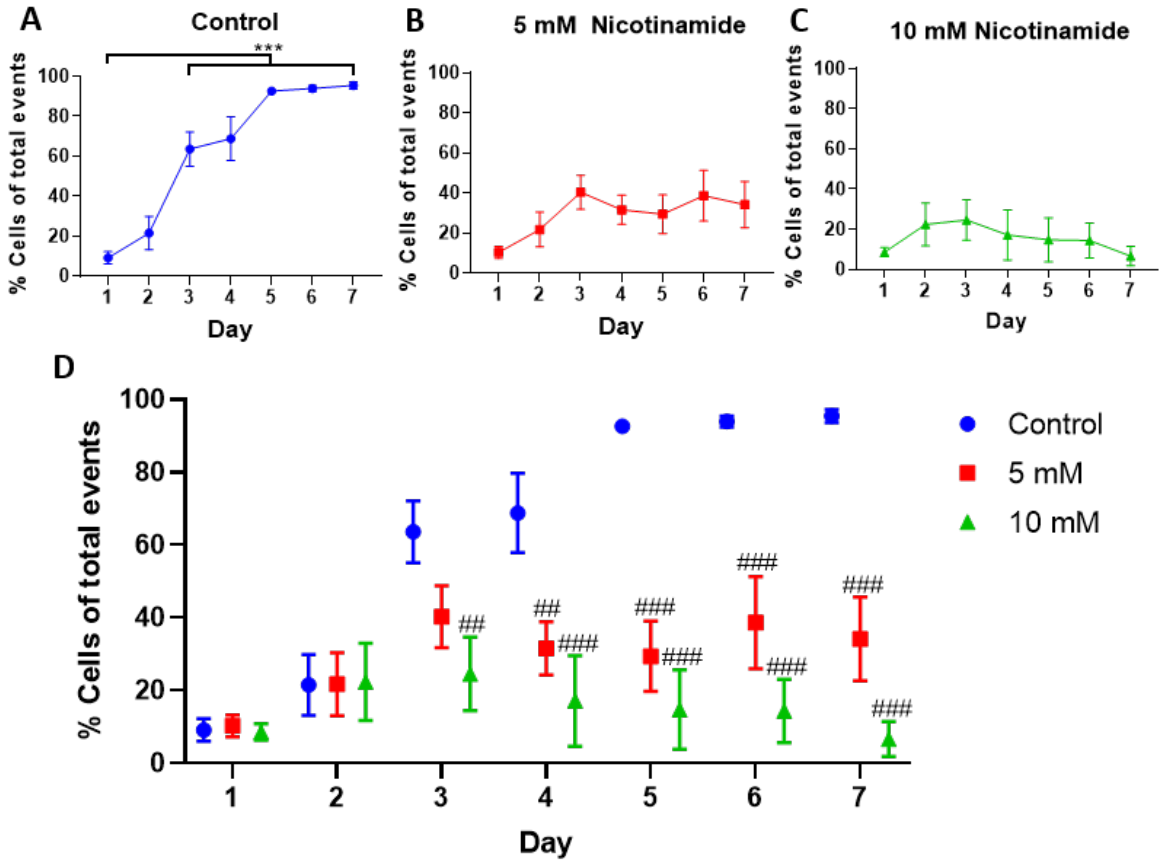


Figure 3.9 Numbers of cells were lower in nicotinamide treated cultures compared to control.
A) Graph showing the percentage cells of total events across days 1-7 of differentiation in control cultures. A significant increase was observed at days 3, 4, 5, 6, and 7 compared to day 1 (*** $p < 0.001$). B) Graph showing the percentage cells of total events across days 1-7 of differentiation in 5 mM nicotinamide treated cultures. No significant difference was observed across 7 days of differentiation. C) Graph showing the percentage cells of total events across days 1-7 of differentiation in 10 mM nicotinamide treated cultures. No significant difference was observed across 7 days of differentiation. D) Graph showing the percentage cells of total events across days 1-7 of differentiation in control (blue circle), 5 mM nicotinamide (red square), and 10 mM nicotinamide (green triangle) treatment conditions. Treatment with 5 mM nicotinamide resulted in a significantly lower percentage cells of total events compared to control at days 4, 5, 6, and 7. Treatment with 10 mM nicotinamide also resulted in a significantly fewer cells as a percentage of total events at days 3, 4, 5, 6 and 7 compared to control (## $p < 0.01$; ### $p < 0.001$).

The application of 5 mM nicotinamide resulted in a significantly lower percentage of cells in samples compared to control between days 4 -7 of differentiation (Tukey's $p < 0.01$; Figure 3.9 D). Similar results were observed with the application of 10 mM nicotinamide with a significant decrease in the percentage of cells compared to control at days 3 to 7 of differentiation (Tukey's $p < 0.01$; Figure 3.9 D). These results show that 5 mM nicotinamide

significantly reduced the number of cells in 46C cultures compared to control from day 4 of differentiation, whilst 10 mM nicotinamide significantly reduced cell numbers from day 3.

3.3.7 Nicotinamide treatment reduced the GFP$^+$ cell population detected by flow cytometry over 7 days of differentiation

Flow cytometry was used to detect GFP$^+$ cells within differentiating 46C cultures over 7 days. Two-way ANOVA showed significant differences across nicotinamide treatment ($F_{(2, 125)} = 181.2$, $p < 0.0001$), days ($F_{(6, 125)} = 36.52$, $p < 0.0001$), and a significant interaction ($F_{(12, 125)} = 21.66$, $p < 0.0001$).

Nicotinamide treatment, at concentrations of both 5 and 10 mM, showed a significantly lower percentage of GFP$^+$ cells compared to control cultures. Control cultures reached a GFP$^+$ peak of 77.5 ± 3.0 percent at day 7, whilst cultures treated with 5 mM nicotinamide reached a peak of 24.3 ± 4.8 percent at day 5, and cultures treated with 10 mM reached a peak of 10.4 ± 1.9 percent at day 3 (Figure 3.10 D). These results show that control cultures contained the highest percentage of GFP$^+$ cells peaking at day 7, whilst 5 and 10 mM nicotinamide treated cultures showed lower percentages of GFP$^+$ cells with peaks at days 5 and 3 respectively.

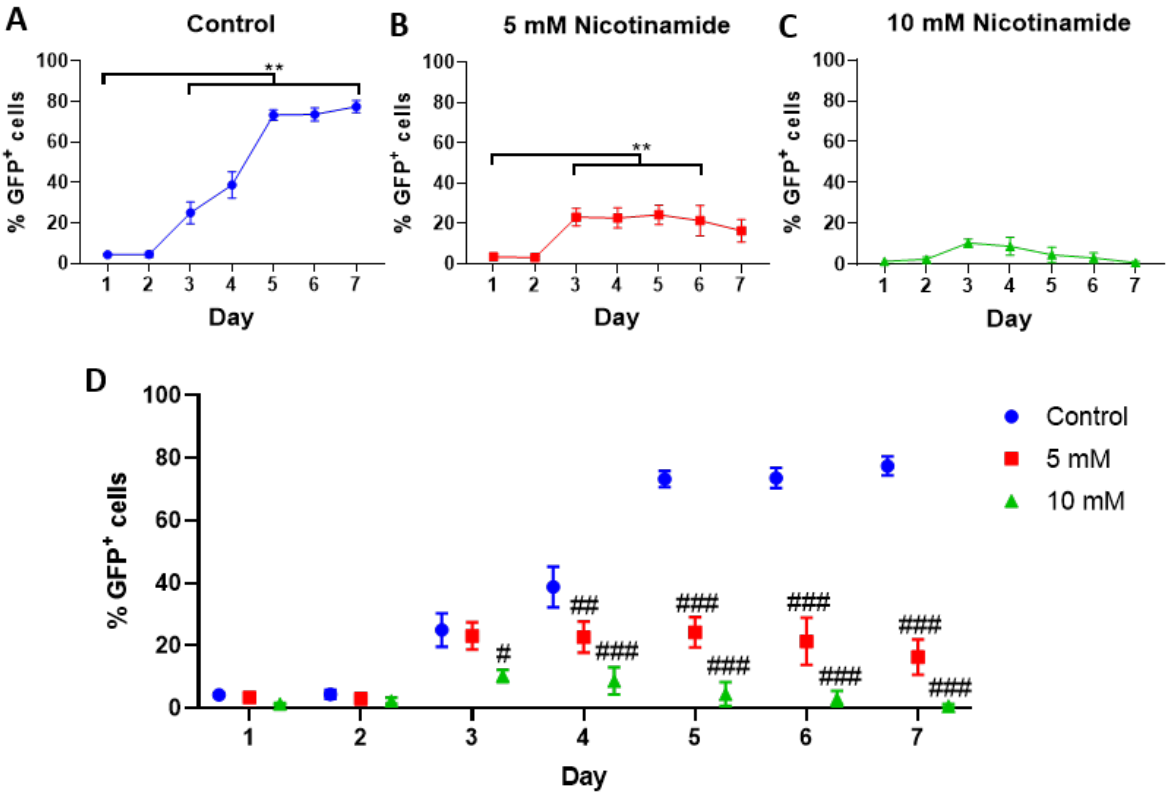


Figure 3.10 The percentage of GFP⁺ cells was reduced in nicotinamide treated cultures
A) Graph showing the percentage of GFP⁺ cells across 7 days of differentiation in control 46C cultures. A significant increase in GFP⁺ cells was observed between day 1 and days 3, 4, 5, 6, and 7.
B) Graph showing the percentage of GFP⁺ cells across 7 days of differentiation in 5 mM nicotinamide treated 46C cultures. A significant increase in GFP⁺ cells was observed between day 1 and days 3, 4, 5, and 6 (** $p < 0.01$).
C) Graph showing the percentage of GFP⁺ cells across 7 days of differentiation in 10 mM nicotinamide treated 46C cultures. No significant differences were observed across days in differentiation.
D) Graph showing the percentage of GFP⁺ cells across 7 days of differentiation in control, 5 mM and 10 mM treated 46C cultures. Treatment with 5 mM nicotinamide significantly reduced numbers of GFP⁺ cells at days 4, 5, 6, and 7 when compared to control cultures. Treatment with 10 mM nicotinamide also significantly reduced GFP⁺ cell numbers compared to control at days 3, 4, 5, 6, and 7 (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$).

An increase in GFP⁺ cells was observed across 7 days of differentiation in control cultures. Post-hoc analysis revealed a significant increase in the percentage of GFP⁺ cells in control cultures comparing day 1 with days 3, 4, 5, 6, and 7 (Dunnett's $p < 0.01$; Figure 3.10 A). These results show that *Sox1-GFP* expression increased over 7 days of differentiation in these 46C control cultures.

An increase in GFP⁺ cells was also seen in differentiating cultures treated with 5 mM nicotinamide. A significant increase in the percentage of GFP⁺ cells in 5 mM treated cultures

was observed between day 1 and days 3, 4, 5, and 6 (Dunnett's $p < 0.01$; Figure 3.10 B). No significant difference in the percentage of GFP^+ cells was found in cultures treated with 10 mM nicotinamide (Figure 3.10 C). These results show that nicotinamide dose-dependently affects *Sox1-GFP* expression in these 46C cultures.

Application of 5 mM nicotinamide to differentiating 46C cells significantly reduced the percentage of GFP^+ cells compared to control at days 4 to 7 of differentiation (Dunnett's $p < 0.01$; Figure 3.10 D). Application of nicotinamide at 10 mM significantly reduced the percentage of GFP^+ cells compared to control at days 3 to 7 of differentiation (Dunnett's $p < 0.05$; Figure 3.10 D). This shows that 5 mM nicotinamide significantly reduced *Sox1-GFP* expression in 46C cultures from day 4 of differentiation, whilst 10 mM nicotinamide significantly reduced *Sox1-GFP* expression from day 3 of differentiation.

3.4 Discussion

3.4.1 Validity of the neuronal differentiation model using 46C cells

The differentiation protocol validated here generated neurons, identified as β-III-tubulin$^+$ cells. As well as expression of this neuronal marker, cells also displayed the typical neuronal morphology of a small cell soma with extending neurites. These cells accounted for 16% of the total cell population. The 46C cell line has also been reported to produce both astrocytes and oligodendrocytes, using the N2B27 medium (Ying *et al.*, 2003). Although the full differentiation protocol took 14 days, in the current study, β-III-tubulin$^+$ cells with neuronal morphology were present in cultures at day 5. Other studies have also assessed the neural commitment of these cells over the initial 6/8 days of differentiation (Ying *et al.*, 2003; Diogo, Henrique and Cabral, 2008) with neuronal morphology and marker expression also apparent at day 5 (Ying *et al.*, 2003).

In mESC cultures assessed at day 7 of differentiation, the antibody for β-III-tubulin used in this PhD thesis also showed some reactivity with cells that did not have a neuronal morphology. These cells were distinctive from neurons, possessing a large nucleus and large flattened cell body with low intensity staining, and accounted for approximately 20% of β-III-tubulin$^+$ stained cells. β-III-tubulin expression has also been observed in non-neuronal cells including: embryonic lens, caudal-ventral somites, and ectodermal cells of the amnion (Lee *et al.*, 1990). Given that cultures in the present study were generated from ESCs, it is possible that non-neuronal cells were present in differentiated cultures. Ying et al. (2003) also observed large, flattened cells in cultures differentiated in N2B27 on laminin. High concentrations of 20 mM nicotinamide have been shown to affect cell morphology (Wang *et al.*, 2012). However, in the current study, non-neuronal β-III-tubulin$^+$ cells were also observed

in control cultures, and numbers did not increase with nicotinamide treatment (data not shown), suggesting that this is not an effect of nicotinamide treatment. Although non-specific staining is not desirable, in this instance, the obvious difference in morphology meant that non-neuronal cells showing weakly positive staining could be readily excluded and therefore did not interfere with analysis or results.

The validation of the neuronal differentiation protocol, discussed in Chapter 3.3.2, confirmed the suitability of the 46C cell line, and the differentiation protocol, to assess the effects of nicotinamide on neural differentiation from ESCs for this PhD thesis.

Human ESCs or iPSCs would provide a more easily clinically translatable tool and the experimental processes described here could be transferred to a human cell line for further assessment and to provide data for more immediate translation to the clinic. However, the use of non-human cell lines and animal models still provides a valuable tool for basic research. The use of yeast, drosophila, zebrafish, rodents and primates still hold a prominent position in research with advantages including detailed characterisation, simplicity and availability. Although not every discovery in non-human cells is translatable, many are, and the understanding of these differences still provides value to research. Mouse and human ESCs share a number of similarities and differences in morphology, gene expression, and electrophysiological properties (Ginis *et al.*, 2004; Wang *et al.*, 2005), although cell-type specific gene expression in midbrain development appears to be conserved (La Manno *et al.*, 2016). Protocols for neuronal differentiation have been translated from mouse to human ESCs and iPSCs (Jaeger *et al.*, 2011; Zhang *et al.*, 2018). Sox1 expression has also been shown to identify neural progenitors in both human and mouse cell lines (Zhang *et al.*, 2018). This supports the possibility that the effects of nicotinamide found in this PhD thesis may be translatable to human stem cells.

3.4.2 Flow Cytometry is a valid measure to assess the differentiation of 46C cells

Immunocytochemistry was initially used to assess the neuronal differentiation of the 46C cell line. However, this method required a long differentiation protocol (14 days) and time-consuming analysis to study the effects of nicotinamide, which has shown to induce neuralisation during the first 7 days of differentiation (Griffin *et al.*, 2013). Therefore, flow cytometry was used to assess the *Sox1-GFP* expression in these cells over the differentiation period. The use of flow cytometry allowed for a more encompassing analysis of cell cultures with the identification of higher numbers of cells in a short timeframe when compared to microscopic analysis.

Initial experiments included the analysis of cells after re-plating at day 7 of differentiation. However, cell numbers and thus GFP expression were dramatically reduced in samples after replating. This was thought to be due to neuronal differentiation within cell cultures, producing neuronal cells that may not have survived the enzymatic detachment and dissociation process used for flow cytometry sample preparation (Barker *et al.*, 1995; Fricker *et al.*, 1996).

Flow cytometry required gating of the cell population to provide an estimate of cell numbers. Gating of the cell population utilised PI and Triton X-100 to distinguish cells from debris within the sample. This experiment utilised PI, in which fluorescence is dramatically enhanced upon DNA binding, as a nuclear marker. However, PI cannot penetrate the intact cell membrane, meaning that it can be used to identify membrane-compromised cells, an indicator of cell death. The use of Triton X-100 to permeabilise cell membranes (Borner *et al.*, 1994; Ahn *et al.*, 1997) alongside addition of PI, allowed for the identification of the cell population which was represented as a shift in fluorescence found only in events above a

specific threshold in forward scatter. Gating events above this threshold allowed for identification of the cell population based solely on forward scatter analysis.

After gating of the cellular population, it was then possible to gate for the GFP^+ population within this. Ideally a GFP^- cell line similar to 46C would be used as a negative control. Other researchers have used the E14tg2a cells (Veraitch *et al.*, 2008; Incitti *et al.*, 2014) from which 46C cells are derived, however, this option was not available. Undifferentiated 46C cells have also been used as a negative GFP control (Diogo, Henrique and Cabral, 2008) which could have provided a viable option for these PhD experiments. Although ideally a GFP^- population would have served to show a fluorescence baseline for each time point and each experimental repeat, the analysis of cells from 1-7 days of differentiation showed a measurable and relative change in GFP expression in control cultures, providing validity in the methods used.

PI could have been used alongside GFP expression to show the proportion of live cells during differentiation. However, to gain accurate cell death data from differentiating flow cytometry samples, more rigorous optimisation would have been required to ensure sample preparation did not affect cell viability. Without this, it would be difficult to ensure that cell death was a result of the treatment condition, rather than sample preparation. Further cell specific markers would also be required to assess whether changes in cell viability were a direct effect of nicotinamide treatment, or an indirect effect due to nicotinamide-induced changes in cell-type populations. Although this may present an interesting aspect to further explore; the assessment of cell viability was not considered as a necessary element in this experimental design which focused solely on GFP expression within cells rather than cell viability.

3.4.3 Cell numbers were lower over 7 days of differentiation in nicotinamide treated cultures

Results from flow cytometry analysis revealed that numbers of cells within control cultures significantly increased from day 3, with cell numbers appearing to plateau from day 5. This increase suggests proliferation within these cultures. Cultures treated with 5 or 10 mM nicotinamide did not show a significant increase compared to day 1 across 7 days of differentiation. Numbers of cells were significantly lower compared to control cultures from day 3 with 10 mM nicotinamide, and from day 4 with 5 mM nicotinamide. This suggests that nicotinamide limited proliferation within these cultures. This is an effect of nicotinamide that has been observed across cell cultures (Audrito *et al.*, 2011; Petin *et al.*, 2019; Malesu *et al.*, 2020) including hESCs which also showed reduced cell growth with application of 10 mM nicotinamide over 4 days (Meng *et al.*, 2018). Lower cell numbers quantified by DAPI staining were also observed in 46C cultures after the 14 day differentiation protocol with nicotinamide applied during the initial 7 days (Griffin *et al.*, 2013). These lower cell numbers and decreased Oct4 expression were still observed on withdrawal of nicotinamide from days 8-14 (Griffin *et al.*, 2017). Nicotinamide has also been shown to downregulate genes associated with stem cell pluripotency (Meng *et al.*, 2018). This suggests that 10 mM nicotinamide reduces the number of proliferating cells within cultures from as early as 3 days into treatment and is not reversible upon removal.

Further assessment of the 46C cultures used in this PhD thesis would be to confirm if nicotinamide increased cell death or decreased proliferation. The numbers of cells staying steady across time in nicotinamide treated cultures suggests a limitation of proliferation, as numbers did not decrease over time. Application of 20 mM nicotinamide has shown cytotoxic effects and wide-spread cell death in 46C cells after 3-4 days (Griffin *et al.*, 2013), however,

the use of 10 mM has not shown toxicity in a number of studies (Vaca *et al.*, 2008; Griffin *et al.*, 2013; Meng *et al.*, 2018). The reduction in cell numbers shown in this PhD thesis may be a result of nicotinamide-induced proliferating cell death through Sirt1 inhibition. Treatment of NG108-15 cells with sirtinol (a Sirt1 inhibitor) killed 79.5% of proliferating cells but only 32.1% of neuronally differentiated cells (Sansone *et al.*, 2013). This suggests that proliferating cells may be vulnerable to nicotinamide-induced Sirt1 inhibition and may explain the reduction in cell numbers shown in this PhD thesis.

The application of PARP inhibitors has been shown to increase apoptosis in a concentration dependent manner (Okuda *et al.*, 2017), therefore this possible effect of nicotinamide should not be excluded without confirmation. However, PARP inhibitors have also decreased cell death with no change in Ki67 expression during the neuralisation process (Cimadamore *et al.*, 2009). These differences highlight the complexity of PARP inhibition and its downstream effects. Although nicotinamide is known to inhibit PARP-1 activity, this may not be the mechanism responsible for the results observed in the 46C cultures used here. Therefore, further analysis of the mechanistic action(s) of nicotinamide is required.

Increased programmed cell death is also observed alongside increased neuralisation, suggesting heightened vulnerability of differentiating cells compared to proliferating ESCs (Cimadamore *et al.*, 2009). This may provide an additional explanation for the lower percentage of cells measured by flow cytometry in nicotinamide treated cultures. Vulnerable cells, that may be more likely to be damaged through sample preparation as a consequence of increased neuralisation or differentiation, would lead to increased debris and thus a decrease in the proportion of cells observed in the flow cytometry data. Therefore, nicotinamide may induce more vulnerable cell populations in these cultures, especially if nicotinamide is able to accelerate differentiation or neuralisation (further discussed in Chapter 3.4.5). If nicotinamide also restricts proliferation, with proliferating cells being less

vulnerable, this may account for the higher in cell numbers in control cultures, but lower cell numbers in nicotinamide treated cultures.

The results presented here suggest that nicotinamide caused a decrease in proliferation of cells within differentiating mESC cultures, however, further analytical techniques to assess proliferation and cell death would assist confirmation of this.

3.4.4 *Sox1-GFP* expression was reduced in nicotinamide treated cultures

GFP expression, measured by OD, increased with days of differentiation in these 46C cultures. OD measures recorded in the current study were generalised marker expression levels across multiple cells, which could be indicative of intensity/expression of GFP per cell, number of cells expressing GFP in the FOI, or size of cells expressing GFP within the FOI.

In control cultures, OD measurements showed an increase in GFP expression, rising steeply between days 5-7 with a significant increase compared to day 1 (Figure 3.6 A). Cultures treated with 5 mM nicotinamide showed a significant increase at day 5 compared to day 1, which peaked, then decreased slightly and plateaued over days 6-7 (Figure 3.6 B). Treatment of cultures with 10 mM nicotinamide showed only a small peak at day 5, with no significant increase in GFP expression, which remained low compared to control cultures (Figure 3.6 C). Lower levels of GFP expression, measured by OD, have also been observed with application of 10 mM nicotinamide, compared to control, in 46C cultures (Griffin *et al.*, 2017).

In this PhD thesis, treatment with 5 mM nicotinamide showed a significant increase at days 5 and 6 when compared to control cultures at day 1 (Figure 3.6 D). The application of 10 mM nicotinamide also showed a significant increase in GFP OD at day 5 compared to control day 1 (Figure 3.6 D). This shows an interaction effect of nicotinamide treatment and days of

differentiation. A slight but not significant increase in GFP OD in nicotinamide treated cultures at day 1 off-set the increases observed in nicotinamide treated cultures when compared within treatment at later days of differentiation. This suggests that nicotinamide may influence the neural differentiation of 46C cultures from as early as 24 hours after application. This is in agreement with Griffin et al. (2017) who observed promotion of neuronal differentiation of 46C cultures in the initial 2 days of differentiation with nicotinamide application.

The increase in GFP expression observed in these 46C cultures is indicative of an increase in *Sox1-GFP* expression as cells become neural progenitors. This expression is then downregulated after neural differentiation. Nicotinamide treated cultures showed lower GFP expression compared to control cultures at days 6 and 7. This decrease in expression may be a result of decreased cell numbers which is evident from DAPI staining. The OD technique was not able to compensate for the decreased cell numbers observed in the cultures treated with nicotinamide (confirmed by flow cytometry Figure 3.9 D). Therefore, flow cytometry was used to provide more detailed analysis of the cell populations and *Sox1-GFP* expression throughout the initial 7 days of differentiation.

When *Sox1-GFP* expression was analysed by flow cytometry, control cultures showed increased GFP expression at day 3 of differentiation (Figure 3.9 A), corresponding with other reports of GFP expression in differentiating 46C cultures (Ying *et al.*, 2003; Incitti *et al.*, 2014). In this PhD thesis, GFP expression in control cultures continued to increase reaching a plateau between days 5-7 with 73-77% of the cell population classed as GFP^+. This is comparable to other studies with GFP^+ 46C cells reaching a maximum of 60% (Incitti *et al.*, 2014) and approximately 75% (Ying *et al.*, 2003) of the population at day 4-5 of differentiation.

In these cells, *Sox1-GFP* expression is associated with neural progenitors, therefore an increase in GFP expression is indicative of neural differentiation. *Sox1* expression is

subsequently downregulated upon neural differentiation (Pevny *et al.*, 1998). A decrease in GFP expression was observed in nicotinamide treated cultures in this PhD thesis, with 5 mM treated cultures reaching a maximum of 24% of cells expression GFP, whilst 10 mM nicotinamide treated cultures reached a maximum of 10% of cells expressing GFP compared to 77% of control cultures. One explanation for reduced GFP expression would be that nicotinamide promoted more non-neural differentiation or inhibited neural differentiation. However, this is unlikely as nicotinamide treated cultures had lower cell numbers compared to control cultures (Figure 3.9 D), without reduced neuronal populations at day 7 (Figure 3.7 D). Suggesting that nicotinamide did not inhibit neuronal differentiation. Another explanation is that nicotinamide inhibited proliferation across cell types, including those expressing GFP. Therefore, GFP expressing cells may have been more proliferative in control cultures, increasing the numbers of GFP^+ cells. Whilst proliferation was inhibited in nicotinamide treated cultures, cell numbers appeared to remain steady, maintaining a balance across proliferation, apoptosis, and differentiation.

In this PhD thesis, control cultures showed an increase in GFP expression (measured by flow cytometry) over 7 days of differentiation, with a plateauing of expression between days 5-7. Incitti et al. (2014) observed a peak of GFP expression at day 5, however, Ying et al. (2003) observed a plateau in GFP expression from day 4-7 (consistent with the observations of this PhD thesis) with a reduction observed from day 8. In this PhD thesis, maximum expression of GFP was reached at day 7 in control cultures, day 5 in 5 mM nicotinamide treated cultures, and day 3 in 10 mM nicotinamide treated cultures. The results described here suggest that maximum GFP expression peaks earlier with nicotinamide treatment. This could be indicative of earlier neural differentiation of these 46C cultures and is further discussed in Chapter 3.4.5.

In these cells, GFP expression is driven by the expression of the *Sox1* gene, however, GFP fluorescence is not simply switched on/off as a result of altered gene expression. Whilst the *Sox1* gene is downregulated with differentiation, GFP protein, with a half-life in mammalian cells of approximately 26 hours, may continue to be expressed for a protracted period. Therefore, a decrease in GFP protein expression may be delayed after downregulation of *Sox1*, resulting in prevailing GFP fluorescence that is not representative of contemporaneous Sox1 expression.

The expression of *Sox1* is not synchronised across cells within in the culture (Ying *et al.*, 2003), and fluorescence intensity of the GFP protein may vary throughout its presence within the cytoplasm. This may account for some of the difficulties encountered when gating the GFP$^+$ population (discussed in Chapter 3.2.5). Other papers have described GFP expression as negative, low, and high (Veraitch *et al.*, 2008). Differences in GFP intensity have also been observed across treatment conditions of 46C cultures, with 10 mM nicotinamide treated cultures being devoid of "strong" GFP expressing cells (Griffin *et al.*, 2017).

3.4.5 Nicotinamide increased the β-III-tubulin$^+$ neuronal population

β-III-tubulin$^+$ cells with neuronal morphology were observed in cultures across treatments at day 4 of differentiation, although numbers were extremely low, with only a few, solitary, neurons with short processes identified across experimental repeats. However, neurons were more obviously visible across all treatment conditions at day 5. This corresponds with Ying et al. (2003) who also observed neuronal morphology and marker expression 5 days into the differentiation protocol.

The number of β-III-tubulin$^+$ cells with neuronal morphology did not differ significantly between control and nicotinamide treated cultures (Figure 3.7), however, a slight increase with 5 mM treatment and a decrease with 10 mM treatment led to a measurable and significant difference in neuron numbers between these nicotinamide concentrations at day 7.

Numbers of total cells were shown to be significantly lower in nicotinamide treated cultures, although numbers of β-III-tubulin$^+$ neurons were not significantly different. This suggests an increase in the neuronal population within the cultures, with neurons constituting a higher percentage of the total cell population. This is consistent with other reports of nicotinamide-induced neuralisation (Cimadamore *et al.*, 2009) and neuronal differentiation and maturation (Griffin *et al.*, 2013, 2017).

The increase in neuronal differentiation could be an indirect effect of nicotinamide-induced reduction in cell numbers, as lower plating densities have shown an increase in neural commitment quantified by *Sox1-GFP* expression in the 46C cell line (Diogo, Henrique and Cabral, 2008). However, treatment with nicotinamide did not show an increase in GFP expression in this PhD thesis. Therefore, the increased neuronal differentiation may be a direct effect of nicotinamide on these cells, rather than an indirect effect through a decreased cell numbers.

Nicotinamide may exert neuronal differentiation/maturation through inhibition of Sirt1 or PARP-1. Sirt1 silencing in NG108-15 cells increased neuronal MAP5 expression (Sansone *et al.*, 2013), whilst PARP-1 inhibitors (including nicotinamide) have shown enhanced ESC neuralisation (Cimadamore *et al.*, 2009). However, NSCs derived from PARP-1 knockout mice showed decreased neuronal, and increased astrocyte (GFAP$^+$) and oligodendrocyte (Olig2$^+$) differentiation (Hong *et al.*, 2019), suggesting that complete PARP-1 inhibition may not be responsible for the increased neuralisation observed in these 46C cultures.

Cimadamore et al. (2009) found that nicotinamide alone did not potentiate neuralisation, but did with the addition of basic fibroblast growth factor and epidermal growth factor. These results together suggest that nicotinamide may be a beneficial addition to differentiation protocols for CRT. Further analytical techniques would be required to explore the mechanisms behind the effects observed in this PhD thesis.

3.4.6 Conclusions and future work

The results observed in this PhD thesis show lower levels of GFP, with earlier maximum expression, in nicotinamide treated cultures compared to control. Nicotinamide also limited 46C cell proliferation but did not significantly alter numbers of β-III-tubulin^{+} neurons at day 7 of differentiation. The results discussed here are consistent with the possibility that nicotinamide may reduce proliferation and enhance cell-cycle exit from ESCs through neuralisation to mature neural differentiation (Griffin *et al.*, 2017).

Although the mechanisms for the effects of nicotinamide observed in this PhD thesis were not explored, decreased proliferation and increased neuralisation have been described through Sirt1 and PARP-1 inhibition. Therefore, nicotinamide may exert these effects through Sirt1 inhibition, which has been shown to increase cell death in proliferating cultures and increase neuronal marker expression (Sansone *et al.*, 2013). Analysis of these cultures using western blot for Sirt1 would reveal changes in Sirt1 expression with nicotinamide treatment.

PARP-1 inhibition has also been shown to decrease NSC proliferation (Hong *et al.*, 2019) and increase neuralisation (Cimadamore *et al.*, 2009). Different PARP inhibitors have also shown variability in their effects on proliferation, differentiation and survival with mouse neural stem/progenitor cells (Okuda *et al.*, 2017). Whilst this highlights the complexity of downstream effects caused by PARP inhibition, it also suggests that nicotinamide-induced PARP-1 inhibition may be a factor in a number of cumulative effects of nicotinamide on a range

of cellular mechanisms. Immunocytochemistry for PARP-1 would confirm whether nicotinamide treatment reduced PARP-1 expression in these cultures and provide insight into this potential mechanism.

Further assessment of these cultures could include the use of proliferation markers such as Ki67 and BrdU to confirm nicotinamide-induced limitation of proliferation in these cultures. Another potential mechanism could be that nicotinamide induced cell death, therefore, viability assays such as TUNEL staining or the MTT assay would able to provide clarification on cell death within these cultures.

Further exploration of Sirt1 and PARP-1 expression in differentiating 46C cultures may assist to elucidate the mechanisms behind the decreased proliferation and increased neuralisation observed in this PhD thesis. It may also be interesting to assess whether nicotinamide is involved in the upregulation of any vital transcription factors involved in dopaminergic differentiation and/or maturation such as sonic hedgehog (Shh), Lmx1b, Nurr1, or Pitx3 (discussed in Chapter 1.3).

The effects observed here, and well-established safety profile of nicotinamide, mean that nicotinamide could be a beneficial addition to the development of clinically transferable protocols for the generation of cells for CRT for PD in line with GMP guidelines. Further work to build on conclusions drawn from this data would be:

I. To assess the effect of nicotinamide on levels of PARP-1 using immunocytochemistry during neural differentiation of 46C cultures.
II. To assess the effect of nicotinamide on the expression of Sirt1 during neural differentiation of 46C cultures using western blot.

4 Nicotinamide and neuroprotection

4.1 Introduction

4.1.1 Nicotinamide may have roles in neuroprotection

It is well known that diet and nutrition are key factors for general health, but there is also a growing body of evidence that it could affect the onset, progression and amelioration of PD (Seidl *et al.*, 2014; Agim and Cannon, 2015). Research over the last century has elucidated the many essential roles that vitamins play in health maintenance, however, their exact effects, biological mechanisms and potential medicinal benefits are still being discovered.

Nicotinamide has been identified as an emerging tool for neuroprotection (Maiese *et al.*, 2003) although the mechanisms behind this are yet to be defined (Chapter 1.10). Potential modes of action for nicotinamide include: mitochondrial function & energy production (NAD^+), oxidative stress, PARPs, and sirtuins (Li, Chong and Maiese, 2006).

4.1.2 6-Hydroxydopamine *in vitro* mechanisms

The inaccessible nature of the brain in general, and more specifically the SNpc, means that studying the molecular mechanisms of PD is often not possible until post-mortem once cells have already been destroyed. *In vitro* models of PD provide a way to visualise dopaminergic neurons and their response to pathology. They also provide a valuable tool for testing potential neuroprotectants that could provide therapy for PD.

The neurotoxin 6-OHDA is commonly used to generate an *in vitro* PD model. It is a synthetic, organic compound that is taken up by catecholaminergic (dopamine, adrenaline, noradrenaline) neurons (Schubert *et al.*, 1973) via the monoamine transporters: serotonin

transporter, noradrenaline transporter (Lin *et al.*, 2011), and in the use of PD models, the dopamine transporter (DAT) present on dopaminergic neurons (Ding *et al.*, 2004; González-Hernández *et al.*, 2004; Redman *et al.*, 2006). Once inside the cell, a series of reactions take place to induce cell death, however, the exact mechanisms behind 6-OHDA toxicity are highly debated. The ability of glial cells to also internalise synaptic dopamine is becoming an area of research, although it is thought that whilst dopamine taken up by neurons is stored, once it is internalised by glia it is broken down by MAO and COMT activity which is higher in glial cells (Meiser, Weindl and Hiller, 2013; Winner *et al.*, 2017; Petrelli *et al.*, 2020). If glial cells are able to internalise dopamine, then 6-OHDA uptake may not be specific to dopamine neurons, however the processing of this toxin may differ between cell types, explaining the selective toxicity.

Cells receiving 6-OHDA treatment undergo apoptosis through a caspase-dependent mechanism (Ding *et al.*, 2004), rather than cell death through necrosis (Lotharius, Dugan and O'Malley, 1999; Callizot *et al.*, 2019). This differs to the toxins MPTP and rotenone which work through other mechanisms to cause toxicity as described in Chapter 1.5.

Some of the proposed mechanisms of 6-OHDA toxicity are highlighted in Figure 4.1. These include mitochondrial dysfunction, production of toxic compounds through auto-oxidation, and oxidative stress resulting from ROS production. The accumulation of these damaging events culminate in cell death (González-Hernández *et al.*, 2004).

Studies have shown that 6-OHDA causes mitochondrial dysfunction (Lotharius, Dugan and O'Malley, 1999; Hanrott *et al.*, 2006) by inhibiting the mitochondrial respiratory chain complexes I and IV (Glinka and Youdim, 1995). Mitochondrial complex I (NADH dehydrogenase) transfers electrons from NADH (producing NAD^+) to coenzyme Q10 in the first step of the mitochondrial transport chain (Wirth *et al.*, 2016).

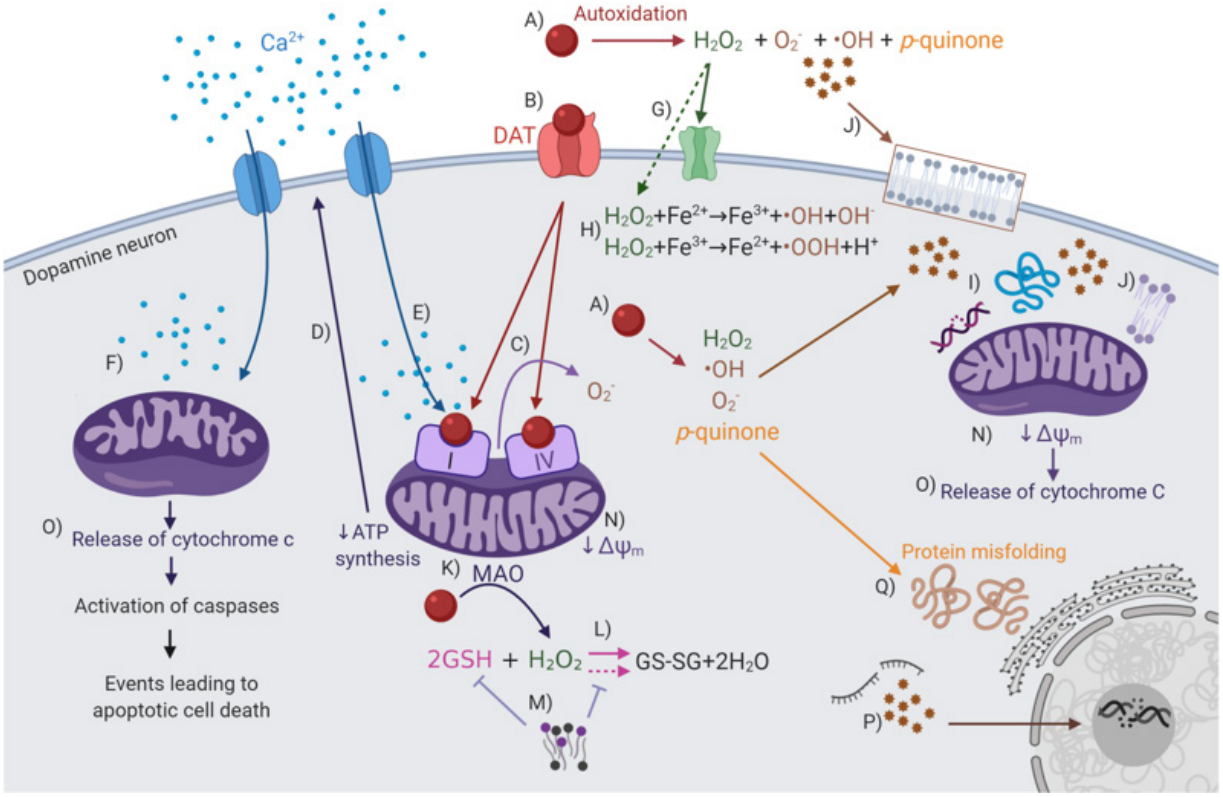


Figure 4.1 Potential toxin mechanisms of 6-hydroxydopamine (6-OHDA).
A) Auto-oxidation of 6-OHDA occurs rapidly under physiological conditions to form hydrogen peroxide (H_2O_2), the reactive oxygen species (ROS) super oxide (O_2^-) and hydroxyl radical (•OH), and 1,4-benzoquinone (commonly known as para- or *p*-quinone; Graham *et al.*, 1978; Soto-Otero *et al.*, 2000). This reaction can happen intra- and/or extracellularly.
B) Due to it structural similarity to dopamine, 6-OHDA enters dopaminergic neurons via the dopamine transporter (DAT; Luthman *et al.*, 1989). Once inside the cell autoxidation of 6-OHDA (A) can occur. Blocking DAT activity can protect dopaminergic neurons from 6-OHDA toxicity (Ding *et al.*, 2004).
C) 6-OHDA has been shown to inhibit complexes I and IV of the mitochondrial respiratory chain (Glinka and Youdim, 1995). Mitochondrial complex I is the enzyme NADH dehydrogenase which transfers electrons from NADH (producing NAD^+) to coenzyme Q10 in the first step of the mitochondrial transport chain (Wirth *et al.*, 2016). Mitochondrial complex IV is the enzyme cytochrome c oxidase which catalyses the transfer of electrons from reduced cytochrome c to O_2 alongside the translocation of H^+ for ATP production (Mansilla *et al.*, 2018). The inhibition of these complexes leads to mitochondrial dysfunction resulting in a series of damaging events including production of O_2^- (Pelicano *et al.*, 2003) contributing to increasing levels of ROS.
D) Mitochondrial dysfunction leads to a reduction in ATP synthesis (Marques, Massari and Tasca, 2019). ATP-dependent pumps maintain a steep concentration gradient of low intracellular Ca^{2+} levels compared to high extracellular levels (Surmeier *et al.*, 2011). A reduction in ATP levels could result in an influx of Ca^{2+} into the cell.
E) Ca^{2+} increases the inhibitory potency of 6-OHDA for mitochondrial complex I. This may cause a feedback amplification with mitochondrial dysfunction resulting in elevated levels of Ca^{2+} which further disrupt mitochondrial function (Glinka, Gassen and Youdim, 1997).
F) Increased intracellular Ca^{2+} can also induce mitochondrial swelling (Kobayashi *et al.*, 2003) and permeability of the mitochondrial membrane (Norenberg and Rama Rao, 2007). Surmeier *et al.* (2011) provide a review on the potential roles of Ca^{2+} in dopaminergic neuron death.
G) H_2O_2 has been cited as highly cell permeable (Ramasarma, 1982; Halliwell, 1992; Nagatsu and Sawada, 2006; Dias, Junn and Mouradian, 2013), however, diffusion rates have been shown to differ across cell types and membranes (Antunes and Cadenas, 2000; Hashida, Sakakura and Makino, 2002; Makino *et al.*, 2004). There are also proposals of channels and aquaporins that may facilitate transportation of hydrogen peroxide across membranes (Bienert *et al.*, 2007; Avola *et al.*, 2018).

H) Intracellular H_2O_2 reacts with Fe^{2+} and Fe^{3+} in the Fenton reaction to produce the ROS hydroxyl radical (•OH), hydroxide ion (OH^-), and hydroperoxyl radical (•OOH; Valko *et al.*, 2007).
I) Increased levels of ROS lead to damage of mitochondrial DNA and disruption of mitochondrial Ca^{2+} regulating proteins (Guo *et al.*, 2013).
J) ROS also lead to lipid peroxidation which consists of damage to lipid membranes including the outer cell membrane and mitochondrial membranes. Lipid peroxidation can lead to structural damage of membranes and production of neurotoxic products (Montine *et al.*, 2002; Guo *et al.*, 2013; Lima *et al.*, 2018).
K) Involvement of the mitochondrial bound enzyme monoamine oxidase (MAO) in 6-OHDA toxicity is debated (Blum *et al.*, 2001). Under normal conditions, MAO breaks down amines such as dopamine and 6-OHDA. MAO inhibitors can increase the toxicity of 6-OHDA by preventing it's breakdown, thereby requiring a lower dose of 6-OHDA for toxicity (Duty and Jenner, 2011). However, MAO inhibitors have also shown protection of TH^+ neurons against 6-OHDA toxicity *in vivo* (Salonen *et al.*, 1996; Cutillas, Ambrosio and Unzeta, 2002). These differences cited in the literature could be due to the specificity of the inhibitor as well as the different 6-OHDA models used. The action of MAO may enhance the toxicity of 6-OHDA as the breakdown of dopamine and 6-OHDA by MAO results in the production of H_2O_2 as a by-product (Blum *et al.*, 2001).
L) H_2O_2 is potentially an important signalling molecule with cellular mechanisms maintaining non-toxic intracellular levels (Armogida, Nisticò and Mercuri, 2012). It is typically inactivated by the action of cytosolic glutathione peroxidase with the co-factor glutathione (GSH) to produce glutathione disulphide (GS-SG) and water (H_2O).
M) GSH levels can be reduced in 6-OHDA treated cell models (Villa *et al.*, 2013; Magalingam, Radhakrishnan and Haleagrahara, 2014) and can also be diminished as a result of lipid peroxidation (J) due to increased levels of ROS (Guo *et al.*, 2018). This results in lower levels of GSH and therefore a reduction in glutathione peroxidase activity to neutralise H_2O_2 (L). Higher levels of H_2O_2 could then lead to further production of ROS (H).
N) Damage to mitochondria arising from C, E, F, I, and J results in a decrease in mitochondrial membrane potential ($\Delta\Psi m$; Ly, Grubb and Lawen, 2003).
O) Mitochondrial swelling (F), ROS mediated damage (I, J) and reduction in $\Delta\Psi m$ (N) lead to the release of cytochrome c (Kobayashi *et al.*, 2003; Gomez-Lazaro *et al.*, 2008; Guo *et al.*, 2013).This in turn activates caspases which are central in apoptotic signalling leading to cell death (Ly, Grubb and Lawen, 2003).
P) Production of ROS resulting from autoxidation of 6-OHDA (A), mitochondrial dysfunction (C) and the Fenton reaction of H_2O_2 (H) causes widespread damage throughout the cell including damage to nuclear DNA and cytosolic RNA (Sanders and Greenamyren, 2013).
Q) Intracellular *p*-quinone produced from autoxidation of 6-OHDA (A) is proposed to disrupt protein folding and impair proteome function contributing to cell death (Farzam *et al.*, 2020).
Created using Biorender.com

Mitochondrial complex IV (cytochrome c oxidase) catalyses the transfer of electrons from reduced cytochrome c to O_2 alongside the translocation of H^+ for ATP production (Mansilla *et al.*, 2018). Dysfunction of the mitochondrial respiratory chain caused by inhibition of complexes I and IV by 6-OHDA results in increased production of ROS (Figure 4.1 C; Pelicano *et al.*, 2003; Wirth *et al.*, 2016), decreases in mitochondrial membrane potential ($\Delta\Psi m$; Figure 4.1 N; Ly, Grubb and Lawen, 2003) and reduced ATP levels (Figure 4.1, C; Villa *et al.*, 2013; Marques, Massari and Tasca, 2019).

A reduction in ATP levels could result in an influx of Ca^{2+} (Figure 4.1 D) further inhibiting mitochondrial complex I (Figure 4.1 E; Glinka, Gassen and Youdim, 1997). Increased intracellular Ca^{2+} can also induce mitochondrial swelling (Figure 4.1 F) leading to the release of cytochrome c (Kobayashi *et al.*, 2003). Cytochrome c release activates caspases which are central to apoptotic signalling leading to cell death (Figure 4.1 O; Ly, Grubb and Lawen, 2003).

MAO breaks down amines including dopamine and 6-OHDA, and its involvement in 6-OHDA toxicity is debated (Blum *et al.*, 2001). A potential contribution of MAO to toxicity is through the production of hydrogen peroxide (H_2O_2) resulting from the breakdown of amines (Figure 4.1 K).

Hydrogen peroxide production is a normal part of cell functioning and it can be considered as a signalling molecule, with cellular mechanisms maintaining non-toxic intracellular levels (Figure 4.1 L; Armogida, Nisticò and Mercuri, 2012). Its toxicity arises from ROS production by interaction with Fe^{2+} and Fe^{3+} in the Fenton reaction (Figure 4.1H; Valko *et al.*, 2007). ROS production and subsequent damage can be further propagated by a reduction in neutralising mechanisms resulting from other toxic compounds involved in 6-OHDA toxicity (Figure 4.1M; Guo *et al.*, 2018). Glutathione (GSH), protects cells against 6-OHDA toxicity (Shimizu *et al.*, 2002), perhaps by reducing H_2O_2 production. Treating cultures with 6-OHDA decreases levels of GSH in RCSN-3 cells (a cell line derived from rat SN; Villa *et al.*, 2013).

Increased ROS is both directly (auto-oxidation; Figure 4.1 A) and indirectly (mitochondrial dysfunction and increased H_2O_2; Figure 4.1 C, H) caused by 6-OHDA, resulting in cell death through oxidative stress (Lotharius, Dugan and O'Malley, 1999). Excessive ROS production leads to lipid peroxidation (Figure 4.1 J; Montine *et al.*, 2002; Guo *et al.*, 2013; Lima *et al.*, 2018), damage to mitochondrial DNA and calcium regulating proteins (Figure 4.1 I; Guo *et al.*, 2013), and damage to nuclear DNA and cytosolic RNA (Figure 4.1 P; Evans, Dizdaroglu

and Cooke, 2004; Sanders and Greenamyren, 2013). This ROS production can occur intracellularly and/or extracellularly resulting in damage to cells.

Auto-oxidation of 6-OHDA occurs rapidly under physiological conditions to form H_2O_2, super oxide (O_2^-), hydroxyl radicals (•OH), and 1,4-benzoquinone (commonly known as *para*-quinone or *p*-quinone; Figure 4.1 A; Graham *et al.*, 1978; Soto-Otero *et al.*, 2000). Intracellular *p*-quinone is proposed to alter protein expression and disrupt protein folding, contributing to cell death (Farzam *et al.*, 2020).

Blocking DAT rescues cells from intracellular 6-OHDA toxicity both *in vitro* (Ding *et al.*, 2004) and *in vivo* (González-Hernández *et al.*, 2004), whilst forced expression of DAT induces vulnerability to 6-OHDA toxicity in cortical neurons (Redman *et al.*, 2006). However, 6-OHDA can also induce toxicity extracellularly: i.e. induce damage in cells in the absence of uptake. If ROS production occurs outside of the cell, then non-specific toxicity can occur. For instance, in PC12 cells, 6-OHDA toxicity remained evident even when DAT was blocked (Hanrott *et al.*, 2006). Extracellular production of H_2O_2 could also contribute to toxicity, with suggestions that H_2O_2 is highly cell permeable (Ramasarma, 1982; Halliwell, 1992; Nagatsu and Sawada, 2006; Dias, Junn and Mouradian, 2013). There are also proposals of channels and aquaporins that may facilitate transportation of hydrogen peroxide across membranes (Bienert *et al.*, 2007; Avola *et al.*, 2018). This could contribute to instances of non-specific 6-OHDA toxicity.

Non-specific toxicity, shown by a reduction in cell numbers, has been reported in VM cultures at concentrations of 6-OHDA between 10 and 100 µM (Michel and Hefti, 1990). This toxicity is not restricted to neurons, with concentrations of 100 µM 6-OHDA for 24 hours resulting in death of approximately 90% of cultured primary rat microglia (Takai *et al.*, 1998). Concentrations as low as 10 µM 6-OHDA have also been shown to induce a range of pro-inflammatory effects on astrocytes including increased expression of iNOS (inducible nitric oxide synthase) and GFAP in the C6 rat astrocyte cell line (Gupta *et al.*, 2015).

The staging of 6-OHDA auto-oxidative events is dependent on the propagating intermediates, substrates and products available throughout auto-oxidation (Gee and Davison, 1989). When 6-OHDA is carried in an ascorbic acid (AA) vehicle this auto-oxidation to neurotoxic metabolites is reduced (Ding *et al.*, 2004). 10 mM AA reduces the rate of oxygen consumption and production of H_2O_2 (Soto-Otero *et al.*, 2000). Production of *p*-quinone is also reduced by AA (Sullivan and Stern, 1981; Soto-Otero *et al.*, 2000). This suggests that the addition of AA to 6-OHDA could help to prevent non-specific toxicity caused by extracellular auto-oxidation.

Methods to create *in vitro* 6-OHDA PD models, and the resulting outcomes, vary across the literature (Table 4.1). Due to the variation described by laboratories across the world, a robust and reproducible 6-OHDA *in vitro* model needed to be developed and specified for the VM cultures used in this PhD research. Once a standardised model was developed it could then be used to test the neuroprotective effects of nicotinamide.

Table 4.1 Table highlighting some *in vitro* PD models using 6-OHDA in primary rodent VM cultures. VM: ventral mesencephalon; E#: embryonic day; P# postnatal day; AA: ascorbic acid. Article reference: 1) Batelli *et al.* (2015) 2) Lotharius, Dugan and O'Malley (1999) 3) Cerruti *et al.* (1993) 4) Rodriguez-Pallares *et al.* (2007) 5) Michel and Hefti (1990) 6) Callizot *et al.* (2019) 7) Izumi *et al.* (2005) 8) Bronstein *et al.*, (1995) 9) Masaki *et al.* (2017) 10) Ding *et al.* (2004) 11) Sun *et al.* (2018)

Conclusion	**6OHDA conc.**	**Vehicle**	**Exposure duration and fixation**	**Culture type**	**Plating density**	**DIV prior**	**Dopaminergic yield**	**Dopamine neuron marker**	**6-OHDA toxicity specificity**	**Article**
6-OHDA roughly reduces %TH^+ of $NeuN^+$ cells from 9.5% to 8% 50 µM and 6.5% 100 µM	10-100 µM	Not stated	24 h then fixed	Mouse E13 mesencephalic region	50,000 cells/cm^2	7	Roughly 9% of $NeuN^+$	TH	Not stated	1
15 µM reduced TH^+ neurons by approximately 55%	5-60 µM	Not stated	Different time points assessed. No recovery period	E14 mouse VM	1.25 x10^3 cells/mm^2	7	1-5% TH^+ of total cells	TH	30-50% neurons also affected	2
Morphological parameters significantly reduced	10 nM	Not stated	48 hr exposure, fixed at 9 DIV	E14 VM rat	300,000 cells per dish	2 or 7	Not stated	Dopamine	Not stated	3
40 µM reduced TH^+ cell numbers by about 60%	10, 40 µM	0.02% saline ascorbate	24 h then fixed	E14 rat VM	1.5x10^5 per cm^2	6	Not stated	TH	Not stated	4
10-100 µM resulted in a dose dependent reduction in TH+ neurons	10-100 µM	20 µM AA 40 µM α-tocopherol Did not affect response to toxin	24 h then fixed/assessed	E15 VM rat	Density after 1 week i*n vitro* = 1.0 to 1.4 x 10" cells per 35 mm dish	6-7	0.5-1% TH^+ of total cells	TH	Non-specific toxicity observed	5
30 µM 24 hr reduced TH^+ numbers by about 30%	10-30 µM	Not stated	24 or 48 h, tests run immediately	E 15 rat VM	40,000 per 96 well 225,000 per 24 well	6	Not stated	TH	Decrease in total cell viability with 48 hr treatment	6
30 µM reduced TH^+ neurons by about 40% 100 µM reduced TH^+ neurons by about 80%	30 µM 100 µM	Not stated	24 h treatment then 48h recovery before fixing	E 16 rat VM	1.3 x 10^5 cells/cm^2	9	Not stated	TH	Not stated	7

6OHDA treatment killed 89 ± 8% TH+ cells (10µM ineffective, 100µM killed non-catecholaminergic cells)	30 µM	0.1% Ascorbate	48 h then fixed	E16 rat VM	100,000 cells/well	4-6	Not stated	TH	Treatment killed 30% TH- neurons	8
Roughly 50% reduction in TH^+ cells	50 µM	Not stated	24 h then fixed	E16 rat VM	130,000 cells/cm^2	9	Not stated	TH	Not stated	9
40 µM reduced TH^+ cell numbers by 53%	40, 100, 500 µM	10 mM diethylenetriaminepentaacetic acid; 0.15% AA; flushed with N_2	15 min then 3-48 hr recovery	P0 rat SN	20,000 per well (0.4cm^2)	3-4	5-6% TH^+ of total cells at 4DIV	TH	100 and 500 µM caused significant loss of GABA neurons	10
Roughly 40% reduction in TH^+ neuron numbers	50 µM	Not stated	24 h then fixed	P0-3 rat ventral midbrain	80,000 cells/well	6-7	Not stated	TH	Not stated	11

4.1.3 Direct effects of nicotinamide through inhibition of PARP-1 and Sirt1

PARP-1 can be considered as cytoprotective due to its role in DNA repair, however overactivation through mechanisms including oxidative stress can lead to depletion of cellular NAD^+ and ATP, and activation of cell death pathways (Virág and Szabó, 2002). One of the main modes of cell death due to 6-OHDA toxicity is through oxidative stress, which could lead to overactivation of PARP-1 as a mediator of this toxicity. However, Callizot et al. (2019) found no protection of a specific PARP-1 inhibitor against 6-OHDA, suggesting that PARP-1 is not central to 6-OHDA mediated toxicity.

Sirt1 is often cited as neuroprotective with *Sirt1*-overexpressing mice showing life span extension and enhanced neural activity (Satoh *et al.*, 2013). Resveratrol has been shown to upregulate Sirt1 providing neuroprotection (Wan *et al.*, 2016; Zhuo Zhang *et al.*, 2019), and has also shown effectiveness in PD models reviewed by Tang (2017). However, a number of the studies showing neuroprotection in this review article use cell lines that may not be considered representative of dopaminergic neurons (as discussed in Chapter 1.5.2). Sirt1 has been found to be crucial in neuroprotection induced by NAMPT overexpression in primary neuronal cultures and transgenic mice (Wang *et al.*, 2011). However, transgenic mice overexpressing *Sirt1* did not show alleviation of MPTP-induced loss of TH^+ neurons (Kitao *et al.*, 2015).

Other studies have shown that inhibition of Sirt1 can be neuroprotective. Inhibition of Sirt1 by application of sirtinol or 5 mM nicotinamide, or knockdown of Sirt1, increased survival of cultured cortical neurons exposed to oxidative stress through application of H_2O_2 (Li *et al.*, 2008). However, 6-OHDA significantly reduced Sirt1 activity in PC12 cells (Zou *et al.*, 2016).

Therefore, if 6-OHDA alone inhibits Sirt1 activity, the addition of nicotinamide as a Sirt1 inhibitor may not exert neuroprotective effects through this mechanism in a 6-OHDA model.

4.1.4 Indirect effects of nicotinamide through nicotinamide derivatives

As described in Chapter 1.10, nicotinamide and associated compounds have shown neuroprotective properties across a variety of neurodegenerative diseases. It has therefore been hypothesised that nicotinamide could provide neuroprotection for dopaminergic neurons. Consistent with this, nicotinamide has shown protection in models of PD. Schulz, Henshaw, Matthews, & Beal (1995) suggest that nicotinamide, given prior to (0.5 hours) and after (every 8 hours for 48 hours) MPTP administration (discussed in Chapter 1.5), in an *in vivo* mouse model reduces neurotoxicity through improved mitochondrial energy production.

However, it is not clear whether such protective effects are mediated by nicotinamide itself, or by one or more derivative molecules. Figure 1.10 described the interconversion of various nicotinamide-related metabolites. Several of these molecules, and the enzymes involved in NAD^+ pathways, have been associated with neuroprotective effects; but, as with nicotinamide, it is often unclear whether these effects are directly attributable to the delivered molecule or depend on generation of a derivative molecule.

Nicotinamide could confer this protection to dopaminergic neurons through increases in pyridine nucleotide levels (NAD^+, NADH, $NADP^+$, NADPH). Administration of NAD^+ in the *in vivo* 6-OHDA mouse model significantly reduced the loss of TH^+ neurons in the SN (Shan *et al.*, 2019). Therefore, it is possible that nicotinamide could exert protective effects by increasing levels of NAD^+. In PC12 cells, NAD^+ levels and Sirt1 activity are reduced by 6-OHDA treatment. Treatment of PC12 cells with the nicotinamide derivative NMN (Figure 1.10) rescues 6-OHDA

induced cell viability attenuating the Sirt1 activity decrease (Zou *et al.*, 2016), and increased cell survival and attenuated rotenone-induced intracellular ATP depletion (Lu *et al.*, 2014).

6-OHDA may decrease levels of GSH (Villa *et al.*, 2013) an important antioxidant (detailed in Chapter 4.1.2). NADPH is utilised by glutathione reductase in the reduction of GS-SG to GSH. Therefore, increases in NADPH could help to maintain levels of GSH, conferring protection against oxidative stress.

Despite evidence of neuroprotection conferred by nicotinamide and associated compounds, there is the potential for nicotinamide to exacerbate neurodegenerative conditions. For example the increase in meat consumption in populations across the globe (and consequently vitamin B_3) correlates with increased incidence of PD (Williams and Ramsden, 2005a), whilst vegan diets are associated with a lower risk (McCarty, 2001). The increased risk with meat intake may be through increased production of N-methylnicotinamide which is toxic to dopaminergic neurons (Williams and Ramsden, 2005b). Also, Harrison, Powell, & Dexter (2019) found that nicotinamide dose-dependently exacerbated behavioural motor defects in the lactacystin rat model of PD. In this study, nicotinamide dose-dependently increased ventricular enlargement induced by lactacystin and exacerbated the loss of TH^+ neurons in the SNpc. They suggest that this could be due to a synergistic inhibitory effect of lactacystin and nicotinamide on proteasome activity and/or nicotinamide-induced inhibition of Sirt1.

4.1.5 Calcitriol and neuroprotection

As discussed in Chapter 1.8, vitamins have widely been shown to promote neuroprotection. One of the most predominantly studied for its neuroprotective properties is vitamin D_3. Calcitriol, the active metabolite of vitamin D_3 (discussed in Chapter 1.9), has shown protection of dopamine neurons from 6-OHDA toxicity in both *in vitro* and *in vivo* models

(Wang *et al.*, 2001; Sanchez *et al.*, 2009; Orme, Bhangal and Fricker, 2013; Lima *et al.*, 2018) and also causes an increase in the release of dopamine in the striatum and SN of rats (4-5 months old; Cass *et al.*, 2014), however, this positive response was not seen in aged (22 months) rats (Cass and Peters, 2017).

Calcitriol has been shown to have a protective effect on dopamine neurons through upregulation of GDNF (Sanchez *et al.*, 2009; Orme, Bhangal and Fricker, 2013) an important factor in dopamine neuron survival and maintenance (Hidalgo-Figueroa *et al.*, 2012; d'Anglemont de Tassigny, Pascual and Lopez-Barneo, 2015). Calcitriol may also offer protection by decreasing oxidative stress and the resulting damage from this change in cell state such as lipid peroxidation (Lima *et al.*, 2018).

4.1.6 Aims of Chapter 4

As described above, there is uncertainty in the potential neuroprotective effects of nicotinamide and its potential roles in the development of PD. The cause of dopaminergic degeneration remains unknown for the majority of PD cases and there are no treatments that can prevent the continual degeneration. Treatments that may halt or slow this neurodegeneration could improve the quality of life for many people with PD. Neuroprotective treatments may also benefit other therapies such as CRT which may be vulnerable to disease progression after initial recovery. This chapter aims to assess the potential of nicotinamide to incite neuroprotection in a PD model. The objectives of this chapter are:

I. The development of an effective *in vitro* model of PD using E14 rat VM cultures treated with 6-OHDA.
II. Assessment of the effects of nicotinamide on dopaminergic neurons (TH^+ populations) within control VM cultures.
III. Assessment of the potential for nicotinamide to provide neuroprotection of TH^+ neurons in the developed *in vitro* 6-OHDA PD model; and comparison with the protective effects of calcitriol and possible combined neuroprotection of both nicotinamide and calcitriol.

4.2 Methods

4.2.1 Assessing neuroprotection of nicotinamide and calcitriol in an *in vitro* 6-OHDA PD model

The *in vitro* PD model described in Chapter 2.5.2 was used to assess potential neuroprotective activity of nicotinamide. Calcitriol was used as a positive, neuroprotective control. VM cultures were obtained as described in Chapter 2.5.1 and plated on to PEI/PDL coated coverslips (0 DIV). Cultures were grown for 7 DIV in their treatment condition, either control (NCM), 10 mM nicotinamide (Griffin *et al.*, 2013), 10 nM calcitriol (Orme, Bhangal and Fricker, 2013), or 10 mM nicotinamide + 10 nM calcitriol. At 7 DIV, 6-OHDA or AA vehicle control was applied as described in Chapter 2.5.2, for 4 hours, and after removal the cells were maintained for a further 48 hours 'recovery'. Cells received their treatment conditions (vitamins) prior to, throughout, and during recovery from 6-OHDA treatment (Figure 4.2). Cultures were assessed using fluorescence microscopy. Cells were fixed at 9 DIV and stained for β-III-tubulin and TH expression as described in Chapter 2.8.

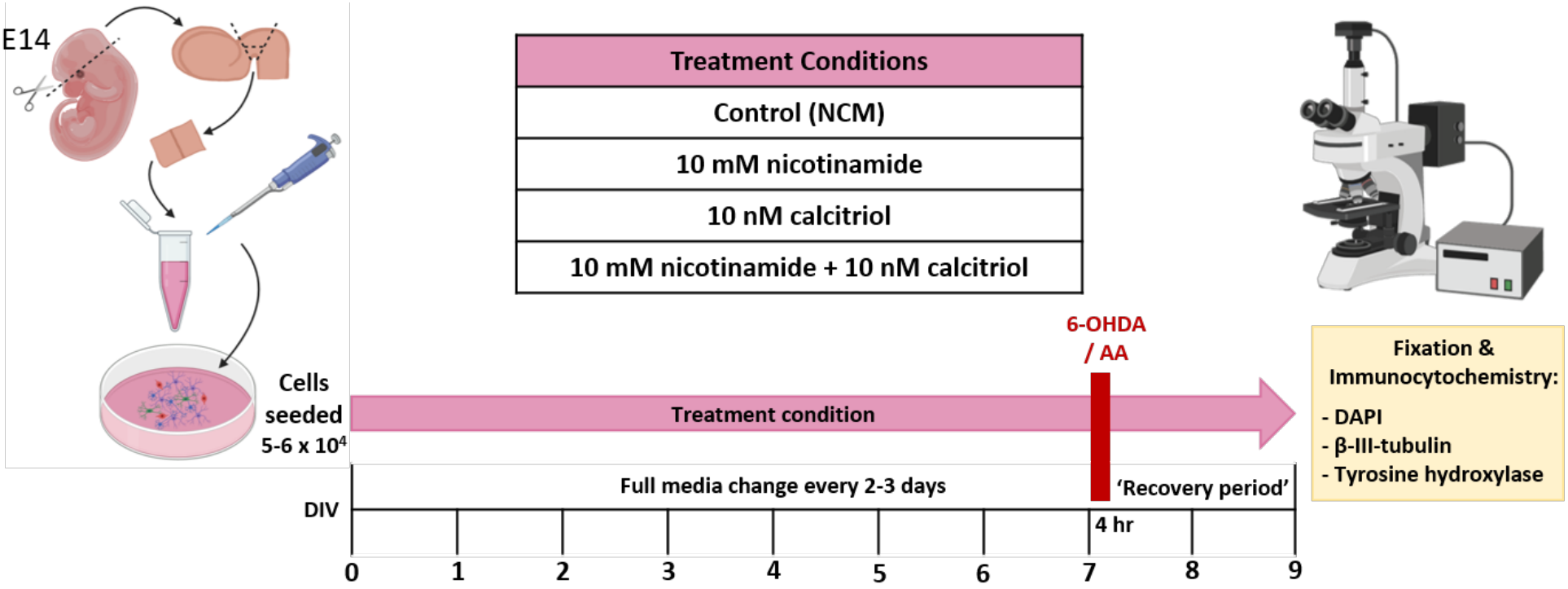


Figure 4.2 Diagram showing the protocol for the *in vitro* 6-OHDA PD model developed here. Cultures were obtained from E14 rat VM and were seeded in micro-drop cultures (Day 0). Cultures received the vitamin treatment conditions displayed in the table throughout the protocol with media changes every 2-3 days. 6-OHDA or AA vehicle was applied on day 7 for 4 hours, after which cultures were washed with NCM and then left for a 48 hour 'recovery period' in their treatment condition. Cells were fixed on day 9 and analysed using immunocytochemistry for β-III-tubulin and tyrosine hydroxylase, with nuclei counterstained with DAPI. 6-OHDA: 6-hydroxydopamine; NCM: neural cell media; AA: ascorbic acid; E14: embryonic day 14. Created with BioRender.com

4.2.2 Acquisition of images for 6-OHDA treated culture analysis

Varying techniques were used to identify and quantify TH^+/ β-III-tubulin$^+$, TH^-/β-III-tubulin$^+$, and TH^-/β-III-tubulin$^-$ cells within VM cultures treated with 6-OHDA. Initial assessment during development of the 6-OHDA model utilised images taken at 40X and 20X magnification (detailed in Chapter 2.9). For final assessment of the effects of vitamins and 6-OHDA on TH^+ cells within VM cultures, images were taken at 10X magnification and the total number of TH^+ cells were quantified within each image.

As TH^+ cells composed a small percentage of cells within each culture, but were usually aggregated within small areas, micrographs taken at 10X magnification ensured representative TH^+ populations were imaged across coverslips. This avoided having many images with zero TH^+ cells, and a few images with a high density of TH^+ cells (observed with 20X and 40X magnifications), which would be likely to skew analyses. FOI were chosen as described in Chapter 2.9, using the DAPI fluorescence channel. Experimental design followed that described in Chapter 2.2, with 3 experimental repeats, 3-4 technical replicates per treatment condition, and 3-4 micrographs per technical replicate.

4.3 Results

4.3.1 Development of the 6-OHDA model

Pilot experiments based on protocols from the literature (Table 4.1), and protocols previously used in the lab, were required to achieve an appropriate and reproducible *in vitro* 6-OHDA model with the primary VM cultures. Based on the literature, concentrations of 25 µM, 50 µM, and 100 µM, and application times of 20 minutes, 2 hours, and 24 hours were tested. Due to time constraints, only one experimental repeat was carried out, therefore, statistical tests were not performed on preliminary 6-OHDA results.

The rapid oxidation of 6-OHDA requires it to be carried in a vehicle to reduce auto-oxidation and subsequent reduction in biological activity and production of *p*-quinone. The vehicle used for this *in vitro* model was AA, the antioxidant effects of which are described above (Chapter 4.1.2). To determine whether there was any effect of AA on TH^+ cells in culture, control cells received either 850 µM AA vehicle or remained untreated, receiving only a media change and subsequent washes. The total number of TH^+ neurons present in 4 micrographs, taken across 3-4 technical replicates, was normalised to the untreated cultures for each experimental repeat (n = 3). Cultures receiving 850 µM AA vehicle showed no significant difference in TH^+ cell numbers compared to untreated cultures (93.7 ± 19.3 vs. 100.0 ± 12.9 in untreated cultures; values normalised; $t = 0.28$, n.s.; Figure 4.3). Therefore, the addition of AA to these VM cultures did not significantly alter the TH^+ cell population.

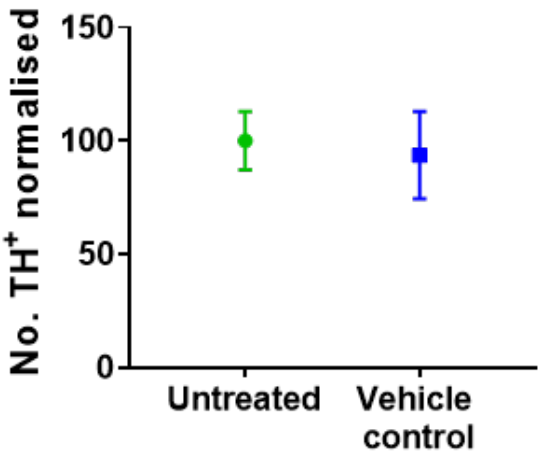


Figure 4.3 Application of ascorbic acid vehicle did not affect TH^+ numbers. Graph showing the total number of TH^+ neurons normalised to control, in untreated cultures (media change only; green circle) and cultures receiving ascorbic acid vehicle control (850 µM; blue square). No significant difference was observed.

The initial pilot experiment compared application of 6-OHDA at 25 µM, 50 µM and 100 µM (in 850 µM AA vehicle) for 20 minutes, 2 hours and 24 hours.

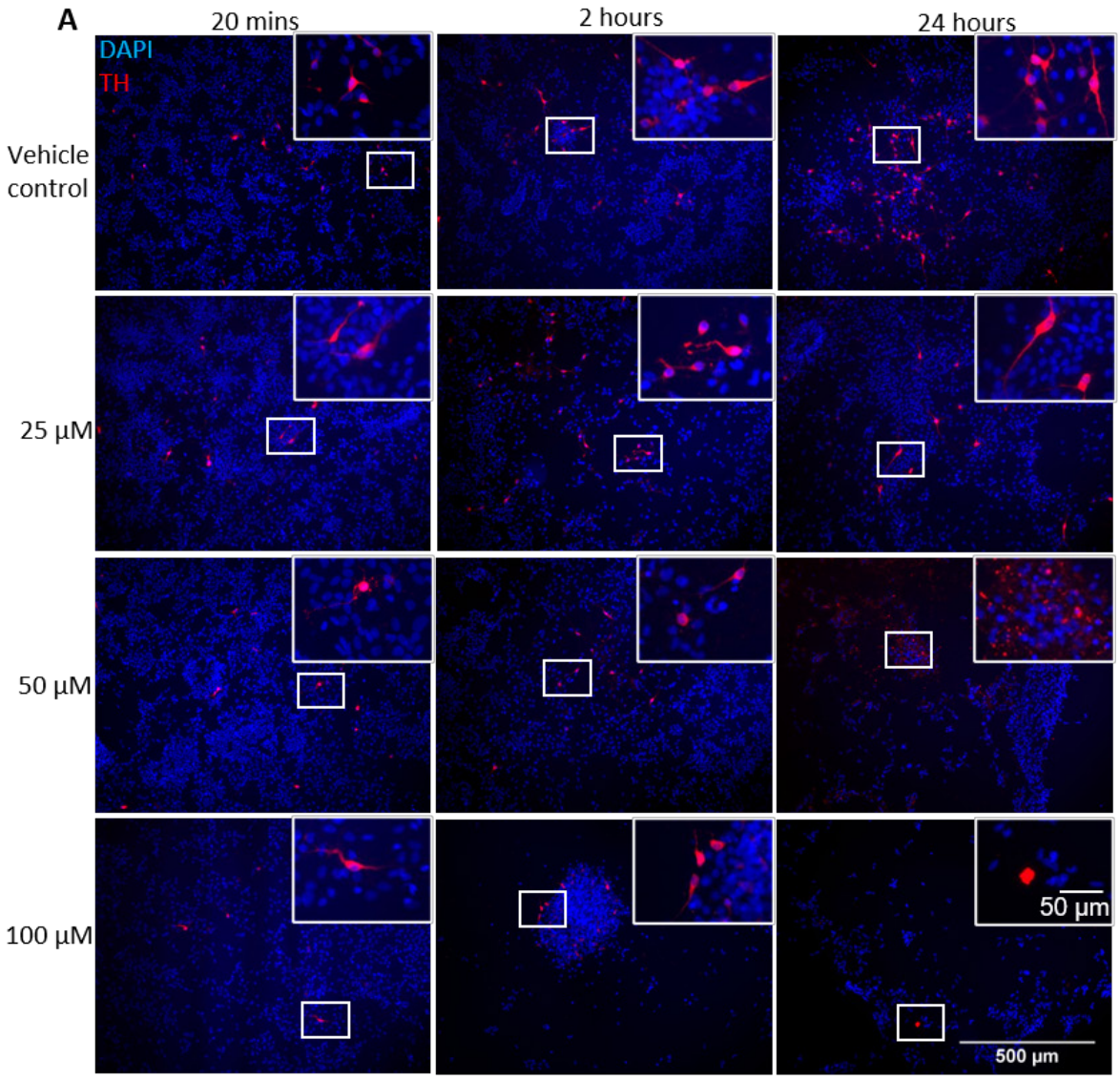
The 20-minute treatment with 25 µM and 50 µM 6-OHDA did not appear to reduce numbers of TH^+ cells whilst cultures treated with 100 µM 6-OHDA for 20 minutes reduced numbers (Figure 4.4 A). However, it was considered unlikely that pipetting could be performed rapidly enough when applying and removing/washing away 6-OHDA across multiple cultures, to reliably replicate this 20-minute timeframe.

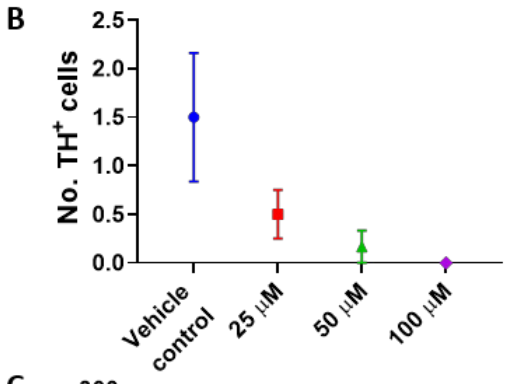
Application of 100 µM 6-OHDA reduced numbers of TH^+ cells dramatically, with very few remaining across cultures at all timepoints (Figure 4.4 A). The toxic effects of 100 µM 6-OHDA also appeared to be non-specific, with total cell numbers ($DAPI^+$ nuclei counts) obviously diminished at 2 hour and 24 hour treatments (Figure 4.4 A).

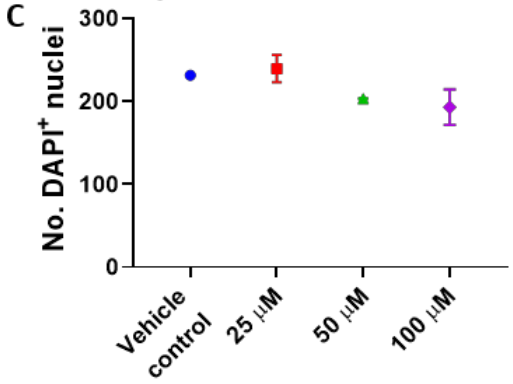
6-OHDA treatment for 2 hours appeared most effective at reducing numbers of TH^+ cells without affecting total cell numbers. Four images from 4 technical replicates were taken at 40X magnification to analyse TH^+ and TH^- cell numbers. At 2 hours, TH^+ numbers were lower across all concentrations of 6-OHDA (Figure 4.4 B). Total cell numbers appeared reduced at 50

μM and 100 μM 6-OHDA suggesting non-specific toxicity, whilst total cell numbers at 25 μM appeared unchanged (Figure 4.4 C). From this pilot data, 25 μM for 2 hours was chosen as the most effective application for an *in vitro* 6-OHDA model for future experiments.

However, when this concentration and exposure time was used for the next experiment, rather than selectively killing TH^+ cells, a large proportion of β-III-tubulin$^+$/TH^- neurons were also destroyed (Figure 4.4 D). As part of troubleshooting it was suspected that small inconsistencies weighing the small amount of 6-OHDA powder (2.5 mg) needed for each experimental repeat could produce inaccuracies in the concentration of 6-OHDA that cells were exposed to, leading to problems in reproducibility. Therefore, a 100 mM 6-OHDA stock in 85 mM AA vehicle was created, aliquoted and stored at -80℃. All future experiments were drawn from these aliquots ensuring more reliable replication of 6-OHDA treatment, compared to preparing from powder each time.


A
20 mins
2 hours
24 hours
DAPI
TH
Vehicle control
25 µM
50 µM
100 µM
50 µm
500 µm


B
No. TH+ cells
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle control
25 µM
50 µM
100 µM


C
No. DAPI+ nuclei
300
200
100
0
Vehicle control
25 µM
50 µM
100 µM

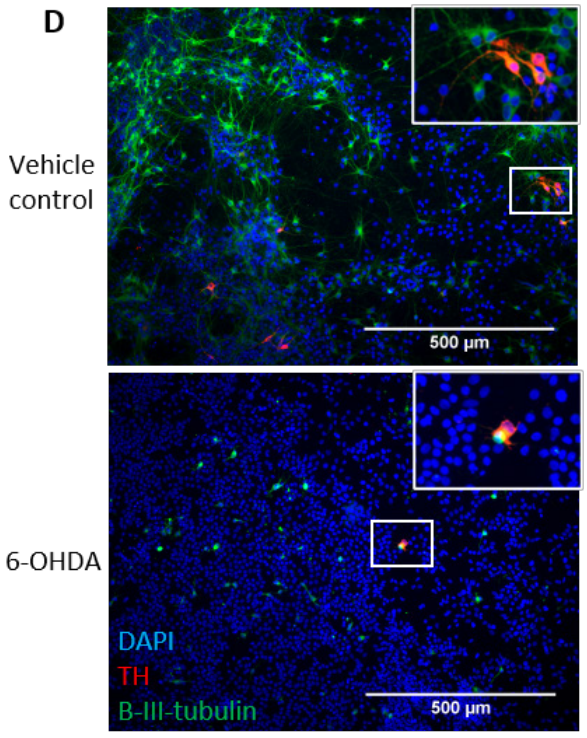

D
Vehicle control
6-OHDA
500 µm
DAPI
TH
B-III-tubulin

Figure 4.4 Pilot test of 6-OHDA toxicity.
A) Representative 10X images showing merge of DAPI and TH immunocytochemistry in VM cultures treated with vehicle control (850 µM AA), or 6-OHDA at concentrations of 25, 50, or 100 µM, for either 20 minutes, 2 hours, or 24 hours. Scale bars hold for all images.
B) Graph showing the average number of TH^+ neurons per 40X micrograph across 6-OHDA treatment conditions for 2 hours.
C) Graph showing the average number of nuclei per 40X micrograph across 6-OHDA treatment conditions for 2 hours. (Graph error bars show SEM of technical replicates).
D) Representative 10X images for vehicle control and 25 µM 6-OHDA treatment for 2 hours. Immunocytochemistry showing DAPI, TH, and β-III-tubulin shows the substantial loss of β-III-$tubulin^+$ neurons in 6-OHDA treated cultures.

As 6-OHDA oxidises in solution it becomes brown/black, due to the production of *p*-quinone, and loses biological effectiveness. To ensure storage was reliable, stock aliquots (100 mM 6-OHDA in 85 mM AA) were left at room temperature and imaged at 1 hour, 48 hours, 1 week and 2 weeks. Only after 2 weeks at room temperature was there a change in colour perceptible by eye (Figure 4.5 A). It was assumed that if thawed 6-OHDA aliquots did not appear transparent and colourless, then degradation had occurred and they would not be used. However, this appearance did not present and no aliquots needed to be discarded.

Stock 6-OHDA was then diluted and tested on VM cultures at 25 µM, 50 µM and 100 µM for 2, 6 and 24 hours. Results showed that 50 µM and 100 µM concentrations, at all time points, reduced TH^+ numbers, with very few remaining across all technical replicates. These concentrations also obviously reduced β-III-$tubulin^+$ and total cells (DAPI) indicating non-specific toxicity (Figure 4.5 B).

To analyse this pilot data, numbers of TH^+/β-III-$tubulin^+$, TH^-/β-III-$tubulin^+$ and TH^-/β-III-$tubulin^-$ cells were quantified from 4 micrographs taken at 20X magnification for 3 technical replicates. These results showed a decrease in the percentage TH^+ neurons of all neurons (i.e. TH^+/β-III-$tubulin^+$ divided by β-III-$tubulin^+$), between vehicle control and 25 µM 6-OHDA treated cultures, at both 2 and 6 hours (Figure 4.5 C). The 6 hour 25 µM treatment also showed a drop in the percentage of β-III-$tubulin^+$ neurons relative to total cells (Figure 4.5 D) suggesting the possibility of non-specific toxicity. It was decided that 25 µM 6-OHDA for 4

hours would be the most suitable option to consistently and significantly reduce TH^+ numbers without causing non-specific neuronal toxicity i.e. a reduction in TH^-/β-III-tubulin$^+$ numbers.

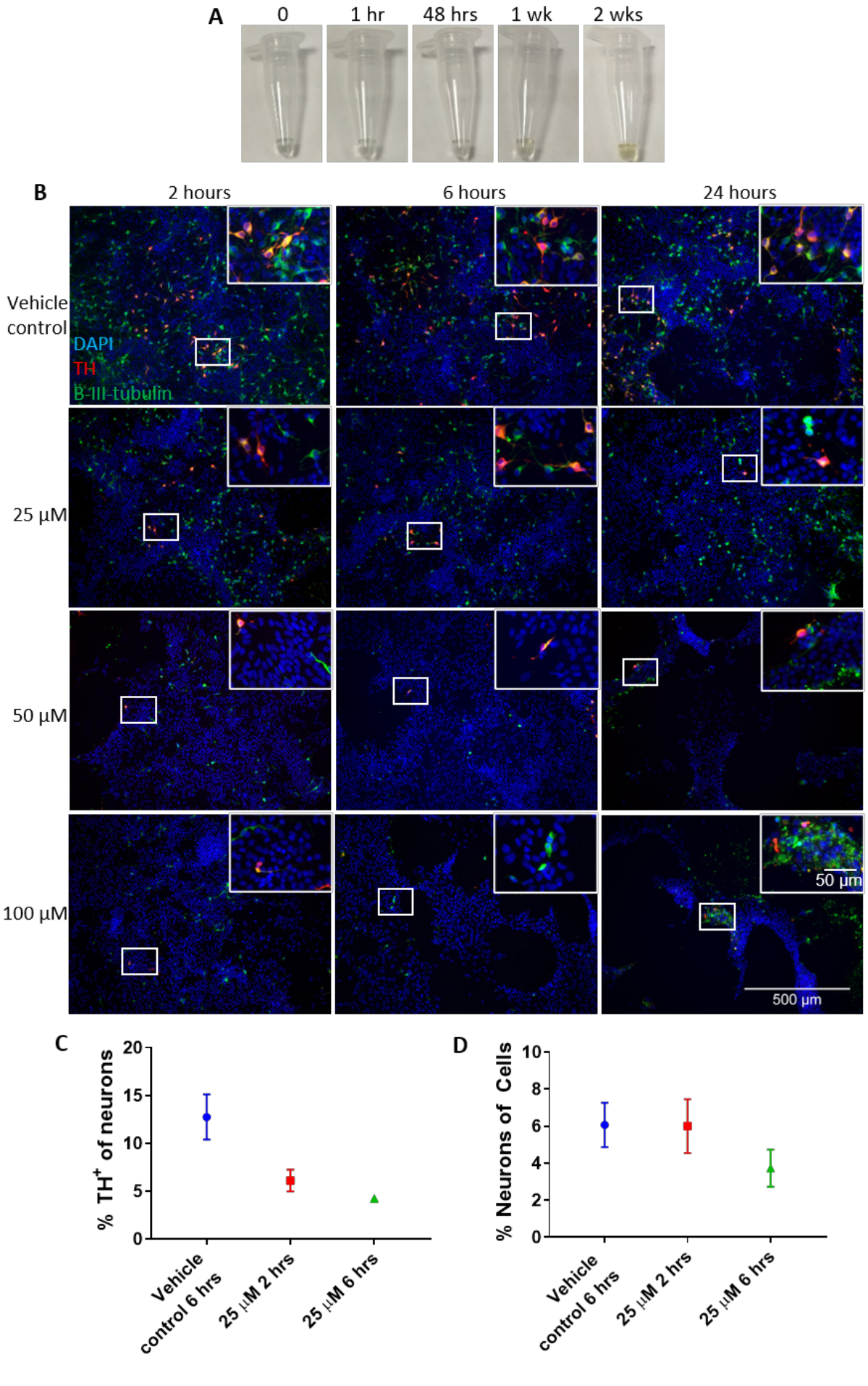

A
0
1 hr
48 hrs
1 wk
2 wks
B
2 hours
6 hours
24 hours
Vehicle control
25 µM
50 µM
100 µM
DAPI
TH
β-III-tubulin
50 µm
500 µm
C
% TH+ of neurons
20
15
10
5
0
Vehicle control 6 hrs
25 µM 2 hrs
25 µM 6 hrs
D
% Neurons of Cells
10
8
6
4
2
0
Vehicle control 6 hrs
25 µM 2 hrs
25 µM 6 hrs

Figure 4.5 Application of 25 µM 6-OHDA (from frozen aliquots) for 4 hours was chosen to create a VM 6-OHDA model.
A) Images of a thawed 6-OHDA aliquot imaged immediately after removal from -80°C storage (0), then 1 hour, 48 hours, 1 week, and 2 weeks left at room temperature. Discolouration was perceptible by eye after 2 weeks.
B) Representative 10X micrographs of VM cultures treated with ascorbic acid vehicle control (850 µM) or 6-OHDA (from freshly thawed aliquot) at concentrations of 25, 50, or 100 µM for 2, 6 or 24 hours. Immunocytochemistry shows DAPI (blue), TH (red), and β-III-tubulin (green). Scale bars hold for all images.
C) Graph showing % TH^+ neurons of total neurons in VM cultures treated with 25 µM 6-OHDA for 2 or 6 hours, compared to cultures receiving vehicle control for 6 hours.
D) Graph showing % β-III-$tubulin^+$ neurons of total cells in VM cultures treated with 25 µM 6-OHDA for 2 or 6 hours, compared to cultures receiving vehicle control for 6 hours. Error bars represent SEM between technical replicates.

4.3.2 Numbers of TH^+ cells in VM cultures across 14 DIV were higher with nicotinamide treatment

In images taken at 40X magnification, DAPI counts showed that nicotinamide treatment was associated with significantly lower numbers of cells in VM cultures at 7 DIV compared to control cultures (220.1 ± 17.81 vs. 283.1 ± 18.8; *t*-test $p < 0.05$; Figure 4.6). This prompted a more thorough characterisation of other cell types ($GFAP^+$ astrocytes, $Nestin^+$ neural stem cells, $Iba1^+$ microglia) present in these cultures. Analyses of VM cultures across multiple experiments were compiled to estimate cell populations, details are described in Chapters 5.2.1 and 5.3.1. This analysis found that TH^+ cells make up approximately 0.66% of control cultures and 1.2% of nicotinamide treated cultures.

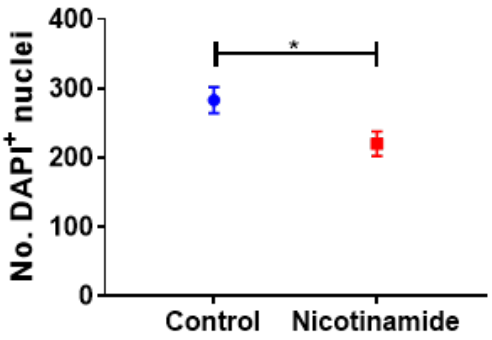


Figure 4.6 Numbers of $DAPI^+$ nuclei were lower in nicotinamide treated VM cultures. Graph showing the average number of $DAPI^+$ nuclei quantified in control (blue circle) and 10 mM nicotinamide (red square) treated VM cultures Nicotinamide treated cultures had significantly lower numbers of nuclei than control after 7 days *in vitro*, * $p < 0.05$. (FOI = 0.073 mm^2).

Micrographs taken at 10X magnification were used to assess numbers of TH^+ neurons within cultures at 1, 4, 7 and 14 DIV. Numbers showed variation across experimental repeats, therefore, the average number of TH^+ cells was normalised to the control at 1 DIV, within each experiment. The number of TH^+ cells varied with both nicotinamide treatment (two-way ANOVA $F_{(1, 78)} = 27.6$, $p < 0.001$) and time in culture (two-way ANOVA $F_{(3, 78)} = 37.16$, $p < 0.001$), with no interaction effects detected (two-way ANOVA $F_{(3, 78)} = 0.69$, n.s.). Post-hoc analysis using Bonferroni's multiple comparison revealed significantly higher numbers of TH^+ neurons in nicotinamide treated cultures compared to control cultures at 1 DIV (166.6 ± 11.3 vs. 100.0 ± 5.7; $p < 0.05$), 4 DIV (302.1 ± 28.2 vs. 206.3 ± 19.9; $p < 0.01$), and 7 DIV (212.4 ± 21.2 vs. 145.0 ± 25.2; $p < 0.05$) with no significant difference at 14 DIV (87.3 ± 14.4 vs. 43.4 ± 9.5; n.s.; Figure 4.7).

Post-hoc analysis using Tukey's multiple comparisons revealed significant differences within control cultures between 1 and 4 DIV (100.0 ± 5.7 vs. 206.3 ± 19.9; $p < 0.001$), and between 7 and 14 DIV (145.0 ± 25.2 vs. 43.4 ± 9.5; $p < 0.01$). In nicotinamide treated cultures, significant differences were found between 1 and 4 DIV (166.6 ± 11.3 vs. 302.1 ± 28.2; $p < 0.001$), between 4 and 7 DIV (302.1 ± 28.2 vs. 212.4 ± 21.2; $p < 0.01$), and between 7 and 14 DIV (212.4 ± 21.2 vs. 87.3 ± 14.4; $p < 0.001$; Figure 4.7).

The morphology of TH^+ cells appeared to change over time in culture (Figure 4.8) with cells exhibiting small somas with few, short processes at 1 DIV. At 4 DIV, TH^+ cells showed a neuronal morphology with small somas and extending, complex processes. This morphology was maintained through to 14 DIV with some TH^+ neurons showing extensive neurites. The addition of nicotinamide to cultures did not appear to effect TH^+ neuron morphology although no quantitative analysis was carried out.

The above results show that TH^+ cell numbers increased between days 1 and 4, and then decreased between days 4-14 in both control and nicotinamide treated VM cultures.

Also, VM cultures treated with nicotinamide contained significantly higher numbers of TH^+ cells than control cultures at days 1, 4 and 7, but not at day 14.

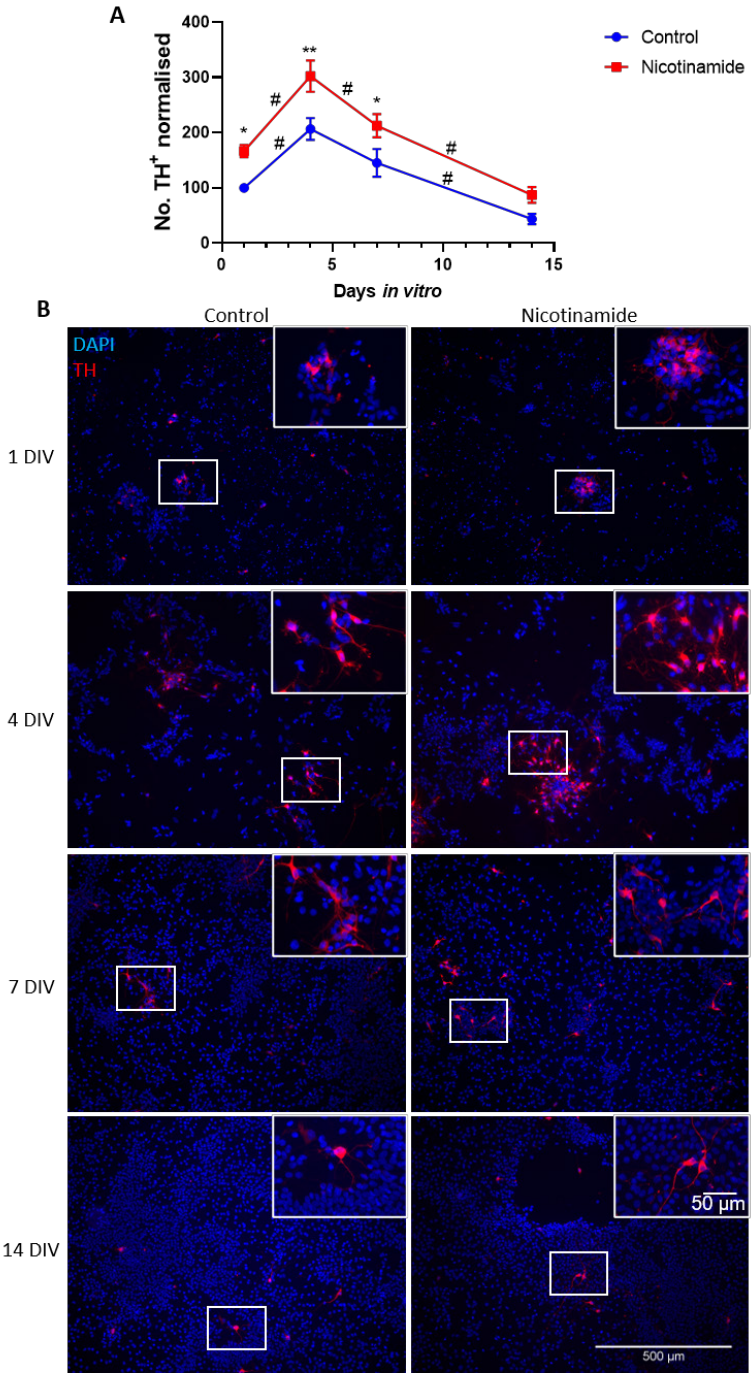


Figure 4.7 Numbers of TH^+ neurons were higher in nicotinamide treated cultures and changed significantly over days *in vitro* (DIV). A) Graph showing the average number of TH^+ cells per 10X micrograph across days 1, 4, 7, and 14 DIV. * Numbers of TH^+ cells were significantly higher in nicotinamide treated cultures (red square) compared to control (blue circle) at 1, 4, and 7 DIV. # Numbers of TH^+ cells also changed across DIV in treatment conditions with a significant increase in TH^+ numbers between days 1 and 4, in both control and nicotinamide treated cultures, a significant decrease was observed between 4 and 7 DIV in nicotinamide treated cultures, and a significant decrease between 7 and 14 in both control and nicotinamide cultures. * $p < 0.05$; ** $p < 0.01$; # $p < 0.05$. B) Representative 10X micrographs of VM cultures in control and nicotinamide treatment conditions across 1, 4, 7, and 14 DIV. Scale bars hold across images.

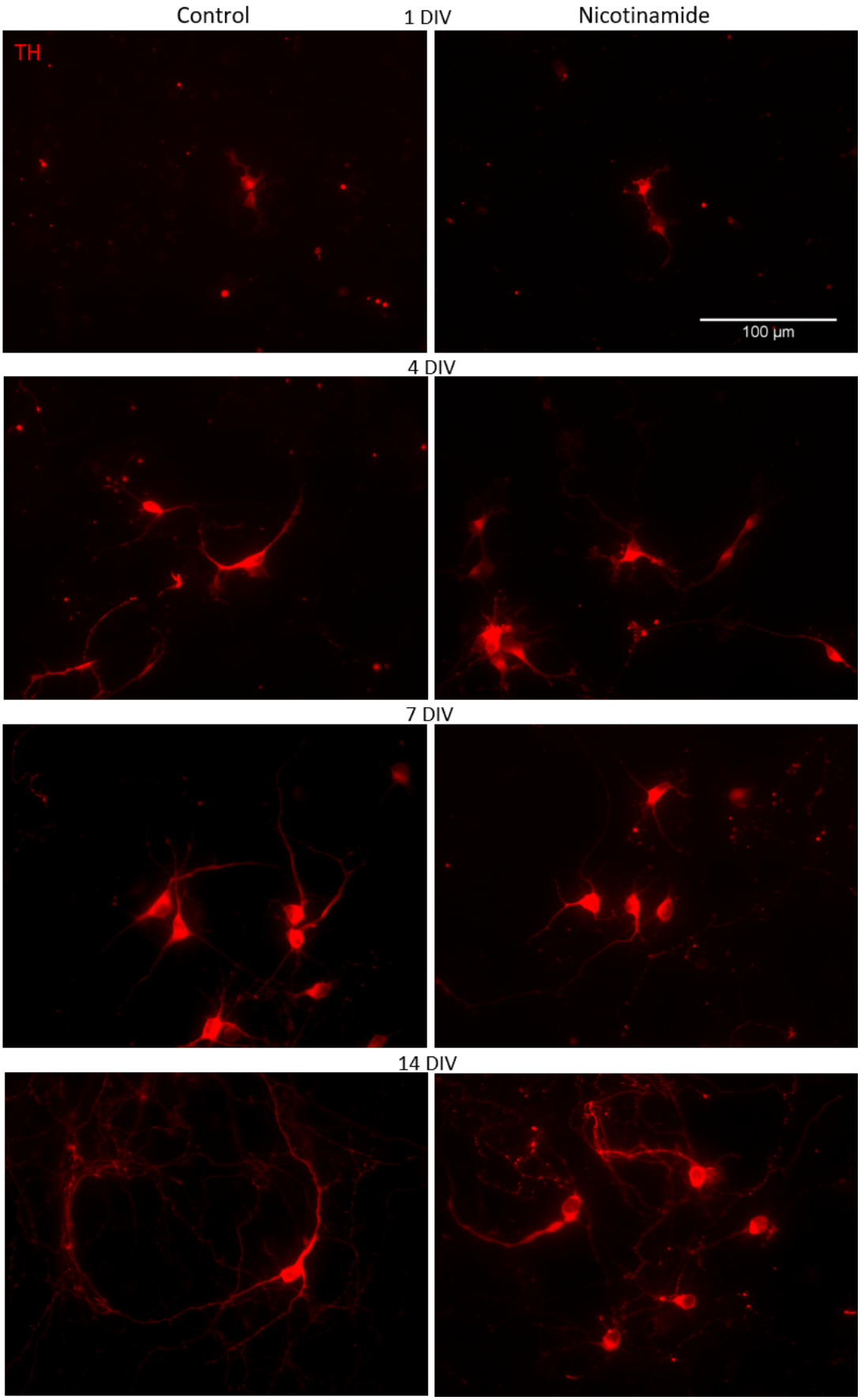


Figure 4.8 Morphology of TH^+ neurons changed over days *in vitro* (DIV). Micrographs showing the morphology of TH^+ cells at days 1, 4, 7, and 14 DIV in both control and nicotinamide treated VM cultures. Scale bar holds across all images.

4.3.3 Numbers of TH^+ cells were also higher in nicotinamide-treated AA vehicle control cultures

For the 6-OHDA treatment experiments, control cultures at 7 DIV were treated for 4 hours with the vehicle used to carry the 6-OHDA, namely NCM containing 850 µM AA. These cultures are referred to as vehicle controls below. Total numbers of TH^+ neurons were counted across 4 micrographs taken at 10X magnification from each technical replicate across 4 experimental repeats. Due to variation across experimental repeats, TH^+ cell counts were normalised to the vehicle control within each experimental repeat.

Firstly, the effects of nicotinamide were investigated within vehicle treated cultures. Cultures treated with nicotinamide and AA vehicle contained significantly more TH^+ cells than control cultures with vehicle (154.2 ± 13.7 vs. 100.0 ± 10.5; $t = 3.15$, $p < 0.01$; Figure 4.9 A).

Numbers of β-III-tubulin$^+$ neurons were also analysed across 10X micrographs and normalised to control due to variation across experimental repeats. Results showed no statistically significant difference in β-III-tubulin$^+$ numbers between vehicle control and nicotinamide treated cultures (100.0 ± 4.1 vs. 90.8 ± 4.3; $t = 1.54$, n.s.; Figure 4.9 B).

These results show that the application of 10 mM nicotinamide also increased numbers of TH^+ neurons within these AA vehicle treated VM cultures, with no significant effects on numbers of β-III-tubulin$^+$/TH^- cells.

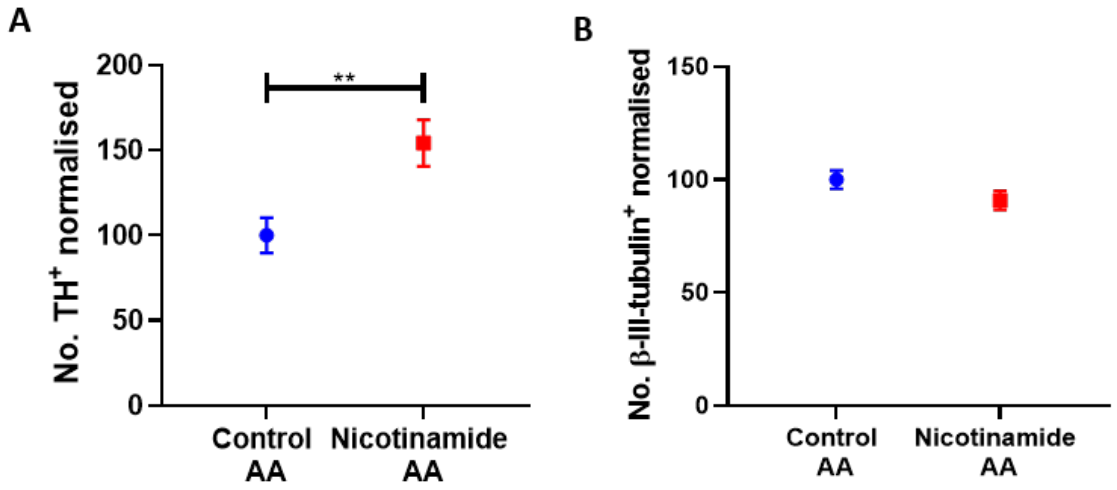


Figure 4.9 Nicotinamide treatment increased numbers of TH^+ neurons, but not total β-III-tubulin$^+$ neurons.
A) Graph showing the number of TH^+ neurons in control (blue circle) and nicotinamide (red square) treated cultures at 9 DIV (with addition of AA, the vehicle control for 6-OHDA). Nicotinamide treated cultures contained significantly higher numbers of TH^+ neurons; ** $p < 0.01$.
B) Graph showing the number of β-III-tubulin$^+$ neurons in control and nicotinamide treated cultures at 9 DIV (with addition of AA, the vehicle control for 6-OHDA).

4.3.4 Calcitriol did not increase numbers of TH^+ cells in cultures

To assess the effects of calcitriol alone and in combination with nicotinamide on TH^+ neurons, total numbers of TH^+ cells were counted across 4 images taken at 10X magnification for each experimental repeat. Numbers were normalised to the vehicle control as described in Chapter 2.11. Results showed no significant difference in the number of TH^+ cells in VM cultures between control and calcitriol alone (Figure 4.10, A). There was also no significant difference in numbers of TH^+ cells in VM cultures treated with nicotinamide alone and nicotinamide in combination with calcitriol when normalised to control (Figure 4.10 A). These results were unexpected due to previous studies showing a positive effect of calcitriol on numbers of TH^+ neurons in VM cultures derived from E12 rat (Orme, Bhangal and Fricker, 2013).

Calcitriol is reported to be air and light sensitive (Sigma-Aldrich, 2009). It was possible that this lack of increase in TH^+ cells could be due to a reduction in biological activity of this

batch of calcitriol over time in storage. Therefore, a new batch of calcitriol was generated. Normalised numbers of TH^+ cells were compared between calcitriol batch 1 (n=3, 4 technical replicates) and calcitriol batch 2 (new calcitriol, n=1, 3 technical replicates). Results showed no statistically significant difference in numbers of TH^+ cells across vehicle control and calcitriol batch 1 or batch 2 (100.0 ± 10.4 vs. 87.4 ± 8.3 vs. 73.9 ± 21.1; one way ANOVA $F_{(2,23)}$ = 0.93, n.s.; Figure 4.10 B).

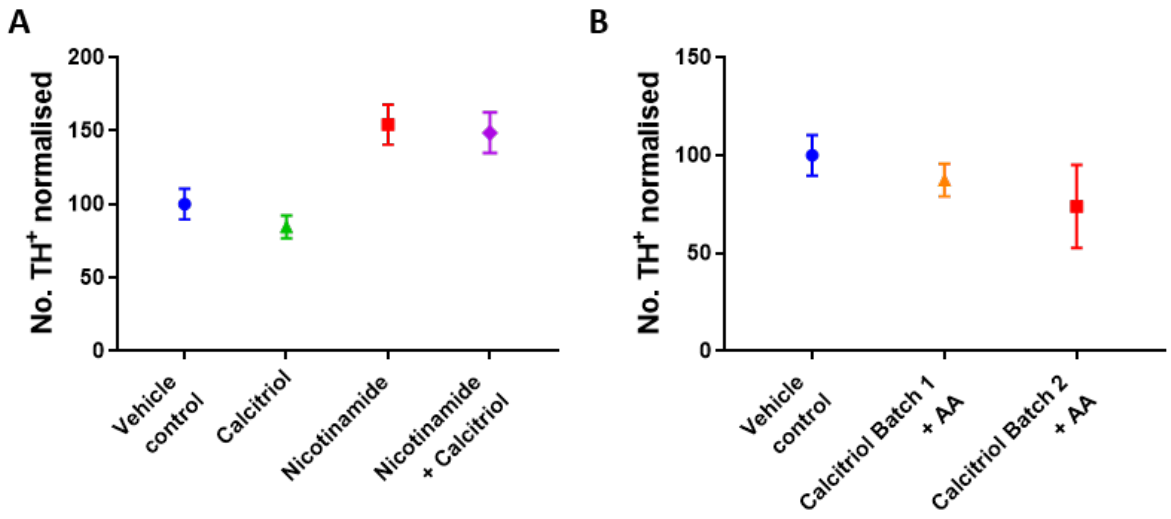


Figure 4.10 Addition of calcitriol did not alter numbers of TH^+ neurons in VM cultures.
A) Graph showing the number of TH^+ neurons normalised to the vehicle control (blue circle), for calcitriol (green triangle), nicotinamide (red square), and combined nicotinamide and calcitriol (purple diamond). No significant difference was observed between control vs. calcitriol, or nicotinamide alone vs. nicotinamide and calcitriol treated cultures.
B) Graph showing the numbers of TH^+ neurons normalised to the vehicle control (blue circle), for batch 1 (orange triangle) and batch 2 (red square) of calcitriol. No significant differences were observed.

4.3.5 Neither nicotinamide nor calcitriol protected TH^+ neurons against 6-OHDA toxicity

The potential protective effects of nicotinamide and/or calcitriol were tested using the 6-OHDA model described in Chapter 2.5.2. As previously described, TH^+ cell numbers were counted in 10X micrographs and normalised to vehicle control. Two-way ANOVA revealed significant effects on numbers of TH^+ cells due to vitamin treatment ($F_{(3, 111)} = 8.7$, $p < 0.001$), 6-OHDA treatment ($F_{(1, 111)} = 107.4$, $p < 0.001$), and with an interaction effect ($F_{(3, 111)} = 4.46$, p

< 0.01). Post-hoc analysis using Bonferroni's multiple comparisons showed significant decreases in numbers of TH^+ cells due to 6-OHDA treatment in control (AA vs. 6-OHDA: 100.0 ± 10.5 vs. 49.1 ± 7.1, $p < 0.01$), nicotinamide (154.2 ± 13.7 vs. 46.1 ± 10.0, $p < 0.001$), calcitriol (84.4 ± 7.7 vs. 38.8 ± 5.0, $p < 0.01$), and combined nicotinamide-calcitriol (148.6 ± 13.8 vs. 62.0 ± 8.0, $p < 0.001$) treated cultures (Figure 4.11 A). These results show that neither nicotinamide nor calcitriol were able to protect TH^+ neurons from 6-OHDA toxicity. There was no significant difference in TH^+ numbers between 6-OHDA treated control, nicotinamide, calcitriol, and combined nicotinamide and calcitriol treated cultures; despite elevated TH^+ numbers in nicotinamide and combined nicotinamide-calcitriol treated vehicle cultures, and no change in calcitriol treated vehicle cultures.

As shown in Figure 4.6, the total number of cells was decreased at 7 DIV in nicotinamide treated cultures. This may affect the toxicity of 6-OHDA in nicotinamide treated cultures. Therefore, the protective potential of 10 mM nicotinamide was also tested when applied only during 6-OHDA treatment and recovery period (i.e. not applied prior to 6-OHDA treatment). One-way ANOVA showed no significant difference between control 6-OHDA, nicotinamide 6-OHDA, or 6-OHDA + nicotinamide with no nicotinamide pre-treatment (Figure 4.11 B).

These results show that 6-OHDA application significantly reduced TH^+ cells numbers in these VM cultures and that the application of the vitamins nicotinamide and calcitriol, both alone and in combination, were unable to prevent this 6-OHDA induced decrease in TH^+ cell numbers.

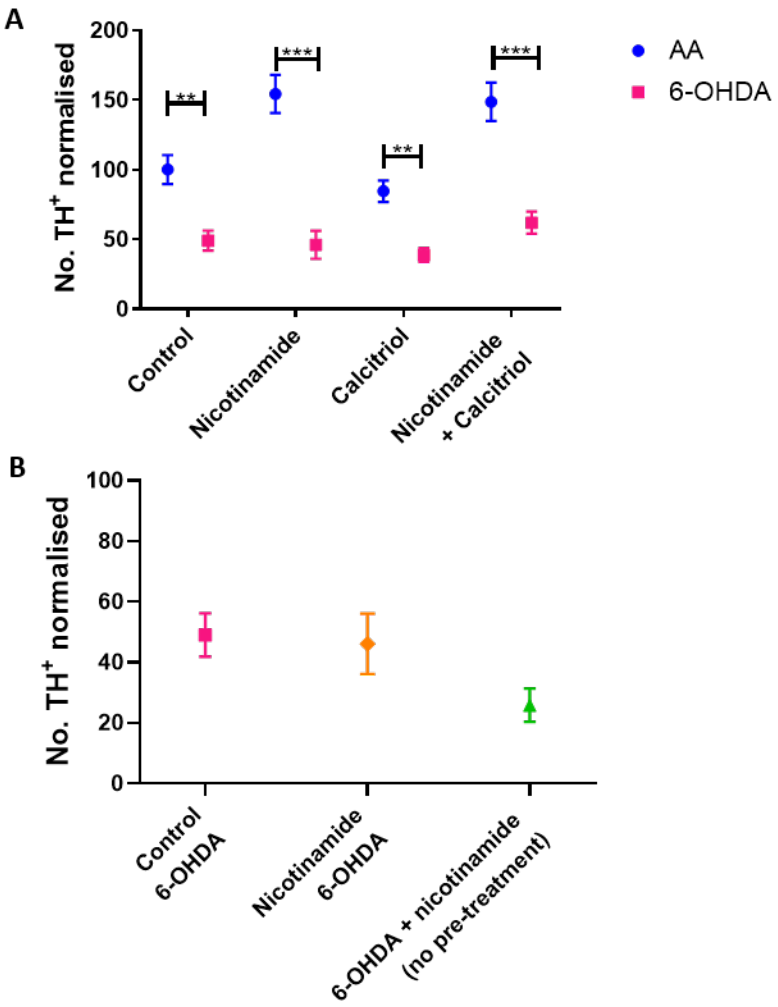


Figure 4.11 6-OHDA treatment reduced numbers of TH$^+$ neurons.
A) Graph showing the number of TH$^+$ neurons normalised to the control in AA vehicle (blue circle) vs. 6-OHDA (pink square treatment across vitamin treatments. Significantly lower numbers of TH$^+$ neurons were observed in 6-OHDA treated cultures compared to AA in all vitamin treatment conditions (nicotinamide, calcitriol, combined nicotinamide and calcitriol); ** $p < 0.01$; *** $p < 0.001$.
B) Graph showing the number of TH$^+$ neurons normalised to the control in 6-OHDA cultures with no nicotinamide (control; pink square), nicotinamide from day 0 (orange diamond), and nicotinamide during and after 6-OHDA treatment (green triangle). No significant differences were observed. AA: ascorbic acid; 6-OHDA: 6-hydroxydopamine.

4.3.6 Application of 6-OHDA also reduced numbers of β-III-tubulin$^+$ cells

The effect of 6-OHDA on β-III-tubulin$^+$ cells was also assessed. For this, the number of β-III-tubulin$^+$ cells in images taken at 10X magnification was quantified and normalised to control as described in Chapter 2.11. Two way ANOVA showed differences in β-III-tubulin$^+$

numbers related to 6-OHDA ($F_{(1, 79)}$ = 135.2, p <0.001) and vitamin ($F_{(3, 79)}$ = 5.82, p < 0.01) treatments, but with no interaction effects (Figure 4.12).

Post-hoc analysis using Bonferroni's multiple comparison revealed significant decreases in the number of β-III-tubulin$^+$ cells in 6OHDA treated cultures compared to AA treated cultures in control (54.1 ± 6.4 vs. 100.0 ± 4.1, p < 0.001), nicotinamide (23.8 ± 8.2 vs. 90.8 ± 4.3, p < 0.001), calcitriol (41.4 ± 6.6 vs. 100.0 ± 7.3, p < 0.001), and nicotinamide-calcitriol combined (33.4 ± 6.0 vs. 74.3 ± 7.8, p < 0.001) treated cultures (Figure 4.12 *).

A significant effect of vitamin treatment was also observed. Post-hoc analysis using Tukey's multiple comparison revealed significantly lower numbers of β-III-tubulin$^+$ cells in nicotinamide-calcitriol combined compared to control (74.3 ± 7.8 vs. 100.0 ± 4.1, p <0.05) and nicotinamide-calcitriol combined compared to calcitriol (74.3 ± 7.8 vs. 100.3 ± 7.3, p < 0.05) in AA vehicle treated cultures. Significantly lower β-III-tubulin$^+$ cell numbers were also observed between control and nicotinamide treated cultures with 6-OHDA application (54.1 ± 6.4 vs. 23.8 ± 8.2, p < 0.01; Figure 4.12 #). However, with 6-OHDA treatment, no significant difference was observed in numbers of β-III-tubulin$^+$ neurons between calcitriol and nicotinamide-calcitriol combined compared to control. This suggests a slight neuroprotective effect of calcitriol on β-III-tubulin$^+$ neurons from 6-OHDA toxicity, compared to treatment with nicotinamide.

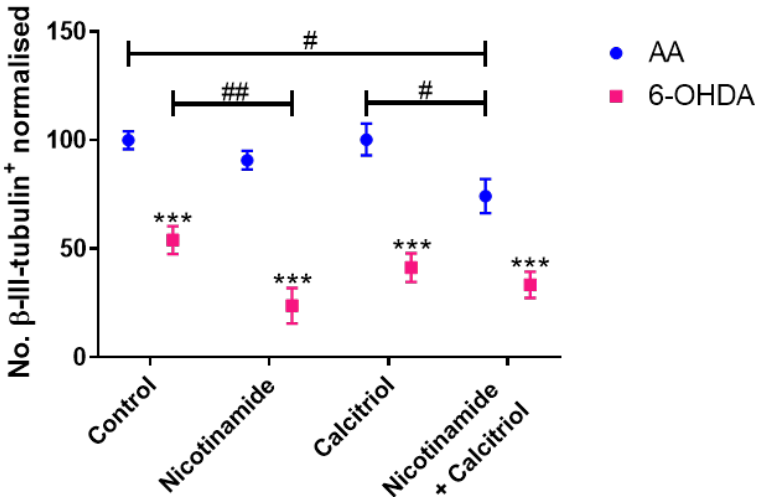


Figure 4.12 6-OHDA treatment reduced numbers of β-III-tubulin$^+$ neurons. Graph showing numbers of β-III-tubulin$^+$ cells normalised to control AA. *Significantly lower numbers of β-III-tubulin$^+$ cells were observed with 6-OHDA (pink square) application compared to AA vehicle (blue symbols) in control, nicotinamide, calcitriol, and combined nicotinamide-calcitriol cultures. #AA vehicle cultures showed significantly lower β-III-tubulin$^+$ cell numbers in combined nicotinamide-calcitriol cultures compared to control and calcitriol alone. 6-OHDA treated cultures showed significantly lower numbers of β-III-tubulin$^+$ cells in nicotinamide compared to control cultures. (***p <0.001; # p < 0.05; ## p < 0.01).

Treatment of cultures with 6-OHDA did not affect the average numbers of cells, counted by DAPI staining at 40X magnification (FOI = 0.073 mm^2), compared to AA in vehicle control or nicotinamide treated cultures (Figure 4.13). These results show that treatment of cultures with 6-OHDA significantly reduced the number of β-III-tubulin$^+$ cells but did not significantly reduce total cell numbers. As previously discussed in Chapter 4.3.2, the number of DAPI$^+$ nuclei was significantly lower in nicotinamide treated cultures compared to control in AA vehicle cultures (221 ± 23.7 vs. 309 ± 23.4), an effect that was also replicated in 6-OHDA treated cultures (189 ± 12.8 vs. 305 ± 13.6). A lack of effect observed in calcitriol treated cultures meant that micrographs for DAPI analysis were only collected for an n of 2, therefore no statistical analysis was carried out.

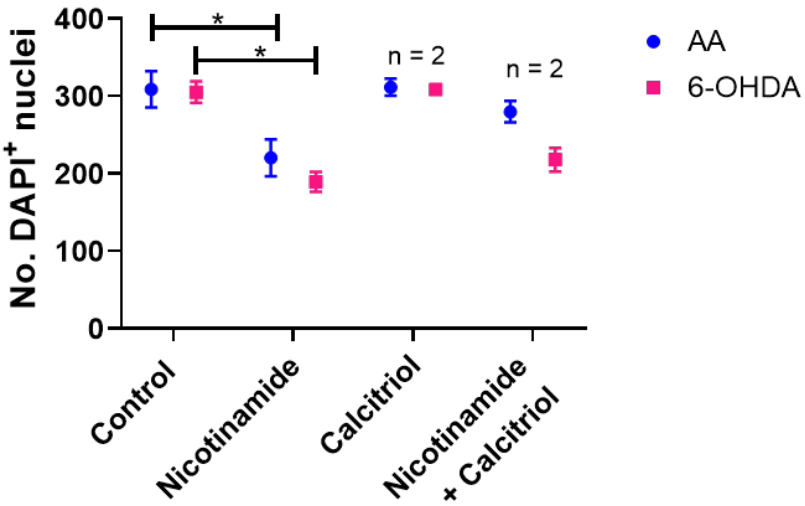


Figure 4.13 Numbers of DAPI$^+$ nuclei were not changed by 6-OHDA treatment. Graph showing average number of DAPI$^+$ nuclei (per 40X micrograph FOI = 0.073 mm^2) in AA vehicle (blue symbol) and 6-OHDA (pink symbol) in control, nicotinamide, calcitriol, and combined nicotinamide-calcitriol treated cultures. No significant difference was observed between AA vehicle and 6-OHDA treatment within control and nicotinamide treated cultures. However, significantly lower numbers of DAPI$^+$ nuclei were observed in nicotinamide treated cultures to control within AA vehicle and 6-OHDA treatments ($p < 0.05$). No statistical analysis was performed on calcitriol and combined nicotinamide-calcitriol cultures as cell counts were only carried out on an n of 2.

4.4 Discussion

4.4.1 Optimising the 6-OHDA *in vitro* model

The results of 6-OHDA toxicity *in vitro* can be highly variable. As discussed in Chapter 4.1.2, the rapid auto-oxidation, and variability of this process dependent on molecular availability, can make 6-OHDA an unpredictable chemical to work with. Another source of variation can arise from the accuracy with which midbrain regions are identified and dissected, and the growth profiles of subsequent cultures.

The final *in vitro* 6-OHDA PD model protocol established and used in this PhD research utilised E14 VM cultures grown for 7 DIV, treated with 25 µM 6-OHDA in an 850 µM AA vehicle for 4 hrs, with cultures fixed 48 hours after removal of the toxin. Results showed a 50% decrease in TH^+ neurons present within cultures treated with 6-OHDA compared to vehicle control. This protocol shares similarities with other 6-OHDA *in vitro* models cited in the literature (Table 4.1). A 50% decrease with 6-OHDA treatment should provide a reasonable testbed for detecting effects of experimental treatments, whether protective, or toxic.

Rat VM from E14-16 is commonly used, and typically cultured for 6-9 DIV before treatment with 6-OHDA. VM cultures contain TH^+ cells/progenitors from the developing SN and VTA. Dissection of the VM at E14 allows for larger numbers of TH^+ cells to be generated in culture as the cells are less developed and can better survive the dissection and culturing process. However it does mean the inclusion of developing VTA TH^+ neurons which are less susceptible to damage than TH^+ cells from the SN (Grant and Clarke, 2002; Healy-Stoffel *et al.*, 2014; Alberico, Cassell and Narayanan, 2015). This could account for the TH^+ population that was resistant to 6-OHDA toxicity found throughout all treatment conditions in preliminary experiments. This type of pattern was also observed by Lotharius et al. (1999) using E14 mouse VM, with a plateau in TH^+ cell numbers at around 70% lower than control despite more than

tripling the 6-OHDA concentration, which generated non-specific toxicity. To avoid the inclusion of VTA dopamine neurons, Ding et al. (2004) used P0 tissue from the SN, meaning that their TH^+ cell population was likely derived solely from the SN. Although this technique may provide a more accurate representation of TH^+ SN neurons, it is a more complicated model to derive and dopaminergic viability may be compromised due to axotomy prior to toxin exposure.

In the literature (Table 4.1), 6-OHDA is typically used at concentrations between 10 and 100 µM. Therefore, the 25 µM concentration used here is reasonable in comparison. Most studies utilise a longer application time, typically 24-48 hours, compared to the 4-hour treatment window used here. However, the majority of studies fix or analyse cells immediately after 6-OHDA exposure, with no recovery window equivalent to the 48 hours used in this research. Rationale for inclusion of a recovery window include a model that may resemble PD more closely, with a singular toxic insult followed by neurodegeneration, compared to prolonged exposure alongside neurodegeneration. It also allows for studying potential neuroprotective effects before, during, and after toxin exposure, rather than neuroprotective effects that can arise from pre-treatment, a treatment method that would be difficult to apply clinically due to the pre-clinical aspect of PD (Chapter 1.1.3). A short exposure combined with removal of the toxin may also reduce non-specific toxicity. Ding et al. (2004) relied on a short time frame (15 minutes) to allow the uptake of 6-OHDA into catecholaminergic neurons, with removal of non-internalised toxin before auto-oxidation within the media and consequently, non-specific toxicity. Cultures were fixed up to 48 hours after removal of 6-OHDA, allowing cells to succumb to toxicity induced by internalised 6-OHDA. In this method, Ding *et al.*, (2004) abolished 6-OHDA toxicity by blocking DAT and preventing internalisation of 6-OHDA. This shows that cell death in their cultures resulted from 6-OHDA toxicity occurring within the cell.

In this PhD research, using the short time frame of 20 minutes 6-OHDA exposure did not induce sufficient loss of TH^+ cells after a further 48 hours *in vitro*. The use of this time frame was also considered to be potentially inconsistent due to pipetting limitations. Application of 25 µM for just 6 hours showed non-specific toxicity of TH^-/β-III-tubulin$^+$ neurons. A number of models within the literature fix cells immediately, but after a longer period of treatment (6-OHDA exposure). This means that extracellular toxicity arising from auto-oxidation of 6-OHDA occurs throughout treatment, and may affect all cell types, rather than restricting toxicity to cells that have internalised it (predominantly DAT^+ cells). Such non-specific toxicity could arise from the formation of toxic compounds such as H_2O_2, *p*-quinone, and ROS within the media, external to cells. For instance Lotharius et al. (1999) found non-specific neurotoxicity at early timepoints after 6-OHDA treatment for 6 hours. Also, Cerruti *et al.* (1993) found a reduction, but not abolition, of 6-OHDA (10 nM for 48 hours) toxicity in TH^+ neurons when DAT was blocked suggesting toxicity arising externally. Only a small proportion of papers report on non-specific toxicity, and in many cases the effects of 6-OHDA on TH^- neurons and non-neuronal cells are not discussed (Table 4.1).

Results from this thesis showed a 50% reduction in TH^+ neurons after 6-OHDA treatment. This reduction was deemed appropriate to study the potential for neuroprotection with a statistically significant decrease in TH^+ neurons that could potentially be rescued by addition of a neuroprotective agent. Other 6-OHDA *in vitro* models have shown reductions in TH^+ populations between 30-90% (Table 4.1).

Reliable replication of 6-OHDA toxicity was not initially possible in this PhD research. This was proposed to be due to the difficultly in reliably weighing the small amount of 6-OHDA powder on each repeat. Similar variability in neuronal response to 6-OHDA treatment was also observed by Ding et al., (2004), who hypothesise that this is due to variations in the actual amount of 6-OHDA in the culture medium. The difficult nature of using *in vitro* 6-OHDA models

can be seen by the variety of methods described in the literature, differing in culture type, concentration of 6-OHDA and exposure time, and the resulting decrease in TH^+ neurons observed. Many reports do not state whether a vehicle was used to decrease auto-oxidation of 6-OHDA, do not declare their starting TH^+ population size, and fail to comment on the absence or presence of non-specific toxicity. Therefore, establishing a protocol for use in this PhD research was necessary.

This protocol and the outcome are comparable to other 6-OHDA *in vitro* models within the literature and was suitable for this research. Although improvements could be made in the selection of SN specific TH^+ neurons and in the reduction of non-specific toxicity, these elements are present across the literature and are difficult to avoid.

4.4.2 Nicotinamide increased numbers of TH^+ neurons

In this thesis, TH was used to identify dopaminergic neurons. However, TH, the rate limiting enzyme required in the synthesis of dopamine (Figure 1.4) is also present in other catecholaminergic neurons (adrenaline, noradrenaline). Adrenaline and noradrenaline neurons arise in the rhombencephalon (Goridis and Rohrer, 2002; Robertson *et al.*, 2013) and therefore should not have been included in VM dissection, allowing for the assumption that the TH^+ cells in these cultures were dopaminergic. TH is a commonly used and reliable marker to identify dopaminergic neurons, especially within VM cultures (Table 4.1). To further confirm dopaminergic neuron populations other markers including DAT, VMAT, Calbindin and GIRK2 could have been used. This would also assist in the identification of SNpc and VTA dopaminergic neurons, further assisting in the translation of results to PD.

Protocols for VM dissection cite TH^+ populations ranging from 'up to 1%' (Michel and Hefti, 1990; Liu and J. Hong, 2003), 2-4% (Gaven, Marin and Claeysen, 2014), to 17-21% (Shimoda *et al.*, 1992) of the total cell population. This variability arises from different

techniques in the obtainment of tissue and culture conditions (such as media) used. This thesis reports a TH^+ population of approximately 0.66% of total cells in control cultures, increasing to 1.2% with nicotinamide treatment. These numbers are low compared to some reported in the literature. This lower number could be the result of the manual dissociation process which can damage dopaminergic cells (Pruszak *et al.*, 2009). However, reducing the intensity of the manual dissociation process led to clumped populations, with β-III-tubulin$^+$ and TH^+ neurons typically growing within spheres, making visual analysis of cell populations more difficult and less accurate (Figure 4.14). Therefore, it was deemed more appropriate to have cultures with a lower dopaminergic density, but that could be analysed effectively. Although avoiding clumped areas during imaging allowed for more accurate analysis of cells within each FOI, it may have led to bias against imaging of neuronal populations: neuronal densities may have been greater in areas that were rejected on the grounds of being difficult to distinguish individual cells. However, the TH^+ populations observed in these VM cultures were still sufficient to detect changes across treatment conditions.

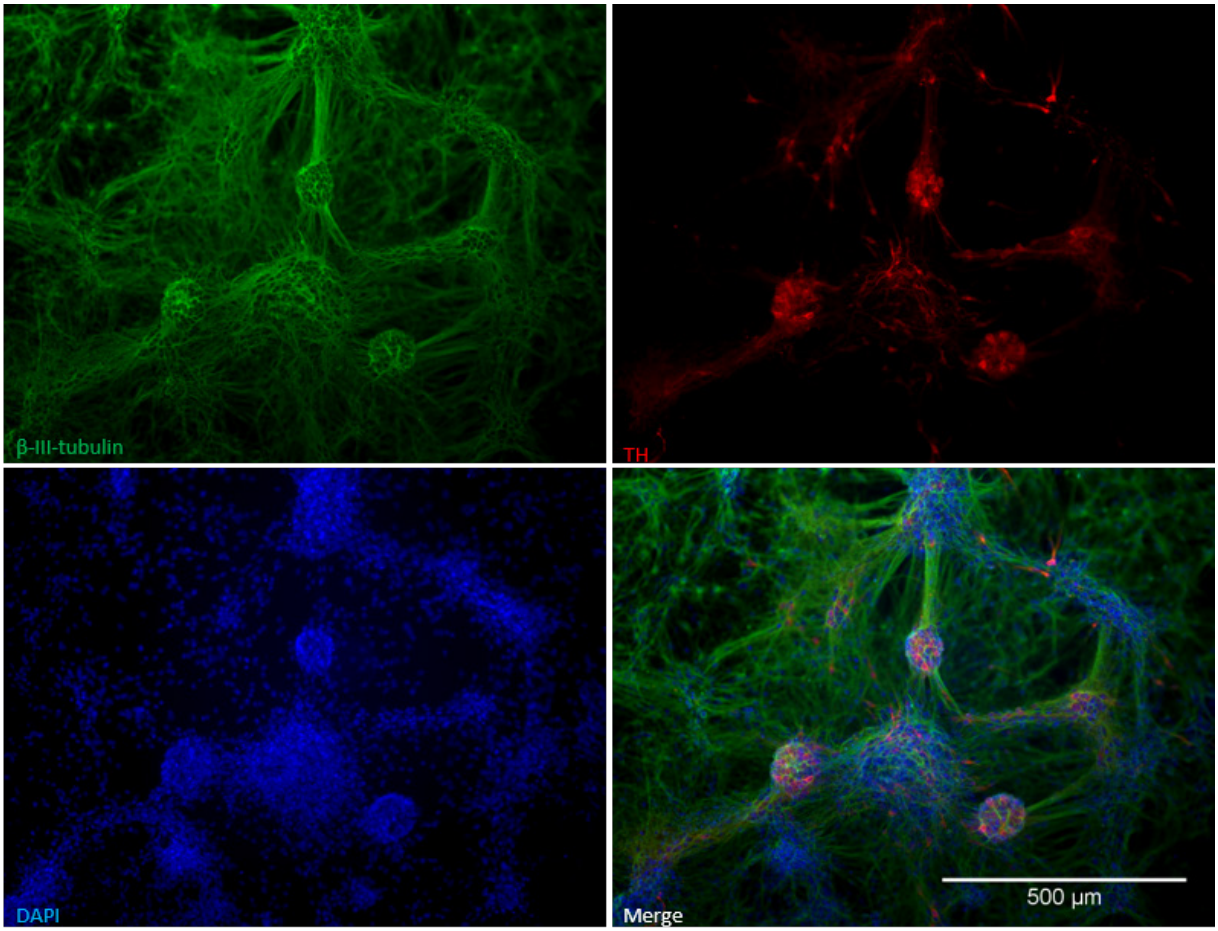


Figure 4.14 Neurons, including TH^+ neurons, tend to grow in clumps or spheres. Fluorescence micrographs of VM cultures with immunocytochemistry for β-III-tubulin (green), tyrosine hydroxylase (TH; red), and DAPI (blue).

Variations in tissue selection (Shimoda *et al.*, 1992), media components, seeding densities, addition of growth factors and decreasing oxygen conditions can be utilised to increase the TH^+ yield (Pruszak *et al.*, 2009). These additional factors were not explored as this thesis focussed on the effects of nicotinamide and calcitriol on the TH^+ populations within these VM cultures. Using a basic medium (NCM) allowed for clarification that the effects were due to the addition of these vitamins, although combining these vitamins with growth factors may provide further increases in TH^+ yield and could be explored for the generation of high yield dopaminergic populations for use in *in vitro* models.

To address the low TH^+ yield found here, different imaging techniques were applied to accurately analyse the TH^+ population. The most effective imaging method to assess the

population spread of TH^+ neurons in these VM cultures was to use a larger FOI for analysis by imaging cultures at 10X magnification.

The number of TH^+ neurons in these VM cultures increased between 1-4 DIV, then decreased between days 4-14 in both control and nicotinamide treatment conditions. This initial increase in TH expression is also reported within the literature. Pruszak et al. (2009) state that maturing dopamine cultures can be observed from 1 to 3 DIV when using E14 rat VM tissue. Gaven, Marin, & Claeysen (2014) found the presence of TH^+ cells in VM cultures derived from E13.5 mouse from 1 DIV with numbers increasing up to 6 DIV, numbers then plateaued until 13 DIV. Bollimpelli & Kondapi (2015) found TH^+ neurons from 3 DIV with maximum expression of TH between 7 and 14 DIV using E14 rat VM, also showing an initial increase in TH expression. Shimoda et al. (1992), using stringent dissection of what they term the mesencephalic dopaminergic region of E14 rats, reported their highest TH^+ densities at 5 DIV, with the percentage of TH^+ cells remaining between 17% and 21% from 4 hours after plating to 10 DIV. However, they state a decrease in the absolute number of cells by 50% and 80% at 7 and 10 DIV respectively, suggesting that they also witnessed a decrease in the number (but not percentage) of TH^+ cells during this time period. VM cultures have been maintained for up to 48 days, however, this protocol used glial feeders and did not report on the numbers of TH^+ neurons at this time point (Lautenschläger *et al.*, 2018).

The initial increase followed by decrease in TH^+ neurons observed in this thesis may be a form of programmed cell death. Neuronal programmed cell death is involved in normal vertebrate brain development and is thought to occur as a result of neurotrophic cell death. The neurotrophic theory is that neurons are overproduced and cell survival is dependent on active cellular connections being made (Dekkers, Nikoletopoulou and Barde, 2013). The changing morphology observed in these VM cultures showed TH expression restricted to small somas after 1 DIV, with neurite extension appearing to increase in number and length at 4 DIV

and stabilising through to 14 DIV. This corresponds with other work showing the development of extensions and further branching and ramification at days 4 and 7 with mouse TH^+ neurons (Gaven, Marin and Claeysen, 2014). Therefore, if TH^+ neurons in these VM cultures did not form suitable synaptic connections, they may have undergone a form of programmed cell death, which could account for decreasing numbers of TH^+ neurons in maturing cultures. The removal of these cells from the embryo, placement into an 'alien' environment, and the absence of the specific striatal neurons that SNpc neurons target (Holmes, Angharad Jones and Greenfield, 1995), would have disrupted signalling and correct formation of the specific synaptic connections required to fully maintain healthy SNpc dopaminergic neuron populations.

The application of 10 mM nicotinamide to these VM cultures significantly increased the number of TH^+ neurons observed, compared to control, at 1, 4 and 7 DIV. The increase in TH^+ neurons did not correspond with an increase in β-III-tubulin$^+$ neurons. This suggests that the effect of nicotinamide could be specific to dopaminergic neurons (TH^+ cells in these cultures).

The observation of a significant effect of both nicotinamide treatment and time on the number of TH^+ neurons with no interaction (Figure 4.7) suggests that an effect of nicotinamide on TH^+ neurons takes place prior to the initial assessment at 1 DIV; however, it is unclear whether this effect could occur pre- and/or post-differentiation. Further exploration of these results using markers of proliferation would help to ascertain whether this increase in TH^+ neurons is due to proliferation and maturation of dopaminergic neuron precursors, or whether this result has arisen from a protective effect of nicotinamide on dopaminergic neurons present in cultures immediately after the plating process.

To the best of my knowledge, the assessment of nicotinamide on TH^+ cells in VM cultures has not been commented on in the literature. However, intraperitoneal injection of

100 mg/kg nicotinamide every 2 days into p1 to p21 mice resulted in a decrease in TH^+ neurons in the SN, with levels of dopamine reduced in the hypothalamus but not striatum (Lee *et al.*, 2009). The differences observed between that report and the results of this thesis potentially arise from vast differences in models used, including *in vivo*/*in vitro*, postnatal/embryonic, and dosage.

4.4.3 Calcitriol did not increase numbers of TH^+ neurons or provide TH^+ neuroprotection against 6-OHDA treatment

Treatment of VM cultures with calcitriol did not increase numbers of TH^+ cells despite previous work in the lab showing an increase in TH^+ cells in VM cultures treated with calcitriol (Orme, Bhangal and Fricker, 2013). These results also did not show a protective effect of calcitriol against 6-OHDA treatment which is contrary to a number of studies which show neuroprotective effects of calcitriol on TH^+ neurons (Wang *et al.*, 2001; Sanchez *et al.*, 2009; Orme, Bhangal and Fricker, 2013; Lima *et al.*, 2018).

The difference observed between this thesis and the work by Orme, Bhangal and Fricker (2013) may be due to the use of different donor ages with E14 used here and E12 used previously. Obtainment of rat VM cultures from E12, E13, and E14 have shown different differentiation potential, after an initial expansion period, with E12 neurospheres generating higher numbers of dopaminergic neurons after differentiation for 6 days (O'Keeffe and Sullivan, 2005). However, tissue derived from older embryos (E14) showed greater neurogenic potential after a longer differentiation period (Hegarty *et al.*, 2014). These studies highlight the effects that donor age and DIV can have on cell populations observed in VM cultures and may account for the differences observed in the effects of calcitriol between this thesis and previous work. However, the vitamin D receptor is expressed in the nucleus of TH^+ cells

between E12-15 and increases after this period, in the rat VM, suggesting that an effect of calcitriol should have been observed in these VM cultures. When comparing β-III-tubulin$^+$ numbers in vehicle control (AA) treated cultures, significantly lower numbers were observed in combined nicotinamide and calcitriol cultures when compared to calcitriol alone (but not compared to control or nicotinamide treated cultures). In 6-OHDA treated cultures, a significant decrease in numbers of β-III-tubulin$^+$ cells was observed in nicotinamide treated cultures compared to control, but not in calcitriol or combined nicotinamide-calcitriol cultures. These results suggest that there may have been an effect of calcitriol on β-III-tubulin$^+$ neurons. However, the meaning and mechanisms of these results are difficult to decipher and would need further exploration.

Due to the inconsistencies between these results and the literature, it was suspected that the calcitriol used could have been degraded in storage. Calcitriol is degraded under hydrolytic, oxidative, thermal and photolytic conditions in as little as 24 hours (Temova and Roškar, 2016). Confirmation of degradation could be ascertained using high performance liquid chromatography. Although care was taken to reduce light and heat exposure during preparation, and final storage was at -80°C; for future work concerning the use of calcitriol, other preparation and storage options could be explored such as storage under inert gas to decrease the risk of oxidation.

4.4.4 Nicotinamide did not provide neuroprotection against 6-OHDA toxicity

Treatment of these VM cultures with 10 mM nicotinamide produced a significant decrease in total cell numbers at 7 DIV (details are further discussed in Chapter 5.3.1). As previously described, 6-OHDA toxicity can vary across different cultures. Reducing cell

numbers could make cells more susceptible to 6-OHDA toxicity by increasing the amount of 6-OHDA that each cell is exposed to, and/or concentration of auto-oxidation-generated toxins per cell. Higher doses of 6-OHDA have a more detrimental effect on the TH^+ population (González-Hernández *et al.*, 2004). To address this and assess the effect of nicotinamide application alongside 6-OHDA application, control cultures were treated with 6-OHDA + nicotinamide starting at 7 DIV, with addition of nicotinamide during the 48-hour recovery process. Using this method allows for observation of the effects of nicotinamide on 6-OHDA toxicity and recovery, without the effects of nicotinamide on VM cultures from exposure during the initial 7 DIV growth period. The number of TH^+ cells at 9 DIV results did not differ significantly from control and nicotinamide 6-OHDA treated cultures. This provided further evidence that nicotinamide was unable to provide neuroprotection against 6-OHDA toxicity for TH^+ cells in VM cultures.

6-OHDA has been found to reduce NAD^+ levels, reduce the NAD^+/NADH ratio and decrease Sirt1 activity, which is attenuated by the addition of NMN (precursor to NAD^+; Zou *et al.*, 2016). However, this was in PC12 cells which (as described in Chapter 1.5.2) do not represent either dopaminergic neurons, or a PD model, as closely as VM cultures do. Also, if 6-OHDA causes toxicity by inhibiting complex I of the electron transport chain (Glinka, Gassen and Youdim, 1997), and therefore the process which converts NADH to NAD^+, then perhaps this process results in the decrease in NAD^+ levels and NAD^+/NADH ratio seen by Zou et al. (2016). Administration of NAD^+ in an *in vivo* 6-OHDA mouse model showed protection of TH^+ neurons in the SN (Shan *et al.*, 2019). Although administration of nicotinamide has been shown to increase levels of NAD^+ (Klaidman, Mukherjee and Adams, 2001; Schöndorf *et al.*, 2018), this mechanism was either not active or was ineffective in the VM 6-OHDA model used in this PhD thesis.

Oxidative stress is another mechanism of the toxic effects of 6-OHDA which can increase levels of H_2O_2 both intra- and extra-cellularly through auto-oxidation (Graham *et al.*, 1978; Soto-Otero *et al.*, 2000). Blocking Sirt1 using 5 mM nicotinamide showed protection of cortical neurons from oxidative stress induced by H_2O_2 (Li *et al.*, 2008). However, this cell model is also not closely comparable to the 6-OHDA VM model used in this thesis. Mechanisms other than oxidative stress also contribute to cell death through 6-OHDA toxicity, therefore, Sirt1 inhibition may not be effective against the myriad of toxic events occurring in these cultures (discussed in Chapter 4.1.2).

Overactivation of PARP-1 can lead to cell death through consumption of NAD^+ and activation of apoptosis and necrosis. Nicotinamide is an inhibitor of PARP-1, although PARP-1 inhibition has shown protective effects in other models of dopaminergic neuron degeneration (Zhang *et al.*, 2000), it has not shown protection from 6-OHDA toxicity (Callizot *et al.*, 2019). Nicotinamide has shown neuroprotection through PARP-1 inhibition in an MPTP model of neurodegeneration (Mandir *et al.*, 1999). However, Harrison et al. (2019) found an exacerbation of toxicity with nicotinamide in the lactacystin model of PD.

Nicotinamide may increase NADH and/or NADPH which are required for the synthesis of tetrahydrobiopterin (BH4), the cofactor essential for TH activity (Thöny, Auerbach and Blau, 2000). If the addition of nicotinamide is able to increase levels of NADH and NADPH, it could allow increased activity of TH. Treatment of PC12 cells with NADH led to increased production of dopamine thought to be induced by the regeneration of BH4 (Vrecko *et al.*, 1997). Also, NADH and NADPH, but not NAD^+ or $NADP^+$, prevent the nitration of tyrosine and subsequent inhibition of TH (Kuhn and Geddes, 2002). This suggests a potential role of nicotinamide through $NADP^+$ and NADPH in dopamine synthesis (Figure 1.10). However, increased levels of cytosolic dopamine may contribute to toxicity (Mosharov *et al.*, 2009), and increased levels of

homovanillic acid (a metabolite of dopamine; Figure 1.4) are observed in the cerebrospinal fluid of PD patients, correlating with motor symptom severity (Stefani *et al.*, 2017).

There is still high debate about the mechanisms of cell death in PD. The outcome of nicotinamide in neuroprotection may be dependent on the choice of PD model. This variability across PD models, and also within the 6-OHDA PD model (described in Chapter 4.4.1) could account for differential effects seen using nicotinamide and its derivatives in neuroprotection studies.

4.4.5 Conclusions and future studies

The results discussed in this chapter have shown the development of an *in vitro* 6-OHDA PD model using E14 VM rat cultures that was comparable to similar models within the literature. This was used to assess potential neuroprotective effects of nicotinamide on TH^+ cells. Nicotinamide did not show any neuroprotective effects against 6-OHDA in these VM cultures. This lack of neuroprotective effect may be due to the 6-OHDA protocol used, although this model was comparable to others in the literature, 6-OHDA toxicity may have been too effective and therefore irreversible for this neuroprotectant. The use of a positive neuroprotective control, calcitriol, was used to analyse this, however, in these experiments calcitriol was also unable to provide a neuroprotective effect. Further optimisation of the 6-OHDA protocol and positive control could be used to further assess the neuroprotective potential of nicotinamide. Another possibility for the lack of neuroprotection may be due to incompatibilities in the mechanisms of action between 6-OHDA and nicotinamide.

However, treatment of normal VM cultures with nicotinamide did show a significant increase in numbers of TH^+ neurons compared to cultures without nicotinamide present. To my knowledge, this effect has not been discussed in the literature. Mechanisms of action could include direct effects of nicotinamide on TH^+ cells by inhibition of PARP-1 or Sirt1, however,

increases in these factors typically relate to cell survival mechanisms. Immunocytochemistry and western blot techniques could be used to analyse the expression levels of PARP-1 and Sirt1 in these cultures.

Nicotinamide could also have exerted this effect by increasing levels of pyridine nucleotides. This mode of action could be explored by assessing the intracellular levels of NAD^+/NADH through luminescence assays.

Further analysis of cultures using A9/A10 specific markers (as highlighted in Table 1.5) would help to confirm the effects of nicotinamide are on dopaminergic neurons and whether this varies between SN/VTA populations.

This increase in TH^+ neurons in cultures treated with nicotinamide could also be an indirect effect arising from the interaction of nicotinamide with other cells present in these VM cultures. This is further explored in Chapter 5.

This chapter shows a positive effect of nicotinamide on the number of TH^+ cells in these cultures, however, the mechanism was not studied. Further experiments would include:

I. Assessment of TH^+ neurons in VM cultures using BrdU to determine whether increased numbers were through proliferation of neural precursors.
II. Analysis of VM cultures using NAD^+/NADH luminescence assays to determine whether effects could be due to nicotinamide induced increases in NAD^+.
III. Assessment of PARP-1 expression in TH^+ neurons using immunocytochemistry.

5 Nicotinamide and microglia

5.1 Introduction

5.1.1 The *in vivo* mechanisms involved in microglial development are still being elucidated

As mentioned in Chapter 1.4.1, microglia, the immune cells of the CNS, arise from yolk sac, rather than neural plate; and have been cultured from rat embryonic brain tissue as early as E10 (Ghosh and Ghosh, 2016). Microglial development in humans, which correlates with rat developmental stages (albeit over longer time periods), suggests that microglia enter the developing brain from the meninges, choroid plexus and ventricles (Verney *et al.*, 2010). Once microglial cells have entered neural tissue they migrate and proliferate to colonise brain regions where their numbers then remain stable throughout healthy ageing (Askew *et al.*, 2017).

This migration and proliferation is thought to be dependent on a number of signalling factors including semaphorins, netrins, macrophage colony stimulating factor (CSF-1), monocyte chemoattractant protein 1, macrophage inflammatory protein, and C-X-C motif chemokine 12 (Bruttger *et al.*, 2015; Mosser *et al.*, 2017). Studies from knockout mice demonstrate the importance of CSF-1 in embryonic development of microglia, with the brains of E16 knockout mice showing almost no microglial cells (Elmore *et al.*, 2014).

Microglia have shown different morphologies and expression patterns dependent on their resident brain region (Tan, Yuan and Tian, 2020). The adult mouse SN has one of the highest microglial populations (Lawson et al., 1990), with these cells exhibiting differences in morphology compared to microglial populations in the bodies of the basal ganglia and in the VTA (De Biase *et al.*, 2017).

Mechanisms behind the colonisation of brain regions by microglia, the differences in homeostatic expression patterns, the maintenance of microglia throughout health, and the myriad of responses during neurodegeneration are highly complex and still being investigated.

5.1.2 *In vitro* models of microglia provide valuable insight, but extrapolation to *in vivo* can be difficult

Microglia possess complex profiles and behaviours which, as previously discussed (Chapter 1.4), are highly responsive to their micro-environment. *In vitro* models can be separated in to three broad categories of stem cell derived, cell lines, and primary cultures, each with advantages and drawbacks (review: Timmerman, Burm, & Bajramovic, 2018). This project used primary cultures to study microglia both in heterogeneous cultures derived from specific brain regions (E14 rat VM and WGE) and in isolated microglial cultures derived from rat mixed glial cultures. This provided two environments in which to study the microglial response to nicotinamide, allowing for exploration of direct and indirect influences. However, it also represented difficulties in the applications of this data as stark differences in microglia morphology were observed in each culture system without application of treatment conditions.

Microglia within purified cultures typically display amoeboid and rod-like morphologies, often associated with a pro-inflammatory phenotype, rather than the ramified morphologies associated with heathy CNS tissue. This is due to the "alien" nature of culture conditions (Kettenmann *et al.*, 2011) such as changes in media (which may contain components that microglia do not typically contact such as serum), atmosphere and O_2/CO_2 balance, pH differences, and 2-dimensional (2D) rather than 3-dimensional (3D) substrate. The mixed glial culture system provides relatively easy access to high purity populations of

microglia (routinely 95-98% Iba1$^+$) and is widely used for microglial research, however, microglial morphologies and behaviours within these cultures may not be representative of *in vivo*, non-pathological microglia. To address part of this problem, 3D culture systems are in development which promote more ramified microglial morphologies, predominant in healthy CNS, by providing an environment that more closely resembles the brain (Watson *et al.*, 2017). These systems attempt to mimic the healthy CNS environment, an important aspect of which is the stiffness of the material. Stiff gel materials have shown an increased pro-inflammatory response compared to softer gels (Prager *et al.*, 2020). This may also contribute to differences observed between microglial cultures grown on a hard glass surface compared to microglia grown on a bedlayer, providing a softer extracellular matrix.

Culturing microglia alongside other neural cell types also increases their ramification (Rosenstiel *et al.*, 2001; Rezaie *et al.*, 2002) with factors released from other cell types influencing microglial phenotype (Timmerman, Burm and Bajramovic, 2018).

As with many cell types, the media used to culture microglia can have drastic influences on phenotype (Bohlen *et al.*, 2017). The vast majority of microglial research uses medium supplemented with 10% foetal bovine serum. Lower serum levels can reduce numbers of microglia within mixed glial cultures (Codeluppi *et al.*, 2011). CNS microglia are separated from the blood due to the BBB, meaning that they do not typically interact with serum components *in vivo*. Therefore, the addition of serum may affect microglial phenotype, essentially simulating haemorrhagic stroke, and serum has been found to disrupt morphology and function (Bohlen *et al.*, 2017).

As discussed in Chapter 1.4.2, a number of markers, which can be associated with specific activation states, have been identified in the expression profiles of microglia (Figure 1.6). These markers can be permanently or transiently expressed, with a number of markers showing upregulation in the different activation states of microglia. Although the use of these

markers is valuable in the identification and profiling of microglia, there is often debate about the phenotypic characterisation of their expression levels. *In vitro* culturing of microglia can contribute to differences in expression patterns meaning that characterisation of microglia based on these markers *in vitro* may not be reliable.

Culturing microglia can provide insights into factors that can influence their phenotype, assisting to identify factors that may able to promote anti-inflammatory or regulatory functions. However, *in vitro* models do not necessarily provide a representative microglial phenotype, meaning that data generated through this pathway must be interpreted with caution.

5.1.3 A number of factors have been shown to influence microglial proliferation

As discussed in Chapter 1.4.3, microglial proliferation is an indicator of inflammatory activation and increased numbers of microglia have been observed at sites of neurodegeneration (Gensel and Zhang, 2015; Mathys *et al.*, 2017). Studying the mechanisms of microglial proliferation, and pathologies associated with it, could help in understanding microglial roles in neurodegeneration. Factors that influence proliferation can also be used in the development of protocols allowing large scale generation of microglia for research, or in the removal of microglia from other cell cultures.

Application of inflammatory activators such as LPS have been shown to increase microglial proliferation. This increase was shown to be attenuated by inhibition of vascular endothelial growth factor (VEGF) signalling (Fukushima *et al.*, 2015). Hydrogen peroxide has also been shown to be important in microglial proliferation in response to β-amyloid peptides (Jekabsone *et al.*, 2006). Interleukin-1 receptor has shown elevated levels in proliferating

microglia with blocking of this receptor impairing proliferation during repopulation after ablation (Bruttger *et al.*, 2015). Neurotrophin-3 and IL-5 have also shown influence in the proliferation of microglia *in vitro* and are present in the developing brain (Navascués *et al.*, 2000). A wide variety of signals can affect microglial proliferation; understanding these mechanisms may assist in the development of neuroprotective strategies.

CSF-1 increases proliferation in microglia, through the upregulation of genes related to proliferation and cell cycle. This stimulating factor also upregulates genes *Arg1*, *Fizz1*, and *Ym1* associated with M2 polarisation, showing that increased proliferation is not necessarily associated with inflammation (Pepe *et al.*, 2017). Inhibition of microglial proliferation has been observed by blocking CSF-1 receptor (Olmos-Alonso *et al.*, 2016) and expression of this receptor is required for microglial proliferation in homeostatic maintenance (Askew *et al.*, 2017).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that induces proliferation and maturation of myeloid progenitors (cells present in bone marrow) (Liva *et al.*, 1999) and has also been shown to induce proliferation of microglia (Lee *et al.*, 1994; Koguchi *et al.*, 2003). Stimulation of astrocyte cultures with factors such as IL-1β, TNF-α, and LPS induce release of GM-CSF (Aloisi *et al.*, 1992; Lee *et al.*, 1994) suggesting that activated astrocytes may release GM-CSF in response to pro-inflammatory stimuli, which is then likely to stimulate microglia.

Treatment of primary microglia with GM-CSF has been shown to increase activation of ERK1 and ERK2 (extracellular signal-regulated kinase 1 and 2, which are part of the family of mitogen-activated protein [MAP] kinases involved in embryogenesis, differentiation, proliferation and cell death). Activation of STAT5A and STAT5B (signal transducer and activator of transcription 5A and 5B, are two of 7 proteins activated by cytokines and growth factors, and are associated with growth, differentiation, apoptosis and immune responses [Santos &

Costa-Pereira, 2011]) is also incurred with GM-CSF treatment, with translocation from cytoplasm to nucleus also observed (Liva *et al.*, 1999). Therefore, these pathways likely mediate proliferation effects of GM-CSF.

Understanding the regulation of microglial proliferation may help in controlling microglia response to injury or insult and may provide therapeutic potential by reducing unwarranted activation or over-activation of microglia which has been implicated in a number of neurodegenerative conditions (Pepe *et al.*, 2017).

5.1.4 Nicotinamide and associated compounds influence microglial activation and proliferation

Nicotinamide and NAD^+ can modulate immune cell function, influencing physiology and pathology in the CNS (Haag *et al.*, 2007). For example, nicotinamide has been shown to reduce demyelination and neurofilament loss after spinal cord injury and is proposed to work through mediating macrophage responses, converting pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages (Yang, He and Wang, 2016; Montserrat- de la Paz *et al.*, 2017). Increasing NAD^+ levels have also been shown to ameliorate autoimmune mediated demyelination in multiple sclerosis (Penberthy, 2009). Alongside this effect, many pathogens, and the toxins they release, target NAD^+ through the release of NADase (Sharma *et al.*, 2016) reducing available levels for host cells, meaning that replenishment is key during illness.

Administration of NMN, a precursor to NAD^+, reduced numbers of $Iba1^+$ microglia, NOX1 (and NADPH oxidase which contributes to generation of intracellular oxidative stress) expression and markers of inflammation (TNF-α and IL-6) in an *in vivo* mouse intracerebral haemorrhage model (Wei *et al.*, 2017).

Nicotinamide treatment 15 mins, 24 hours and 48 hours after traumatic brain injury in juvenile rats reduced the number of $Iba1^+$ microglia cells present 72 hours post-lesion (Smith *et al.*, 2019) although the proposed mechanisms are not detailed.

5.1.5 NADPH is involved in microglial activation

Nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) is an extracellular facing, membrane-bound enzyme responsible for the production of free radicals ($NADPH + 2O_2 \leftrightarrow NADP^+ + 2O_2^- + H^+$) which are involved in cell signalling and pathogen destruction (Bedard and Krause, 2007). NOX2, also known as $gp91^{phox}$, is part of the NADPH oxidase enzyme complex found on phagocytes including microglia. NOX2 is expressed by both neurons and microglia, and is highly responsive to cortical impact injury with microglia increasing and sustaining higher expression levels after injury, whilst neurons decrease expression levels with gradual return after injury (Cooney, L Bermudez-Sabogal and Byrnes, 2013).

NOX2 expression increases in experimental autoimmune encephalomyelitis (a model of CNS demyelinating disease), with microglial activation accompanied by overexpression of NADPH oxidase (Filippo *et al.*, 2016).

NADPH oxidase activation stimulates microglial proliferation (Jekabsone *et al.*, 2006) whilst inhibitors of NADPH oxidase prevent microglia proliferation (Mander, Jekabsone and Brown, 2006). The NADPH inhibitor apocynin reduced microglial activation (identified as $OX\text{-}6^+$ also known as major histocompatibility complex class II) whilst the NADPH activator angiotensin II increased numbers of activated microglia in 6-OHDA treated VM cultures (Rodriguez-Pallares *et al.*, 2007). Microglial exposure to neuromelanin results in enhanced NADPH oxidase dependent ROS production (W. Zhang *et al.*, 2011) and NADPH oxidase has been implicated in PD (Belarbi *et al.*, 2017). Niacin restriction has also been shown to

upregulate NADPH oxidase, increasing ROS production (in cultured human keratinocytes), an effect that was attenuated by addition of nicotinamide to culture medium (Benavente and Jacobson, 2008).

Administration of NADPH to MPTP treated mice attenuated MPTP-induced elevation of Iba1 and levels of inflammasome proteins. The authors suggest that this could be due to p38 MAPK inhibition induced by NADPH (Zhou *et al.*, 2018). Nicotinamide may influence NADPH oxidase through involvement with the pyridine nucleotides (Figure 1.10) or 'consumers' of these including PARPs and CD38.

5.1.6 PARPs are involved in microglial activation

PARP1 has been shown to be essential for TNF-α induced microglial proliferation and morphological changes associated with inflammatory activation. Microglia derived from PARP-$1^{-/-}$ mice showed lower basal levels of MMP-9 (matrix metalloproteinase 9); which is released by microglia and degrades extracellular matrix. Blocking of PARP-1 activity by DPQ (3,4-dihydro-5-[4-(1-piper-idinyl)butoxy]-1(2h)-isoquinolinone) also reduced expression levels of MMP-9 in microglia cultures derived from wildtype mice, and co-cultures of microglia with neurons showed that neurotoxicity is mediated by MMP-9 through PARP-1 activity (Kauppinen and Swanson, 2005). As previously discussed, nicotinamide is an inhibitor of PARP activity (Suzuki *et al.*, 2010) suggesting that nicotinamide may be able to influence microglial activation through PARP-1 inhibition.

5.1.7 CD38 is a consumer of NAD^+

NAD^+ is 'consumed' by CD38, a transmembrane glycoprotein that catalyses NAD^+ into nicotinamide, adenosine diphosphate-ribose (ADPR) and cyclic ADPR (Hamblin, 2003). Its

expression increases with pro-inflammatory microglial activation (Mayo *et al.*, 2008). CD38 plays a major role in the age-related decline of NAD^+ levels, with expression increasing with age and correlated with decreased levels of NAD^+ (Camacho-Pereira *et al.*, 2016). CD38 is also involved in microglial activation-induced cell death, a mechanism by which microglia reduce numbers after a pro-inflammatory response (Mayo *et al.*, 2008). This suggests that NAD^+ levels may be depleted by microglial activation, which could have adverse effects for other neural cells.

The effects of nicotinamide on microglia is not well covered in the literature, however, there appears to be a link between nicotinamide, its derivatives, and pathways inhibited by nicotinamide in microglial activation and the immune system in general (Ma *et al.*, 2016; Cetina Biefer, Vasudevan and Elkhal, 2017). Given the potential role of microglia in neurodegeneration, and more specifically PD; and suggestions of a neuro-immunomodulatory effect of nicotinamide, this area of research could assist in the development of novel neuroprotective mechanisms.

5.1.8 Aims of Chapter 5

Data presented in Chapter 4.3.2, showed that nicotinamide treated VM cultures had significantly lower total numbers of cells, as observed with the nuclear stain DAPI. This led to the investigation of the effects of nicotinamide on different cell types present in these cultures. Neurons have historically taken precedence in the study of neurodegeneration, and more specifically PD. However, it is becoming increasingly apparent that glia may play significant roles in both degeneration and protection in neurodegenerative disease, and compounds may affect disease progression through these supporting cell types.

Nicotinamide and associated compounds may also have a role in microglial activation and inflammatory response having been shown to reduce inflammation in other disease models. Neuroinflammation is implicated in the degeneration of dopaminergic neurons in PD, therefore, reducing neuroinflammation could help to slow the progression of PD and may also have a role in the outcome of other therapies such as CRT. Due to the wide-ranging influences of nicotinamide, both directly and indirectly, the potential mechanisms are not well described. Furthermore, due to the adaptive nature of microglia, it is important to analyse their response to compounds across multiple culture systems to fully explore the effects it may induce.

This chapter will discuss:

I. The identification of cell types within VM cultures derived from E14 rat.
II. The effects of nicotinamide on $GFAP^+$ astrocytes and $Nestin^+$ cells present within VM cultures.
III. The effects of nicotinamide on microglia in three culture systems (VM, WGE, and isolated microglial cultures).

5.2 Methods

5.2.1 Characterisation and quantification of cell types within VM cultures

To estimate the percentage of each cell type within VM cultures, cell counts were collated from multiple experiments following immunocytochemistry for β-III-tubulin, TH, Nestin, GFAP and Iba1. Images were taken using different magnifications dependent on the cell type analysed and subsequent image analysis technique used. To collate the cell counts, data were converted to number of cells per mm^2 by multiplying the number of cells per image by the area of the micrograph for the magnification at which the image was taken (described in Chapter 2.9). The number of cells per mm^2 was then averaged across all experiments for both control and nicotinamide treated cultures. Since this technique contains different imaging techniques and does not allow for normalisation or error representation, the results are given as approximate values.

5.2.2 Exploring the effect of nicotinamide on the proliferation of microglia within VM cultures

The effects of nicotinamide on the proliferation of microglia within VM cultures was initially assessed by fixing cells at 1, 4, 7, and 14 DIV. The results from this experiment led to more detailed analysis of the effects of nicotinamide on microglial proliferation by changing the experimental treatment (+/- 10 mM nicotinamide) at 7 DIV. This is explained in Figure 5.1. Cultures were grown in control conditions with neural cell media (NCM), or with additional 10 mM nicotinamide in nicotinamide supplemented NCM (NSM). Cells were then either fixed at 7 DIV (NCM; NSM), continued with the same media (NCM→NCM; NSM→NSM), or swapped

to the other media for the following 7 DIV (NCM→NSM; NSM→NCM), then fixed at 14 DIV. Immunocytochemistry for Iba1 and DAPI was performed as described in Chapter 2.8.

Due to the low quantity and wide-spread distribution of microglia within VM cultures, micrographs were taken at 10X magnification, to allow for broad capture of microglia across the cultures, for image analysis as described in Chapter 2.9.

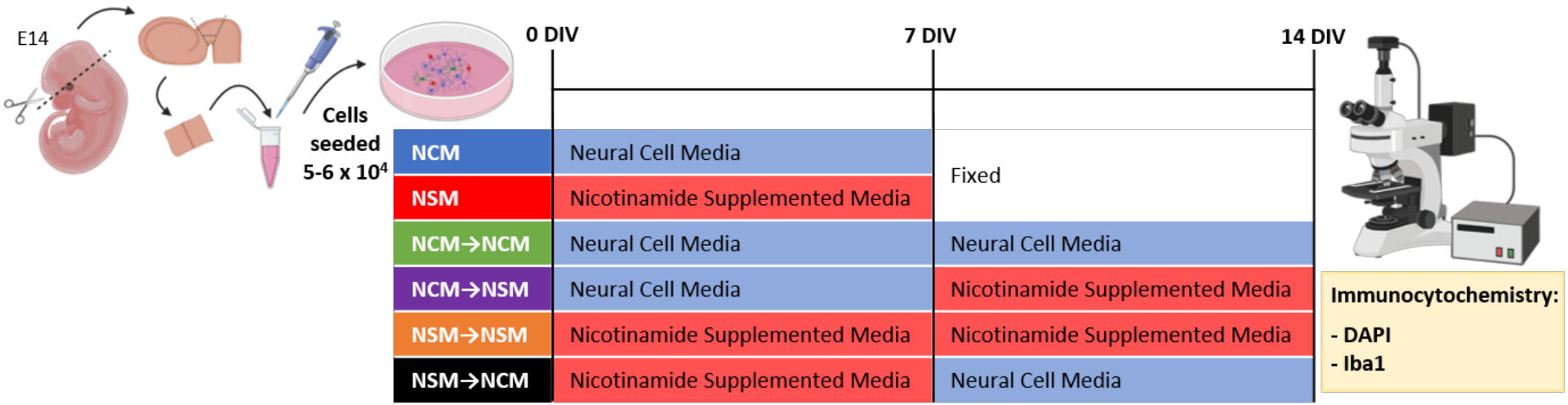


Figure 5.1 Diagram describing the treatment groups used when exploring the effects of nicotinamide on microglial proliferation by switching the addition or omission of 10 mM nicotinamide to ventral mesencephalon (VM) cultures. Cultures derived from embryonic day 14 rat VM were grown in control conditions (neural cell media; NCM; blue), or with additional 10 mM nicotinamide (NSM; red). Cells were then either fixed at 7 days *in vitro* (DIV; NCM/NSM; blue/red respectively), continued with the same media (NCM→NCM, green; NSM→NSM, orange), or swapped to the other media for the following 7 DIV (NCM→NSM, purple; NSM→NCM, black), then fixed at 14 DIV. Immunocytochemistry for Iba1 and DAPI was performed to quantify the microglia population. Created with BioRender.com

5.2.3 Treatment of pure microglial cultures with nicotinamide and GM-CSF

To observe the effects of nicotinamide and GM-CSF on the proliferation of microglia, pure microglial cultures were obtained from mixed glial cultures as described in Chapter 2.6. Cells were plated onto PDL coated coverslips (Chapter 2.3.2) in 24-well plates at a density of 2.2 x 10^4 cells/well. Cells were immediately exposed to treatment conditions by flooding the wells with the appropriate media. Concentrations of 1, 5 and 10 mM nicotinamide were used alongside application of 20 ng/mL GM-CSF as outlined in Figure 5.2. These pure microglial cultures were maintained for 5 days with full media changes on days 2 and 4 to replenish treatment conditions.

Cultures were fixed and stained for Iba1 using the immunocytochemistry protocol described in Chapter 2.8. This experiment contained 3 experimental repeats, with each treatment condition containing 3 technical replicates. Results were quantified using cell counts and optical density (OD) measurements.

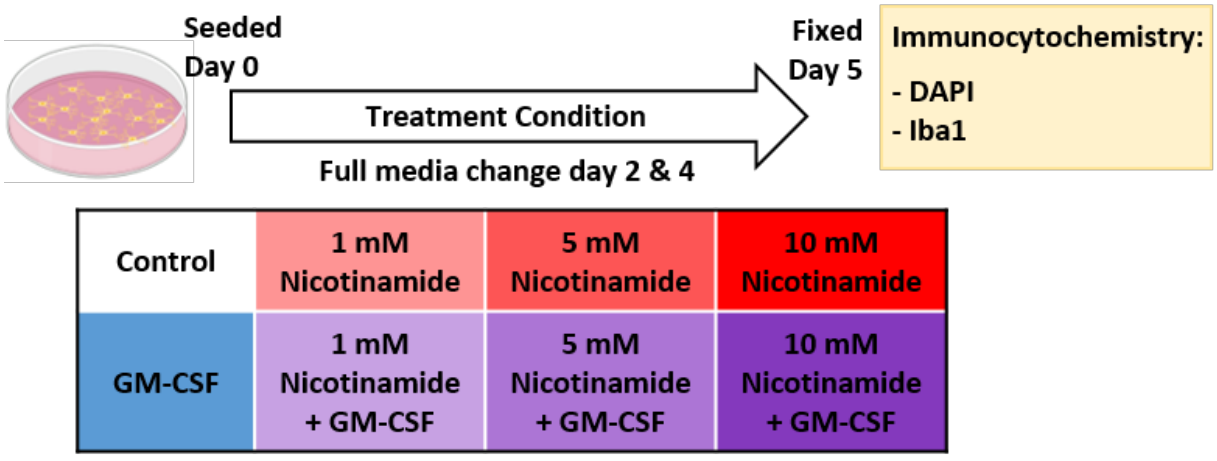


Figure 5.2 Diagram showing treatment conditions of isolated microglial cultures treated with GM-CSF (blue) and/or concentrations of nicotinamide (purple/red respectively). Cultures received treatment from day 0, had a full media change on days 2 and 4; then were fixed on day 5 for immunocytochemistry for DAPI and Iba1. Created with BioRender.com.

For cell counts, 4 micrographs were taken at 10X magnification. Cells were evenly dispersed over the coverslip, so this imaging technique allowed capture of representative numbers of cells within the culture.

For OD measurements, 4 micrographs were taken at 40X magnification. This technique was deemed more appropriate for analysing Iba1 expression as it provides a more detailed image of individual microglial cells. OD measurements were carried out as described in Chapter 2.10.

5.3 Results

5.3.1 Nicotinamide altered VM culture cell populations

Data presented in Figure 4.6, shows that nicotinamide treated VM cultures had significantly reduced total numbers of cells, as observed with the nuclear stain DAPI. This led to the investigation of the effects of nicotinamide on different cell types present in these cultures. This data is explored in the following sections.

VM cultures at 7 DIV were assessed for the presence of markers for predominant neural cell types: β-III-tubulin (neurons), GFAP (astrocytes), Iba1 (microglia) and Nestin (NSCs). The number of nuclei associated with expression of each marker was quantified to approximate the number of each specific cell type per mm^2 as described in Chapter 5.2.1. These values were then used to estimate the proportions of each cell type as shown in Figure 5.3 A. Under standard conditions, NSCs (identified by Nestin) made up approximately 46% of cells. β-III-tubulin$^+$ neurons accounted for approximately 13% of cells. GFAP$^+$ astrocytes for 2%, and Iba1$^+$ microglia were approximately 0.85% of culture populations. The remaining 38% of nuclei were negative for these markers (i.e. could not be accounted for by this technique).

The equivalent data for VM cultures treated with 10 mM nicotinamide for 7 DIV is shown in Figure 5.3 B. Nestin$^+$ NSCs comprised approximately 34% of nicotinamide treated cultures, β-III-tubulin$^+$ neurons made up around 14% of cultures. GFAP$^+$ astrocytes were also estimated at 2%, with Iba1$^+$ microglia making up 0.15% of nicotinamide treated cultures. The remaining 50% of cells did not stain for the above markers.

Cultures were observed under brightfield and phase contrast microscopy to potentially identify unstained cells. Figure 5.3 (C, D) shows phase contrast and fluorescence micrographs from VM cultures at 7 DIV grown in control conditions. It was not possible to definitively

identify unstained cell types, however, some cells exhibited large nuclei and a flattened appearance indicative of astrocytes, although these were negative for GFAP staining.

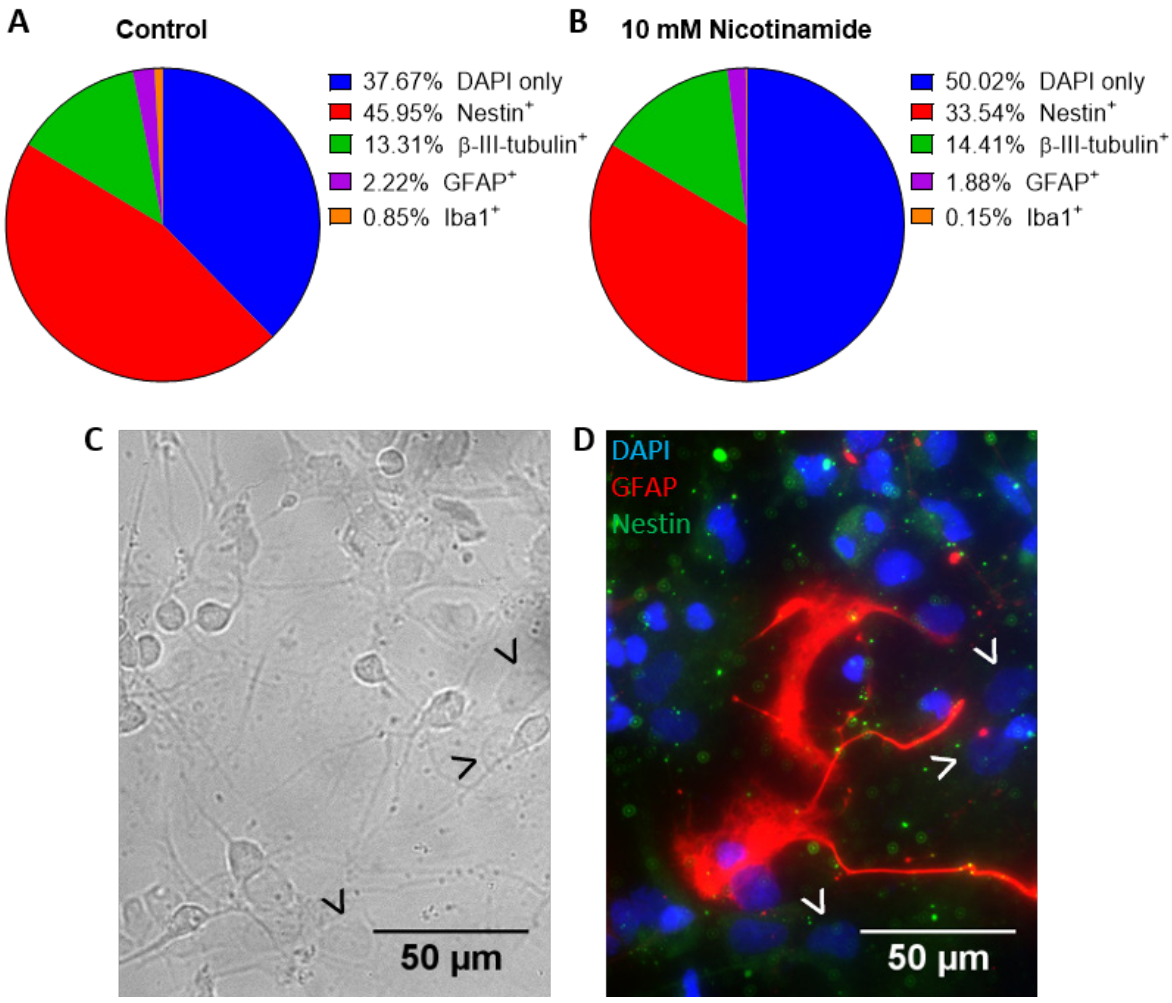


Figure 5.3 Cell types identified within VM cultures at 7 days *in vitro*.
A) Pie chart showing the approximate neural cell proportions of VM control cultures calculated from immunocytochemistry for Nestin, GFAP, BIII-tub and Iba1. DAPI only indicates negative for cell-type specific markers.
B) Pie chart showing the approximate make up of VM cultures treated with nicotinamide, calculated from immunocytochemistry for Nestin, GFAP, BIII-tub and Iba1.
C) Phase contrast micrograph of VM cultures showing morphology of the cells.
D) Immunocytochemistry for DAPI (blue), GFAP (red), and Nestin (green). Some GFAP$^-$ cells exhibited astrocytic morphology of a larger, dimmer nuclei in phase contrast micrographs (> arrowheads).

5.3.2 Nicotinamide did not affect numbers of GFAP$^+$ astrocytes in VM cultures

GFAP$^+$ astrocytes were quantified within VM cultures at 7 DIV. Numbers of GFAP$^+$ cells were counted from micrographs taken at 40X magnification across 3-4 technical replicates

from 3 experimental repeats. Results showed no difference in GFAP$^+$ cell numbers between control and nicotinamide treated cultures (5.64 ± 1.58 vs. 3.61 ± 1.06; t = 1.06, n.s.; Figure 5.4).

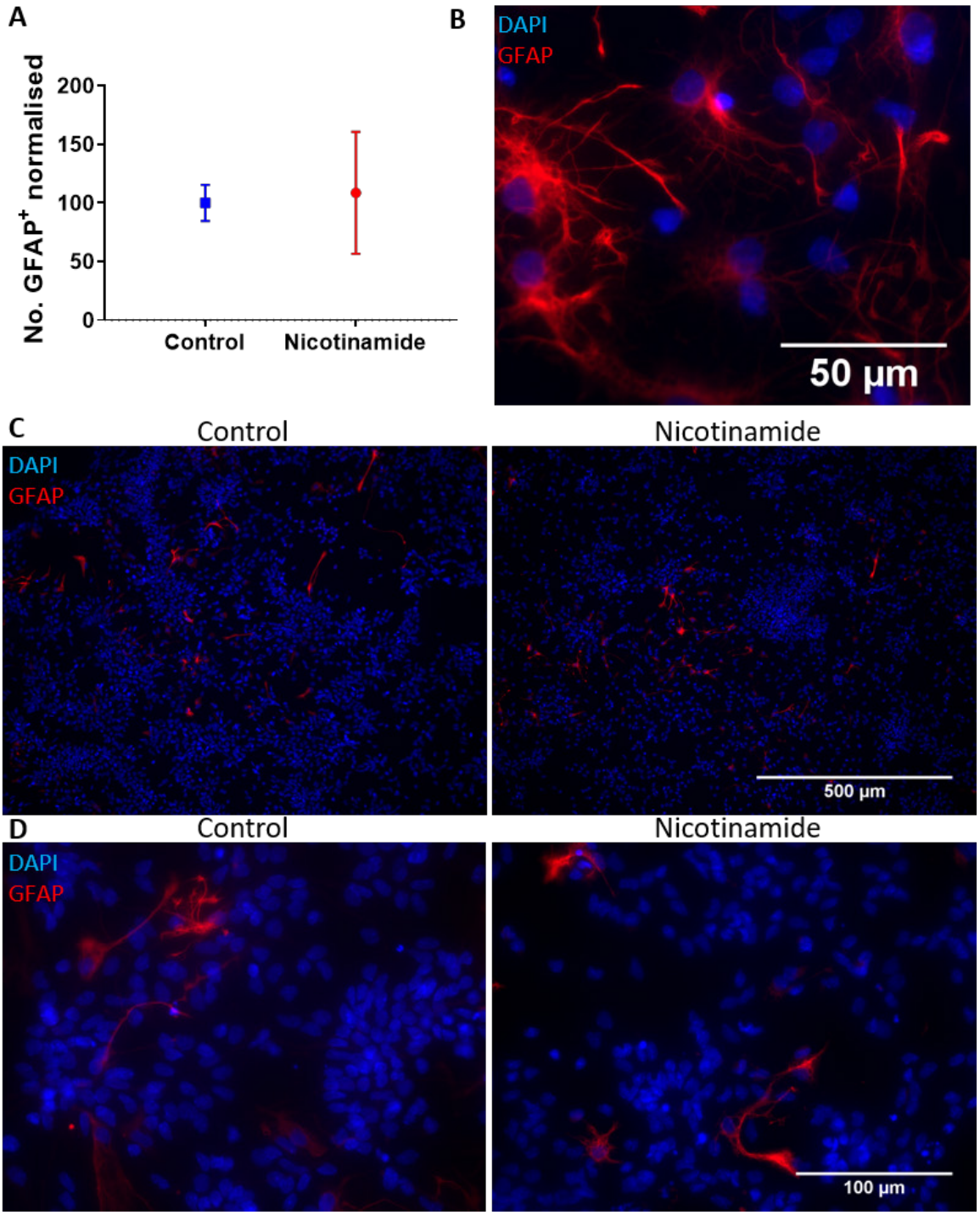


Figure 5.4 GFAP$^+$ astrocytes did not differ between control and nicotinamide treated VM cultures. A) Graph showing the number of GFAP$^+$ astrocytes normalised to control in control (blue square) and nicotinamide (red circle) treated cultures at 7 DIV. B) Micrograph showing morphology of astrocytes in control cultures. C) Representative micrographs showing numbers and spread of GFAP$^+$ astrocytes in control and nicotinamide treated VM cultures. D) Representative micrographs showing morphology of GFAP$^+$ astrocytes between control and nicotinamide treated cultures.

Analysis revealed that GFAP$^+$ cells accounted for approximately 2% of the total cell population. This number was lower than expected for VM cultures as similar cultures have reported 50% GFAP$^+$ cells (Liu and J. Hong, 2003), and phase microscopy of these cultures showed GFAP$^-$ cells with astrocyte morphology. To assess whether there was a larger population of astrocytes present, but not presenting as GFAP$^+$, cultures were co-immunostained with the originally-used GFAP antibody (supplied by DAKO) and another GFAP antibody (supplied by Biolegend). The DAKO GFAP antibody immunostained fewer cells than the Biolegend GFAP antibody and co-localisation occurred in some, but not all, Biolegend GFAP$^+$ cells Figure 5.5 However, the Biolegend GFAP antibody also appears to have labelled fewer cells than anticipated.

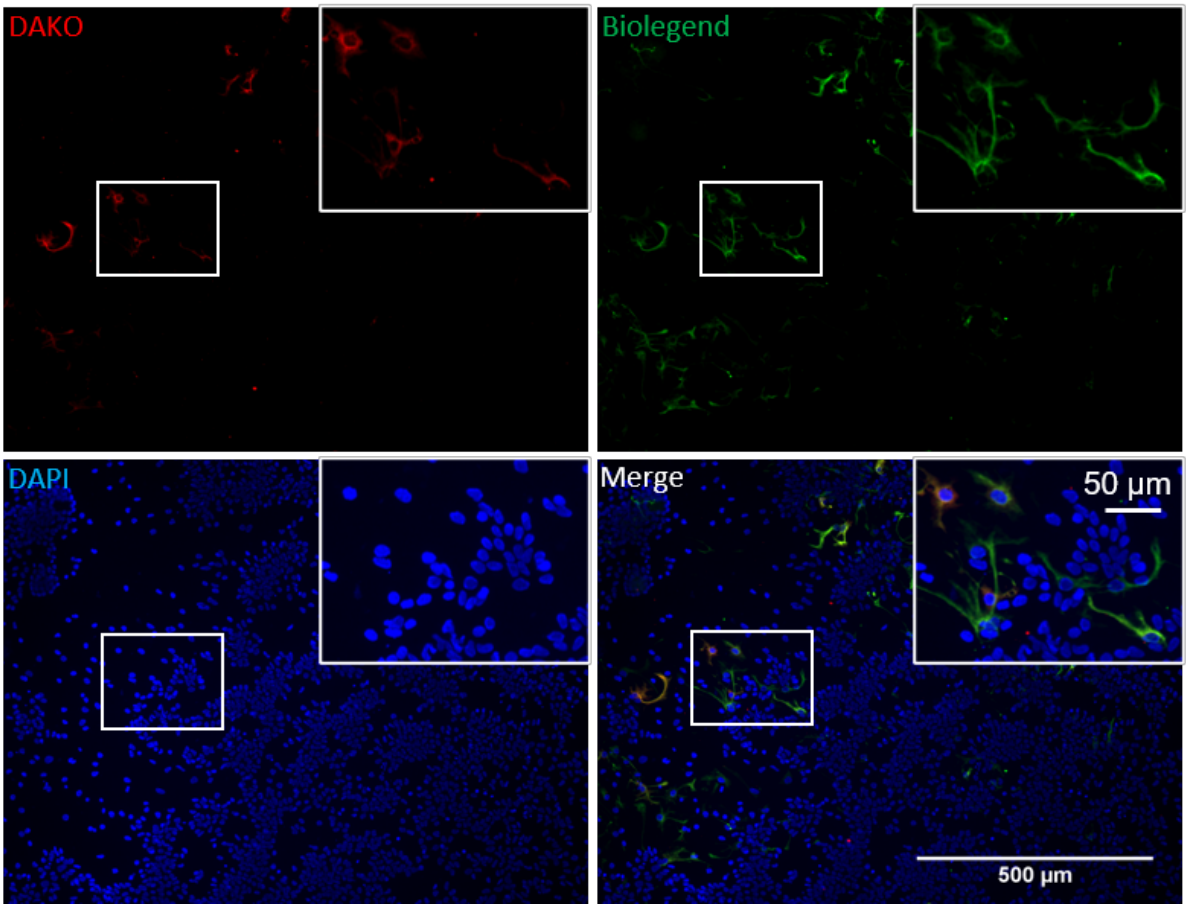


Figure 5.5 GFAP antibodies from DAKO and Biolegend show different immunoreactivity. Representative micrographs of control VM cultures show the different immunoreactivity of GFAP antibodies supplied by DAKO (red) and Biolegend (green). The merge shows some co-localisation, however, the Biolegend antibody appeared to label more cells than the DAKO antibody.

5.3.3 Lower numbers of Nestin$^+$ cells were present when VM cultures were treated with nicotinamide

Numbers of Nestin$^+$ cells were counted from 4 micrographs taken at 40X magnification across 3-4 technical replicates and 3 experimental repeats. The average number of Nestin$^+$ cells for each technical replicate was normalised as described in Chapter 2.11. These results showed a significantly lower number of Nestin$^+$ cells in 10 mM nicotinamide treated VM cultures compared with control cultures at 7 DIV ($t = 3.37$, $p < 0.05$; Figure 5.6).

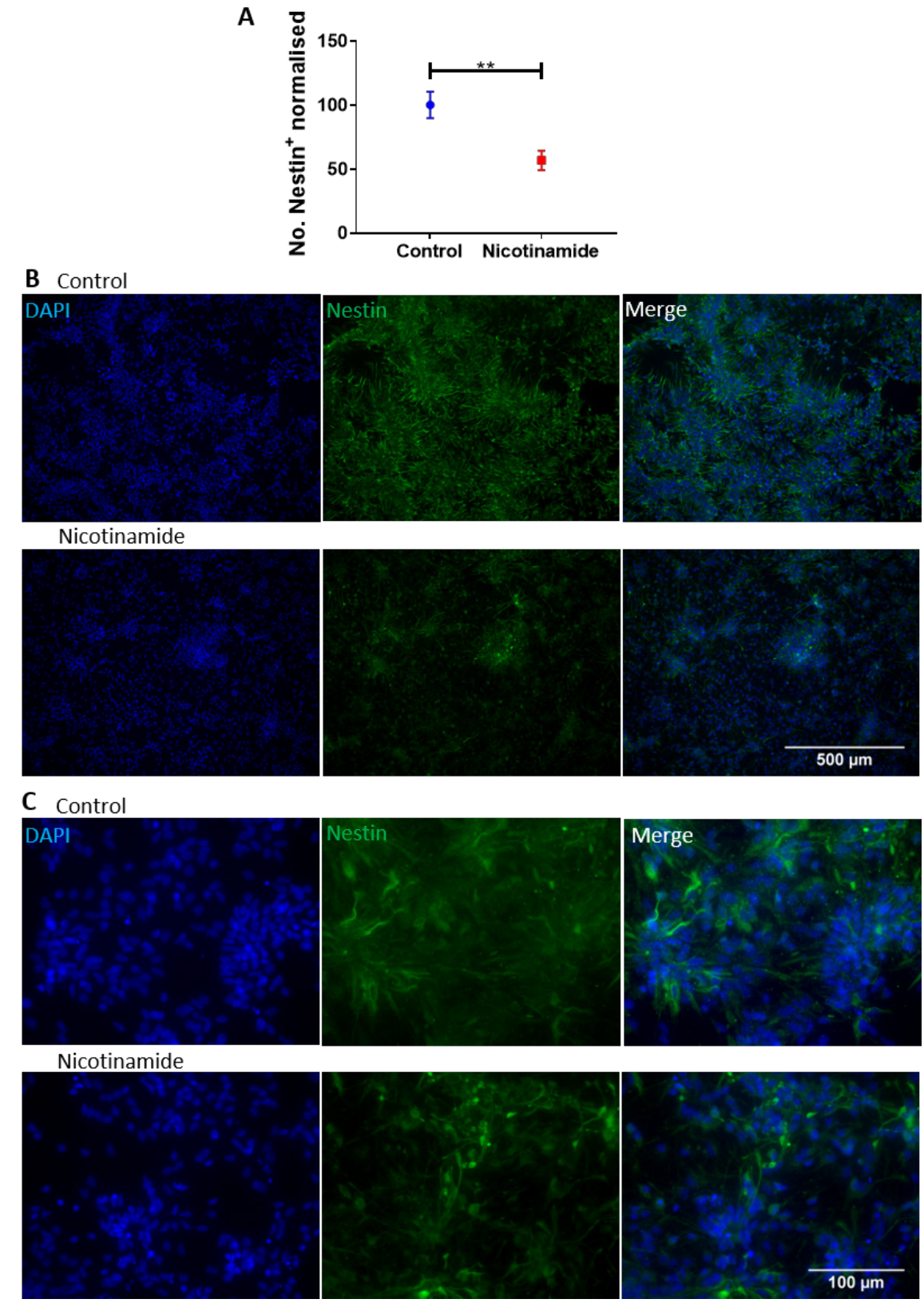


Figure 5.6 Numbers of Nestin$^+$ cells were lower in nicotinamide treated cultures.
A) Graph showing the number of Nestin$^+$ cells normalised to the control in control (blue circle) and nicotinamide treated (red square) VM cultures at 7 DIV.
B) Representative micrographs taken at 10X magnification showing the distribution of Nestin$^+$ cells in control and nicotinamide treated cultures.
C) Representative micrographs taken at 40X magnification showing morphology of Nestin$^+$ cells in control and nicotinamide treated cultures.

5.3.4 Nicotinamide restricted the proliferation of microglia in VM cultures

As microglia make up < 1% of VM cultures at 7 DIV (Chapter 5.3.1), micrographs were taken at 10X magnification, as this was more likely to capture representative proportions of $Iba1^+$ cells than micrographs at 40X magnification. The average number of $Iba1^+$ microglia was counted from 4 micrographs taken across 3-4 technical replicates from 3 experimental repeats. Initial experiments showed obviously lower numbers of $Iba1^+$ microglia in VM cultures at 7 DIV (versus control; discussed below). To explore this observation, further experiments were performed to quantify $Iba1^+$ microglia at 1, 4 and 14 DIV.

Cell counts showed an exponential increase in $Iba1^+$ cell numbers in control VM cultures across 1, 4, 7, and 14 DIV (Figure 5.7 A). This increase was not seen in nicotinamide treated cultures and the growth curves produced from control and nicotinamide treated cultures were significantly different (Malthusian growth goodness of fit R^2 = 0.84 vs. 0.12; $137.7_{(2,83)}$ $p < 0.0001$).

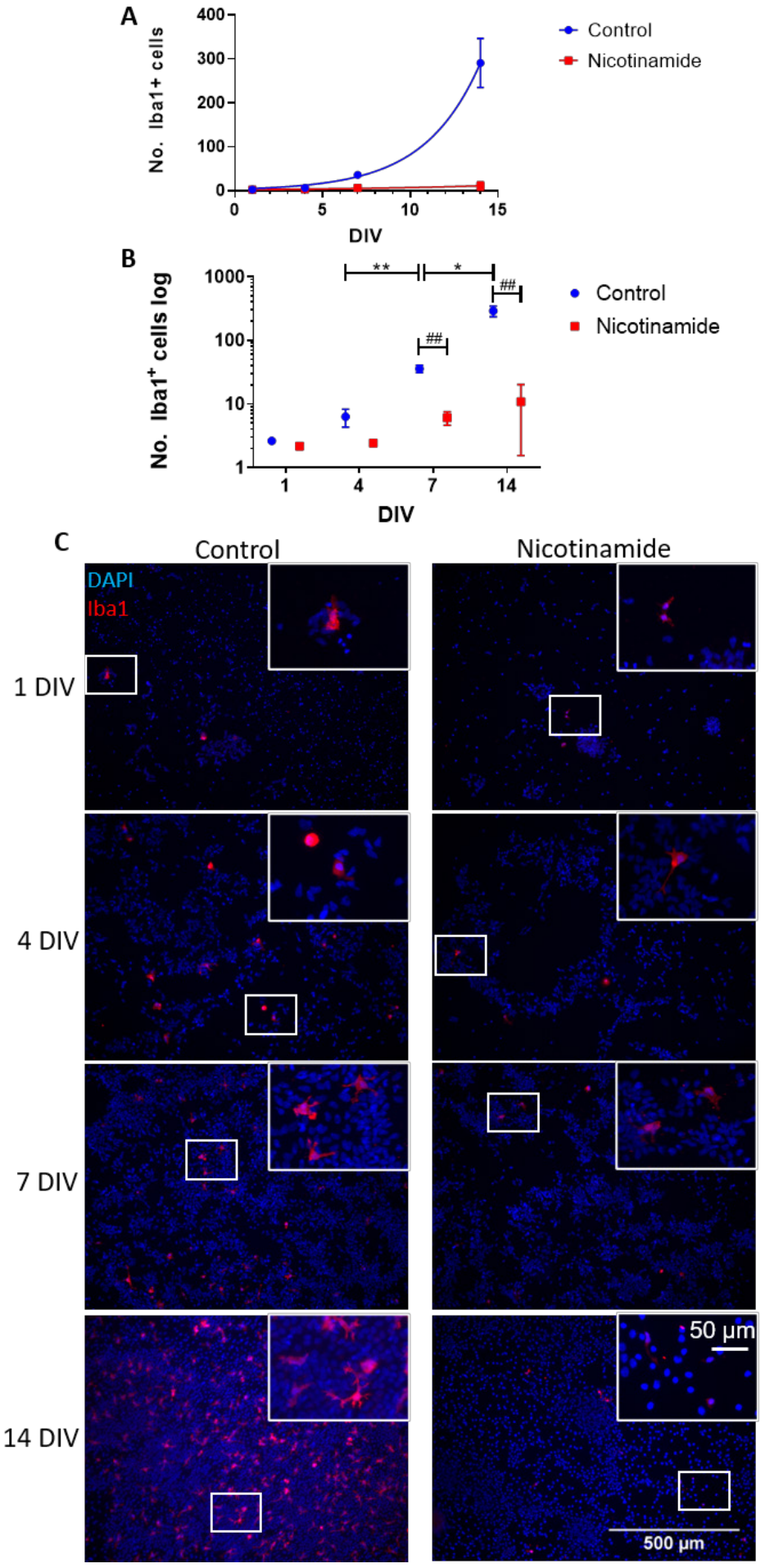

A
No. Iba1+ cells
400
300
200
100
0
0
5
10
15
DIV
Control
Nicotinamide
B
No. Iba1+ cells log
1000
100
10
1
1
4
7
14
DIV
**
*
##
##
Control
Nicotinamide
C
Control
Nicotinamide
DAPI
Iba1
1 DIV
4 DIV
7 DIV
14 DIV
50 µm
500 µm

Figure 5.7 Nicotinamide restricted proliferation of Iba1$^+$ microglia in VM cultures. A) Graph showing the exponential increase in numbers of Iba1$^+$ microglia per 10X micrograph (1.16 mm^2) in control (blue circle) VM cultures across 14 DIV, whilst numbers of Iba1$^+$ microglia showed very little increase in nicotinamide treated cultures (red square). B) Graph showing the number of Iba1$^+$ cells (log scale) per 10X micrograph (1.16 mm^2) in control (blue circle) and nicotinamide (red square) treated cultures. * In control cultures, numbers of Iba1$^+$ cells were significantly increased at day 7 vs. day 4, and day 14 vs. day 7. # Numbers of Iba1$^+$ cells were significantly lower in nicotinamide treated cultures compared to control at days 7 and 14. C) Representative 10X micrographs showing immunocytochemistry for DAPI and Iba1 in control and nicotinamide treated cultures at 1, 4, 7, and 14 DIV. Scale bar holds across images. * $p < 0.05$; ** $p < 0.01$; ## $p < 0.01$. DIV: days *in vitro*.

In control cultures, numbers of Iba1$^+$ microglia increased significantly over time (one way ANOVA, effects of DIV, $F_{(3, 8)} = 24.56$, $p < 0.001$; Figure 5.7 B, C). Individual *t*-tests showed significant increases in numbers of Iba1$^+$ cells between 4 and 7 DIV (6.31 ± 1.97 vs. 35.8 ± 4.58, $t = 5.9$, $p < 0.01$), and 7 and 14 DIV (35.8 ± 4.58 vs. 290.6 ± 55.7, $t = 4.6$, $p < 0.05$). These results show that microglia are highly proliferative in these VM cultures over 14 DIV.

When treated with nicotinamide, one-way ANOVA showed that numbers of Iba1$^+$ microglia did not significantly increase over time (DIV; Figure 5.7 B, C). Numbers of Iba1$^+$ microglia in control and nicotinamide treated VM cultures did not differ at 1 DIV (2.63 ± 0.13 vs. 2.18 ± 0.27, $t = 1.52$, n.s.) or 4 DIV (6.31 ± 1.97 vs. 2.44 ± 0.13, $t = 1.97$, n.s.). However, numbers were significantly lower in nicotinamide treated cultures compared to control cultures at 7 DIV (6.08 ± 1.44 vs. 35.8 ± 4.58, $t = 6.19$, $p < 0.01$) and 14 DIV (10.9 ± 9.32 vs. 290.6 ± 55.7, $t = 4.95$ $p<0.01$; Figure 5.7 B, C). This suggests that nicotinamide inhibits the proliferation of microglia in these VM cultures.

5.3.5 VM microglial proliferation was restricted during application of nicotinamide

To further test the effects of nicotinamide on the proliferation of microglia, VM cultures were grown in neural cell media (NCM) or nicotinamide supplemented NCM (NSM). Cells were then either fixed at 7 DIV (NCM; NSM), continued with the same media

(NCM→NCM; NSM→NSM), or swapped to the other media for the following 7 DIV (NCM→NSM; NSM→NCM) as described in Chapter 5.2.2. Numbers of Iba1$^+$ microglia were counted within 10X micrographs from 3-4 technical replicates and 3 experimental repeats. Numbers were then normalised to the 7 DIV NCM as described in Chapter 2.11.

Results from these VM cultures showed that, consistent with data shown in Chapter 5.3.4, cultures treated with nicotinamide (NSM) show significantly lower numbers of Iba1$^+$ microglia than control (NCM) cultures at 7 DIV (Figure 5.8 A, B, C).

Also consistent with Chapter 5.3.4, numbers of Iba1$^+$ cells increased significantly from 7 DIV to 14 DIV in cultures treated with NCM only (Tukey's: $p < 0.0001$; Figure 5.8, A, D). However, when cultures were swapped to nicotinamide supplemented media at 7 DIV (NCM→NSM) and grown to 14 DIV, numbers of Iba1$^+$ microglia did not increase significantly compared to control at 7 DIV (NCM; Tukey's: n.s.; Figure 5.8 A, B, F); that is, a further 7 days' culture did not lead to increased or decreased microglial numbers, when nicotinamide was added. At 14 DIV, cultures treated with only NCM (NCM→NCM) had significantly higher numbers of Iba1$^+$ microglia than cultures swapped to nicotinamide treatment at 7 DIV (NCM→NSM; Tukey's: $p < 0.0001$; Figure 5.8 A, D, F).

Consistent with data shown in Chapter 5.3.4, these results showed no significant difference between numbers of Iba1$^+$ microglia in nicotinamide treated cultures at 7 (NSM) and 14 DIV (NSM→NSM; Tukey's: n.s.; Figure 5.8 A, C, E). However, when nicotinamide treated cultures were swapped to NCM at 7 DIV and grown to 14 DIV (NSM→NCM), numbers of Iba1$^+$ microglia significantly increased compared to nicotinamide treated cultures stopped at 7 DIV (NSM; Tukey's: $p < 0.0001$; Figure 5.8 A, C, G); that is, a further 7 day's culture without nicotinamide, resulted in increased numbers of Iba1$^+$ cells. Cultures that were swapped from NSM to NCM at 7 DIV (NSM→NCM) also had significantly higher numbers of Iba1$^+$ microglia

than cultures grown with nicotinamide for 14 DIV (NSM→NSM; Tukey's: $p < 0.0001$; Figure 5.8).

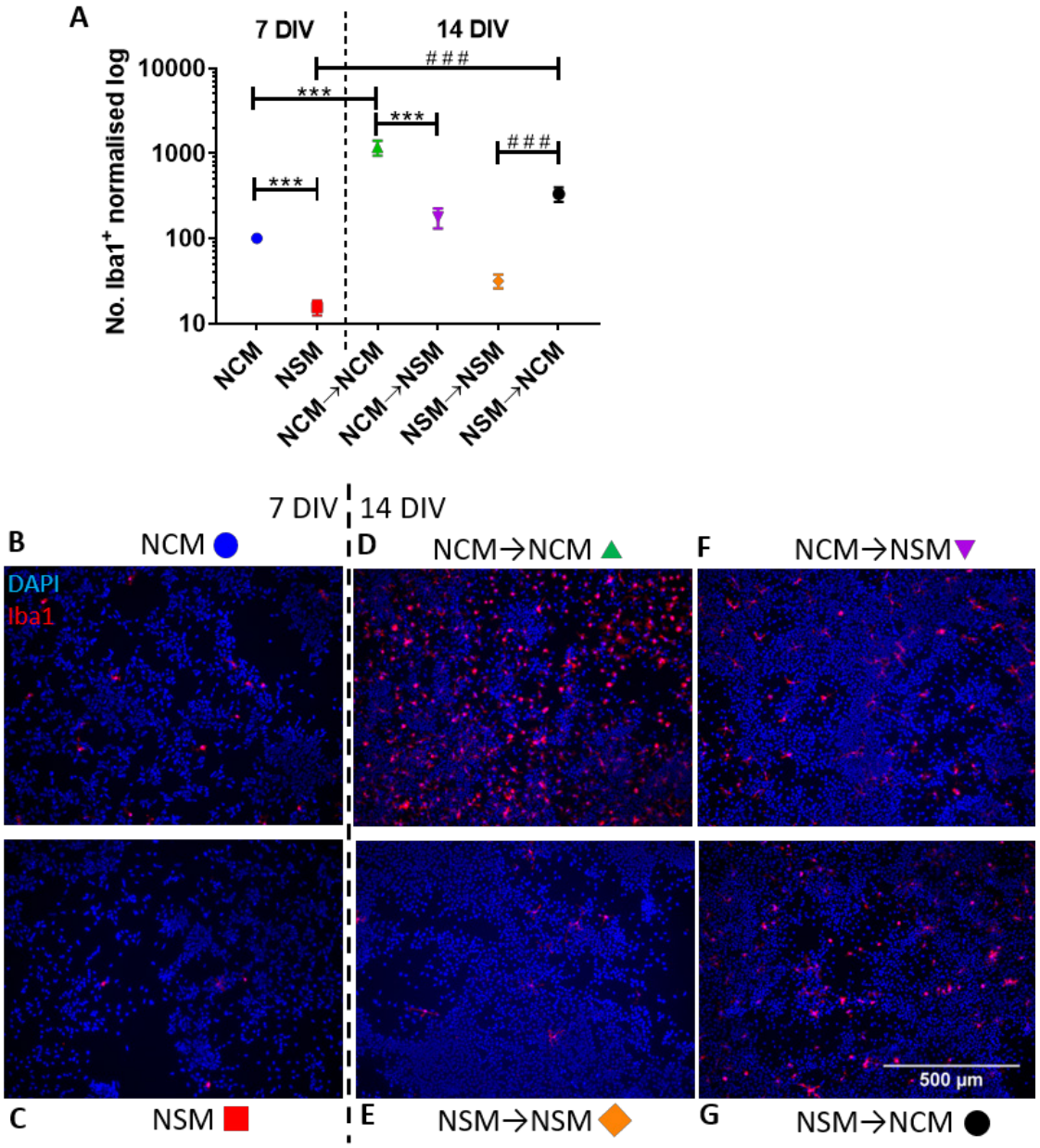


Figure 5.8 Nicotinamide only restricts proliferation during application. A) Graph showing the number of Iba1$^+$ cells normalised to control at 7 DIV (NCM; blue circle; B) for the treatment conditions described in Figure 5.1. * numbers of Iba1$^+$ cells were lower in nicotinamide treated cultures (NSM; red square; C) compared to control (NCM; blue circle; B) at 7 DIV (left of dashed line). Numbers of Iba1$^+$ were higher in control cultures at 14 DIV (NCM→NCM; green triangle; D) compared to control at 7 DIV (NCM; blue circle; B). NCM→NCM cultures also showed higher numbers of Iba1$^+$ cells at 14 DIV compared to control cultures that were swapped to nicotinamide treatment at day 7 (NCM→NSM; purple triangle; F). # Nicotinamide treated cultures that were swapped to control media at day 7 and grown to 14 DIV (NSM→NCM; black circle; G) showed higher numbers of Iba1$^+$ cells compared to nicotinamide treated cultures fixed at 7 DIV (NSM; red square; C). NSM→NCM cultures also showed higher numbers of Iba1$^+$ cells than cultures that were treated with nicotinamide for 14 days (NSM→NSM; orange diamond; E). *** $p < 0.001$; ### $p < 0.001$.

These results show that treatment of these VM cultures with 10 mM nicotinamide only inhibits microglia proliferation during application and the effects cease once this dose of nicotinamide is removed.

5.3.6 The E14 VM contained more microglia than the E14 whole ganglionic eminence

The effects of nicotinamide on microglia were also tested in cultures derived from the whole ganglionic eminence (WGE). Four micrographs at 10X magnification were taken from each of 3-4 technical replicates, across 3 experimental repeats. Numbers of $Iba1^+$ microglia were counted and averaged across each technical replicate.

Firstly, the WGE was compared to VM in terms of numbers of microglia present. Comparisons between cultures grown from E14 VM and E14 WGE at 7 DIV, showed the average number of $Iba1^+$ cells was significantly higher in VM than WGE (35.76 ± 4.58 vs. 2.77 ± 2.21; *t*-test $p < 0.01$; Figure 5.9 A).

Comparing numbers of $Iba1^+$ microglia between 7 and 14 DIV in VM and WGE suggested a difference in growth rates between cultures grown from these regions. Simple linear regression analysis comparing $Iba1^+$ numbers across 7 and 14 DIV for cultures derived from VM and WGE confirmed a significant difference between these slopes ($R^2 = 0.84$ vs. 0.27; $F_{(1, 8)} = 19.64$, $p < 0.01$; Figure 5.9 B). These results suggest a significant difference in the rate of increase of $Iba1^+$ microglia between cultures derived from E14 VM and WGE, although the use of more timepoints would be useful to confirm this.

When assessing the effect of nicotinamide on WGE derived cultures, no significant difference was observed (two-way ANOVA n.s.; Figure 5.10). This shows that nicotinamide did not exert significant effects on the number of $Iba1^+$ microglia present in WGE cultures, and

neither did time (7 vs 14 DIV), although Iba1$^+$ numbers were low in both control and treatment conditions at both timepoints.

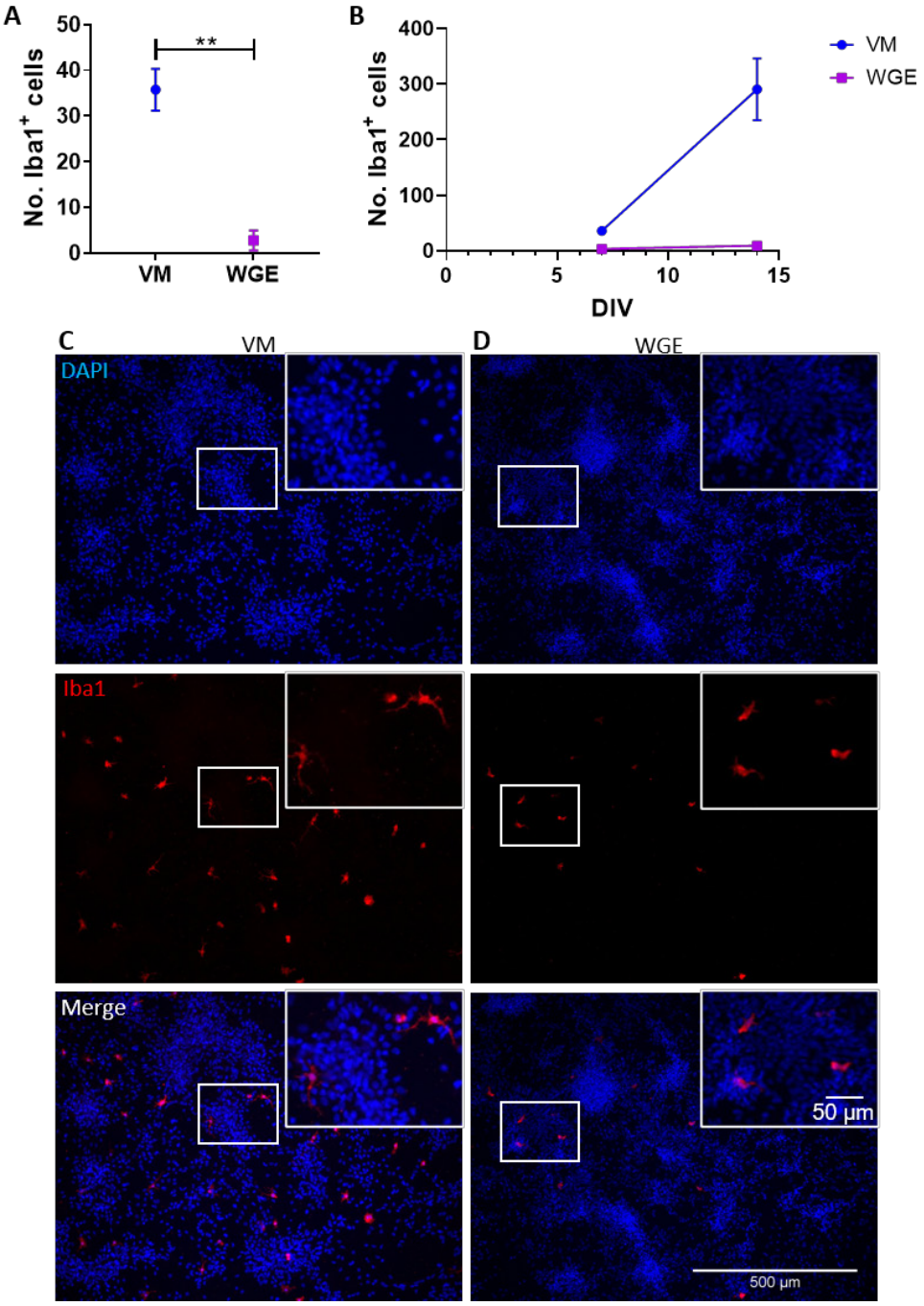


Figure 5.9 VM cultures contained more Iba1$^+$ microglia than WGE cultures at 7 DIV.
A) Graph showing the average number of Iba1$^+$ microglia per 10X micrograph (FOI = 1.16 mm^2) in cultures derived from E14 rat VM (blue circle) and WGE (purple square), after 7 DIV. VM cultures contain significantly more Iba1$^+$ cells than WGE cultures (** $p < 0.01$).
B) Graph showing simple linear regression representing growth between 7 and 14 DIV in VM (blue circle) and WGE (purple square) derived cultures. A significant difference was observed between the slopes ($p < 0.01$).
C) Representative 10X fluorescence micrograph showing DAPI and Iba1 immunocytochemistry for VM cultures at 7 DIV. D) Representative 10X fluorescence micrograph showing DAPI and Iba1 immunocytochemistry for WGE cultures at 7 DIV. Scale bars hold across all images. DIV: days *in vitro*; VM: ventral mesencephalon; WGE: whole ganglionic eminence.

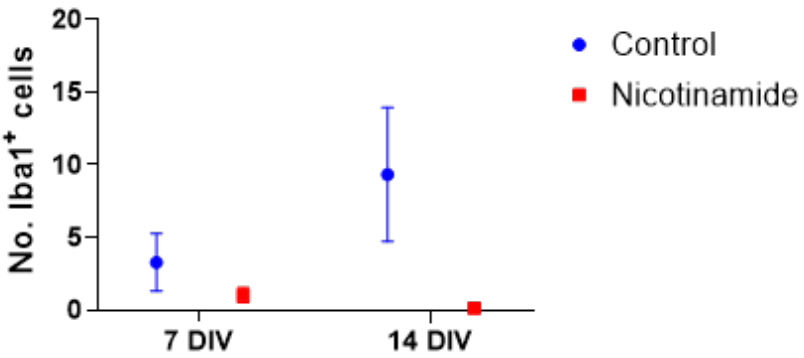


Figure 5.10 Nicotinamide did not significantly affect numbers of Iba1$^+$ cells in WGE cultures. Graph showing the average number of Iba1$^+$ cells per 10X micrograph (FOI = 1.16 mm2) in control (blue circle) and nicotinamide treated (red square) WGE cultures at 7 and 14 days in vitro (DIV). No significant effect was observed using two-way ANOVA.

5.3.7 Varied microglia morphology was present within VM cultures

Iba1$^+$ microglia within VM cultures displayed a range of morphologies including amoeboid, primary branching and secondary branching (Figure 5.11 A, B, C respectively). The morphology of microglia in both control and nicotinamide treated VM cultures changed over time (Figure 5.11 control: D-G; nicotinamide: H-K). At 1 DIV, nearly all microglia were amoeboid shape or close to an amoeboid shape with short branching. At 4 DIV, different shapes were becoming apparent with some microglia developing primary branches. By 7 DIV more complex primary and secondary branching was observed alongside some amoeboid morphology. This variety of shapes continued to be displayed at 14 DIV. Treatment of VM cultures with 10 mM nicotinamide did not appear to affect the morphology of Iba1$^+$ microglia compared to control cultures.

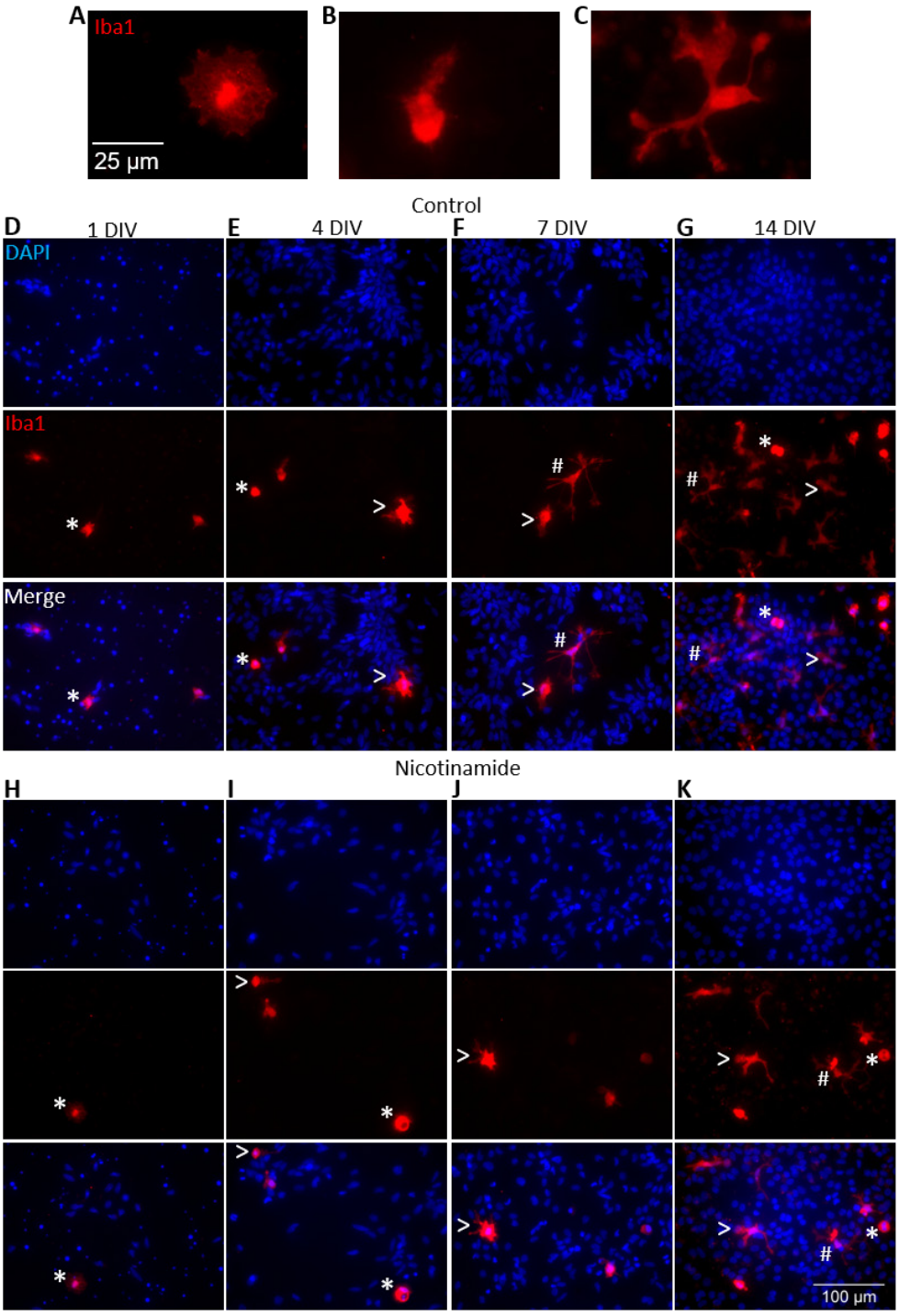


Figure 5.11 Iba1^{+} microglia displayed various morphologies in VM cultures. A) Fluorescence micrograph showing amoeboid morphology. B) Fluorescence micrograph showing an example of primary branching with simple projections extending from the soma. C) Fluorescence micrograph showing an example of secondary branching with more complex processes containing further branching extending from the soma. Scale bar holds across A-C. D) Fluorescence micrographs showing DAPI and Iba1 immunocytochemistry for control VM cultures at 1 DIV. E) Immunocytochemistry for control VM cultures at 4 DIV. F) Immunocytochemistry for control VM cultures at 7 DIV. G) Immunocytochemistry for control VM cultures at 14 DIV. H) Immunocytochemistry DAPI and Iba1 for nicotinamide treated VM cultures at 1 DIV. I) Immunocytochemistry for nicotinamide treated VM cultures at 4 DIV. J) Immunocytochemistry for nicotinamide treated VM cultures at 7 DIV. K) Immunocytochemistry for nicotinamide treated VM cultures at 14 DIV. * represents examples of amoeboid morphology; > represents examples of primary branching; # represents examples of secondary branching. Scale bar hold across D-K. DIV: Days *in vitro*; VM: ventral mesencephalon.

5.3.8 Nicotinamide reduced numbers of Iba1$^+$ cells in microglia cultures derived from primary mixed glia

The effect of nicotinamide was also tested on primary microglia cultures derived from mixed glial cultures. These isolated microglia cultures were seeded and grown for 5 days in control conditions or with 1, 5 or 10 mM nicotinamide. Numbers of Iba1$^+$ microglia were quantified across 4 micrographs taken at 10X magnification for each of 3 technical replicates and 3 experimental repeats. Numbers of Iba1$^+$ microglia were then normalised as described in Chapter 2.11. Microglia grown in control conditions displayed different morphology to microglia within E14 VM cultures. This morphology, shown in Figure 5.12 is characteristic of pure 2D microglial cultures with mostly amoeboid or large, flattened 'fried egg' morphology, some primary branching, rod-like or unipolar/bipolar morphologies (1 or 2 long, extending processes), and very limited secondary branching morphologies.

Numbers of Iba1$^+$ microglia were significantly affected by concentration of nicotinamide (one-way ANOVA $F_{(3,32)} = 0.62$, $p < 0.01$). Post-hoc analysis revealed numbers were significantly reduced with application of 10 mM nicotinamide compared to control (Dunnett's: $p < 0.001$; Figure 5.13). The morphology of Iba1$^+$ microglia in nicotinamide treated cultures also appeared to be affected, with smaller cell bodies observed with increasing concentrations (Figure 5.13). At 10 mM, microglial cell bodies were barely larger than their nuclei, possibly indicating apoptotic or pyknotic cells, suggestive of toxicity. This shows that application of 10 mM nicotinamide to primary microglial cultures for 5 days reduced the number of microglia in cultures, and suggests that nicotinamide treatment can affect microglial morphology.

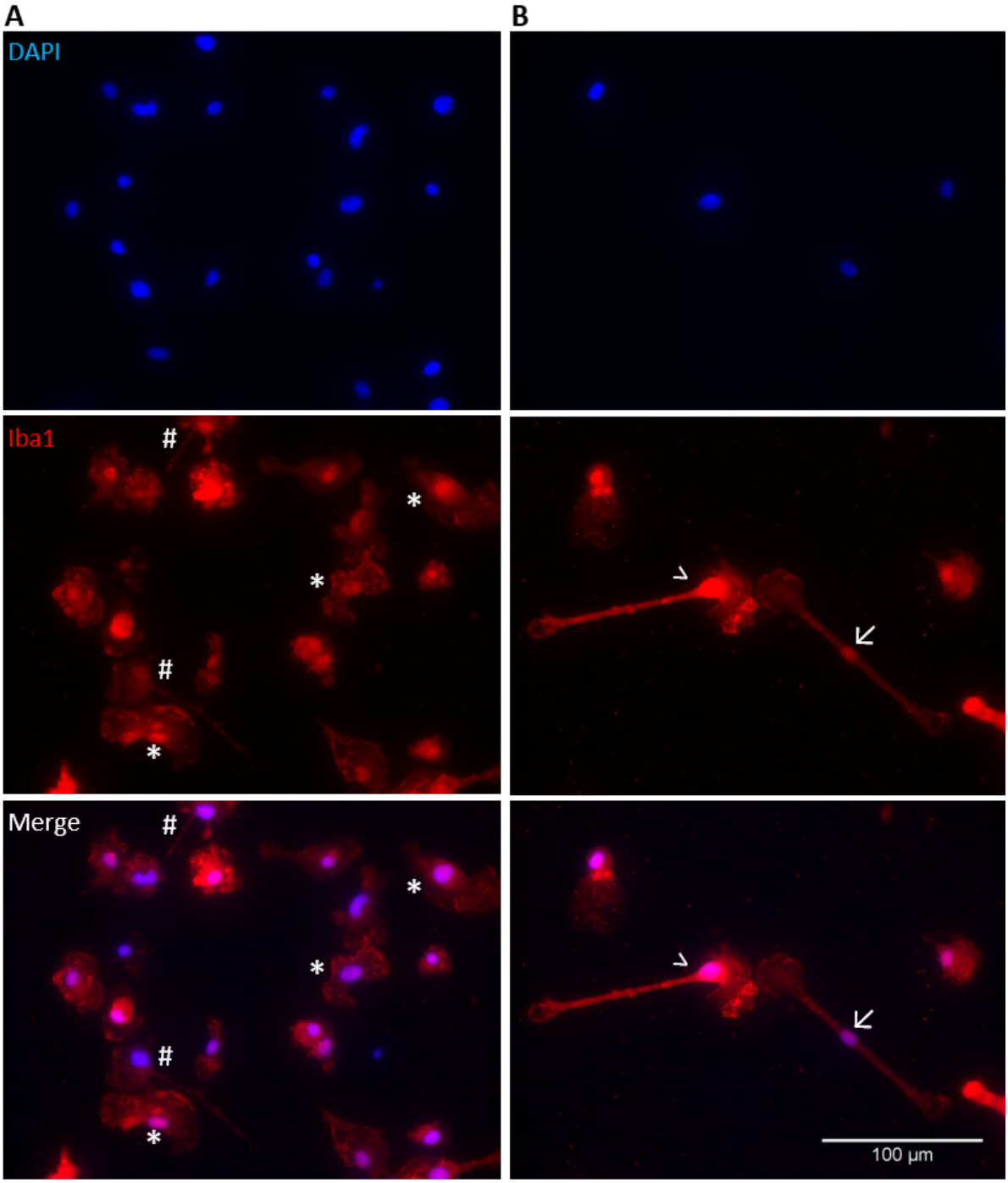


Figure 5.12 Morphology of microglia isolated from mixed glial cultures. Representative fluorescence micrographs of microglia isolated from mixed glial cultures grown for 5 days in control conditions with immunocytochemistry for DAPI (blue) and Iba1 (red). A) Iba1^{+} microglia displaying large, flattened 'fried egg' morphology (*) and some showing primary branching (#). B) Iba1^{+} microglia displaying unipolar (>) and bipolar (→) morphologies, the processes typically end with large flattened 'bulbs'. Scale bar holds across all images.

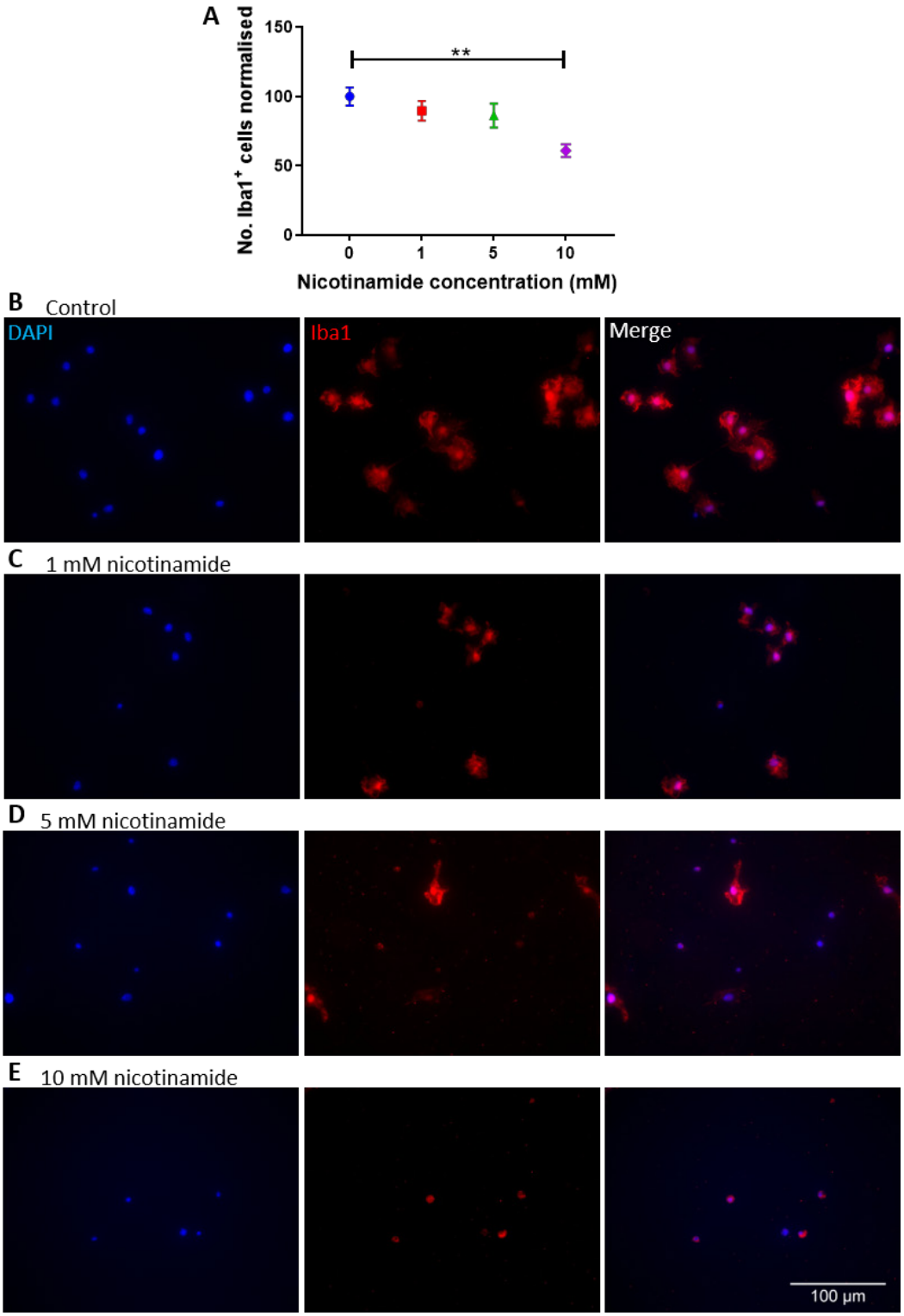


Figure 5.13 The number of Iba1$^+$ cells was reduced in isolated microglial cultures treated with 10 mM nicotinamide. A) Graph showing the number of Iba1+ microglia normalised to control in isolated microglial cultures in control (0; blue circle), and with 1 mM (red square), 5 mM (green triangle), and 10 mM (purple diamond) nicotinamide treatment. 10 mM nicotinamide significantly reduced numbers of Iba1$^+$ microglia in these cultures (** $p < 0.01$). B-E) Representative 40X fluorescence micrographs showing DAPI and Iba1 immunoreactivity control (B), 1 mM nicotinamide (C), 5 mM nicotinamide (D), and 10 mM nicotinamide (E). The morphology of Iba1 expression appears to change with nicotinamide treatment with cell size appearing to decrease with increasing nicotinamide concentrations (B-E). 10 mM nicotinamide treated cells (E) appear rounded and possibly pyknotic. Scale bar holds across images.

5.3.9 In isolated microglial cultures, GM-CSF increased numbers of Iba1$^+$ microglia and counteracted nicotinamide

Numbers of Iba1$^+$ microglia were quantified and normalised as described in Chapter 2.11. The addition of GM-CSF for 5 days to primary microglial cultures significantly increased numbers of Iba1$^+$ cells by about 45% compared to control (t = 2.59; p < 0.05; Figure 5.14).

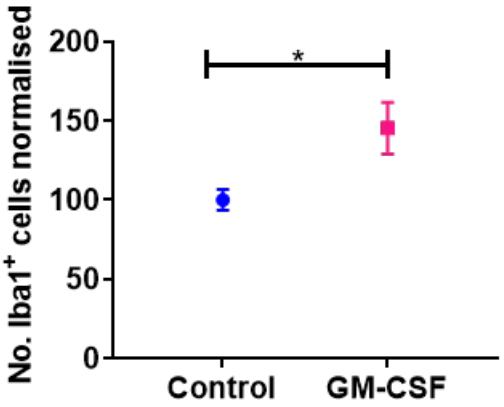


Figure 5.14 GM-CSF increased the number of Iba1$^+$ cells in isolated microglial cultures. Graph showing the number of Iba1$^+$ cells normalised to control in control and GM-CSF treated isolated microglial cultures. Numbers of Iba1$^+$ microglia were significantly higher with application of GM-CSF (20 ng/mL; pink square) compared to control (blue circle) after 5 days in culture (* p < 0.05).

The addition of GM-CSF alongside nicotinamide resulted in no significant difference in Iba1$^+$ cell numbers across all concentrations of nicotinamide, versus GM-CSF alone (1 mM, 5 mM, 10 mM; one-way ANOVA: n.s.; Figure 5.15). This shows that GM-CSF counteracted the reduction in Iba1$^+$ microglia numbers induced by nicotinamide treatment described in Chapter 5.3.8.

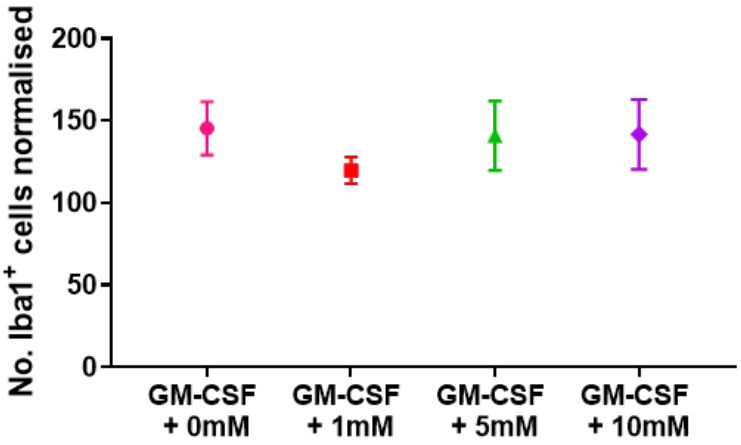


Figure 5.15 Nicotinamide treatment alongside GM-CSF did not affect the number of Iba1$^+$ cells in isolated microglial cultures. Graph showing the average number of Iba1$^+$ cells normalised to the control (Figure 5.14 blue circle) in isolated microglial cultures treated with GMCSF alone (pink circle) and alongside nicotinamide at concentrations of 1 mM (red square), 5 mM (green triangle), and 10 mM (purple diamond). No significant difference was observed across these conditions using one-way ANOVA.

5.3.10 Numbers, morphology, and expression of Iba1$^+$ microglia were differentially affected by GM-CSF and nicotinamide

The morphology of Iba1$^+$ cells showed stark differences between control and GM-CSF treated cultures (Figure 5.16 A, B). Cultures treated with GM-CSF appeared to have larger cell areas (as judged by membrane covering the substrate; cell volume could not be determined) with stronger intensity of Iba1 expression. Optical density (OD) measurements (described in Chapter 2.10) provided a rapid, facile, and high throughput assessment of these cultures. The OD of whole micrographs can be influenced by cell numbers, morphology and/or levels of Iba1 expression. Results were normalised as described in Chapter 2.11. Results showed a significant effect of nicotinamide on the OD of these isolated microglial cultures (two-way ANOVA, nicotinamide as factor, $F_{(3,64)} = 3.84$, $p < 0.05$). GM-CSF also had a significant impact on the OD of these cultures (two-way ANOVA, GM-CSF as factor, $F_{(1,64)} = 16.4$, $p < 0.001$). Post-hoc analysis revealed a significant increase in OD in GM-CSF treated microglial cultures compared to control (Sidak's: $p < 0.001$; Figure 5.16, A-C). This suggests an increase in the area covered

by cell membrane and/or the intensity of Iba1 staining when GM-CSF is applied to microglia cultures. This increase could be due to factors including numbers of $Iba1^+$ cells, morphology of $Iba1^+$ cells, and expression intensity of Iba1.

Cultures treated with 1 mM, 5 mM or 10 mM nicotinamide alongside GM-CSF did not show a significant difference in OD compared to cultures receiving nicotinamide alone (Tukey's: n.s.; Figure 5.16, C-G). However, OD was significantly decreased when comparing cultures treated with GM-CSF plus nicotinamide (1, 5 and 10 mM) versus GM-CSF alone (Tukey's: $p < 0.01$; $p < 0.05$; $p < 0.01$ respectively; Figure 5.16 C-G). This suggests that nicotinamide treatment may counteract the increase in OD incurred by GM-CSF treatment.

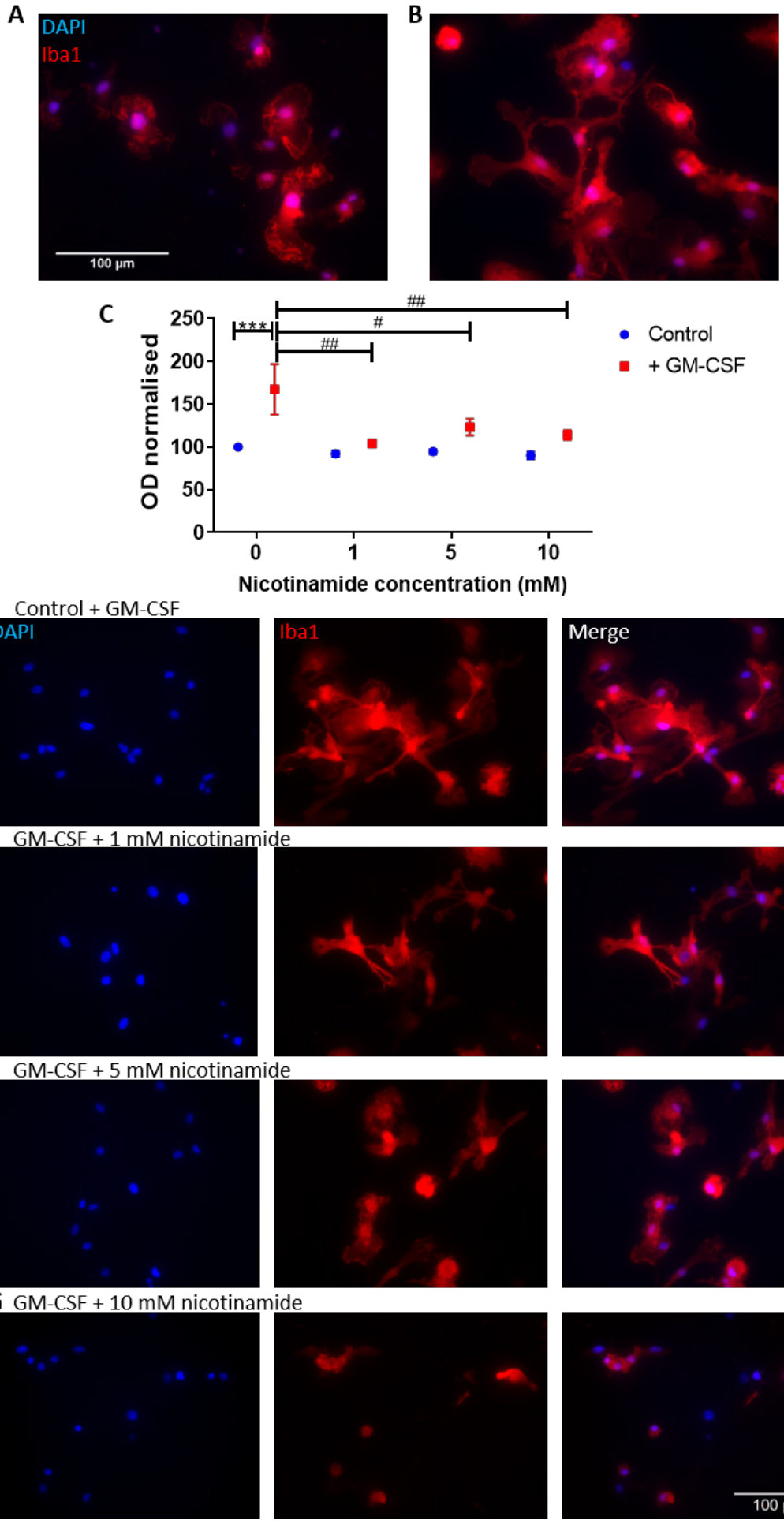

A
DAPI
Iba1
100 µm
B
C
OD normalised
250
200
150
100
50
0
0
1
5
10
Nicotinamide concentration (mM)
***
#
##
##
Control
+ GM-CSF
D Control + GM-CSF
DAPI
Iba1
Merge
E GM-CSF + 1 mM nicotinamide
F GM-CSF + 5 mM nicotinamide
G GM-CSF + 10 mM nicotinamide
100 µm

Figure 5.16 Application of GM-CSF to isolated microglia cultures increased Iba1 expression measured by optical density (OD), an effect that was counteracted by nicotinamide treatment.
A-B) Representative 40X micrographs (FOI = 0.073mm^2) showing DAPI and Iba1 immunoreactivity in control (A) and GM-CSF treated (B) isolated microglial cultures. Iba1 expression appears more intense in GM-CSF treated cultures (B), with potentially larger cell bodies and more identifiable processes compared to control (A). Scale bar holds across A and B.
C) Graph showing the OD, normalised to control, for Iba1 immunofluorescence in micrographs taken at 40X magnification, control (0, blue circle) and nicotinamide alone at concentrations of 1, 5 and 10 mM (blue circles), and GM-CSF alone (0, red square) and GM-CSF with nicotinamide at concentrations of 1, 5, and 10 mM (red squares). * Significantly higher Iba1 OD was observed in GM-CSF alone treated cultures compared to control. # Significantly lower Iba1 OD was observed in cultures treated with nicotinamide alongside GM-CSF compared to GM-CSF alone. *** $p < 0.001$; # $p < 0.05$; ## $p < 0.01$.
D-G) Representative 40X fluorescence micrographs showing immunoreactivity for DAPI and Iba1 in isolated microglial cultures grown for 5 days. D) GM-CSF alone, E) GM-CSF with 1 mM nicotinamide, F) GM-CSF with 5 mM nicotinamide, G) GM-CSF with 10 mM nicotinamide. It is apparent that cell morphology changes with increased nicotinamide treatment, with 10 mM nicotinamide and GM-CSF treated cells showing smaller, rounded cell bodies and some potentially pyknotic cells. Scale bar holds across images D-G.

5.4 Discussion

5.4.1 Identification of cell types within VM cultures

As shown Figure 4.6, overall cell numbers in VM cultures were significantly reduced with nicotinamide application. This reduction in cell numbers was not due to a reduction in β-III-tubulin$^+$ neurons. This led to more detailed characterisation of the cell types present within these VM cultures. Control cultures predominantly stained for Nestin$^+$ cells (46%), followed by β-III-tubulin$^+$ cells (13%), then GFAP$^+$ cells (2%) and Iba1$^+$ cells (0.85%).

Despite using multiple neural cell type-specific markers, it was not possible to identify ~40% of cells. On morphological/microscopic examination at least some of these cells were consistent with oligodendroglial morphologies, or astrocyte morphologies, although the latter were not immune-positive for the DAKO GFAP antibody (discussed later in Chapter 5.4.2).

One explanation for these cells being negative for the markers tested, is the possibility that they are non-neural cells, for example fibroblasts, which could have contaminated the culture from incomplete removal of meningeal tissue (Pruszak *et al.*, 2009). Fibroblasts are difficult to positively identify, with few reliable antibodies available (Goodpaster *et al.*, 2008). Vimentin can be used to identify fibroblasts, however this intermediate filament protein is also expressed in astrocytes (Kamphuis *et al.*, 2012). Although it is difficult to confirm the presence or absence of fibroblasts in these VM cultures, steps were taken during dissection to reduce the risk of fibroblast contamination. For example, VM tissue was moved to fresh dissection media after removal of meningeal tissue for further trimming before collection, and tissue sections were washed 3 times with fresh dissection medium before dissociation.

The cell populations of VM cultures described in the literature are displayed in Table 5.1, although many papers using E14 rat VM cultures do not report on the presence or absence of non-neuronal populations. The proportions of astrocytes is often reported, based on GFAP

staining, including use of the DAKO GFAP antibody discussed in Chapter 5.3.2. Microglial populations are reported in less than half of the studies identified here, and in one case, the marker used for identification was not reported. None of the papers shown in Table 5.1 report on Nestin$^+$ neural cell populations, or the presence of neural stem cells, based on any markers. The percentage of cells remaining uncharacterised in these papers was up to 29%, at 7 DIV (Callizot *et al.*, 2019). None of these studies maintained cultures for 14 DIV.

Differences in cell populations can arise from differences in dissection methods (which cell types are initially present) and media composition (which can influence survival, proliferation, and differentiation). These VM cultures contained a lower percentage of neurons than reported by others in the literature. The literature reports neuronal populations within VM cultures as being 40-97% of cells at 7 DIV. However, Cheung, Hickling, & Beart (1997) reported 97% (± 14 SEM) MAP2$^+$ (microtubule-associated protein 2) neurons alongside 13% (± 1 SEM) GFAP$^+$ cells at 7 DIV. There is some difficulty reconciling these numbers suggesting inaccuracies in staining and/or counting methods. MAP2 has since been shown to be present in immature glial cells (Blümcke *et al.*, 2001) suggesting that non-neuronal cells may have been incorrectly identified as neurons. These numbers are also reported as the percentage of stained cells without suggestion of a population that was not immune-positive for any of TH, MAP2, or GFAP.

The lower percentage neuronal population reported here could arise from tougher manual dissociation, or from the staining/imaging techniques used as discussed in Chapter 4.4.2.

Nicotinamide treated cultures showed a change in the proportions of cell types present within the VM cultures. The reduction in Nestin$^+$ cells is discussed later in Chapter 5.4.3. The reduction in Iba1$^+$ microglia is discussed in Chapter 5.4.4.

Table 5.1 Table showing examples of VM culture populations from the literature. * indicates that no antibody was specified. Article number references: 1: Cheung, Hickling and Beart (1997); 2: Zhang *et al.* (2014); 3: Callizot *et al.* (2019); 4: Liu and J. Hong (2003); 5: Gaven, Marin and Claeysen (2014).

Dopamine neurons	Neurons	Astrocytes	Microglia	Neural stem cells	VM culture details	Media	Article no.
TH^+: 5% of $MAP2^+$	$MAP2^+$: 85%	$GFAP^+$: 0.5%	Not stated	Not stated	E14-16 Rat 1.9×10^6 per 24 well 1 DIV	Neurobasal, B27, L-glutamine (500 mM) and penicillin (200 units/ ml) and streptomycin (200 mg/ml)	1
TH^+: 5% of $MAP2^+$	$MAP2^+$: 77%	$GFAP^+$: 3%	Not stated	Not stated	3 DIV		
TH^+: 5% of $MAP2^+$	$MAP2^+$: 87%	$GFAP^+$: 10%	Not stated	Not stated	5 DIV		
TH^+: 5% of $MAP2^+$	$MAP2^+$: 97%	$GFAP^+$: 13%	Not stated	Not stated	7 DIV		
TH^+: 2-3% of neurons	Neu-N: 40%	Astroglia*: 48%	OX-42: 11% of total cells	Not stated	E14 Rat 5×10^5 per 24 well 7 DIV	Minimum essential medium, 10% heat-inactivated foetal bovine serum, 10% heat-inactivated horse serum, 1 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µM non-essential amino acids, 50 U/mL penicillin, 50 µg/mL streptomycin	2
Not stated, suggested as above	Neu-N: 45%	Astroglia*: 54%	OX-42: 1% (suppressed by LME)	Not stated		As above, 1.5 mM Leu-Leu methyl ester hydrobromedia (LME) added 24 hr after seeding to supress microglial proliferation	

TH^+: 6-8% of neuronal population	Neurons: 70% of total cells, "mainly GABAergic"	Not stated	Microglia*: ~1% of total cells	Not Stated	E15 Rat 2.25 x 10^5 per 24 well 6 DIV	Neurobasal, 2% B27, 2 mM L-glutamine, 2% PS solution, 10 ng/mL brain-derived neurotrophic factor, 1 ng/mL glial-derived neurotrophic factor	3
TH^+: up to 1%	$MAP2^+$: 40%	$GFAP^+$: 50% (DAKO)	$OX\text{-}42^+$: 10% of total cells	Not stated	E13-14 Rat 5 x 10^5 per 24 well 7 DIV	Minimum essential medium, 10% heat-inactivated foetal bovine serum, 10% heat-inactivated horse serum, nonessential amino acids (1X), 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin–streptomycin (50 U/mL, 50 µg/mL), 1 g/L D-glucose	4
DAT^+ or $TH^=$: 2-4% of whole cell population	MAP2 in addition to: $Serotonin^+$: <1%; $GAD67^+$ (GABA): 50%; Glutamatergic*: 40%	$GFAP^+$: 2-3%	Not stated	Not stated	E13.5 mouse 6 x 10^5 per 24 well Up to 13 DIV TH^+ peak at 6 DIV	In sterile water, mix successively 40 ml of 5x Dulbecco's Modified Eagle/F12-Ham, 1 mL 1 M HEPES, 2 mL 200 mM L-Glutamine, 2 mL penicillin-streptomycin, 4 mL 30% glucose, 3 mL 7.5% $NaHCO_3$	5

5.4.2 Nicotinamide does not appear to affect GFAP$^+$ astrocytes

Astrocytes typically make up 13-50% of E14 rat VM cultures at 7 DIV in the literature (Table 5.1). VM cultures described here contained approximately 2% GFAP$^+$ astrocytes of the total cell population. This low GFAP$^+$ population alongside a large unstained population could mean that the majority of astrocytes present in these VM cultures did not express GFAP or the antibody used did not detect the GFAP isoform that was expressed.

GFAP is a type III intermediate filament protein and is considered to be a marker of fully differentiated astrocytes that is upregulated in instances of injury and damage (Hol and Capetanaki, 2017). Although GFAP is widely used as an astrocyte marker indicative of reactive astrocytes, astrocyte expression patterns are not as simple as being either GFAP$^+$ or GFAP$^-$ (Liddelow and Barres, 2017). Other markers, including Nestin, are also expressed in astrocytes in response to damaging stimuli (Cho *et al.*, 2006; Luna *et al.*, 2010). GFAP expression can vary dependent on culture preparation, with astrocytes in directly seeded primary culture of mouse cortex showing the weakest GFAP expression compared to subcultured astrocytes, after being placed on a rotary shaker (Du *et al.*, 2010). Medium composition such as the presence of serum (Codeluppi *et al.*, 2011), growth factors, and metabolites from other cell types can also affect expression patterns in cultured astrocytes (Lange *et al.*, 2017). The culturing process used here, with only 7 DIV, and the presence of other neural cell types may have subdued GFAP expression in these VM cultures. Cultures grown from E14 WGE showed no GFAP$^+$ cells in micrographs (taken at 40X magnification; although a few clusters were visible across the entire coverslip) at 7 DIV, however, micrographs (also taken at 40X magnification) from cultures at 14 DIV showed approximately 11% GFAP$^+$ of total cells in control cultures (data not shown). This suggests that GFAP expression increases with time.

However, other studies have reported a decrease in GFAP$^+$ astrocytes, and an increase in calbindin D28k immunoreactive astrocytes, from day 1-9 in astrocytes derived from postnatal mice, suggesting that GFAP may not be consistently expressed in astrocytes (Xu, 2018). Zengli Zhang *et al.* (2019) also found different expression patterns for the astrocyte markers GFAP, S100-β, and NDRG2 (N-Myc downstream-regulated gene 2) in tissue slices obtained from different mouse brain regions, suggesting that the choice of astrocyte markers should be tailored to different brain regions.

Another possibility for the lower than expected GFAP$^+$ population is the differences in antibody specificity for GFAP isoforms. Nine variants of GFAP are described across human, mouse, and rat, with different expression patterns across brain regions (Kamphuis *et al.*, 2012). The DAKO anti-GFAP antibody was used for astrocyte identification in VM cultures in the majority of this PhD thesis, however, it detected low numbers of GFAP$^+$ astrocytes. Figure 5.5 shows the differences in GFAP staining between GFAP antibodies supplied by DAKO and Biolegend. This shows that the DAKO GFAP antibody did not identify all GFAP$^+$ astrocytes in these VM cultures, resulting in the lower than expected numbers observed. The differences between these antibodies may arise from the generation of the antibody, although both the DAKO and Biolegend immunogens are derived using bovine spinal cord. However, DAKO is polyclonal, and Biolegend monoclonal; from this it would be expected that the DAKO antibody would recognise more epitopes than the Biolegend antibody, which is why it was initially chosen. The differences observed also could be because different antibodies may be selective for a particular isoform, or isoforms, of GFAP, with different specificities also found between human and mouse GFAP (Lin, Messing and Perng, 2017).

From the results presented here, 10 mM nicotinamide did not have an effect on GFAP$^+$ astrocytes in these VM cultures. However, further analysis using other astrocyte markers or cultures containing pure astrocyte populations should be used to test this conclusion, given

the uncertainty as to whether all astrocytes were reliably identified within these cultures. The data reported here does however correspond with data showing that nicotinamide did not affect the differentiation of glial ($GFAP^+$ and neural/glial antigen-2 $[NG2]^+$) cells from mESCs (Griffin *et al.*, 2017). However, nicotinamide is an inhibitor of PARP-1, and PARP-1 knockout mice have shown increased differentiation of $GFAP^+$ astrocytes and oligodendrocytes (Hong *et al.*, 2019), suggesting that nicotinamide could have an effect on glial populations.

5.4.3 Nicotinamide reduced numbers of $Nestin^+$ cells in VM cultures

$Nestin^+$ cells made up the highest percentage of identified cells in both control and nicotinamide treated VM cultures at 7 DIV. Nestin is a class 6 intermediate filament protein and although it is commonly regarded as a NSC marker, it is also expressed in a number of nervous tissue tumours, and in non-neural cells, including, retina, muscle, skin, teeth and liver (review: Gilyarov, 2008). Due to the origin of the VM cultures used here, it is likely that the $Nestin^+$ population is of neural lineage. $Nestin^+$ cells have been shown to differentiate into both neurons and astrocytes (Andressen *et al.*, 2001) and Nestin expression has been identified in neuronal and astrocyte populations after damage (Gilyarov, 2008; Luna *et al.*, 2010; Cho *et al.*, 2013). Proliferating microglia have also been shown to transiently express Nestin after their depletion *in vivo* (Elmore *et al.*, 2014; Huang *et al.*, 2018).

Nicotinamide treatment over 7 DIV reduced the number of $Nestin^+$ cells in these VM cultures. Here, these cells are thought to represent the NSC population, although, as shown above, Nestin expression has been reported in neurons, astrocytes, and microglia. In these VM cultures, co-expression of Nestin and GFAP was observed, although incidence was very low with few presentations across cultures (<15 across experimental repeats; data not

included). NSCs are a proliferative population, and so nicotinamide may be expected to limit their proliferation.

As discussed in Chapter 3, nicotinamide has been shown to reduce the proliferation capacity of a number of cell types. Treatment of the mouse NSC line C17.2 with nicotinamide dose dependently reduced proliferation, with a significant decrease seen from concentrations of 20 mM and above (Wang *et al.*, 2012). Although this was a mouse cell line, it correlates with the effects seen by nicotinamide in these primary rat NSCs. Nicotinamide has also been found to decrease the number of Oct4 (an ESC marker) expressing cells in mESCs (Griffin *et al.*, 2017). These results show that nicotinamide limited proliferation of Nestin$^+$ cells in these VM cultures, correlating with results discussed in Chapter 3, with nicotinamide also reducing proliferation in the mESCs used in this PhD project.

5.4.4 Nicotinamide limits the proliferation of Iba1$^+$ microglia in VM cultures

As shown in Chapter 5.3.4, the number of Iba1$^+$ microglia in VM cultures was significantly lower with nicotinamide treatment. This effect also observed in an *in vivo* traumatic brain injury rat model, with significantly lower numbers of Iba1$^+$ microglia present in nicotinamide treated rats compared to saline-treated (Smith *et al.*, 2019). These results suggest that nicotinamide lowers microglia *in vivo*, with this effect being replicated in the *in vitro* cultures employed here.

By observing the number of microglia present in these VM cultures across 1, 4, 7 and 14 DIV it was possible to show that nicotinamide limited the proliferation of Iba1$^+$ microglia. Furthermore, by addition or removal of nicotinamide treatment after 7 DIV, it was shown that

proliferation of microglia is only limited during application of nicotinamide, with the effect being reversible upon removal of nicotinamide.

As previously discussed, microglia can transiently express Nestin, raising the possibility that these effects could be due to a decrease in the differentiation of microglia from $Nestin^+$ progenitors, as distinct from microglia being generated by proliferation of microglial cells themselves. Elmore et al. (2014) suggest that microglia repopulation *in vivo* occurs through proliferation and differentiation of $Nestin^+$ cells. However, a fate-mapping study failed to find microglia derived from $Nestin^+$ cells (Huang *et al.*, 2018) and Askew et al. (2017) found no evidence of Nestin expression in proliferating microglia involved in homeostatic maintenance. Much of the literature also coincides with the idea that microglia are highly proliferative, with general consensus suggesting proliferation rather than differentiation is responsible for establishing and maintaining the brain microglial population (Navascués *et al.*, 2000; Ginhoux and Prinz, 2015; Askew *et al.*, 2017; Huang *et al.*, 2018). The use of a marker such as Ki67 or BrdU, combined with Nestin or Iba1, would help to definitively determine whether the increase in microglia observed in these VM cultures is due to proliferation rather than differentiation.

This effect of nicotinamide limiting microglial proliferation was seen in VM cultures, which contain a number of other neural cell types, hindering the ability to determine whether nicotinamide acts directly on microglia. To further explore the effects of nicotinamide on microglia alone, high purity microglia cultures derived from mixed glia cultures were treated with nicotinamide at concentrations of 1, 5 and 10 mM.

5.4.5 Nicotinamide reduced numbers of microglia in cultures derived from mixed glia

Nicotinamide treatment of microglia derived from mixed glial cultures showed reduced numbers of $Iba1^+$ microglia after 5 days with 10 mM nicotinamide treatment, but not with lower concentrations. It is not clear whether this is due to a restriction in proliferation, or promotion of cell death. The change in morphology exhibited by these microglia suggests that this could be due to toxicity. Microglia treated with 10 mM nicotinamide were typically much smaller than those in control cultures, with the $Iba1^+$ cell body being barely larger than the nucleus, resembling the early effects of pyknosis (Figure 5.13). However, these observations do not represent definitive evidence of a toxic effect.

To assess the toxicity of nicotinamide, further analyses could be performed. Possibilities include staining with TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) or PI, or measures of metabolic activity such as an MTT assay. These assays could confirm if 10 mM nicotinamide is toxic to isolated microglial cultures derived from mixed glia.

To further explore the nicotinamide-induced decrease in microglial numbers observed in microglial cultures, and the limitation of microglia proliferation observed in VM cultures. The effect of nicotinamide alongside GM-CSF, a cytokine known to promote microglial proliferation, was tested.

5.4.6 GM-CSF counteracted the nicotinamide-induced decrease in numbers of microglia, but not Iba1 expression

To assess potential mechanisms of nicotinamide-induced reduction in proliferation in VM cultures, and reduced numbers of microglia in cultures derived from mixed glia, GM-CSF was used to induce proliferation within microglia cultures. GM-CSF is released by astrocytes

after inflammatory activation (Aloisi *et al.*, 1992; Lee *et al.*, 1994), suggesting that GM-CSF could be a source of the microglial proliferation observed in these VM cultures. In cultures derived from mixed glia, application of GM-CSF for 5 days significantly increased both numbers of $Iba1^+$ microglia and expression of Iba1 measured by OD. Application of nicotinamide alongside GM-CSF reduced levels of Iba1 expression measured by OD, but did not reduce numbers of microglia, an effect that was observed with application of 10 mM nicotinamide alone. This suggests that nicotinamide did not counteract the increased proliferation induced by GM-CSF (Figure 5.14), but did counteract the increase in Iba1 expression measured by OD (Figure 5.16). This may suggest that GM-CSF and nicotinamide affect microglial proliferation and Iba1 expression through separate mechanisms.

The combination of nicotinamide and GM-CSF treatment of microglial cultures did not prevent GM-CSF-induced microglial proliferation. This suggests that the proliferation observed in VM cultures may not be associated with stimulation from endogenous GM-CSF, which is released by astrocytes after inflammatory activation, because if this was the case, then nicotinamide would not be expected to inhibit proliferation. The low numbers of $GFAP^+$ cells within VM cultures at 7 DIV suggests that either numbers of astrocytes were low or that astrocytes were not mature or not activated in a way that promotes GFAP expression. This supports the idea that GM-CSF released by astrocytes may not be the cause of microglial proliferation in VM cultures. To investigate this, levels of GM-CSF in VM cultures could be tested.

GM-CSF induces increased proliferation of microglia through phosphorylation of ERK1, ERK2, STAT5A, and STAT5B (Liva *et al.*, 1999). The literature does not suggest that nicotinamide is associated with these pathways, although, recently published data has shown that nicotinamide treatment of retinas abolished a toxin-induced increase in ERK phosphorylation. The authors suggest that ERK phosphorylation is part of a self-protective

response of cells (Sugano *et al.*, 2019). Therefore, preventing the activation of ERK may not be a direct effect of nicotinamide, but may be attributed to the overall protective role that nicotinamide showed in this disease model. The results found in this PhD thesis suggest that nicotinamide and GM-CSF involve separate pathways in control of proliferation, with no evidence in the literature suggesting an overlap of mechanisms.

5.4.7 Nicotinamide (10 mM) induced different microglial responses between VM cultures and microglia cultures

Studying microglia within VM cultures has advantages over 2D monocultures as the former more closely resembles the intact brain environment, however, the mechanisms behind effects of treatment are harder to elucidate as there are other cell types present within the cultures. Studying microglia as a monoculture can help to uncover how these cells respond to treatment, as confounding elements are omitted, and direct effects can be determined. In this PhD project, microglia in VM cultures and isolated microglia in cultures derived from mixed glia seemed to respond differently to treatment with 10 mM nicotinamide. However, even without nicotinamide treatment, microglia cultured within these two systems still showed differences in morphology (Figure 5.11 and Figure 5.12).

Microglia are very sensitive to changes in their environment (Kettenmann *et al.*, 2011) making it difficult to know whether *in vitro* observations are predictive of *in vivo* behaviours. Microglia derived from mixed glia cultures displayed morphologies typical of isolated microglia grown in a 2D culture system (Tamashiro, Dalgard and Byrnes, 2012) with mostly large and flattened, or rod-like appearance. However, VM cultures displayed microglia with morphology closer to microglia grown in 3D *in vitro* cultures (Figure 5.17), which are frequently highly ramified, with the small cell soma which earned *micro*glia their name, and

more closely resembling morphologies displayed *in vivo* (Miller *et al.*, 2019). This increase in ramification is observed when microglia are cultured with a bedlayer of cells, including epithelial cells and astrocytes (Rosenstiel *et al.*, 2001; Rezaie *et al.*, 2002). Microglia are influenced by signalling from other neural cell types, consequently, removal of neural cell interactions can affect microglia phenotypes (review: Timmerman, Burm, & Bajramovic, 2018). The morphological differences observed between these cultures have been frequently reported in the literature. Morphological differences are even found *in vivo* between the pars reticula and pars compacta of the SN (De Biase *et al.*, 2017). This adaptive nature of microglia make them difficult to study and to extrapolate observations from *in vitro* models (Mosser *et al.*, 2017).

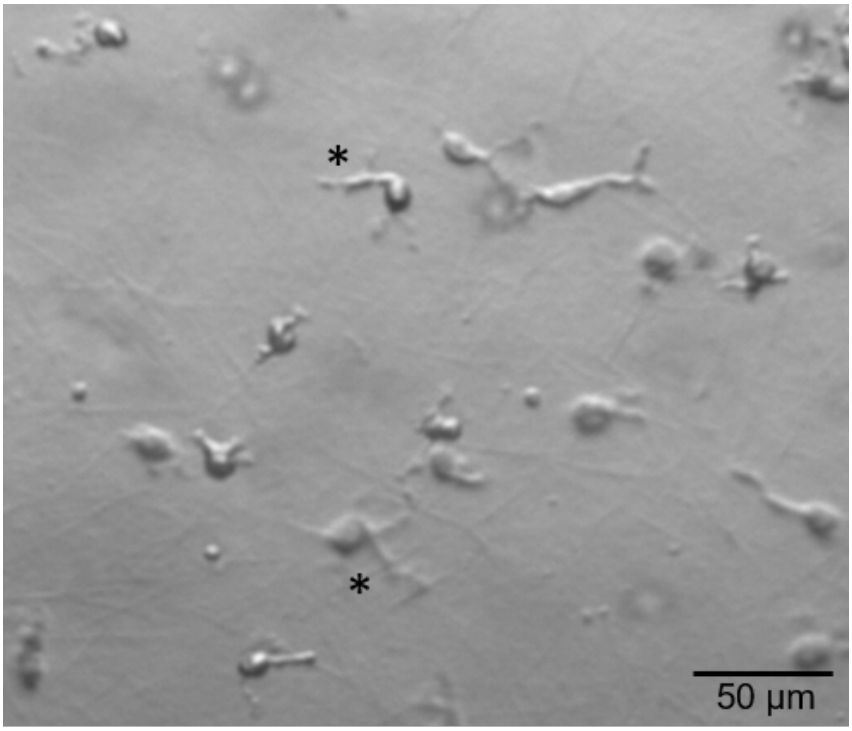


Figure 5.17 Phase contract micrograph showing primary rat microglia grown in a collagen hydrogel providing a 3-dimensional substrate. *Microglia display more complex morphology with small cell soma, multiple branches, and secondary branching systems, more closely resembling morphology displayed by microglia *in vivo*. Cells were highly motile, with small somas typical of microglia in histological sections. Image courtesy of Dr Stuart Jenkins (Keele University).

Nicotinamide treatment reduced numbers of Iba1^{+} microglia in VM cultures by limiting proliferation. The microglia in these cultures still displayed the morphologies that were

present, and progressed over time, in control cultures despite 14 days of 10 mM nicotinamide treatment. However, after 5 days of 10 mM nicotinamide treatment, $Iba1^+$ microglia in cultures derived from mixed glia cultures had differing morphologies compared to control cultures. Microglia treated with 10 mM nicotinamide tended to have very small, rounded cell bodies with Iba1 expression being barely larger than the nucleus, indicative of toxicity.

One potential explanation for the apparent toxicity of 10 mM nicotinamide in isolated microglia cultures, but not VM cultures, is the difference in cell numbers. Microglia derived from mixed glia cultures were plated at 20,000 cells per well, evenly dispersed, compared to 50,000 cells in a 50 µL micro-drop used for VM cultures. These cell densities were used based on protocols established within the laboratory for each culture system. This reduction in total cell numbers for isolated microglial cultures may mean that individual cells are exposed to higher amounts of nicotinamide; for the same nicotinamide concentration in each cultures system, there is more nicotinamide per cell in the lower density microglial cultures.

Each culture system was also grown in a different medium, specified from previous laboratory protocols unique to each cell model. As discussed in Chapter 5.4.2 with astrocytes, different culture medium can also produce different phenotypes in microglial cultures (Bohlen *et al.*, 2017). The addition of serum can also affect microglia within cultures, inhibiting the development of ramified morphology (Navascués *et al.*, 2000). Codeluppi et al. (2011) found that astrocyte cultures (derived from adult rat spinal cord) grown with lower levels of serum resulted in lower microglial populations. Similarly, microglial cultures exposed to serum showed increased proliferation compared to defined media (Bohlen *et al.*, 2017). Microglia morphology in 2D cultures is also affected by serum. Microglial cultures isolated from mixed glial cultures (P0-2 rats), grown with 10% FBS showed larger, flatter morphologies with increased area of Iba1 expression, and fewer processes per cell, compared to microglia grown in defined medium without FBS (Montilla *et al.*, 2020).

Ideally, a single medium that could be used by both culture systems would assist in isolating the specific effects of nicotinamide. To simplify issues arising from differences in culture systems, only Iba1 expression and cell numbers were analysed. Other factors such as expression of, what are considered, M1 and M2 specific markers would be more difficult to compare across such different cultures.

Another potential explanation for the differences observed in microglia between different culture settings is the potential for secondary effects of nicotinamide through other cell types found in the VM cultures. Microglia react to many environmental cues including factors released by other neural cell types (Jeffrey J.Bajramovic, 2011; Tan, Yuan and Tian, 2020). Microglia within VM cultures may be directly influenced by nicotinamide, or the reduced proliferation may be in response to cues released by other neural cell types influenced by nicotinamide treatment; although this possibility requires further exploration. The potential effects of other cell types on microglia may also have been observed in the differences seen between VM and WGE cultures. The significantly lower numbers of $Iba1^{+}$ microglia observed in nicotinamide treated VM cultures at 14 DIV was not observed in WGE, however, numbers of $Iba1^{+}$ microglia were very low in WGE cultures, making it more difficult to observe a genuine effect. The difference between VM and WGE microglia is further discussed in Chapter 5.4.9.

Microglia also show heterogeneity in terms of density, morphology and gene expression across CNS regions (Tan, Yuan and Tian, 2020) and differences in gene expression have been shown between postnatal (P3-9) and 2 month old mice (Matcovitch-Natan *et al.*, 2016). Therefore, the regional and age differences in the collection of microglia between VM cultures (E14) and microglia derived from mixed glia (P0-3) could also play a role in the differences observed across culture systems in this PhD thesis. However, there is limited evidence that intrinsic factors are responsible for differences observed between microglia

(Tan, Yuan and Tian, 2020), suggesting the differences observed across the culture systems used here are due to the different environments that the cells are exposed to. Even spleen macrophages and blood monocytes adapt their membrane characteristics to those displayed by microglia when cultured with astrocytes (Schmidtmayer *et al.*, 1994).

PARP-1 inhibition in *in vitro* BV2 microglia and primary microglial cultures attenuated the release of pro-inflammatory factors after inflammatory stimulation, but did not decrease basal levels of iNOS expression or NF-κB activity (i.e. levels of iNOS and NF- κB prior to application of inflammatory stimulation; Stoica *et al.*, 2014). Nicotinamide may not affect microglia under physiological conditions, but may instead prevent action in response to certain inflammatory stimuli. Fukushima, *et al.* (2015) found that inhibiting VEGF signalling attenuated LPS-induced microglial proliferation, however application of this inhibitor alone did not change basal proliferation levels. To explore this, the effect of nicotinamide on isolated microglia cultures could be assessed alongside application of pro-inflammatory factors such as LPS.

5.4.8 Mechanisms of nicotinamide-restricted proliferation of microglia may be direct or in-direct

Possibilities in the different response to nicotinamide seen here between $Iba1^+$ microglia in VM cultures and $Iba1^+$ microglia in cultures derived from mixed glia cultures could be due to the presence of other cell types within VM cultures. This raises problems in distinguishing between direct or indirect effects of nicotinamide on microglia. Nicotinamide may act indirectly on microglia present in VM cultures, potentially through nicotinamide-induced release of factors from neurons, astrocytes or NSCs which are present in VM cultures but excluded from microglial cultures derived from mixed glia. However, nicotinamide-

induced limitation of microglia proliferation could be a direct effect, while the proliferation of microglia within control VM cultures could be in response to factors from other cell types present in VM cultures.

A direct mechanism for nicotinamide-induced reduction in proliferation observed in VM cultures could be through inhibition of PARP-1. Microglia cultures derived from PARP-$1^{-/-}$ mice were resistant to TNF-α induced proliferation (Kauppinen and Swanson, 2005) and, as previously discussed, nicotinamide is an inhibitor of PARP-1 activity. PARP-1 inhibition also protected against neuronal degeneration in an *in vivo* traumatic brain injury mouse model, with reductions in the release of inflammatory associated factors in microglia cultures derived from primary cortex and BV2 cells (Stoica *et al.*, 2014). To further explore these possibilities, microglial PARP-1 expression in VM cultures could be explored. The nature of nicotinamide-induced limitation of microglial proliferation found in VM cultures suggests that this could be in response to PARP-1 inhibition.

Factors that induce microglial proliferation could be present within VM cultures. The possibility of GM-CSF released by astrocytes does not appear to be the cause of this proliferation as nicotinamide was unable to prevent GM-CSF induced proliferation. However, astrocytes and neurons secrete a number of other factors that can affect microglia (Goshi *et al.*, 2020) with CSF-1, TGF-β2, and cholesterol suggested as essential to microglia survival (Bohlen *et al.*, 2017). Further analysis of VM and isolated microglial culture conditioned medium could help to elucidate factors that may be influencing microglial proliferation.

The response of microglia to other cells present in culture may be a factor in the lower numbers and slower proliferation rate of microglia observed in cultures derived from WGE compared to VM, given their different cellular compositions (further discussed in Chapter 5.4.9).

5.4.9 The developing VM contains more microglia than the developing WGE

The presence of microglia in VM cultures and their implications in disease models are not often commented on. In this PhD thesis, the microglial populations of cultures derived from two brain regions associated with PD were assessed. VM development produces the SN, and the WGE eventually forms the striatum. This allowed for assessment of two individual but related brain regions. Studies have shown that the adult mouse SN, and basal ganglia in general, have one of the highest adult microglial populations (De Biase *et al*., 2017; Lawson *et al*., 1990*)*.

Numbers of Iba1^{+} microglia in WGE were significantly lower than VM at 7 and 14 DIV. This difference correlates with studies showing different regional distributions of microglia (Tan, Yuan and Tian, 2020). Linear regression (Figure 5.9 B) showed differences in the proliferation rate of microglia in VM and WGE cultures. This could be due to differences in the initial proportions of microglia at seeding (if each developing brain region contains different densities of microglia at E14), region specific cues from other cell types derived from these tissues, or differences in microglial specialisation which have occurred due to their development within each region. Differences in the number of microglia between VM and WGE may arise from the process of microglia infiltration and migration within the developing brain. Migration of microglial cells is still underway in the postnatal brain (Mosser *et al.*, 2017) suggesting that microglia have not fully populated all regions of the brain at E14, when these cultures were established.

Microglia are present in the developing rat brain from E10 (Ghosh and Ghosh, 2016). Amoeboid microglia are suggested to enter the developing brain from the meninges, choroid plexus and ventricles (Verney *et al.*, 2010), with observations of microglia arising earlier in the

mesencephalon, from which VM cultures are derived, than frontal lobes, which contain the developing WGE (Wierzba-Bobrowicz *et al.*, 1998). This could account for the higher numbers of microglia observed in VM cultures at 7 DIV and could be confirmed by analysis of WGE cultures from 1 DIV. However, it does not explain the slower proliferation rate observed in WGE between 7 and 14 DIV compared with VM.

Data was not collected from 1 and 4 DIV in WGE cultures, however, this would be an interesting point to investigate and would help to clarify differences for microglial proliferation in different brain regions. The difference in proliferation rate observed between 7 and 14 DIV across VM and WGE cultures suggests that these differences are from region specific stimuli, but do not clarify extrinsic or intrinsic factors.

After microglial ablation in the adult mouse, cortex, cerebellum and spinal cord showed different repopulation rates (Bruttger *et al.*, 2015). An increase in microglial numbers compared to control was observed 7 days after ablation, with numbers stabilising to control levels after 14 days (Elmore *et al.*, 2014). Repopulation exhibits the same heterogeneity across basal ganglia regions. The sparse distribution of ablation surviving microglia and the homogeneity observed during repopulation suggest that environmental cues, rather than intrinsic programming, are required for microglial region specificity (De Biase *et al.*, 2017). De Biase et al. (2017) suggest that microglial regional specificity is in place in the basal ganglia from the second postnatal week, arising from external factors. The results from this PhD project showed regional differences in population densities in tissue derived from E14 brain regions (after 7 DIV; rat gestation is typically 21 days).

Marshall, Demir, Steindler, & Laywell (2008) found that astrocyte monolayers cultured from the postnatal (P2-3), but not adult (4 month old), subventricular zone were able to repeatedly produce large numbers of microglia through addition of “microglial proliferation media” (media supplemented with 10% FBS and 20 ng/mL GM-CSF) to these monolayers.

Monolayers derived from the postnatal subventricular zone also showed higher repeated microglial yield than those derived from cortex or cerebellum. This study suggests extrinsic factors in microglia proliferation but do not clarify the potential of intrinsic factors involved in these results. However, later research using mixed cultures derived from P2-3 mouse subependymal zone or cortex suggested intrinsic proliferation characteristics of microglia derived from each area (Marshall *et al.*, 2014).

Region specific analysis of postnatal mouse basal ganglia tissues showed homogeneity of microglial distributions at P6, with regional differences observed at P12. Ki67 staining (a marker of proliferation) suggested that these changes are from proliferation, rather than differentiation, of microglia in each region (De Biase *et al.*, 2017). Using markers of proliferation could be another route to examining the microglial differences observed in these VM and WGE cultures.

Microglia expression profiles also vary across the CNS (Tan, Yuan and Tian, 2020). Expression profiles and morphological analysis were not carried out in this PhD thesis. Immunocytochemical stains, analysis of expression levels, and morphology can be difficult to accurately measure with microglia. These results are sometimes ambiguous, especially *in vitro*, due to the complexity and variation of features that are innate to microglia. Flow cytometry could be utilised to further explore potential differences in microglia expanded from VM and WGE cultures, however, it may be difficult to produce large enough sample sizes.

Despite decades of research it is not clear whether differences in microglia observed across CNS regions are due to intrinsic or extrinsic factors with research highlighting evidence for both. These results show evidence for differences in microglia proliferation within cultures derived from different embryonic brain regions, however, further analysis is required to elucidate potential mechanisms.

5.4.10 Conclusions and future study

The results of this chapter suggest that nicotinamide is able to limit the proliferation of both NSCs and microglia but does not appear to influence $GFAP^+$ astrocytes. Further research could use other astrocyte markers or purified astrocyte cultures to fully assess the potential effects of nicotinamide on astrocytes. Further analysis of NSC and microglial populations could involve the use of proliferation markers such as Ki67 or BrdU to confirm this reduction in proliferation for either or both cell types. This would be especially useful in isolated microglial populations alongside the use of TUNEL, PI or MTT to explore the possibility of a toxic effect of 10 mM nicotinamide in these cultures. Further analysis could also include the use of markers showing M1 or M2 polarisation, although the use of these *in vitro* may not be easy to extrapolate to *in vivo* characterisation of microglia, due to controversy around whether *in vitro* marker expression is representative of *in vivo* phenotypes/expression profiles.

Further assessment of microglial populations could also explore the mechanisms for the exponential growth observed in VM but not WGE cultures. Potential avenues could look for measurement of factors that promote microglial proliferation. This would help to distinguish between external or intrinsic stimuli that may be responsible for these differences. It is not clear whether nicotinamide is limiting basal microglial proliferation rates or if it is limiting proliferation in response to other stimuli. Application of nicotinamide alongside factors that are known to promote proliferation may also be useful to determine the mechanisms behind the response observed in this PhD thesis.

Results from these further experiments could also provide information on whether nicotinamide has a direct or indirect effect on microglia and how this could be utilised in therapies for neurodegeneration. For instance, the use of nicotinamide may be applicable to PD either through control of the inflammatory response observed in the SN of PD brains and

thought to contribute to or exacerbate SN degeneration (Chapter 1.4.4); or through the potential to limit the immune response that is observed in CRT to ensure integration and protection of grafted dopaminergic neurons (Tomov, 2020).

Further experiments to build on the results discussed here would include:

I. Further assessment of microglial proliferation in VM and WGE cultures through the use of BrdU.
II. Assessment of VM microglia using immunocytochemistry for pro- and anti-inflammatory markers including iNOS and Arg1.
III. Assessment of isolated microglia cultures using TUNEL assay to assess apoptosis with nicotinamide treatment.
IV. Assessment of NAD^+/NADH levels using luminescence assays in isolated microglial cultures.
V. Assessment of PARP-1 expression in microglia in both VM and isolated microglia cultures with nicotinamide treatment.

6 General conclusions and discussion

6.1 Overview of thesis

Despite decades of research, the exact pathology, cause, and an effective treatment for, PD are still being elucidated. The overarching aim of this thesis was to explore the implications that nicotinamide may have on neural cells regarding differentiation, neuroprotection, and neuroimmunomodulation, which may be beneficial to uncovering the pathology of, or potential novel treatments for, PD.

Chapter 3 discussed the use of nicotinamide in the neural and neuronal differentiation of stem cells. The application of 5 mM and 10 mM nicotinamide to mESC cultures resulted in lower numbers of cells compared to controls in differentiating cultures. This suggests that nicotinamide is able to limit the proliferation of these cells, and this same effect of 10 mM nicotinamide has been observed in hESCs (Meng *et al.*, 2018). Alongside reduced proliferation, the application of nicotinamide may also promote accelerated neural maturation (Griffin *et al.*, 2013, 2017), observed in this PhD thesis by an earlier peak in *Sox1-GFP* expression with nicotinamide treatment during differentiation. Furthermore, cultures treated with nicotinamide did not show significantly lower numbers of β-III-tubulin^{+} neurons despite having significantly fewer cell numbers, suggesting that nicotinamide may be selective for the promotion of a neuronal fate; with a greater percentage of neurons within these cultures.

Chapter 4 explored the potential of nicotinamide as a neuroprotectant through an *in vitro* PD model using VM-derived cultures and 6-OHDA. Despite suggestions that nicotinamide may be neuroprotective for PD (Seidl *et al.*, 2014; Agim and Cannon, 2015), 10 mM nicotinamide failed to show neuroprotection against 6-OHDA-induced toxicity in dopaminergic neurons. However, the results from this model do not mean that nicotinamide cannot provide neuroprotection in PD, and further research could provide insight into the role

of nicotinamide, whether beneficial or detrimental, to PD (Williams and Ramsden, 2005a). Significantly higher numbers of TH^+ neurons were observed in 10 mM nicotinamide treated cultures at 1, 4, and 7 DIV. Further exploration of this effect using markers of proliferation may help to uncover whether this effect is through neuroprotection of TH^+ neurons that are present in the cultures from day 0, or whether it promotes the differentiation of dopaminergic neurons from precursors. If increased markers of proliferation are not found within TH^+ neurons in nicotinamide treated cultures, this may suggest that nicotinamide is able to offer some form of neuroprotection. Although a neuroprotective effect was not found from 6-OHDA toxicity, nicotinamide may provide neuroprotection from other forms of cytotoxicity which may also occur in PD degeneration. Further exploration of this field may assist in the development of novel treatments for PD (further discussed in Chapters 6.1.2 and 6.1.3).

Chapter 5 explored the effects of nicotinamide on other cells of the CNS including $GFAP^+$ astrocytes, $Nestin^+$ neural stem cells, and microglia. Results showed no significant difference in the number of $GFAP^+$ astrocytes in 10 mM nicotinamide treated cultures, however, overall numbers were low and, due to fluctuating expression, there is uncertainty about the suitability of GFAP as a marker for astrocyte populations (Lin, Messing and Perng, 2017). Astrocytes represent a complex cell population, and to be able to effectively assess whether nicotinamide exerts and effect on these cells, further analysis would be needed. A variety of markers including vimentin (Bramanti *et al.*, 2010), Aldh1l1, and glutamate transporter, their expression levels, and morphological differences between protoplasmic (type 1; large cell bodies with small processes) and fibrous (type 2; small soma with numerous, long processes) astrocytes could be assessed using immunocytochemistry. Transcriptome and proteomic analyses would also be able to further explore any potential changes in astrocytes with nicotinamide application (Khakh and Deneen, 2019).

Significantly lower numbers of Nestin$^+$ cells were observed in 10 mM nicotinamide treated VM cultures after 7 DIV. This supports data from Chapter 3 that nicotinamide can limit the proliferation of stem cells without reducing numbers of neurons; a characteristic that may assist in the delivery of CRT for PD. To my knowledge, the presence of Nestin$^+$ stem cells in VM cultures is not commonly commented on in the literature. This may represent a novel finding in the characterisation of cell types within these cultures and the potential for nicotinamide to limit the proliferation of cell types (that may not be desired) in these cultures without affecting the β-III-tubulin$^+$ neuronal population.

Nicotinamide also had a significant impact on the proliferation of Iba1$^+$ microglia within VM cultures. Numbers of Iba1$^+$ microglia showed an exponential increase over 14 DIV in control cultures, which was significantly limited with 10 mM nicotinamide treatment. Further exploration of this effect by addition or removal of nicotinamide (half-way through the 14 day culture period) confirmed that nicotinamide treatment did not induce microglial cell death in these cultures, but halted proliferation, an effect that was released upon removal of nicotinamide treatment. To my knowledge this represents a novel and potentially therapeutically useful finding surrounding how the microglial population within normal VM cultures behave, and the effect that nicotinamide has on these cells.

To further explore the effects of nicotinamide on microglia, isolated microglial cultures were treated with nicotinamide concentrations of 1, 5, and 10 mM. Nicotinamide showed a dose-dependent decrease in the number of Iba1$^+$ microglia in isolated cultures, with significantly lower numbers observed with 10 mM treatment for 5 days. The morphology of isolated microglia treated with 10 mM nicotinamide suggested a toxic effect although further analysis, and perhaps longer-term culture, would be needed to confirm this.

To address the potential mechanism by which nicotinamide limited proliferation of Iba1$^+$ microglia in VM cultures, isolated microglia were treated with nicotinamide alongside

GM-CSF, a stimulator of microglial proliferation associated with M2a activation (Figure 1.6). Nicotinamide did not prevent the GM-CSF-induced increase in numbers of $Iba1^+$ microglia in cultures, suggesting that nicotinamide may limit proliferation through a different mechanism than that by which GM-CSF promotes proliferation. However, another possible explanation is that the concentration of GM-CSF overrode the influence of nicotinamide. Further analysis using different concentrations of GM-CSF may help to ascertain whether nicotinamide has a direct inhibitory effect on GM-CSF-induced proliferation. To my knowledge, the literature does not suggest that the mechanistic action of GM-CSF overlaps with the factors that nicotinamide is known to influence. Therefore other mechanisms should be explored for nicotinamide, including sirtuin or PARP inhibition.

Additional exploration of the mechanisms for nicotinamide-induced limitation of microglial proliferation could utilise pro-inflammatory factors such as LPS, which stimulates an M1 activation state (Figure 1.6). Markers of microglial activation states could also assist to determine more specific effects of nicotinamide on microglia; although these can be difficult to accurately study in *in vitro* cultures, they could help to understand the mechanisms by which nicotinamide limits proliferation. The limitation of microglial proliferation may be a useful tool in the treatment of diseases involving pro-inflammatory activation which is typically associated with an increase in proliferation and can result in exacerbated damage (Mathys *et al.*, 2017). Further research into the novel effects of nicotinamide-limited microglial proliferation found in this PhD thesis may assist in the development of immunomodulatory therapies that could help to treat PD; or be used alongside future therapies such as CRT to assist with graft integration by modifying/limiting microglial proliferation/activation and thus a negative host response to the transplant.

Results from Chapter 5 also showed lower numbers of microglia in cultures derived from the E14 WGE (from which the striatum develops) compared to those derived from E14

VM (from which the SN develops), both of which are areas of interest in PD. Microglia show regional heterogeneity across the brain and this also varies with age (Tan, Yuan and Tian, 2020). Microglia within WGE cultures also appeared to proliferate at a slower rate than those within VM cultures. Further analysis of WGE cultures at 1 and 4 DIV could confirm the differences observed here and further analysis of cells types and secreted factors may shed light on the environmental influences that drive microglial proliferation, how this could be affected by nicotinamide treatment, and whether this may be utilised in PD research.

Nicotinamide, associated compounds, and their interactions could play a role in neurodevelopment, neuroprotection, and neurodegeneration. However, the mechanisms and interactions are complex. Determining the most effective way to utilise the beneficial effects of nicotinamide and its derivatives still requires more work. Novel results from this thesis may be able to contribute to our understanding of the mechanisms behind PD and the development of future therapies.

6.2 The mechanistic actions of nicotinamide are yet to be elucidated

A clear effect of nicotinamide was observed in all culture systems (mESCs, primary VM, and primary microglia) used in this PhD project. The mechanistic action of nicotinamide has been attributed to inhibition of PARPs, sirtuins, kinase activity, and increased levels of pyridine nucleotides (Li, Chong and Maiese, 2006; Meng *et al.*, 2018). Understanding the functions and mechanisms of nicotinamide may provide a valuable tool for future therapies.

The novel observation that nicotinamide-restricted proliferation of microglia in VM cultures, could be explained through direct inhibition of PARPs or sirtuins by nicotinamide, or potentially through increased levels of NAD^+. For instance, the proliferation of rat T cells, cultured *in vitro*, was found to be inhibited by NAD^+, but not nicotinamide (Bortell *et al.*, 2001), highlighting a potential pathway by which nicotinamide exerts this effect. To explore the possibility that nicotinamide exerts the observed effects through increases in NAD^+ levels, an experiment was designed during this PhD project to measure the intracellular levels of NAD^+/NADH, using a luminescence assay, in both VM and isolated microglial cultures. Due to the COVID-19 pandemic and laboratory closure, these experiments were not able to be completed, however, some preliminary data was obtained suggesting that nicotinamide treatment may dose-dependently increase intracellular levels of NAD^+/NADH in both VM and microglial cultures. This warrants investigation. Further optimisation of the protocol, and more experimental repeats, could clarify whether nicotinamide is definitively able to increase intracellular levels of NAD^+/NADH, and whether this effect is observed in both VM and high purity microglial cultures. Analysing these cultures separately may also assist in discerning between the effects observed in the multi-cellular VM cultures, and the effects observed in high purity microglial cultures.

The exponential increase in the number of microglia over time in standard VM cultures may have been due to factors released by other cell types within these cultures. For instance, factors released by astrocytes have been shown to induce microglial proliferation (Goshi *et al.*, 2020). This idea was supported by the difference in growth rate observed between 7 and 14 DIV in VM compared to WGE cultures. To explore external factors released by astrocytes that may influence the difference in growth rate observed between cultures, and the mechanism by which nicotinamide may limit proliferation, isolated microglial cultures were treated with nicotinamide alongside GM-CSF, a molecule released by astrocytes and known to promote microglial proliferation. As expected, GM-CSF was able to induce proliferation in isolated microglial cultures and may be a factor that is released in VM cultures to stimulate microglial proliferation. However, nicotinamide did not limit GM-CSF-induced proliferation, despite resulting in the microglial cells' morphology resembling pyknotic cells. Although further evidence would be required before reaching a firm conclusion, the results suggested that GM-CSF promotes proliferation through a mechanism separate to which nicotinamide inhibits proliferation. An experimental procedure utilising conditioned medium from VM and WGE cultures may reveal whether factors released from different cell types present in these cultures can contribute to differences in proliferation rates. Further analysis of the culture medium from VM and WGE cultures may be able to reveal specific factors released by cells that could contribute to the proliferation of microglia observed in standard primary cell cultures; from this it may be possible to deduce potential mechanisms/pathways by which nicotinamide limits proliferation.

Nicotinamide and its derivatives have broad, wide-ranging actions and the potential to influence many cellular mechanisms (Li, Chong and Maiese, 2006). A broad and inclusive method to study the potential mechanisms of nicotinamide in these cultures could be through the use of transcriptome and proteome analysis. This may assist to highlight changes in the

expression profiles of cells exposed to nicotinamide, the pathways that nicotinamide may influence, and behaviours that are associated with these pathways. This type of analysis may also help to explain the observed effects of nicotinamide on proliferation and differentiation observed here.

6.3 Nicotinamide may aid in the development of CRT for PD

The results discussed in this PhD thesis may be relevant in the development of CRT as a treatment for PD. Nicotinamide treated mESCs showed reduced proliferation over the differentiation period observed here. Addition of nicotinamide to differentiating stem cell cultures may help to prevent the presence of proliferating cells within grafts, an aspect that is essential for developing CRT as a safe and effective treatment for PD, by reducing the chances of teratoma formation (Hagell and Brundin, 2001). Despite reducing total cell numbers through diminished proliferation, nicotinamide did not alter the number of β-III-tubulin^{+} neurons after 7 days of differentiation, resulting in a higher percentage neuronal population. Therefore, the addition of nicotinamide to dopaminergic differentiation protocols may help to increase the percentage neuron yield. Nicotinamide treated cultures also showed an earlier peak in *Sox1-GFP* expression measured with flow cytometry. This is in agreement with previously observed data using different analysis methods, suggesting enhanced maturation of neuronal populations from stem cells using nicotinamide (Griffin *et al.*, 2017). This effect may also be beneficial in reducing the protocol length required to differentiate transplantable cells from stem cells. This would benefit the clinical translation of CRT by enabling a higher throughput system for the development of suitable grafts. It may also assist in developing the potential of iPSCs by reducing the time between cell retrieval and grafting.

As well as reducing the proliferation of stem cells, nicotinamide also limited the proliferation of microglia, a behaviour that is associated with pro-inflammatory activation. In this regard, nicotinamide could provide immunosuppressive benefit when combined with the grafting procedure. Grafting cells is thought to illicit an immune response from both the graft itself, and the procedure of graft placement. Limiting this immune response is believed to be

beneficial to graft survival (Moriarty, Parish and Dowd, 2018), however immune suppression can have serious side effects and may not be suitable long-term (Kovarik, 2013). Nicotinamide could be administered with the graft or potentially incorporated into a bio-material to protect cells from a pro-inflammatory immune response and graft infiltration by host microglia. Nicotinamide treated VM cultures also showed higher numbers of TH^+ neurons compared to control at 1 ,4 and 7 DIV. This effect may be through nicotinamide-induced promotion of dopaminergic differentiation, or protection of dopamine neurons present at cell seeding, potentially be reducing the numbers of reactive microglia in the cultures. Although this mechanism requires further exploration, it indicates another function of nicotinamide that could benefit CRT for PD.

Furthermore, nicotinamide is well-described and food and drug administration approved meaning it can be included in GMP grade protocols for the generation of dopaminergic neurons, or in combination with bio-materials suitable for CRT for PD. Nicotinamide can also cross the BBB and high doses are generally well tolerated with plasma concentrations reaching the millimolar range after oral supplementation (Dragovic *et al.*, 1995). However, further investigation is needed to understand how orally administered nicotinamide may influence levels of nicotinamide in cerebrospinal fluid, or the striatum (typically the site of graft placement), and whether this is able to influence microglia, providing therapeutic benefit.

6.4 Nicotinamide may offer a neuroprotective therapy for PD

If nicotinamide could influence microglia *in vivo*, it may be possible for nicotinamide to provide neuroprotection against dopaminergic neuron degeneration in PD patients. Although nicotinamide did not show neuroprotection against 6-OHDA in the *in vitro* model used here, PARP-1 inhibition (an effect of nicotinamide) has shown neuroprotection in an *in vivo* traumatic brain injury model (Stoica *et al.*, 2014). However, PARP-1 inhibition did not show neuroprotection in 6-OHDA treated VM cultures (Callizot *et al.*, 2019) which is consistent with the lack of neuroprotection observed here. Direct use of nicotinamide has shown protection of SNpc neurons and striatal dopamine levels in two MPTP mouse models of PD (Anderson, Bradbury and Schneider, 2008) and numerous other studies have also highlighted the potential of nicotinamide and/or its derivatives in neuroprotection (discussed in Chapters 1.10 and 4.4.4). To my knowledge, nicotinamide has not been tested as a potential neuroprotectant in an *in vitro* VM 6-OHDA PD model, representing a novel finding here and one that correlates with a lack of nicotinamide-induced neuroprotection also observed in an *in vivo* lactacystin PD model (Harrison, Powell and Dexter, 2019). Further analysis of cell cultures treated with nicotinamide and 6-OHDA may elucidate why neuroprotection was not observed here.

Although more information is needed, these results suggest that microglia may not be critically involved in the degeneration of TH^+ neurons after 6-OHDA application in VM cultures, as reduced microglial numbers did not alter levels of neuronal survival in nicotinamide treated cultures. Assessment of the microglial population in these cultures after 6-OHDA treatment may reveal their reaction to 6-OHDA and 6-OHDA-induced neuron degeneration. Furthermore, by limiting their proliferation using nicotinamide treatment, it may be possible

to observe whether 6-OHDA and associated neuron degeneration influence microglial proliferation, and whether nicotinamide would be effective at reducing this reaction.

Nicotinamide strongly limited microglia proliferation in VM cultures, a finding that I believe to be novel. Further exploration of this effect could involve the use of markers indicative of M1/M2 polarisation to assess whether nicotinamide can influence phenotype as well as proliferation. Although this can be difficult to study using *in vitro* cultures, methods including immunocytochemistry and PCR may be beneficial. Building on this, flow cytometry and immunohistochemistry could be used to explore *in vivo* effects of nicotinamide on microglial populations. Exploring the effects of nicotinamide *in vivo*, with and without different inflammatory stimuli could assess whether nicotinamide may be a suitable neuroprotective strategy for PD. Exposure to certain anti-inflammatory medication is associated with a decreased risk of PD, although the exact role/mechanism, and reasoning behind the specificity of certain medications is not clear (Rees *et al.*, 2011; Racette *et al.*, 2018).

If biomarkers for preclinical PD become available, it may be possible to utilise nicotinamide as a neuroprotective strategy before substantial loss of dopamine neurons from the SNpc occurs. This may help to prevent or limit progression of PD, reducing the need for more drastic therapies such as DBS or CRT.

However, there is also the potential for nicotinamide to be involved in the degeneration aspects of PD, with increased meat consumption (and therefore increased nicotinamide intake) correlating with an increased risk of PD, at a population level (McCarty, 2001; Hill and Williams, 2017). In these VM cultures, nicotinamide did not exert a neurotoxic effect at concentrations of 10 mM, a dose that greatly exceeds the estimated cerebrospinal fluid concentration of 15 µM (Spector, 1979), and plasma concentration of 1.4 mM after oral administration of 10 g (Dragovic *et al.*, 1995). However, it does not rule out the possibility that

high levels of nicotinamide may be a contributing factor alongside other genetic or environmental influences. Also, in this PhD, additional nicotinamide was only applied for up 2 weeks which would not account for the potential of accumulative negative impacts of increased nicotinamide throughout a lifetime potentially contributing to late-onset PD.

The results shown in this PhD thesis discuss exciting and novel applications of nicotinamide, especially surrounding PD research. This opens possibilities of the use of nicotinamide in therapies for PD, providing groundwork for assessing the potential influences of nicotinamide in neurodegeneration, neuroprotection, and neurorestoration.

References

Abd-El-Basset, E. and Fedoroff, S. (1995) 'Effect of bacterial wall lipopolysaccharide (LPS) on morphology, motility, and cytoskeletal organization of microglia in cultures', *Journal of Neuroscience Research*, 41(2), pp. 222–237. doi: 10.1002/jnr.490410210.

Adams, J. and Audhya, T. (2011) 'Nutritional and metabolic status of children with autism vs. neurotypical children, and the association with autism severity', *Nutrition & Metabolism*, 8, pp. 1–32. doi: 10.1186/1743-7075-8-34.

Agim, Z. S. and Cannon, J. R. (2015) 'Dietary Factors in the Etiology of Parkinson's Disease', *BioMed Research International*. Hindawi Limited, 2015, pp. 1–16. doi: 10.1155/2015.

Ahn, J. M. *et al.* (1997) 'Triton X-100 Induces Apoptosis in Human Hepatoma Cell Lines', *Yonsei Medical Journal*, 38(1), pp. 52–59.

Akiyama, H. and McGeer, P. L. (1990) 'Brain microglia constitutively express β-2 integrins', *Journal of Neuroimmunology*. Elsevier, 30(1), pp. 81–93. doi: 10.1016/0165-5728(90)90055-R.

Alano, C. C. *et al.* (2010) 'NAD+ depletion is necessary and sufficient for poly(ADP-ribose) polymerase-1-mediated neuronal death', *Journal of Neuroscience*. Society for Neuroscience, 30(8), pp. 2967–2978. doi: 10.1523/JNEUROSCI.5552-09.2010.

Alberico, S. L., Cassell, M. D. and Narayanan, N. S. (2015) 'The Vulnerable Ventral Tegmental Area in Parkinson's Disease', *Basal Ganglia*, 5(2–3), pp. 51–55. doi: 10.1016/j.physbeh.2017.03.040.

Aloisi, F. *et al.* (1992) 'Production of hemolymphopoietic cytokines (IL-6, IL-8, colony-stimulating factors) by normal human astrocytes in response to IL-1 beta and tumor necrosis factor-alpha.', *Journal of immunology*. American Association of Immunologists, 149(7), pp. 2358–2366.

Amé, J. C., Spenlehauer, C. and De Murcia, G. (2004) 'The PARP superfamily', *BioEssays*, 26(8), pp. 882–893. doi: 10.1002/bies.20085.

Andersen, J. K. (2004) 'Oxidative stress in neurodegeneration: cause or consequence?', *Nature Medicine*, 10 Suppl(July), pp. S18-25. doi: 10.1038/nrn1434\rnrn1434 [pii].

Anderson, D. W., Bradbury, K. A. and Schneider, J. S. (2008) 'Broad neuroprotective profile of nicotinamide in different mouse models of MPTP-induced parkinsonism', *European Journal of Neuroscience*. John Wiley & Sons, Ltd, 28(3), pp. 610–617. doi: 10.1111/j.1460-9568.2008.06356.x.

Anderson, S. R. and Vetter, M. L. (2018) 'Developmental roles of microglia: A window into mechanisms of disease', *Developmental Dynamics*. John Wiley & Sons, Ltd, 248(1), p. dvdy.1. doi: 10.1002/dvdy.1.

Andressen, C. *et al.* (2001) 'Nestin-Specific Green Fluorescent Protein Expression in Embryonic Stem Cell-Derived Neural Precursor Cells Used for Transplantation', *Stem Cells*, 19(5), pp. 419–424. doi: 10.1634/stemcells.19-5-419.

Anis, E. *et al.* (2018) 'Evaluation of phytomedicinal potential of perillyl alcohol in an in vitro Parkinson's Disease model', *Drug Development Research*, 79(5), pp. 218–224. doi: 10.1002/ddr.21436.

Antunes, F. and Cadenas, E. (2000) 'Estimation of H2O2 gradients across biomembranes', *FEBS Letters*. No longer published by Elsevier, 475(2), pp. 121–126. doi: 10.1016/S0014-5793(00)01638-0.

Arenas, E., Denham, M. and Villaescusa, J. C. (2015) 'How to make a midbrain dopaminergic neuron', *Development (Cambridge, England)*. Company of Biologists Ltd, 142(11), pp. 1918–1936. doi: 10.1242/dev.097394.

Armogida, M., Nisticò, R. and Mercuri, N. B. (2012) 'Therapeutic potential of targeting hydrogen peroxide metabolism in the treatment of brain ischaemia', *British Journal of*

Pharmacology. Wiley-Blackwell, 166(4), pp. 1211–1224. doi: 10.1111/j.1476-5381.2012.01912.x.

Asai, H. *et al.* (2015) 'Depletion of microglia and inhibition of exosome synthesis halt tau propagation', *Nature Neuroscience*. Nature Publishing Group, 18(11), pp. 1584–1593. doi: 10.1038/nn.4132.

Ashourian, N. and Mousdicas, N. (2006) 'Pellagra-like dermatitis', *New England Journal of Medicine*, 354(15), p. 1614. doi: 10.1056/NEJMicm050641.

Askew, K. *et al.* (2017) 'Coupled Proliferation and Apoptosis Maintain the Rapid Turnover of Microglia in the Adult Brain', *Cell Reports*. Elsevier B.V., 18(2), pp. 391–405. doi: 10.1016/j.celrep.2016.12.041.

Aubert, J. *et al.* (2003) 'Screening for mammalian neural genes via fluorescence-activated cell sorter purification of neural precursors from Sox1–gfp knock-in mice', *PNAS*, 100(1), pp. 11836–11841.

Audrito, V. *et al.* (2011) 'Nicotinamide blocks proliferation and induces apoptosis of chronic lymphocytic leukemia cells through activation of the p53/miR-34a/SIRT1 tumor suppressor network', *Cancer Research*, 71(13), pp. 4473–4483. doi: 10.1158/0008-5472.CAN-10-4452.

Avalos, J. L., Bever, K. M. and Wolberger, C. (2005) 'Mechanism of sirtuin nhibition by nicotinamide: Altering the NAD + cosubstrate specificity of a Sir2 enzyme', *Molecular Cell*. Cell Press, 17(6), pp. 855–868. doi: 10.1016/j.molcel.2005.02.022.

Avola, R. *et al.* (2018) 'New insights on Parkinson's disease from differentiation of SH-SY5Y into dopaminergic neurons: An involvement of aquaporin4 and 9', *Molecular and Cellular Neuroscience*. Elsevier Inc, 88(2017), pp. 212–221. doi: 10.1016/j.mcn.2018.02.006.

Bach, J.-P. *et al.* (2011) 'Projected Numbers of People With Movement Disorders in the Years 2030 and 2050', *Movement Disorders*. John Wiley & Sons, Ltd, 26(12), pp. 2286–2290.

doi: 10.1002/mds.23860.

Bain, G. *et al.* (1995) 'Embryonic Stem Cells Express Neuronal Properties in Vitro', *Developmental Biology*. Academic Press, 168(2), pp. 342–357. doi: 10.1006/dbio.1995.1085.

Balan, V. *et al.* (2008) 'Life span extension and neuronal cell protection by Drosophila nicotinamidase', *Journal of Biological Chemistry*, 283(41), pp. 27810–27819. doi: 10.1074/jbc.M804681200.

Balestrino, R. and Schapira, A. H. V. (2020) 'Parkinson disease', *European Journal of Neurology*. Blackwell Publishing Ltd, 27(1), pp. 27–42. doi: 10.1111/ene.14108.

Banasik, M., Stedeford, T. and Strosznajder, R. P. (2012) 'Natural inhibitors of poly(ADP-ribose) polymerase-1', *Molecular Neurobiology*, 46(1), pp. 55–63. doi: 10.1007/s12035-012-8257-x.

Barker, R. A. *et al.* (1995) 'A comparative study of preparation techniques for improving the viability of nigral grafts using vital stains, in vitro cultures, and in vivo grafts', *Cell Transplantation*. Cell Transplant, 4(2), pp. 173–200. doi: 10.1016/0963-6897(95)90032-2.

Barker, R. A. *et al.* (2013) 'Fetal dopaminergic transplantation trials and the future of neural grafting in Parkinson's disease', *The Lancet Neurology*, 12(1), pp. 84–91. doi: 10.1016/S1474-4422(12)70295-8.

Barker, R. A. *et al.* (2019) 'Designing stem-cell-based dopamine cell replacement trials for Parkinson's disease', *Nature Medicine*. Nature Publishing Group, 25, pp. 1045–1053. doi: 10.1038/s41591-019-0507-2.

Batelli, S. *et al.* (2015) 'The parkinson's disease-related protein dj-1 protects dopaminergic neurons in vivo and cultured cells from alpha-synuclein and 6-hydroxydopamine toxicity', *Neurodegenerative Diseases*, 15(1), pp. 13–23. doi: 10.1159/000367993.

Bear, M. F., Connors, B. W. and Paradiso, M. A. (2007) *Neuroscience: Exploring the Brain*. Third. Baltimore: Lippincott Williams & Wilkins.

Bedard, K. and Krause, K. H. (2007) 'The NOX family of ROS-generating NADPH oxidases: Physiology and pathophysiology', *Physiological Reviews*. American Physiological Society, 87(1), pp. 245–313. doi: 10.1152/physrev.00044.2005.

Bedford, P. *et al.* (2018) 'Considering cell therapy product "good manufacturing practice" status', *Frontiers in Medicine*. Frontiers Media S.A., 5, p. 118. doi: 10.3389/fmed.2018.00118.

Belarbi, K. *et al.* (2017) 'NADPH oxidases in Parkinson's disease: a systematic review.', *Molecular neurodegeneration*. BioMed Central, 12(84), pp. 1–18. doi: 10.1186/s13024-017-0225-5.

Bellou, V. *et al.* (2016) 'Environmental risk factors and Parkinson's disease: An umbrella review of meta-analyses', *Parkinsonism and Related Disorders*. Elsevier Ltd, 23(2015), pp. 1–9. doi: 10.1016/j.parkreldis.2015.12.008.

Belloy, M. E., Napolioni, V. and Greicius, M. D. (2019) 'A Quarter Century of APOE and Alzheimer's Disease: Progress to Date and the Path Forward', *Neuron*, 101(5), pp. 820–838. doi: 10.1016/j.neuron.2019.01.056.

Benavente, C. A. and Jacobson, E. L. (2008) 'Niacin restriction upregulates NADPH oxidase and reactive oxygen species (ROS) in human keratinocytes', *Free Radical Biology and Medicine*. NIH Public Access, 44(4), pp. 527–537. doi: 10.1016/j.freeradbiomed.2007.10.006.

Bentea, E., Verbruggen, L. and Massie, A. (2017) 'The Proteasome Inhibition Model of Parkinson's Disease', *Journal of Parkinson's Disease*. IOS Press, 7(1), pp. 31–63. doi: 10.3233/JPD-160921.

Berg, D. *et al.* (2015) 'MDS research criteria for prodromal Parkinson's disease', *Movement Disorders*, 30(12), pp. 1600–1611. doi: 10.1002/mds.26431.

Berger, N. A. *et al.* (1987) 'Role of nicotinamide adenine dinucleotide and adenosine triphosphate in glucocorticoid-induced cytotoxicity in susceptible lymphoid cells', *Journal of*

Clinical Investigation. American Society for Clinical Investigation, 79(6), pp. 1558–1563. doi: 10.1172/JCI112989.

Bernal, A. and Arranz, L. (2018) 'Nestin-expressing progenitor cells: function, identity and therapeutic implications', *Cellular and Molecular Life Sciences*. Springer International Publishing, 75(12), pp. 2177–2195. doi: 10.1007/s00018-018-2794-z.

Bezard, E., Gross, C. E. and Brotchie, J. M. (2003) 'Presymptomatic compensation in Parkinson's disease is not dopamine-mediated', *Trends in Neurosciences*, 26(4), pp. 215–221. doi: 10.1016/S0166-2236(03)00038-9.

De Biase, L. M. *et al.* (2017) 'Local Cues Establish and Maintain Region-Specific Phenotypes of Basal Ganglia Microglia', *Neuron*. Cell Press, 95(2), pp. 341–356. doi: 10.1016/j.neuron.2017.06.020.

Bieganowski, P. and Brenner, C. (2004) 'Discoveries of nicotinamide riboside as a nutrient and conserved NRK genes establish a preiss-handler independent route to NAD+ in fungi and humans', *Cell*. Cell Press, 117(4), pp. 495–502. doi: 10.1016/S0092-8674(04)00416-7.

Bienert, G. P. *et al.* (2007) 'Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 282(2), pp. 1183–1192. doi: 10.1074/jbc.M603761200.

Bilimoria, P. M. and Stevens, B. (2015) 'Microglia function during brain development: New insights from animal models', *Brain Research*. Elsevier, 1617, pp. 7–17. doi: 10.1016/J.BRAINRES.2014.11.032.

Bissonette, G. B. and Roesch, M. R. (2016) 'Development and function of the midbrain dopamine system: What we know and what we need to', *Genes, Brain and Behavior*. Blackwell Publishing Ltd, 15(1), pp. 62–73. doi: 10.1111/gbb.12257.

Bitterman, K. J. *et al.* (2002) 'Inhibition of silencing and accelerated aging by

nicotinamide, a putative negative regulator of yeast Sir2 and human SIRT1', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 277(47), pp. 45099–45107. doi: 10.1074/jbc.M205670200.

Björklund, A. and Dunnett, S. B. (2007) 'Dopamine neuron systems in the brain: an update', *Trends in Neurosciences*, 30(5), pp. 194–202. doi: 10.1016/j.tins.2007.03.006.

Björklund, A., Lindvall, O. and Nobin, A. (1975) 'Evidence of an incerto-hypothalamic dopamine neurone system in the rat', *Brain Research*. Elsevier, 89(1), pp. 29–42. doi: 10.1016/0006-8993(75)90131-6.

Blum, D. *et al.* (2001) 'Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: Contribution to the apoptotic theory in Parkinson's disease', *Progress in Neurobiology*, 65(2), pp. 135–172. doi: 10.1016/S0301-0082(01)00003-X.

Blum, K. *et al.* (2008) 'Attention-deficit-hyperactivity disorder and reward deficiency syndrome', *Neuropsychiatric Disease and Treatment*. Dove Press, pp. 893–917. doi: 10.2147/ndt.s2627.

Blümcke, I. *et al.* (2001) 'Distinct Expression Pattern of Microtubule-Associated Protein-2 in Human Oligodendrogliomas and Glial Precursor Cells', *Journal of Neuropathology and Experimental Neurology*, 60(10), pp. 984–993.

Boche, D., Perry, V. H. and Nicoll, J. A. R. (2013) 'Review: Activation patterns of microglia and their identification in the human brain', *Neuropathology and Applied Neurobiology*, 39(1), pp. 3–18. doi: 10.1111/nan.12011.

Bogan, K. L. and Brenner, C. (2008) 'Nicotinic Acid, Nicotinamide, and Nicotinamide Riboside: A Molecular Evaluation of NAD + Precursor Vitamins in Human Nutrition', *Annual Review of Nutrition*. Annual Reviews, 28(1), pp. 115–130. doi: 10.1146/annurev.nutr.28.061807.155443.

Bohlen, C. J. *et al.* (2017) 'Diverse requirements for microglial survival, specification,

and function revealed by defined-medium cultures', *Neuron*, 94(4), pp. 759–773. doi: 10.1016/j.neuron.2017.04.043.

Bolam, J. P. and Pissadaki, E. K. (2012) 'Living on the edge with too many mouths to feed: Why dopamine neurons die', *Movement Disorders*. Wiley-Blackwell, 27(12), pp. 1478–1483. doi: 10.1002/mds.25135.

Bollimpelli, V. S. and Kondapi, A. K. (2015) 'Enriched rat primary ventral mesencephalic neurons as an in-vitro culture model', *NeuroReport*, 26(12), pp. 728–734. doi: 10.1097/WNR.0000000000000420.

Borgemeester, R. W. K., Lees, A. J. and van Laar, T. (2016) 'Parkinson's disease, visual hallucinations and apomorphine: A review of the available evidence', *Parkinsonism & Related Disorders*, 27, pp. 35–40. doi: 10.1016/j.parkreldis.2016.04.023.

Borner, M. M. *et al.* (1994) 'The detergent Triton X-100 induces a death pattern in human carcinoma cell lines that resembles cytotoxic lymphocyte-induced apoptosis', *FEBS Letters*, 353, pp. 129–132.

Bortell, R. *et al.* (2001) 'Nicotinamide Adenine Dinucleotide (NAD) and Its Metabolites Inhibit T Lymphocyte Proliferation: Role of Cell Surface NAD Glycohydrolase and Pyrophosphatase Activities', *The Journal of Immunology*, 167(4), pp. 2049–2059. doi: 10.4049/jimmunol.167.4.2049.

Braak, H. *et al.* (2006) 'Stanley Fahn Lecture 2005: The staging procedure for the inclusion body pathology associated with sporadic Parkinson's disease reconsidered.', *Movement disorders : official journal of the Movement Disorder Society*, 21(12), pp. 2042–51. doi: 10.1002/mds.21065.

Braidy, N. *et al.* (2013) 'Serum nicotinamide adenine dinucleotide levels through disease course in multiple sclerosis', *Brain Research*, 1537, pp. 267–272. doi: 10.1016/j.brainres.2013.08.025.

Braidy, N. *et al.* (2014) 'Mapping NAD+ metabolism in the brain of ageing Wistar rats: Potential targets for influencing brain senescence', *Biogerontology*, 15(2), pp. 177–198. doi: 10.1007/s10522-013-9489-5.

Bramanti, V. *et al.* (2010) 'Biomarkers of glial cell proliferation and differentiation in culture', *Frontiers in Bioscience - Scholar*, 2 S(2), pp. 558–570. doi: 10.2741/s85.

Brederlau, A. *et al.* (2006) 'Transplantation of Human Embryonic Stem Cell-Derived Cells to a Rat Model of Parkinson's Disease: Effect of In Vitro Differentiation on Graft Survival and Teratoma Formation', *Stem Cells*, 24(6), pp. 1433–1440. doi: 10.1634/stemcells.2005-0393.

Bronstein, D. M. *et al.* (1995) 'Glia-dependent neurotoxicity and neuroprotection in mesencephalic cultures', *Brain Research*, 704(1), pp. 112–116. doi: 10.1016/0006-8993(95)01189-7.

Brooks, D. J. (2000) 'Dopamine agonists : their role in the treatment of Parkinson ' s disease', *Journal of Neurology, Neurosurgery & Psychiatry*, 68(6), pp. 685–690. doi: 10.1136/jnnp.68.6.685.

Broome, S. T. *et al.* (2020) 'Dopamine: an immune transmitter', *Neural Regeneration Research*, 15(12), pp. 2173–2185. doi: 10.4103/1673-5374.284976.

Bruttger, J. *et al.* (2015) 'Genetic Cell Ablation Reveals Clusters of Local Self-Renewing Microglia in the Mammalian Central Nervous System', *Immunity*. Cell Press, 43(1), pp. 92–107. doi: 10.1016/j.immuni.2015.06.012.

Buchholz, D. E. *et al.* (2013) 'Rapid and Efficient Directed Differentiation of Human Pluripotent Stem Cells Into Retinal Pigmented Epithelium', *STEM CELLS Translational Medicine*. Wiley, 2(5), pp. 384–393. doi: 10.5966/sctm.2012-0163.

Burke, R. E., Dauer, W. T. and Vonsattel, J. P. G. (2008) 'A critical evaluation of the Braak staging scheme for Parkinson's disease', *Annals of Neurology*. NIH Public Access, pp. 485–491.

doi: 10.1002/ana.21541.

Burkert, R., McGrath, J. and Eyles, D. (2003) 'Vitamin D receptor expression in the embryonic rat brain', *Neuroscience Research Communications*. Wiley Subscription Services, Inc., A Wiley Company, 33(1), pp. 63–71. doi: 10.1002/nrc.10081.

Bussian, T. J. *et al.* (2018) 'Clearance of senescent glial cells prevents tau-dependent pathology and cognitive decline', *Nature*. Nature Publishing Group, 562(7728), pp. 578–582. doi: 10.1038/s41586-018-0543-y.

Callizot, N. *et al.* (2019) 'Necrosis, apoptosis, necroptosis, three modes of action of dopaminergic neuron neurotoxins', *PLoS ONE*, 14(4), pp. 1–19. doi: 10.1371/journal.pone.0215277.

Camacho-Pereira, J. *et al.* (2016) 'CD38 Dictates Age-Related NAD Decline and Mitochondrial Dysfunction through an SIRT3-Dependent Mechanism.', *Cell metabolism*. NIH Public Access, 23(6), pp. 1127–1139. doi: 10.1016/j.cmet.2016.05.006.

Carlsson, A. (1987) 'Brain Neurotransmitters in Aging and Dementia: Similar Changes Across Diagnostic Dementia Groups', *Gerontology*. Karger Publishers, 33(3–4), pp. 159–167. doi: 10.1159/000212870.

Cass, W. A. *et al.* (2014) 'Calcitriol promotes augmented dopamine release in the lesioned striatum of 6-hydroxydopamine treated rats', *Neurochemical Research*, 39(8), pp. 1467–1476. doi: 10.1007/s11064-014-1331-1.

Cass, W. A. and Peters, L. E. (2017) 'Reduced ability of calcitriol to promote augmented dopamine release in the lesioned striatum of aged rats', *Neurochemistry International*, 108, pp. 222–229. doi: 10.1016/j.physbeh.2017.03.040.

Castelo-Branco, G. *et al.* (2003) 'Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 100(22), pp.

12747–52. doi: 10.1073/pnas.1534900100.

Castilho, R. F., Hansson, O. and Brundin, P. (2000) 'Improving the survival of grafted embryonic dopamine neurons in rodent models of Parkinson's disease', *Progress in Brain Research*, 127, pp. 203–231. doi: 10.1016/S0079-6123(00)27011-8.

Castro-Portuguez, R. and Sutphin, G. L. (2020) 'Kynurenine pathway, NAD+ synthesis, and mitochondrial function: Targeting tryptophan metabolism to promote longevity and healthspan', *Experimental Gerontology*. Elsevier BV, 132, p. 110841. doi: 10.1016/j.exger.2020.110841.

Cenci, M. A. and Konradi, C. (2010) 'Maladaptive striatal plasticity in l-DOPA-induced dyskinesia', *Progress in Brain Research*. Elsevier B.V., 183, pp. 209–233. doi: 10.1016/S0079-6123(10)83011-0.

Cerruti, C. *et al.* (1993) 'Protection by BTCP of cultured dopaminergic neurons exposed to neurotoxins', *Brain Research*, 617(1), pp. 138–142. doi: 10.1016/0006-8993(93)90624-V.

Cetina Biefer, H. R., Vasudevan, A. and Elkhal, A. (2017) 'Aspects of tryptophan and nicotinamide adenine dinucleotide in immunity: A new twist in an old tale', *International Journal of Tryptophan Research*. SAGE Publications Ltd. doi: 10.1177/1178646917713491.

Chen, C., Turnbull, D. M. and Reeve, A. K. (2019) 'Mitochondrial dysfunction in Parkinson's disease—cause or consequence?', *Biology*. MDPI AG, 8(2), pp. 1–26. doi: 10.3390/biology8020038.

Chen, L., He, D.-M. and Zhang, Y. (2009) 'The differentiation of human placenta-derived mesenchymal stem cells into dopaminergic cells in vitro.', *Cellular & molecular biology letters*, 14(3), pp. 528–36. doi: 10.2478/s11658-009-0015-3.

Cheng, H. C., Ulane, C. M. and Burke, R. E. (2010) 'Clinical progression in Parkinson disease and the neurobiology of axons', *Annals of Neurology*. NIH Public Access, 67(6), pp. 715–725. doi: 10.1002/ana.21995.

Cheung, N. ., Hickling, Y. . and Beart, P. . (1997) 'Development and survival of rat embryonic mesencephalic dopaminergic neurones in serum-free, antioxidant-rich primary cultures', *Neuroscience Letters*, 233(1), pp. 13–16. doi: 10.1016/S0304-3940(97)00613-7.

Chhor, V. *et al.* (2013) 'Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia In vitro', *Brain, Behavior, and Immunity*. Academic Press Inc., 32, pp. 70–85. doi: 10.1016/j.bbi.2013.02.005.

Cho, B. P. *et al.* (2006) 'Pathological dynamics of activated microglia following medial forebrain bundle transection', *Glia*, 53(1), pp. 92–102. doi: 10.1002/glia.20265.

Cho, J. M. *et al.* (2013) 'Characterization of nestin expression in astrocytes in the rat hippocampal CA1 region following transient forebrain ischemia', *Anatomy & Cell Biology*, 46(2), pp. 131–140. doi: 10.5115/acb.2013.46.2.131.

Choi, D. K., Koppula, S. and Suk, K. (2011) 'Inhibitors of microglial neurotoxicity: Focus on natural products', *Molecules*, 16(2), pp. 1021–1043. doi: 10.3390/molecules16021021.

Christie, V. B. *et al.* (2008) 'Synthesis and evaluation of synthetic retinoid derivatives as inducers of stem cell differentiation', *Organic & Biomolecular Chemistry*. The Royal Society of Chemistry, 6(19), p. 3497. doi: 10.1039/b808574a.

Cicchetti, F. *et al.* (2002) 'Neuroinflammation of the nigrostriatal pathway during progressive 6-OHDA dopamine degeneration in rats monitored by immunohistochemistry and PET imaging', *European Journal of Neuroscience*. John Wiley & Sons, Ltd, 15(6), pp. 991–998. doi: 10.1046/j.1460-9568.2002.01938.x.

Cimadamore, F. *et al.* (2009) 'Nicotinamide Rescues Human Embryonic Stem Cell-Derived Neuroectoderm from Parthanatic Cell Death', *Stem Cells*, 27(8), pp. 1772–1787. doi: 10.1038/jid.2014.371.

Clayton, E. L. *et al.* (2017) 'Early microgliosis precedes neuronal loss and behavioural impairment in mice with a frontotemporal dementia-causing CHMP2B mutation', *Human*

Molecular Genetics. Oxford University Press, 26(5), pp. 873–887. doi: 10.1093/hmg/ddx003.

Codeluppi, S. *et al.* (2011) 'Influence of rat substrain and growth conditions on the characteristics of primary cultures of adult rat spinal cord astrocytes', *Journal of Neuroscience Methods*. Elsevier B.V., 197(1), pp. 118–127. doi: 10.1016/j.jneumeth.2011.02.011.

Coleman, M. P. and Freeman, M. R. (2010) 'Wallerian Degeneration, Wld S , and Nmnat', *Annual Review of Neuroscience*, 33(1), pp. 245–267. doi: 10.1146/annurev-neuro-060909-153248.

Collier, T. J., Kanaan, N. M. and Kordower, J. H. (2017) 'Aging and Parkinson's disease: Different sides of the same coin?', *Movement Disorders*. John Wiley and Sons Inc., pp. 983–990. doi: 10.1002/mds.27037.

Cooney, S. J., L Bermudez-Sabogal, S. and Byrnes, K. R. (2013) 'Cellular and temporal expression of NADPH oxidase (NOX) isotypes after brain injury', *Journal of Neuroinflammation*, 10(155), pp. 1–13.

Cooper, O. *et al.* (2010) 'Differentiation of human ES and Parkinson's disease iPS cells into ventral midbrain dopaminergic neurons requires a high activity form of SHH, FGF8a and specific regionalization by retinoic acid.', *Molecular and cellular neurosciences*. NIH Public Access, 45(3), pp. 258–66. doi: 10.1016/j.mcn.2010.06.017.

Cooper, S., Robison, A. J. and Mazei-Robison, M. S. (2017) 'Reward Circuitry in Addiction', *Neurotherapeutics*. Springer New York LLC, 14(3), pp. 687–697. doi: 10.1007/s13311-017-0525-z.

Corder, E. H. *et al.* (1993) 'Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families', *Science*, 261(5123), pp. 921–923. doi: 10.1126/science.8346443.

Cossu, G. *et al.* (2016) 'Levodopa and neuropathy risk in patients with Parkinson disease: Effect of COMT inhibition.', *Parkinsonism & related disorders*, 27, pp. 81–4. doi:

10.1016/j.parkreldis.2016.04.016.

Costa, C. M. *et al.* (2019) 'Levels of cortisol and neurotrophic factor brain-derived in Parkinson's disease', *Neuroscience Letters*. Elsevier, 708(May), p. 134359. doi: 10.1016/j.neulet.2019.134359.

Crain, J. M., Nikodemova, M. and Watters, J. J. (2013) 'Microglia express distinct M1 and M2 phenotypic markers in the postnatal and adult CNS in male and female mice', *Journal of Neuroscience Research*, 91(9), pp. 1143–1151. doi: 10.1016/j.immuni.2010.12.017.Two-stage.

Cseh, A. M. *et al.* (2017) 'Poly(adenosine diphosphate-ribose) polymerase as therapeutic target: Lessons learned from its inhibitors', *Oncotarget*, 8(30), pp. 50221–50239. doi: 10.18632/oncotarget.16859.

Cui, X. *et al.* (2010) 'Maternal vitamin D deficiency alters the expression of genes involved in dopamine specification in the developing rat mesencephalon', *Neuroscience Letters*. doi: 10.1016/j.neulet.2010.09.057.

Cutillas, B., Ambrosio, S. and Unzeta, M. (2002) 'Neuroprotective effect of the monoamine oxidase inhibitor PF 9601N [N-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine] on rat nigral neurons after 6-hydroxydopamine-striatal lesion', *Neuroscience Letters*. Elsevier Ireland Ltd, 329(2), pp. 165–168. doi: 10.1016/S0304-3940(02)00614-6.

d'Anglemont de Tassigny, X., Pascual, A. and Lopez-Barneo, J. (2015) 'GDNF-based therapies, GDNF-producing interneurons, and trophic support of the dopaminergic nigrostriatal pathway. Implications for parkinson's disease', *Frontiers in Neuroanatomy*. Frontiers Media SA, 9(FEB), pp. 1–15. doi: 10.3389/fnana.2015.00010.

Damani, M. R. *et al.* (2011) 'Age-related alterations in the dynamic behavior of microglia', *Aging Cell*. NIH Public Access, 10(2), pp. 263–276. doi: 10.1111/j.1474-9726.2010.00660.x.

Daubner, S. C., Le, T. and Wang, S. (2011) 'Tyrosine Hydroxylase and Regulation of Dopamine Synthesis', *Archives of Biochemistry and Biophysics*, 508(1), pp. 1–12. doi: 10.1016/j.abb.2010.12.017.Tyrosine.

Davis, E. J., Foster, T. D. and Thomas, W. E. (1994) 'Cellular forms and functions of brain microglia.', *Brain research bulletin*, 34(1), pp. 73–8.

Dawson, T. M., Ko, H. S. and Dawson, V. L. (2010) 'Genetic Animal Models of Parkinson's Disease', *Neuron*. NIH Public Access, 66(5), pp. 646–661. doi: 10.1016/j.neuron.2010.04.034.

Dekkers, M. P. J., Nikoletopoulou, V. and Barde, Y. A. (2013) 'Death of developing neurons: New insights and implications for connectivity', *Journal of Cell Biology*. The Rockefeller University Press, 203(3), pp. 385–393. doi: 10.1083/jcb.201306136.

Deuschl, G. (2006) 'A Randomized Trial of Deep-Brain Stimulation for Parkinson', *New England Journal of Medicine*, 355, pp. 896–908. doi: 10.1056/NEJMoa060281.

Devinsky, O. *et al.* (2013) 'Glia and epilepsy: excitability and inflammation'. doi: 10.1016/j.tins.2012.11.008.

Dias, V., Junn, E. and Mouradian, M. M. (2013) 'The role of oxidative stress in parkinson's disease', *Journal of Parkinson's Disease*. I O S Press, 3(4), pp. 461–491. doi: 10.3233/JPD-130230.

Dickson, D. W. *et al.* (2010) 'Evidence in favor of Braak staging of Parkinson's disease', *Movement Disorders*. John Wiley & Sons, Ltd, 25(S1), pp. S78–S82. doi: 10.1002/mds.22637.

Ding, Y. M. *et al.* (2004) 'Effects of 6-hydroxydopamine on primary cultures of substantia nigra: specific damage to dopamine neurons and the impact of glial cell line-derived neurotrophic factor', *Journal of Neurochemistry*, 89(3), pp. 776–787. doi: 10.1111/j.1471-4159.2004.02415.x.

Diogo, M. M., Henrique, D. and Cabral, J. M. S. (2008) 'Optimization and integration of

expansion and neural commitment of mouse embryonic stem cells', *Biotechnology and Applied Biochemistry*. John Wiley & Sons, Ltd, 49(2), pp. 105–112. doi: 10.1042/BA20070011.

Doi, D. *et al.* (2014) 'Isolation of human induced pluripotent stem cell-derived dopaminergic progenitors by cell sorting for successful transplantation', *Stem Cell Reports*. Cell Press, 2(3), pp. 337–350. doi: 10.1016/j.stemcr.2014.01.013.

Donat, C. K. *et al.* (2017) 'Microglial Activation in Traumatic Brain Injury', *Frontiers in Aging Neuroscience*, 9(June), p. 208. doi: 10.3389/fnagi.2017.00208.

Dorsey, E. R. *et al.* (2007) 'Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030', *Neurology*, 69, pp. 384–386. doi: 10.1212/01.wnl.0000271777.50910.73.

Dragovic, J. *et al.* (1995) 'Nicotinamide pharmacokinetics in patients', *Radiotherapy and Oncology*, 36(3), pp. 225–228. doi: 10.1016/0167-8140(95)01581-Z.

Drexler, H. G. and Uphoff, C. C. (2002) 'Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention', *Cytotechnology*, 39(2), pp. 75–90. doi: 10.1023/A:1022913015916.

Du, F. *et al.* (2010) 'Purity, cell viability, expression of GFAP and bystin in astrocytes cultured by different procedures', *Journal of Cellular Biochemistry*, 109(1), pp. 30–37. doi: 10.1002/jcb.22375.

Duan, W.-M. *et al.* (1998) 'Quinolinic acid-induced inflammation in the striatum does not impair the survival of neural allografts in the rat', *European Journal of Neuroscience*. John Wiley & Sons, Ltd, 10(8), pp. 2595–2606. doi: 10.1046/j.1460-9568.1998.00279.x.

Dunnett, S. B. and Björklund, A. (1992) 'Staging and dissection of rat embryos', in Dunnett, S. B. and Björklund, A. (eds) *Neural transplantation: A practical approach*. 1st edn. New York: Oxford University Press, pp. 1–18.

Duty, S. and Jenner, P. (2011) 'Animal models of Parkinson's disease: A source of novel

treatments and clues to the cause of the disease', *British Journal of Pharmacology*, 164(4), pp. 1357–1391. doi: 10.1111/j.1476-5381.2011.01426.x.

Ekdahl, C. T., Kokaia, Z. and Lindvall, O. (2009) 'Brain inflammation and adult neurogenesis: The dual role of microglia', *Neuroscience*, 158(3), pp. 1021–1029. doi: 10.1016/j.neuroscience.2008.06.052.

Eliasson, M. J. L. *et al.* (1997) 'Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia', *Nature Medicine*. Nat Med, 3(10), pp. 1089–1095. doi: 10.1038/nm1097-1089.

Elmore, M. R. P. *et al.* (2014) 'Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain', *Neuron*. Cell Press, 82(2), pp. 380–397. doi: 10.1016/j.neuron.2014.02.040.

Elobeid, A. *et al.* (2016) 'Altered Proteins in the Aging Brain', *Journal of Neuropathology and Experimental Neurology*. Oxford University Press, 75(4), p. 316. doi: 10.1093/jnen/nlw002.

Elyasi, L. *et al.* (2018) 'Induction of cross-tolerance between protective effect of morphine and nicotine in 6-hydroxydopamine-induce neurotoxicity in SH-SY5Y human dopaminergic neuroblastoma cells', *International Journal of Neuroscience*, pp. 1–10. doi: 10.1080/00207454.2018.1494169.

Erceg, S. *et al.* (2008) 'Differentiation of human embryonic stem cells to regional specific neural precursors in chemically defined medium conditions.', *PloS one*. Public Library of Science, 3(5), p. e2122. doi: 10.1371/journal.pone.0002122.

Evans, M. D., Dizdaroglu, M. and Cooke, M. S. (2004) 'Oxidative DNA damage and disease: Induction, repair and significance', *Mutation Research - Reviews in Mutation Research*. Elsevier, 567(1), pp. 1–61. doi: 10.1016/j.mrrev.2003.11.001.

Fabbrini, G. *et al.* (2007) 'Levodopa-induced dyskinesias', *Movement Disorders*, 22(10),

pp. 1379–1389. doi: 10.1002/mds.21475.

Fan, Y., Winanto and Ng, S. Y. (2020) 'Replacing what's lost: A new era of stem cell therapy for Parkinson's disease', *Translational Neurodegeneration*. BioMed Central Ltd., 9(2), pp. 1–10. doi: 10.1186/s40035-019-0180-x.

Fan, Z. *et al.* (2015) 'Influence of microglial activation on neuronal function in Alzheimer's and Parkinson's disease dementia', *Alzheimer's and Dementia*, 11(6), pp. 608-621.e7. doi: 10.1016/j.jalz.2014.06.016.

Farrer, L. A. (1997) 'Effects of Age, Sex, and Ethnicity on the Association Between Apolipoprotein E Genotype and Alzheimer Disease', *JAMA*, 278(16), p. 1349. doi: 10.1001/jama.1997.03550160069041.

Farzam, A. *et al.* (2020) 'A functionalized hydroxydopamine quinone links thiol modification to neuronal cell death', *Redox Biology*, 28(101377). doi: 10.1016/j.redox.2019.101377.

Fazl, A. and Fleisher, J. (2018) 'Anatomy, Physiology, and Clinical Syndromes of the Basal Ganglia: A Brief Review', *Seminars in Pediatric Neurology*. W.B. Saunders, 25, pp. 2–9. doi: 10.1016/j.spen.2017.12.005.

Feldman, J. L. *et al.* (2015) 'Kinetic and structural basis for Acyl-group selectivity and NAD^+ dependence in sirtuin-catalyzed deacylation', *Biochemistry*. American Chemical Society, 54(19), pp. 3037–3050. doi: 10.1021/acs.biochem.5b00150.

Filippo, M. Di *et al.* (2016) 'Persistent activation of microglia and NADPH drive hippocampal dysfunction in experimental multiple sclerosis OPEN', *Scientific reports*, 6(20926). doi: 10.1038/srep20926.

Foster, H. D. and Hoffer, A. (2004) 'The two faces of L-DOPA: Benefits and adverse side effects in the treatment of Encephalitis lethargica, Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis', *Medical Hypotheses*. Churchill Livingstone, 62(2), pp. 177–181.

doi: 10.1016/S0306-9877(03)00318-9.

Fox, S. H. *et al.* (2011) 'The Movement Disorder Society Evidence-Based Medicine Review Update: Treatments for the motor symptoms of Parkinson's disease', *Mov Disord*, 26 Suppl 3, pp. S2-41. doi: 10.1002/mds.23829.

Fredericks, D. *et al.* (2017) 'Parkinson's disease and Parkinson's disease psychosis: a perspective on the challenges, treatments, and economic burden.', *The American journal of managed care*, 23(5 Suppl), pp. S83–S92.

Freed, C. R. *et al.* (1992) 'Survival of Implanted Fetal Dopamine Cells and Neurologic Improvement 12 to 46 Months after Transplantation for Parkinson's Disease', *New England Journal of Medicine*. Massachusetts Medical Society , 327(22), pp. 1549–1555. doi: 10.1056/NEJM199211263272202.

Freed, C. R. *et al.* (2001) 'Transplantation of Embryonic Dopamine Neurons for Severe Parkinson's Disease', *New England Journal of Medicine*. Massachusetts Medical Society , 344(10), pp. 710–719. doi: 10.1056/NEJM200103083441002.

Fricker, R. A. *et al.* (1996) 'A comparative study of preparation techniques for improving the viability of striatal grafts using vital stains, in vitro cultures, and in vivo grafts', *Cell Transplantation*. Cell Transplant, 5(6), pp. 599–611. doi: 10.1016/S0963-6897(96)00087-5.

Fricker, R. A. *et al.* (2018) 'The Influence of Nicotinamide on Health and Disease in the Central Nervous System', *International Journal of Tryptophan Research*. SAGE Publications Ltd, 11, pp. 1–11. doi: 10.1177/1178646918776658.

Fukushima, S. *et al.* (2015) 'Robust increase of microglia proliferation in the fornix of hippocampal axonal pathway after a single LPS stimulation', *Journal of Neuroimmunology*. Elsevier, 285, pp. 31–40. doi: 10.1016/J.JNEUROIM.2015.05.014.

Fukushima, T. *et al.* (2002) 'Possible role of 1-methylnicotinamide in the pathogenesis

of Parkinson's disease', *Experimental and Toxicologic Pathology*. Elsevier GmbH, 53(6), pp. 469–473. doi: 10.1078/0940-2993-00214.

Fukushima, T. (2005) 'Niacin metabolism and Parkinson's disease.', *Environmental health and preventive medicine*, 10(1), pp. 3–8. doi: 10.1265/ehpm.10.3.

Fukuwatari, T. and Shibata, K. (2013) 'Nutritional aspect of tryptophan metabolism', *International Journal of Tryptophan Research*, 6(SUPPL.1), pp. 3–8. doi: 10.4137/IJTR.S11588.

Galvin, J. E., Lee, V. M.-Y. and Trojanowski, J. Q. (2001) 'Clinical and Pathological Implications', *Arch Neurol*, 58, pp. 186–190.

Gantz, S. C. *et al.* (2018) 'The Evolving Understanding of Dopamine Neurons in the Substantia Nigra and Ventral Tegmental Area', *Annual Review of Physiology*, 80(1), pp. 219–241. doi: 10.1146/annurev-physiol-021317-121615.

Gao, H. M. and Hong, J. S. (2008) 'Why neurodegenerative diseases are progressive: uncontrolled inflammation drives disease progression', *Trends in Immunology*. NIH Public Access, 29(8), pp. 357–365. doi: 10.1016/j.it.2008.05.002.

Gasbarri, A. *et al.* (1996) 'The projections of the retrorubral field A8 to the hippocampal formation in the rat', *Experimental Brain Research*, 112(2), pp. 244–252. doi: 10.1007/BF00227643.

Gates, M. A. *et al.* (2006) 'Re-examining the ontogeny of substantia nigra dopamine neurons', *European Journal of Neuroscience*, 23(5), pp. 1384–1390. doi: 10.1111/j.1460-9568.2006.04637.x.

Gaven, F., Marin, P. and Claeysen, S. (2014) 'Primary culture of mouse dopaminergic neurons', *Journal of Visualized Experiments*, (91), pp. 1–11. doi: 10.3791/51751.

Gee, P. and Davison, A. J. (1989) 'Intermediates in the aerobic autoxidation of 6-hydroxydopamin: Relative importance under different reaction conditions', *Free Radical Biology and Medicine*, 6, pp. 271–284.

Gensel, J. C. and Zhang, B. (2015) 'Macrophage activation and its role in repair and pathology after spinal cord injury', *Brain Research*. Elsevier B.V., 1619, pp. 1–11. doi: 10.1016/j.brainres.2014.12.045.

German, D. C. *et al.* (1992) 'Midbrain Dopaminergic Cell Loss in Parkinson's Disease and MPTP-Induced Parkinsonism: Sparing of Calbindin-D25k—Containing Cells', *Annals of the New York Academy of Sciences*, 648(1), pp. 42–62. doi: 10.1111/j.1749-6632.1992.tb24523.x.

Ghosh, B. *et al.* (2019) 'Partial Reconstruction of the Nigrostriatal Circuit along a Preformed Molecular Guidance Pathway', *Molecular Therapy - Methods and Clinical Development*. Cell Press, 14, pp. 217–227. doi: 10.1016/j.omtm.2019.06.008.

Ghosh, P. and Ghosh, A. (2016) 'Morphological and behavioural variation in CNS innate defence cell microglia is development and age sensitive', *Neuroimmunology and Neuroinflammation*, 3(2), p. 38. doi: 10.20517/2347-8659.2015.32.

Gilyarov, A. V. (2008) 'Nestin in central nervous system cells', *Neuroscience and Behavioral Physiology*, 38(2), pp. 165–169. doi: 10.1007/s11055-008-0025-z.

Ginhoux, F. *et al.* (2013) 'Origin and differentiation of microglia', *Frontiers in Cellular Neuroscience*, 7(April), pp. 1–14. doi: 10.3389/fncel.2013.00045.

Ginhoux, F. and Prinz, M. (2015) 'Origin of microglia: Current concepts and past controversies', *Cold Spring Harbor Perspectives in Biology*, 7(8), pp. 1–15. doi: 10.1101/cshperspect.a020537.

Ginis, I. *et al.* (2004) 'Differences between human and mouse embryonic stem cells', *Developmental Biology*. Academic Press Inc., 269(2), pp. 360–380. doi: 10.1016/j.ydbio.2003.12.034.

Giri, B. *et al.* (2019) 'Niacin Ameliorates Neuro-Inflammation in Parkinson ' s Disease via GPR109A', *International Journal of Molecular Sciences*, 20, p. 4559.

Glinka, Y., Gassen, M. and Youdim, M. B. H. (1997) 'Mechanism of 6-hydroxydopamine

neurotoxicity', *Journal of Neural Transmission, Supplement*. Springer, Vienna, pp. 55–66. doi: 10.1007/978-3-7091-6842-4_7.

Glinka, Y. Y. and Youdim, M. B. H. (1995) 'Inhibition of mitochondrial complexes I and IV by 6-hydroxydopamine', *European Journal of Pharmacology: Environmental Toxicology and*, 292(3–4), pp. 329–332. doi: 10.1016/0926-6917(95)90040-3.

Goetz, C. G. *et al.* (2005) 'Evidence-based medical review update: Pharmacological and surgical treatments of Parkinson's disease: 2001 to 2004', *Movement Disorders*, 20(5), pp. 523–539. doi: 10.1002/mds.20464.

Gomez-Lazaro, M. *et al.* (2008) '6-Hydroxydopamine activates the mitochondrial apoptosis pathway through p38 MAPK-mediated, p53-independent activation of Bax and PUMA', *Journal of Neurochemistry*, 104(6), pp. 1599–1612. doi: 10.1111/j.1471-4159.2007.05115.x.

González-Hernández, T. *et al.* (2004) 'Expression of dopamine and vesicular monoamine transporters and differential vulnerability of mesostriatal dopaminergic neurons', *Journal of Comparative Neurology*, 479(2), pp. 198–215. doi: 10.1002/cne.20323.

Gonzalez, R. *et al.* (2013) 'Deriving dopaminergic neurons for clinical use. A practical approach.', *Scientific reports*, 3, p. 1463. doi: 10.1038/srep01463.

Goodpaster, T. *et al.* (2008) 'An immunohistochemical method for identifying fibroblasts in formalin-fixed, paraffin-embedded tissue', *Journal of Histochemistry and Cytochemistry*, 56(4), pp. 347–358. doi: 10.1369/jhc.7A7287.2007.

Goridis, C. and Rohrer, H. (2002) 'Specification of catecholaminergic and serotonergic neurons', *Nature Reviews Neuroscience*, 3(7), pp. 531–541. doi: 10.1038/nrn871.

Goshi, N. *et al.* (2020) 'A primary neural cell culture model to study neuron, astrocyte, and microglia interactions in neuroinflammation', *Journal of Neuroinflammation*, 17(1). doi: 10.1186/s12974-020-01819-z.

Grabert, K. *et al.* (2016) 'Microglial brain region-dependent diversity and selective regional sensitivities to aging', *Nature Neuroscience*. Nature Publishing Group, 19(3), pp. 504–516. doi: 10.1038/nn.4222.

Graham, D. G. *et al.* (1978) 'Autoxidation versus Covalent Binding of Quinones as the Mechanism of Toxicity of Dopamine, 6-Hydroxydopamine, and Related Compounds toward C1300 Neuroblastoma Cells in Vitro', *Molecular Pharmacology*, 14(4), pp. 644–653.

Grant, R. J. and Clarke, P. B. S. (2002) 'Susceptibility of ascending dopamine projections to 6-hydroxydopamine in rats: Effect of hypothermia', *Neuroscience*. Pergamon, 115(4), pp. 1281–1294. doi: 10.1016/S0306-4522(02)00385-8.

Gray, J. and Ross, M. E. (2011) 'Neural tube closure in mouse whole embryo culture.', *Journal of visualized experiments : JoVE*, (56), pp. 3–6. doi: 10.3791/3132.

Green, S. *et al.* (1991) 'N-methylation of pyridines in Parkinson's disease', *The Lancet*, 338(8759), pp. 120–121. doi: 10.1016/0140-6736(91)90113-4.

Greenamyre, J. T., Betarbet, R. and Sherer, T. B. (2003) 'The rotenone model of Parkinson's disease: Genes, environment and mitochondria', *Parkinsonism and Related Disorders*, 9(SUPPL. 2), pp. 59–64. doi: 10.1016/S1353-8020(03)00023-3.

Grehan, S., Tse, E. and Taylor, J. M. (2001) 'Two distal downstream enhancers direct expression of the human apolipoprotein E gene to astrocytes in the brain', *Journal of Neuroscience*, 21(3), pp. 812–822. doi: 10.1523/jneurosci.21-03-00812.2001.

Griffin, S. M. *et al.* (2013) 'Nicotinamide promotes neuronal differentiation of mouse embryonic stem cells in vitro', *NeuroReport*, 24(18), pp. 1041–1046. doi: 10.1097/WNR.0000000000000071.

Griffin, S. M. *et al.* (2017) 'Nicotinamide alone accelerates the conversion of mouse embryonic stem cells into mature neuronal populations', *PLoS ONE*. Public Library of Science, 12(8), p. e0183358. doi: 10.1371/journal.pone.0183358.

Grünewald, A. *et al.* (2016) 'Mitochondrial DNA Depletion in Respiratory Chain-Deficient Parkinson Disease Neurons', *Annals of Neurology*. John Wiley and Sons Inc., 79(3), pp. 366–378. doi: 10.1002/ana.24571.

Guerreiro, S. *et al.* (2020) 'CD38 in Neurodegeneration and Neuroinflammation', *Cells*. MDPI AG, 9(2), p. 471. doi: 10.3390/cells9020471.

Guo, C. Y. *et al.* (2013) 'Oxidative stress, mitochondrial damage and neurodegenerative diseases', *Neural Regeneration Research*, 8(21), pp. 2003–2014. doi: 10.3969/j.issn.1673-5374.2013.21.009.

Guo, J. D. *et al.* (2018) 'Damage to dopaminergic neurons by oxidative stress in Parkinson's disease (Review)', *International Journal of Molecular Medicine*. Spandidos Publications, 41(4), pp. 1817–1825. doi: 10.3892/ijmm.2018.3406.

Gupta, S. *et al.* (2015) '6-Hydroxydopamine and lipopolysaccharides induced DNA damage in astrocytes: Involvement of nitric oxide and mitochondria', *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, 778, pp. 22–36. doi: 10.1016/j.mrgentox.2014.12.007.

Haag, F. *et al.* (2007) 'Extracellular NAD and ATP: Partners in immune cell modulation', *Purinergic Signalling*, 3(1–2), pp. 71–81. doi: 10.1007/s11302-006-9038-7.

Haage, V. *et al.* (2019) 'Comprehensive gene expression meta-analysis identifies signature genes that distinguish microglia from peripheral monocytes/macrophages in health and glioma', *Acta Neuropathologica Communications*. BioMed Central, 7(1), p. 20. doi: 10.1186/s40478-019-0665-y.

de Haas, A. H., Boddeke, H. W. G. M. and Biber, K. (2008) 'Region-specific expression of immunoregulatory proteins on microglia in the healthy CNS', *Glia*. John Wiley & Sons, Ltd, 56(8), pp. 888–894. doi: 10.1002/glia.20663.

Haber, S. N. (2014) 'The place of dopamine in the cortico-basal ganglia circuit',

Neuroscience. Elsevier Ltd, 282, pp. 248–257. doi: 10.1016/j.neuroscience.2014.10.008.

Hagell, P. *et al.* (2002) 'Dyskinesias following neural transplantation in Parkinson's disease', *Nature Neuroscience*. Nature Publishing Group, 5(7), p. 627. doi: 10.1038/nn863.

Hagell, P. and Brundin, P. (2001) 'Cell survival and clinical outcome following intrastriatal transplantation in Parkinson disease.', *Journal of neuropathology and experimental neurology*, 60(8), pp. 741–752.

Hallett, P. J. *et al.* (2014) 'Long-Term Health of Dopaminergic Neuron Transplants in Parkinson's Disease Patients', *Cell Reports*. The Authors, 7(6), pp. 1755–1761. doi: 10.1016/j.celrep.2014.05.027.

Halliwell, B. (1992) 'Reactive Oxygen Species and the Central Nervous System', *Journal of Neurochemistry*, 59(5), pp. 1609–1623. doi: 10.1111/j.1471-4159.1992.tb10990.x.

Halliwell, B. (2006) 'Oxidative stress and neurodegeneration: where are we now?', *Journal of Neurochemistry*. Blackwell Publishing Ltd, 97(6), pp. 1634–1658. doi: 10.1111/j.1471-4159.2006.03907.x.

Hamblin, T. J. (2003) 'CD38: what is it there for?', *Blood*, 102(6), pp. 1939–1940.

Hanrott, K. *et al.* (2006) '6-Hydroxydopamine-induced apoptosis is mediated via extracellular auto-oxidation and caspase 3-dependent activation of protein kinase Cδ', *Journal of Biological Chemistry*, 281(9), pp. 5373–5382. doi: 10.1074/jbc.M511560200.

Harrison, I. F., Powell, N. M. and Dexter, D. T. (2019) 'The histone deacetylase inhibitor nicotinamide exacerbates neurodegeneration in the lactacystin rat model of Parkinson's disease', *Journal of Neurochemistry*, 148(1), pp. 136–156. doi: 10.1111/jnc.14599.

Hashida, K., Sakakura, Y. and Makino, N. (2002) 'Kinetic studies on the hydrogen peroxide elimination by cultured PC12 cells: Rate limitation by glucose-6-phosphate dehydrogenase', *Biochimica et Biophysica Acta - General Subjects*, 1572(1), pp. 85–90. doi: 10.1016/S0304-4165(02)00282-9.

Hayley, S. *et al.* (2015) 'Brain region-specific gene expression profiles in freshly isolated rat microglia'. doi: 10.3389/fncel.2015.00084.

He, X. B. *et al.* (2015) 'Vitamin C facilitates dopamine neuron differentiation in fetal midbrain through TET1- and JMJD3-dependent epigenetic control manner', *Stem Cells*. doi: 10.1002/stem.1932.

Healy-Stoffel, M. *et al.* (2014) 'Differential effects of intrastriatal 6-hydroxydopamine on cell number and morphology in midbrain dopaminergic subregions of the rat', *Brain Research*. Elsevier B.V., 1574, pp. 113–119. doi: 10.1016/j.brainres.2014.05.045.

Hegarty, S. V. *et al.* (2014) 'Ventral midbrain neural stem cells have delayed neurogenic potential in vitro', *Neuroscience Letters*. Elsevier, 559, pp. 193–198. doi: 10.1016/j.neulet.2013.12.009.

Henchcliffe, C. and Beal, M. F. (2008) 'Mitochondrial biology and oxidative stress in Parkinson disease pathogenesis.', *Nature clinical practice. Neurology*, 4(11), pp. 600–609. doi: 10.1038/ncpneuro0924.

Herceg, Z. and Wang, Z. Q. (2001) 'Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death', *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, 477(1–2), pp. 97–110. doi: 10.1016/S0027-5107(01)00111-7.

Herrera, A. J. *et al.* (2000) 'The Single Intranigral Injection of LPS as a New Model for Studying the Selective Effects of Inflammatory Reactions on Dopaminergic System', *Neurobiology of Disease*, 7(4), pp. 429–447. doi: 10.1006/nbdi.2000.0289.

Hidalgo-Figueroa, M. *et al.* (2012) 'GDNF is predominantly expressed in the PV+ neostriatal interneuronal ensemble in normal mouse and after injury of the nigrostriatal pathway.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 32(3), pp. 864–872. doi: 10.1523/JNEUROSCI.2693-11.2012.

Hill, L. J. and Williams, A. C. (2017) 'Meat intake and the dose of vitamin B3 - nicotinamide: Cause of the causes of disease transitions, health divides, and health futures?', *International Journal of Tryptophan Research*, 10(1). doi: 10.1177/1178646917704662.

Hirsch, L. *et al.* (2016) 'The Incidence of Parkinson's Disease: A Systematic Review and Meta-Analysis', *Neuroepidemiology*. S. Karger AG, 46(4), pp. 292–300. doi: 10.1159/000445751.

Hol, E. M. and Capetanaki, Y. (2017) 'Type III intermediate filaments desmin, glial fibrillary acidic protein (GFAP), vimentin, and peripherin', *Cold Spring Harbor Perspectives in Biology*, 9(12), pp. 1–17. doi: 10.1101/cshperspect.a021642.

Holmes, C., Angharad Jones, S. and Greenfield, S. A. (1995) 'The influence of target and non-target brain regions on the development of mid-brain dopaminergic neurons in organotypic slice culture', *Developmental Brain Research*. Elsevier, 88(2), pp. 212–219. doi: 10.1016/0165-3806(95)00112-Q.

Hong, S. *et al.* (2019) 'Defective neurogenesis and schizophrenia-like behavior in PARP-1-deficient mice', *Cell Death and Disease*. Springer US, 10(12). doi: 10.1038/s41419-019-2174-0.

Horsfall, L. *et al.* (2013) 'Time trends in incidence of Parkinson's disease diagnosis in UK primary care', *Journal of Neurology*, 260(5), pp. 1351–1357. doi: 10.1007/s00415-012-6804-z.

Howes, O. D. *et al.* (2017) 'The Role of Genes, Stress, and Dopamine in the Development of Schizophrenia', *Biological Psychiatry*. Elsevier USA, 81(1), pp. 9–20. doi: 10.1016/j.biopsych.2016.07.014.

Hu, X. *et al.* (2020) 'Modeling Parkinson's Disease Using Induced Pluripotent Stem Cells', *Stem Cells International*, 2020. doi: 10.1155/2020/1061470.

Hu, Y. *et al.* (2013) 'Overexpression of CD38 Decreases Cellular NAD Levels and Alters

the Expression of Proteins Involved in Energy Metabolism and Antioxidant Defense', *Journal of Proteome Research*, 13(2), pp. 786–795. doi: 10.1021/pr4010597.

Huang, Y. *et al.* (2018) 'Repopulated microglia are solely derived from the proliferation of residual microglia after acute depletion', *Nature Neuroscience*. Springer US, 21(4), pp. 530–540. doi: 10.1038/s41593-018-0090-8.

Huot, P., Lévesque, M. and Parent, A. (2007) 'The fate of striatal dopaminergic neurons in Parkinson's disease and Huntington's chorea', *Brain*, 130, pp. 222–232. doi: 10.1093/brain/awl332.

Hwang, E. S. and Song, S. B. (2020) 'Possible Adverse Effects of High-Dose Nicotinamide : Mechanisms and Safety Assessment', *Biomolecules*, 10(687), pp. 1–21.

Idelson, M. *et al.* (2009) 'Directed Differentiation of Human Embryonic Stem Cells into Functional Retinal Pigment Epithelium Cells', *Cell Stem Cell*. Elsevier, 5(4), pp. 396–408. doi: 10.1016/j.stem.2009.07.002.

Imai, S.-I. and Guarente, L. (2016) 'It takes two to tango: NAD + and sirtuins in aging/longevity control', *Nature Publishing Group Aging and Mechanisms of Disease*, 2(16017). doi: 10.1038/npjamd.2016.17.

Incitti, T. *et al.* (2014) 'Sorting of Sox1-GFP Mouse Embryonic Stem Cells Enhances Neuronal Identity Acquisition upon Factor-Free Monolayer Differentiation', *BioResearch Open Access*, 3(3), pp. 127–135. doi: 10.1089/biores.2014.0009.

Ito, D. *et al.* (1998) 'Microglia-specific localisation of a novel calcium binding protein, Iba1', *Molecular Brain Research*, 57, pp. 1–9.

Ito, T. K. *et al.* (2020) 'A nonrandomized study of single oral supplementation within the daily tolerable upper level of nicotinamide affects blood nicotinamide and NAD+ levels in healthy subjects', *Translational Medicine of Aging*. Elsevier Ltd, 4, pp. 45–54. doi: 10.1016/j.tma.2020.04.002.

Izumi, Y. *et al.* (2005) 'P-Quinone Mediates 6-Hydroxydopamine-Induced Dopaminergic Neuronal Death and Ferrous Iron Accelerates the Conversion of P-Quinone Into Melanin Extracellularly', *Journal of Neuroscience Research*, 79(6), pp. 849–860. doi: 10.1002/jnr.20382.

Jacobs, F. M. J. *et al.* (2007) 'Retinoic acid counteracts developmental defects in the substantia nigra caused by Pitx3 deficiency', *Development*, 134(14).

Jaeger, I. *et al.* (2011) 'Temporally controlled modulation of FGF/ERK signaling directs midbrain dopaminergic neural progenitor fate in mouse and human pluripotent stem cells', *Development*. Company of Biologists, 138(20), pp. 4363–4374. doi: 10.1242/dev.066746.

Jeffrey J.Bajramovic (2011) 'Regulation of Innate Immune Responses in the Central Nervous System', *CNS & Neurological Disorders - Drug Targets*, 10, pp. 4–24.

Jekabsone, A. *et al.* (2006) 'Fibrillar beta-amyloid peptide Aβ 1-40 activates microglial proliferation via stimulating TNF-α release and H 2 O 2 derived from NADPH oxidase: a cell culture study', *Journal of Neuroinflammation*, 3(24). doi: 10.1186/1742-2094-3-24.

Jellinger, K. A. (2019) 'Is Braak staging valid for all types of Parkinson's disease?', *Journal of Neural Transmission*. Springer-Verlag Wien, pp. 423–431. doi: 10.1007/s00702-018-1898-9.

Jęśko, H. *et al.* (2017) 'Sirtuins and Their Roles in Brain Aging and Neurodegenerative Disorders', *Neurochemical Research*. Springer US, 42(3), pp. 876–890. doi: 10.1007/s11064-016-2110-y.

Jessen, K. R. (2004) 'Glial cells', *International Journal of Biochemistry and Cell Biology*, 36(10), pp. 1861–1867. doi: 10.1016/j.biocel.2004.02.023.

Jiang, W. *et al.* (2014) 'Dairy foods intake and risk of Parkinson's disease: a dose-response meta-analysis of prospective cohort studies.', *European journal of epidemiology*. Eur J Epidemiol, 29(9), pp. 613–619. doi: 10.1007/s10654-014-9921-4.

Johannessen, J. N. *et al.* (1985) 'IV. Differences in the metabolism of MPTP in the rodent and primate parallel differences in sensitivity to its neurotoxic effects', *Life Sciences*. Life Sci, 36(3), pp. 219–224. doi: 10.1016/0024-3205(85)90062-1.

Johns, P. (2014) *Clinical neuroscience : an illustrated colour text*. 2nd edn. China: Churchill Livingstone Elsevier.

Johnson, I. P. (2015) 'Age-related neurodegenerative disease research needs aging models', *Frontiers in Aging Neuroscience*. Frontiers Media S.A., 7, p. 168. doi: 10.3389/fnagi.2015.00168.

Kaakkola, S. (2010) 'Problems with the Present Inhibitors and a Relevance of New and Improved COMT Inhibitors in Parkinson's Disease', in, pp. 207–225. doi: 10.1016/B978-0-12-381326-8.00009-0.

Kamphuis, W. *et al.* (2012) 'GFAP isoforms in adult mouse brain with a focus on neurogenic astrocytes and reactive astrogliosis in mouse models of Alzheimer disease', *PLoS ONE*, 7(8). doi: 10.1371/journal.pone.0042823.

Kastner, A. *et al.* (1992) 'Is the Vulnerability of Neurons in the Substantia Nigra of Patients with Parkinson's Disease Related to Their Neuromelanin Content?', *Journal of Neurochemistry*, 59(3), pp. 1080–1089. doi: 10.1111/j.1471-4159.1992.tb08350.x.

Kasuya, M. (1975) 'The effect of vitamin E on the toxicity of alkyl mercurials on nervous tissue in culture', *Toxicology and Applied Pharmacology*. Academic Press, 32(2), pp. 347–354. doi: 10.1016/0041-008X(75)90225-2.

Kauppinen, T. M. and Swanson, R. A. (2005) 'Poly(ADP-Ribose) Polymerase-1 Promotes Microglial Activation, Proliferation, and Matrix Metalloproteinase-9-Mediated Neuron Death', *The Journal of Immunology*, 174(4), pp. 2288–2296. doi: 10.4049/jimmunol.174.4.2288.

Kefalopoulou, Z. *et al.* (2014) 'Long-term clinical outcome of fetal cell transplantation for Parkinson disease: two case reports.', *JAMA neurology*. Europe PMC Funders, 71(1), pp.

83–7. doi: 10.1001/jamaneurol.2013.4749.

Kermer, P. *et al.* (2015) 'BAG1 is Neuroprotective in In Vivo and In Vitro Models of Parkinson's Disease', *Journal of Molecular Neuroscience*, 55(3), pp. 587–595. doi: 10.1007/s12031-014-0396-2.

Kettenmann, H. *et al.* (2011) 'Physiology of Microglia', *Physiological Reviews*. American Physiological Society Bethesda, MD, 91(2), pp. 461–553. doi: 10.1152/physrev.00011.2010.

Khakh, B. S. and Deneen, B. (2019) 'The Emerging Nature of Astrocyte Diversity', *Annual Review of Neuroscience*, 42, pp. 187–207. doi: 10.1146/annurev-neuro-070918-050443.

Khaliq, Z. M. and Bean, B. P. (2010) 'Pacemaking in dopaminergic ventral tegmental area neurons: Depolarizing drive from background and voltage-dependent sodium conductances', *Journal of Neuroscience*. Society for Neuroscience, 30(21), pp. 7401–7413. doi: 10.1523/JNEUROSCI.0143-10.2010.

Kikuchi, T. *et al.* (2017) 'Human iPS cell-derived dopaminergic neurons function in a primate Parkinson's disease model', *Nature*. Nature Publishing Group, 548(7669), pp. 592–596. doi: 10.1038/nature23664.

Kim, J., Basak, J. M. and Holtzman, D. M. (2009) 'The Role of Apolipoprotein E in Alzheimer's Disease', *Neuron*, pp. 287–303. doi: 10.1016/j.neuron.2009.06.026.

Kirkeby, A. *et al.* (2012) 'Generation of Regionally Specified Neural Progenitors and Functional Neurons from Human Embryonic Stem Cells under Defined Conditions', *Cell Reports*. Cell Press, 1(6), pp. 703–714. doi: 10.1016/j.celrep.2012.04.009.

Kirkeby, A. *et al.* (2017) 'Predictive Markers Guide Differentiation to Improve Graft Outcome in Clinical Translation of hESC-Based Therapy for Parkinson's Disease', *Cell Stem Cell*. Cell Press, 20(1), pp. 135–148. doi: 10.1016/j.stem.2016.09.004.

Kirkeby, A. and Barker, R. A. (2019) 'Parkinson disease and growth factors — is GDNF good enough?', *Nature Reviews Neurology*. Springer US, 15(6), pp. 312–314. doi: 10.1038/s41582-019-0180-6.

Kiss, L. E. and Soares-Da-Silva, P. (2014) 'Medicinal chemistry of catechol O-methyltransferase (COMT) inhibitors and their therapeutic utility', *Journal of Medicinal Chemistry*, 57(21), pp. 8692–8717. doi: 10.1021/jm500572b.

Kitani, T., Okuno, S. and Fujisawa, H. (2003) 'Growth phase-dependent changes in the subcellular localization of pre-B-cell colony-enhancing factor', *FEBS Letters*. Elsevier, 544(1–3), pp. 74–78. doi: 10.1016/S0014-5793(03)00476-9.

Kitao, Y. *et al.* (2015) 'Transgenic supplementation of SIRT1 fails to alleviate acute loss of nigrostriatal dopamine neurons and gliosis in a mouse model of MPTP-induced parkinsonism', *F1000Research*, 4(0), pp. 1–8. doi: 10.12688/f1000research.6386.1.

Klaidman, L. K., Mukherjee, S. K. and Adams, J. D. (2001) 'Oxidative changes in brain pyridine nucleotides and neuroprotection using nicotinamide', *Biochimica et Biophysica Acta - General Subjects*, 1525(1–2), pp. 136–148. doi: 10.1016/S0304-4165(00)00181-1.

Klein, M. O. *et al.* (2019) 'Dopamine: Functions, Signaling, and Association with Neurological Diseases', *Cellular and Molecular Neurobiology*, 39, pp. 31–59. doi: 10.1007/s10571-018-0632-3.

Knörle, R. (2018) 'Neuromelanin in Parkinson's Disease: from Fenton Reaction to Calcium Signaling', *Neurotoxicity Research*. Neurotoxicity Research, 33(2), pp. 515–522. doi: 10.1007/s12640-017-9804-z.

Kobayashi, T. *et al.* (2003) 'Calcium-induced mitochondrial swelling and cytochrome c release in the brain: Its biochemical characteristics and implication in ischemic neuronal injury', *Brain Research*. Elsevier, 960(1–2), pp. 62–70. doi: 10.1016/S0006-8993(02)03767-8.

Koguchi, K. *et al.* (2003) 'Microglial Cell Cycle-Associated Proteins Control Microglial

Proliferation in Vivo and in Vitro and Are Regulated by GM-CSF and Density-Dependent Inhibition', *Journal of Neuroscience Research*, 74(6), pp. 898–905. doi: 10.1002/jnr.10829.

Kordower, J. H. *et al.* (2016) 'Parkinsonian monkeys with prior levodopa-induced dyskinesias followed by fetal dopamine precursor grafts do not display graft-induced dyskinesias', *Journal of Comparative Neurology*, 00, pp. 1–15. doi: 10.1002/cne.24081.

Korzhevskii, D. E. and Kirik, O. V. (2016) 'Brain Microglia and Microglial Markers', *Neuroscience and Behavioral Physiology*, 46(3), pp. 284–290. doi: 10.1007/s11055-016-0231-z.

Kovarik, J. (2013) 'From Immunosuppression to Immunomodulation: Current Principles and Future Strategies', *Pathobiology*. Karger Publishers, 80(6), pp. 275–281. doi: 10.1159/000346960.

Krack, P. *et al.* (2003) 'Five-year follow-up of bilateral stimulation of the subthalamic nucleus in advanced Parkinson's disease.', *N Engl J Med*, 349(20), pp. 1925–1934. doi: 10.1056/NEJMoa035275.

Kramer, M. L. and Schulz-Schaeffer, W. J. (2007) 'Presynaptic α-synuclein aggregates, not Lewy bodies, cause neurodegeneration in dementia with lewy bodies', *Journal of Neuroscience*. Society for Neuroscience, 27(6), pp. 1405–1410. doi: 10.1523/JNEUROSCI.4564-06.2007.

Krasemann, S. *et al.* (2017) 'The TREM2-APOE Pathway Drives the Transcriptional Phenotype of Dysfunctional Microglia in Neurodegenerative Diseases', *Immunity*, 47, pp. 566–581. doi: 10.1016/j.immuni.2017.08.008.

Krashia, P. *et al.* (2017) 'On the properties of identified dopaminergic neurons in the mouse substantia nigra and ventral tegmental area', *European Journal of Neuroscience*. Blackwell Publishing Ltd, 45(1), pp. 92–105. doi: 10.1111/ejn.13364.

Kriks, S. *et al.* (2011) 'Dopamine neurons derived from human ES cells efficiently

engraft in animal models of Parkinson's disease', *Nature*, 480(7378), pp. 547–551. doi: 10.1038/nature10648.

Kuhn, D. M. and Geddes, T. J. (2002) 'Reduced nicotinamide nucleotides prevent nitration of tyrosine hydroxylase by peroxynitrite', *Brain Research*, 933(1), pp. 85–89. doi: 10.1016/S0006-8993(02)02307-7.

Lange, S. C. *et al.* (2017) 'Primary cultures of astrocytes: Their value in understanding astrocytes in health and disease', *Neurochemical research*, 37(11), pp. 2569–2588. doi: 10.1016/j.physbeh.2017.03.040.

Langston, J. W. *et al.* (1999) 'Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure.', *Annals of neurology*, 46(4), pp. 598–605.

Larsson, S. C. *et al.* (2017) 'No clear support for a role for vitamin D in Parkinson's disease: A Mendelian randomization study', *Movement Disorders*. John Wiley and Sons Inc., 32(8), pp. 1249–1252. doi: 10.1002/mds.27069.

Lashuel, H. A. *et al.* (2013) 'The many faces of α-synuclein: from structure and toxicity to therapeutic target', *Nature Reviews Neuroscience*, 14(1), pp. 38–48. doi: 10.1016/j.immuni.2010.12.017.Two-stage.

Lautenschläger, J. *et al.* (2018) 'An easy-to-implement protocol for preparing postnatal ventral mesencephalic cultures', *Frontiers in Cellular Neuroscience*, 12(44), pp. 1–10. doi: 10.3389/fncel.2018.00044.

Lavado-Roldán, A. and Fernández-Chacón, R. (2016) 'Two for the Price of One: A Neuroprotective Chaperone Kit within NAD Synthase Protein NMNAT2', *PLoS Biology*, 14(7), pp. 1–5. doi: 10.1371/journal.pbio.1002522.

Lawson, L. J. *et al.* (1990) 'Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain.', *Neuroscience*, 39(1), pp. 151–170.

Le, W. D. *et al.* (1999) 'Selective agenesis of mesencephalic dopaminergic neurons in Nurr1- deficient mice', *Experimental Neurology*. Academic Press Inc., 159(2), pp. 451–458. doi: 10.1006/exnr.1999.7191.

Lee, J. Y. *et al.* (2009) 'Nicotinamide reduces dopamine in postnatal hypothalamus and causes dopamine-deficient phenotype', *Neuroscience Letters*, 461(2), pp. 163–166. doi: 10.1016/j.neulet.2009.06.005.

Lee, M. K. *et al.* (1990) 'The expression and posttranslational modification of a neuron-specific β-tubulin isotype during chick embryogenesis', *Cell Motility and the Cytoskeleton*, 17(2), pp. 118–132. doi: 10.1002/cm.970170207.

Lee, S. C. *et al.* (1994) 'GM-CSF promotes proliferation of human fetal and adult microglia in primary cultures', *Glia*, 12(4), pp. 309–318. doi: 10.1002/glia.440120407.

Lehmann, S., Loh, S. H. Y. and Martins, L. M. (2017) 'Enhancing NAD+ salvage metabolism is neuroprotective in a PINK1 model of Parkinson's disease', *Biology Open*, 6(2), pp. 141–147. doi: 10.1242/bio.022186.

Li, F., Chong, Z. Z. and Maiese, K. (2006) 'Cell Life Versus Cell Longevity: The Mysteries Surrounding the NAD+ Precursor Nicotinamide', *Current medicinal chemistry*. NIH Public Access, 13(8), p. 883.

Li, Y. *et al.* (2008) 'SirT1 inhibition reduces IGF-I/IRS-2/Ras/ERK1/2 signaling and protects neurons', *Cell metabolism*, 8(1), pp. 38–48. doi: 10.1016/j.cmet.2008.05.004.SirT1.

Liang, C. L., Sinton, C. M. and German, D. C. (1996) 'Midbrain dopaminergic neurons in the mouse: Co-localization with calbindin-D(28K) and calretinin', *Neuroscience*. Elsevier Ltd, 75(2), pp. 523–533. doi: 10.1016/0306-4522(96)00228-X.

Liddelow, S. A. and Barres, B. A. (2017) 'Reactive Astrocytes: Production, Function, and Therapeutic Potential', *Immunity*. Elsevier Inc., 46(6), pp. 957–967. doi: 10.1016/j.immuni.2017.06.006.

Lima, L. A. R. *et al.* (2018) 'Vitamin D protects dopaminergic neurons against neuroinflammation and oxidative stress in hemiparkinsonian rats', *Journal of Neuroinflammation*. Journal of Neuroinflammation, 15(1), pp. 1–11. doi: 10.1186/s12974-018-1266-6.

Lin, N. H., Messing, A. and Perng, M. Der (2017) 'Characterization of a panel of monoclonal antibodies recognizing specific epitopes on GFAP', *PLoS ONE*. Public Library of Science, 12(7), pp. 1–21. doi: 10.1371/journal.pone.0180694.

Lin, Z. *et al.* (2011) 'Monoamine transporters: Vulnerable and vital doorkeepers', *Progress in Molecular Biology and Translational Science*. Elsevier B.V., 98, pp. 1–46. doi: 10.1016/B978-0-12-385506-0.00001-6.

Lindvall, O. *et al.* (1990) 'Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease.', *Science (New York, N.Y.)*, 247(4942), pp. 574–577. doi: 10.1126/science.2105529.

Liu, B. and Hong, J.-S. (2003) 'Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention.', *The Journal of Pharmacology and Experimental Therapeutics*, 304(1), pp. 1–7. doi: 10.1124/jpet.102.035048.logical.

Liu, B. and Hong, J. (2003) 'Primary Rat Mesencephalic Neuron–Glia, Neuron-Enriched, Microglia-Enriched, and Astroglia-Enriched Cultures', in Wang, J. Q. (ed.) *Drugs of Abuse: Neurological reviews and protocols*. Totowa: Humana Press, pp. 387–395.

Liu, C. C. *et al.* (2013) 'Apolipoprotein e and Alzheimer disease: Risk, mechanisms and therapy', *Nature Reviews Neurology*. Nature Publishing Group, 9(2), pp. 106–118. doi: 10.1038/nrneurol.2012.263.

Liu, D. J. *et al.* (2014) 'SIRT1 knockdown promotes neural differentiation and attenuates the heat shock response', *Journal of Cellular Physiology*, 229(9), pp. 1224–1235.

doi: 10.1002/jcp.24556.

Liva, S. M. *et al.* (1999) 'Signal transduction pathways induced by GM-CSF in microglia: Significance in the control of proliferation', *Glia*, 26(4), pp. 344–352. doi: 10.1002/(SICI)1098-1136(199906)26:4<344::AID-GLIA8>3.0.CO;2-L.

Ljungberg, M. C. *et al.* (2012) 'CREB-activity and NMNAT2 transcription are down-regulated prior to neurodegeneration, while NMNAT2 over-expression is neuroprotective, in a mouse model of human tauopathy', *Human Molecular Genetics*, 21(2), pp. 251–267. doi: 10.1093/hmg/ddr492.

Llames, S. *et al.* (2015) 'Feeder Layer Cell Actions and Applications', *Tissue Engineering - Part B: Reviews*. Mary Ann Liebert Inc., 21(4), pp. 345–353. doi: 10.1089/ten.teb.2014.0547.

Long, A. N. *et al.* (2015) 'Effect of nicotinamide mononucleotide on brain mitochondrial respiratory deficits in an Alzheimer's disease-relevant murine model', *BMC Neurology*, 15(19). doi: 10.1186/s12883-015-0272-x.

Lopes, F. M. *et al.* (2017) 'Mimicking Parkinson's Disease in a Dish: Merits and Pitfalls of the Most Commonly used Dopaminergic In Vitro Models', *NeuroMolecular Medicine*, 19(2–3), pp. 241–255. doi: 10.1007/s12017-017-8454-x.

Losada-Perez, M. (2018) 'Glia: from "just glue" to essential players in complex nervous systems: a comparative view from flies to mammals', *Journal of Neurogenetics*. Informa UK Limited, trading as Taylor & Francis Group, 32(2), pp. 78–91. doi: 10.1080/01677063.2018.1464568.

Lotharius, J., Dugan, L. L. and O'Malley, K. L. (1999) 'Distinct mechanisms underlie neurotoxin-mediated cell death in cultured dopaminergic neurons', *Journal of Neuroscience*, 19(4), pp. 1284–1293. doi: 10.1523/jneurosci.19-04-01284.1999.

Lu, L. *et al.* (2014) 'Nicotinamide mononucleotide improves energy activity and survival rate in an in vitro model of Parkinson's disease', *Experimental and Therapeutic Medicine*, 8(3),

pp. 943–950. doi: 10.3892/etm.2014.1842.

Luna, G. *et al.* (2010) 'Expression profiles of nestin and synemin in reactive astrocytes and Müller cells following retinal injury: A comparison with glial fibrillar acidic protein and vimentin', *Molecular Vision*, 16, pp. 2511–2523.

Luthman, J. *et al.* (1989) 'Effects of d-amphetamine and methylphenidate on hyperactivity produced by neonatal 6-hydroxydopamine treatment', *Psychopharmacology*, 99(4), pp. 550–557. doi: 10.1007/BF00589907.

Ly, J. D., Grubb, D. R. and Lawen, A. (2003) 'The mitochondrial membrane potential (Δψm) in apoptosis; an update', *Apoptosis*, 8, pp. 115–128. doi: 10.1023/A.

Ma, Y. *et al.* (2002) 'Dyskinesia after fetal cell transplantation for parkinsonism: A PET study', *Annals of Neurology*. Wiley Subscription Services, Inc., A Wiley Company, 52(5), pp. 628–634. doi: 10.1002/ana.10359.

Ma, Y. *et al.* (2016) 'Anti-Inflammation Effects and Potential Mechanism of Saikosaponins by Regulating Nicotinate and Nicotinamide Metabolism and Arachidonic Acid Metabolism', *Inflammation*, 39(4), pp. 1453–1461. doi: 10.1007/s10753-016-0377-4.

Magalingam, K. B., Radhakrishnan, A. and Haleagrahara, N. (2014) 'Protective effects of flavonol isoquercitrin, against 6-hydroxy dopamine (6-OHDA)-induced toxicity in PC12 cells', *BMC Research Notes*, 7(49), pp. 1–8. doi: 10.1186/1756-0500-7-49.

Mahlknecht, P., Seppi, K. and Poewe, W. (2015) 'The concept of prodromal Parkinson's disease', *Journal of Parkinson's Disease*. IOS Press, 5(4), pp. 681–697. doi: 10.3233/JPD-150685.

Mahul-Mellier, A. L. *et al.* (2020) 'The process of Lewy body formation, rather than simply α-synuclein fibrillization, is one of the major drivers of neurodegeneration', *Proceedings of the National Academy of Sciences of the United States of America*, 117(9), pp. 4971–4982. doi: 10.1073/pnas.1913904117.

Maiese, K. *et al.* (2003) 'Nicotinamide: necessary nutrient emerges as a novel cytoprotectant for the brain.', *Trends in pharmacological sciences*. Elsevier, 24(5), pp. 228–32. doi: 10.1016/S0165-6147(03)00078-6.

Makino, N. *et al.* (2004) 'A metabolic model describing the H2O2 elimination by mammalian cells including H2O2 permeation through cytoplasmic and peroxisomal membranes: Comparison with experimental data', *Biochimica et Biophysica Acta - General Subjects*, 1673(3), pp. 149–159. doi: 10.1016/j.bbagen.2004.04.011.

Malagelada, C. and Greene, L. A. (2008) 'PC12 Cells as a model for parkinson's disease research', in Nass, R. and Przedborski, S. (eds) *Parkinson's Disease: Molecular and therapeutic insights from model systems*. Academic Press, pp. 375–387. doi: 10.1016/B978-0-12-374028-1.00029-4.

Malesu, R. *et al.* (2020) 'Nicotinamide for skin cancer chemoprevention: Effects of nicotinamide on melanoma: In vitro and in vivo', *Photochemical and Photobiological Sciences*. Royal Society of Chemistry, 19(2), pp. 171–179. doi: 10.1039/c9pp00388f.

Mander, P. K., Jekabsone, A. and Brown, G. C. (2006) 'Hydrogen Peroxide from NADPH Oxidase Microglia Proliferation Is Regulated by', *The Journal of Immunology*, 176, pp. 1046–1052. doi: 10.4049/jimmunol.176.2.1046.

Mandir, A. S. *et al.* (1999) 'Poly(ADP-ribose) polymerase activation mediates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 96(10), pp. 5774–5779. doi: 10.1073/pnas.96.10.5774.

La Manno, G. *et al.* (2016) 'Molecular Diversity of Midbrain Development in Mouse, Human, and Stem Cells', *Cell*. Cell Press, 167(2), pp. 566-580.e19. doi: 10.1016/j.cell.2016.09.027.

Mansilla, N. *et al.* (2018) 'The complexity of mitochondrial complex iv: An update of

cytochrome c oxidase biogenesis in plants', *International Journal of Molecular Sciences*. MDPI AG, 19(3), p. 662. doi: 10.3390/ijms19030662.

Marques, N. F., Massari, C. M. and Tasca, C. I. (2019) 'Guanosine Protects Striatal Slices Against 6-OHDA-Induced Oxidative Damage, Mitochondrial Dysfunction, and ATP Depletion', *Neurotoxicity Research*. Neurotoxicity Research, 35(2), pp. 475–483. doi: 10.1007/s12640-018-9976-1.

Marshall, G. P. *et al.* (2008) 'Subventricular Zone Microglia Possess a Unique Capacity for Massive In Vitro Expansion', *Glia*, 56(16), pp. 1799–1808. doi: 10.1002/glia.20730.

Marshall, G. P. *et al.* (2014) 'Microglia from neurogenic and non-neurogenic regions display differential proliferative potential and neuroblast support', *Frontiers in Cellular Neuroscience*. Frontiers Media SA, 8, p. 108. doi: 10.3389/fncel.2014.00180.

Masaki, Y. *et al.* (2017) 'Protective effect of Nrf2–ARE activator isolated from green perilla leaves on dopaminergic neuronal loss in a Parkinson's disease model', *European Journal of Pharmacology*, 798, pp. 26–34. doi: 10.1016/j.ejphar.2017.02.005.

Massudi, H. *et al.* (2012) 'Age-associated changes in oxidative stress and NAD+ metabolism in human tissue', *PLoS ONE*, 7(7), pp. 1–9. doi: 10.1371/journal.pone.0042357.

Mastroeni, D. *et al.* (2009) 'Microglial responses to dopamine in a cell culture model of Parkinson's disease', *Neurobiology of Aging*. NIH Public Access, 30(11), pp. 1805–1817. doi: 10.1016/j.neurobiolaging.2008.01.001.

Matcovitch-Natan, O. *et al.* (2016) 'Microglia development follows a stepwise program to regulate brain homeostasis', *Science*, 353(6301). doi: 10.1126/science.aad8670.

Mathys, H. *et al.* (2017) 'Temporal Tracking of Microglia Activation in Neurodegeneration at Single-Cell Resolution', *Cell Reports*. Elsevier B.V., 21(2), pp. 366–380. doi: 10.1016/j.celrep.2017.09.039.

Mayo, L. *et al.* (2008) 'Dual role of CD38 in microglial activation and activation-induced

cell death.', *Journal of immunology*. NIH Public Access, 181(1), pp. 92–103. doi: 10.4049/jimmunol.181.1.92.

McCarty, M. F. (2001) 'Does a vegan diet reduce risk for Parkinson's disease?', *Medical Hypotheses*, 57(3), pp. 318–323. doi: 10.1054/mehy.2001.1321.

McGeer, P. L. *et al.* (1988) 'Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains', *Neurology*, 38(8), pp. 1285–1285. doi: 10.1212/WNL.38.8.1285.

McKeon, A. and Benarroch, E. E. (2018) 'Glial fibrillary acid protein: Functions and involvement in disease', *Neurology*, 90(20), pp. 925–930. doi: 10.1212/WNL.0000000000005534.

Medeiros, M. S. *et al.* (2016) 'Iron and Oxidative Stress in Parkinson's Disease: An Observational Study of Injury Biomarkers', *PloS one*. Public Library of Science, 11(1), p. e0146129. doi: 10.1371/journal.pone.0146129.

Mehra, S., Sahay, S. and Maji, S. K. (2019) 'α-Synuclein misfolding and aggregation: Implications in Parkinson's disease pathogenesis', *Biochimica et Biophysica Acta - Proteins and Proteomics*. Elsevier, 1867(10), pp. 890–908. doi: 10.1016/j.bbapap.2019.03.001.

Meiser, J., Weindl, D. and Hiller, K. (2013) 'Complexity of dopamine metabolism', *Cell Communication and Signaling*. BioMed Central, 11(1), p. 34. doi: 10.1186/1478-811X-11-34.

Menassa, D. A. and Gomez-Nicola, D. (2018) 'Microglial Dynamics During Human Brain Development.', *Frontiers in immunology*. Frontiers Media SA, 9, p. 1014. doi: 10.3389/fimmu.2018.01014.

Meng, Y. *et al.* (2018) 'Nicotinamide Promotes Cell Survival and Differentiation as Kinase Inhibitor in Human Pluripotent Stem Cells', *Stem Cell Reports*. Cell Press, 11(6), pp. 1347–1356. doi: 10.1016/j.stemcr.2018.10.023.

Michel, P. P. and Hefti, F. (1990) 'Toxicity of 6-hydroxydopamine and dopamine for

dopaminergic neurons in culture', *Journal of Neuroscience Research*, 26(4), pp. 428–435. doi: 10.1002/jnr.490260405.

Michishita, E. *et al.* (2005) 'Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins', *Molecular Biology of the Cell*. American Society for Cell Biology, 16(10), pp. 4623–4635. doi: 10.1091/mbc.E05-01-0033.

Miller, E. B. *et al.* (2019) 'In vivo imaging reveals transient microglia recruitment and functional recovery of photoreceptor signaling after injury', *Proceedings of the National Academy of Sciences of the United States of America*, 116(33), pp. 16603–16612. doi: 10.1073/pnas.1903336116.

Mizuguchi, M. *et al.* (1995) 'Neuronal localization of CD38 antigen in the human brain', *Brain Research*. Elsevier, 697(1–2), pp. 235–240. doi: 10.1016/0006-8993(95)00885-T.

Mogi, M. *et al.* (2000) 'Increase in level of tumor necrosis factor-α in 6-hydroxydopamine- lesioned striatum in rats is suppressed by immunosuppressant FK506', *Neuroscience Letters*. Elsevier, 289(3), pp. 165–168. doi: 10.1016/S0304-3940(00)01275-1.

Montilla, A. *et al.* (2020) 'Functional and Metabolic Characterization of Microglia Culture in a Defined Medium', *Frontiers in Cellular Neuroscience*. Frontiers Media S.A., 14, p. 22. doi: 10.3389/fncel.2020.00022.

Montine, T. J. *et al.* (2002) 'Lipid peroxidation in aging brain and Alzheimer's disease', *Free Radical Biology and Medicine*, 33(5), pp. 620–626. doi: 10.1016/S0891-5849(02)00807-9.

Montserrat- de la Paz, S. *et al.* (2017) 'Niacin and its metabolites as master regulators of macrophage activation', *Journal of Nutritional Biochemistry*. Elsevier Inc., 39, pp. 40–47. doi: 10.1016/j.jnutbio.2016.09.008.

Moon, H. E. and Paek, S. H. (2015) 'Mitochondrial Dysfunction in Parkinson's Disease.', *Experimental neurobiology*. Korean Society for Brain and Neural Science, 24(2), pp. 103–16. doi: 10.5607/en.2015.24.2.103.

Moriarty, N., Parish, C. L. and Dowd, E. (2018) 'Primary tissue for cellular brain repair in Parkinson's disease: Promise, problems and the potential of biomaterials', *European Journal of Neuroscience*, (April), pp. 1–15. doi: 10.1111/ejn.14051.

Morroni, F. *et al.* (2018) 'Comparison of Adaptive Neuroprotective Mechanisms of Sulforaphane and its Interconversion Product Erucin in *in Vitro* and *in Vivo* Models of Parkinson's Disease', *Journal of Agricultural and Food Chemistry*, 66(4), pp. 856–865. doi: 10.1021/acs.jafc.7b04641.

Mosharov, E. V. *et al.* (2009) 'Interplay between Cytosolic Dopamine, Calcium, and α-Synuclein Causes Selective Death of Substantia Nigra Neurons', *Neuron*. NIH Public Access, 62(2), pp. 218–229. doi: 10.1016/j.neuron.2009.01.033.

Mosser, C. A. *et al.* (2017) 'Microglia in CNS development: Shaping the brain for the future', *Progress in Neurobiology*, 149–150, pp. 1–20. doi: 10.1016/j.pneurobio.2017.01.002.

Mylne, A. Q. N. *et al.* (2009) 'Trends in Parkinson's disease related mortality in England and Wales, 1993-2006', *European Journal of Neurology*, 16(9), pp. 1010–1016. doi: 10.1111/j.1468-1331.2009.02715.x.

Nagatsu, T. and Sawada, M. (2006) 'Molecular mechanism of the relation of monoamine oxidase B and its inhibitors to Parkinson's disease: Possible implications of glial cells', *Journal of Neural Transmission, Supplement*, (71), pp. 53–65. doi: 10.1007/978-3-211-33328-0_7.

Nakatsuji, N., Nakajima, F. and Tokunaga, K. (2008) 'HLA-haplotype banking and iPS cells', *Nature Biotechnology*, 26(7), pp. 739–740. doi: 10.1038/nbt0708-739.

Navascués, J. *et al.* (2000) 'Entry, dispersion and differentiation of microglia in the developing central nervous system', *Anais da Academia Brasileira de Ciencias*, 72(1), pp. 91–102. doi: 10.1590/S0001-37652000000100013.

Newmark, H. L. and Newmark, J. (2007) 'Vitamin D and Parkinson's disease - A

hypothesis', *Movement Disorders*, 22(4), pp. 461–468. doi: 10.1002/mds.21317.

NIH (no date) *Optical Density Calibration*. Available at: https://imagej.nih.gov/ij/docs/examples/calibration/index.html.

Nimmerjahn, A., Kirchhoff, F. and Helmchen, F. (2005) 'Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo.', *Science*. American Association for the Advancement of Science, 308(5726), pp. 1314–1318. doi: 10.1126/science.1110647.

Norenberg, M. D. and Rama Rao, K. V. (2007) 'The Mitochondrial Permeability Transition in Neurologic Disease', *Neurochemistry International*, 50(0), pp. 983–997. doi: 10.1016/j.physbeh.2017.03.040.

Norman, A. W. (2008) 'From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health.', *The American journal of clinical nutrition*, 88(2), pp. 491S-499S.

Noyce, A. J. *et al.* (2012) 'Meta-Analysis of Early Nonmotor Features and Risk Factors for Parkinson Disease', *Annals of Neurology*, 72(6), pp. 893–901. doi: 10.1002/ana.23687.

O'Keeffe, G. W. and Sullivan, A. M. (2005) 'Donor age affects differentiation of rat ventral mesencephalic stem cells', *Neuroscience Letters*, 375(2), pp. 101–106. doi: 10.1016/j.neulet.2004.10.083.

O'Regan, G. *et al.* (2017) 'Glucocerebrosidase Mutations in Parkinson Disease', *Journal of Parkinson's Disease*. IOS Press, 7(3), pp. 411–422. doi: 10.3233/JPD-171092.

Obeso, J. A. *et al.* (2004) 'How does Parkinson's disease begin? The role of compensatory mechanisms', *Trends in Neurosciences*, 27(3), pp. 125–127. doi: 10.1016/j.tins.2003.12.006.

Odum, E. P. and Wakwe, V. C. (2012) 'Plasma concentrations of water-soluble vitamins in metabolic syndrome subjects', *Nigerian Journal of Clinical Practice*, 15(4), pp. 442–447. doi: 10.4103/1119-3077.104522.

Oertel, W. and Schulz, J. B. (2016) 'Current and experimental treatments of Parkinson disease: A guide for neuroscientists.', *Journal of neurochemistry*, 139, pp. 1–13. doi: 10.1111/jnc.13750.

Ogawa, S. K. and Watabe-Uchida, M. (2018) 'Organization of dopamine and serotonin system: Anatomical and functional mapping of monosynaptic inputs using rabies virus', *Pharmacology Biochemistry and Behavior*. Elsevier, 174, pp. 9–22. doi: 10.1016/j.pbb.2017.05.001.

Okuda, A. *et al.* (2017) 'Poly(ADP-ribose) polymerase inhibitors activate the p53 signaling pathway in neural stem/progenitor cells', *BMC Neuroscience*. BioMed Central, 18(1), pp. 1–18. doi: 10.1186/s12868-016-0333-0.

Olanow, C. W. *et al.* (2003) 'A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease', *Annals of Neurology*. Wiley Subscription Services, Inc., A Wiley Company, 54(3), pp. 403–414. doi: 10.1002/ana.10720.

Olmos-Alonso, A. *et al.* (2016) 'Pharmacological targeting of CSF1R inhibits microglial proliferation and prevents the progression of Alzheimer's-like pathology', *Brain*, 139(3), pp. 891–907. doi: 10.1093/brain/awv379.

Ono, Y. *et al.* (2007) 'Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells.', *Development (Cambridge, England)*, 134(17), pp. 3213–3225. doi: 10.1242/dev.02879.

Orihuela, R., Mcpherson, C. A. and Harry, G. J. (2016) 'Microglial M1/M2 polarization and metabolic states', *British Journal of Pharmacology 649 British Journal of Pharmacology*, 173, pp. 649–665. doi: 10.1111/bph.13139.

Orme, R. P., Bhangal, M. S. and Fricker, R. A. (2013) 'Calcitriol Imparts Neuroprotection In Vitro to Midbrain Dopaminergic Neurons by Upregulating GDNF Expression', *PLoS ONE*,

8(4), p. 62040. doi: 10.1371/journal.pone.0062040.

Orsomando, G. *et al.* (2012) 'Simultaneous Single-Sample Determination of NMNAT Isozyme Activities in Mouse Tissues', *PLoS ONE*. Public Library of Science, 7(12), p. 53271. doi: 10.1371/journal.pone.0053271.

Pansky, B. (1982) 'Early Nervous System Development: The Neural Tube and Neural Crest', in *Review of Medical Embryology*. Alameda: Embryome Sciences.

Paolicelli, R. C. *et al.* (2011) 'Synaptic pruning by microglia is necessary for normal brain development', *Science*, 333(6048), pp. 1456–1458. doi: 10.1126/science.1202529.

Paris, I. *et al.* (2008) 'The catecholaminergic RCSN-3 cell line: A model to study dopamine metabolism', *Neurotoxicity Research*, 13(3–4), pp. 221–230. doi: 10.1007/BF03033505.

Park, H. R. *et al.* (2013) 'Oxazolopyridines and thiazolopyridines as monoamine oxidase B inhibitors for the treatment of Parkinson's disease', *Bioorganic & Medicinal Chemistry*, 21(17), pp. 5480–5487. doi: 10.1016/j.bmc.2013.05.066.

Parkinson's UK (2019) *Statistics for journalists | Parkinson's UK*. Available at: https://www.parkinsons.org.uk/about-us/statistics-journalists (Accessed: 18 January 2019).

Parkinson, J. (2002) 'An Essay on the Shaking Palsy (Originally published in 1817)', *Journal of Neuropsychiatry*, 14(2), pp. 223–236. doi: 10.1176/appi.neuropsych.14.2.223.

Parmar, M., Grealish, S. and Henchcliffe, C. (2020) 'The future of stem cell therapies for Parkinson disease', *Nature Reviews Neuroscience*. Springer US, 21(2), pp. 103–115. doi: 10.1038/s41583-019-0257-7.

Parsons, R. B. *et al.* (2002) 'Expression of nicotinamide N-methyltransferase (E.C. 2.1.1.1) in the Parkinsonian brain', *Journal of Neuropathology and Experimental Neurology*, 61(2), pp. 111–124. doi: 10.1093/jnen/61.2.111.

Parsons, X. H. *et al.* (2011) 'Efficient derivation of human cardiac precursors and

cardiomyocytes from pluripotent human embryonic stem cells with small molecule induction', *Journal of Visualized Experiments*. Journal of Visualized Experiments, (57), p. e3274. doi: 10.3791/3274.

Pearce, R. K. B. *et al.* (1997) 'Alterations in the distribution of glutathione in the substantia nigra in Parkinson's disease', *Journal of Neural Transmission*. Springer-Verlag, 104(6–7), pp. 661–677. doi: 10.1007/BF01291884.

Pelicano, H. *et al.* (2003) 'Inhibition of mitochondrial respiration: A novel strategy to enhance drug-induced apoptosis in human leukemia cells by a reactive oxygen species-mediated mechanism', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 278(39), pp. 37832–37839. doi: 10.1074/jbc.M301546200.

Penberthy, W. T. (2009) 'Nicotinic acid-mediated activation of both membrane and nuclear receptors towards therapeutic glucocorticoid mimetics for treating multiple sclerosis', *PPAR Research*, 2009. doi: 10.1155/2009/853707.

Peng, C. *et al.* (2011) 'Pitx3 is a critical mediator of GDNF-induced BDNF expression in nigrostriatal dopaminergic neurons', *Journal of Neuroscience*. Society for Neuroscience, 31(36), pp. 12802–12815. doi: 10.1523/JNEUROSCI.0898-11.2011.

Pepe, G. *et al.* (2017) 'Selective proliferative response of microglia to alternative polarization signals', *Journal of Neuroinflammation*. BioMed Central, 14(1), p. 236. doi: 10.1186/s12974-017-1011-6.

Perry, V. H., Hume, D. A. and Gordon, S. (1985) 'Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain', *Neuroscience*. Pergamon, 15(2), pp. 313–326. doi: 10.1016/0306-4522(85)90215-5.

Perry, V. H. and Teeling, J. (2013) 'Microglia and macrophages of the central nervous system: The contribution of microglia priming and systemic inflammation to chronic neurodegeneration', *Seminars in Immunopathology*, 35(5), pp. 601–612. doi:

10.1007/s00281-013-0382-8.

Petin, K. *et al.* (2019) 'NAD metabolites interfere with proliferation and functional properties of THP-1 cells', *Innate Immunity*, 25(5), pp. 280–293. doi: 10.1177/1753425919844587.

Petrelli, F. *et al.* (2020) 'Dysfunction of homeostatic control of dopamine by astrocytes in the developing prefrontal cortex leads to cognitive impairments', *Molecular Psychiatry*. Springer Nature, 25(4), pp. 732–749. doi: 10.1038/s41380-018-0226-y.

Pevny, L. H. *et al.* (1998) 'A role for SOX1 in neural determination', *Development*, 125(10), pp. 1967–1978.

Phatnani, H. and Maniatis, T. (2015) 'Astrocytes in neurodegenerative disease', *Cold Spring Harbor Perspectives in Biology*. Cold Spring Harbor Laboratory Press, 7(6), pp. 1–18. doi: 10.1101/cshperspect.a020628.

Pike, N. B. (2005) 'Flushing out the role of GPR109A (HM74A) in the clinical efficacy of nicotinic acid', *Journal of Clinical Investigation*. American Society for Clinical Investigation, 115(12), pp. 3400–3403. doi: 10.1172/JCI27160.

Pilleri, M. and Antonini, A. (2015) 'Therapeutic strategies to prevent and manage dyskinesias in Parkinson's disease', *Expert Opinion on Drug Safety*, 14(2), pp. 281–294. doi: 10.1517/14740338.2015.988137.

Poewe, W. (2008) 'When a Parkinson's disease patient starts to hallucinate', *Practical Neurology*, 8(4), pp. 238–241. doi: 10.1136/jnnp.2008.152579.

Politis, M. *et al.* (2010) 'Serotonergic Neurons Mediate Dyskinesia Side Effects in Parkinson's Patients with Neural Transplants', *Science Translational Medicine*, 2(38).

Polymeropoulos, M. H. *et al.* (1997) 'Mutation in the α-synuclein gene identified in families with Parkinson's disease', *Science*. American Association for the Advancement of Science, 276(5321), pp. 2045–2047. doi: 10.1126/science.276.5321.2045.

Postuma, R. B. *et al.* (2016) 'The new definition and diagnostic criteria of Parkinson's disease', *The Lancet Neurology*. Elsevier, 15(6), pp. 546–548. doi: 10.1016/S1474-4422(16)00116-2.

Poulin, J. F. *et al.* (2018) 'Mapping projections of molecularly defined dopamine neuron subtypes using intersectional genetic approaches', *Nature Neuroscience*. Nature Publishing Group, 21(9), pp. 1260–1271. doi: 10.1038/s41593-018-0203-4.

Prager, J. *et al.* (2020) 'Stiffness-matched biomaterial implants for cell delivery: clinical, intraoperative ultrasound elastography provides a "target" stiffness for hydrogel synthesis in spinal cord injury', *Journal of Tissue Engineering*, 11. doi: 10.1177/2041731420934806.

Pringsheim, T. *et al.* (2014) 'The prevalence of Parkinson's disease: A systematic review and meta-analysis', *Movement Disorders*, 29(13), pp. 1583–1590. doi: 10.1002/mds.25945.

Pruszak, J. *et al.* (2009) 'Isolation and culture of ventral mesencephalic precursor cells and dopaminergic neurons from rodent brains', *Current Protocols in Stem Cell Biology*, (SUPPL.11), pp. 1–21. doi: 10.1002/9780470151808.sc02d05s11.

Racette, B. A. *et al.* (2018) 'Immunosuppressants and risk of Parkinson disease', *Annals of Clinical and Translational Neurology*. Wiley-Blackwell, 5(7), pp. 870–875. doi: 10.1002/acn3.580.

Rafiepour, K. *et al.* (2018) 'Phytohormone Abscisic Acid Protects Human Neuroblastoma SH-SY5Y Cells Against 6-Hydroxydopamine-Induced Neurotoxicity Through Its Antioxidant and Antiapoptotic Properties', *Rejuvenation Research*, p. rej.2018.2062. doi: 10.1089/rej.2018.2062.

Ramasarma, T. (1982) 'Generation of Hydrogen Peroxide in DMFC', *Biochimica et Biophysica Acta*, 694(1), pp. 69–93. doi: 10.1149/ma2009-02/5/317.

Ransohoff, R. M. (2016) 'How neuroinflammation contributes to neurodegeneration', *Science*. American Association for the Advancement of Science, pp. 777–783. doi:

10.1126/science.aag2590.

Redman, P. T. *et al.* (2006) 'A vital role for Kv channels in dopamine transporter-mediated 6-hydroxydopamine neurotoxicity', *Neuroscience*, 143(1), pp. 1–13. doi: 10.1161/CIRCULATIONAHA.110.956839.

Rees, K. *et al.* (2011) 'Anti-hypertensive drugs as disease-modifying agents for Parkinson's disease: evidence from observational studies and clinical trials', *Cochrane Database of Systematic Reviews*, (11), pp. 1–48. doi: 10.1002/14651858.cd008535.pub2.

Reubinoff, B. E. *et al.* (2001) 'Neural progenitors from human embryonic stem cells', *Most*, 19(December), pp. 1134–1140. doi: 10.1038/nbt1201-1134.

Revazova, E. S. *et al.* (2007) 'Patient-Specific Stem Cell Lines Derived from Human Parthenogenetic Blastocysts', *Cloning and Stem Cells*, 9(3), pp. 432–449. doi: 10.1089/clo.2007.0033.

Reyes, S. *et al.* (2012) 'GIRK2 expression in dopamine neurons of the substantia nigra and ventral tegmental area', *Journal of Comparative Neurology*, 520(12), pp. 2591–2607. doi: 10.1002/cne.23051.

Rezaie, P. *et al.* (2002) 'Motility and ramification of human fetal microglia in culture: An investigation using time-lapse video microscopy and image analysis', *Experimental Cell Research*, 274(1), pp. 68–82. doi: 10.1006/excr.2001.5431.

Rizzo, G. *et al.* (2016) 'Accuracy of clinical diagnosis of Parkinson disease', *Neurology*. Lippincott Williams and Wilkins, 86(6), pp. 566–576. doi: 10.1212/WNL.0000000000002350.

Robertson, S. D. *et al.* (2013) 'Developmental origins of central norepinephrine neuron diversity', *Nature Neuroscience*. NIH Public Access, 16(8), pp. 1016–1023. doi: 10.1038/nn.3458.Developmental.

Rodriguez-Pallares, J. *et al.* (2007) 'Mechanism of 6-hydroxydopamine neurotoxicity: The role of NADPH oxidase and microglial activation in 6-hydroxydopamine-induced

degeneration of dopaminergic neurons', *Journal of Neurochemistry*, 103(1), pp. 145–156. doi: 10.1111/j.1471-4159.2007.04699.x.

Rogers, G. *et al.* (2017) 'Parkinson's disease: Summary of updated NICE guidance', *The BMJ*, 358, pp. 1–7. doi: 10.1136/bmj.j1951.

Rosenstiel, P. *et al.* (2001) 'From theory to therapy: Implications from an in vitro model of ramified microglia', *Microscopy Research and Technique*, 54(1), pp. 18–25. doi: 10.1002/jemt.1116.

Rossi, A. *et al.* (2018) 'Projection of the prevalence of Parkinson's disease in the coming decades: Revisited.', *Movement disorders : official journal of the Movement Disorder Society*. NIH Public Access, 33(1), pp. 156–159. doi: 10.1002/mds.27063.

Roy, S. *et al.* (2014) 'Effect of maternal micronutrients (folic acid and vitamin B12) and omega 3 fatty acids on indices of brain oxidative stress in the offspring.', *Brain & development*. Elsevier, 36(3), pp. 219–27. doi: 10.1016/j.braindev.2013.03.004.

Sable, P. *et al.* (2014) 'Maternal micronutrient imbalance alters gene expression of BDNF, NGF, TrkB and CREB in the offspring brain at an adult age', *International Journal of Developmental Neuroscience*, 34. doi: 10.1016/j.ijdevneu.2014.01.003.

Salonen, T. *et al.* (1996) 'Monoamine oxidase B inhibitor selegiline protects young and aged rat peripheral sympathetic neurons against 6-hydroxydopamine-induced neurotoxicity', *Acta Neuropathologica*, 91(5), pp. 466–474. doi: 10.1007/s004010050453.

Sanchez, B. *et al.* (2009) '1,25-Dihydroxyvitamin D3 administration to 6-hydroxydopamine-lesioned rats increases glial cell line-derived neurotrophic factor and partially restores tyrosine hydroxylase expression in substantia nigra and striatum', *Journal of Neuroscience Research*, 87(3), pp. 723–732. doi: 10.1002/jnr.21878.

Sanders, L. H. and Greenamyren, J. T. (2013) 'Oxidative damage to macromolecules in human Parkinson disease and the rotenone model', *Free Radical Biology and Medicine*.

Elsevier, 62, pp. 111–120. doi: 10.1016/j.freeradbiomed.2013.01.003.

Sansone, L. *et al.* (2013) 'SIRT1 silencing confers neuroprotection through IGF-1 pathway activation', *Journal of Cellular Physiology*, 228(8), pp. 1754–1761. doi: 10.1002/jcp.24334.

Santiago, J. A., Littlefield, A. M. and Potashkin, J. A. (2016) 'Integrative transcriptomic meta-analysis of Parkinson's disease and depression identifies NAMPT as a potential blood biomarker for de novo Parkinson's disease', *Scientific Reports*, 6(September), pp. 1–10. doi: 10.1038/srep34579.

Santos, C. I. and Costa-Pereira, A. P. (2011) 'Signal transducers and activators of transcription-from cytokine signalling to cancer biology', *Biochimica et Biophysica Acta - Reviews on Cancer*. Elsevier B.V., 1816(1), pp. 38–49. doi: 10.1016/j.bbcan.2011.03.003.

Sasaki, Y., Araki, T. and Milbrandt, J. (2006) 'Stimulation of nicotinamide adenine dinucleotide biosynthetic pathways delays axonal degeneration after axotomy', *Journal of Neuroscience*. Society for Neuroscience, 26(33), pp. 8484–8491. doi: 10.1523/JNEUROSCI.2320-06.2006.

Satoh, A. *et al.* (2013) 'Sirt1 extends life span and delays aging in mice through the regulation of Nk2 Homeobox 1 in the DMH and LH', *Cell Metabolism*, 18(3), pp. 416–430. doi: 10.1016/j.cmet.2013.07.013.

Savica, R. *et al.* (2016) 'Time Trends in the Incidence of Parkinson Disease.', *JAMA neurology*. NIH Public Access, 73(8), pp. 981–989. doi: 10.1001/jamaneurol.2016.0947.

Schafer, D. P. *et al.* (2012) 'Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner.', *Neuron*. NIH Public Access, 74(4), pp. 691–705. doi: 10.1016/j.neuron.2012.03.026.

Schapira, A. H. V. *et al.* (1990) 'Mitochondrial Complex I Deficiency in Parkinson's Disease', *Journal of Neurochemistry*, 54(3), pp. 823–827. doi: 10.1111/j.1471-

4159.1990.tb02325.x.

Schapira, A. H. V. (2011) 'Monoamine Oxidase B Inhibitors for the Treatment of Parkinson's Disease', *CNS Drugs*. Springer International Publishing, 25(12), pp. 1061–1071. doi: 10.2165/11596310-000000000-00000.

Schmidtmayer, J. *et al.* (1994) 'Blood monocytes and spleen macrophages differentiate into microglia-like cells on monolayers of astrocytes: Membrane currents', *Glia*, 12(4), pp. 259–267. doi: 10.1002/glia.440120403.

Schneider, C. A., Rasband, W. S. and Eliceiri, K. W. (2012) 'NIH Image to ImageJ: 25 years of image analysis', *Nature Methods*. Nature Publishing Group, 9(7), pp. 671–675. doi: 10.1038/nmeth.2089.

Schöndorf, D. C. *et al.* (2018) 'The NAD+ Precursor Nicotinamide Riboside Rescues Mitochondrial Defects and Neuronal Loss in iPSC and Fly Models of Parkinson's Disease', *Cell Reports*, 23(10), pp. 2976–2988. doi: 10.1016/j.celrep.2018.05.009.

Schrag, A., Ben-Shlomo, Y. and Quinn, N. (2002) 'How valid is the clinical diagnosis of Parkinson's disease in the community?', *Journal of neurology, neurosurgery, and psychiatry*. BMJ Publishing Group Ltd, 73(5), pp. 529–34. doi: 10.1136/JNNP.73.5.529.

Schubert, P. *et al.* (1973) 'Selective uptake of 3H-6-hydroxydopamine by neurones of the central nervous system', *Experimental Brain Research*. Springer-Verlag, 17(5), pp. 539–548. doi: 10.1007/BF00234867.

Schultz, M. B. and Sinclair, D. A. (2016) 'Why NAD(+) Declines during Aging: It's Destroyed.', *Cell metabolism*. NIH Public Access, 23(6), pp. 965–966. doi: 10.1016/j.cmet.2016.05.022.

Schulz, J. B. *et al.* (1995) 'Coenzyme Q10 and nicotinamide and a free radical spin trap protect against MPTP neurotoxicity', *Experimental Neurology*. doi: 10.1016/0014-4886(95)90033-0.

Schwarcz, R. *et al.* (2012) 'Kynurenines in the mammalian brain: When physiology meets pathology', *Nature Reviews Neuroscience*, 13(7), pp. 465–477. doi: 10.1038/nrn3257.

Seidl, S. E. *et al.* (2014) 'The emerging role of nutrition in Parkinson's disease.', *Frontiers in aging neuroscience*, 6(March), p. 36. doi: 10.3389/fnagi.2014.00036.

Semba, R. D. (2012) 'The discovery of the vitamins', *International Journal for Vitamin and Nutrition Research*, 82(5), pp. 310–315. doi: 10.1024/0300-9831/a000124.

Seppi, K. *et al.* (2011) 'The Movement Disorder Society Evidence-Based Medicine Review Update: Treatments for the non-motor symptoms of Parkinson's disease', *Movement Disorders*, 26(S3), pp. S42–S80. doi: 10.1002/mds.23884.

Shaerzadeh, F. *et al.* (2020) 'Microglia senescence occurs in both substantia nigra and ventral tegmental area', *Glia*. John Wiley and Sons Inc., (March), pp. 1–18. doi: 10.1002/glia.23834.

Shakhova, O. and Sommer, L. (2010) 'Neural crest-derived stem cells', *StemBook*. Edited by F. Gage and F. Watt, pp. 1–20. doi: 10.3824/stembook.1.51.1.

Shan, C. *et al.* (2019) 'Protective effects of β- nicotinamide adenine dinucleotide against motor deficits and dopaminergic neuronal damage in a mouse model of Parkinson's disease', *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. Elsevier, 94(December 2018), p. 109670. doi: 10.1016/j.pnpbp.2019.109670.

Sharma, O. *et al.* (2016) 'NAD+-Glycohydrolase Promotes Intracellular Survival of Group A Streptococcus', *PLoS Pathogens*, 12(3), pp. 1–21. doi: 10.1371/journal.ppat.1005468.

Shen, H., Hoffer, B. J. and Wang, Y. (2009) 'Restoration of Nigrostriatal Pathway in Parkinson's Animals by the Bridge Transplantation Technique', *Journal of Experimental and Clinical Medicine*. Elsevier, pp. 12–16. doi: 10.1016/S1878-3317(09)60005-X.

Shen, M. and Yen, A. (2009) 'Nicotinamide cooperates with retinoic acid and 1,25-dihydroxyvitamin D 3 to regulate cell differentiation and cell cycle arrest of human

myeloblastic leukemia cells', *Oncology*, 76(2), pp. 91–100. doi: 10.1159/000188664.

Shi, Y. *et al.* (2019) 'Microglia drive APOE-dependent neurodegeneration in a tauopathy mouse model.', *The Journal of experimental medicine*. Rockefeller University Press, 216(11), pp. 2546–2561. doi: 10.1084/jem.20190980.

Shimizu, E. *et al.* (2002) 'Roles of endogenous glutathione levels on 6-hydroxydopamine-induced apoptotic neuronal cell death in human neuroblastoma SK-N-SH cells', *Neuropharmacology*. Pergamon, 43(3), pp. 434–443. doi: 10.1016/S0028-3908(02)00108-9.

Shimoda, K. *et al.* (1992) 'A high percentage yield of tyrosine hydroxylase-positive cells from rat E14 mesencephalic cell culture', *Brain Research*, 586(2), pp. 319–331. doi: 10.1016/0006-8993(92)91642-R.

Shin, E. *et al.* (2012) 'Serotonergic and dopaminergic mechanisms in graft-induced dyskinesia in a rat model of Parkinson's disease', *Neurobiology of Disease*, 47(3), pp. 393–406. doi: 10.1016/j.nbd.2012.03.038.

Sigma-Aldrich (2009) *1α,25-Dihydroxyvitamin D3*, *Product Information*.

Simmnacher, K. *et al.* (2020) 'Modeling Cell-Cell Interactions in Parkinson's Disease Using Human Stem Cell-Based Models', *Frontiers in Cellular Neuroscience*. Frontiers Media S.A., 13, p. 571. doi: 10.3389/fncel.2019.00571.

Simola, N., Morelli, M. and Carta, A. R. (2007) 'The 6-hydroxydopamine model of Parkinson's disease', *Neurotoxicity Research*, 11(3–4), pp. 151–167. doi: 10.1007/BF03033565.

Simón-Sánchez, J. *et al.* (2009) 'Genome-wide association study reveals genetic risk underlying Parkinson's disease', *Nature Genetics*. Nature Publishing Group, 41(12), pp. 1308–1312. doi: 10.1038/ng.487.

Singleton, A. B., Farrer, M. J. and Bonifati, V. (2013) 'The genetics of Parkinson's

disease: progress and therapeutic implications.', *Movement disorders : official journal of the Movement Disorder Society*. NIH Public Access, 28(1), pp. 14–23. doi: 10.1002/mds.25249.

Smith, A. C. *et al.* (2019) 'Effects of nicotinamide on spatial memory and inflammation after juvenile traumatic brain injury', *Behavioural Brain Research*, 364, pp. 123–132. doi: 10.1016/j.bbr.2019.02.024.

Smith, J. A. *et al.* (2012) 'Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases', *Brain Research Bulletin*, 87(1), pp. 10–20. doi: 10.1016/j.brainresbull.2011.10.004.

Soilu-Hänninen, M. *et al.* (2012) 'A randomised, double blind, placebo controlled trial with vitamin D3 as an add on treatment to interferon b-1b in patients with multiple sclerosis', *Journal of Neurology, Neurosurgery & Psychiatry*, 85(5), pp. 565–571. doi: 10.1136/jnnp-2011-301876.

Son, M. J. M. Y. *et al.* (2013) 'Nicotinamide overcomes pluripotency deficits and reprogramming barriers', *Stem Cells*, 31(6), pp. 1121–1135. doi: 10.1002/stem.1368.

Soto-Otero, R. *et al.* (2000) 'Autoxidation and neurotoxicity of 6-hydroxydopamine in the presence of some antioxidants: Potential implication in relation to the pathogenesis of Parkinson's disease', *Journal of Neurochemistry*, 74(4), pp. 1605–1612. doi: 10.1046/j.1471-4159.2000.0741605.x.

Soulet, D. and Rivest, S. (2008) 'Microglia', *Current Biologyiology*, 18(12), pp. 506–508.

Spaans, S. K. *et al.* (2015) 'NADPH-generating systems in bacteria and archaea.', *Frontiers in microbiology*. Frontiers Media SA, 6, p. 742. doi: 10.3389/fmicb.2015.00742.

Spector, R. (1979) 'Niacin and Niacinamide Transport in the Central Nervous System. in Vivo Studies', *Journal of Neurochemistry*, 33(4), pp. 895–904. doi: 10.1111/j.1471-4159.1979.tb09919.x.

Spector, R. and Johanson, C. E. (2007) 'Vitamin transport and homeostasis in

mammalian brain: Focus on vitamins B and E', *Journal of Neurochemistry*, 103(2), pp. 425–438. doi: 10.1111/j.1471-4159.2007.04773.x.

Stahl, K., Skare, Ø. and Torp, R. (2009) 'Organotypic cultures as a model of Parkinson's disease. A twist to an old model', *TheScientificWorldJournal*, 9(February), pp. 811–821. doi: 10.1100/tsw.2009.68.

Stefani, A. *et al.* (2017) 'Homovanillic acid in CSF of mild stage Parkinson's disease patients correlates with motor impairment', *Neurochemistry International*. Elsevier Ltd, 105, pp. 58–63. doi: 10.1016/j.neuint.2017.01.007.

Stein, L. R. *et al.* (2014) 'Expression of nampt in hippocampal and cortical excitatory neurons is critical for cognitive function', *Journal of Neuroscience*. Society for Neuroscience, 34(17), pp. 5800–5815. doi: 10.1523/JNEUROSCI.4730-13.2014.

Steinbeck, J. A. *et al.* (2015) 'Optogenetics enables functional analysis of human embryonic stem cell-derived grafts in a Parkinson's disease model', *Nature Biotechnology*, 33(2), pp. 204–209. doi: 10.1038/nbt.3124.Optogenetics.

Stern, M. B. and Siderowf, A. (2010) 'Parkinson's at risk syndrome: Can Parkinson's disease be predicted?', *Movement Disorders*. Wiley Subscription Services, Inc., A Wiley Company, 25(S1), pp. S89–S93. doi: 10.1002/mds.22719.

Stoica, B. A. *et al.* (2014) 'PARP-1 inhibition attenuates neuronal loss, microglia activation and neurological deficits after traumatic brain injury', *Journal of Neurotrauma*. Mary Ann Liebert Inc., 31(8), pp. 758–772. doi: 10.1089/neu.2013.3194.

Streit, W. J. and Xue, Q.-S. (2009) 'Life and Death of Microglia', *Journal of Neuroimmune Pharmacology*, 4, pp. 371–379. doi: 10.1007/s11481-009-9163-5.

Stubberfield, C. R. and Cohen, G. M. (1988) 'NAD+ depletion and cytotoxicity in isolated hepatocytes', *Biochemical Pharmacology*. Biochem Pharmacol, 37(20), pp. 3967–3974. doi: 10.1016/0006-2952(88)90081-0.

Subramaniam, M. and Roeper, J. (2016) 'Subtypes of Midbrain Dopamine Neurons', in Steiner, H. and Tseng, K. Y. (eds) *Handbook of Behavioral Neuroscience*. Elsevier B.V., pp. 317–334. doi: 10.1016/B978-0-12-802206-1.00016-7.

Sugano, E. *et al.* (2019) 'N-Methyl-N-Nitrosourea-Induced Photoreceptor Degeneration Is Inhibited by Nicotinamide via the Blockade of Upstream Events before the Phosphorylation of Signalling Proteins', *BioMed Research International*. Hindawi Limited, 2019, pp. 1–10. doi: 10.1155/2019/3238719.

Sullivan, S. G. and Stern, A. (1981) 'Effects of superoxide dismutase and catalase on catalysis of 6-hydroxydopamine and 6-aminodopamine autoxidation by iron and ascorbate', *Biochemical Pharmacology*, 30(16), pp. 2279–2285. doi: 10.1016/0006-2952(81)90099-X.

Sun, X. *et al.* (2018) 'Guanabenz promotes neuronal survival via enhancement of ATF4 and parkin expression in models of Parkinson disease', *Experimental Neurology*. Academic Press, 303, pp. 95–107. doi: 10.1016/J.EXPNEUROL.2018.01.015.

Surmeier, D. J. *et al.* (2011) 'The role of calcium and mitochondrial oxidant stress in the loss of substantia nigra pars compacta dopaminergic neurons in Parkinson's disease', *Neuroscience*, 198, pp. 221–231. doi: 10.1016/j.neuroscience.2011.08.045.

Suzuki, E. *et al.* (2010) 'Protective effect of nicotinamide against poly(ADP-ribose) polymerase-1-mediated astrocyte death depends on its transporter-mediated uptake', *Life Sciences*, 86, pp. 676–682. doi: 10.1016/j.lfs.2010.02.019.

Takai, N. *et al.* (1998) 'Involvement of caspase-like proteinases in apoptosis of neuronal PC12 cells and primary cultured microglia induced by 6-hydroxydopamine', *Journal of Neuroscience Research*, 54(2), pp. 214–222. doi: 10.1002/(SICI)1097-4547(19981015)54:2<214::AID-JNR9>3.0.CO;2-H.

Tam, W. Y. and Ma, C. H. E. (2014) 'Bipolar/rod-shaped microglia are proliferating microglia with distinct M1/ M2 phenotypes', *Scientific Reports*, 4(7279). doi:

10.1038/srep07279.

Tamashiro, T. T., Dalgard, C. L. and Byrnes, K. R. (2012) 'Primary Microglia Isolation from Mixed Glial Cell Cultures of Neonatal Rat Brain Tissue', *Journal of Visualized Experiments*, (66), p. e3814. doi: 10.3791/3814.

Tan, Y. L., Yuan, Y. and Tian, L. (2020) 'Microglial regional heterogeneity and its role in the brain', *Molecular Psychiatry*. Springer Nature, 25(2), pp. 351–367. doi: 10.1038/s41380-019-0609-8.

Tang, B. L. (2017) 'Sirtuins as modifiers of Parkinson's disease pathology', *Journal of Neuroscience Research*, 95(4), pp. 930–942. doi: 10.1002/jnr.23806.

Taylor, C. J. *et al.* (2012) 'Generating an iPSC bank for HLA-matched tissue transplantation based on known donor and recipient hla types', *Cell Stem Cell*. Cell Press, 11(2), pp. 147–152. doi: 10.1016/j.stem.2012.07.014.

Temova, Ž. and Roškar, R. (2016) 'Stability-Indicating HPLC-UV Method for Vitamin D3 Determination in Solutions, Nutritional Supplements and Pharmaceuticals', *Journal of Chromatographic Science*, 54(7), pp. 1180–1186. doi: 10.1093/chromsci/bmw048.

Thanvi, B., Lo, N. and Robinson, T. (2007) 'Levodopa-induced dyskinesia in Parkinson's disease: clinical features, pathogenesis, prevention and treatment.', *Postgraduate medical journal*. BMJ Group, 83(980), pp. 384–8. doi: 10.1136/pgmj.2006.054759.

Thomas, B. and Beal, M. F. (2007) 'Parkinson's disease', *Human Molecular Genetics*, 16(R2), pp. R183–R194. doi: 10.1093/hmg/ddm159.

Thompson, L. H. *et al.* (2009) 'Reconstruction of the nigrostriatal dopamine pathway in the adult mouse brain', *European Journal of Neuroscience*. John Wiley & Sons, Ltd, 30(4), pp. 625–638. doi: 10.1111/j.1460-9568.2009.06878.x.

Thöny, B., Auerbach, G. and Blau, N. (2000) 'Tetrahydrobiopterin biosynthesis, regeneration and functions', *Biochemical Journal*, 347(1), pp. 1–16. doi: 10.1042/0264-

6021:3470001.

Timmerman, R., Burm, S. M. and Bajramovic, J. J. (2018) 'An overview of in vitro methods to study microglia', *Frontiers in Cellular Neuroscience*. Frontiers Media S.A., pp. 1–12. doi: 10.3389/fncel.2018.00242.

Toledo-Aral, J. J. *et al.* (2003) 'Trophic restoration of the nigrostriatal dopaminergic pathway in long-term carotid body-grafted parkinsonian rats', *Journal of Neuroscience*. Society for Neuroscience, 23(1), pp. 141–148. doi: 10.1523/jneurosci.23-01-00141.2003.

Tolosa, E. *et al.* (1998) 'disease treatment History of levodopa and dopamine agonists in Parkinson ' s disease treatment', *Neurology*, 50, pp. S2–S10.

Tomov, N. (2020) 'Glial cells in intracerebral transplantation for Parkinson ' s disease', *Neural Regeneration Research*, 15(7), pp. 1173–1178. doi: 10.4103/1673-5374.270296.

Trounson, A. and McDonald, C. (2015) 'Stem Cell Therapies in Clinical Trials: Progress and Challenges', *Cell Stem Cell*. Elsevier Inc., 17(1), pp. 11–22. doi: 10.1016/j.stem.2015.06.007.

Uchihara, T. *et al.* (1995) 'ApoE immunoreactivity and microglial cells in Alzheimer's disease brain', *Neuroscience Letters*, 195(1), pp. 5–8. doi: 10.1016/0304-3940(95)11763-M.

Ulatowski, L. M. and Manor, D. (2015) 'Vitamin E and neurodegeneration', *Neurobiology of Disease*, 84, pp. 78–83. doi: 10.1016/j.nbd.2015.04.002.

Ulrich, J. D. *et al.* (2018) 'ApoE facilitates the microglial response to amyloid plaque pathology', *Journal of Experimental Medicine*. Rockefeller University Press, 215(4), pp. 1047–1058. doi: 10.1084/jem.20171265.

Vaca, P. *et al.* (2008) 'Nicotinamide induces differentiation of embryonic stem cells into insulin-secreting cells', *Experimental Cell Research*, 314(5), pp. 969–974. doi: 10.1016/j.yexcr.2007.11.019.

Valko, M. *et al.* (2007) 'Free radicals and antioxidants in normal physiological functions

and human disease', *International Journal of Biochemistry and Cell Biology*, 39(1), pp. 44–84. doi: 10.1016/j.biocel.2006.07.001.

Vatassery, G. T. (1992) 'Vitamin E: Neurochemistry and Implications for Neurodegeneration in Parkinson's Disease', *Annals of the New York Academy of Sciences*, 669, pp. 97–110.

Vatassery, G. T., Smith, W. E. and Quach, H. T. (1989) 'Ascorbic acid, glutathione and synthetic antioxidants prevent the oxidation of vitamin E in platelets.', *Lipids*, 24(12), pp. 1043–7.

Veenstra, T. D. *et al.* (1998) '1,25-Dihydroxyvitamin D3 receptors in the central nervous system of the rat embryo', *Brain Research*, 804(2), pp. 193–205. doi: 10.1016/S0006-8993(98)00565-4.

in 'T Veld, B. A. *et al.* (2001) 'Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease', *New England Journal of Medicine*, 345(21), pp. 1515–1521. doi: 10.1056/NEJMoa010178.

Veraitch, F. S. *et al.* (2008) 'The Impact of Manual Processing on the Expansion and Directed Differentiation of Embryonic Stem Cells', *Biotechnology and Bioengineering*, 99(5), pp. 1216–1229. doi: 10.1002/bit.21673.

Verderio, C. *et al.* (2001) 'Evidence of a role for cyclic ADP-ribose in calcium signalling and neurotransmitter release in cultured astrocytes', *Journal of Neurochemistry*, 78(3), pp. 646–657. doi: 10.1046/j.1471-4159.2001.00455.x.

Verney, C. *et al.* (2010) 'Early microglial colonization of the human forebrain and possible involvement in periventricular white-matter injury of preterm infants', *Journal of Anatomy*. John Wiley & Sons, Ltd, 217(4), pp. 436–448. doi: 10.1111/j.1469-7580.2010.01245.x.

Villa, A. *et al.* (2009) 'Generation and properties of a new human ventral

mesencephalic neural stem cell line', *Experimental Cell Research*. Elsevier Inc., 315(11), pp. 1860–1874. doi: 10.1016/j.yexcr.2009.03.011.

Villa, M. *et al.* (2013) 'One-electron reduction of 6-hydroxydopamine quinone is essential in 6-hydroxydopamine neurotoxicity', *Neurotoxicity Research*, 24(1), pp. 94–101. doi: 10.1007/s12640-013-9382-7.

Virachit, S. *et al.* (2019) 'Levels of glial cell line-derived neurotrophic factor are decreased, but fibroblast growth factor 2 and cerebral dopamine neurotrophic factor are increased in the hippocampus in Parkinson's disease', *Brain Pathology*, 29(6), pp. 813–825. doi: 10.1111/bpa.12730.

Virág, L. and Szabó, C. (2002) 'The Therapeutic Potential of Poly (ADP-Ribose)', *Pharmacological Reviews*, 54(3), pp. 375–429.

Vrablik, T. L. *et al.* (2009) 'Nicotinamidase modulation of NAD+ biosynthesis and nicotinamide levels separately affect reproductive development and cell survival in C. elegans', *Development*, 136(21), pp. 3637–3646. doi: 10.1242/dev.028431.

Vrecko, K. *et al.* (1997) 'NADH stimulates endogenous dopamine biosynthesis by enhancing the recycling of tetrahydrobiopterin in rat phaeochromocytoma cells', *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 1361(1), pp. 59–65. doi: 10.1016/S0925-4439(97)00016-1.

Wagers, A. J. and Weissman, I. L. (2004) 'Plasticity of adult stem cells', *Cell*, 116(5), pp. 639–648. doi: 10.1016/S0092-8674(04)00208-9.

Wakade, C. *et al.* (2014) 'Upregulation of GPR109A in Parkinson's disease', *PLoS ONE*, 9(10), pp. 1–10. doi: 10.1371/journal.pone.0109818.

Wakade, C. *et al.* (2018) 'Niacin modulates macrophage polarization in Parkinson's disease', *Journal of Neuroimmunology*, 320, pp. 76–79. doi: 10.1016/j.jneuroim.2018.05.002.

Wallen-Mackenzie, A. *et al.* (2003) 'Nurr1-RXR heterodimers mediate RXR ligand-

induced signaling in neuronal cells', *Genes and Development*. Cold Spring Harbor Laboratory Press, 17(24), pp. 3036–3047. doi: 10.1101/gad.276003.

Wan, D. *et al.* (2016) 'Resveratrol provides neuroprotection by inhibiting phosphodiesterases and regulating the cAMP/AMPK/SIRT1 pathway after stroke in rats', *Brain Research Bulletin*. Elsevier Inc., 121, pp. 255–262. doi: 10.1016/j.brainresbull.2016.02.011.

Wang, B. *et al.* (2018) 'The Neuroprotection of Low-Dose Morphine in Cellular and Animal Models of Parkinson's Disease Through Ameliorating Endoplasmic Reticulum (ER) Stress and Activating Autophagy', *Frontiers in Molecular Neuroscience*, 11, p. 120. doi: 10.3389/fnmol.2018.00120.

Wang, H. *et al.* (2012) 'Different effects of histone deacetylase inhibitors nicotinamide and trichostatin A (TSA) in C17.2 neural stem cells', *Journal of Neural Transmission*, 119(11), pp. 1307–1315. doi: 10.1007/s00702-012-0786-y.

Wang, J.-Y. *et al.* (2001) 'Vitamin D3 attenuates 6-hydroxydopamine-induced neurotoxicity in rats', *Brain Research*, 904, pp. 67–75.

Wang, K. *et al.* (2005) 'Electrophysiological Properties of Pluripotent Human and Mouse Embryonic Stem Cells', *Stem Cells*. Wiley, 23(10), pp. 1526–1534. doi: 10.1634/stemcells.2004-0299.

Wang, P. *et al.* (2011) 'Nicotinamide phosphoribosyltransferase protects against ischemic stroke through SIRT1-dependent adenosine monophosphate-activated kinase pathway', *Annals of Neurology*, 69(2), pp. 360–374. doi: 10.1002/ana.22236.

Wang, W.-Y. *et al.* (2015) 'Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease', *Annals of Translational Medicine*, 3(10), p. 136. doi: 10.3978/j.issn.2305-5839.2015.03.49.

Wang, X. and Michaelis, E. K. (2010) 'Selective neuronal vulnerability to oxidative stress in the brain', *Frontiers in Aging Neuroscience*. Frontiers Media SA, 2, pp. 1–13. doi:

10.3389/fnagi.2010.00012.

Wang, Y. *et al.* (2000) 'Vitamin D(3) attenuates cortical infarction induced by middle cerebral arterial ligation in rats.', *Neuropharmacology*, 39(5), pp. 873–80.

Wang, Y. *et al.* (2019) 'An overview of Sirtuins as potential therapeutic target: Structure, function and modulators', *European Journal of Medicinal Chemistry*. Elsevier Masson SAS, 161, pp. 48–77. doi: 10.1016/j.ejmech.2018.10.028.

Watson, P. M. D. *et al.* (2017) 'Bioengineered 3D Glial Cell Culture Systems and Applications for Neurodegeneration and Neuroinflammation', *SLAS DISCOVERY: Advancing the Science of Drug Discovery*. Oxford University Press, 22(5), pp. 583–601. doi: 10.1177/2472555217691450.

Webster, R. A. (2001) 'Dopamine (DA)', in Webster, R. A. (ed.) *Neurotransmitters, Drugs, and Brain Function*. Chichester: John Wiley and Sons, Inc., pp. 137–161.

Wei, C.-C. *et al.* (2017) 'Nicotinamide mononucleotide attenuates brain injury after intracerebral hemorrhage by activating Nrf2/HO-1 signaling pathway OPEN', *Scientific reports*, 7(717), pp. 1–13. doi: 10.1038/s41598-017-00851-z.

Weir, S. *et al.* (2018) 'Short- and long-term cost and utilization of health care resources in Parkinson's disease in the UK', *Movement Disorders*, 33(6), pp. 974–981. doi: 10.1002/mds.27302.

Wierzba-Bobrowicz, T. *et al.* (1998) 'The comparison of microglia maturation in different structures of the human nervous system', *Folia Neuropathologica*. Termedia Publishing House Ltd., 36(3), pp. 152–160.

Williams, A. C. and Ramsden, D. B. (2005a) 'Nicotinamide: A double edged sword', *Parkinsonism & Related Disorders*, 11(7), pp. 413–420. doi: 10.1016/j.parkreldis.2005.05.011.

Williams, A. C. and Ramsden, D. B. (2005b) 'Nicotinamide homeostasis: A xenobiotic pathway that is key to development and degenerative diseases', *Medical Hypotheses*, 65(2),

pp. 353–362. doi: 10.1016/j.mehy.2005.01.042.

Williams, E. O. *et al.* (2016) 'Sirtuin 1 Promotes Deacetylation of Oct4 and Maintenance of Naive Pluripotency.', *Cell reports*. NIH Public Access, 17(3), pp. 809–820. doi: 10.1016/j.celrep.2016.09.046.

Winner, B. M. *et al.* (2017) 'Metabolism of dopamine in nucleus accumbens astrocytes is preserved in aged mice exposed to MPTP', *Frontiers in Aging Neuroscience*. Frontiers Media S.A., 9, p. 410. doi: 10.3389/fnagi.2017.00410.

Wirth, C. *et al.* (2016) 'Structure and function of mitochondrial complex i', *Biochimica et Biophysica Acta - Bioenergetics*. Elsevier B.V., 1857(7), pp. 902–914. doi: 10.1016/j.bbabio.2016.02.013.

Wolz, M. *et al.* (2012) 'Immediate effects of deep brain stimulation of the subthalamic nucleus on nonmotor symptoms in Parkinson's disease', *Parkinsonism and Related Disorders*. Elsevier Ltd, 18(8), pp. 994–997. doi: 10.1016/j.parkreldis.2012.05.011.

Wyss-Coray, T. (2016) 'Ageing, neurodegeneration and brain rejuvenation', *Nature*. Nature Publishing Group, pp. 180–186. doi: 10.1038/nature20411.

Xia, Y. *et al.* (2019) 'Microglia as modulators of exosomal alpha-synuclein transmission', *Cell Death and Disease*. Nature Publishing Group, 10(3), p. 174. doi: 10.1038/s41419-019-1404-9.

Xicoy, H., Wieringa, B. and Martens, G. J. M. (2017) 'The SH-SY5Y cell line in Parkinson's disease research: a systematic review', *Molecular Neurodegeneration*. Molecular Neurodegeneration, 12(10), pp. 1–11. doi: 10.1186/s13024-017-0149-0.

Xu, J. (2018) 'New Insights into GFAP Negative Astrocytes in Calbindin D28k Immunoreactive Astrocytes', *Brain Sciences*, 8(143), pp. 1–13. doi: 10.3390/brainsci8080143.

Xu, Q. *et al.* (2006) 'Profile and regulation of apolipoprotein E (ApoE) expression in the CNS in mice with targeting of green fluorescent protein gene to the ApoE locus', *Journal of*

Neuroscience, 26(19), pp. 4985–4994. doi: 10.1523/JNEUROSCI.5476-05.2006.

Yang, F. *et al.* (2017) 'Dietary antioxidants and risk of Parkinson's disease in two population-based cohorts', *Movement Disorders*. John Wiley and Sons Inc., 32(11), pp. 1631–1636. doi: 10.1002/mds.27120.

Yang, H. *et al.* (2007) 'Nutrient-sensitive mitochondrial NAD+ levels dictate cell survival', *Cell*, 130, pp. 1095–1107. doi: 10.1016/j.cell.2007.07.035.

Yang, R., He, J. and Wang, Y. (2016) 'Activation of the niacin receptor HCA2 reduces demyelination and neurofilament loss, and promotes functional recovery after spinal cord injury in mice', *European Journal of Pharmacology*. Elsevier, 791, pp. 124–136. doi: 10.1016/j.ejphar.2016.08.020.

Yang, T. *et al.* (2007) 'Syntheses of Nicotinamide Riboside and Derivatives: Effective Agents for Increasing Nicotinamide Adenine Dinucleotide Concentrations in Mammalian Cells', *Journal of Medicinal Chemistry*. UTC, 50(26), pp. 6458–6461. doi: 10.1021/jm701001c.

Ying, Q. L. *et al.* (2003) 'Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture', *Nature Biotechnology*, 21(2), pp. 183–186. doi: 10.1038/nbt780.

Youdim, M. B. H. and Bakhle, Y. S. (2006) 'Monoamine oxidase: Isoforms and inhibitors in Parkinson's disease and depressive illness', *British Journal of Pharmacology*. Wiley-Blackwell, pp. S287–S296. doi: 10.1038/sj.bjp.0706464.

Zecca, L. *et al.* (2001) 'Substantia nigra neuromelanin: Structure, synthesis, and molecular behaviour', *Journal of Clinical Pathology - Molecular Pathology*. BMJ Publishing Group, 54(6), pp. 414–418. doi: 10.1136/mp.54.6.414.

Zecca, L. *et al.* (2008) 'Human neuromelanin induces neuroinflammation and neurodegeneration in the rat substantia nigra: Implications for Parkinson's disease', *Acta Neuropathologica*, 116(1), pp. 47–55. doi: 10.1007/s00401-008-0361-7.

Zeng, X. S. *et al.* (2018) 'Cellular and molecular basis of neurodegeneration in Parkinson disease', *Frontiers in Aging Neuroscience*, 10(APR), pp. 1–16. doi: 10.3389/fnagi.2018.00109.

Zhang, J. *et al.* (2000) 'GPI 6150 prevents H2O2 cytotoxicity by inhibiting poly(ADP-ribose) polymerase', *Biochemical and Biophysical Research Communications*. Academic Press Inc., 278(3), pp. 590–598. doi: 10.1006/bbrc.2000.3816.

Zhang, M. *et al.* (2018) 'Highly efficient methods to obtain homogeneous dorsal neural progenitor cells from human and mouse embryonic stem cells and induced pluripotent stem cells', *Stem Cell Research & Therapy*. BioMed Central Ltd., 9(1), p. 67. doi: 10.1186/s13287-018-0812-6.

Zhang, W. *et al.* (2005) 'Aggregated alpha-synuclein activates microglia: a process leading to disease progression in Parkinson's disease', *The FASEB Journal*, 19(6), pp. 533–542. doi: 10.1096/fj.04-2751com.

Zhang, W. *et al.* (2010) 'Neuronal protective role of PBEF in a mouse model of cerebral ischemia', *Journal of Cerebral Blood Flow and Metabolism*. SAGE Publications, 30(12), pp. 1962–1971. doi: 10.1038/jcbfm.2010.71.

Zhang, W. *et al.* (2011) 'Neuromelanin activates microglia and induces degeneration of dopaminergic neurons: implications for progression of Parkinson's disease', *Neurotoxicity Research*, 19(1), pp. 63–72. doi: 10.1007/s12640-009-9140-z.

Zhang, W. *et al.* (2014) 'Role and Mechanism of Microglial Activation in Iron-Induced Selective and Progressive Dopaminergic Neurodegeneration', *Molecular Neurobiology*, 49(3), pp. 1153–1165. doi: 10.1007/s12035-013-8586-4.

Zhang, Y. *et al.* (2011) 'Inhibition of Sirt1 promotes neural progenitors toward motoneuron differentiation from human embryonic stem cells', *Biochemical and Biophysical Research Communications*. Elsevier Inc., 404(2), pp. 610–614. doi: 10.1016/j.bbrc.2010.12.014.

Zhang, Zhuo *et al.* (2019) 'Sirt1 improves functional recovery by regulating autophagy of astrocyte and neuron after brain injury', *Brain Research Bulletin*, 150, pp. 42–49. doi: 10.1016/j.brainresbull.2019.05.005.

Zhang, Zengli *et al.* (2019) 'The Appropriate Marker for Astrocytes: Comparing the Distribution and Expression of Three Astrocytic Markers in Different Mouse Cerebral Regions', *BioMed Research International*, p. 9605265. doi: 10.1155/2019/9605265.

Zhou, Y. *et al.* (2018) 'Reduced Nicotinamide Adenine Dinucleotide Phosphate Inhibits MPTP-Induced Neuroinflammation and Neurotoxicity', *Neuroscience*. doi: 10.1016/j.neuroscience.2018.08.032.

Zhu, X.-H. *et al.* (2015) 'In vivo NAD assay reveals the intracellular NAD contents and redox state in healthy human brain and their age dependences', *Proceedings of the National Academy of Sciences*, 112(9), pp. 2876–2881. doi: 10.1073/pnas.1417921112.

Zietlow, R., Dunnett, S. B. and Fawcett, J. W. (1999) 'The effect of microglia on embryonic dopaminergic neuronal survival *in vitro* : diffusible signals from neurons and glia change microglia from neurotoxic to neuroprotective', *European Journal of Neuroscience*. John Wiley & Sons, Ltd, 11(5), pp. 1657–1667. doi: 10.1046/j.1460-9568.1999.00583.x.

Zou, X. D. *et al.* (2016) 'NAMPT protects against 6-hydroxydopamine-induced neurotoxicity in PC12 cells through modulating SIRT1 activity', *Molecular Medicine Reports*, 13(5), pp. 4058–4064. doi: 10.3892/mmr.2016.5034.

Appendix A

The Influence of Nicotinamide on Health and Disease in the Central Nervous System

Rosemary A Fricker, Emma L Green, Stuart I Jenkins and Síle M Griffin

School of Medicine and Institute for Science and Technology in Medicine, Keele University, Staffordshire, UK.

International Journal of Tryptophan Research
Volume 11: 1–11

DOI: 10.1177/1178646918776658
SAGE

ABSTRACT: Nicotinamide, the amide form of vitamin B_3 (niacin), has long been associated with neuronal development, survival, and function in the central nervous system (CNS), being implicated in both neuronal death and neuroprotection. Here, we summarise a body of research investigating the role of nicotinamide in neuronal health within the CNS, with a focus on studies that have shown a neuroprotective effect. Nicotinamide appears to play a role in protecting neurons from traumatic injury, ischaemia, and stroke, as well as being implicated in 3 key neurodegenerative conditions: Alzheimer's, Parkinson's, and Huntington's diseases. A key factor is the bioavailability of nicotinamide, with low concentrations leading to neurological deficits and dementia and high levels potentially causing neurotoxicity. Finally, nicotinamide's potential mechanisms of action are discussed, including the general maintenance of cellular energy levels and the more specific inhibition of molecules such as the nicotinamide adenine dinucleotide-dependent deacetylase, sirtuin 1 (SIRT1).

KEYWORDS: Nicotinamide, vitamin B_3, neuronal death, Alzheimer's disease, Parkinson's disease, Huntington's disease, mitochondria, neurodegeneration, neuroprotection, niacin

RECEIVED: July 31, 2017. **ACCEPTED:** October 29, 2017.

TYPE: Review

FUNDING: R.A.F. has been funded for research into the role of vitamins in neuroprotection by (www.parkinsons.org.uk), grant number G-0911. S.M.G. and E.L.G. have been funded by Queen Elizabeth Hospital Birmingham Charity Ref. 16-3-053 and ACORN studentship grants from Keele University.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

CORRESPONDING AUTHOR: Rosemary A Fricker, School of Medicine and Institute for Science and Technology in Medicine, Keele University, Keele, Staffordshire ST5 5BG, UK. Email: r.a.fricker@keele.ac.uk

Introduction

There is a growing body of evidence that diet and nutrition play a direct role in maintaining neuronal health. In particular, dietary factors can influence the onset and progression of Parkinson's disease (PD), and potentially its amelioration.[1,2] The emerging pattern from this body of research is that there are clear consequences to an imbalance in dietary factors on the production and maintenance of mature neurons.

Our research and that of others suggest that vitamins are essential both for the formation of neurons and their survival. Here, we review nicotinamide and associated active metabolites. We discuss nicotinamide's role in the maintenance of mature central nervous system (CNS) neurons; its influence on neuronal health and survival during ageing, injury, and disease; and its potential as a therapeutic for neurodegenerative disease.

Vitamins and Their Role in Health

During the last century, a new class of nutritional supplements was identified. These 'vitamins' were defined as biologically active organic compounds essential for normal health and growth, which cannot, or can only partially, be synthesised by the human body. Grouped by their biological and chemical activity, 13 classes of vitamins (Table 1) are currently recognised, having diverse biochemical functions such as regulation of cell and tissue growth, mineral metabolism, acting as coenzymes in metabolism, and directing cell differentiation.[3] Thus, vitamins are essential for the development and maintenance of the body, with their deficiencies leading to conditions affecting multiple systems, such as pellagra, scurvy, rickets, bleeding disorders, and vulnerability to infections.[4] If untreated, vitamin deficiencies can lead to significant ill health and potentially death.

Nicotinamide, Nicotinamide Adenine Dinucleotide, and Neuronal Health

Nicotinamide, the water-soluble amide form of vitamin B_3, is a key component of the metabolic pathway involved in the production of nicotinamide adenine dinucleotide (NAD+). One source of nicotinamide is the diet, via intake of eggs, meat, fish, and mushrooms. A second source of nicotinamide is the metabolism of endogenous tryptophan, an essential amino acid. Nicotinamide can also be generated from niacin via the formation of NAD+.

Nicotinamide is stored in only small quantities in the liver, with most being either excreted or catabolised to provide other key metabolic products. It is difficult to achieve adverse effects from excessive intake, even with pharmacologically high doses, but overdose can cause hepatotoxicity in rare cases.[5]

The enzyme, nicotinamide phosphoribosyltransferase (NAMPT), catalyses the synthesis of nicotinamide mononucleotide (NMN) from nicotinamide (Figure 1). Its role in the metabolic pathway for the biosynthesis of NAD (oxidised form NAD+; reduced form NADH) suggests its importance in cells that are sensitive to decreases in NAD levels, such as neurons.[6] NAD homeostasis has also been found to be altered with ageing[7–10]; thus, by influencing levels of NAD+ within neurons,

Table 1. The thirteen recognised classes of vitamins and their roles.

VITAMIN	OTHER NAMES	EXAMPLES OF PHYSIOLOGICAL FUNCTIONS
Vitamin A	Retinol, retinoic acid, retinal, carotenoid	Growth, maintenance of skin, bone development, maintenance of myelin, maintenance of vision
Vitamin B_1	Thiamine	Growth, appetite, digestion, nerve activity, energy production
Vitamin B_2	Riboflavin	Growth and development of foetus, redox systems, and respiratory enzymes; maintenance of mucosal, epithelial, and eye tissues
Vitamin B_3	Nicotinamide, niacinamide, nicotinic acid, niacin	Maintenance of NAD and NADP, coenzyme in lipid catabolism, oxidative deamination
Vitamin B_5	Pantothenic acid	Lipid metabolism, protein metabolism, part of coenzyme A in carbohydrate metabolism
Vitamin B_6	Pyridoxine, pyridoxol, adermine	Growth; protein, CHO, and lipid metabolism; coenzyme in amino acid metabolism
Vitamin B_7	Biotin, protective factor X	Growth; maintenance of skin, hair, bone marrow, and sex glands; biosynthesis of aspartate and unsaturated fatty acids
Vitamin B_9	Folic acid, folacin, folinic acid	Synthesis of nucleic acid, differentiation of embryonic nervous system
Vitamin B_{12}	Cobalamin	Coenzyme in nucleic acid, protein, and lipid synthesis; maintenance of epithelial cells and nervous system
Vitamin C	Ascorbic acid	Absorption of iron, antioxidant, growth, wound healing, formation of cartilage, dentine, bone and teeth, maintenance of capillaries
Vitamin D	Vitamin D_3, cholecalciferol, calcitriol	Normal growth, Ca and P absorption, maintains and activates alkaline phosphatase in bone, maintains serum calcium and phosphorus levels
Vitamin E	Tocopherol, Tokopharm, tocotrienols	Antioxidant, growth maintenance, aids absorption of unsaturated fatty acids, maintains muscular metabolism and integrity of vascular system and central nervous system
Vitamin K	Prothrombin factor, menaquinones	Blood-clotting mechanisms, electron transport mechanisms, growth, prothrombin synthesis in liver

nicotinamide may play a key role in neuronal maturation and neuroprotection.

The enzyme NMN adenylyltransferase (NMNAT) converts NMN to NAD+ (Figure 1). Three isozymes, NMNAT1, 2, and 3, are localised to the nucleus, cytoplasm, or mitochondria, respectively.[11] An increase in NMNAT activity has been shown to lead to axonal protection in cultured neurons undergoing Wallerian degeneration, through a rise in nuclear NAD levels, leading to activation of the NAD-dependent protein deacetylase sirtuin 1 (SIRT1),[12,13] implicating nicotinamide indirectly in neuroprotection.

In humans, nicotinamide undergoes some level of degradation, primarily through *N*-methylation to *N*-methyl nicotinamide via activity of the enzyme nicotinamide *N*-methyltransferase (NNMT). As mentioned above, the remaining metabolism of nicotinamide produces the NAD coenzymes in both the oxidised and reduced forms (NAD+ and NADH) in addition to nicotinamide adenine nucleotide phosphate, which is vital in mitochondrial respiration to produce adenosine triphosphate (ATP), as well as being implicated in more than 200 enzymatic reactions including those conferring cell protective and antioxidant roles (Figure 1).[14–16]

NAD+ can also be generated via tryptophan metabolism within the liver and kidneys[17] and from dietary nicotinic acid and niacin. Tryptophan can be metabolised into small amounts of nicotinic acid mononucleotide (NAMN) that can then be converted to NAD+. However, 60 mg of tryptophan is required to yield the equivalent amount of NAMN generated from 1 mg of niacin.[18] Therefore, tryptophan is not a necessary supplement to many Western, niacin-rich diets,[19] although tryptophan alone can be enough to prevent niacin deficiency.[17] Tryptophan metabolism is a 9-step process and the first part of this, known as the kynurenine pathway,[17] is altered in a number of neurodegenerative diseases including PD, Huntington's disease (HD), and Alzheimer's disease (AD)[20,21] as well as other neurological disorders.[22] This disruption may increase the production of neurotoxins[21–23] while also reducing NAD+ levels, leaving neurons more susceptible to damage. Thus, the finely balanced relationship between nicotinamide and NAD+ may greatly influence neuronal health.

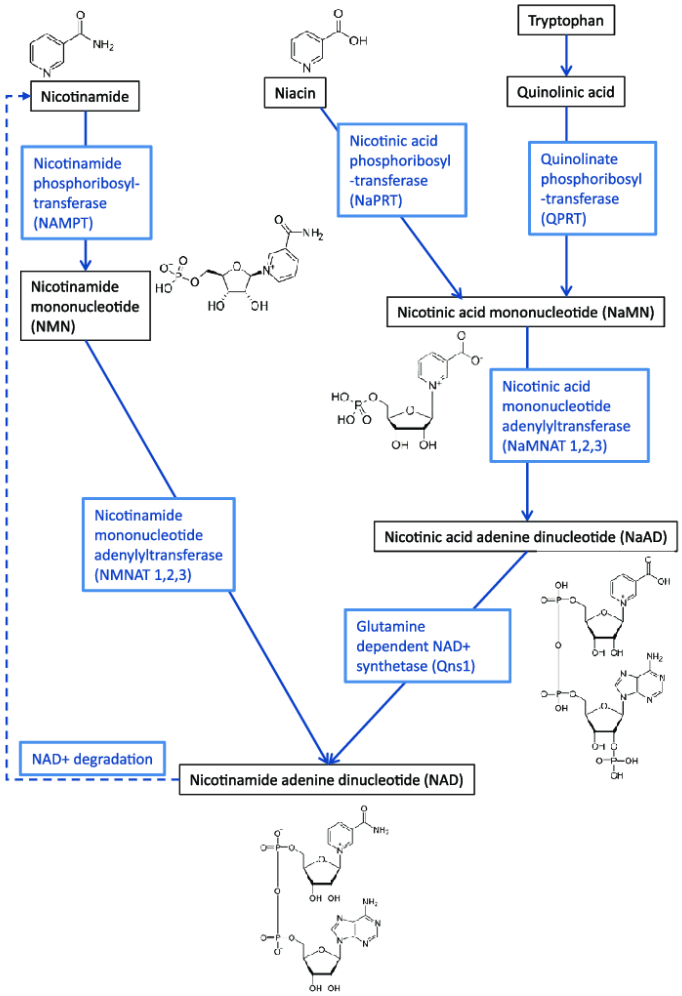


Figure 1. Simplified schematic representation of the key pathways for the metabolism of nicotinamide, niacin, and tryptophan in the production of NAD+.

Nicotinamide in the Peripheral Nervous System

Nicotinamide has been linked with Wallerian degeneration, ie, the axon degeneration that occurs distal to an injury or severance within axons of the peripheral nervous system. In peripheral nerve explants from a mouse mutant with slowed Wallerian degeneration (the *Wlds* mutation), Wlds acts to protect severed neurons, in conjunction with *Nmnat1* and *SirT1*, with *Nmnat* overexpression also able to rescue degenerating axons.[24,25] More recent studies suggest that NMNAT acts as a molecular chaperone to prevent protein misfolding and so protect key processes within neurons.[26] Another piece of evidence to suggest a role for nicotinamide in axon degeneration is the observation that NMN accumulates after nerve injury but prior to peripheral axon degeneration, and this can be ameliorated by inhibition of NAMPT, involved in the conversion of nicotinamide to NMN.[27]

Nicotinamide has been proposed to be a key player in peripheral neuropathy within the eye. Overexpression of *Nmnat1* protected retinal ganglion cells (neurons) from axonal degeneration and cell death after ischaemic insult and chronic elevation of intraocular pressure, a model for glaucoma.[28] In an aged mouse *in vivo* model, oral delivery of nicotinamide or enhanced expression of the *Nmnat1* gene prevented both retinal ganglion cell soma loss and thinning of the retinal nerve fibre layer.[29] Similarly, in a mouse model of diabetes-induced neuropathy, sensory nerve endings within the cornea were

protected through administration of nicotinamide riboside.[30] This effect could not be attributed to control of glucose alone, suggesting that other subcellular mechanisms are involved.

Another disease affecting the retinal pigment epithelium (RPE), and thus indirectly the photoreceptors transmitting sensory information to the optic nerve, is age-related macular degeneration (AMD). In a recent study, nicotinamide was shown to ameliorate the disease phenotype in RPE cells generated from pluripotent stem cell lines derived from patients with AMD, with nicotinamide and its associated pathways being proposed as targets for therapies for AMD.[31]

Nicotinamide in the CNS

A number of studies indicate that nicotinamide is essential for the growth and maintenance of the CNS, acting to promote neuronal differentiation and neuronal survival, respectively. For example, nicotinamide appears to enhance and accelerate the conversion of embryonic stem cells to neural progenitors[32,33] and neuronal differentiation from precursors[34] suggesting a key role in neural development.

There is a wealth of evidence to suggest that NAD+ metabolism has a direct influence on neuronal survival in the CNS.[35,36] NAD+ is an important substrate that acts on 3 major classes of enzymes: the sirtuin family (SIRTs), the poly(ADP-ribose) polymerases (PARPs) and related adenosine diphosphate (ADP)-ribose transferases (ARTs), and the cyclic ADP-ribose (cADPR) synthases, CD38 and CD157. A by-product of SIRT, PARP, and ART activity is nicotinamide. Nicotinamide can inhibit the activity of these enzymes through binding to NAD+. In addition, neurons contain only low levels of the enzyme NAMPT, required for the first step in the conversion of nicotinamide to NAD+, potentially lowering its availability in these cells. NAD+ levels decrease with ageing[10] and this may be linked to lowered levels of NAMPT (for an excellent review see the work by Verdin[35]). Further evidence to support this comes from studies where the class of aminopropyl carbazole chemicals P7C3 has been found to exert neuroprotective effects in models of PD,[37] stroke,[38] and amyotrophic lateral sclerosis[39] through activation of NAMPT.[40]

There is evidence that nicotinamide can freely cross the blood-brain barrier in both directions.[41] Interestingly, one study has suggested that this transport is not affected in neurodegenerative disease, indicating that systemic nicotinamide could be given as a treatment without fear of reduced access to the CNS.[42] The NNMT messenger RNA (mRNA) is expressed in multiple CNS regions including the spinal cord, temporal lobe, medulla, cerebellum, and within the basal ganglia in the subthalamic nucleus, caudate nucleus, and the dopamine neurons of the substantia nigra, of particular relevance to PD.[43] These findings highlight the capacity of nicotinamide to influence neuronal differentiation and health, fuelling interest in potential applications as a neuroprotective agent.

The Role of Nicotinamide in Neuronal Injury, Ischaemia, and Stroke

Since the turn of the century, nicotinamide has been recognised as a key player in neuroprotection and neurorestoration in animal models of ischaemia.[44,45] Nicotinamide has been shown to protect neuronal cells in a rodent model of ischaemic stroke and this effect is concentration dependent. In the early stages of developing cerebral infarction in the ischaemic brain, decreased levels of NAD+ are observed, preceding neuronal apoptosis. Studies have shown that intraperitoneal injection of 500 mg/kg nicotinamide up to 2 hours after ischaemia decreased the infarct volume of rats and improved both sensory and motor behaviour when compared with non-treated animals.[46]

Prolonged hypoxia followed by re-oxygenation (reperfusion) of neural tissue leads to impairment of NAD+/NADH recycling, termed hyperoxidation. Pre-treatment with nicotinamide can improve neuronal function, reduce NADH levels, and restore ATP levels.[47] A similar effect was observed when the PARP-1 inhibitor PJ-34 was applied. Nicotinamide can inhibit PARP activity, consequently enhancing NAD production, and this may be one mechanism of neuroprotection (discussed later in this review). Niacin metabolism may lead to long-term restoration of the blood and oxygen supply to damaged neurons. Niacin given 24 hours after induction of experimental stroke in the rat significantly increased levels of high-density lipoprotein cholesterol. This in turn promoted angiogenesis, arteriogenesis, and local cerebral blood flow, reducing functional deficits.[44]

Traumatic brain injury (TBI) is an area where nicotinamide may have a role as a therapeutic agent. Although the initial impact causing the trauma is highly damaging, the secondary sequelae create much of the lasting damage, through mechanisms such as inflammation, free radical generation, and excitotoxic cell death. Nicotinamide's wide ranging influence on different cellular processes has made it a molecule worth exploring in TBI. Vonder Haar et al showed that infusion of nicotinamide via osmotic minipumps, starting 30 minutes following a controlled cortical impact injury, significantly reduced the lesion size. This neuroprotection was correlated with improvement of sensory, motor, and cognitive skills, with animals showing improved scores on the bilateral tactile adhesive removal task, locomotor placing task, and reference memory paradigm of the Morris water maze, respectively.[48] A more recent study showed further improvements in reducing cortical neuron loss after a contusion injury when nicotinamide was co-administered with progesterone. Results showed a significant decrease in cavitation, degenerating neurons and reactive astrocytes. Transcriptional profiling suggested a reduction in genes in both inflammatory and immune pathways. Progesterone and nicotinamide–co-treated animals reached higher scores on adhesive removal and forelimb placing tasks, compared with groups that received either treatment alone.[49]

The Role of Nicotinamide in Neurodegenerative Disease

Alzheimer's disease

Alzheimer's disease is one of the most common neurodegenerative diseases, affecting perhaps 30 million people worldwide,[50] who suffer slow cognitive decline. The characteristic pathology of AD involves the presence of amyloid β (Aβ) plaques and neurofibrillary tangles.[51] The exact cause of AD is unknown, although genetic, environmental, and developmental factors are thought to be involved.[52] Cases are highest in developed countries and are expected to rise, with the biggest increase occurring in developing countries.[53] There is an urgent need to identify neuroprotective, and ideally neuro-regenerative, treatments for AD, but clinical trials have thus far failed to reliably demonstrate efficacy in a substantial number of patients. However, several lines of evidence suggest that nicotinamide or related molecules may offer therapeutic benefits for patients with AD.[54–61]

For example, severe tryptophan/niacin deficiency leads to the syndrome pellagra, where patients can develop neurological deficits manifesting as dementia, and described as 'premature ageing'.[62] Symptoms are similar to AD and include psychosis, disorientation, memory loss, and confusion, which can all be combated by niacin supplementation.[63] Although pellagra is seen primarily in young people in areas where diets are based mainly on corn (eg, Africa and India), it also occurs in adulthood in Western societies, eg, in alcoholics who are usually deficient in numerous vitamins or as a consequence of eating disorders such as anorexia nervosa.[14] Niacin deficiency in ageing populations has been linked with dementia. In a study between 1993 and 2002 in the Chicago community, dietary levels of niacin were shown to be inversely related to the onset of AD, measured through at least 2 clinical cognitive evaluations.[64] It is not clear from these data whether niacin or nicotinamide may be the more important metabolite.

Nicotinamide and niacin produce cellular and molecular effects that may be relevant to AD. Elevated total cholesterol and low-density lipoproteins are linked directly to the pathology of AD.[44] Cholesterol in neurons contributes to Aβ formation and accumulation, and it has been suggested that increased levels of membrane cholesterol can make hippocampal neurons more sensitive to insults such as tau toxicity.[65] Niacin decreases cholesterol levels both in the serum and intracellularly, which may offer protection in AD. Niacin upregulates peroxisome proliferator–activated receptor γ (PPARG) mRNA expression, promoting cholesterol efflux and so reducing cellular levels. Niacin also has been shown to upregulate liver X receptors, stimulation of which facilitates clearance of Aβ42,[44] and may improve memory in an AD mouse model.[66]

Within the neuron, NAD+ serves as a substrate for the synthesis of cADPR, used in calcium signalling, important for synaptic plasticity. This is particularly important in the hippocampus, a structure critical for learning and memory. Thus, through maintaining levels of NAD+, nicotinamide could protect against age-dependent neuronal degeneration in the hippocampus. Interestingly, however, Young and Kirkland showed that decreased niacin intake and cADPR levels actually led to enhanced ability for spatial learning in adult male rats. When the diet was supplemented with nicotinamide, spatial learning ability then decreased.[67] This suggests that nicotinamide's relationship with hippocampal neurons and learning and memory may be more complex than predicted. Interestingly, the enzyme Nmnat2, which is involved in the conversion of nicotinamide to NAD+, has been linked to neuroprotection against tauopathy in a mouse model of dementia. *Nmnat2* transcription was seen to be downregulated prior to neurodegeneration in a transgenic mouse model possessing a mutation associated with frontotemporal dementia. Injection of adeno-associated viruses overexpressing *Nmnat2* in the hippocampus of these mice from 6 weeks of age reduced the extent of neurodegeneration observed at 5 months.[68] Lower levels of *Nmnat2* mRNA and protein have also been observed in patients with AD, and its activity is linked to tau clearance.[69] Green et al[55] report that oral nicotinamide selectively reduces phosphoThr231-tau in a mouse model of AD, through a mechanism similar to SirT1 inhibition. This increased levels of microtubule stability–associated proteins and reduced cognitive deficits but did not affect Aβ pathology.

Mitochondrial dysfunction and bioenergetic deficits interrupt synaptic plasticity and impair learning and memory. These mechanisms are increasingly proposed to be key to AD.[56] Neuronal mitochondrial function can be improved through increased NAD+ and the activity of SIRT1 and SIRT3.[56] This has been achieved in a mouse model of AD, where nicotinamide treatment diminished learning and memory impairment.[59] Nicotinamide has also been shown to reduce oxidative stress in *ex vivo* and *in vivo* rat models of AD.[61,70]

The evidence outlined above has underpinned several clinical trials for AD, using nicotinamide or NADH. In 1996, NADH was reported to improve mini mental state examination scores in patients with AD, although this was an open-label trial for 8 to 12 weeks, with only 17 subjects and no controls.[71] Rainer et al[72] failed to detect improved cognitive effects of NADH in patients with dementia (including AD). In 2004, a randomised double-blind clinical trial using NADH with patients with AD reported a halt in cognitive decline and superior verbal fluency (compared with placebo; n = 12 in treatment group).[73] A 2017 nicotinamide clinical trial (Safety Study of Nicotinamide to Treat Alzheimer's Disease; NCT00580931) reported no increase in adverse events, supporting the relative safety of high (1500 mg, twice daily) nicotinamide doses.[74] No improvements in the monitored cognitive functions were detected as the number of patients was small (n = 15) and the time-course was relatively short (24 weeks).[74] A further clinical trial is investigating the effects of nicotinamide on the phosphorylation of tau (Nicotinamide as an Early Alzheimer's Disease Treatment [NEAT]; NCT03061474) and is due to be completed in February 2019.

Parkinson's disease

One particular neurodegenerative disorder that may be influenced by diet and nutrition is PD. A significant hallmark of this disorder is the death of midbrain dopamine neurons within the substantia nigra, leading to an imbalance in activity within the basal ganglia circuitry deep within the brain, manifesting in reduced movement (akinesia), rigidity, and tremor. About 95% of cases of PD cannot currently be attributed to genetic defects; therefore, science research has focussed on environmental factors that may influence the health of mature substantia nigra dopamine neurons.

Parkinson's disease is characterised by neuronal inclusions comprising α-synuclein aggregates. Although the cause of the disease is currently unknown, one hypothesis is that the dopamine neurons are compromised through oxidative stress, and more recently, it has been suggested that this oxidative stress may originate in the gastro-intestinal tract, leading to neuronal damage. People with PD have been shown to have increased intestinal permeability as well as α-synuclein aggregates and higher levels of oxidative stress in the gastro-intestinal tract. Although there is only limited evidence for a direct link between diet and PD, these data suggest that diets high in saturated fat from animal sources may have a negative impact on neuronal health, whereas unsaturated fats and foods containing antioxidants may be protective, by reducing inflammation and oxidation.[75]

Vitamin B_3 intake has been suggested to play both protective and detrimental roles in PD.[14] NAD+ levels are found to be decreased in patients with PD[76] and a reduced risk of PD is associated with higher consumption of foods containing niacin.[77,78] The patients with PD taking niacin supplementation for other disorders reported an easing of the symptoms, although doses were stopped due to adverse side effects.[79,80]

NADH is fundamental for the normal functioning of mitochondrial complex 1, which is established to be defective in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism[81] and idiopathic PD.[82,83] NADH is also integral to the production of tetrahydrobiopterin,[84] a co-factor necessary for tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis, also deficient in PD. In addition, NADH is linked to reduced glutathione, an important antioxidant shown to be insufficient at the early stages of PD.[15,85] Thus, the ability for nicotinamide to increase levels of NADH or ATP might be therapeutic for compromised midbrain dopamine neurons. In 2 mouse models of PD, nicotinamide has demonstrated neuroprotective properties by attenuating striatal dopamine depletion and protecting substantia nigra pars compacta neurons in acute MPTP-treated mice.[16]

Levels of nicotinamide- NNMT have been shown to be increased in the cerebrospinal fluid and within specific neuron populations including the midbrain dopamine neurons, of patients with PD, suggesting a role in pathogenesis.[43,86] Higher NNMT levels have been proposed to cause an increase in conversion of nicotinamide to *N*-methyl nicotinamide, structurally related to the toxin *N*-methyl-4 phenylpyridinium (MPP+), the active MPTP derivative that selectively destroys dopamine neurons.[85] In addition, increased NNMT activity leads to lowered mitochondrial complex 1 activity and impaired mitochondrial function, resulting in neurodegeneration.[15,87] Given this, a high level of nicotinamide obtained from meat-rich diets has been proposed as a nutritional factor that, in excess, may predispose dopamine neurons to mitochondrial stress, triggering neuronal apoptosis and leading to PD.[62,88] In support of this theory, our own work has shown that cultured stem cell–derived neurons respond positively to supplementation with nicotinamide within a dose range of 5 to 10 mM *in vitro*, but that a 20-mM dose is highly toxic to all neurons.[32]

Altering the course of PD through dietary means is difficult, and further experimentation is needed to determine whether this modulation is impactful; however, potential exists for supplements such as prebiotics to beneficially modify the gut milieu, reducing constipation in individuals with PD. Due to the potential role of the gastrointestinal barrier in exposure to injurious factors, therapeutic intervention through whole foods, dietary patterns, and supplemental nutrition (probiotics, prebiotics, and synbiotics) may positively impact intestinal milieu and result in reduced inflammation and oxidation and reduced risk for PD.

Huntington's disease

Similar to PD, neurodegeneration in HD has been associated with impaired mitochondrial function. Loss of GABAergic medium spiny projection neurons in the striatum is thought to occur through the mutant form of the protein huntingtin interfering with normal cellular processes such as respiration and energy metabolism, leading to neuronal dysfunction. Potential downstream effects on the neurons include altered protein trafficking and synaptic transmission, excitotoxicity through overstimulation of excitatory glutamatergic receptors, altered calcium levels, generation of free radicals, and neuronal apoptosis. For over 2 decades, it has been known that mitochondrial toxins such as malonate or 3-nitropropionic acid (3-NP) administered to rodents can induce striatal neuron degeneration.[89–91] Administration of nicotinamide can attenuate the extent of striatal lesions produced by malonate and this effect has been seen both with a continual release paradigm (via infusion pump over 7 days) or when nicotinamide was given intraperitoneally just prior to and following the lesion surgery.[92] There was an additive effect on neuroprotection when nicotinamide was delivered in combination with coenzyme Q_{10}, an essential component of the electron transport chain and a free radical scavenger, suggesting that multiple pathways may be involved in the neuroprotection observed.[92]

Metabolic toxins such as malonate provide a crude insult to medium spiny striatal neurons. More recent research has assessed the effect of nicotinamide in a transgenic model of HD (the B6.HDR6/1 mouse model that expresses a mutant form of the human HD gene where exon 1 contains an expanded stretch of approximately 125 CAG repeats), thought to more closely replicate the disease process. Nicotinamide delivered either through an osmotic minipump or in the drinking water increased brain levels of brain-derived neurotrophic factor (BDNF) and PPARG (PGC-1α) concomitantly improving movement control, measured in the open-field, rotarod, and balance beam tasks. Interestingly, behavioural improvement was not associated with any decrease in abnormal aggregation of Huntington protein and did not prevent late-stage weight loss.[93] Conversely, nicotinamide administered to an alternate transgenic model (YAC128 mice, expressing the entire human HD gene with exon 1 containing 128 CAG repeats) did not lead to an improvement in motor behaviours and in wild-type control animals actually worsened their motor performance.[94] The latter study compared nicotinamide directly against resveratrol to investigate their effects as either a SirT1 inhibitor or activator, respectively. In contrast to nicotinamide, resveratrol improved motor performance. Interestingly, both molecules had a positive effect on cultured striatal and cortical neurons derived from YAC128 mice, suggesting that opposed actions on SirT1 could lead to a similarly positive survival outcome.[94]

Mechanisms for neuroprotection by nicotinamide

Rather than just being a nutritional factor, nicotinamide has been shown to both prevent and reverse the injury of neuronal and endothelial cells. Nicotinamide can support DNA stability and can maintain membrane integrity, preventing cellular injury, phagocytosis, apoptosis, and vascular clot formation.[45]

One mechanism by which nicotinamide may act is simply to restore ATP levels within neurons. For instance, MPTP, used to create a model of PD, inhibits the mitochondrial complex 1 selectively within dopaminergic neurons, leading to ATP depletion and formation of reactive oxygen species (ROS), ultimately leading to cell death. Anderson et al[16] propose that subacute MPTP exposure leading to an energy crisis in the neurons can be ameliorated by nicotinamide, through restoration of intracellular NAD+ and ATP levels.

Nicotinamide's neuroprotective capacity has been linked to PARP, an enzyme implicated in DNA repair and cell death that, in excess, causes depletion of both NAD+ and ATP. PARP is activated in response to DNA damage, possibly through oxygen-free radicals, where it catalyses NAD+ into ADP-ribose and nicotinamide.[95] Overactivation of PARP leads to NAD+ depletion and lowers ATP levels.[62] Nicotinamide can inhibit the activity of PARP, thereby protecting cells from oxidative stress, apoptosis, and necrotic forms of cell death.

The importance of nicotinamide in replenishment of NAD+ levels also links with the transmembrane glycoprotein CD38. Through consumption of NAD+, CD38 synthesises and hydrolyses cADPR and ADP-ribose[96] and may have a role in regulating NAD levels.[97] The activity of this NADase has been found to increase with age and is responsible for age-related decline of NAD.[98] Cells overexpressing CD38 not only have lower NAD levels but also have a reduction in proteins associated with antioxidant defence, leaving them more susceptible to oxidative stress.[99] CD38 is also implicated in the degradation of NMN[98] meaning that an increase in activity could not only degrade NAD but also lower production levels, necessitating the need for extra nicotinamide. Another protein responsible for NAD depletion is sterile alpha and TIR motif containing 1 (SARM1). After axonal damage expression of SARM1 mediates axon degeneration while depleting NAD+, however, degeneration can be blocked by expression of NMNAT1 and Nampt.[100,101]

An alternative mechanism of action for nicotinamide is through inhibition of the NAD-dependent deacetylase SIRT1. SIRT1 is expressed throughout the CNS, predominantly within the nucleus of neurons, and is found within brain regions that are susceptible to neurodegeneration seen with ageing.[102] It has been associated with the conversion of neural progenitors to a neuronal fate[103] and then to specific neuronal phenotypes, including motor neurons of the spinal cord.[104] Changes in SIRT1 levels have also been implicated in neuronal ageing and neurodegeneration.[12,105] Increased SIRT1 levels have been associated with neuroprotection in AD through targeting both Aβ and tau proteins. In PD, SIRT1 may protect dopamine neurons through activating heat shock factor 1 (HSF1) levels in dopamine neurons, and in HD, through activation of CREB-regulated transcription coactivator 1 (TORC1) and subsequently BDNF levels.[105] However, experimental data have to be interpreted in the context of NAD levels within neuronal populations as sirtuins require NAD for their bioactivity and therefore SIRT1 may show opposite effects when present with either low or high levels of NAD, respectively.

Nicotinamide has also been shown to influence DNA degradation via a number of cell pathways, eg, activating protein kinase B (Akt), which phosphorylates the forkhead transcription factor (FKHRL1), inhibiting apoptosis. Activation of Akt maintains mitochondrial membrane polarisation, preventing the release of cytochrome C, thus averting cellular injury.[45] Nicotinamide can also act as an inhibitor of caspase 1, caspase 3, and caspase 8 during cellular injury, in turn preventing the release of cytochrome C and inhibiting apoptosis.[45,106]

Nicotinamide may also act to prevent neurodegeneration through altering calcium signalling. Calcium signalling plays a major role in many neuronal processes including axon elongation and response to external stimuli. However, there is evidence from studies on Wallerian degeneration and neurodegenerative disease that axonal degeneration leads to an inability to control

calcium levels, and that a rise in calcium within the axon creates neurotoxicity, causing neuronal death.[107] Aberrant calcium signalling has also been implicated in the mechanism by which neuroinflammation causes neurodegeneration, suggesting that calcium receptors may be a target for neuroprotective therapies.[108] Nicotinamide has been shown to be an inhibitor of cADPR in sea urchin eggs by interfering with the mobilising activity of the key intracellular signalling molecules: β-NAD+, cyclic GMP, and nitric oxide – modulators of cADPR synthesis.[109]

Nicotinamide and NAD also have implications in immune cell modulation[110] and with activated microglia found at sites of neurodegeneration,[111] these molecules could work through anti-inflammatory means. GPR109A, an anti-inflammatory G-protein receptor present on macrophages, has been found in higher levels in patients with PD[76]; however, treatment with niacin reduces these expression levels.[80]

More evidence for the mechanisms by which nicotinamide confers neuroprotection comes from studies of the vascular system. A body of evidence suggests that vascular ageing and associated endothelial breakdown are linked to an increase in ROS within vascular cells such as the progenitors that are required for endothelial repair.[112] Lowering levels of ROS, eg, through dietary caloric restriction may stimulate ROS-dependent protective pathways, such as those involving SIRT1, and have anti-inflammatory effects on endothelial cells.[113] Nicotinamide itself has been investigated in mouse models as a treatment option for pre-eclampsia. Increased dietary intake of nicotinamide was shown to improve the health of both mothers and pups, decreasing blood pressure and endotheliosis. The authors suggest that the mode of action of nicotinamide was through restoration of foetal ATP synthesis, mostly likely through inhibition of cADPR.[114]

Clearly, there are a number of mechanisms implicated for nicotinamide's role in neurodegeneration or neuroprotection, due to its activity in so many cellular processes conferring energy generation and cellular protection and repair, and evidenced from numerous body systems as well as the CNS. Teasing out specific downstream effects of nicotinamide's activity remains a challenge, but one worth exploring.

Summary

There is a growing body of evidence that nicotinamide is implicated in neuronal differentiation and health, neuronal injury, and neurodegeneration in the CNS. Changes in nicotinamide levels have been linked with AD, PD, and HD, and nicotinamide treatment in animal models has shown amelioration of neurodegeneration and associated behavioural recovery. Equally, there is evidence of nicotinamide being used as a restorative agent in animal models of neuronal injury and ischaemia.

The plethora of intracellular systems influenced by nicotinamide levels makes it difficult to determine precise mechanisms of action by this dietary metabolite. However, it is becoming clear that nicotinamide should be titrated to balanced levels in the CNS to avoid neural sequelae caused by either too little or too much nicotinamide within mature neurons.

With this in mind, supporting neuronal health through good dietary supplementation and management of small bioactive molecules such as nicotinamide appears an exciting and achievable prospect.

Acknowledgements

This concise review article was specifically commissioned for the special issue of the *International Journal of Tryptophan Research* on tryptophan metabolism and neurodegenerative diseases. We summarise global research into the mechanisms by which the active tryptophan metabolite nicotinamide may cause or prevent neurodegenerative disease, with specific focus on the CNS. Thus, this article aims to help readers to identify high-quality evidence to develop an informed opinion on the potential of nicotinamide to prevent or combat neuronal death.

Author Contributions

RAF wrote the first draft of the manuscript. SMG, ELG, and SIJ contributed to the writing of the manuscript and agree with manuscript results and conclusions. ELG and SIJ made critical revisions and approved final version. All authors reviewed and approved the final manuscript.

Disclosures and Ethics

As a requirement of publication, authors have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality, and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. The external blind peer reviewers report no conflicts of interest.

REFERENCES

1. Seidl SE, Santiago JA, Bilyk H, Potashkin JA. The emerging role of nutrition in Parkinson's disease. *Front Aging Neurosci*. 2014;6:36. doi:10.3389/fnagi.2014.00036.
2. Agim ZS, Cannon JR. Dietary factors in the etiology of Parkinson's disease. *Biomed Res Int*. 2015;2015:672838. doi:10.1155/2015/672838.
3. Kraemer K, Semba RD, Eggersdorfer M, Schaumberg DA. Introduction: the diverse and essential biological functions of vitamins. *Ann Nutr Metab*. 2012;61:185–191. doi:10.1159/000343103.
4. Semba RD. The discovery of the vitamins. *Int J Vitam Nutr Res*. 2012;82:310–315. doi:10.1024/0300-9831/a000124.
5. Knip M, Douek IF, Moore WPT, et al. Safety of high-dose nicotinamide: a review. *Diabetologia*. 2000;43:1337–1345. doi:10.1007/s001250051536.
6. Imai S. Nicotinamide phosphoribosyltransferase (Nampt): a link between NAD biology, metabolism, and diseases. *Curr Pharm Des*. 2009;15:20–28. doi:10.2174/138161209787185814.
7. Braidy N, Poljak A, Grant R, et al. Mapping NAD+ metabolism in the brain of ageing Wistar rats: potential targets for influencing brain senescence. *Biogerontology*. 2014;15:177–198. doi:10.1007/s10522-013-9489-5.

8. Massudi H, Grant R, Braidy N, Guest J, Farnsworth B, Guillemin GJ. Age-associated changes in oxidative stress and NAD+ metabolism in human tissue. *PLoS ONE*. 2012;7:e42357. doi:10.1371/journal.pone.0042357.
9. Zhu X-H, Lu M, Lee B-Y, Ugurbil K, Chen W. In vivo NAD assay reveals the intracellular NAD contents and redox state in healthy human brain and their age dependences. *Proc Natl Acad Sci U S A*. 2015;112:2876–2881. doi:10.1073/pnas.1417921112.
10. Gomes AP, Price NL, Ling AJY, et al. Declining NAD+ induces a pseudohypoxic state disrupting nuclear-mitochondrial communication during aging. 2013;155:1624–1638. doi:10.1016/j.cell.2013.11.037.
11. Jayaram HN, Kusumanchi P, Yalowitz JA. NMNAT expression and its relation to NAD metabolism. *Curr Med Chem*. 2011;18:1962–1972.
12. Araki T, Sasaki Y, Milbrandt J. Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science*. 2004;305:1010–1013. doi:10.1126/science.1098014.
13. Bedalov A, Simon JA. Neuroscience. NAD to the rescue. *Science*. 2004;305:954–955. doi:10.1126/science.1102497.
14. Williams A, Ramsden D. Nicotinamide: a double edged sword. *Park Relat Disord*. 2005;11:413–420. doi:10.1016/j.parkreldis.2005.05.011.
15. Williams AC, Cartwright LS, Ramsden DB. Parkinson's disease: the first common neurological disease due to auto-intoxication? *QJM*. 2005;98:215–226. doi:10.1093/qjmed/hci027.
16. Anderson DW, Bradbury KA, Schneider JS. Broad neuroprotective profile of nicotinamide in different mouse models of MPTP-induced parkinsonism. *Eur J Neurosci*. 2008;28:610–617. doi:10.1111/j.1460-9568.2008.06356.x.
17. Fukuwatari T, Shibata K. Nutritional aspect of tryptophan metabolism. *Int J Tryptophan Res*. 2013;6:3–8. doi:10.4137/IJTR.S11588.
18. Authority EFS. Scientific opinion on dietary reference values for niacin. *EFSA J*. 2014;12:3759. doi:10.2903/j.efsa.2014.3759.
19. Richard DM, Dawes MA, Mathias CW, Acheson A, Hill-Kapturczak N, Dougherty DM. L-tryptophan: basic metabolic functions, behavioral research and therapeutic indications. *Int J Tryptophan Res*. 2009;2:45–60.
20. Szabó N, Kincses ZT, Toldi J, Vécsei L. Altered tryptophan metabolism in Parkinson's disease: a possible novel therapeutic approach. *J Neurol Sci*. 2011;310:256–260. doi:10.1016/j.jns.2011.07.021.
21. Maddison DC, Giorgini F. The kynurenine pathway and neurodegenerative disease. *Semin Cell Dev Biol*. 2015;40:134–141. doi:10.1016/j.semcdb.2015.03.002.
22. Davis I, Liu A. What is the tryptophan kynurenine pathway and why is it important to neurotherapeutics? *Expert Rev Neurother*. 2015;15:719–721. doi:10.1586/14737175.2015.1049999.
23. Okuda S, Nishiyama N, Saito H, Katsuki H. 3-Hydroxykynurenine, an endogenous oxidative stress generator, causes neuronal cell death with apoptotic features and region selectivity. *J Neurochem*. 2002;70:299–307. doi:10.1046/j.1471-4159.1998.70010299.x.
24. Coleman MP, Freeman MR. Wallerian degeneration, WldS, and Nmnat. *Annu Rev Neurosci*. 2010;33:245–267. doi:10.1146/annurev-neuro-060909-153248.
25. Conforti L, Gilley J, Coleman MP. Wallerian degeneration: an emerging axon death pathway linking injury and disease. *Nat Rev Neurosci*. 2014;15:394–409. doi:10.1038/nrn3680.
26. Brazill JM, Li C, Zhu Y, Zhai RG. NMNAT: it's an NAD+ synthase . . . It's a chaperone . . . It's a neuroprotector. *Curr Opin Genet Dev*. 2017;44:156–162. doi:10.1016/j.gde.2017.03.014.
27. Stefano M, Di Orsomando G, Mori V, et al. A rise in NAD precursor nicotinamide mononucleotide (NMN) after injury promotes axon degeneration. *Cell Death Differ*. 2015;22:731–742. doi:10.1038/cdd.2014.164.
28. Zhu Y, Zhang L, Sasaki Y, Milbrandt J, Gidday JM. Protection of mouse retinal ganglion cell axons and soma from glaucomatous and ischemic injury by cytoplasmic overexpression of Nmnat1. *Invest Ophthalmol Vis Sci*. 2013;54:25–36. doi:10.1167/iovs.12-10861.
29. Williams PA, Harder JM, Foxworth NE, et al. Vitamin B3 modulates mitochondrial vulnerability and prevents glaucoma in aged mice. *Science*. 2017;355:756–760. doi:10.1120/science.aal0092.
30. Trammell SAJ, Schmidt MS, Weidemann BJ, et al. Nicotinamide riboside is uniquely and orally bioavailable in mice and humans. *Nat Commun*. 2016;7:12948. doi:10.1038/ncomms12948.
31. Saini JS, Corneo B, Miller JD, et al. Nicotinamide ameliorates disease phenotypes in a human iPSC model of age-related macular degeneration. *Cell Stem Cell*. 2017;20:635–647.e7. doi:10.1016/j.stem.2016.12.015.
32. Griffin SM, Pickard MR, Orme RP, Hawkins CP, Fricker RA. Nicotinamide promotes neuronal differentiation of mouse embryonic stem cells in vitro. *Neuroreport*. 2013;24:1041–1046. doi:10.1097/WNR.0000000000000071.
33. Griffin M, Pickard MR, Orme RP, Hawkins CP. Nicotinamide alone accelerates the conversion of mouse embryonic stem cells into mature neuronal populations. 2017;12:e0183358.
34. Hernandez-Martinez J-M, Forrest CM, Darlington LG, Smith RA, Stone TW. Quinolinic acid induces neuritogenesis in SH-SY5Y neuroblastoma cells independently of NMDA receptor activation. *Eur J Neurosci*. 2017;45:700–711. doi:10.1111/ejn.13499.
35. Verdin E. NAD+ in aging, metabolism, and neurodegeneration. *Science*. 2015;350:1208–1213. http://science.sciencemag.org/content/sci/350/6265/1208.full.pdf. Accessed October 17, 2017.
36. Katsyuba E, Auwerx J. Modulating NAD+ metabolism, from bench to bedside. *EMBO J*. 2017;36:2670–2683. doi:10.15252/embj.201797135.
37. De Jesús-Cortés H, Miller AD, Britt JK, et al. Protective efficacy of P7C3-S243 in the 6-hydroxydopamine model of Parkinson's disease. *NPJ Parkinsons Dis*. 2015;1:15010. doi:10.1038/npjparkd.2015.10.
38. Loris ZB, Pieper AA, Dalton Dietrich W. The neuroprotective compound P7C3-A20 promotes neurogenesis and improves cognitive function after ischemic stroke. *Exp Neurol*. 2017;290:63–73. doi:10.1016/j.expneurol.2017.01.006.
39. Tesla R, Parker H, Xu P, Drawbridge J, Jo S, Huntington P. Neuroprotective efficacy of aminopropyl carbazoles in a mouse model of amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A*. 2012;109:17016–17021. doi:10.1073/pnas.1213960109/-/DCSupplemental.
40. Wang G, Han T, Nijhawan D, et al. P7C3 neuroprotective chemicals function by activating the rate-limiting enzyme in NAD salvage. *Cell*. 2014;158:1324–1334. doi:10.1016/j.cell.2014.07.040.P7C3.
41. Spector R. Niacinamide transport through the blood-brain barrier. *Neurochem Res*. 1987;12:27–31.
42. Hankes LV, Coenen HH, Rota E, et al. Effect of Huntington's and Alzheimer's diseases on the transport of nicotinic acid or nicotinamide across the human blood-brain barrier. *Adv Exp Med Biol*. 1991;294:675–678.
43. Parsons RB, Smith M-L, Williams AC, Waring RH, Ramsden DB. Expression of nicotinamide N-methyltransferase (E.C. 2.1.1.1) in the Parkinsonian brain. *J Neuropathol Exp Neurol*. 2002;61:111–124. http://www.ncbi.nlm.nih.gov/pubmed/11853016.
44. Chen J, Chopp M. Niacin, an Old Drug, has New Effects on Central Nervous System Disease. *Open Drug Discov J*. 2010;2:181–186.
45. Maiese K, Chong ZZ. Nicotinamide: necessary nutrient emerges as a novel cytoprotectant for the brain. *Trends Pharmacol Sci*. 2003;24:228–232. doi:10.1016/S0165-6147(03)00078-6.
46. Mokudai T, Ayoub IA, Sakakibara Y, Lee EJ, Ogilvy CS, Maynard KI. Delayed treatment with nicotinamide (vitamin B(3)) improves neurological outcome and reduces infarct volume after transient focal cerebral ischemia in Wistar rats. *Stroke*. 2000;31:1679–1685. doi:10.1161/01.STR.31.7.1679.
47. Shetty PK, Galeffi F, Turner DA. Nicotinamide pre-treatment ameliorates NAD(H) hyperoxidation and improves neuronal function after severe hypoxia. *Neurobiol Dis*. 2014;62:469–478. doi:10.1016/j.nbd.2013.10.025.
48. Vonder Haar C, Anderson GD, Hoane MR. Continuous nicotinamide administration improves behavioral recovery and reduces lesion size following bilateral frontal controlled cortical impact injury. *Behav Brain Res*. 2011;224:311–317. doi:10.1016/j.bbr.2011.06.009.
49. Peterson TC, Hoane MR, McConomy KS, et al. A combination therapy of nicotinamide and progesterone improves functional recovery following traumatic brain injury. *J Neurotrauma*. 2015;32:765–779. doi:10.1089/neu.2014.3530.
50. Kepp KP. Ten challenges of the amyloid hypothesis of Alzheimer's disease. *J Alzheimers Dis*. 2017;55:447–457. doi:10.3233/JAD-160550.
51. Kumar A, Singh A, Ekavali E. A review on Alzheimer's disease pathophysiology and its management: an update. *Pharmacol Rep*. 2015;67:195–203. doi:10.1016/j.pharep.2014.09.004.
52. Anand R, Gill KD, Mahdi AA. Therapeutics of Alzheimer's disease: past, present and future. *Neuropharmacology*. 2014;76:27–50. doi:10.1016/j.neuropharm.2013.07.004.
53. Ferri CP, Prince M, Brayne C, et al. Global prevalence of dementia: a Delphi consensus study. *Lancet*. 2005;366:2112–2117. doi:10.1016/S0140-6736(05)67889-0.
54. Braidy N, Guillemin G, Grant R. Promotion of cellular NAD+ anabolism: therapeutic potential for oxidative stress in ageing and Alzheimer's disease. *Neurotox Res*. 2008;13:173–184. doi:10.1007/BF03033501.
55. Green KN, Steffan JS, Martinez-Coria H, et al. Nicotinamide restores cognition in Alzheimer's disease transgenic mice via a mechanism involving sirtuin inhibition and selective reduction of Thr231-phosphotau. *J Neurosci*. 2008;28:11500–11510. doi:10.1523/JNEUROSCI.3203-08.2008.
56. Kerr JS, Adriaanse BA, Greig NH, et al. Mitophagy and Alzheimer's disease: cellular and molecular mechanisms. *Trends Neurosci*. 2017. doi:10.1016/j.tins.2017.01.002.
57. Chi Y, Sauve AA. Nicotinamide riboside, a trace nutrient in foods, is a vitamin B3 with effects on energy metabolism and neuroprotection. *Curr Opin Clin Nutr Metab Care*. 2013;16:657–661. doi:10.1097/MCO.0b013e32836510c0.
58. Gong B, Pan Y, Vempati P, et al. Nicotinamide riboside restores cognition through an upregulation of proliferator-activated receptor-γ coactivator 1α regulated β-secretase 1 degradation and mitochondrial gene expression in Alzheimer's mouse models. *Neurobiol Aging*. 2013;34:1581–1588. doi:10.1016/j.neurobiolaging.2012.12.005.
59. Liu D, Pitta M, Jiang H, et al. Nicotinamide forestalls pathology and cognitive decline in Alzheimer mice: evidence for improved neuronal bioenergetics and autophagy procession. *Neurobiol Aging*. 2013;34:1564–1580. doi:10.1016/j.neurobiolaging.2012.11.020.

60. Long AN, Owens K, Schlappal AE, Kristian T, Fishman PS, Schuh RA. Effect of nicotinamide mononucleotide on brain mitochondrial respiratory deficits in an Alzheimer's disease-relevant murine model. *BMC Neurol*. 2015;15:19. doi:10.1186/s12883-015-0272-x.
61. Turunc Bayrakdar E, Armagan G, Uyanikgil Y, Kanit L, Koylu E, Yalcin A. Ex vivo protective effects of nicotinamide and 3-aminobenzamide on rat synaptosomes treated with Aβ(1–42). *Cell Biochem Funct*. 2014;32:557–564. doi:10.1002/cbf.3049.
62. Williams AC, Hill LJ, Ramsden DB. Nicotinamide, NAD(P)(H), and methyl-group homeostasis evolved and became a determinant of ageing diseases: hypotheses and lessons from pellagra. *Curr Gerontol Geriatr Res*. 2012;2012:302875. doi:10.1155/2012/302875.
63. Morris MC, Schneider JA, Tangney CC. Thoughts on B-vitamins and dementia. *J Alzheimers Dis*. 2006;9:429–433.
64. Morris MC, Evans DA, Bienias JL, et al. Dietary niacin and the risk of incident Alzheimer's disease and of cognitive decline. *J Neurol Neurosurg Psychiatry*. 2004;75:1093–1099. doi:10.1136/jnnp.2003.025858.
65. Nicholson AM, Ferreira A. Increased membrane cholesterol might render mature hippocampal neurons more susceptible to -amyloid-induced calpain activation and tau toxicity. *J Neurosci*. 2009;29:4640–4651. doi:10.1523/JNEUROSCI.0862-09.2009.
66. Vanmierlo T, Rutten K, Dederen J, et al. Liver X receptor activation restores memory in aged AD mice without reducing amyloid. *Neurobiol Aging*. 2011;32:1262–1272. doi:10.1016/j.neurobiolaging.2009.07.005.
67. Young GS, Kirkland JB. The role of dietary niacin intake and the adenosine-5'-diphosphate-ribosyl cyclase enzyme CD38 in spatial learning ability: is cyclic adenosine diphosphate ribose the link between diet and behaviour? *Nutr Res Rev*. 2008;21:42–55. doi:10.1017/S0954422408945182.
68. Ljungberg MC, Ali YO, Zhu J, et al. CREB-activity and NMNAT2 transcription are down-regulated prior to neurodegeneration, while NMNAT2 overexpression is neuroprotective, in a mouse model of human tauopathy. *Hum Mol Genet*. 2012;21:251–267. doi:10.1093/hmg/ddr492.
69. Ali YO, Allen HM, Yu L, et al. NMNAT2: HSP90 complex mediates proteostasis in proteinopathies. *PLoS Biol*. 2016;14:e1002472. doi:10.1371/journal.pbio.1002472.
70. Turunc Bayrakdar E, Uyanikgil Y, Kanit L, Koylu E, Yalcin A. Nicotinamide treatment reduces the levels of oxidative stress, apoptosis, and PARP-1 activity in Aβ(1–42)-induced rat model of Alzheimer's disease. *Free Radic Res*. 2014;48:146–158. doi:10.3109/10715762.2013.857018.
71. Birkmayer JGD. Coenzyme nicotinamide adenine dinucleotide: new therapeutic approach for improving dementia of the Alzheimer type. *Ann Clin Lab Sci*. 1996;26:1–9.
72. Rainer M, Kraxberger E, Haushofer M, Mucke HAM, Jellinger KA. No evidence for cognitive improvement from oral nicotinamide adenine dinucleotide (NADH) in dementia. Short communication. *J Neural Transm*. 2000;107:1475–1481. doi:10.1007/s007020070011.
73. Demarin V, Podobnik Sarkanji S, Storga-Tomic D, Kay G. Treatment of Alzheimer's disease with stabilized oral nicotinamide adenine dinucleotide: a randomized, double-blind study. *Drugs Exp Clin Res*. 2004;30:27–33.
74. Phelan MJ, Mulnard RA, Gillen DL, Schreiber SS. Phase II clinical trial of nicotinamide for the treatment of mild to moderate Alzheimer's disease. *J Geriatr Med Gerontol*. 2017;3:021. doi:10.23937/2469-5858/1510021.
75. Rasmussen HE, Piazza BR, Forsyth CB, Keshavarzian A. Nutrition and gastrointestinal health as modulators of Parkinson's disease. In: *Pharma-Nutrition*. Cham: Springer; 2014:213.
76. Wakade C, Chong R, Bradley E, Thomas B, Morgan J. Upregulation of GPR109A in Parkinson's disease. *PLoS ONE*. 2014;9:e109818. doi:10.1371/journal.pone.0109818.
77. Hellenbrand W, Boeing H, Robra BP, et al. Diet and Parkinson's disease. II: a possible role for the past intake of specific nutrients. Results from a self-administered food-frequency questionnaire in a case-control study. *Neurology*. 1996;47:644–650.
78. Fall PA, Fredrikson M, Axelson O, Granérus AK. Nutritional and occupational factors influencing the risk of Parkinson's disease: a case-control study in southeastern Sweden. *Mov Disord*. 1999;14:28–37. doi:10.1002/1531-8257(199901)14.
79. Alisky JM. Niacin improved rigidity and bradykinesia in a Parkinson's disease patient but also caused unacceptable nightmares and skin rash – a case report. *Nutr Neurosci*. 2005;8:327–329. doi:10.1080/10284150500484638.
80. Wakade C, Chong R, Bradley E, Morgan JC. Low-dose niacin supplementation modulates GPR109A, niacin index and ameliorates Parkinson's disease symptoms without side effects. *Clin Case Rep*. 2015;3:635–637. doi:10.1002/ccr3.232.
81. Nicklas WJ, Vyas I, Heikkila RE. Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *Life Sci*. 1985;36:2503–2508.
82. Mizuno Y, Ohta S, Tanaka M, et al. Deficiencies in complex I subunits of the respiratory chain in Parkinson's disease. *Biochem Biophys Res Commun*. 1989;163:1450–1455. doi:10.1016/0006-291X(89)91141-8.
83. Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. *J Neurochem*. 1990;54:823–827.
84. Lovenberg W, Levine R, Robinson D, Ebert M, Williams A, Calne D. Hydroxylase cofactor activity in cerebrospinal fluid of normal subjects and patients with Parkinson's disease. *Science*. 1979;204:624–626. doi:10.1126/science.432666.
85. Williams AC, Ramsden DB. Autotoxicity, methylation and a road to the prevention of Parkinson's disease. *J Clin Neurosci*. 2005;12:6–11. doi:10.1016/j.jocn.2004.10.002.
86. Aoyama K, Matsubara K, Kondo M, et al. Nicotinamide-N-methyltransferase is higher in the lumbar cerebrospinal fluid of patients with Parkinson's disease. *Neurosci Lett*. 2001;298:78–80. doi:10.1016/S0304-3940(00)01723-7.
87. Parsons RB, Smith SW, Waring RH, Williams AC, Ramsden DB. High expression of nicotinamide N-methyltransferase in patients with idiopathic Parkinson's disease. *Neurosci Lett*. 2003;342:13–16. doi:10.1016/S0304-3940(03)00218-0.
88. Williams AC, Ramsden DB. Nicotinamide homeostasis: a xenobiotic pathway that is key to development and degenerative diseases. *Med Hypotheses*. 2005;65:353–362. doi:10.1016/j.mehy.2005.01.042.
89. Beal MF, Brouillet E, Jenkins B, Henshaw R, Rosen B, Hyman BT. Age-dependent striatal excitotoxic lesions produced by the endogenous mitochondrial inhibitor malonate. *J Neurochem*. 1993;61:1147–1150. doi:10.1111/j.1471-4159.1993.tb03633.x.
90. Beal MF, Brouillet E, Jenkins BG, et al. Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. *J Neurosci*. 1993;13:4181–4192.
91. Meldrum A, Page KJ, Everitt BJ, Dunnett SB. Age-dependence of malonate-induced striatal toxicity. *Exp Brain Res*. 2000;134:335–343. doi:10.1007/s002210000465.
92. Beal MF, Henshaw DR, Jenkins BG, Rosen BR, Schulz JB. Coenzyme Q10 and nicotinamide block striatal lesions produced by the mitochondrial toxin malonate. *Ann Neurol*. 1994;36:882–888. doi:10.1002/ana.410360613.
93. Hathorn T, Snyder-Keller A, Messer A. Nicotinamide improves motor deficits and upregulates PGC-1α and BDNF gene expression in a mouse model of Huntington's disease. *Neurobiol Dis*. 2011;41:43–50. doi:10.1016/j.nbd.2010.08.017.
94. Naia L, Rosenstock TR, Oliveira AM, et al. Comparative mitochondrial-based protective effects of resveratrol and nicotinamide in Huntington's disease models. *Mol Neurobiol*. 2017;54:5385–5399.
95. Mandir AS, Przedborski S, Jackson-Lewis V, et al. Poly(ADP-ribose) polymerase activation mediates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism. *Proc Natl Acad Sci U S A*. 1999;96:5774–5779.
96. Gelman L, Deterre P, Gouy H, Boumsell L, Debré P, Bismuth G. The lymphocyte surface antigen CD38 acts as a nicotinamide adenine dinucleotide glycohydrolase in human T lymphocytes. *Eur J Immunol*. 1993;23:3361–3364. doi:10.1002/eji.1830231245.
97. Chini EN. CD38 as a regulator of cellular NAD: a novel potential pharmacological target for metabolic conditions. *Curr Pharm Des*. 2009;15:57–63.
98. Camacho-Pereira J, Tarragó MG, Chini CCS, et al. CD38 dictates age-related NAD decline and mitochondrial dysfunction through an SIRT3-dependent mechanism CD38 dictates age-related NAD decline and mitochondrial dysfunction through an SIRT3-dependent mechanism. *Cell Metab*. 2016;23:1127–1139. doi:10.1016/j.cmet.2016.05.006.
99. Hu Y, Wang H, Wang Q., Deng H. Overexpression of CD38 decreases cellular NAD levels and alters the expression of proteins involved in energy metabolism and antioxidant defense. *J Proteome Res*. 2013;13:786–795. doi:10.1021/pr4010597.
100. Gerdts J, Brace EJ, Sasaki Y, et al. SARM1 activation triggers axon degeneration locally via NAD+ destruction. 2015;348:453–457. doi:10.1126/science.1258366. SARM1.
101. Gerdts J, Summers DW, Milbrandt J, DiAntonio A. Axon self destruction: new links among SARM1, MAPKs, and NAD+ metabolism. *Neuron*. 2016;89:449–460.
102. Zakhary SM, Ayubcha D, Dileo JN, et al. Distribution analysis of deacetylase SIRT1 in rodent and human nervous systems. *Anat Rec*. 2010;293:1024–1032. doi:10.1002/ar.21116.
103. Prozorovski T, Schulze-Topphoff U, Glumm R, et al. Sirt1 contributes critically to the redox-dependent fate of neural progenitors. *Nat Cell Biol*. 2008;10:385–394. doi:10.1038/ncb1700.
104. Zhang Y, Wang J, Chen G, Fan D, Deng M. Inhibition of Sirt1 promotes neural progenitors toward motoneuron differentiation from human embryonic stem cells. *Biochem Biophys Res Commun*. 2011;404:610–614. doi:10.1016/j.bbrc.2010.12.014.
105. Donmez G. The neurobiology of sirtuins and their role in neurodegeneration. *Trends Pharmacol Sci*. 2012;33:494–501. doi:10.1016/j.tips.2012.05.007.
106. Lin S-H, Chong ZZ, Maiese K. Nicotinamide: a nutritional supplement that provides protection against neuronal and vascular injury. *J Med Food*. 2001;4:27–38. doi:10.1089/10966200152053686.
107. Wang JT, Medress ZA, Barres BA. Axon degeneration: molecular mechanisms of a self-destruction pathway. *J Cell Biol*. 196:7–18. doi:10.1083/jcb.201108111.

108. Fairless R, Williams SK, Diem R. Dysfunction of neuronal calcium signalling in neuroinflammation and neurodegeneration. *Cell Tissue Res*. 2014;357:455–462. doi:10.1007/s00441-013-1758-8.
109. Sethi JK, Empson RM, Galione A. Nicotinamide inhibits cyclic ADP-ribose-mediated calcium signalling in sea urchin eggs. *Biochem J*. 1996;319:613–617. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1217811&tool=pmcentrez&rendertype=abstract.
110. Haag F, Adriouch S, Braß A, et al. Extracellular NAD and ATP: partners in immune cell modulation. *Purinergic Signal*. 2007;3:71–81. doi:10.1007/s11302-006-9038-7.
111. Liu B, Hong J-S. Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. *J Pharmacol Exp Ther*. 2003;304:1–7. doi:10.1124/jpet.102.035048.logical.
112. Marín C, Yubero-Serrano EM, López-Miranda J, Pérez-Jiménez F. Endothelial aging associated with oxidative stress can be modulated by a healthy Mediterranean diet. *Int J Mol Sci*. 2013;14:8869–8889. doi:10.3390/ijms14058869.
113. Ungvari Z, Parrado-Fernandez C, Csiszar A, De Cabo R. Mechanisms underlying caloric restriction and life span regulation: implications for vascular aging (historical perspective). *Circ Res*. 2008;102:519–528. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2424221/pdf/nihms50295.pdf. Accessed October 27, 2017.
114. Li F, Fushima T, Oyanagi G, et al. Nicotinamide benefits both mothers and pups in two contrasting mouse models of preeclampsia. *Proc Natl Acad Sci U S A*. 2016;113:13450–13455. doi:10.1073/pnas.1614947113.