

AN *IN VITRO* MODEL OF THE IMPACT OF CHEMOTHERAPY ON NEURAL STEM CELLS AND THE PROTECTION PROVIDED BY CELLS IN THE NEUROGENIC NICHE

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This thesis is dedicated to my parents, my sons and my husband in appreciation of their many contributions to my well-being. I am blessed to have such great role models as my family.

Abstract

Chemotherapy has been highly successful in treating many forms of cancer; however there are increasing reports that this treatment causes cognitive declines in cancer survivors. These effects have been called “chemobrain” and while not affecting all patients, can persist for many years after the completion of treatment. The symptoms of chemobrain include a decline in concentration, memory and attention which are associated with a lower quality of life and in ability to return to work. Very little is known about the mechanisms behind these changes, or even the brain regions that are affected. Animal studies have found that systemic chemotherapy causes a decrease in the proliferation of neural stem cells (NSCs) in the subgranular zone (SGZ) neurogenic niche of the hippocampus and a decline in spatial memory. As hippocampal neurogenesis is required for a number of memory functions including the consolidation of long term memory, a decline in neurogenesis is likely to be one of the causes of the cognitive decline experienced by patients after chemotherapy. Previous animal work has shown that chronic treatment with the antidepressant fluoxetine prevents the decrease in neurogenesis and the associated cognitive decline. In the absence of fluoxetine chemotherapy spares dividing cells which are in contact with the surface of blood vessels in the SGZ of the hippocampus. This project used *in vitro* techniques to firstly look at whether fluoxetine has a direct effect on the sensitivity of neural cells to chemotherapy or whether treatment of astrocytes cells with fluoxetine produces an indirect effect on sensitivity. Secondly the effect of contact between NSCs and either astrocytes or endothelial cells was investigated to see if these cell types could provide protection to NSCs from chemotherapy. In the third part of the project, fluorescence activated cell sorting (FACS) and measurements of oxygen consumption after different

drug treatments were used to investigate DNA damage, apoptosis and changes in the cell cycle and the metabolic response of different cell types to chemotherapy respectively.

To do this we evaluated the relative sensitivity of primary NSCs, neural N2a cells, endothelial cells HBMEC, primary astrocytes, C6 astrocytes and 3T3 cells to the chemotherapy drug 5-fluorouracil (5-FU). NSCs were found to be more sensitive to 5-FU than other cell types. A concentration of 5 μM 5-FU was found to reduce NSC viability by 50% but largely to spare astrocytes and endothelial cells. FACS analysis showed that 5-FU was causing DNA damage and inducing apoptosis with some cells arresting in the G1 stage of the cell cycle. Measurements of oxygen consumption were not conclusive in explaining the differences in sensitivity between different cell types.

Treatment with fluoxetine, either directly to neural cell cultures or via conditioned media from fluoxetine treated astrocytes or endothelial cells, was not found to be protective against 5-FU treatment. In contrast co-culture of NSCs with astrocytes or endothelial cells, protected NSCs from chemotherapy treatment. This effect was specific to cells in the neurogenic niche as co-culture with 3T3 fibroblasts did not provide protection. Direct cell contact between neural cells and astrocytes or endothelial cells was required for protection as neither conditioned media nor co-culture separated by a porous membrane was found to be effective.

To investigate the mechanism behind astrocyte or endothelial contact dependent protection of neural cells, the gap junction protein Cx34 was stained for and found to be present on neural, astrocytic and endothelial cells. Fluorescent dye loading of marked cells showed that gap junctions were functional and dye could pass between them.

Blocking gap junctions with the gap junction inhibitor carbenoxolone (CBX) abolished the protection provided by contact with astrocytes or endothelial cells. I hypothesise that the protection provided *in vivo* by fluoxetine may be mediated by its anti-inflammatory effect on the brain and is not a direct effect on cells in the stem cell niche. Contact with astrocytes or endothelial cells may provide Ca^{2+} buffering via gap junctions and in this way protect the more sensitive neural cells from the effects of chemotherapy.

Presentations

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Contents

1 Chapter General introduction 1

1.1 Chemotherapy 2

1.1.1 Clinical studies 3

1.1.2 Neuroimaging studies 11

1.1.3 Animal studies 13

1.2 5-Fluorouracil 18

1.2.1 Mechanisms of action of 5-fluorouracil 19

1.2.2 *In vitro* chemotherapy studies on stem cells 22

1.3 Mechanisms of chemotherapy-induced cognitive impairments 22

1.3.1 Oxidative stress 23

1.3.2 Effects on myelin and white matter 25

1.3.3 Inflammation 26

1.4 Adult hippocampal neurogenesis 27

1.5 Stem cells 27

1.5.1 Neural stem cells 29

1.6 Adult neurogenesis 30

1.6.1 Hippocampus 37

1.7 Vascular niches 39

1.7.1 Role of growth factors 42

1.7.2 Interactions with other cells – astrocytes, endothelial cells and neurons which influence the rate of neurogenesis 44

1.8 The involvement of neurogenesis in learning and memory 49

1.8.1 Impact of chemotherapy on hippocampal neurogenesis 50

1.8.2 Is it possible to protect hippocampal neurogenesis from chemotherapy? 52

1.9 Antidepressants 53

1.9.1 Fluoxetine 54

1.10 Development of in vitro co-culture model 54

1.11 Aims and objectives 56

2 Chapter The sensitivity of different cell types within the hippocampal stem cell niche to chemotherapy 58

2.1 Introduction 59

2.1.1 Cell types used 60

2.1.2 Characterisation of suitable cell culture medium for all cells 63

2.2 Materials and methods 64

2.2.1 Cell culture 64

2.2.2 Counting cells 67

2.2.3 Cell types 67

2.2.4 Optimisation of the primary astrocytes culture method 70

2.2.5 Use of different extracellular molecules (ECMs) 75

2.2.6 Immunocytochemistry 76

2.2.7 Growth dynamics (growth rate) 77

2.2.8 MTT assay 77

2.2.9 Statistical analysis 79

2.3 Results 79

2.3.1 Optimisation of primary astrocyte cultures 79

2.3.2 Growth rate 82

2.3.3 Determination of a standard medium for all cell types which would be suitable for co-culture 82

2.3.4 The sensitivity of different cell types within the hippocampal stem cell niche to 5-FU chemotherapy 86

2.4 Discussion 89

2.4.1 Primary astrocyte culture 89

2.4.2 Growth rate 90

2.4.3 Determination of suitable medium 91

2.4.4 Determining the relative sensitivity of N2a cells and other components of the neurogenic niche to 5-FU 92

3 Chapter Investigation of the mechanisms underlying protection of neural cells in co-culture assays **97**

3.1 Introduction 98

3.1.1 The role of stem cell niche cells in controlling neurogenesis 98

3.1.2 Protective role of niche cells to NSCs 101

3.1.3 Direct and indirect co-culture models 103

3.1.4 Gap junctions 104

3.1.5 Aims 108

3.2 Material and methods 109

3.2.1 Co-culture of N2a cells on different cell monolayers 109

3.2.2 Production of CM 112

3.2.3 Effect of FLX in protecting N2a cells from the effects of 5-FU 114

3.2.4 Transwell assay 115

3.2.5 Immunocytochemical detection of the gap junction protein Cx43 ... 117

3.2.6 Treatment with CBX 118

3.2.7 Dye Coupling 119

3.2.8 Statistical analysis 120

3.3 Results 121

3.3.1 Co-culture of different cellular components of the stem cell niche with neural cells to determine if any cell types can protect neural stem cells from chemotherapy 121

3.3.2 The effects of CM, from different cell types within the neurogenic niche, on N2a cell number after 5-FU treatment 126

3.3.3 The effect of the antidepressant FLX on N2a proliferation 128

3.3.4 Co-culture of N2a cells with either astrocytes or endothelial cell in a transwell assay to test for the impact of secreted factors in protecting neural cells from 5-FU 131

3.3.5 Identification of GJs between N2a cells and other cell types 135

3.4 Discussion 145

3.4.1 To determine whether co-culture of N2a cells with other cellular components of the neurogenic niche protects the N2a cells from the effects of 5-FU 145

3.4.2 To determine if direct contact with other niche cell types protects N2a cells 149

3.4.3 To determine if CM from endothelial or astrocytes can protect N2a cells from 5-FU 149

3.4.4 CM from FLX-treated C6 150

3.4.5 Conclusion 155

4 Chapter Investigation of DNA damage, apoptosis and changes in the cell cycle by chemotherapy using flow cytometry **156**

4.1 Introduction 157

4.1.1 Cell cycle 157

4.1.2 Phosphorylated histone H2AX (pH2AX) 159

4.1.3 Cell death pathways 161

4.1.4 Alternative modes of cell death 167

4.2 Materials and Method 170

4.2.1 DNA damage and cell cycle analysis 170

4.2.2 Flow cytometric analysis of apoptosis 172

4.2.3 Statistical Analysis 173

4.3 Results 175

4.3.1 DNA damage (pH2AX) flow cytometry analysis 175

4.3.2 Cell cycle 180

4.3.3 Apoptosis 185

4.4 Discussion 191

4.4.1 DNA damage 192

4.4.2 Cell cycle 193

4.4.3 Apoptosis.....194

5 Evaluation of the of cellular oxygen consumption rate in C6 and N2a.....196

5.1 Introduction.....197

5.1.1 Mitochondria.....197

5.1.2 Mitochondria and neuronal cells.....198

5.1.3 The Warburg effect (aerobic glycolysis).....199

5.2 Methods.....200

5.2.1 Respiration Measurements.....200

5.2.2 Data analysis.....202

5.3 Results.....204

5.3.1 Comparison of oxygen consumption rates in N2a and C6 cells.....204

5.4 Discussion.....208

5.4.1 Oxygen consumption rate.....208

6 General discussion.....211

6.1 Outline of experiments done in different chapters.....215

6.2 Principle discoveries.....216

6.3 Conclusion.....225

6.4 Future directions and points to consider.....226

7 References:.....228

8 Appendices.....288

8.1 Appendix I.....289

8.2 Appendix II 294

List of figures

Figure 1.1 Chemical structure of 5-Fluorouracil 18

Figure 1.2 Mechanisms of action of the antimetabolite 5-FU 21

Figure 1.3 Symmetrical and asymmetrical cell divisions 28

Figure 1.4 Hierarchy of stem cells 30

Figure 1.5 Diagram of hippocampal neurogenesis 36

Figure 1.6 The hippocampal circuitry 38

Figure 1.7 Neurovascular interaction 42

Figure 1.8 Schematic illustration of the cellular organization in the brain 46

Figure 1.9 Section of hippocampus showing the dentate gyrus 52

Figure 2.1 Phase contrast image of 3T3 cells grown in monolayer. Scale bar: 40 .. 68

Figure 2.2 Photomicrographs of mouse hippocampal NSCs in culture 70

Figure 2.3 Astrocyte cultures at different time points after isolation 74

Figure 2.4 Structures of MTT and coloured formazan product. 78

Figure 2.5 Fluorescence images of primary hippocampal astrocyte cell cultures. . 80

Figure 2.6 Immunocytochemistry staining of primary astrocyte cells derived from hippocampus and spinal cord using papain digestion. 81

Figure 2.7 Growth curves of different cell lines and primary cells cultured in complete medium for 12, 24, 48, 72 and 95 hours 82

Figure 2.8 Phase contrast images of C6, Endothelial and N2a cells showing cell morphology after growing in different media. 84

Figure 2.9 Cell viability as measured by MTT assay of C6, N2a and endothelial cells cultured in RPMI-1640 or DMEM media and incubated for 24 hours. 85

Figure 2.10 The effect of different concentrations of 5-FU (0, 5, 10, 15 and 20 µM) 88

Figure 2.11 The viability as measured by MTT assay of (A) N2a and (B) NSCs after 24 hours exposure to different concentrations of 5-FU (0, 5, 10, 15, and 20). 89

Figure 3.1 Schematic representation of connexins, connexons, and GJs. 107

Figure 3.2 Co-culture model systems used for the analysis of cell-to-cell interactions. 111

Figure 3.3 Scheme for standardized CM production. N2a with CM from C6 or endothelial cells. 114

Figure 3.4 Transwell assay setup: 117

Figure 3.5 Fluorescent microscope images of different cell types co-cultured with N2a cells after 48 hours 5-FU treatment......123

Figure 3.6 Effect of 5 µM 5-FU on the survival of N2a cells when cultured alone or co-cultured with C6 astrocytes, primary hippocampal astrocytes, primary spinal cord astrocytes, endothelial cells or 3T3 cells.....125

Figure 3.7 The effect of astrocyte or endothelial CM in protecting N2a cells from 5-FU.....127

Figure 3.8 Evaluation of the cell viability by MTT assay of N2a cells in different concentrations of FLX (0.1, 0.5, 1, 5, and 10µM) for 48 hours.....128

Figure 3.9 Effect of 10 µM FLX conditioned glial media on neural cells......131

Figure 3.10 Representative fluorescence images of cells in Transwell co-culture system.....133

Figure 3.11 Transwell co-culture of C6 astrocytes; primary hippocampal astrocytes; primary spinal cord astrocytes and N2a cells......134

Figure 3.12 Immunofluorescence staining of for Cx43 in different cell types......136

Figure 3.13 The effects of 50 µM CBX on the viability of C6, endothelial and N2a cells.....139

Figure 3.14 Representative fluorescence micrographs of co-cultures of N2a cells and other cell types in the absence (control) and presence of 50 µM CBX. 141

Figure 3.15 Treatment with the GJ inhibitor CBX blocks the protection from 5-FU provided by co-culture with astrocytes or endothelial cells......142

Figure 3.16 N2a cells formed functional GJCs, with C6 and endothelial cells as shown by dye transfer.....144

Figure 3.17 Effects of inhibition of heterocellular coupling between N2a cells and C6.....145

Figure 4.1 Cell cycle: potential targets for chemotherapeutic drugs. 158

Figure 4.2 The distinct morphological steps of apoptosis 163

Figure 4.3 A representation of the two major apoptotic pathways; the extrinsic and the intrinsic apoptosis pathways 166

Figure 4.4 Graphs showing the different stages of Weasel Software used to analyse flow cytometry data in treated cells. 177

Figure 4.5 Bar chart showing the proportion of gated cells (%) stained for pH2AX (DNA damage marker).....179

Figure 4.6 Graph readings illustrating the various stages of Weasel Software used to analyse flow cytometry data in treated cells. 181

Figure 4.7 Cell cycle progression (G0/G1, S or G2/M), ... 184

Figure 4.8 Dot-plots for apoptosis assay following 48 hours of drug treatment in endothelial cells. 189

Figure 4.9 Bar chart showing the proportion of gated cells (%) stained either positively and negatively for caspase 3/7 (apoptotic marker) 190

Figure 4.10 Variable sizes of neurospheres appear a few days after keeping NSCs in the medium, ... 191

Figure 5.1 Clark oxygen electrode. 202

Figure 5.2 Oxygen consumption rates (OCR) of N2a and C6 cells. 206

Figure 5.3 Effect of FCCP after 5-FU on OCR in C6 and N2a. 208

Figure 6.1 Suggested buffering of Ca^{2+} ions by astrocytes via GJ between astrocytes and neural cells. 222

Abbreviations

5-FU	5-Fluorouracil
ANOVA	Analysis of variance
AraC	Cytosine arabinoside
ATP	Adenosine Triphosphate
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BrdU	5-Bromo-2-deoxyuridine
°C	degrees Celsius
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CBX	Carbenoxolone
CH_2THF	5,10-Methylene-tetrahydrofolate
CM	Conditioned media
CMF	Cyclophosphamide, Methotrexate, and 5-fluorouracil

CNS	Central nervous system
CSF	Cerebrospinal fluid
Cx	Connexin
DAPI	4',6-diamidino-2-phenylindole
DCX	Doublecortin
DMEM	Dulbecco’s Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DSBs	Double stranded breaks
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate
ECM	Extracellular matrix
EGF	Epidermal growth factor
EPO	Erythropoietin

FdUMP	Fluoro-deoxyuridine monophosphate
FdUTP	Fluoro-deoxyuridine triphosphate
FGF-2	Fibroblast growth factor-2
FLX	Fluoxetine
FUTP	Fluorouridine triphosphate
GFAP	Glial fibrillary acidic protein
GJs	Gap Junctions
GJC	Gap Junction Communication
HBMECs	Human brain microvascular endothelial cells
IGF-1	Insulin like growth factor
LCV	Leucovorin/calcium folinate
LTP	Long term potentiation
M	micro (1×10^{-6})
MRI	Magnetic resonance imaging
MTHF	Methylenetetrahydrofolate

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	Methotrexate
MW	Molecular weight
MWM	Morris water maze
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
$Na_2S_2O_4$	Sodium dithionite
NeuN	Neuronal Nuclei
NGF	Nerve growth factor
NH_4HCO_3	Ammonium bicarbonate
NLR	Novel location recognition
NSC	Neural stem cell
OCR	Oxygen consumption rate
PBS	Phosphate Buffered Saline

PDGF	Platelet-derived growth factor
PEDF	Pigment epithelium-derived
Pen/Strep	Penicillin and streptomycin
PET	Positron emission tomography
PFA	Paraformaldehyde
pH	Hydrogen ion concentration
pH2AX	Phosphorylated histone H2AX
PI	Propidium iodide
RNA	Ribonucleic acid
RNAse	Ribonuclease
ROS	Reactive oxygen species
Rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute medium
SEM	Standard error of the mean
SFM	Serum free medium

SGZ	Subgranular zone
SSRI	Selective serotonin reuptake inhibitor
SVZ	Subventricular zone
TMP	thymidine monophosphate
TNF-α	Tumor necrosis factor-α
TS	Thymidine synthetase
VEGF	Vascular endothelial growth factor
v/v	volume per volume
ΔO_2	cell respiration

1 Chapter General introduction

1.1 Chemotherapy

Cancer is a primary cause of death worldwide which accounts for 13% of all deaths per year (Thun et al., 2010). The latest global statistics on cancer incidence and mortality in 2008 show an incidence of 12.7 million leading to 7.6 million deaths (Jemal et al., 2011). Recent statistics from Cancer Research UK showed that over two million people are suffering from cancer in the UK at the moment and that this is expected to increase by 3% a year (Coleman et al., 2011).

Cancer is treated in a variety of ways often involving surgery to remove the primary tumour followed by adjuvant chemotherapy, radiation, hormonal treatments, immunotherapy and gene therapy to remove all traces of the cancer. Since the 1950's, chemotherapy has been one of the common adjuvant treatments for most cancer cases (Seigers and Fardell, 2011). There are different classes of chemotherapy drugs but they nearly all work by disrupting aspects of cell division and in this way have a significant effect on the rapidly dividing cells of the cancer. However chemotherapy is non selective and not only kills cancer cells (Ménard et al., 2008), but also affects non cancer cells which are dividing and in this way causes several unwanted side effects. It is now thought that some cells such as stem cells are more sensitive to chemotherapy than cancer cells and due to this are particularly affected by chemotherapy treatment (Han et al., 2008).

Clinical studies have found that chemotherapeutic agents used to treat brain, breast, lung, prostate, and ovarian cancers can cause a number of neurological side effects including vascular complications, mood disorders and peripheral neuropathies in both

children and adults (Meyers et al., 2005, Yang and Moon, 2013). Some of these produce short-term effects such as diarrhoea, hair loss, myelosuppression, immunosuppression, and gastro-intestinal tract toxicity which generally disappear on discontinuation of the chemotherapy treatment (Schagen et al., 2002, Verburg et al., 2000, Sieklucka-Dziuba et al., 1998, Boehmke and Dickerson, 2005). In this thesis I am particularly concerned about the cognitive side effects of chemotherapy which can persist for long periods of time after the end of treatment and can have long term effects on patient quality of life and ability to work (Wefel and Schagen, 2012, Matsuda et al., 2005, Scherwath et al., 2006).

1.1.1 Clinical studies

Numerous patient reports have described cognitive decline after chemotherapy treatment (Wigmore, 2013b). These neurological changes are associated with a wide range of different types of chemotherapeutic agents either used alone or in combination. Cognitive impairments associated with chemotherapy are often mild to moderate and vary in duration from months to years (Schagen et al., 2001, Janelsins et al., 2011). Some patients may experience little or no decline in cognitive performance while other patients suffer from more serious cognitive impairments (Paquet et al., 2013, Mehlsen et al., 2009). The proportion of cancer survivors reporting or being diagnosed with cognitive impairment varies between 16% to 75% (Tchen et al., 2003, Vardy et al., 2007). This variability is likely to be due to a lack of consistency in the use and type of neuropsychological testing, differences in the sample and control groups, different statistical analyses and inconsistencies between qualitative and quantitative studies. For example many patients report post chemotherapy cognitive impairments, often

manifesting as difficulties in word and spatial recall but direct psychometric testing often finds that only a sub set of these patients are significantly below expected population norms (Wigmore, 2013a). It is unclear at present why this is the case but it has been suggested that performance under test conditions may not adequately measure real life situations. Functional magnetic resonance imaging studies of post chemotherapy patients, undergoing testing, has shown that they use more extensive cortical areas than untreated individuals, probably to compensate for reduced cognitive ability (Silverman et al., 2007).

Drugs which have been associated with cognitive decline include methotrexate (MTX), BCNU, melphalan, fludarabine, cytarabine, 5-fluorouracil (5-FU), levamisole, cisplatin, and capecitabine (Staat and Segatore, 2005). In addition, a number of studies have also observed that myelin damage can occur after chemotherapy treatment and that this is also associated with deficits in cognitive performance. All of these chemotherapy agents are used to treat a variety of common cancers including breast, gastrointestinal tract and prostate (Fremouw et al., 2012).

Many factors have been found to increase the risk of developing cognitive decline associated with chemotherapy, these include exposure to high doses of chemotherapy, additive or synergistic effects of multi-agent chemotherapy, administration of chemotherapy either concurrent or subsequent to cerebral radiation, intra-arterial administration with blood brain barrier (BBB) disruption, and intrathecal administration (Wefel and Schagen, 2012).

It is difficult to determine the exact time when these impairments are manifested, as published studies have tested cognitive performance at different time periods after the completion of treatment. The duration of cognitive effects has also been reported to vary with a study by Ahles and colleagues (Ahles et al., 2002), finding that cognitive deficits by some individuals persist for 5-10 years after the completion of treatment. However, other studies have failed to detect any cognitive impairment after receiving chemotherapy (Collins et al., 2009).

According to Dubois and co-authors (Dubois et al., 2014), the cognitive side effects of chemotherapy, include deficits in working memory, language ability, reaction time, organizational skills, specific elements of thinking or attention /concentration, processing speed and executive functions (Seigers et al., 2013a). These effects have been called "chemobrain" or "chemofog" by patients suffering from this condition (Acharya et al., 2015a, Argyriou et al., 2011). Symptoms, which can persist for long periods, can be a cause of considerable distress for patients and prevent their return to their previous academic, occupational or social activities. Patients generally show recovery of cognitive ability after 5 years (Stanton, 2006).

Although "chemobrain" was first studied and reported by breast cancer survivors who had received chemotherapy treatment, the same symptoms have also been reported by patients treated for other cancers including head and neck, gastrointestinal, colorectal and prostate cancers (Huhtala et al., 2009, Santini et al., 1989, Ilson, 2003, Rich et al., 2004, Shin et al., 2005). It is important for patients who have received chemotherapy to be informed about chemobrain and its symptoms to enable them to manage the effects. Many studies have reported that a wide range of chemotherapeutic agents, including

antimetabolites such as cytosine arabinoside (Ara-C), 5-FU, MTX, DNA cross-linking agents such as BCNU and cisplatin and even anti-hormonal agents can cause mild to moderate cognitive dysfunction (Wefel and Schagen, 2012).

An early study by Peterson et al in 1980 found an association between chemotherapy and cognitive dysfunctions and showed that up to 86% of cancer patients experienced these symptoms (Peterson and Popkin, 1980). A later study by Silberfarb (1993) recognized a possible relation between cognitive deficits and various types of chemotherapy (Silberfarb, 1983). In a more recent clinical study by Monje and colleagues, (Monje and Dietrich, 2012), they reported that between 20–40% of breast cancer patients suffer from these cognitive impairments after chemotherapy treatment. The nature, degree and timing of these effects differ depending upon the type of cancer and treatment, and also the age, sex, health, the hormonal status, and cognitive state (depression, anxiety and fatigue) of patients (Long et al., 2011, Wigmore, 2012). These cognitive declines have been confirmed by psychometric testing in patients who complain of cognitive deficits.

Based on the results of these studies, the most consistent impairments observed were in verbal memory and on tasks which involve strategic or executive processes (Jansen et al., 2005). These impairments are related directly to functions performed by the hippocampus and frontal lobes of the brain (Squire, 1992, Stuss et al., 1986).

In the early 1980s, the first clinical studies were performed to examine the cognitive side effects after adjuvant chemotherapy. These first findings increased interest and led

to the study of the long-term side effect of chemotherapy by both cross sectional and longitudinal studies.

Studies of breast cancer patients who had received cyclophosphamide and 5-FU showed that 75% of these patients suffered from problems with memory and concentration compared to controls 6 months after completion treatment (Wieneke and Dienst, 1995). A study of breast cancer and lymphoma survivors examined 5 years after treatment, reported that 39% had cognitive dysfunction compared to 14% in a group of patients treated with local therapy (Ahles and Saykin, 2001). Further clinical studies (Monje and Dietrich, 2012, Schagen et al., 1999) reported that around 20–40% of breast cancer patients suffer from cognitive impairments after the cessation treatment. These clinical studies have shown cognitive declines in the speed of information processing, verbal and visual memory, attention, mental flexibility, the ability to concentrate, multitasking the ability to organize information and increases in fatigue, confusion, among breast cancer patients 5 years after administration of cyclophosphamide, MTX and 5-FU (Ahles et al., 2002, Jansen et al., 2008).

1.1.1.1 Cross sectional studies

The first attempts to investigate the symptoms of chemobrain in cancer survivors were cross sectional in design and consistently found greater cognitive impairments in patients who had received chemotherapy compared to healthy controls. In these studies the cognitive status of the patients before treatment was unknown.

Cross sectional studies, which compare cancer patients after receiving chemotherapy with patients treated only with surgery or a healthy group, have observed that cognitive

impairments appear shortly after chemotherapy in the treated group and can persist for up to 10 years post treatment (Fardell et al., 2010). The first cross-sectional studies conducted by Wieneke and Dienst in 1995 (Wieneke and Dienst, 1995), found that 75% of cancer survivor complained of poor attention /concentration and impaired memory performance 6 months after completion treatment. Similar studies have found a 25% reduction in performance in visual tests during the same time period (Hurria et al., 2006), and in some cases up to 4 years later (Kreukels et al., 2008).

In addition, several studies have found that breast cancer survivors who received high-dose chemotherapy complain of more significant cognitive problems compared with patients receiving standard-dose chemotherapy (Yang et al., 2011a, van Dam et al., 1998). A study by Schagen et al. of 39 breast cancer patients treated with systemic chemotherapy which were compared to age matched breast cancer patients treated with local therapy, found that the systemically treated group suffered from more problems with concentration and memory two years later, compared to those experiencing local therapy (Schagen et al., 1999). From these different studies, the effects of chemotherapy are dose-dependent and can persist for many years (Castellon et al., 2004, Yamada et al., 2010, Koppelmans et al., 2012). In contrast to these findings, a study conducted by Donovan et al. in 2005, failed to find significant differences in cognitive ability in breast cancer patients treated with standard-dose adjuvant chemotherapy and radiotherapy compared to those treated only with radiotherapy in relation to episodic memory, attention, motor performance and language (Donovan et al., 2005b). The problem with this study was that the radiotherapy group was significantly older than those in the chemotherapy group and so may have experienced age related cognitive difficulties (Charlton et al., 2006).

Furthermore, issues arise with cross sectional studies in comparing chemotherapy patients with the normal population due to their different experience of diagnosis and other aspects of treatment. Therefore, longitudinal studies where the same patient is tested before and after treatment have largely superseded these cross-sectional studies.

1.1.1.2 Longitudinal studies

Longitudinal studies study individual patients before during and after treatment are a better type of study as they use the base line cognitive scores of patients before chemotherapy to compare with post treatment cognition and do not require a separate control group. They enable the quantification of pre-treatment levels of cognition; which can identify individuals with pre-existing cognitive deficits and identify changes at various time-points to identify temporal changes in cognition.

A study on women with breast carcinoma, showed that 35% of them had some form of cognitive impairment before the start of systemic chemotherapy treatment as compared with an untreated group (Wefel et al., 2004). This was a surprising result and the reasons underlying this are still unclear. Possible suggested mechanisms are the impact of the tumour itself including the release of inflammatory cytokines or the stress of diagnosis.

Despite finding that a proportion of patients suffer from low pre-treatment cognitive scores, nearly all longitudinal studies have found significant cognitive declines associated with the period of chemotherapy treatment (Ahles et al., 2010, Bender et al., 2006, Biglia et al., 2012, Collins et al., 2009, Deprez et al., 2011, Galalae et al., 2005, Hedayati et al., 2012, Hermelink et al., 2007, Jansen et al., 2011, Quesnel et al., 2009,

Schagen et al., 2006, Vearncombe et al., 2009, Wefel et al., 2004, Whitney et al., 2008). For example a longitudinal study by Vardy and colleagues on colorectal cancer patients treated with 5-FU and oxaliplatin found that 20% of patients still suffered from neuropsychological changes 6 months after stopping chemotherapy (Vardy et al., 2007).

A few studies have however failed to detect cognitive decline after treatment (Anderson-Hanley et al., 2003, Tager et al., 2010b, Jenkins et al., 2006). The reason for these contradictory views is not known. It has been suggested that additional factors may affect cognitive performance such as stress and the emotional state of patients although this has been allowed for in most studies. For example, patients might be worrying about their treatment and in some cases the cancer itself may lead to a decline in mental functions (Fremouw et al., 2012). In addition, the presence of other factors including interactions between genetics, the type of chemotherapy treatment, dose level, frequency of dosing, environment, the family history of individuals, their level of education, ethical issues and methodological including the relatively small samples sizes used in many studies (Briones and Woods, 2011), differences in age of the patients, nature and location of the tumours, additional anti-cancer treatments (e.g. hormonal), intensity of the adjuvant treatment (Squire, 2004, Long et al., 2011). Furthermore, lack of appropriate control groups, study designs, or statistical measures have confounded some studies. All of these can make studies on the cognitive effects of chemotherapy in humans more difficult (Seigers et al., 2013a). Therefore, the type of chemotherapy, the length of time for treatment and particularly the tests used in evaluating results of patient studies must be taken into account (Donovan et al., 2005a, Hermelink, 2011, Tager et al., 2010a).

Chemotherapy associated cognitive decline is likely to be a major health problem in the future because, with the increasing age of populations, the incidence of cancer and the need for cancer treatment will increase and lead to greater numbers of patients who experience the cognitive effects of treatment (Fardell et al., 2011). At the same time, improvements in cancer treatment are helping to increase the number of cancer survivors. These factors make it likely that cognitive impairments will become increasingly common in cancer survivors, and researchers are currently investigating the causes and means to improve cognition in patients suffering from the side effects of chemotherapy treatment. Some of clinical studies on chemobrain are listed on (Table 1.1) (Appendix I).

1.1.2 Neuroimaging studies

Neuroimaging of the brain has enabled structural and functional changes to be detected and correlated with cognitive changes (Schagen et al., 2001, Deprez et al., 2011). Such techniques include PET (positron emission tomography), which provides a measure of neural metabolism (Ahles and Saykin, 2007) and magnetic resonance imaging (MRI), which produces high resolution images which can detect atrophy and some pathologies that have enabled researchers to describe structural changes in grey and white matter (Koppelmans et al., 2012).

In important studies by de Ruiter and Raffa the brains of cancer survivors, who had been treated with adjuvant chemotherapy, were imaged before and after chemotherapy treatment and compared with healthy untreated individuals (de Ruiter et al., 2012, Raffa and Tallarida, 2010). The results of these studies showed that chemotherapy can cause

significant reductions in both grey and white matter volumes (Lepage et al., 2014a). For example, several MRI cross-sectional studies on chemotherapy treated cancer survivors, found a decrease in grey matter in frontal structures and the hippocampus, as well as a decrease in white matter integrity compared to controls (Inagaki et al., 2007, Deprez et al., 2011, Abraham et al., 2008). Other important studies (Silverman et al., 2007, de Ruiter et al., 2011, Ferguson et al., 2007), using PET have found a decrease in resting metabolism and cerebral blood flow in the basal ganglia, inferior frontal gyrus and cerebellum during memory tests in chemotherapy treated cancer survivors 5-10 years earlier. Functional studies have indicated that larger brain areas are used in cognitive tasks by chemotherapy treated patients as compared to non-treated individuals. Brain scans by MRI for patients who received 5-FU and experienced leukoencephalopathy, showed changes in periventricular white matter and extensive myelin loss of cerebral white matter on brain biopsy (Choi et al., 2001).

Longitudinal studies have shown similar changes in grey matter density in bilateral frontal, temporal (including hippocampus), cerebellar regions and right thalamus one month after chemotherapy with only partial recovery in these structures one year later (McDonald et al., 2010, Silverman et al., 2007, Collins et al., 2009). Another study showed decreases in the superior and middle frontal lobe gyri, parahippocampal gyrus, cingulate gyrus and precuneus one year after chemotherapy treatment but this effect had disappeared by 5 years (Fardell et al., 2011). However, some studies have failed to find any changes after chemotherapy treatment (Yoshikawa et al., 2005).

Perry and Schmidt (2006) reviewed the neurotoxicity associated with different types of chemotherapeutic agents including MTX, 5-FU, cytarabine, and cisplatin. In several

cases, mild to moderate necrosis was found in the white matter of the brain. These injuries could indicate a risk of permanent damage when MTX is combined with radiotherapy (Perry and Schmidt, 2006).

Other MRI studies have demonstrated that headache, nausea and changes in mental status are associated with alteration in cerebral white matter in cancer patients receiving adjuvant chemotherapy (Kastrup and Diener, 2008, Onujiogu et al., Rajasekhar and George, 2007, Soussain et al., 2009).

1.1.3 Animal studies

Animal studies have found that chemotherapy, as well as killing dividing cells including neural stem cells (NSC) (see section on hippocampal neurogenesis) also caused oxidative stress, prevented neuronal differentiation, activated microglia, induced apoptosis, changed levels of histone protein phosphorylation and chromatin remodelling and lead to the aberrant expression of neurotrophins and neurogenic proteins in the brain (Seigers and Fardell, 2011, Seigers et al., 2016). As will be discussed later, these molecular changes affect the rate of hippocampal neurogenesis which in turn is correlated with cognition, learning and memory (Mustafa et al., 2008b, Briones and Woods, 2011, Christie et al., 2012).

Chemotherapy can also have specific toxic effects on the central nervous system (CNS) (Soffietti et al., 2014). A recent study showed that chemotherapy agents are more toxic to healthy brain cells than to the cancer cells (Han et al., 2008). Han and colleagues (Han et al., 2008), found that CNS progenitor cells and oligodendrocytes are very susceptible to damage by 5-FU *in vitro* and *in vivo*. Exposure for more than 5 days to 5-

FU cause delayed damage to CNS white-matter tracts. This was one of the first animal studies to provide information on white matter damage in those treated with chemotherapy (Han et al., 2008). These results are probably applicable to a wide range of chemotherapy agents as a number of studies have reported that as well as 5-FU, carmustine, cisplatin, and cytarabine, are toxic to CNS progenitor cells and oligodendrocytes. Based on this evidence, neurotoxicity to normal CNS cells and NSCs can be considered as a factor causing cognitive dysfunction in cancer survivors after chemotherapy (Dietrich et al., 2006a). The impact of chemotherapy may affect different cognitive domains as a study by Winocur et al. in 2011 demonstrated that administration of 5-FU to mice reduced spatial memory but not cued memory (Winocur et al., 2011). Seigers et al, in an animal study, found a decrease in the density of the corpus callosum after MTX treatment (Seigers et al., 2009b).

1.1.3.1 The use of animal models to study the chemobrain phenomenon

Animal models have been used to investigate the association between chemotherapy and cognition and to try to determine the underlying molecular mechanisms behind the cognitive declines found after chemotherapy (Seigers and Fardell, 2011). In addition, these studies offer an opportunity to look for similarities between patient reports of memory and concentration decline and those found in animal models after chemotherapy to see if they are associated with changes in hippocampal and frontal cortical brain regions. Animal studies also make it possible to control for other features of cancer pathology associated with the tumour itself and to eliminate psychological factors, such as depression or stress, likely to be found in human patients. These models

can also be used to test therapeutic strategies to prevent and treat cognitive deficiencies. A limitation of animal models using rodents is their relatively short lifespan which makes investigation of long term cognitive dysfunction difficult.

Most animal studies have found that chemotherapy produces a cognitive decline in one or more cognitive tests (Eijkenboom and Van Der Staay, 1999, Foley et al., 2008, Madhyastha et al., 2002, Mustafa et al., 2008b).

Clinical studies have several methodological problems, particularly in choosing controls and eliminating extraneous variables which may be caused by variations in age, education and the psychological effects of cancer diagnosis and treatment. Therefore, the use of animal models as research tools has provided faster results with greater experimental control and allowed more detailed investigations of the impact of chemotherapy (Schagen and Vardy, 2007).

Animal models reviewed by Seigers et al. and Wigmore, show that a wide range of chemotherapeutic agents induce cognitive dysfunction (Seigers et al., 2013b, Wigmore, 2013b). These animal studies showed more or less similar effects to those found in patient studies and demonstrated behavioural changes associated with hippocampal and frontal cortical regions after receiving chemotherapy (Reuter-Lorenz and Cimprich, 2013, Seigers et al., 2013b, Wilson and Weber, 2013b). For example, animal studies in our laboratory have demonstrated deficits in spatial learning tasks after using 5-FU (Mustafa et al., 2008a, ElBeltagy et al., 2010a).

These behavioural studies are similar to the results found by other investigators using a range of behavioural tests including the Morris water maze (MWM) and novel object

recognition (NOR) (Seigers et al., 2008, Winocur et al., 2006, Mustafa et al., 2008b, Lyons et al., 2012), inhibitory avoidance conditioning test (Liedke et al., 2009, Reiriz et al., 2006), contextual conditioned response (ELBeltagy et al., 2010c), fear conditioning (MacLeod et al., 2007), object location recognition as well as cue-specific and rule learning (ELBeltagy et al., 2010c, Mustafa et al., 2008b). The chemotherapy drugs used include 5-FU, MTX, doxorubicin (DOX), cisplatin and cyclophosphamide (see Table 1.2, Appendix I).

Recent animal investigations are shown in Appendix I (Table 1.2). These studies have illustrated that rodents treated, with chemotherapy agents (5-FU; MTX; Cyclophosphamide; DOX; Oxaliplatin; thioTEPA; Ara-C; Temozolomide; BCNU; Cisplatin; Cytarabine; Paclitaxel) as well as other cytotoxic drugs (methylazoxymethanol acetate; 30-azido-deoxythymidine, show a decline in their ability to perform a number of behavioural tasks. For example, Winocur and colleagues showed that animals receiving a combination of MTX and 5-FU chemotherapy had significant deficits in spatial memory and other cognitive tasks (Winocur et al., 2006).

Eijkenboom and Van Der Staay in 1999 showed that rats injected with vincristine in the dorsal hippocampus had impaired spatial learning in the MWM which was linked to a wide spread lesion in the dorsal hippocampus (Eijkenboom and Van Der Staay, 1999).

In conclusion as summarised in (Table 1.2), nearly all animal studies have found cognitive impairment after systemic chemotherapy exposure. An exception is a study which found no impairment on rat behaviour after treatment with 5-FU (Lee et al., 2006). In contrast, they found an improvement in performance in the MWM after 5-FU

or cyclophosphamide treatment. No explanation has been provided for these contrary results.

A number of investigators using different chemotherapy agents have suggested that a reduction in adult hippocampal neurogenesis is one of the key mechanisms behind the cognitive decline found after chemotherapy (Yang et al., 2010, Christie et al., 2012, ELBeltagy et al., 2012, Lyons et al., 2011, Mondie et al., 2010).

In vivo studies have been supported by *in vitro* studies of different cell types using both primary and cell lines. These have shown that NSCs are one of the cell types which are particularly sensitive to chemotherapeutic agents (Dietrich et al., 2006a, Wick et al., 2004a). However the reasons behind this are unclear and may involve the effects of inflammation or corticosteroid levels within the brain or interactions with other cell types within the neurogenic niche in which stem cells are found.

Interestingly it has been shown that transplantation of human NSCs which have not experienced chemotherapy into rodents treated with chemotherapy produced a cognitive improvement (Acharya et al., 2015b). This is unlikely to be a viable treatment for chemotherapy induced cognitive impairment in patients but demonstrates that the survival and role of NSCs is critical in the mechanisms behind chemobrain.

In summary both clinical and animal studies have demonstrated that systemic chemotherapy produces long term cognitive decline in behaviours requiring the hippocampus and frontal cortex. This project is primarily concerned with the impact of chemotherapy on adult hippocampal neurogenesis and specifically the effect of 5-FU on NSCs and other cell types found in the hippocampal neurogenic niche.

1.2 5-Fluorouracil

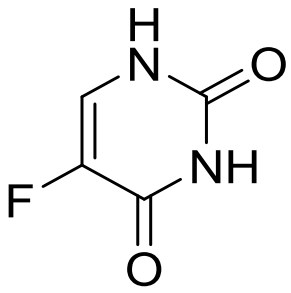


Figure 1.1 Chemical structure of 5-Fluorouracil.

This thesis focuses on the use of a single chemotherapy compound, 5-FU. 5-FU was synthesised and developed as an antimetabolite chemotherapy agent by Heidelberger in collaboration with Duschinski et al. in 1957 at the Roche Research laboratories. It is an analogue of uracil with a fluorine atom substitution at the C-5 position in place of a hydrogen atom. Addition of this fluorine was known to convert a non-toxic molecule to one with increased toxicity (Heidelberger et al., 1957) (Figure 1.1). This drug is a pyrimidine analogue, and is regarded as one of the most important chemotherapeutic agents used for the treatment of breast, head and neck, gastrointestinal, prostate and colorectal cancers (Huhtala et al., 2009, Ghoshal and Jacob, 1997, De Angelis et al., 2006, Fumoleau et al., 2003, Posner et al., 2000). It can be used alone or in combination with other cytotoxic drugs and hormones (Singh et al., 2012, Conte et al., 2007, Sandström et al., 2006).

Antimetabolites such as 5-FU prevent progress in the cell cycle by disrupting the nucleotide synthesis that is required for ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) synthesis (Dreshfield et al., 1996).

5-FU has a low molecular weight (130.08 Daltons); and can cross the BBB via simple diffusion reaching a concentrations in the cerebrospinal fluid (CSF) of between 11-50% of the serum concentration (Bourke et al., 1973). Although generally well tolerated, treatment with 5-FU can cause side effects including pancytopenia and diarrhoea, anorexia, nausea, and taste changes, when administered at high doses (Jansen et al., 2005, Takii et al., 2014, Stoeltzing et al., 2003). Like other chemotherapy agents it can also cause myelosupression, mucosities of the gastrointestinal tract and thrombophlebilits of peripheral veins (Cwikiel et al., 1996). 5-FU can cause adverse effects on brain functions such as memory by damage and apoptosis to microglia, oligodendrocytes, and NSCs, axons as well as changes in neurotransmitter levels (Yang and Moon, 2013). In addition, as will be discussed later, 5-FU can reduce the number of proliferating cells in the subgranular zone (SGZ) of the dentate gyrus (de Ruiter et al., 2012).

1.2.1 Mechanisms of action of 5-fluorouracil

To determine how 5-FU impact on the brain, it is necessary to understand how it mediates its actions on cells.

5-FU can inhibit the growth of mammalian cells and two main modes of action have been proposed. First 5-FU as an analogue of uracil easily enters cells where it is converted into three active metabolites– fluoro-deoxyuridine monophosphate (FdUMP), fluoro-deoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). The main action of 5-FU is its inhibition of the enzyme thymidylate synthase (TS) (Shin et al., 2005). Thymidine is made by the conversion of deoxyuridine monophosphate

(dUMP) to thymidine monophosphate (TMP). This process is catalysed by thymidylate synthase (TS) in the presence of the reduced folate N5,N10-methylenetetrahydrofolate (MTHF) (Valeriote and Santelli, 1984). When FdUMP binds to the nucleotide-binding site of TS, this prevents the binding of the normal substrate dUMP to TS, resulting in inhibition of deoxythymidine monophosphate (dTMP) synthesis (Santi et al., 1974). Inhibition of TS produces a deficiency in thymidine which disrupts DNA synthesis (Longley et al., 2003, Van Triest and Peters, 1999) (Figure 1.2). Although 5-FU has a short plasma half-life (< 30 minutes), it can suppress TS levels for several days (Longley et al., 2004).

The endogenous folate 5,10-methylene tetrahydropholate (CH_2THF) potentiates the action of TS by facilitating the binding of dUMP to TS and is sometimes included in 5-FU treatment (Parker and Cheng, 1990).

Administration of intravenous 5-FU combined with leucovorin (LV, 5-formylTHF), a precursor of 5,10-methyleneTHF, which directly contributes to the intracellular pool of 5,10-methyleneTHF and enhances the formation and stabilizes the inhibition of the TS (Kerr, 1989) (Figure 1.2). TS suppression causes nucleotide pool imbalances that result in S-phase cell cycle arrest and apoptosis (Longley et al., 2002). Lack of TS can lead to cell apoptosis which is termed thymidine death. Although the mechanism of cell apoptosis is still unclear it has been hypothesized that incorrect DNA synthesis and inefficient repair could therefore lead to activation of pro-apoptotic signalling pathways (Ahmad et al., 1998).

The second mechanism of 5-FU toxicity is thought to be due to the misincorporation of FdUTP and FUTP into DNA and RNA strands, respectively (Valeriote and Santelli, 1984, Aherne et al., 1996). These cause major interruptions in normal DNA and RNA function and enhance cell death by initiating p53-mediated apoptosis.

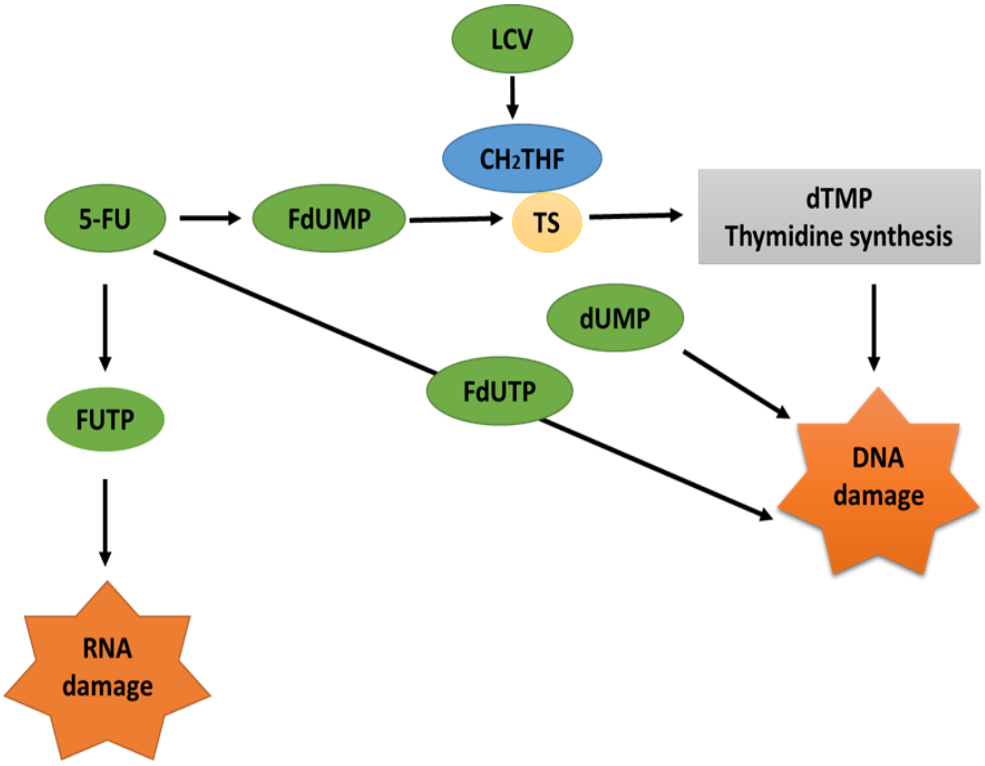


Figure 1.2 Mechanisms of action of the antimetabolite 5-FU. Normally dUMP is converted to dTMP which is necessary for the synthesis of the DNA pyrimidine base, thymidine. This is catalysed by TS and facilitated by the binding of CH_2THF to TS. The major active metabolite of 5-FU, FdUMP, competitively binds to TS (also facilitated by the binding of CH_2THF inhibiting the production of dTMP, leading to DNA damage and cell death. This disruption also causes a build-up of dUMP. Both dUMP and another active metabolite of 5-FU, FdUTP get incorporated into the DNA causing lethal damage. The third active metabolite of 5-FU, FUTP, is miss incorporated into RNA affecting cellular metabolism and viability. LCV increases intracellular levels of CH_2THF, enhancing binding of FdUMP to TS, enhancing the effects of the 5-FU metabolite. Diagram modified from Longley (Longley et al., 2003).

In conclusion it can be seen that 5-FU has the potential to affect cells in several different ways which could impact on NSC proliferation and their interactions with other cells in the neurogenic niche.

1.2.2 *In vitro* chemotherapy studies on stem cells

It would be most useful if the *in vivo* effects of chemotherapy on NSCs could be modelled on cells *in vitro*. This would reduce the number of animals required in investigations and make it possible to investigate changes and interactions between defined types of cell. In the mid-90's, primary NSCs were isolated from the adult hippocampus (Kilpatrick et al., 1995, Ray et al., 1995). These cells were cultured either as free floating neurospheres or monolayers (Palmer et al., 1999, Reynolds and Weiss, 1992a). Using these techniques it is now possible to examine the neurotoxic effects of chemotherapy on these cell populations by measuring their viability and levels of apoptosis (Shimoyama et al., 2002, Wick et al., 2004b, Peters et al., 2000b).

1.3 Mechanisms of chemotherapy-induced cognitive impairments

Both patient and animal studies have provided strong evidence that systemic chemotherapy affects brain structure and function and that the cognitive changes can be ascribed directly to chemotherapy treatment. However we still have little understanding about the possible mechanisms behind cognitive impairment induced by chemotherapy. Suggested mechanisms include inhibition of hippocampal neurogenesis (Ahles, 2012), oxidative damage (Nadin et al., 2006, Blasiak et al., 2004), elevated levels of inflammatory cytokines in brain tissue (Burstein, 2007), direct damage to neurons and white matter (Stewart et al., 2006).

1.3.1 Oxidative stress

Oxidative stress, defined as an imbalance between the production of reactive oxygen species (ROS) (free radicals) and antioxidant defences, has been associated with cancer, inflammation, aging, drug action, and drug toxicity (Sies, 1985). Oxidative stress can cause damage to DNA in neuronal cells and may occur through exposure to exogenous toxins (Ozben, 2007) such as DOX which is commonly used to treat breast cancer and has been associated with the induction of oxidative stress (Quiles et al., 2002). Other antineoplastic agents, used to treat breast cancer, which have been found to produce oxidative stress include the anthracyclines, cyclophosphamide, and 5-FU (Chen et al., 2007b). Additionally, a number of cytostatics, including carboplatin (Geller et al., 2001, Husain et al., 2003), cyclophosphamide (Oboh and Ogunruku, 2010), cytarabine (Koros and Kitraki, 2009), and MTX (Rajamani et al., 2006) are also associated with increased oxidative stress.

DOX is known to produce intracellular ROS (Aluise et al., 2011) which are mostly produced by the respiratory chain within mitochondria. Production of ROS can cause mutations in the mitochondrial DNA. Because chemotherapeutic agents in general disrupt DNA, it is likely that mitochondrial DNA is also affected by chemotherapy treatment due to ROS formation and oxidative stress. Experimental, administration of DOX to mice caused an elevation in tumor necrosis factor-α (TNF-α), which can induce the expression of nitric oxide synthases. Nitric oxide synthases can then lead to increased oxidative stress (Chen et al., 2007b). DOX treatment also causes increased levels of pro inflammatory cytokines in the brain which in turn lead to mitochondrial dysfunction, oxidative stress, and neuronal death. These processes suggest a

relationship between DNA damage and cognitive difficulties, may contribute to the cognitive impairment experienced by patients undergoing chemotherapy (Joshi et al., 2010, Tangpong et al., 2006). Oxidative DNA damage has been linked with neurodegenerative disorders that are characterized by cognitive symptoms (Keller et al., 2005; Wang et al., 2014), further suggesting a relationship between DNA damage and cognitive difficulties.

Oxidative reactions have also been correlated with 5-FU cytotoxicity, which was mediated by a reduction in the activities of superoxide dismutase and glutathione peroxidase. These findings showed that apoptosis and free radical injury might be involved in 5-FU induced cerebral white matter injury (Hara et al., 1990, Szczepańska et al., 1988). These authors also found that oxidative DNA damage in patient groups was associated with reduced grey matter density in temporal and mesencephalic regions.

Studies of early-stage breast cancer patients have found increased oxidative DNA damage, as compared to healthy controls, six years after the end of treatment (Conroy et al. (2012). Similarly, increasing levels of TNF-α, decrease hippocampal volume at early stages in breast cancer patients (Kesler et al., 2013). A study by Keller et al. in 2005 found elderly patients with mild cognitive impairment had higher levels of oxidative DNA damage in the brain on autopsy than age matched controls (Keller et al., 2005). Konat and colleagues investigated the role of oxidative stress in the development of cognitive dysfunction after cyclophosphamide and DOX treatment of rats. Rats showed impaired learning behaviour after the treatment with chemotherapy. However, this effect was absent when the chemotherapy was given in combination with antioxidants

such as N-acetyl cysteine (Konat et al., 2008). Interestingly astrocytes are also known to protect neurons against oxidative stress by releasing antioxidants (Park et al., 2001).

1.3.2 Effects on myelin and white matter

Chemotherapy agents, particularly 5-FU, have been found to decrease myelin thickness and deregulate the expression of the Olig2 gene which is essential for differentiation of functional oligodendrocytes, in the corpus callosum of rats (Han et al., 2008). *In vivo* studies have shown that different types of chemotherapy including 5-FU, carmustine, cisplatin, and cytarabine, affect oligodendrocytes precursors (Dietrich et al., 2006a). In addition, MTX also caused degeneration of white matter and white matter necrosis (Gregorios et al., 1989). Han et al in 2008, have suggested that damage to white matter including loss of oligodendrocytes could decrease the speed of information processing, and therefore could contribute to the cognitive decline observed in patients after chemotherapy (Han et al., 2008). A study by Madden et al. in 2012 found that age related decreases in white matter integrity contributed to a decrease in communication efficiency between networks and caused impairment in cognition (Madden et al., 2004). Research on mice treated with 5-FU showed significantly reduced myelin integrity that correlated with functional alterations in impulse conduction within the auditory system (Han et al., 2008).

In addition, oligodendrocyte precursor cells and non-dividing mature oligodendrocytes which are required for the maintenance of white matter tracts were also affected by chemotherapy and demyelination has been reported after completing treatment (Dietrich et al., 2006b).

1.3.3 Inflammation

Chemotherapy can cause both central and peripheral inflammatory reactions resulting in elevated levels of pro inflammatory cytokines. Cytokines are proteins that are released by activated immune cells in response to inflammation, stress, or direct injury to neurons (Maier, 2003). Both inflammation and cytokines can cause cognitive dysfunction and are able to reduce hippocampal neurogenesis (Ekdahl et al., 2009, Hein and O'Banion, 2009, Wu et al., 2012).

Different chemotherapy agents including etoposide, 5FU, and DOX increase the production of proinflammatory cytokine by cells *in vitro* (Elsea et al., 2008). Clinical studies on breast cancer patients receiving taxenes and DOX showed transient increases in proinflammatory cytokines (Pusztai et al., 2004, Janelsins et al., 2012). The increase in cytokine levels by DOX has been associated with cognitive dysfunction. However, MTX has anti-inflammatory properties and may not cause cognitive decline by this mechanism (Hobl et al., 2010). In addition, Jansen et al in 2005 showed that chemotherapy can also cause anaemia and increases in the inflammatory response (Jansen et al., 2005).

Animal studies have shown that increasing levels of TNF-a can produce oxidative stress and focal areas of inflammation (Joshi et al., 2010). However, blocking antibodies raised against TNF-a, prevent these inflammatory changes (Tangpong et al., 2006). The role inflammation in producing cognitive decline after systemic chemotherapy is still unclear and there is currently a lack of patient studies looking at the long-term effects of a variety of agents on cytokine levels (Monje et al., 2003).

1.4 Adult hippocampal neurogenesis

A persistent reduction in adult hippocampal neurogenesis is one of the possible mechanisms behind the experience of "chemobrain" by patients after treatment with chemotherapy and following section will describe the process of hippocampal neurogenesis.

Though the above mechanisms provide potential explanations as to how chemotherapy can affect neurogenesis and white matter integrity, it is likely that the true process involves a combination of these and others yet to be hypothesized. A number of factors must be taken into account including the type of agent, genetic variations and age when considering the overall effect of chemotherapy on cognition as it pertains to neurogenesis and oligogenesis.

1.5 Stem cells

Stem cells are undifferentiated cells with the ability to undergo symmetric or asymmetric divisions. Symmetric divisions produce daughter cells both of which maintain full stem cell characteristics, while asymmetric divisions produce one new stem cell and one differentiated cell (Brand and Livesey, 2011, Ulloa-Montoya et al., 2005) (Figure 1.3). Stem cells have been classified according to their developmental potential as totipotent, pluripotent, multipotent or unipotent. Totipotency is the ability to form all cell types of the conceptus, including the extra-embryonic and embryonic tissues. Such cells have the capability to produce all cell types as they can form the whole organism (Mitalipov and Wolf, 2009). Pluripotent stem cells are restricted to form a limited number of tissues normally from one of the three germ layers (ectoderm,

mesoderm and endoderm) but not the whole organism (Gage, 2000, Nakada et al., 2011). Pluripotent stem cells can differentiate into many if not all cell types. Multipotent stem cells have a limited differentiation capacity but can still give rise to multiple cells types. Unipotent stem cells, or progenitor cells, can give rise to only one cell type (Leeb et al., 2010). Many adult stem cells remain in a non-proliferative, quiescent state for most of their lifetime, dividing asymmetrically to produce a new stem cell and a progenitor cell committed to a more restricted fate. Division of these cells helps to maintain and repair tissues (Fuchs et al., 2004).

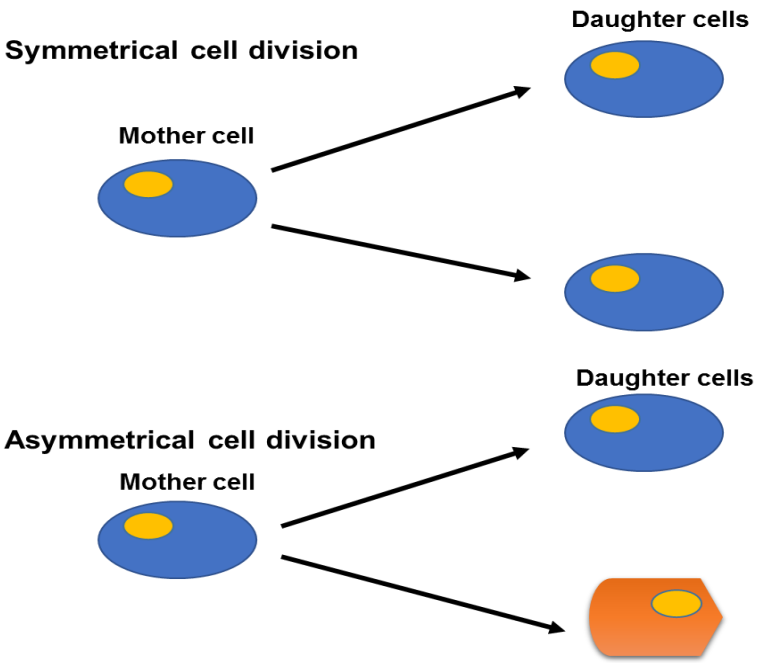


Figure 1.3 Symmetrical and asymmetrical cell divisions. In symmetrical cell division, one mother cell divides to give two identical daughter cells which can either be both stem cells or both daughter cells can start to differentiate. In asymmetrical cell division, one mother cell divides to give one daughter cell identical to mother cell to maintain stem cell numbers and one differentiated cell.

1.5.1 Neural stem cells

NSCs are the fundamental ancestor cells of all neural and glial cells within the nervous system. These cells are multipotent stem cells which have the ability to self-renew and to produce cells which differentiate into neurons, astroglia, oligodendrocytes (Monje, 2008, Palm et al., 2013, Ryan and Nolan, 2016) (Figure 1.4). The discovery, in the 1960s, that NSCs continue to proliferate and generate new neurons in the adult brain provides the possibility to find cures for millions of patients suffering from degenerative disorders (Dougherty et al., 2005). The mechanisms of proliferation and differentiation of these cells are not clear but their continued division and differentiation also makes them susceptible to external factors. It is important to identify NSC in order to study them. *In vitro* they are defined as being able to self-renew and produce neurons and glia while *in vivo* they can be recognised by their radial shape, and the expression of glial fibrillary acidic protein (GFAP) and nestin (Alvarez-Buylla et al., 2002).

Reynolds in 1992, isolated NSC from the striatum of adult mouse brains and demonstrated that they grew as neutrospheres in culture (Guo et al., 2010, Reynolds and Weiss, 1992b). Recent studies have found that NSCs can be obtained directly from neuroectodermal structures or indirectly from embryonic stem cells under appropriate conditions (Yip et al., 2003).

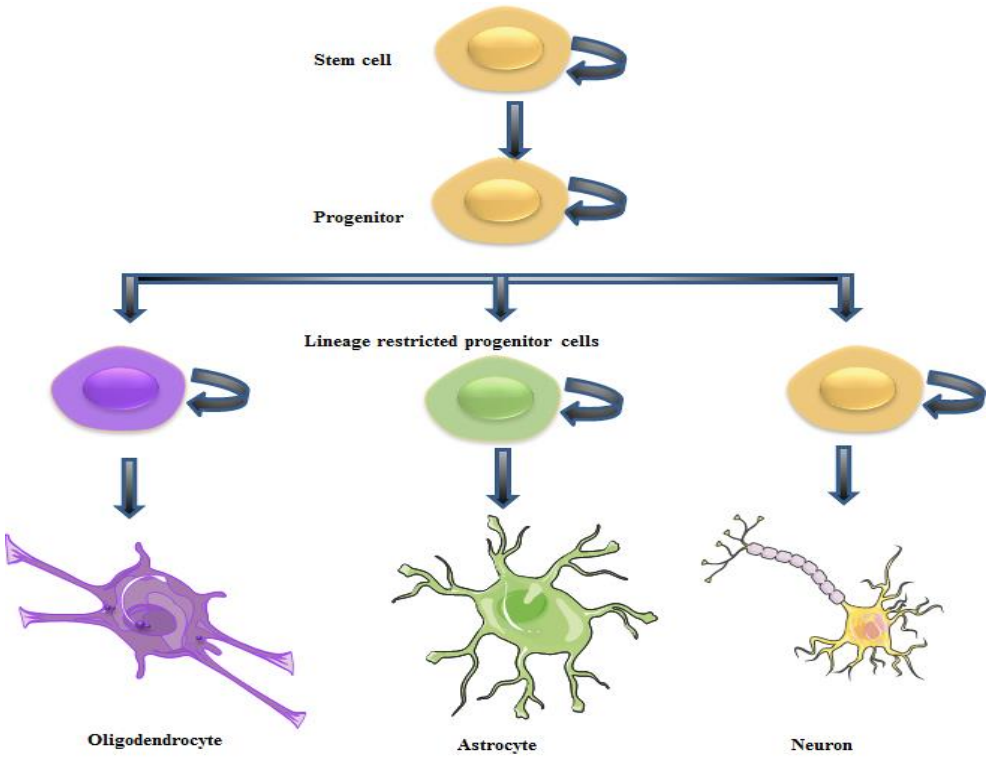


Figure 1.4 Hierarchy of stem cells. The progeny of the NSC is the neural progenitor cell, which differentiates into lineage restricted progenitor cells. These give rise to oligodendrocytes, astrocytes and neurons.

1.6 Adult neurogenesis

Neurogenesis is the process of new neuron formation from NSCs (Ming and Song, 2011). At the beginning of the 20th century it was generally believed that new neurons were produced only during the embryonic and perinatal stages of brain development in mammals (y Cajal, 1959). This view was challenged by Joseph Altman in 1965, when he claimed that newly generated neurons continue to be formed throughout life in specific regions within the brains of rodents (Altman and Das, 1965). These observations were initially ignored until the generation of new neurons was observed in the brains of songbirds (Paton and Nottebohm, 1984) and Altman's observations have

now been confirmed in all mammalian brains including humans (Gould et al., 1997). The two main brain regions, in which adult neurogenesis occurs, are on the inside edge of the blades of the dentate gyrus, the SGZ, within the hippocampus and a strip of tissue in the lateral walls of the lateral ventricles, the subventricular zone (SVZ). Additional regions including the hypothalamus, the olfactory bulbs and the cortex have also been suggested (Lie et al., 2004, Fowler et al., 2008).

During the 1960s and 70s, tritiated thymidine was used to mark dividing cells within the adult brain. After several months, the products of these divisions were identified, on morphological grounds, as neurons. From these observations it was concluded that NSCs were dividing and producing mature neurons in the process of adult neurogenesis (Kaplan and Hinds, 1977, Altman and Das, 1965). In the 1990s, these studies were followed up when tritiated thymidine was replaced by the thymidine analogue 5-bromo-2-deoxyuridine (BrdU), which like tritiated thymidine is incorporated into replicating DNA, but can be combined with immunohistochemical markers of neuronal differentiation. More recently cell proliferation has been quantified by immunostaining for the nuclear protein Ki-67 which is expressed at all stages of the cell cycle in dividing cells , but is absent in resting cells in the G0 cell phase (Kempermann et al., 1997, Kim et al., 2009b, Scholzen and Gerdes, 2000). While the function of this protein is still unknown, Ki-67 was demonstrated to be an effective endogenous mitotic marker for adult-born SVZ cells and has been used to quantify the number of dividing cells *in vivo* and *in vitro* (Shen et al., 2008b, Bullwinkel et al., 2006, Tramontin et al., 2003).

Fred Gage was the first researcher to use BrdU *in vivo* in combination with the neuronal marker NeuN to identify mature neuron production in the dentate gyrus of the

hippocampus of rodents (Kuhn et al., 1996), which has conclusively proved that new neurons are continuously produced in the adult mammalian hippocampus. It is estimated that approximately 9000 new neurons per day are produced in the rat hippocampus (Cameron et al., 1993). The rate of hippocampal neurogenesis varies between species being lower in humans (Gould et al., 1997, Eriksson et al., 1998, Spalding et al., 2013) and mice (Snyder et al., 2009, Kempermann et al., 1997, Jessberger and Gage, 2014) when compared to rats. There are several factors such as age, genetics, excitatory input, ionizing radiation exposure, physiological stimuli, and environmental conditions which regulate the rate of cell proliferation in the hippocampus (Yang and Moon, 2013, Ryan and Nolan, 2016).

Adult neurogenesis has been found in other vertebrates including birds (Barnea and Nottebohm, 1994), reptiles (Lopez-Garcia, 1993), and fish (Kempermann et al., 2003). Of the two main ‘‘neurogenic’’ brain regions in mammals (Venere et al., 2012), the SGZ of the dentate gyrus of the hippocampus and the SVZ of the lateral ventricles, the SVZ is considered the largest neurogenic region. Cells isolated from adult mouse SVZ can give rise to both neurons and glia *in vitro* demonstrating that they are multipotent stem cells (Doetsch et al., 1999) and these give rise to 30-60,000 new neurons per day in the olfactory bulb of rodents (Goldberg and Hirschi, 2009). In both the SVZ and SGZ neurogenesis starts with large astrocyte like stem cells (Dayer et al., 2003) which are similar to subventricular radial glia-like astrocytes found during embryonic brain development. These similarities include morphology and expression of GFAP and the glycoprotein CD133 (Sultan et al., 2013, Basak and Taylor, 2009).

New neurons produced by the division of NSCs in the SVZ join a network of migrating cells called the rostral migratory stream. These cells migrate forward into the olfactory bulb and differentiate into interneurons and oligodendrocytes (Mirzadeh et al., 2008, Taupin, 2006). Cells delivered by the rostral migratory stream replace cells lost through apoptosis in the olfactory bulb. In contrast, neurons produced by NSCs division in SGZ migrate the much shorter distance into the dentate gyrus and differentiate into granule cell neurons which send their axons to the CA3 region of the hippocampus. The production of new granule cells for the dentate gyrus does not replace lost cells but is thought to be important for learning and memory (Bonaguidi et al., 2012, Kohman and Rhodes, 2013).

It is now recognized that the cellular and extracellular environment around NSCs constitutes a specialized niche microenvironment which influences the behaviour of NSCs. In the adult SVZ, the cellular components of this stem cell niche include putative NSCs (type B cells), transit amplifying cells (type C cells), neuroblasts (type A cells), ependymal cells and the vascular endothelium (Conover and Notti, 2008). These interact to control the relatively slow proliferation of NSCs which divide to produce the much more rapidly dividing transit amplifying and progenitor cells (Tavazoie et al., 2008, Ohira, 2011). Type B and Type C cells form a tubular network through which Type A neuroblasts migrate towards the olfactory bulbs while continuing to divide within the rostral migratory stream (Ming and Song, 2011, Shen et al., 2008a).

Contact between particular cell types appears to be important in controlling the proliferation and differentiation of cells during neurogenesis. Proliferating cells are

frequently observed on the surface of small micro blood vessels and this interaction will be discussed in a subsequent section (Shen et al., 2008a).

The process of new neuron production goes through several stages which start with NSC proliferation followed by proliferation of progenitors and migration of differentiating cells to the appropriate location and finally integration into the neural circuitry. All of these steps are regulated by both intrinsic and extrinsic factors (Palmer et al., 1997, Haslinger et al., 2009).

Examples of extrinsic factors include physical exercise, task learning, seizures, and chronic treatment with antidepressants, all of which have been shown to increase the rate of neurogenesis (Seri et al., 2001, Parent et al., 1997, Ryan and Nolan, 2016). Similarly growth factors, neurotrophins, cytokines, and hormones are considered as major up regulators of adult neurogenesis which can increase cell proliferation and differentiation. In contrast, aging, cytotoxic drugs and stress significantly decrease the proliferation of cells in hippocampus (Chesnokova et al., 2016). Adult neurogenesis is also regulated by pathological stimuli such as focal or global ischemia and hypoxia both of which increase neurogenesis. Some of these factors have been tested *in vitro* where they have been shown to promote the survival, proliferation and differentiation of NSCs (Lindvall and Kokaia, 2015, Nakatomi et al., 2002).

Growth factors including fibroblast growth factor (FGF) and epidermal growth factor (EGF) increase proliferation in the SVZ-olfactory bulb pathway and are also thought to be also involved in neuronal differentiation. For example, FGF supports neurogenesis and increases neuron production in the olfactory bulb while EGF instructs SVZ-derived

cells towards an astrocytic fate (Jin et al., 2003). Other growth factors such as brain-derived neurotrophic factor (BDNF) also increase neurogenesis in the olfactory bulb while vascular endothelial growth factor (VEGF) promotes SVZ-derived neuron survival (Ahmed et al., 1995, Jin et al., 2004, Zigova et al., 1998). It is likely that the expression and levels of these growth factors in the immediate environment of neural stem and progenitor cells is important in regulating cell proliferation and survival and that they apply to both SGZ and SVZ.

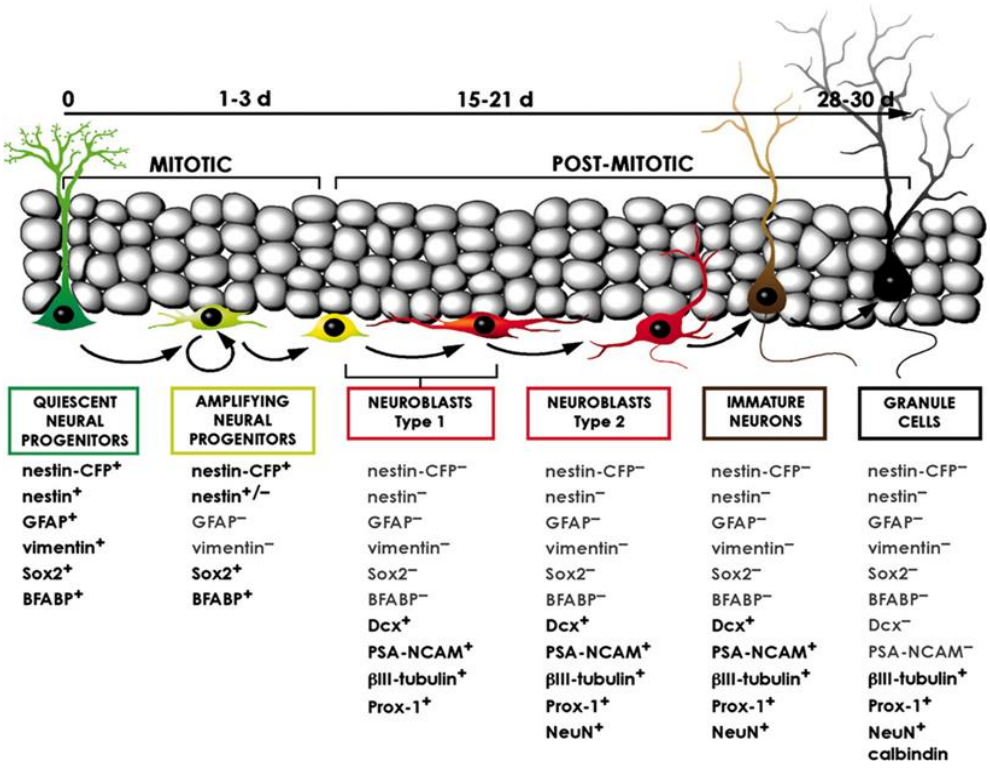


Figure 1.5 Diagram of hippocampal neurogenesis. The dentate gyrus is shown as white ovals and stem and progenitor cells are shown in colour as they progress through differentiation. Neurogenesis starts with the relatively quiescent NSCs (green with red nuclei) which have a radial morphology which extends across the dentate gyrus. Division of NSCs produces amplifying neural progenitors (green with green nuclei). These cells adopt a horizontal morphology within the SGZ and their rapid rate of division produces clusters of mitotic cells. Within 1 to 3 days, these cells start to differentiate and migrate up through the granule cell layer before completing their differentiation into immature granule cell neurons. Further maturation sees them extend dendrites into the molecular layer and axons which grow across the hilus to synapse with cells in the CA3 region of the hippocampus. The whole process of neurogenesis takes 4 to 6 weeks and once integrated, cells can survive for at least several years. Diagram modified from Ekdahl (Ekdahl et al., 2009).

As well as location and morphology, the progress of hippocampal neurogenesis can be followed by the expression of different markers which are restricted to particular stages

of the process. Radial glia-like stem cells, also known as B cells express nestin and the astrocyte marker GFAP (Fukuda et al., 2003). The non-radial transit amplifying progenitors which arise from asymmetric division of NSCs, are highly proliferative and start to express the immature neural marker, doublecortin (Suh et al., 2009). This is a microtubule associated protein which is expressed initially in dividing progenitors and continues to be expressed as they stop dividing and migrate into the granule cell layer. Differentiation into mature neurons is marked by expression of proteins such as NeuN (Toni and Sultan, 2011) (Figure 1.5).

Extensive work over the past 10 years had shown both SVZ and SGZ-derived NSCs can be cultured *in vitro* as free-floating cell clusters, termed "neurospheres". These can be maintained as undifferentiated cells in the presence of EGF and basic fibroblast growth factor (bFGF) or allowed to settle as an adherent monolayer on the cell culture dish surface where they will differentiate. When maintained as neurospheres this behaviour indicates that these EGF-responsive cells have the characteristics of stem cells in that they continue to proliferate, self-renew and can produce differentiated neurons and glia (Reynolds and Weiss, 1992b). Neurospheres contain more than one type of cell and as well as true NSCs it is thought that many of the cells within the neurosphere are transit amplifying neural progenitors (Gage, 2000).

1.6.1 Hippocampus

Hippocampus, this term is derived from the Greek hippokampos: hippos mean horse and kampos refers to the sea (El Falougy and Benuska, 2006) and the human hippocampus has the appearance of a seahorse when dissected out. The hippocampus is composed of different sub regions including the dentate gyrus and the cornu ammonis

(CA), presubiculum, and subiculum. The dentate gyrus has upper and lower blades which enclose the hilus, while the CA region is divided to fields- CA1, CA2, and CA3 (Andersen, 2007) (Figure 1.6). The CA3 region is thought to have a central role in memory formation (Nakashiba et al., 2009, Kesner et al., 2008).

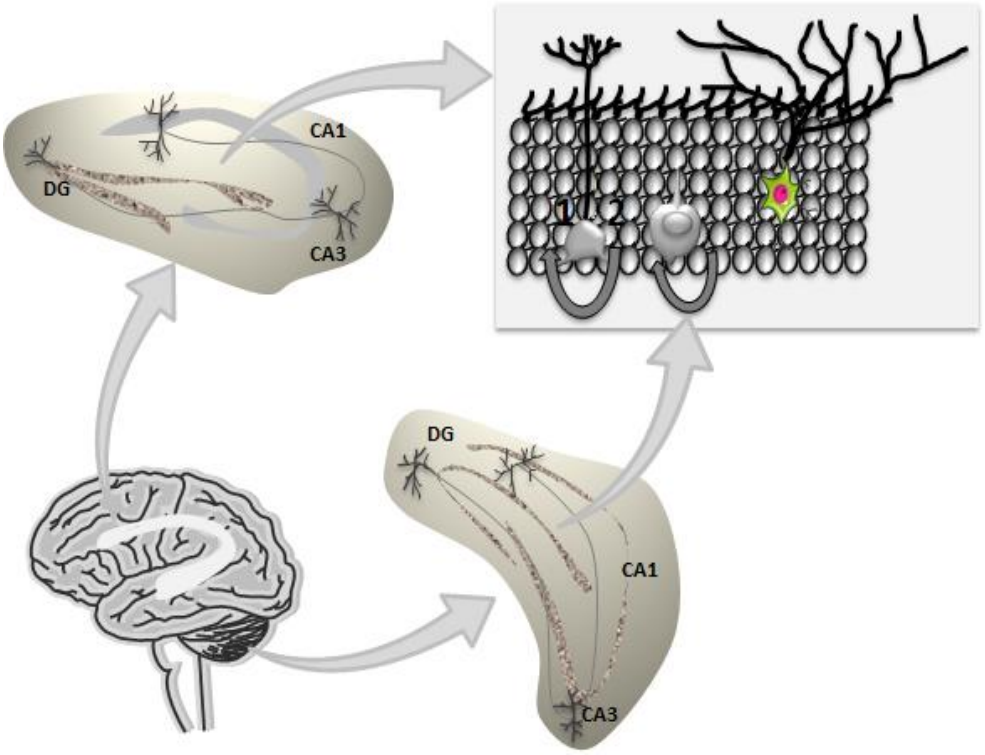


Figure 1.6 The hippocampal circuitry. The hippocampus in the human is located within the temporal lobe, extending axons through the fornix to the mammillary bodies. It is composed of 3 distinct subregions—the DG, CA1, and CA3—each responsible for specific components of information processing, diagram modified from Dias (Dias et al., 2014).

The hippocampus is considered to be a main component of the limbic system and plays a functional role in the formation and recovery of specific types of memory. It also acts to regulate emotion, and as such is thought to be an important brain region involved in depression (Monje, 2008). The dentate gyrus is composed of three layers of cells: molecular, granular and SGZ (Toni and Sultan, 2011). It is in the SGZ that division of

neural stem and progenitor cells produces thousands of new neurons each day (Wilson and Weber, 2013a). It has been known since the 1950s, when the patient HM who had his inferior temporal lobes removed was investigated, that the hippocampus is required for the consolidation of short into long term memories. It is now generally accepted that hippocampal neurogenesis is an essential component of this process (Lyons et al., 2012, Deng et al., 2010). Increasing hippocampal neurogenesis, in rodents, is associated with an improvement in long-term recognition memory (Van Praag et al., 1999, Duan et al., 2008). In contrast to this, factors which reduce hippocampal neurogenesis, including cranial radiation therapy, increasing levels of the gluco- corticoid stress hormones (Abramets et al., 2013) and certain inflammatory states (Monje and Dietrich, 2012) reduce hippocampal dependent memory formation. As described previously, systemic chemotherapy has been associated with memory deficits in both patients and animal models (Ahles, 2012). Systemic treatment with 5-FU reduces the number of proliferating cells in the rat SGZ and produces memory deficits (Mignone and Weber, 2006, ELBeltagy et al., 2010b). As hippocampal neurogenesis is required for the consolidation of long term memory, this is likely to be one of the causes of the cognitive decline experienced by patients after chemotherapy.

1.7 Vascular niches

Schofield (Schofield, 1977) introduced the term ‘stem-cell niche’ for the first time in a review on hematopoietic stem cells (Scadden, 2006). Niches can be defined as a microenvironment which is composed of stem cells and the neighbouring cellular components. In the brain, stem cell niches may include glial, endothelial cells and non-cellular components such as secreted molecules and the extracellular signals that

regulate the behaviour of adult NSCs and their development (Morrens et al., 2012, Yang et al., 2011c).

In the past the vascular system was considered as simply a channel for the passage of blood and nutrients to organs. However, now the vascular system has been identified as a structure providing support to stem cells within different organs which is important for maintaining homeostasis in health and disease (Schildge et al., 2014). Many studies have shown that the vascular system, in addition to providing adjacent cells with nutrients, also provides non-nutritional signals, such as chemokines, growth factors and cytokines that regulate growth, morphogenesis and homeostasis. One distinctive feature of the vascular system is its ability to form specialized 'vascular niches' which influence the behaviour of cells in the immediate surrounding area (Goldberg and Hirschi, 2009). A study by Kamei et al. in 2012 showed that endothelial cells and astrocytes are the main components of vascular neurogenic niches (Kamei et al., 2012). These cells release factors including VEGF, BDNF and bFGF. In addition, direct contact with NSCs enables cell surface receptor and cell surface ligands such as the Notch receptor signalling system to stimulate the self-renewal of NSCs, regulate neurogenesis and niche homeostasis (Imayoshi et al., 2010). Czéh et al. found that in human post-mortem brain samples, low levels of BDNF were associated with low levels of neurogenesis, indicating the essential nature of this factor for neurogenesis (Czéh et al., 2005). The secretion of soluble and membrane bound factors from astrocytes has been found to support the proliferation, and maturation of developing neurons, and improve cell survival during neurogenesis (Barkho et al., 2006) (Figure 1.7). Palmer et al. (Palmer et al., 2000) was the first to give a role to a vascular niche in adult hippocampal neurogenesis. He showed that many dividing cells in the SGZ, which

were known to be neural progenitors, were associated with blood vessels, a feature confirmed by other authors (Sun et al., 2010). It is thought that the neurogenic stem cell niche within the SGZ receives instructions from the vascular system to provide essential support for stem cell activities and also regulates stem cell proliferation, differentiation and fate determination (Ninkovic and Götz, 2007). This association is also found in the SVZ where the vasculature consists of an extensive network of interconnected blood vessels (Hagg, 2005) suggesting it is a general feature of neurogenic regions. In the process of adult neurogenesis, these niches provide a specialized microenvironment that controls the production of new neurons (Lim and Alvarez-Buylla, 1999). In the adult SVZ and SGZ, several main elements are thought to contribute to the stem cell niche and promote neurogenesis. These include direct cell–cell interactions; close contact with blood vessels; and a rich extracellular matrix (ECM) and specialized basal lamina (Ihrie and Álvarez-Buylla, 2011, Calabrese et al., 2007).

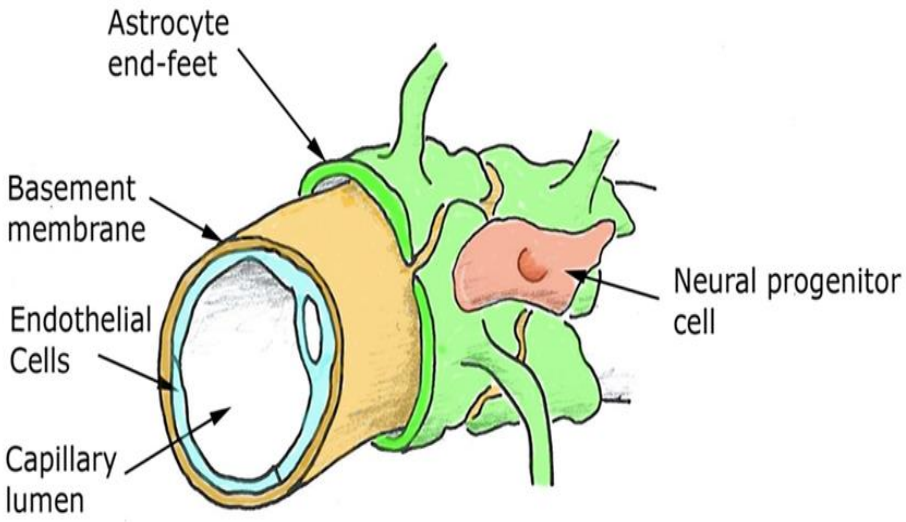


Figure 1.7 Neurovascular interaction. Endothelial cells form the inner cellular lining of blood vessels and these cells sit on a basement membrane. Astrocytes contact blood vessels via astrocyte end-feet and together with endothelial cells make the BBB. Dividing neural progenitor cells in contact with blood vessels could be close to or in contact with any or all of these features.

1.7.1 Role of growth factors

These factors function to regulate some aspects of neuronal differentiation.

1.7.1.1 Vascular endothelial growth factor

VEGF is a hypoxia-induced angiogenic protein that has neurotrophic and neuroprotective effects in the CNS (Greenberg and Jin, 2005). More recent studies have shown that VEGF has a functional role in exerting trophic effects and is protective of neurons because VEGF can enhance neurite outgrowth and the survival of sympathetic

(Khaibullina et al., 2004), dorsal root ganglion (Sondell et al., 2000), and cortical neurons in culture (Jin, Mao et al. 2006). VEGF also protects both HN33 (mouse hippocampal neuron×neuroblastoma) and cortical neurons from cell death induced by hypoxic conditions (Jin et al., 2002). On the other hand, reduced concentrations of VEGF cause the death of cultured cortical and hippocampal neurons (Oosthuyse et al., 2001). VEGF plays an important role in reducing the impact of neurological disorders such as traumatic brain injury, amyotrophic lateral sclerosis, and stroke (Hermann and Zechariah, 2009).

As many dividing cells are in contact with blood vessels in the SGZ (Sun, Zhou et al. 2010), the role of endothelial cells in promoting of neural stem/progenitor cell proliferation by the release of various growth factors has been studied (Louissaint Jr et al., 2002). These studies showed that activation of VEGF receptors, particularly VEGFR-2 (Flk-1) promoted most of the neuronspecific effects of VEGF (de Almodovar et al., 2009). Most studies have observed that pro-neurogenic stimuli increase VEGFR expression in the hippocampus (de Almodovar et al., 2009), for example hippocampal VEGF and VEGFR expression is upregulated by antidepressant treatment (Fournier et al., 2012). In contrast, reduced hippocampal neurogenesis, caused by aging or stress, is linked with reduced levels of VEGF and Flk-1 in the hippocampus. During CNS development, VEGFs secreted by cells which are located in the ventricular zone, stimulate vessels to grow toward them and further support neurogenesis (Ihrie and Álvarez-Buylla, 2011).

1.7.1.2 Brain-derived neurotrophic factor

BDNF belongs to the nerve growth factor family and binds to the TrkB receptor (Kozisek et al., 2008). It enhances the growth and development of immature neurons, as well as the survival and function of adult neurons (Gonul et al., 2005). BDNF can act as an antidepressant, and protects neurons from stress induced damage (Russo-Neustadt et al., 2001) as well as enhancing learning (Hashimoto et al., 2004), angiogenesis and neurogenesis (Boldrini et al., 2012). It is also up regulated by physical activity (Vivar et al., 2012). BDNF can be released by neurons, astrocytes and endothelial cells (Friedman et al., 1998). *In vitro* studies have shown that BDNF secreted by astrocytes, is neuroprotective (Babu et al., 2009). Endothelial-derived BDNF acts as a chemoattractant and a survival factor for neuronal progenitors (Snapyan et al., 2009). Studies of post-mortem human brain tissue have found an increase in BDNF levels in individuals treated with antidepressants (Chen et al., 2001), while *in vivo* studies have found that antidepressant drugs increase serum BDNF levels (De Foubert et al., 2004).

1.7.2 Interactions with other cells – astrocytes, endothelial cells and neurons which influence the rate of neurogenesis

1.7.2.1 Glia

Rudolf Virchow in 1858, introduced the term glia (Verkhratsky and Butt, 2007). Glia were believed to be an accessory cell population that simply supported neurons. However glial cells, which form the largest cell population in the brain, are now known to play many important roles in the CNS (Wang and Bordey, 2008). The term includes astrocytes, micro glia and oligodentrocytes, all of which are associated with adult

neurogenesis in some way (Nedergaard et al., 2003). In most of the adult brain, astrocytes play an essential role in supporting neuron function by supplying energy substrates and removing neural waste including metabolic by products. They also release neurotransmitters during synaptic transmission and have a role in optimizing the environment of neurons by maintaining ion and pH homeostasis (Kim et al., 2011, Chew et al., 2014). Astrocytes often have elaborated processes that make them appear star shaped and are classified into two subtypes protoplasmic and fibrous depending on their cellular morphology and anatomical location. Protoplasmic astrocytes are found in all grey matter (Oberheim et al., 2006). In contrast, fibrous astrocytes can be found in all white matter where they contact nodes of Ranvier. Neuroanatomical reports suggest that both protoplasmic and fibrous astrocytes have close contact with blood vessels (Sofroniew and Vinters, 2010) and communicate with each other via gap junctions (GJs) (Allen and Barres, 2009, Abbott et al., 2006). Astrocytes can be found between the endothelial cells of the brain blood vessels and neurons (Mathiisen et al., 2010) and maintain the BBB by extending astrocyte end-feet onto the surface of blood vessels (Kajitani et al., 2012, Tavazoie et al., 2008).

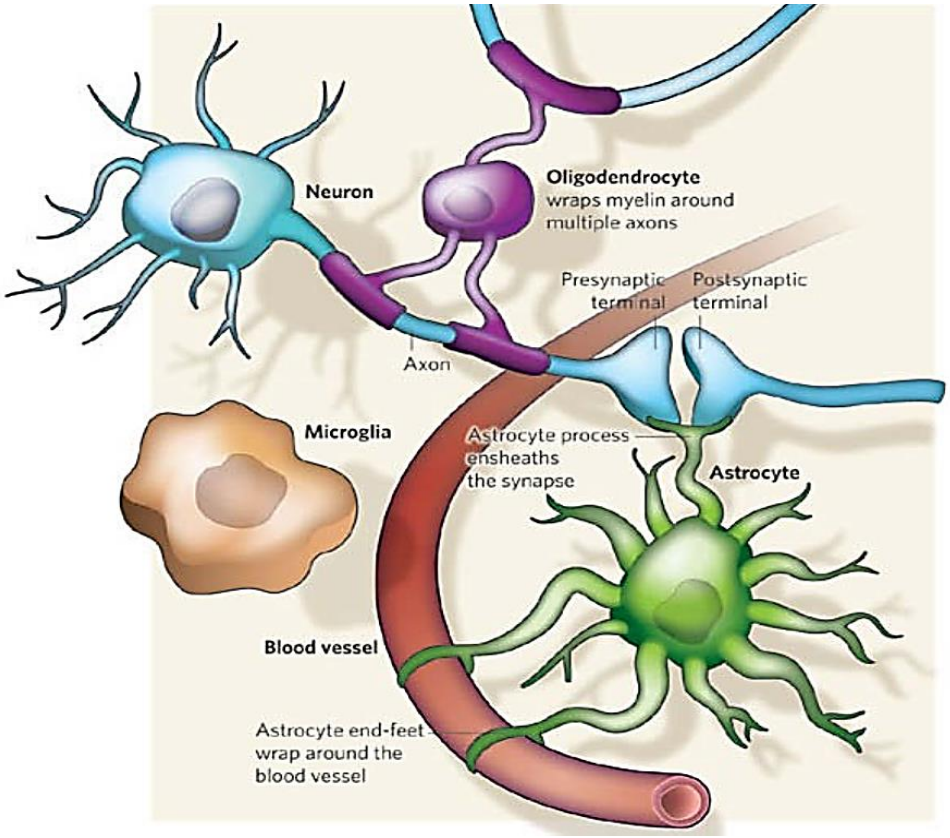


Figure 1.8 Schematic illustration of the cellular organization in the brain. The signal transducing neurons (blue) and their interaction with the major glial cell types including astrocytes (green), oligodendrocytes (purple) and microglial cells (brown). Adapted from Macmillan Publishers Ltd: Nature, (Allen and Barres, 2009).

1.7.2.1.1 Roles of astroglia in the neurogenesis

1.7.2.1.1.1 Astrocytes

Astrocytes are derived during development from NSCs but continue to proliferate in many different regions of adult CNS (Alvarez-Buylla and Lim, 2004). During both embryonic and adult neurogenesis specialised, astrocytes form radial glia-like stem cells which divide to produce new neurons as well as providing a component of the microenvironment for neurogenesis (Környei et al., 2005).

Astrocytes, obtained from the adult hippocampus, when co-cultured with NSCs, increase the proliferation of NSCs and cause them to differentiate into neurons (Taupin, 2006, Suh et al., 2009). However astrocytes derived from non-neurogenic regions such as the adult spinal cord do not have this function (Lie et al., 2002), supporting the view that astrocytes are regionally specified and can play an important role in adult neurogenesis. This role may involve cell-cell contact and / or the secretion of soluble factors (Barkho et al., 2006, Suh et al., 2009, Song et al., 2002).

A number of studies have shown that astrocytes function as sensors and regulators of the CNS microenvironment (Doetsch, 2003). However, the molecular signals associated with astrocytes that regulate the ability of NSCs to self-renew and differentiate are still elusive. One suggestion is the secreted signalling molecule, sonic hedgehog (Shh) which is thought to be act as a morphogen and mitogen in neurogenic regions (Stolp and Molnár, 2015, Jiao and Chen, 2008).

Studies have shown that the C6 glioma cell line has astrocytic properties and expresses GFAP one of the main markers for astrocytes (Naus et al., 1992, Czéh et al., 2005). Therefore, we have used this cell line as well as primary astrocytes in this study. C6 rat glioma cell lines (ATCCCRL-2199) was cloned from N-nitrosomethylurea treated rat glioma cells after a series of animal and culture passages. This cell line expresses the glucocorticoid receptor, S100 protein, glyceryl phosphate dehydrogenase, somatotrphin and GFAP (Benda et al., 1968).

1.7.2.2 Endothelial cells

Endothelial cells form the inner lining of blood vessels and are basic elements of the BBB, (Hirschi et al., 1999) where they regulate the exchange of hormones, metabolites and small molecules between capillaries and the surrounding parenchyma (Cleaver and Melton, 2003). Endothelial cells have the ability to synthesize basal lamina elements and secrete some growth factors such as BDGF, and VEGF that have a crucial role in neurogenesis (Xiong et al., 2013).

The neurogenic regions of the adult mammalian brain have dense vascular plexuses which creates a specific microenvironment for NSCs (Sun et al., 2010). This component of the vascular niche is thought to promote fate specification, differentiation, quiescence, and proliferation of NSCs while endothelial-secreted factors play a role in the survival/self-renewal and migration of NSCs (Cleaver and Melton, 2003, Shin et al., 2014).

Endothelial cells, when co-cultured with NSCs increase neurite outgrowth, maturation and promote neuronal migration and enhance self-renewal via symmetric cell division which produces large numbers of nestin^{+} precursors (Shen et al., 2004, Goldberg and Hirschi, 2009). Neural stem or progenitor cells taken from the stroke boundary, when cultured with cerebral endothelial cells show a significant increase in their proliferation, neuronal differentiation and capillary tube formation. This is thought to be due to the release of specific growth factors, such as pigment epithelium-derived factor (PEDF) or VEGF by the endothelial cells (Guzman et al., 2008). Adding conditioned media (CM) from endothelial cells to NSCs promotes their differentiation into neurons and

astrocytes. These results indicate that endothelial cells can play a significant role in neurogenesis (Paris et al., 2001).

1.8 The involvement of neurogenesis in learning and memory

It is thought that neurogenesis plays a significant role in stress, depression and learning and memory (Aimone et al., 2010, Dranovsky and Hen, 2006). For example, rodent studies have shown that neurogenesis in the SGZ is reduced by stress (David et al., 2010, Mirescu and Gould, 2006). Hippocampal neurogenesis provides the cellular plasticity which is required for the formation of trace memories. Rats exposed to cranial irradiation (Yau et al., 2015, Duan et al., 2008) show reduced hippocampal neurogenesis and poorer performance in hippocampus-dependent tasks in particular spatial learning tested in the MWM (Madsen et al., 2003, Snyder et al., 2005). Studies have shown the involvement of neurogenesis in situations involving stress, depression and learning and memory (Aimone et al., 2010, Dranovsky and Hen, 2006). Rodent studies showed that neurogenesis in the SGZ is reduced by stress caused by factors such as isolation, footshock or physical restraint (David et al., 2010, Mirescu and Gould, 2006).

In humans there are fewer studies about the function of adult neurogenesis but these confirm the role of the hippocampus in learning and memory, and the deficits produced by cranial irradiation (Maguire et al., 1996).

During the process of neurogenesis a certain amount of cell death occurs as neural progenitors differentiate. This appears to be important in the formation of new circuits

and memory formation (Abrous et al., 2005) but excessive cell death is correlated with poorer cognition.

1.8.1 Impact of chemotherapy on hippocampal neurogenesis

Human clinical and animal model studies have found that treatment with chemotherapy or irradiation impacts both normal and cancer cells including the neural precursor cells, which are found in both the SGZ and SVZ of adult brains (Noble, 2000, Yang and Moon, 2013). Even at low doses chemotherapy increases cell death and decreases cell division in the SVZ, SGZ and corpus callosum of mice and rats (Dietrich et al., 2006a, Mignone and Weber, 2006, Han et al., 2008). Chemotherapy also, affects non-dividing mature oligodendrocytes and with time may lead to demyelination (Seigers et al., 2009a). *In vitro* studies have shown that neural cells are susceptible to chemotherapeutic agents even at doses below that required to kill tumour cells (Dietrich et al., 2006a, Mignone and Weber, 2006). This difference in sensitivity between different cell types may relate to the degree of mitochondrial respiration that is present within these cells and this will be investigated in this project by measuring the oxygen consumption rate.

The effect of 5-FU on cell proliferation and cell survival during hippocampal neurogenesis has been investigated immediately after treatment and six weeks after the end of treatment. The findings showed a progressive decline in the rate of cell division and the survival of newly generated cells after treatment had stopped (ELBeltagy et al., 2012).

In vivo studies on using chemotherapeutic agents such as carmustine, cisplatin, 5-FU, thioTEPA (triethylenethiophosphoramide) cyclophosphamide, paclitaxel, DOX and cytarabine have shown a persistent suppression of cell division in the SVZ, corpus callosum and dentate gyrus (Dietrich et al., 2006a, Mustafa et al., 2008b, Mignone and Weber, 2006, Janelsins et al., 2010). The results of animal studies have shown that chemotherapy impairs learning and memory functions which require the hippocampal and frontal lobes of the brain (Winocur et al., 2011).

Recent results from our group have confirmed that many proliferating cells in the SGZ are in close contact with small blood vessels (Figure1.9). After chemotherapy, proliferating cells in contact with blood vessels, survive better than cells some distance from blood vessels (Maqbool PhD thesis, 2015). This suggests that association with blood vessels provides protection from chemotherapy. In the present project I have used a number of *in vitro* models to study the protective effect of different combinations of neural and non-neural cells.

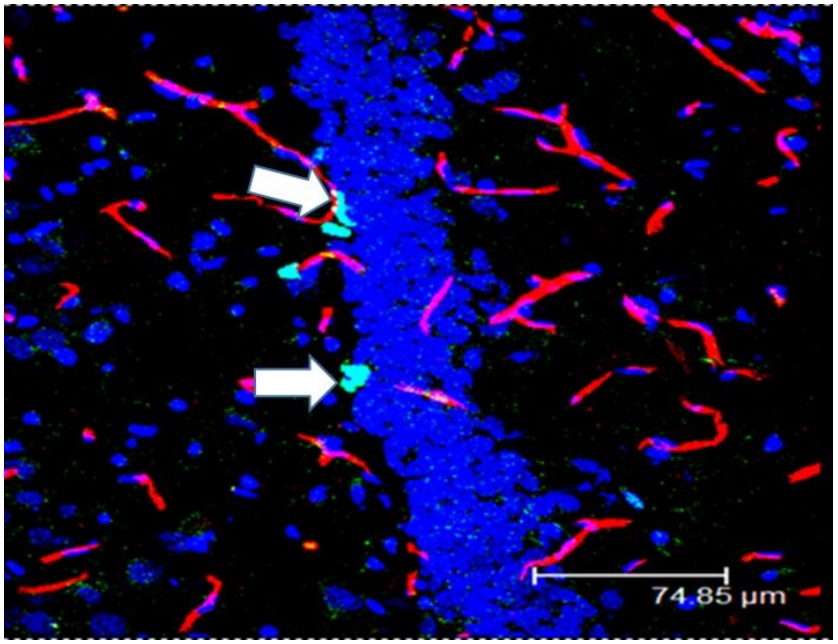


Figure 1.9 Section of hippocampus showing the dentate gyrus: (Blue/ DAPI); blood vessels (RECA Red); dividing cells (Ki67 Green). The upper arrow shows dividing cells on the surface of a blood vessel while the lower arrow shows dividing cells some distance from blood vessels, Treatment with 5-FU causes loss of cells in the later position but spares dividing cells which are in contact with blood vessels (picture courtesy of Dr Mqbool).

1.8.2 Is it possible to protect hippocampal neurogenesis from chemotherapy?

A number of cognitive enhancers have been tested on cancer patients and animal models treated with chemotherapy. Erythropoietin (EPO) a glycoprotein hormone which enhances the generation of red blood cells within the bone marrow has been tested as a neuroprotectant after brain trauma, stroke and epilepsy. EPO improving anemia induce by chemotherapy and radiation (Rosenzweig et al., 2004). It appears to reduce inflammation and oxidative stress as well as inhibiting apoptosis (Ponce et al., 2013, Subirós et al., 2012) and improves hippocampal dependent memory in both human and animal subjects (Ransome and Turnley, 2007, Adamcio et al., 2008, Brines

et al., 2000). Administration of IGF-1 before and following a high dose of cyclophosphamide in animals significantly improved neural cell proliferation (Janelsins et al., 2010).

Moreover, EPO has been shown to improve cognition in a small group of cancer patients with anemia (Massa et al., 2006, Rosenzweig et al., 2004), and an *in vivo* study by Bianchi, showed that EPO prevented the decreases in nerve conduction velocity induced by cisplatin (Bianchi et al., 2007).

1.8.2.1 Exercise

Physical exercise improves cognition after cognitive decline (Cotman et al., 2007) and increases hippocampal neurogenesis (Van Praag et al., 2005). Providing animals with exercise wheels prevents the cognitive decline found after treatment with 5-FU and oxaliplatin (Fardell et al., 2012).

1.9 Antidepressants

There are several types of antidepressant including tricyclic antidepressants, serotonin selective reuptake inhibitors (SSRI), and lithium. The chronic administration of antidepressants increases cell proliferation and neurogenesis in the SGZ (Sairanen et al., 2005). Antidepressants appear to be able to counteract the negative effect of stress on neurogenesis. The mechanisms behind this are unclear but chronic antidepressant treatment increases expression of cAMP response element binding protein (CREB), BDNF, bcl-2 and microtubule-associated protein (MAP) kinases that are known to increase cell proliferation and survival pathways (Vinet et al., 2004).

1.9.1 Fluoxetine

The SSRI antidepressant FLX was introduced in 1974 (Fuller et al., 1974) and has become one of the first choices for depression treatment. FLX acts mainly by inhibiting the reuptake of serotonin (De Foubert et al., 2004) but also increases the extracellular concentrations of serotonin, dopamine and norepinephrine in the hypothalamus, cortex, and prefrontal cortex (Bymaster et al., 2002). FLX, can reduce inflammation and prevent apoptosis in the brain (Encinas et al., 2006, Lim et al., 2009).

Studies in our lab on rodents given the chemotherapy agents 5-FU or MTX have shown that co-treatment with FLX prevents the reduction in neurogenesis and cognition normally found with chemotherapy treatment (Lyons et al., 2011).

The exact mechanism by which FLX up-regulates hippocampal neurogenesis is still uncertain, but it has been suggested that it increases the proliferation of rapidly dividing type 2 progenitors in the SGZ while leaving the slowly dividing NSC unaffected (Encinas et al., 2006). Antidepressant treatment increases the expression of BDNF in limbic structures, especially the hippocampus (Russo-Neustadt et al., 2001). As a result, this can lead to an increase in the rate of neurogenesis and the survival of new neurons (Malberg et al., 2000). Therefore, we looked at FLX to determine whether this antidepressant could be protective to neural cells exposed to chemotherapy.

1.10 Development of *in vitro* co-culture model

It is possible to mimic aspects of *in vivo* tissue conditions by co-cultures consisting of more than one cell type including primary cells and cell lines. This can be done when

the cell types are in contact or separated by a permeable membrane which prevents cell contact but allows substances to pass between the cells. This type of co-culture model has been used to study the effects of soluble, and secreted products on adjacent cell types (Dodson et al., 1997). In this study we were interested in whether protection from chemotherapy can be provided by non-neural cells found in the neurogenic niche and if so, whether this involves direct or indirect cell to cell communication.

1.11 Aims and objectives

Chemotherapy has been found to cause long term cognitive decline in a proportion of patients receiving this treatment. It has been found that chemotherapy reduces hippocampal neurogenesis, which is known to be required for aspects of memory processing. The aim of this thesis was use *in vitro* techniques to study the impact of the chemotherapy agent 5-FU on NSCs and other cell types in the neurogenic niche. This was followed by investigation into the interactions between neural cells and astrocytes and endothelial cells. The investigation started by measuring the relative sensitivity of each cell type within the neurogenic niche. This was followed by co-culturing of neural cells with either endothelial or astrocytes to determine if co-culture would protect NSCs from the effects of 5-FU chemotherapy. Having demonstrated protection, it was investigated whether cell contact was required for protection. These experiments were completed by examining the role of GJs in protecting NSC from chemotherapy.

In parallel with these experiments we used fluorescent cell sorting to investigate the effect of 5-FU on apoptosis, DNA damage and cell cycle markers in all the cell types used. Chemotherapy may affect metabolism and this may relate to different sensitivity of different cell types. This investigated by measuring mitochondrial function.

The objectives of these investigations were:

1. To determine the relative sensitivity of NSCs and other components of the neurogenic niche (using both primary and cell lines of endothelial and glial cells) to the chemotherapy agent 5-FU using MTT assays.

2. To determine if a single culture media recipe can be used for neural, endothelial and glial cells.
3. To determine the influence of cellular interactions between neural cells, astrocytes and endothelial cells in protecting neural cells from chemotherapy. This was followed by studying whether cell-cell was required to enable protection.
4. To determine whether astrocytes from spinal cord and hippocampus respond differently to chemotherapy and have different abilities to protect neural cells.
5. To test whether the antidepressant FLX is neuroprotective to NSCs.
6. To test whether CM of FLX treated C6 astrocyte cells are protective against chemotherapy agents.
7. To investigate whether GJs are present between neural cells and other cells in co-culture.
8. To block GJs and see if this effect the protection provided by other cell types in co-culture
9. To measure the effect of chemotherapy in causing DNA damage and apoptosis and changing cell cycle parameters in different cell types.
10. To use oxygen consumption as a measure of the impact of chemotherapy on different cell lines.

The outcome of this project should provide new information on how chemotherapy affects hippocampal neurogenesis and how glia and endothelial cells may modify this response.

2 Chapter The sensitivity of different cell types within the hippocampal stem cell niche to chemotherapy

2.1 Introduction

In this chapter the effects of 5-FU on the viability of primary cells and cell lines from different tissues was determined. These were C6 cells, an astrocyte cell line; primary hippocampal astrocytes; N2a cells, a neural cell line; primary rodent NSCs; Human brain microvascular endothelial cells (HBMECs) an endothelial cell line and a mouse cell line not from the hippocampal stem cell niche, 3T3 fibroblasts, which were used as a control. Our hypothesis was that while chemotherapy would impact all cell types; there would be differences in their sensitivity to chemotherapy. Co-culture of other cell types with NSCs, might reveal protective effects against chemotherapy. In this way association between NSCs and other cell types in the neurogenic niche may modulate the effects of chemotherapy on hippocampal neurogenesis.

These ideas are based upon previous work from our laboratory which showed that 5-FU significantly reduces the proliferation of hippocampal NSCs in the SGZ of the rat dentate gyrus (ELBeltagy et al., 2012). This results in a decrease in hippocampal neurogenesis which is associated with a decline in spatial memory in animals treated with chemotherapy (Lyons et al., 2011, Mustafa et al., 2008b, ELBeltagy et al., 2012), a situation which is likely to replicate the cognitive decline experienced by many patients after chemotherapy (Wigmore, 2012). However not all dividing cells in the SGZ of the dentate gyrus are equally affected by chemotherapy. Cells closely associated with capillaries in the SGZ appear to be spared from the effects of chemotherapy and continue to divide (Maqbool PhD thesis, see Figure 1.9).

2.1.1 Cell types used

2.1.1.1 N2a

In the late 1960's, the N2a cell line was derived from a spontaneous mouse neuroblastoma (Goshima et al., 1993, Klebe and Ruddle, 1969). These cells are small flattened and adherent. Under normal culture conditions they divide rapidly but when cAMP is added, the cells differentiate into mature neurons (Wu et al., 2009, Tremblay et al., 2010, Munoz et al., 2003). Although when reimplanted *in vivo* they form tumours (Augusti-Tocco and Sato, 1969), they are frequently used as a model of NSCs (Hargreaves et al., 2006, Fowler et al., 2001). N2a cells have been used to study neurodegenerative diseases and neurotoxicity (Alais et al., 2008, Lepage et al., 2014b). In this study mouse primary hippocampal NSCs were also used as a comparison to determine which cell type is more sensitive to 5-FU.

2.1.1.2 Primary hippocampal NSCs

Primary hippocampal stem cells were isolated from the brains of neonatal mice and cultured as floating balls of cells (neurospheres) (Doetsch, 2003, Gritti et al., 2008). Although maintaining NSCs as neurospheres prevents their differentiation into neurons, the *in vitro* culture of neurospheres has its own challenges as the neurosphere contains a mixture of stem cells, progenitor cells, and more differentiated cells (Lobo et al., 2003, Suslov et al., 2002, Bez et al., 2003). However, regular subculture of neurospheres and addition of the growth factors such as EGF and bFGF stimulates proliferation, prevents differentiation and maintains the cells in state similar to proliferating neural stem and progenitor cells in the SGZ (Galli et al., 2003, Xiong et al., 2011). Removal of these

growth factors initiates differentiation (Reynolds and Weiss, 1992b, Vukicevic et al., 2010). As N2a cells are mouse-derived, we have used primary NSCs derived from mouse hippocampus. Primary NSCs were subjected to the same experiments as the N2a cells in order to make comparisons between them as recent studies by Dietrich and Han (Han et al., 2008, Dietrich et al., 2006a), using the cytotoxic agents Ara-C and 5-FU found that primary cells were more sensitive than transformed cells (Han et al., 2008, Dietrich et al., 2006a).

2.1.1.3 Astrocyte cell line (C6)

The C6 rat glioma cell line derives from a N-nitrosomethylurea-induced rat glioma (Benda et al., 1968). C6 cells have been widely used as a model of astrocytes, because they express many of the receptor types, ion channels, and transport systems which mimic that of astrocytes (Brismar, 1995).

2.1.1.4 Primary astrocytes

Comparing primary astrocytes with an astrocyte cell line provided an oportunity to determine the response of these cells to chemotherapy (McCARTHY and De Vellis, 1980). Primary cells are generally regarded as being closer than immortalized cell lines to the *in vivo* situation (Saura, 2007). It is also important to realize that there may be region-dependent differences in the astrocytic phenotype that could have a profound impact on the behaviour and interactions of these cells. For this reason both primary hippocampal and primary spinal cord derived astrocytes obtained from neonates were used.

In vitro, astrocytes have been shown to support other glial populations and neurons. For example astrocytes in neuronal cultures buffer glutamate and thus make neurones resistant to high concentrations of external glutamate (Rosenberg and Aizenman, 1989). In addition, astrocytes secret trophic factors that can support motor neuron survival and stimulate stem cell differentiation into neurones (Song et al., 2002, Albrecht et al., 2002). Astrocytes have been shown to make cancer cells resistant to chemotherapy by forming GJs between these cell types. Astrocytes are thought to buffer calcium ion levels in cancer cells and to reduce calcium levels in cancer cells by allowing the flow of ions into the astrocytes (Lin et al., 2010, Chen et al., 2015).

Astrocytes are a common non-neuronal cell type present in neuronal primary cultures (Banker, 1998, Petters et al., 2014). To obtain pure astrocyte cultures, a commonly used method is the "shaking method". With this protocol, a primary mixed glial culture obtained from 1-2 day old neonatal rats is agitated to remove microglia and oligodendrocytes and obtain a high concentration of pure astrocytes. An alternative method uses enzymes for tissue dissociation (Quintana et al., 2011, Raff et al., 1979). In this thesis, this method was modified in three ways for the isolation of astrocyte cultures from 1-2 day neonatal rats as described in the material methods.

To assess the purity of the astrocytes obtained using these methods, cultures were immunostained for the astrocyte marker, GFAP. GFAP is a cytoskeletal intermediate filament subunit frequently used as specific marker for astrocytes (Kivell et al., 2000) and is frequently used as a marker to quantify the proportion of astrocytes in primary cultures (Kivell et al., 2000, Velasco et al., 2003, Petters and Dringen, 2014).

2.1.1.5 Fibroblast (3T3)

The mouse fibroblast cell line 3T3 (Todaro and Green, 1963) was derived from Swiss mouse embryos and has been used widely to study the control of cell division and viral transformation in tissue culture (Aaronson and Todaro, 1968). They may be proliferated indefinitely in serial culture and stop growing when they reach confluence but remain viable in a resting state for long periods (Green and Kehinde, 1974). In the present study, we used 3T3s as non-neural controls to compare with other cells from the neurogenic niche.

2.1.1.6 Human brain microvascular endothelial cells (HBMECs)

HBMEC cells, isolated from human brain, were obtained from ScienCell Research Laboratories and used at low (<10) passage numbers. HBMECs display a typical cobblestone appearance in monolayer culture with large dark nuclei. These cells are checked for purity and that they were the expected cell type, by expression of the endothelial cell specific markers Von Willebrand Factor, Occludin and ICAM-1using western blotting (carried out by Dr Beazley-Long, School of Life Sciences, University of Nottingham).

2.1.2 Characterisation of suitable cell culture medium for all cells

As the different cells used in this project are normally grown in different cell culture media, it was necessary to establish a common media which suited all cell types so that co-culture studies could be carried out. This was further complicated by the fact that

primary NSCs which are maintained as floating neurospheres required a specific substrate to attach to such as laminin.

DMEM or RPMI-1640 cell culture media are recommended for most cells. RPMI-1640 is richer than DMEM and recommended for HBMECs (Elmorsy and Smith, 2015, Elsheikha et al., 2013, Lin et al., 2013). To determine whether using this type of media affected our cell lines, a cell viability assay (MTT) was used to compare cell viability in DMEM and RPMI- 1640. Different cells exhibit different morphologies, and all cells were examined to check for morphological changes when different media was used. Based on these studies we selected RPMI-1640 as a suitable culture medium for all cell types. Use of this single media enabled us to culture all our cell lines in one medium.

2.2 Materials and methods

2.2.1 Cell culture

All cell cultures were performed under sterile conditions in a class II laminar flow cabinet (Thermo scientific, UK). All cell lines were cultured under standard cell culture conditions at 37 °C and 5 % CO2 until 80-90 % confluent.

Cell lines were cultured in growth media [10% fetal bovine serum (FBS) in RPMI-1640 or DMED, L-glutamine (at a concentration of 200mM) and 100 units/ml penicillin / streptomycin (Pen/Strep) (Sigma Aldrich UK)]. 50 ml of FBS,5ml of 200mM L-glutamine (final 2mM) and 5ml of NEAA were added to a 500ml bottle of DMEM via 0.22 μm filters (Sartorius) and stored at 4ºC. Aliquots of 50ml of FBS were prepared and stored at -20ºC. A solution of 10X trypsin/EDTA (0.5g/l porcine trypsin and 0.2g/l EDTA) was purchased also sterile from Sigma Aldrich, and 1 ml aliquots of

trypsin/EDTA were prepared and stored at -20 ºC. Each time when required one aliquot was diluted in phosphate buffered saline (PBS, Sigma Aldrich) at 1ml in 9ml (final concentration of trypsin 0.05g/l, EDTA at 0.02g/l. Growth medium should refreash every 3 days until ~90% confluent, and then passaged. While, mNSCs were grown in NSC maintenance medium [DMEM/F12 and Neurobasal Medium in 1:1 ratio, bFGF (Invitrogen/Fisher, PHG0026), EGF (Sigma-Aldrich, E9644), Glutamine, N2, and B27 supplements (gibco by Life technology, UK) and Pen/Strep] in 25 cm2 non treated flasks and left in the incubator at 5% CO2, 100% humidity, 37C° for several days. The above components were kept as stock and EGF and FGF (both 20ng/ml) were added fresh prior to use).

2.2.1.1 Cells passage (subculture)

Cells at 80-90% confluence were dissociated using trypsin–EDTA (Sigma Aldrich, UK). The medium was aspirated, and the cells washed with phosphate buffered saline (PBS) (Sigma Aldrich, UK) to remove FBS, which would inhibit the activity of the trypsin. Then 2 ml of pre-warmed trypsin–EDTA solution was added, the flasks were returned to the incubator for 5 minutes at 37 °C. After incubation, to dislodge the cells, the flask was gently rocked. Then 5ml of culture medium plus 10% FBS were added to block further trypsin activity and dissociate the cells into a suspension. This was centrifuged at 1000 rpm for 5 minutes. The supernatant, containing the trypsin, was aspirated and the cells re-suspended in 5 ml of fresh growth medium (pre-warmed to 37 ºC). Finally, the cell suspension was transferred to a new flask, either a T25 (with 5 ml fresh media) or T75 (with 15 ml fresh media) with a 1:5 dilution in fresh media. If the

cells are to be used in an experiment, they were counted (Bio-Rad, cell counter Hemel Hempstead, UK) and plated out onto suitable plates.

2.2.1.2 Cryopreservation of cells

Confluent, cells were trypsinised and counted as described above. After centrifugation for 5 minutes at 1000 rpm, the supernatant was aspirated. The pellet gently resuspended in 1 ml of freezing solution, [90 % (v/v) FBS and 10 % (v/v) dimethylsulphoxide (DMSO)] and counted. DMSO (Sigma, D2650, USA) was used as a cryoprotectant against ice crystal formation. Each 1×10^6 cells were placed in pre-labelled cryo-vials (Sarstedt, UK) which were cooled slowly in a container of isopropanol at -80 °C overnight and were transferred to liquid nitrogen for long term storage.

2.2.1.3 Resuscitation of cells from liquid nitrogen

Frozen vials of cells were removed from the liquid nitrogen store (Dewar Dilvac, UK), the top of the vial was loosened to release the pressure of any evaporating nitrogen. The cells were thawed quickly in a water bath at 37 °C. Before opening the tube, the outside of the vial was wiped with 70 % IMS. The thawed cells were transferred to a sterilin tube containing approximately 10ml of growth medium, centrifuged at 1000 rpm for 5 minutes and, the supernatant aspirated. Cells were resuspended in approximately 1ml of growth medium and directly transferred into the appropriate culture flask. The growth medium was changed after 24 hours to remove any residual DMSO.

2.2.2 Counting cells

To evaluate total cell number and the percentage viability, a haemocytometer (Hawksley, UK), trypan blue (Sigma Aldrich, RNBF 2697, UK) and an automated cell counter was used.

Trypan blue a vital stain which stains nonviable cells blue was used to distinguish living and dead cells when counted on a haemocytometer slide. A TC20 automated cell counter (Bio-Rad, Hemel Hempstead, UK) was also used to assess cell viability by suspending cells in an equal volume of trypan blue solution (10µl of 0.4% trypan blue). This mixture was loaded into the manufacturer's slides and counted.

2.2.3 Cell types

A number of different primary and immortal cell lines were used for this project.

2.2.3.1 Culturing and proliferation of cell lines

2.2.3.1.1 Mouse neuroblastoma (N2a) cells

N2a cells are an immortal mouse neuroblastoma cell line which are adherent and grow as a monolayer on uncoated cell culture plastic (European Collection of Cell Cultures). They were originally maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS), L-glutamine (2mM) and penicillin / streptomycin (Pen/Strep) [(100 U/ml and 100mg/ml, respectively) (Sigma Aldrich)].

2.2.3.1.2 Human brain microvascular endothelial cells (HBMECs)

HBMECs (ScienCell Research Laboratories) were used at passages 15–35. Cells were maintained in completed RPMI 1640 medium (Sigma-Aldrich) containing 10% FBS, 2mM glutamine,1 mM sodium pyruvate (Sigma-Aldrich), 1× nonessential amino acids (gibco), 1×MEM vitamins, and 100 units/ml Pen/Strep (Sigma-Aldrich) as previously described by (Elsheikha et al., 2013).

2.2.3.1.3 Fibroblast (3T3)

Fibroblasts (3T3) were kindly provided by Monika Owen, (FRAME Laboratory, University of Nottingham) and initially grown in DMEM with 10% FBS, 2 mM L-glutamine and 100 units/ml Pen/Strep (Figure 2.1).

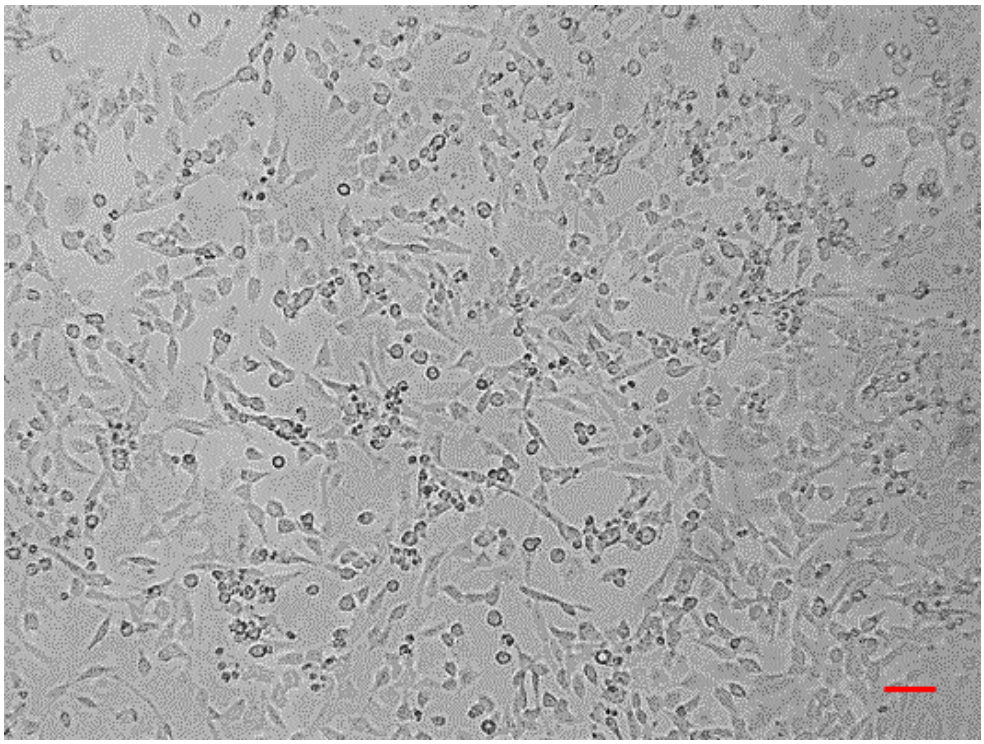


Figure 2.1 Phase contrast image of 3T3 cells grown in monolayer. Scale bar: 40 µm.

2.2.3.1.4 Glioma cell lines (C6)

The C6 rat glioma cell line was obtained from the American Type Culture Collection (Rockville, MD. U.S.A.). These cells are adherent and when grown in the T-75 cm2 flasks, they form stable monolayers. C6 cells were initially cultured in DMEM with 10% FBS, 2 mM L-glutamine and 100 units/ml Pen/Strep.

2.2.3.2 Primary cells

2.2.3.2.1 Culturing of primary mouse hippocampal NSCs

These cells were kindly provided by Annabelle Chambers, (University of Nottingham). Frozen NSCs which had previously been isolated from mouse hippocampus, were removed from liquid nitrogen and thawed rapidly at 37˚C for 2-3 minutes. Cells were resuspended with 5ml pre-warmed NSC medium consisting of [DMEM/F12, Neurobasal Medium, N2 supplement, B27 supplement (gibco by Life technology, UK), Pen/Strep, with addition of EGF (Sigma-Aldrich, E9644), FGF-2 (Invitrogen/Fisher, PHG0026)] and centrifuged at 1000rpm for 5 minutes. The supernatant was aspirated and 1ml NSC maintenance medium was added and the pellet resuspended, and the cells counted before transfer to untreated T25 tissue culture flasks, NSC medium added and cultured at 37˚C, and 5% CO_2. NSCs between passages 5-12 were used in all studies. Hippocampal stem cells were cultured as free-floating neurospheres with media changes every 4-5 days. Care was taken to maintain small neurospheres to retain as high a proportion of immature cells within the neurospheres as possible (Suslov et al., 2002), as shown in Figure (2.2). The solution was transferred to a 30ml tube, followed by centrifugation at 1000 rpm for 5 minutes. Supernatant was aspirated, and the pellet

resuspended with 1ml StemPro Accutase (gibco by Life Technologies) per T25 flask and tritureated to a single-cell suspension. Cells were agitated every 5 minutes to prevent the neurospheres settling down and kept at 37°C. After 15 minutes, 10ml of fresh neurobasal media was added to stop the action of accutase, followed by centrifugation at 1000rpm for 5 minutes. NSCs were also cultured as an adherent monolayer by plating the cells in tissue culture flasks or plates precoated with laminin (Palmer et al., 2000), as shown in Figure (2.2).

A B

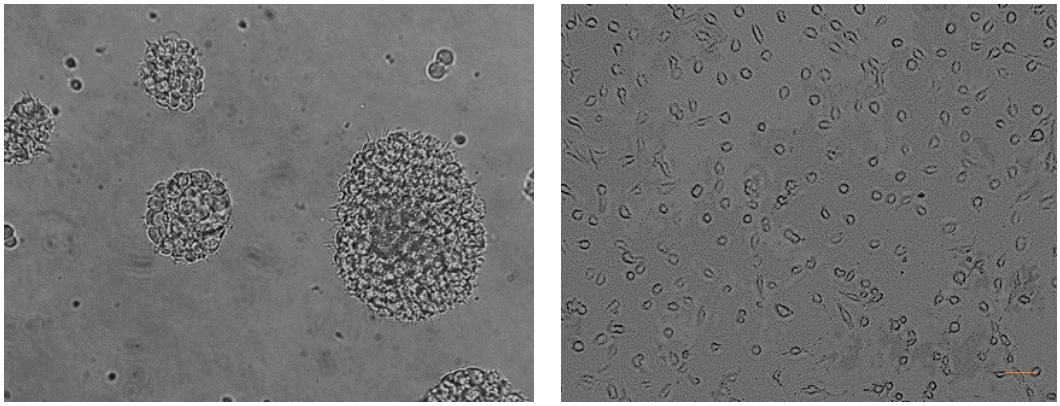


Figure 2.2 Photomicrographs of mouse hippocampal NSCs in culture. Floating NSCs cultured in serum-free medium in the presence of bFGF and EGF, neurosphere is shown at a higher magnification, 25X (A). NSCs after seeding them on coated wells, Laminin (B). Scale bar 50 μm.

2.2.4 Optimisation of the primary astrocytes culture method

Three different protocols were used for purification of astrocytes from the hippocampus. Collagenase protocol: Primary astrocytes were isolated from the hippocampi of 1-2 day old neonatal rats. Brains were removed from the skull meninges

and blood vessels were carefully removed using fine forceps and the hippocampi dissected from the brains of 8 animals. Tissue was minced into small pieces, transferred to 5ml of chilled sterile media Leibovitz medium (L-15) containing gentamycin, triturated slowly ten times using a Pasteur pipette and then incubated with 8ml L-15 and 1 ml 1.33 % collagenase for 10 minutes to obtain a single-cell suspension (Liu et al., 1998). Cells were centrifuged and the pellet subsequently incubated with trypsin for 10 minutes and dissociated by passages through needles of increasing gauges (16-, 19-, and 25-gauge) with a 10mL syringe (BD Microlane UK). Cells were plated at the equivalent of 2 brains per T-75 flask in DMEM with 10% FBS, fed with new media by half-changing the media every two or three days. After 8 days, macrophages and oligodendrocytes were removed by shaking the flask at 100 rpm at 37 °C for 16 hours. After the cells reached confluence (10 days), the confluent cells were treated with10µM Ara-C (Sigma, UK) overnight to kill dividing microglia followed by washing twice with fresh media. These steps may need to be repeated to further eliminate other cell types. Adherent cells were trypsinised (0.25%, 10 minutes at 37˚C) followed by 5 minutes of centrifugation. The cells were counted and plated either for immunostaining or co-culture experiments. % purity was determined by counting the number of cells positive for the astrocyte marker GFAP. The percentage of positive cells in 4 fields in 3 separate coverslips were counted for every isolation. Astrocytes were used between passages 2-6 after which their viability decreases (Liu et al., 1998).

A limitation of this method is the use of Ara-C, an antimitotic agent used to kill microglia, fibroblasts and other contaminating cells. However as we were using the chemotherapy agent 5-FU, the prior exposure to Ara-C might affect subsequent results.

Dispase protocol: As described above, primary cultures of astrocytes were isolated from the hippocampi of 1-2 day old rat pups. Tissue was transferred to 3 ml of sterile Hank's Balanced Salt Solution (HBSS) (Gibco, UK 14170-088). The tissue was washed with 5ml of collection buffer [100 mL modified HBSS add 0.5 mL BSA (20%)] repeated three times. Tissue was allowed to completely settle to the bottom of the tube and then centrifuged. The supernatant was removed and the pellet enzymatically dissociated in 15 mL of neutral protease (DISPASE) (Roche 1284908) and incubated in a water bath (35-37 °C) for 30 minutes with gentle mixing every 5 minutes. After centrifugation, the supernatant was removed and put aside in an incubator. The remaining cell mass was triturated carefully with a flame-polished glass pipette until homogenous and further dissociated in 15 mL of enzyme solution and incubated in water bath for 15 minutes with mixing every 3 minutes followed by centrifugation at 800-900rpm for 10 minutes. The pellet was dissociated in 0.05% DNase (Roche 1284932) (diluted in HBSS) and incubated for 15 minutes at 37°C in the water bath and centrifuged. Cells were resuspended in culture media. The number and viability of cells was determined with a Neubauer chamber. The cell suspension was then plated onto poly-L-ornithine (Sigma, P4957) coated flasks in DMEM/10% FBS overnight. The following day a flame-polished glass pipette was used to carefully wash off the less adherent cells (neurons) by gentle application of a PBS wash and media change. Glia cells attach more strongly to poly-L-ornithine (Dr Beazley-Long personal communication). The culture medium was changed every 3 days. It took approximately 21 days for the culture to become confluent. The limitation of this method was the lengthy waiting times and low yield, with only 60-70% of the cells immunoreactive for GFAP.

Papain protocol: Tissue isolated from neonatal hippocampus or spinal cord was removed and transferred to ice cold dissection buffer: Ca^{+2} and Mg^{+2}-free Hank's HEPES-buffered saline solution (Gibco, UK), 22 mM glucose, 20 mM HEPES (Sigma, H 3375), 100 units.ml−1 penicillin, 100 µg.ml−1 streptomycin, 0.1 mM -L--serine, 0.5 mM L-glutamine and 1 mM sodium pyruvate (pH adjusted to 7.2 with NaOH). The tissue was enzymatically digested in 15 µg/ml papain (Worthington, USA) for 45 minutes at 37°C. The suspension was centrifuged at 1250 rpm for 5 minutes. Tissue was subsequently mechanically dissociated by repeated trituration through fire-polished Pasteur pipettes in the presence of 20 mg/ml DNase in medium containing DMEM (Sigma Aldrich, D6429), Paisley, UK), 4500 mg.l−1 glucose, 4 mM L-glutamine, 110 mg L-pyruvate, 10% FBS (Gibco, 10500), 50 µg/ml -L-proline (Gibco), 100 units.ml−1 penicillin, and 100 µg/ml streptomycin and centrifuged at 750 rpm for 5 minutes. The supernatant was kept in the 50 ml tube and the pellet resuspended in 10 ml media and centrifuged at 750 rpm for 3 minutes. This protocol was repeated a further three times. The combined supernatants were centrifuged at 1250 rpm for 5 minutes, the pellet resuspended in 4 ml media and transfered to T75 cm^2 flasks. After 4-5 days, the cells were shaken at 100 rpm/min for 16 hours to remove less adherent neurons and microglia, followed by a change of media and the attached astrocytes allowed to reach confluence, as shown in Figure (2.3). Cells were trypsinised (0.25%) and plated into 75 cm2 flasks. Using this method, 95% of the cells were frequently positive for GFAP (James et al., 2011).

We found this method significantly faster, more convenient and provided a higher yield of astrocytes and so this protocol was used in for all subsequent experiments.

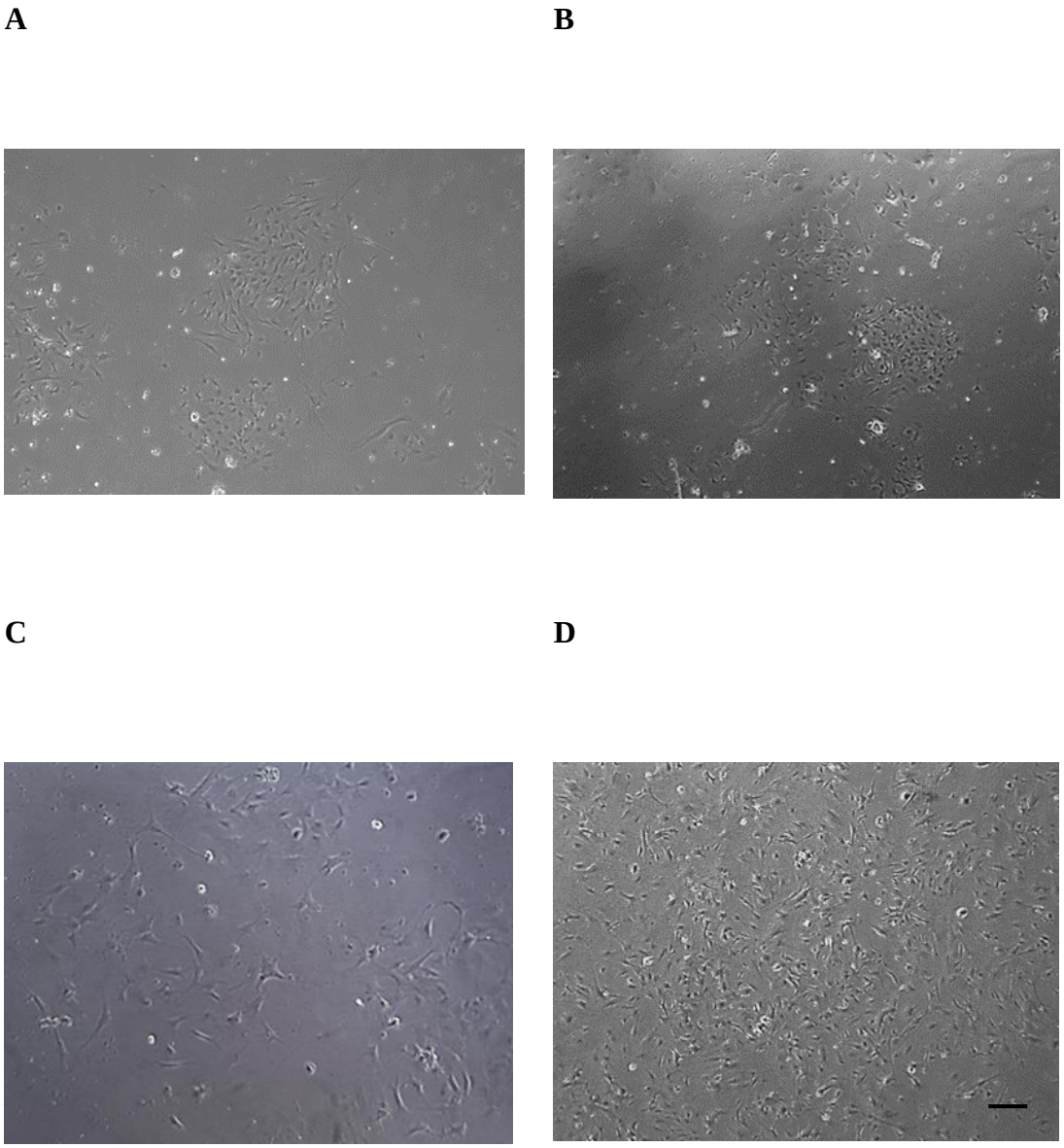


Figure 2.3 Astrocyte cultures at different time points after isolation. One day after plating of mixed hippocampal cells (A). 3 days after plating of hippocampal cells, an astrocytic layer is forming (B). 7 days after plating the astrocyte layer is completely confluent (C). After removing microglia and other cells by vigorous shaking, the astrocytes are nearly confluent 2 weeks after plating (D). Scale bar 40 μm.

2.2.5 Use of different extracellular molecules (ECMs)

Some primary cells and cell lines require a protein coating to attach, grow and divide on regular tissue culture plastic. This applies to NSCs which only adhere from floating neurospheres onto a surface coated with Poly L-Lysine (Sigma- Aldrich, P1274-100MG), Poly-L-ornithine, and Laminin**.**

2.2.5.1 Laminin

Laminin is an epithelial cell adhesion glycoprotein, composed of 3 chains designated α1, β1, and γ1. Laminin is present in in the NSCs niche during development (Kerever et al., 2007) where it plays important role in adhesion, differentiation, migration, and growth of many cells *in vitro*.

Laminin (laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane, Sigma- Aldrich, L2020) was thawed and diluted with DMEM/F12 (50 µl/5ml) culture ware was incubated with this at 37°C overnight. On the next day, the laminin solution was removed, and the coating washed with DMEM/F12 twice before seeding with cells.

2.2.5.2 Poly L-Lysine (PLL)

A stock solution of 1 mg/ml PLL (Sigma- Aldrich, UK) was prepared by dissolving 100 mg of PLL powder in 100 ml of H_2O; 1ml aliquots were stored at –20°C until used. 1ml of stock solution was diluted ten-fold in H_2O was used to coat cover slips or flasks and left in the laminar flow hood for 30 minutes. After aspirating the solution, the coated surfaces were washed three times and dried under UV light for 45 minutes to allow cross linking and stored at –20°C.

2.2.5.3 Poly-L-Ornithine

Polyornithine was diluted in sterile H_2O, to prepare a stock of 0.2mg/ml, stored at -20. This was Diluted 1:4 (=0.05mg/ml) in sterile H_2O before use. Plastic culture dishes were incubated with poly-L-ornithine overnight at 37 °C. The next day, the coating solution was aspirated, washed twice with deionized water and once with PBS prior to use.

2.2.6 Immunocytochemistry

Primary astrocytes were plated onto PLL coated coverslips (13mm) and grown until confluent. The cells were washed with PBS fixed with 4% paraformaldehyde (PFA) in PBS for 5-10 minutes at room temperature and washed three times with PBS for 5 minutes each. The cells were permeabilized and blocked with PBS containing 0.3% Triton X-100 and 5% goat serum at room temperature. The cells were then incubated with primary antibody, polyclonal anti-GFAP, (Dako, Z0334) diluted 1:500 with blocking buffer overnight at 4ºC. The next day, the cells were washed with PBS three times for 5 minutes each. Secondary antibody, goat anti rabbit (Alexa Fluor 488– Life Technologies, A11034)) diluted 1:500 with biotin diluents and incubated for 1 hour at 37ºC in the dark. After three washes with PBS, the nuclei were counterstained with (1:1000) DAPI (diamidinophenylindole, Sigma, USA, D9564, 1 mg/ml stock) for 5 minutes, washed again with PBS mounted with DABCO and coverslipped for fluorescence microscopy and photographed using a Nikon EFD-3 Fluorescence microscope and SPOT Insight QE camera with Spot advanced software (Image Solution Ltd). Controls in which the primary antibody was omitted had no detectable staining. To calculate the purity of the astrocyte preparation, the number of GFAP-positive cells

per field was counted under a fluorescent microscope at 40X magnification. Then, the percentage of positive GFAP to the total number of cells was calculated for each slide. Experiments were repeated in triplicate and for each experiment at least three coverslips were viewed and at least four fields were counted per coverslip.

2.2.7 Growth dynamics (growth rate)

To produce growth curves cells ($5x10^4$) were grown on 13mm coverslips and cultured for 12, 24, 48, 72or 96 hours. At each time point cells were trypsinised off the dishes by standard methods and counted using a haemocytometer. All experiments were conducted a minimum of three times and duplicates were done for each experiment. The growth curve results were plotted as mean ± SEM values.

2.2.8 MTT assay

The MTT assay is a colorimetric assay (Mosmann, 1983), based on the ability of viable cells to reduce an (3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazolium salt (MTT) metabolically to a purple formazan product. This occurs when MTT is cleaved by the activity of NAD(P) H-dependent dehydrogenase enzyme in metabolically active cells (Figure 2.4). The MTT assay was used to quantify viable cell number and provides a sensitive, quantitative measure of their rate of growth. Cells were cultured in 24-well plates (Coster, UK) at different densities depending upon cell type, (C6, primary astrocyte, HBMEC and neural 5×10^4, 8×10^4, 6×10^4 and 1×10^5 cell/well, respectively) and allowed to proliferate for 24 hours. After that, 0.5 ml of treated media [concentrations ranging from 0µM (Control) to 20 µM (5, 10, 15, and 20 µM)] were added to each well and incubated for 24 and 48 hours. 50 µl of MTT

solution (Sigma Aldrich, M5655- 5mg/ml in PBS) was added to each well and incubated at 37°C for 30 minutes. The reaction was stopped by adding 1 ml of solubilisation solution, DMSO (Sigma Aldrich Company Ltd, UK) to each well with pipetting (formazan crystals are solubilised in the DMSO). Then, 200 µl aliquots from each well were transferred to a 96-well plate (Nunclon, UK) and the solubilized formazan evaluated by spectrophotometery (Molecular Devices, SpectraMaz 340PC) at an absorbance of 570 nm, each reading was repeated four times per condition (Kim et al. 2011). The percentage viability was calculated as [(absorbance of drug-treated) sample/ (control absorbance)]× 100. Non-drug treated cells were used as controls. The MTT assay was chosen because it is a simple, rapid, precise, semi-automated assay and sensitive for testing cytotoxic drugs (Alley et al., 1988, Sumantran, 2011, Ciapetti et al., 1993, Fotakis and Timbrell, 2006). This assay has been used to determine the sensitivity of wide number of cancer cells exposed to chemotherapy agents (Van de Loosdrecht et al., 1994, Sargent and Taylor, 1989, Pieters et al., 1988).

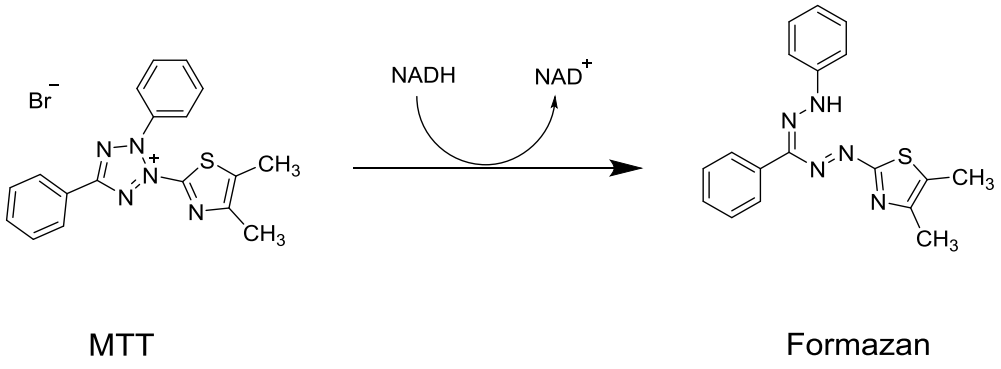


Figure 2.4 Structures of MTT and coloured formazan product.

The MTT protocol for NSCs was as above except that free-floating neurospheres were dissociated with accutase for 15 minutes and centrifuged. The single cell suspension

obtained were plated on pre-coated with 0.5ml laminin (50 µl/5ml) in 24-well plates at a density of 1×10^5 cells/well to allow them to grow as an adherent monolayer overnight and treated.

2.2.9 Statistical analysis

All graphs and statistical analysis were performed in Prism v7.0 (GraphPad, San Diego, USA), where n= 4. Data were considered significant if $p < 0.05$, and expressed as mean+ SEM optical density, as a percentage of control optical density. Cell viability was analysed firstly for normalisation (D'Agostino test), and then using one-way ANOVA (non-parametric or parametric, depending on normalisation results). Where significant multiple comparisons were performed. Data was analysed with a normal One-way ANOVA and Dunnett's post-hoc multiple comparison test. The suitable growth medium analysis was performed using unpaired two-tailed Student's t-test, and considered significant where $p \leq 0.05$.

2.3 Results

2.3.1 Optimisation of primary astrocyte cultures

Isolation of primary astrocyte cells with trypsin digestion and using Ara-C and collagenase was discontinued due to the need for Ara-C. Isolation of primary astrocytes with Dispase was tried four times but was unsuccessful due to low yields (60-70% astrocytes), weak adherence and slow growth (growth arrest after 7 days) (Figure 2.5). However, isolation by papain digestion was successful and was used for all further experiments.

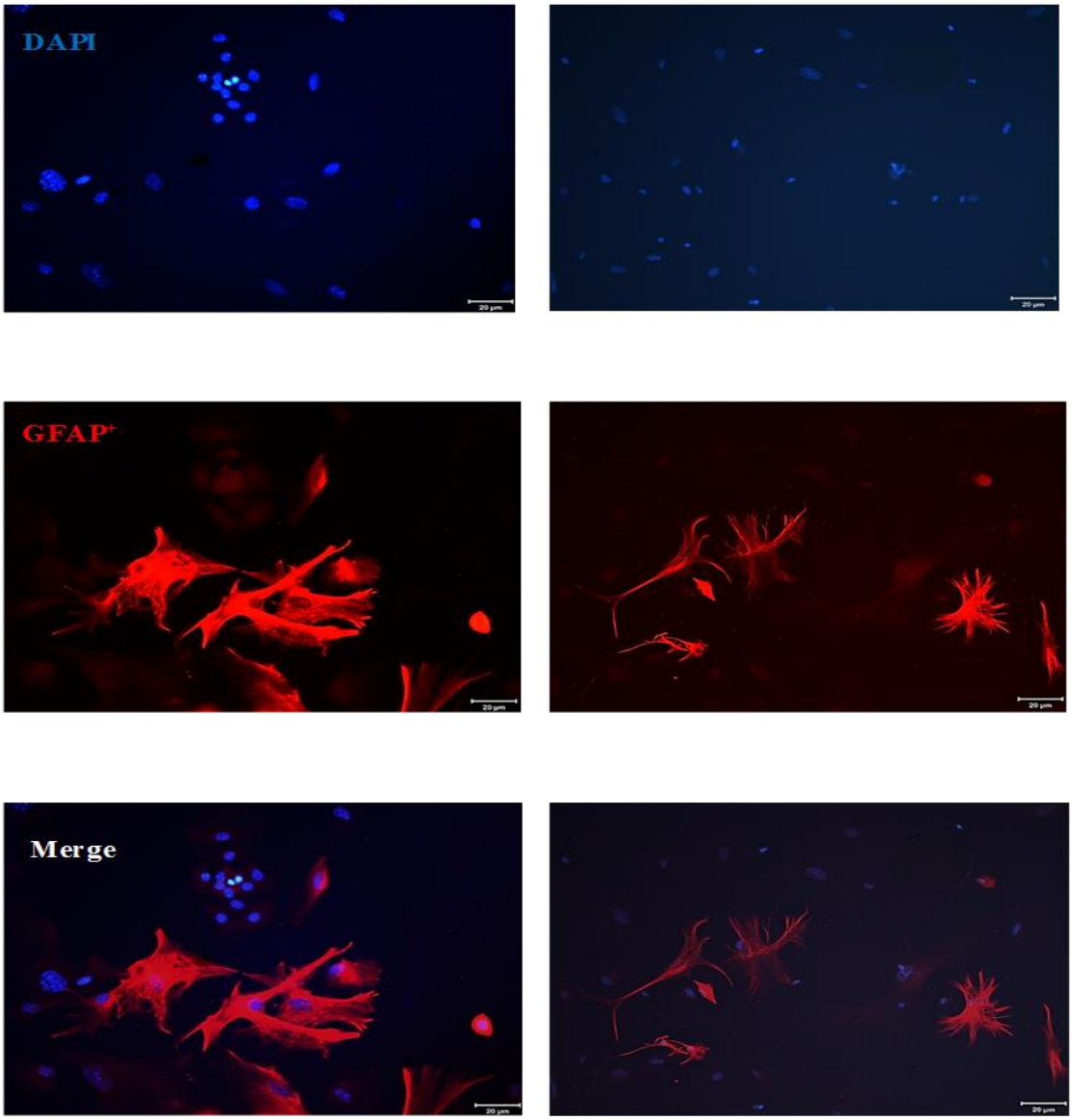


Figure 2.5 Immunocytochemical staining of primary hippocampal astrocyte cell cultures. Primary astrocyte cell cultures obtained using collagenase digestion (Panel left). Primary astrocyte cell cultures obtained from protease (Panel right). Representative images show astrocytes growing on coated coverslips within 24-well tissue culture plates after 3 days of culture. Images are representative of 4 independent experiments and selected from triplicate samples. Scale bar 20 μm.

Primary astrocytes obtained using the papain protocol, had a typical star-shaped morphology with some having long branched processes after 2 days in culture (Figure

2.6 B). The cultures became confluent after 12-14 days. The % purity of astrocytes was determined by immunostaining for the astrocyte marker, GFAP. Over 95% of cells were $GFAP^+$ (Figure 2.6) with the staining evenly distributed throughout the cytoplasm. This confirmed that the primary cells isolated from the hippocampus and spinal cord were astrocytes and that the protocol produced a high purity of these cells.

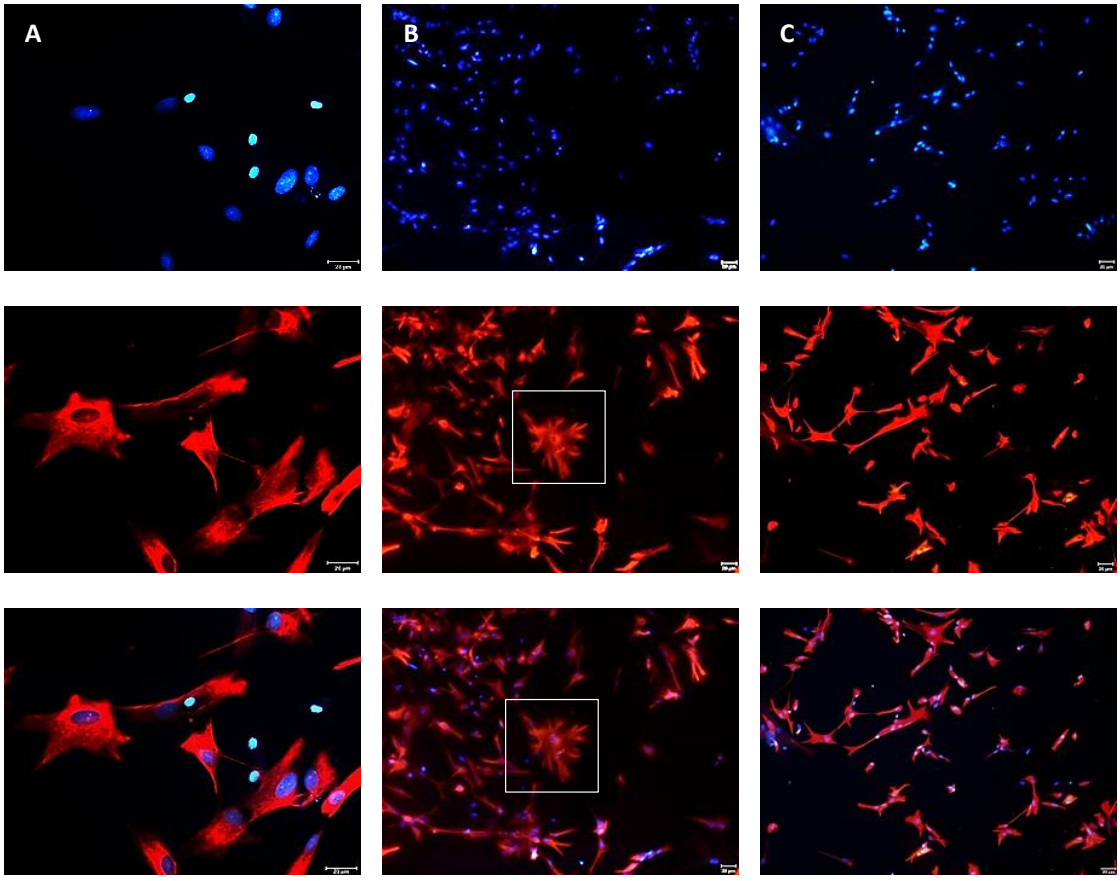


Figure 2.6 Immunocytochemistry staining of primary astrocyte cells derived from hippocampus and spinal cord using papain digestion. GFAP positive cells (red) and cell nuclei counter stained blue with DAPI. Astrocytes from hippocampus, the white square shows a star- shaped astrocyte (A and B). Astrocytes from spinal cord (C). Magnification: higher magnification (40X) A, low-magnification overview (20X) B and C. The scale bar 20 μm.

2.3.2 Growth rate

Both cell lines and primary cells grew rapidly over 48 hours. C6 and 3T3 cells, grew faster than primary cells (Figure 2.7). The growth rate of neural cells was slower than glia. All cells showed an exponential growth rate, with maximum growth at 48 hours which was chosen as the time to cultivate cells before treatment.

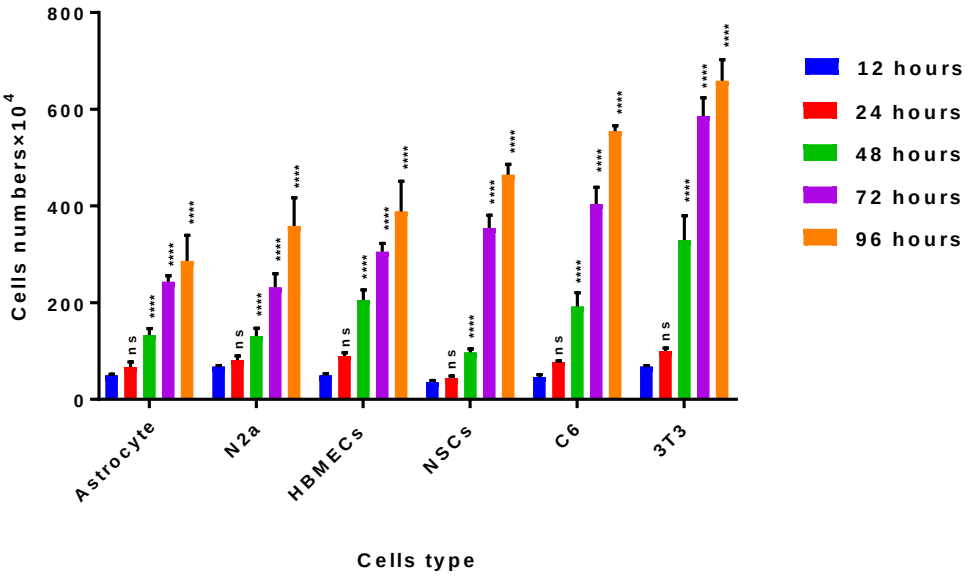


Figure 2.7 Growth curves of different cell lines and primary cells for a maximum of 4 days in culture. A significant increase in cell number was obvious by day 3 in culture. Cell numbers are presented as mean ± SEM values. n=3. Statistical significance was determined by Two-way ANOVA.

2.3.3 Determination of a standard medium for all cell types which would be suitable for co-culture

To test the impact of different media on the growth of different types of cell, which are normally gown in either DMEM or RPMI-1640, each cell type was seeded into 24-well

plates and grown in DMEM or RPMI-1640 for 24 hours followed by an MTT assay. No morphological changes were observed between cells cultured in either media (Figure 2.8). In addition, the results showed no significant difference in the number of N2a cells when cultured in DMEM compared to RPMI-1640. However, C6 (glial cells) and HBMECs grew significantly better when cultured in RPMI-1640 compared with those in DMEN (Figure 2.9). This is likely to be due to RPMI-1640 being a richer media. Based on these results, it was decided to grow all cell types in RPMI-1640.

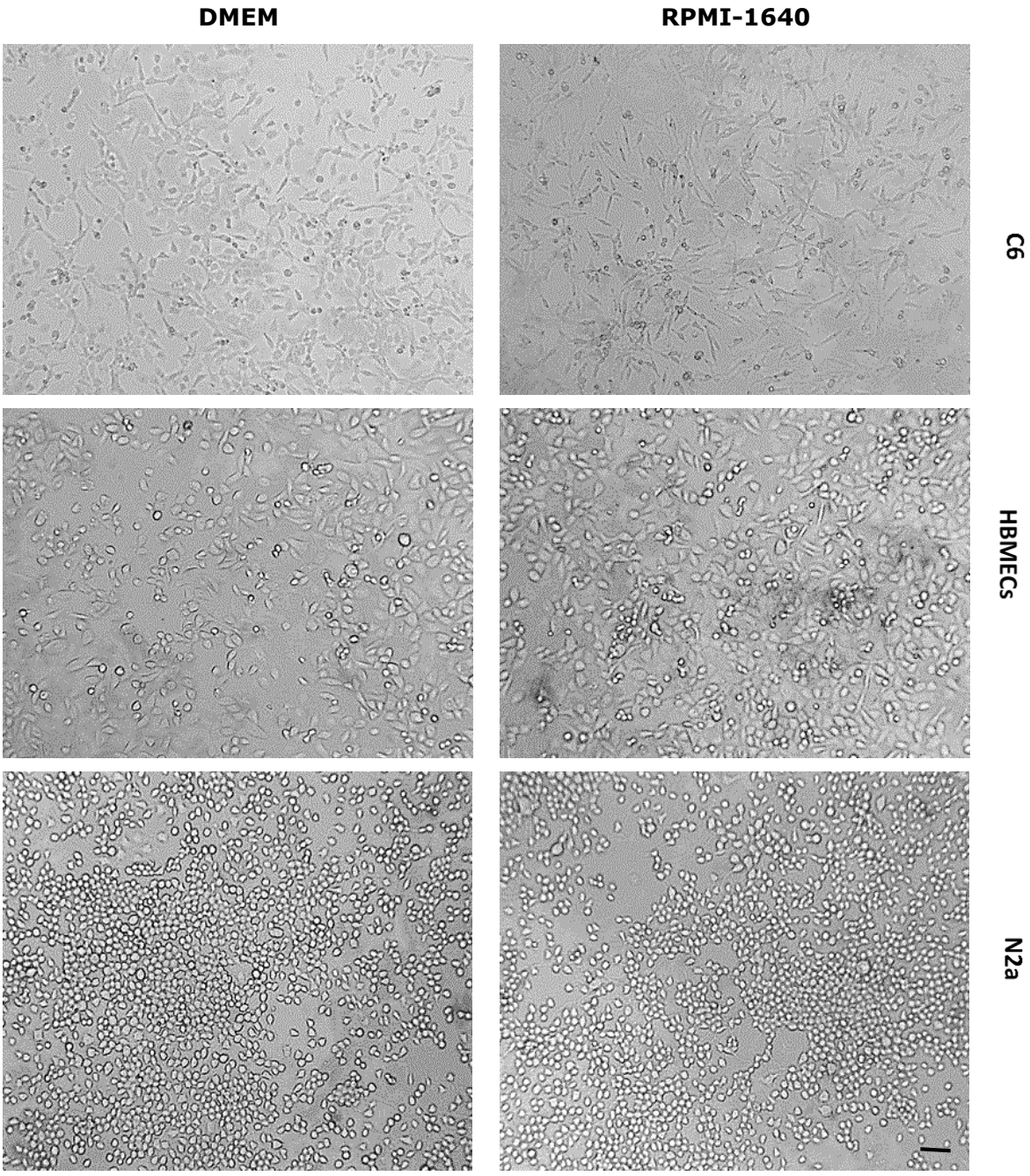


Figure 2.8 Phase contrast images of C6, HBMEC and N2a cells showing cell morphology after growing in different media. C6, HBMEC and N2a cells were seeded at a density of 5×10^4, 6×10^4 and 1×10^5 cells/well on 24 well plates and incubated for 24 hours before growing on DMEM or RPMI-1640. After a further 24 hours of incubation the morphology and the degree of confluence was analysed. Under all conditions normal morphology and confluence were maintained. Scale bar 50 μm.

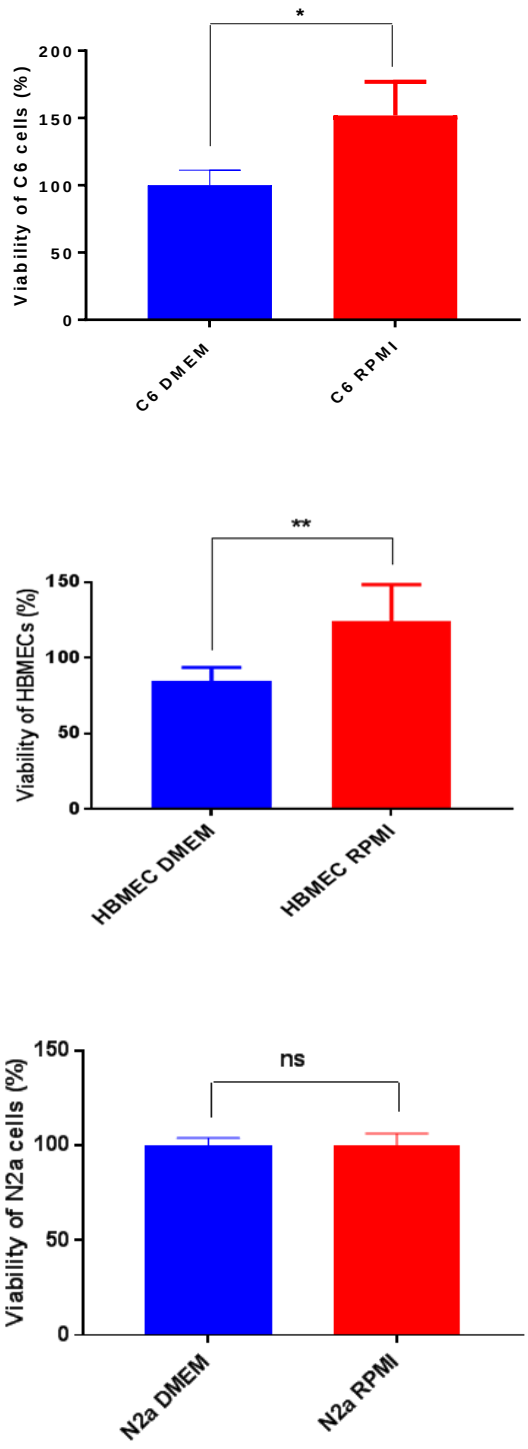


Figure 2.9 Cell viability as measured by MTT assay of C6, HBMECs and N2a cultured in RPMI-1640 or DMEM media and incubated for 24 hours. C6 cells and HBMECs show a significant increase in viability in RPMI-1640 as compared to DMEM. Results analysed by t-test, data presented as mean ± S.E.M and results are taken from three independent experiments looking at 16 wells in each experiment.

2.3.4 The sensitivity of different cell types within the hippocampal stem cell niche to 5-FU chemotherapy

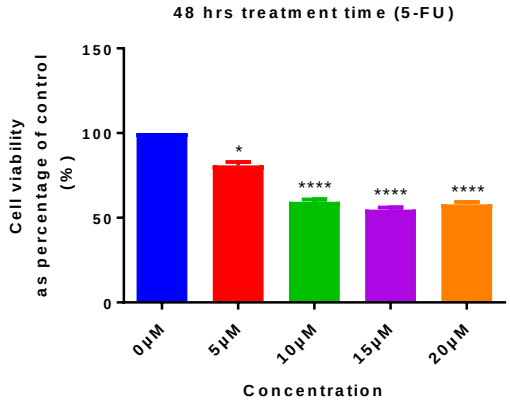
The effect of different concentrations of 5-FU was tested on N2a, C6, HBMEC, and 3T3 cell lines and primary astrocytes and mouse hippocampal NSCs. Cells were incubated with 5-FU from 0-20 μM. Cells were visualized under the microscope after 48 hours of being seeded (prior to the 5-FU treatment) and appeared attached and viability assessed by MTT assay of three replicates in each experiment.

As shown in (Figure 2.10) the lowest concentration of 5-FU used (5μM) significantly reduced the viability of all cell lines and primary cells after 48 hours ($p<0.001$). However, the impact of this dose and the steepness of the dose response curve varied across the cell lines and primary cells. NSC and N2a cells showed the greatest sensitivity to 5-FU, with a reduction to 50% viability while C6 astrocytes and brain endothelial cells showed greater resistance maintaining at least 80% of the viability of untreated cells after 48 hours (Figure 2.10). Cell viability of N2a and NSCs was significantly decreased at 5 μM 5-FU but higher concentrations (10, 15 and 20 μM) did not cause significantly more reduction in cell viability. This could be due to the chemotherapy induced differentiation of neural cells (Kempermann et al., 2006, Galli et al., 2003), and these differentiated cells are less sensitive to chemotherapy treatment as they are no longer proliferating.

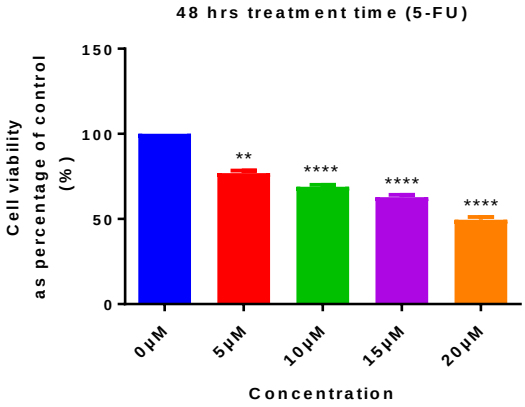
Based on these finding, 5μM of 5-FU for 48 hours incubation was chosen as the concentration to be used in future experiments as this had little effect on astrocytes and endothelial cells but was significantly toxic to neural cells. We also tested the

sensitivity of neural cells after 24 hours exposure to 5-FU. The effect of 5-FU on neural cell viability was time and concentration-dependent. After 24 hours of treatment, the highest concentration of 5-FU (20 μM) significantly decreased the N2a and mouse hippocampal NSC but not the other cell types (Figure 2.11).

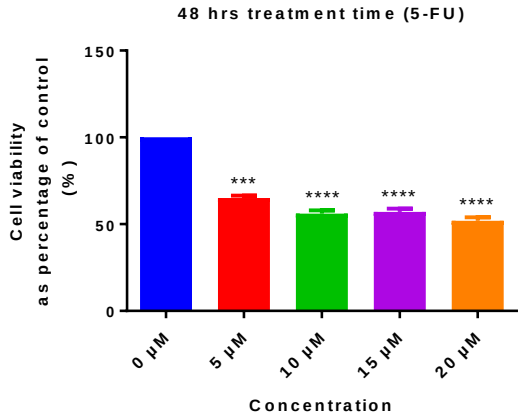
(A) C6



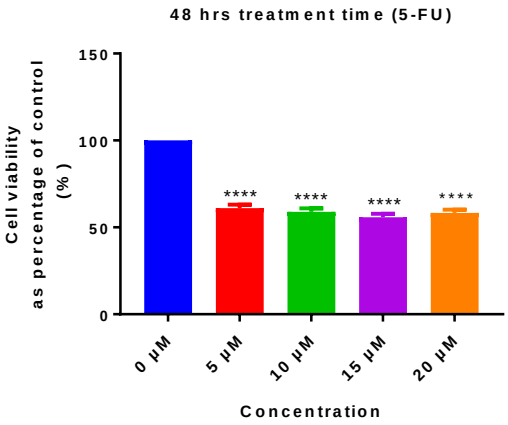
(B) HBMECs



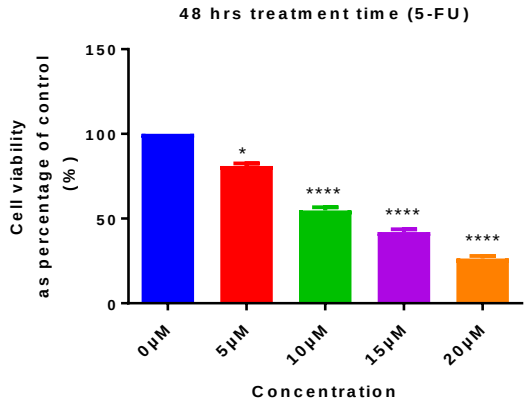
(C) 3T3



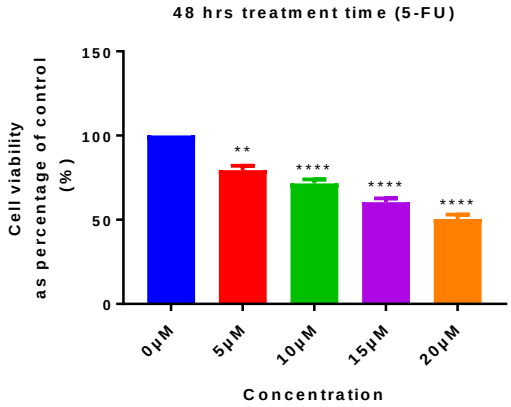
(D) N2a



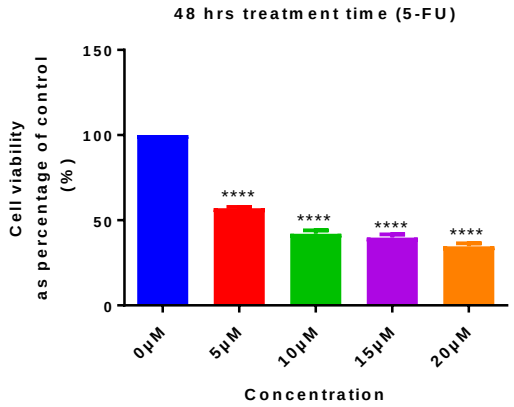
(E) Hip. Astrocyte



(F) Sp. Astrocyte



(G) NSCs



(H)

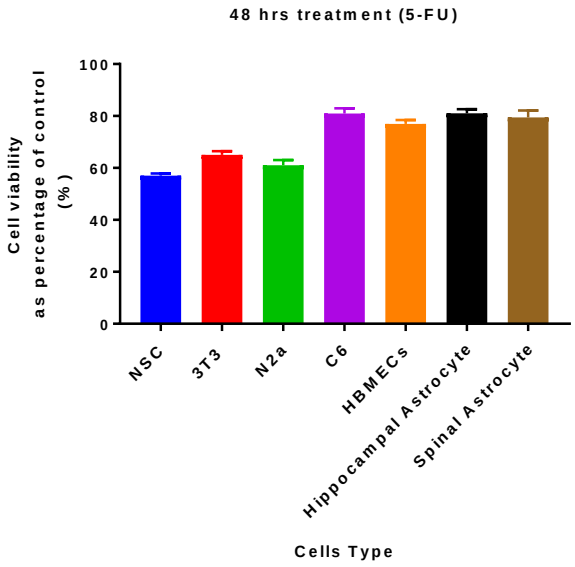


Figure 2.10 The effect of different concentrations of 5-FU (0, 5, 10, 15 and 20 µM) on C6 (A), HBMECs (B), 3T3 (C), N2a (D), Hippocampal astrocytes (E), Spinal astrocytes (F) and NSCs (G) cell viability as measured by MTT assay after 48 hours treatment. Comparison of cell viability across different cell types at 5 µM (5-FU) (H). There was a significant difference in the cell viability between the groups. All concentrations of 5-FU significantly reduced the viability of all cell types. Data is presented as the mean ±S.E.M; (*P<0.05, ** P<0.01, ***P<0.0001, ****P<0.00001, One-way ANOVA, n=4) followed by Dunnett's multiple test was used to compare the mean of control groups with treated groups.

(A) N2a **(B) NSCs**

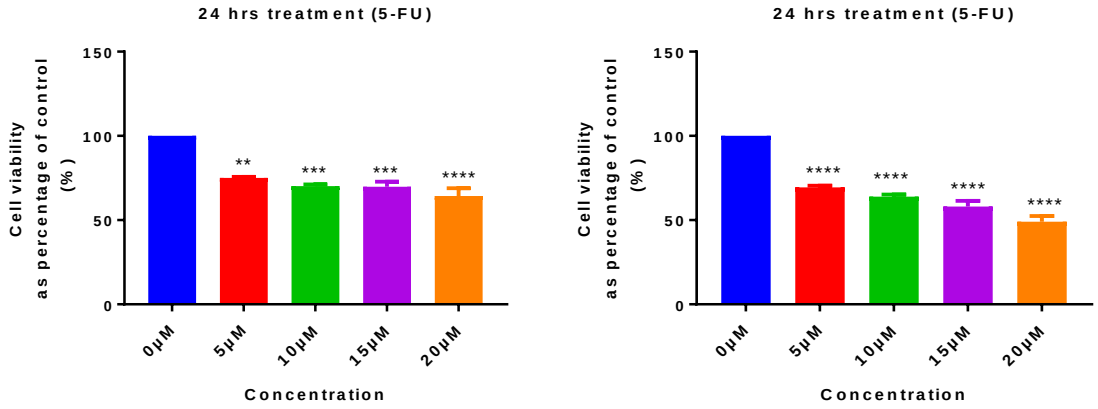


Figure 2.11 The viability as measured by MTT assay of (A) N2a and (B) NSCs after 24 hours exposure to different concentrations of 5-FU (0, 5, 10, 15, and 20). 5-FU significantly decreased N2a and NSC viability after 24 hour. Values are reported as mean ±S.E.M; (**P<0.01, ***P<0.0001, ****P<0.00001, One-way ANOVA, n=3-8)

2.4 Discussion

2.4.1 Primary astrocyte culture

Hippocampal and spinal astrocytes were successfully isolated by papain digestion from neonatal rat CNS based on previous methods (James et al., 2011). This protocol produced almost pure cultures of primary astrocytes. This method was found to yield viable cells which continued to proliferate after isolation. The viability of cells obtained by this method was 90% as determined by trypan blue exclusion. Shaking on an orbital shaker at 100 rpm was used to remove other cell types such as oligodendrocytes, neuroblasts and microglia. This protocol was simple and reproducible and avoided the use of cytotoxic agents such as Ara-C and produced higher viabilities, yields and

purities than the first and second methods described in the materials and methods. This was despite attempts to modify these methods by using shorter digestion times with trypsin and removing Ara-C from the protocol. Attempts to increase the adherence of cells by pre-coating tissue culture flasks with poly-L-ornithine did not improve yields.

Primary astrocytes obtained by this method required 12-14 days to become confluent. It was found that passaging these cells more than 4 times decreased their proliferation. Immunostaining for GFAP was found to be an effective method to assess the purity of these cells and cultures were over 95% positive for GFAP (Figure 2.6). Morphologically, many of these cells showed long, branched processes while others had short, unbranched processes after 2 days in culture.

2.4.2 Growth rate

The rate of the proliferation of cells is an important factor influencing the results of viability assays. Therefore, growth curves were plotted for both the cell lines and primary cells (Figure 2.7). The C6, HBMEC, and 3T3 lines are adherent cell lines which grow as a monolayer. The normal growth rate of the cells was determined over a time period of 4 days. The cells were counted every 24 hours to calculate the cells/well. As shown in the (Figure 2.7), these cells replicate after~ 24 hours of culturing and continued to grow exponentially even after 96 hours. These results suggest that drug treatment after 24 or 48 hours incubation following the initial seeding of cells was appropriate for subsequent viability assays. As expected of a cancer cell line, the glia cells (C6) demonstrated a higher rate of proliferation as compared to primary astrocytes (Figure 2.7). Neural cells start to divide after 48 hours. In contrast, Ray et al. in 1993

suggest that the doubling rate for neural cells is approximately 4 -7 days (Ray et al., 1993).

Primary neural stem cells showed a lower growth rate compared to glia and endothelial cells. Such differences in the growth patterns of primary neural and glia cells was expected as studies have observed that neural cells are largely dependent on surrounding cells such as astrocytes and endothelial cells *in vitro* and *in vivo* for survival and maintenance (Hirobe et al., 2002). Therefore, in mono-culture, neural cell growth is much slower due to the absence of contributing paracrine growth factors and mitogens secreted by surrounding neurogenic cells *in vivo* or in co-culture.

2.4.3 Determination of suitable medium

To establish *in vitro* co-cultures with two different cell types and to compare the effects of 5-FU across different cell types it was advantageous to grow all cell types in the same culture media.

In *in vitro* culture, ECMs can improve cellular behaviour and survival. The cell response depends on the cell phenotype and on the type of ECMs that have been used. In addition, ECMs can help cells to grow in the absence of serum and other growth factors (Kleinman et al., 1987). Laminin was chosen as a suitable substrate for monoculture and co-culture systems of NSCs *in vitro*. Laminin supports cell adherence for different types of cells including epithelial, endothelial, neural, muscle, and liver cells *in vitro*.

The different cells and cell lines we used have traditionally been grown in either DMEM or RPMI-1640. RPMI-1640 has been used for HBMECs while DMEM has been used for N2a and C6 astrocytes. The first investigation was to determine if one type of media could be used for growing all three (N2a, HBMEC and astrocytic) types of cells. Cell morphology was checked in both media (figure 2.8) and showed no sign of abnormal morphology for any of the cells in either media. Cell viability was measured by MTT assay for each cell type growing in both media. The results for N2a cells showed that there was no significant difference in viability in RPMI-1640 or DMEM (Figure 2.9). However, C6 and HBMECs showed increased viability in RPMI-1640 compared to DMEM probably due to the higher nutrient content of this media. From these investigations all cell types were subsequently cultured in RPMI-1640.

2.4.4 Determining the relative sensitivity of N2a cells and other components of the neurogenic niche to 5-FU

In this study, we compared the effects produced by different concentrations of 5-FU (5, 10, 15 and 20μM) *in vitro* on the viability of N2a, C6, HBMEC and 3T3 cells and primary astrocytes and NSCs for 24 and 48 hours. As a cytotoxic compound, 5-FU was expected to significantly reduce cell viability (Shimoyama et al., 2002, Peters et al., 2000a).

The literature contains the results of treating a number different cell lines and primary cells with 5-FU. These studies demonstrate that the sensitivity of different cell types to 5-FU is quite variable.

Lamberti et al. in 2012, studied the effect of DOXO and 5-FU on rat cardiocytes (H9c2) and a human colon adenocarcinoma (HT-29) cell line. He has found 5-FU cause growth inhibition and cell death in both cell lines. 50% inhibitory concentration value of 5-FU was 4 μM and 400 μM in HT29 and H9c2, respectively. These results suggest that the most resistant cells appear to be H9c2 (Lamberti et al., 2012).

5-FU treatment produced a 50% decrease in viability in HeLa cells and lymphocytes were treated with 20μM and 35μM respectively (Ahmed and Jamil, 2011). Astrocytes were relatively resistant to 5-FU (Kim et al., 2011, Lin et al., 2010). This is in agreement with our study. Other studies have found that 5-FU at concentrations of 3.2μM and 1.3μM significantly decreased the viability of COLO-205 and HT-29 human colon cancer cell lines (Wiebke et al., 2003). Concentrations of between 0.5 and 5 μM decreased the viability of primary oligodendrocytes (Dietrich et al., 2006a). 0.5 μM significantly decreased the viability of the mouse melanoma cell line F3.CD which had metastasised to the brain (Joo et al., 2009). However, embryonic stem cells are particularly sensitive to apoptosis induced by 5-FU (Kim et al., 2009a) and NSCs, which proliferate in the neurogenic regions of the adult brain, are also particularly affected by 5-FU (Han et al., 2008). This line with our results.

Wang et al. in 2004, have found 1.2μM inhibit 50 % of viability of MCF7 cells treated with 5-FU for 48 hours (Wang et al., 2004a). However, other studies have found 10μM (Hernández-Vargas et al., 2006) and 23μM decrease the viability by 50% after treatment for 48 hours (Raymond et al., 1997).

Clinical study by Han et al suggest that 5-FU in plasma and CSF concentrations in the range 0.3-71.0 μM (Han et al., 2008), Therefore, the dose we have used is within the rang that described clinically.

This would mean that therapeutic doses of chemotherapy are likely to reduce hippocampal neurogenesis, a result confirmed by animal studies (Lyons et al., 2011, ELBeltagy et al., 2012). This effect on hippocampal neurogenesis is likely to be one of the causes for the prolonged cognitive decline found by many patients after chemotherapy (Monje and Palmer, 2003, Laack and Brown, 2004), a cognitive effect which has also been produced in animal models (Mignone and Weber, 2006). NSCs in the hippocampal SGZ stem cell niche are maintained and supported by other cellular components of the niche which includes astrocytes and endothelial cells (Morrens et al., 2012, Yang et al., 2011c). The response of these other cell types to chemotherapy could have a significant effect on the sensitivity and survival of the NSCs in this location. As a first step in understanding these interactions the differential sensitivity of neural, astrocytic and endothelial cells was assessed when these cell types were maintained in a common media. Immortal cell lines are easier to handle than primary cells and for this reason the N2a and C6 cell lines were used as models for NSCs and astrocytes respectively. As these lines are likely to be more resistant to chemotherapy as compared to primary cells, primary hippocampal NSCs and primary astrocytes were also tested for their sensitivity to 5-FU. Astrocytes are reported to vary in phenotype in different brain regions and for this reason primary astrocytes from hippocampus and spinal cord were isolated for testing.

NSCs were more sensitive and had a steeper dose response compared to N2a cells. The first results using 48 hours exposure to 5µM 5-FU reduced the viability of N2a and NSC cells to 50% (Fig 2.10). Further experiments which used 24 hours exposure to 5-FU, showed that both N2a cells and NSCs showed a dose response but relatively high doses (20µM) were required to reduce viability to approximately 50% (Figure 2.11). NSCs again showed a steeper dose response and reduced viability compared with N2a cells with this protocol. From these results it was decided to use 48 hours exposure to chemotherapy to test the viability of the other cell types being considered. As seen in Figure (2.10); chemotherapy reduced the viability of all cell types with C6 astrocytes, primary astrocytes from hippocampus and spinal cord and 3T3 cells being the most resistant to treatment and retaining approximately 80% viability after 48 hours treatment with 5µM 5-FU. Endothelial cells were slightly more sensitive with 75% viability after 5µM 5-FU treatment.

These results demonstrate that neural cells, whether primary NSC or from a cell line (N2a) are more sensitive than other cell types found in the neurogenic niche. Both N2a and primary NSC cells show similar sensitivity to chemotherapy. Primary astrocytes from hippocampus and spinal cord showed no difference in sensitivity to chemotherapy. Taking these results together, systemic chemotherapy treatment with 5-FU is likely to significantly reduce NSCs in the hippocampal stem cell niche in comparison to astrocytes or endothelial cells, as shown in Figure (2.10).

It can be concluded from this study, that 5-FU affects proliferation in the SGZ *in vivo*. NSC grown *in vitro* are also sensitive to the cytotoxic effects of 5-FU (ELBeltagy et al., 2012). Optimization of media and culture conditions to more closely resemble the stem

cell niche in the hippocampus will be necessary to further evaluate the effects *in vitro* that may be later tested *in vivo*. The importance of *in vitro* experiments for dissecting *in vivo* variables is crucial for faster and more controlled evaluation of conditions. N2a cells serve as a NSC model that respond to cytotoxic conditions and may mimic the *in vivo* effects of 5-FU under the appropriate conditions. *In vivo* experiments with animal models are deemed necessary to prove that the effects observed *in vitro* are occurring *in vivo* and vice versa.

3 Chapter Investigation of the mechanisms underlying protection of neural cells in co-culture assays

3.1 Introduction

The data presented in the previous chapter demonstrated that RPMI is a suitable culture media for co-culturing N2a or NCS with other cell types found in the neurogenic niche of the hippocampus. The cell types used for co-culture were C6 astrocytes, endothelial cell together and the non-neurogenic cell line, 3T3 fibroblasts used as a control. Co-culture of different cell types has been widely used to study the role of cellular communication and interactions important in the growth and physiology of tissues as this approach is thought to more closely mimic the *in vivo* cellular environment. In this study, co-cultures of pairs of different cell types were used to investigate the interactions between them during exposure to chemotherapy. As previously described NSCs, are the starting point for the process of adult hippocampal neurogenesis. The proliferative and differentiation changes required for neurogenesis are under the influence of surrounding cells which provide the microenvironment of the stem cell niche and give signals which direct stem cell behaviour (Sun et al., 2015b, Shen et al., 2008a). The stem cell niche within the hippocampus is found in a narrow strip, 2- 3 cells wide, called the SGZ found on the inside of the dentate gyrus. This region contains blood vessels, astrocytes, mature neurons and pericytes, found on the outside of blood vessels which are involved in angiogenesis (Guo et al., 2010).

3.1.1 The role of stem cell niche cells in controlling neurogenesis

Many dividing NSCs, in the SGZ, are in contact with blood vessels (Palmer et al., 2000). The cell types found in this location might contribute by cell–cell contacts or humoral factors to controlling NSC proliferation and survival. The cell associated with

blood vessels include the endothelial cells forming the capillary walls and astrocytes whose end-feet, on the surface of blood vessels, contribute to the BBB (Abbott et al., 2006, Kajitani et al., 2012). From the early stages of development and continuing throughout life, there is a dependent relationship between the vascular and nervous systems and it has been suggested that the close association between blood vessels and NSCs which is present in the SGZ, is important for maintaining adult neurogenesis (Palmer et al., 2000). Understanding the nature and regulation of this microenvironment (niche) could contribute to our understanding of the regulation of neurogenesis and how NSCs may be protected by this association.

In the hippocampus, regulation of NSCs by adjacent cells could occur through direct cell-to-cell signalling via GJs (Kunze et al., 2009), paracrine signals such as Wnt (Lie et al., 2005), neurotransmitters, most notably glutamate (Cameron et al., 1995) and gamma-Aminobutyric acid (GABA) (Ge et al., 2006, Wang et al., 2005), as well as systemic factors, including growth factors and hormones (Ehret et al., 2015). GJs, which have been described between a number of different cell types in the nervous system, may provide a key mechanism to support cell to cell communication or interactions by allowing substances to pass between cells. In addition, direct cell to cell contact between components of the neurogenic niche may facilitate other forms of communication such as Notch receptor signalling or protease activated G protein receptors (Chuang et al., 2015, Tung and Lee, 2012).

3.1.1.1 Endothelial cells

Endothelial cells which form the lining of blood vessels, have been shown to play a role in the proliferation, survival and fate of NSCs (Xiong et al., 2013) and regulate the differentiation and migration of NSCs within the neurogenic niche (Shin et al., 2014). Endothelial cells derived from different sources such as bovine pulmonary artery and mouse brain, secrete factors that promote NSC self-renewal and neurogenesis *in vitro* (Shen et al., 2004). Key factors produced by endothelial cells include VEGF (Shen et al., 2004, Schänzer et al., 2004), EGF (Doetsch et al., 2002), bFGF (Kilpatrick and Bartlett, 1995), BDNF (Cheng et al., 2003), PEDF (Ramírez-Castillejo et al., 2006) that support neurogenesis. In addition, these cells also secrete signalling molecules such as Wnts and Shh which are known to affect NSC proliferation and differentiation (Goldman and Chen, 2011). Co-culture of endothelial cells from bovine pulmonary artery, mouse brain vascular smooth muscle cells or 3T3 fibroblasts with mouse cerebral cortex NSCs, showed that only the endothelial cells supported NSC proliferation and differentiation into neurons (Shen et al., 2004). This study showed that endothelial cells may have a specific inductive role in maintaining and directing NSCs to a neural fate. Similarly a study by Leventhal et al. which co-cultured endothelial cells, astrocytes or fibroblasts, with rat neural precursors from the SVZ, found that only the endothelial cells increased neural cell survival and new neuron productions (Leventhal et al., 1999).

3.1.1.2 Astrocytes

Astrocytes are the main components of the neurogenic niche in the adult dentate gyrus. Astrocytes have a crucial role in the survival and activation of radial-glial like stem cells in the hippocampus (Song et al., 2012). *In vitro* studies have shown that astrocytes have a role in neuronal attachment and growth, secreting adhesion and ECM proteins, as well as a range of growth factors and cytokines (Wang and Cynader, 1999, Tabernero et al., 2002). Studies by Charles et al. in 2011 demonstrated that astrocytes play an important role in neural support, invasion and angiogenesis (Charles et al., 2011).

3.1.2 Protective role of niche cells to NSCs

In our study, we were interested in determining what effect, if any; cells within the neurogenic niche have on neural cell survival when treated with chemotherapy.

3.1.2.1 Endothelial cells

Co-culture studies with endothelial cells has shown that these cells can provided protection to stem cells and tumour cells, for example from radiation damage (Paris et al., 2001, Garcia-Barros et al., 2003). Preclinical studies report that cerebral endothelial cells isolated from the edge of regions damaged by stroke, when co-cultured with neural stem/ progenitor cells (NSPCs), significantly increased neural progenitor cell proliferation, neuronal differentiation and capillary tube formation (Guzman et al., 2008). These studies indicate that endothelial cells can play a significant role in the cell survival and proliferation of both neural and non-neural cells. Seigers et al. in 2010

showed that MTX, significantly decreased the density of blood vessels in the hippocampus after three weeks of treatment (Seigers et al., 2010). While a study by Han et al suggest that 5-FU caused a transitory increase in apoptosis in endothelial cells (Han et al., 2008). These findings indicate that a reduction in endothelial cells in the neurogenic niche may lead to a reduction in hippocampal neurogenesis which could contribute to the development of cognitive deficiencies after chemotherapy treatment.

3.1.2.2 Astrocytes

Astrocytes obtained from adult hippocampus increase the proliferation of adult hippocampal neural precursors in co-culture and to protect neurons against the toxicity of oxidative stress (Ozog et al., 2002, Mattson and Rychlik, 1990). Any defect in astrocytic functions, such as maintenance of antioxidant defence and cellular energy levels, has been shown to strongly effect neuronal survival (Anderl et al., 2009). It has been suggested that the increase in hippocampal neurogenesis, brought about by treatment with the antidepressant FLX *in vivo*, may be mediated by increased expression and secretion of neurotrophic/growth factors from astrocytes (Boku et al., 2013). These factors may also have protective functions and for this reason we treated C6 astrocytes with FLX and tested the CM from these cells for protection against chemotherapy. According to studies by Chan et al in 2015 direct contact between astrocytes and some types of cancer cells and NSC significantly decreases cell apoptosis induced by chemotherapy drugs (Chen et al., 2015). To test if C6 astrocytes could have a protective effect on neural cells in this way when treated with 5-FU, we have used both direct and indirect co-culture models.

3.1.3 Direct and indirect co-culture models

Direct co-culture allows cells from different types, to make contact while indirect co-culture prevents contact between cells grown in close proximity. Using these models it is possible to distinguish between interactions based on the secretion of diffusible factors and those requiring direct membrane contact between cells. Diffusible substances may also participate in two-way communication, in which paracrine factors are secreted by both cell types that affect each other while interactions requiring direct cell contact can act through membrane bound ligands and receptors or gap junctions (Grellier et al., 2009).

3.1.3.1 Conditioned media

Many cell types including astrocytes and endothelial cells are known to secrete trophic factors which can influence NSC behaviour and survival. CM in which particular cells have been grown; will contain everything that has been secreted by the cells including growth factors, metabolites and cytokines. CM from C6 (C6-CM) and endothelial cells (HBMEC-CM) was used to detect secreted diffusible factors that might influence the survival of NSCs treated with chemotherapy. Secreted factors include Nerve growth factor (NGF), interleukin-1, Platelet-derived growth factor (PDGF), bFGF, laminin, and protease inhibitors (Selak et al., 1986, Davis et al., 1985, Westermann et al., 1988, Richardson et al., 1988, Houlgatte et al., 1989). CM obtained from the glial cell lines, B49 (Schubert et al., 1974) and R33 (Albert et al., 1982), the Schwannoma cell line JS1 (Kimura et al., 1990), and PC 12 pheochromocytoma increased the survival of dopaminergic neurons in a dose dependent manner (Engele et al., 1991).

CM obtained from astrocyte cultures (astrocyte-conditioned medium, ACM) promotes neuronal growth and reduces apoptosis (Zhu et al., 2006, Wang and Cynader, 1999). ACM has been used to investigate the role of astrocytes in neuroprotection and astrocyte–neuronal interactions (Alanazi et al., 2016). These studies showed that ACM protected primary hippocampal neurons from damage induced by corticosterone (Zhu et al., 2006). The exact mechanism of these interactions between glial components and neurons are elusive, but soluble factors from ACM enhance neural, cell growth and activity (Xu et al., 2005).

3.1.3.2 Transwell assay

Non-contact co-culture can be carried out using the transwell system where a 0.4 μm microporous membrane separates the two cell types and so blocks cell to cell contact, but allows small molecules to pass through.

3.1.4 Gap junctions

In a tissue, many cells connect to each other through cell-cell junctions. There are four main cell junctions including adherence junctions, tight junctions, GJs, and desmosomes. From those types of cell-cell junctions, GJs are the only cell-cell junction that allows the transfer of low molecular weight (1-2 kDa) molecules between cells. GJs are expressed on NSCs and are important in neural development and hippocampal neurogenesis (Zhang et al., 2017, Zhou and Jiang, 2014, Kunze). Astrocytes also express GJs where they are important in maintaining ionic homeostasis (Liu et al., 2017). Of particular relevance to this project, NSCs and astrocytes are connected by GJ

which have been shown to be important in NSC survival during adult neurogenesis (Liebmann et al., 2013, Rozental et al., 1998).

The situation with endothelial cells may be different from that between astrocytes and NSCs. NSCs are known to make direct contacts with the endothelial cells forming blood vessels (Tavazoie et al., 2008). In the SVZ these contacts are devoid of astrocyte end-feet and have been described as proliferative hotspots where NSCs show higher rates of proliferation. However while endothelial cells themselves form GJ with other endothelial cells (Yu et al., 2015). GJ have not been described between endothelial cells and NSCs. GJ are not the only contact dependent communication between endothelial and NSCs. Signalling is also mediated by receptors and membrane bound ligands found on the surface of endothelial and NSCs (Tung and Lee, 2012).

3.1.4.1 Connexins and GJs

As GJs are potentially important in communication between NSCs and other cells in the neurogenic niche this section will cover their structure and function. GJs are aggregates of inter-cellular channels, which connect the cytoplasm of adjacent cells. Structurally, GJs are composed of integral membrane proteins called connexins (Cx) (Figure 3.1) which form hexagonal arrangements within the cell membrane called connexons or hemichannels. The hemichannel in one cell is aligned and joined to a hemichannel in the adjacent cell to form a pore which allows the passage of small molecular weight molecules between cells (Fonseca et al., 2002). Clusters of these channels form GJs. The large family of Cx proteins are generally named after their molecular weight e.g Cx45. GJs in which both cells use the same Cx molecules are called homomeric while

those formed by different Cx are called heteromeric connexons (Meşe et al., 2007). GJ channels show different selectivity and permeability to the passing molecules according to the Cx subtype (Evans et al., 2006). GJ have a large pore diameter that allows the transfer of substance up to a molecular mass of 1.8 kilodalton (kDa) including cytosolic metabolites, ions (Na^+, K^+), signalling molecules such as adenosine triphosphate (ATP), metabolites (glucose, amino acids, nucleotides), second messengers (Ca^{2+}, cAMP, IP3) and dyes (calcein) (Kumar and Gilula, 1996, King and Lampe, 2005). A variety of techniques have been used to investigate the functional role and confirm the presence of gap junctional coupling. These include electrical conductance between coupled cells and the transfer of low molecular weight dyes between cells (dye coupling) (MacVicar and Dudek, 1981, Dermietzel and Spray, 1993). Both neurons and astrocytes express Cx43 (Ramos et al., 2017) and transgenic mice lacking Cx43 show abnormalities in neuronal migration during development (Giaume and Naus, 2013). GJs play significant regulatory roles in embryonic development, electrical coupling, metabolic transport in non-vascularized tissue, apoptosis, differentiation, and tissue homeostasis (Zhang et al., 2013, Sáez et al., 2003). Blocking GJ between astrocytes and NSCs makes the NSCs more susceptible to glutamate induced neurotoxicity (Ozog et al., 2002).

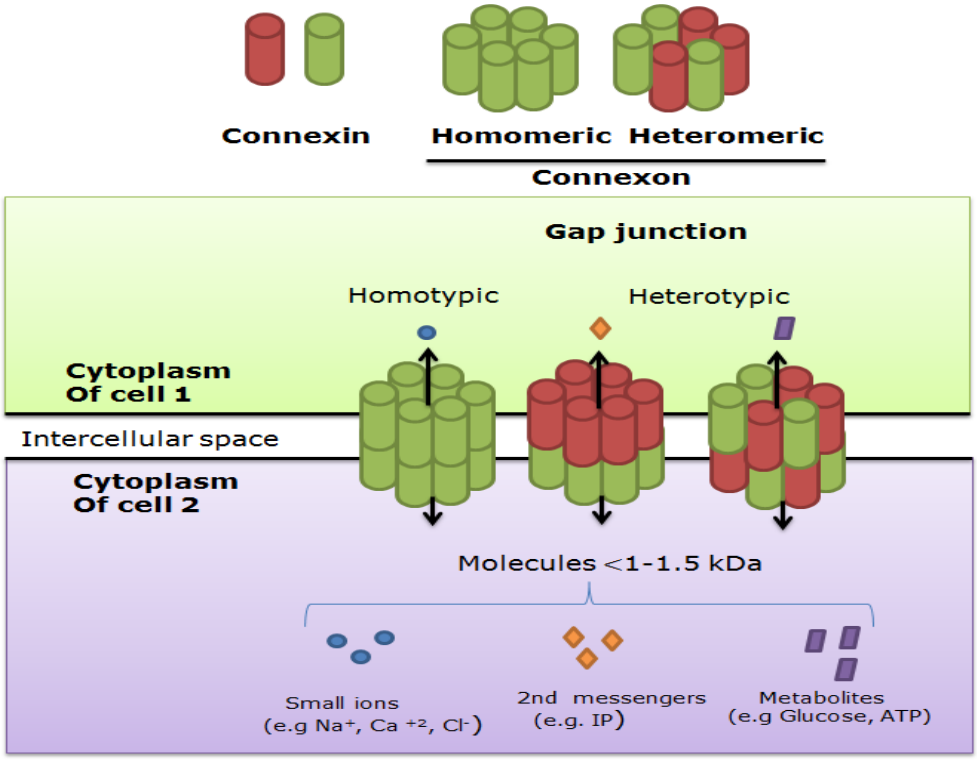


Figure 3.1 Schematic representation of connexins, connexons, and GJs. Diagram modified from Wong (Wong et al., 2004).

3.1.4.2 Gap junction inhibitor

Carbenoxolone (CBX) is a water-soluble synthetic GJ blocker (Davidson and Baumgarten, 1988). A number of studies have shown that blocking GJC between astrocytes and neurons increases the vulnerability of the neurons to oxidative stress (Ozog et al., 2002, Blanc et al., 1998). These results have been interpreted as showing that neuronal/ astrocytic GJC stabilizes intracellular calcium homeostasis and dissipates oxidative stress factors. CBX has been used in numerous studies both *in vivo* and *in vitro* to block GJs and has been found to interfere with synaptic function (Frantseva et al., 2002).

3.1.4.3 Dye coupling

Dye transfer or "dye coupling" is a powerful method to demonstrate the functionality of GJ connections between cells (Vaney, 2002, Arumugam et al., 2005). GJ functionality can be measured in co-culture using the fluorescence dye, calcein-AM which can pass through GJs into connected cells. The calcein AM kit provides a simple, rapid, and accurate method to measure cell viability. Calcein AM is a non-fluorescent, hydrophobic compound that easily permeates intact, live cells. The hydrolysis of calcein AM by intracellular esterases produces calcein, a hydrophilic, strongly fluorescents compound that is well-retained in the cell cytoplasm. Treatment with CBX prevents this dye transfer (Bani-Yaghoub et al., 1999).

3.1.5 Aims

As proliferating NSCs in contact with blood vessels are protected from 5-FU treatment, NSCs were co-cultured with either astrocytes or endothelial cells to see if either of these cells could provide protection. Co-cultures were set up which either allowed contact between the two cell types or separated them with a permeable membrane. This was done to determine if soluble factors or cell to cell contact was required for protection. CM from astrocytes or endothelial cells was tested for its protective actions. NSCs were treated with FLX or CM from astrocytes treated with FLX were also tested for their resistance to 5-FU. All cell types were stained for the GJ protein Cx43 and the functionality of GJ was tested by dye transfer between NSCs and astrocytes and endothelial cells. Finally, the GJ inhibitor CBX was used to see if blocking GJ affected the protection provided by astrocytes and endothelial cells.

3.2 Material and methods

3.2.1 Co-culture of N2a cells on different cell monolayers

To determine whether astrocytes or endothelial cells found in the neurogenic niche, can provide protection for NSCs from the effects of 5-FU, NSCs were seeded onto monolayer of astrocytes, endothelial cells or 3T3 fibroblasts.

The different cell lines were used in these experiments were N2a neural cells (Li et al., 2011), C6 astrocytic cells (Hur et al., 2004), HBMECs endothelial cells (Stins et al., 1997) and (3T3) mouse fibroblasts (Takeuchi et al., 2008). All cells were grown in RPMI-1640 containing 10% FBS, 2mM L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, 1% MEM vitamins and 5 ml Pen/Strep (Sigma Aldrich, USA).

For direct contact co-cultures, C6, HBMECs and 3T3 cells were grown in T75 flasks and were trypsinized when 80-90% confluent and plated into a 4-well chamber slide (Lab Tek- Nuna Thermo scientific, UK) and allowed to adhere overnight. To distinguish between N2a cells and C6, HBMECs or 3T3 cells in co-cultures, N2a cells were stained with the vital cytoplasmic stain (PKH26 red fluorescent cell linker- Sigma Aldrich, USA, see details below) before co-culturing. Stained single-cell suspensions of N2a cells were grown on top of confluent C6, HBMECs or 3T3 cells at a relatively low density (2.5×10^4cell/ml) or ratio of 1:2 based upon the previous literature (Kim et al., 2011) (Figure 3.2). One day after plating the N2a cells, media containing 5 µM 5-FU replaced the existing media and the co-cultures were incubated for a further 48 hours. Following this the media was aspirated carefully and the cells fixed in 4% PFA for 10 minutes. The cells were washed with PBS and all cell nuclei were visualized by staining

blue with DAPI. The cells were washed in PBS three times for 5 minutes. The plastic rim was removed from the slide and the cells mounted under coverslips with DABCO. Four different fields from three replicate wells were selected randomly for photographing and the total cell number together with stained N2a cells were counted blind at 40X magnification. All slides were viewed using a Nikon EFD-3 fluorescence microscope and photographed using a Spot Insight QE camera.

In each experiment there were 4 wells: -

N2a on their own with no chemotherapy

N2a on their own with chemotherapy

N2a co-cultured with no chemotherapy

N2a co-cultured with chemotherapy

The same protocol of co-culture was performed using primary astrocytes isolated as described previously. Rat astrocyte cells were split at a ratio of 1:2 and used at passage (2-6). Confluent primary astrocytes grown in T75 flasks were trypsinised and cultured on 4-well chamber slide at density 1×10^5. Next day, mouse hippocampal NSC, stained with PKH26, were seeded on to laminin coated wells at density 5×10^4 in astrocyte culture medium. The NSCs medium was changed to co-culture media which is DMEM/F12 supplemented with 1%FBS. Then, the cells were treated with 5μM 5-FU for 48 hours. Negative controls consisted of monocultures of NSCs. After 48 hours treatment in culture the number of cells stained red with PKH26 indicated the number of surviving neural cells were counted. Co-cultures were maintained for 5 days before fixing cells and immunostaining. For each group, cells were counted in four non-

overlapping optical fields of the microscope, the quantification for each group was repeated for three separate experiment.

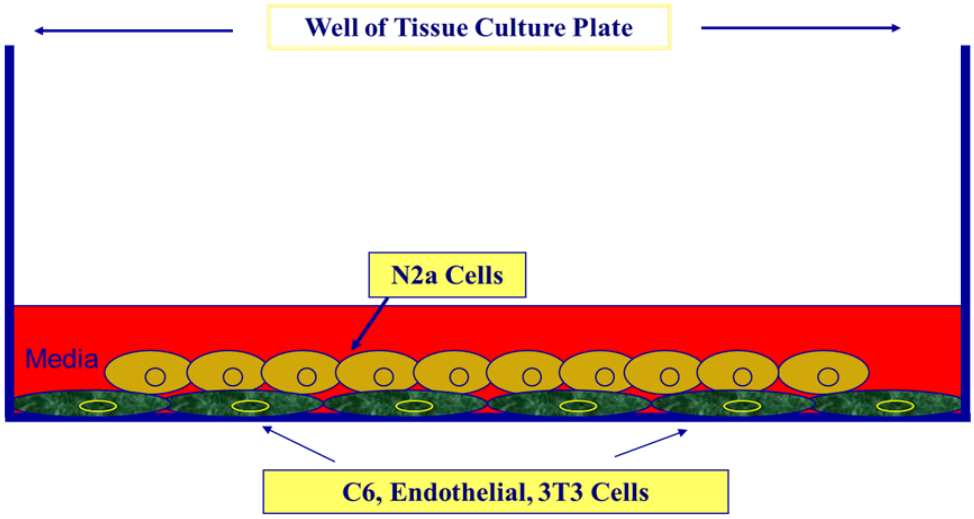


Figure 3.2 Co-culture model systems used for the analysis of cell-to-cell interactions. Different cell types were in direct contact. In this system, cells can be seeded directly on to chamber slide

3.2.1.1 The staining of N2a cells with the PKH26 red fluorescent dye

Cells were stained according to the manufacturer's instructions (Sigma-Aldrich-USA) with some modification. PKH26 is a lipophilic dye that labels the cell membrane and has low or no cytotoxicity (Fox et al., 1999, Samlowski et al., 1991, Chawla et al., 2006).

N2a cells were trypsinised, washed and resuspended at a density of 2×10^6 of cells in 500 µl of diluent C (250 µl for each 1 million of cells, dilution buffer provided in

PKH26 cell labelling kit). The, cell suspension was mixed with an equal volume of labelling solution containing PKH26 with dilution buffer (1 µl of PKH26 to 250 µl of diluent C) with gentle pipetting to ensure complete dispersion. This aqueous solution is designed to maintain cell viability, while maximizing dye solubility and staining efficiency during the labelling step. The cells/ dye suspension was incubated for 5 minutes at room temperature with gentle agitation every 1 minute. Because the cell-to-dye ratio can affect staining intensity, it is important that all cells are exposed to the same concentration of dye at the same time (Chung et al., 2014). The staining reaction was stopped by adding 2 ml of FBS and incubation at 37˚C for 1 minute. This was followed by centrifuging the cells at 400G for 5 minutes. After carefully removing the supernatant, the pellet was resuspended in complete media and transferred to a new tube to prevent carry-over of dye adsorbed on the tube walls. Stained cells were washed in 10ml of co-culture medium for three times and centrifuged (Schmidt et al., 2006). Then, the stained cells were seeded on top of the monolayer of supporting cell for 24hr. It was observed that the dye did not diffuse to adjacent unstained cells.

3.2.2 Production of CM

In order to evaluate the effect of secreted astrocyte (C6) and HBMEC derived factors on N2a survival, we produced CM from these cells which was then added to cultures of N2a cells which could be treated with 5-FU.

C6-CM and HBMEC-CM was used to test protection against the effects of 5-FU in 24-well plates. N2a cells were cultured at an initial density 5×10^4 cell/well in complete RPMI-1640 and incubated for 24 hours. C6 or HBMECs were cultured in T75 flasks in

complete RPMI-1640. CM was obtained from C6 and endothelial monolayers grown in RPMI for 24 hours during which they were in serum-free medium (SFM). Growing cells in serum free media eliminates any activity of the serum on the secreted growth factors. The media was aspirated from the cells and centrifuged at 2000 rpm for 5 minutes followed by filtration [0.22µm Millipore (Sartorius Stedim, 18555-K, Germany)] to remove any cells from the CM. The CM was mixed with fresh culture media containing 20% FBS (1:1) to produce a final FBS concentration of 10% as described in the literature (Környei et al., 2005). This media was transferred immediately onto N2a cells plated 24 hours before (Figure 3.3). When CM was used the treated cells were exposed to 5µM 5-FU with CM for 48 hours. Protection of N2a from the effect of 5-FU was quantified by using standard MTT assay.

In each experiment there were 4 groups: -

N2a cultivated in fresh medium with no chemotherapy

N2a cultivated in fresh medium with chemotherapy

N2a cultivated in endothelial/ glial cell CM with no chemotherapy

N2a cultivated in endothelial/ glial cell CM with chemotherapy

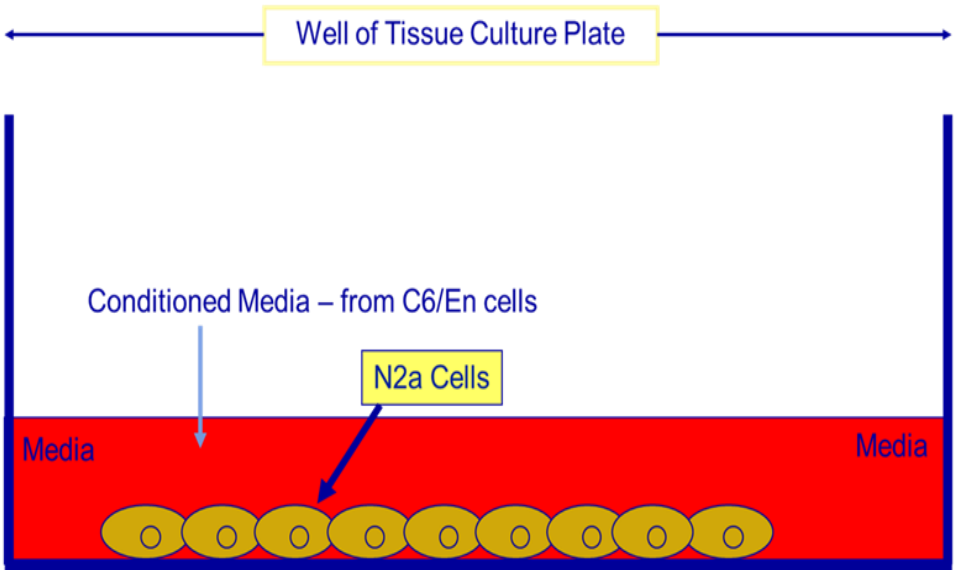


Figure 3.3 Scheme for standardized CM production. N2a with CM from C6 or endothelial cells.

3.2.3 Effect of FLX in protecting N2a cells from the effects of 5-FU

The effect of FLX on N2a viability was measured by MTT assay. This was followed by exposing C6 astrocytes to FLX. C6 cells were grown to confluence; the media was changed to serum– free RPMI-1640 one day before treatment with FLX to eliminate the effects of serum as proposed by Allaman et al. C6 cells were exposed to FLX at a concentration of 10 µM for 24 hours. The concentration of 10µM FLX was chosen based on previous studies (Allaman et al., 2011). The media from these cells was collected and used as C6-CM+FLX. The effect of this CM was tested on N2a cells. CM+FLX media was mixed (1:1) with fresh culture medium prior to being put onto N2a cells. Cell viability was measured by the MTT assay. All data shown are representative of at least three different experiments.

In each experiment there were 4 groups: -

N2a cultivated in fresh medium

N2a cultivated in fresh medium with chemotherapy

N2a cultivated in C6/ Primary astrocyte CM+FLX

N2a cultivated in C6/ Primary astrocyte CM+FLX with chemotherapy

The effect of CM obtained from primary astrocyte cultures on NSCs was investigated using the same protocol as that used with the CM from cell lines except that the NSCs were seeded into 24-well plates coated with laminin in NeuroBasal/DMEM/F12. Astrocyte cells were grown in complete medium DMEM until 70–80% cell density. One day before taken CM, the media was changed to SFM. This media was transferred to NSCs in culture after mixing with NSC media/DMEM (1:1).

3.2.4 Transwell assay

To determine whether direct physical contact between N2a and C6 cells was required to provide protection against chemotherapy, we established an *in vitro* co-culture model using a transwell insert (Corning Incorporated costar, 3460, USA). The insert has a microporous (0.4 μm) membrane which separates the chamber into upper and lower parts. A confluent flask of C6 cells was washed, trypsinised and centrifuged at 1000 rpm for 5 minutes. The cells were counted using a cell counter. Transwells were seeded in a two-step process.

First 1×10^5 (per filter) C6 cells were cultured on the lower side of the membrane. These inserts were aseptically handled with sterile forceps. For the culture, 300 µl of cell suspension was added and transferred to 6-well plates. In order that the medium did not dry out during incubation, 1ml media was added to the empty wells. The cells were incubated for two days to adhere and grow to confluence on the transwell in an upside-down position.

Then, the insert was transferred to a 12-well plate and turned upside down. On the upper side of the membrane, N2a cells stained with PKH26 at a density of 5×10^4 (cells per filter) were added in 600 µl media without any coating on the transwell membrane. This co-culture model allowed C6 and N2a cells to share same growth medium and allowed only soluble factors to freely diffuse between cells but prevented physical contact. To determine the effect of 5-FU on NSCs, we treat the cells with 5 µM 5-FU for 48 hours as described in chapter 2. After this, the inserts were fixed with 4% PFA for 5 minutes, followed by 3 washes in PBS, care was taken not to dislodge cells. Then, the cells were counter stained with DAPI. Filters membranes with the cells were then cut out of the inserts using scalpel blades, and transferred directly onto microscope slides, and cover slips mounted using DABCO.

The protocol of culturing primary cells in transwells was similar to that described above with the following changes. When primary NSCs were cultured with primary astrocytes, transwell filters were coated, on both sides, with laminin. Astrocytes were cultured on the lower side of insert, in an inverted position, at a density of 5×10^5 cell/filter in a six-well plate and allowed to attach. Then, the medium was changed from DMEM (10% FBS) to serum-free medium (DMEM/F12) in order to culture them in the

same media as NSCs. The cells were incubated for 24 hours. The inserts were transferred to 12-well plates. NSCs were cultured on upper side of the membrane in SFM. The culture media used for co-culture was DMEM/F12 with 1% Pen/Strep in serum free media. Serum was not used as addition of serum causes NSCs to differentiate. During the co-culture process, the cells were monitored and photographed using an inverted phase contrast microscope. For controls, NSCs were grown on the membrane with no cells on the lower side. This assay is illustrated in (figure 3.4) below.

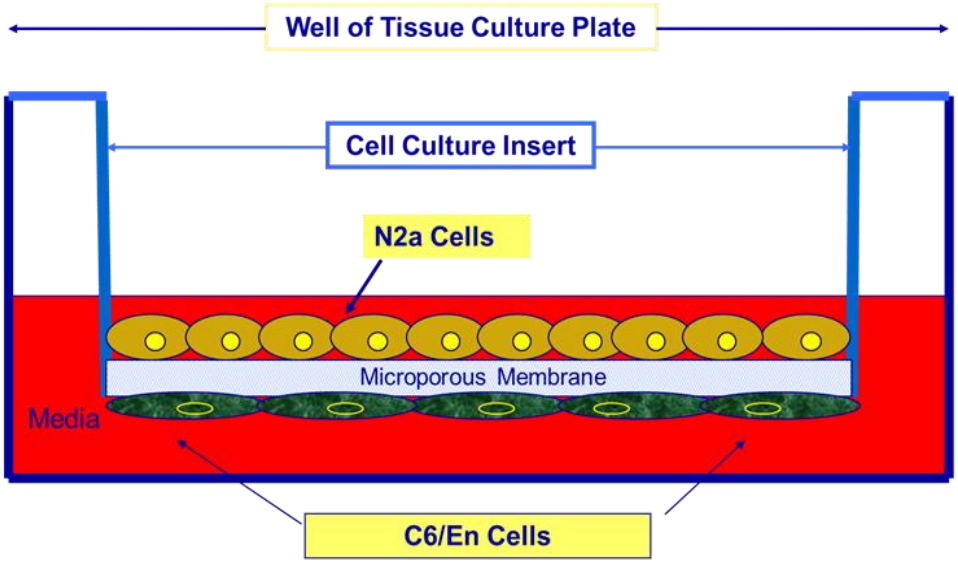


Figure 3.4 Transwell assay setup: The transwell is divided into an upper and a lower side by a microporous membrane (pore size 0.4 μm) allowing only soluble factors to pass through the membrane. The arrangement allows non-contact interaction between two different cell types to be studied. The upper chamber contained primary NSCs cells or N2a cells stained with PKH26 and the lower chamber contained C6 or astrocyte cells.

3.2.5 Immunocytochemical detection of the gap junction protein Cx43

C6, astrocytes, HBMECs, N2a and NSCs cells were seeded onto 13 mm coverslip slides and grown to 60-70 % confluency. The growth medium was removed, and the cells

washed with PBS, followed by fixation in 4 % PFA for 5-10 minutes and washing with PBS to remove excess fixative. Cells were permeabilised with Triton which was then removed by 3 washes in PBS for 5 minutes each. Following permeabilisation, the cells were incubated with primary rabbit polyclonal Cx43 (Cell Signalling Technology, Cat. No. 3512) at 1:100 (3 % goat serum (Biological Industries, *In Vitro*, Denmark), 2 % bovine serum albumin (BSA) (Sigma-Aldrich), and 0.3 % Triton X-100 in PBS) and incubated at 4°C overnight. The goat serum and BSA included in the incubation buffer blocks nonspecific binding of antibody. The next day, the cells were washed and incubated with secondary antibody (Goat anti-Rabbit Alexa Fluor 488– Life Technologies, A11034) diluted 1:500 in blocking agent for 1hour at room temperature before washing and nuclear staining with DAPI (Sigma-Aldrich) and mounting the coverslips in fluorescence mounting medium (Dako, Denmark). Negative controls were obtained by following the same procedure, except for omission of the primary antibody. The cells were visualized under a non-inverted Optiphot fluorescence microscope.

3.2.6 Treatment with CBX

The effect of CBX on C6, HBMEC and N2a viability was measured by MTT assay to determine the toxicity of CBX. Then, to determine whether the neuroprotection mediated by astrocytes occurred by gap junction communication (GJC), we carried out co-culture in the presence of CBX (50 μM) (Sigma, Aldrich, UK), a potent inhibitor of GJC. After cells attached, C6 and N2a cells were pretreated with CBX for 1 hour and then incubated with 5μM 5-FU for 48 hours in the presence of CBX. Controls consisted of cultures treated with either CBX or 5-FU on their own. Surviving neural cells were quantified by counting PKH26 stained cells.

In each experiment there were 8 wells: -

N2a on their own with no chemotherapy

N2a on their own with chemotherapy

N2a on their own with CBX

N2a on their own with chemotherapy and CBX

N2a co-cultured with no chemotherapy and no CBX

N2a co-cultured with chemotherapy

N2a co-cultured no chemotherapy and CBX

N2a co-cultured with CBX

3.2.7 Dye Coupling

Dye coupling experiments were carried out to estimate the functionality of GJs between individual C6 cells and the surrounding N2a cells or primary NSC and astrocytes. Dye coupling was assessed by pre-loading as described by Goldberg et al (Goldberg et al., 1995) with some modifications.

The dye used was the acetoxymethyl (AM) ester derivative of the fluorescent indicator calcein (Calcein-AM, 3-6 Di amino methyl) fluorescein, tetraacetoxy methyl ester, C-3100; Molecular Probes by Life technology, USA. Calcein-AM (MW: 623). Calcein-AM is non-fluorescent, electrically neutral and a highly lipophilic agent. The lipophilic characteristic of calcein-AM is due to its lipophilic acetoxymethyl group (AM) which allows calcein-AM to easily permeate the plasma membrane (Wang et al., 1993, Lichtenfels et al., 1994, Thomas et al., 2004). Calcein AM is a fluorogenic substrate

that is cleaved in viable cells to form a green fluorescent negatively charged membrane-impermeable product, calcein unable to pass through the cell membrane except via GJs. Dye coupling was evaluated after 4 hours by detecting the presence of green fluorescent calcein in adjacent cells (Fonseca et al., 2006).

C6 cells were preloaded with 5mM (Calcein AM, Invitrogen) for 20 minutes at 37°C. Cells were washed with SFM three times to remove excessive dye. Cells were passaged, counted and plated on coverslips. PKH26 was used to stain N2a cells as previously described.

As controls, cells were cultured with 3T3 and CBX (50 µM). In addition, the calcein stained cells alone (0 h of co-culture) and PKH26 stained N2a cells alone were used as controls. The level of coupling was determined by counting calcein positive, PKH26 negative cells coupled to a calcein positive cell. Cells were fixed in 4% PFA for 10 minutes and viewed at 20X and 40X using a fluorescence microscope. On each coverslip, 10 non-overlapping areas were counted. One experimental group consisted of cell counts from three coverslips and each series of experiments was repeated three times.

3.2.8 Statistical analysis

The parametric data which represent three to five times experimental repeats was analysed by using GraphPad Prism v7.0. Quantitative data were expressed as mean ± SEM and co-culture analysed with Two-way ANOVA was performed followed by Tukey multiple comparison test was undertaken to check the significance of difference by comparing the mean of the control group with that of experimental group.

Differences of $P < 0.05$ were considered significant. Statistically significant differences are indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

In microscopy experiments, cells were counted on three coverslips (4 fields of view counted per coverslip) per treatment from three independent experiments. Data were expressed as a percentage of the total number of cells counted per field.

3.3 Results

3.3.1 Co-culture of different cellular components of the stem cell niche with neural cells to determine if any cell types can protect neural stem cells from chemotherapy

From the dose response curves shown in Figure (2.10) in Chapter 2, it was found that a dose of 5 µM 5-FU has a small effect on the survival of astrocytic cells, but a much larger effect on N2a cells. This dose was therefore used to test the ability of astrocytes or endothelial cells to protect neural cells from the effects of chemotherapy. Astrocytes or endothelial cells were cultured as a monolayer and stained N2a cells seeded onto this layer prior to treatment with 5-FU. The survival of N2a cells after chemotherapy treatment on their own or in combination with other cell types was then investigated by counting the numbers of marked N2a cells (Figure 3.5). The results are shown in (Figure 3.6). As expected from the dose response results, N2a cells on their own showed a significant drop in cell viability when treated with 5 µM 5-FU.

Co-culture of N2a cells with astrocytic or endothelial cells showed a significant improvement in N2a cell viability after chemotherapy treatment. Based on these results, C6 astrocytes and endothelial cells have a significant protective effect on N2a cells

when exposed to 5 µM 5- FU. To evaluate if this effect were specific to C6 astrocytes and HBMEC endothelial cells or was a general effect of co-culturing N2A cells with another cell type, N2a cells were co-cultured with the non-neural 3T3 fibroblast cell line. The results of co-culture of N2a cells with 3T3 fibroblasts showed that these cells had no protective effect on the viability of N2A cells when treated with 5 µM 5-FU (Figure 3.6).

To determine if these results were limited to cell lines or astrocytes from particular regions of the CNS we used the co-culture system to determine the ability of primary astrocytes to support neural cell survival. Primary astrocytes were isolated either from the hippocampus or spinal cord of neonatal rats. These results mimicked those obtained using the C6 astrocyte cell line, both primary hippocampal and spinal astrocytes provided protection for N2a cells against 5-FU (Figure 3.6).

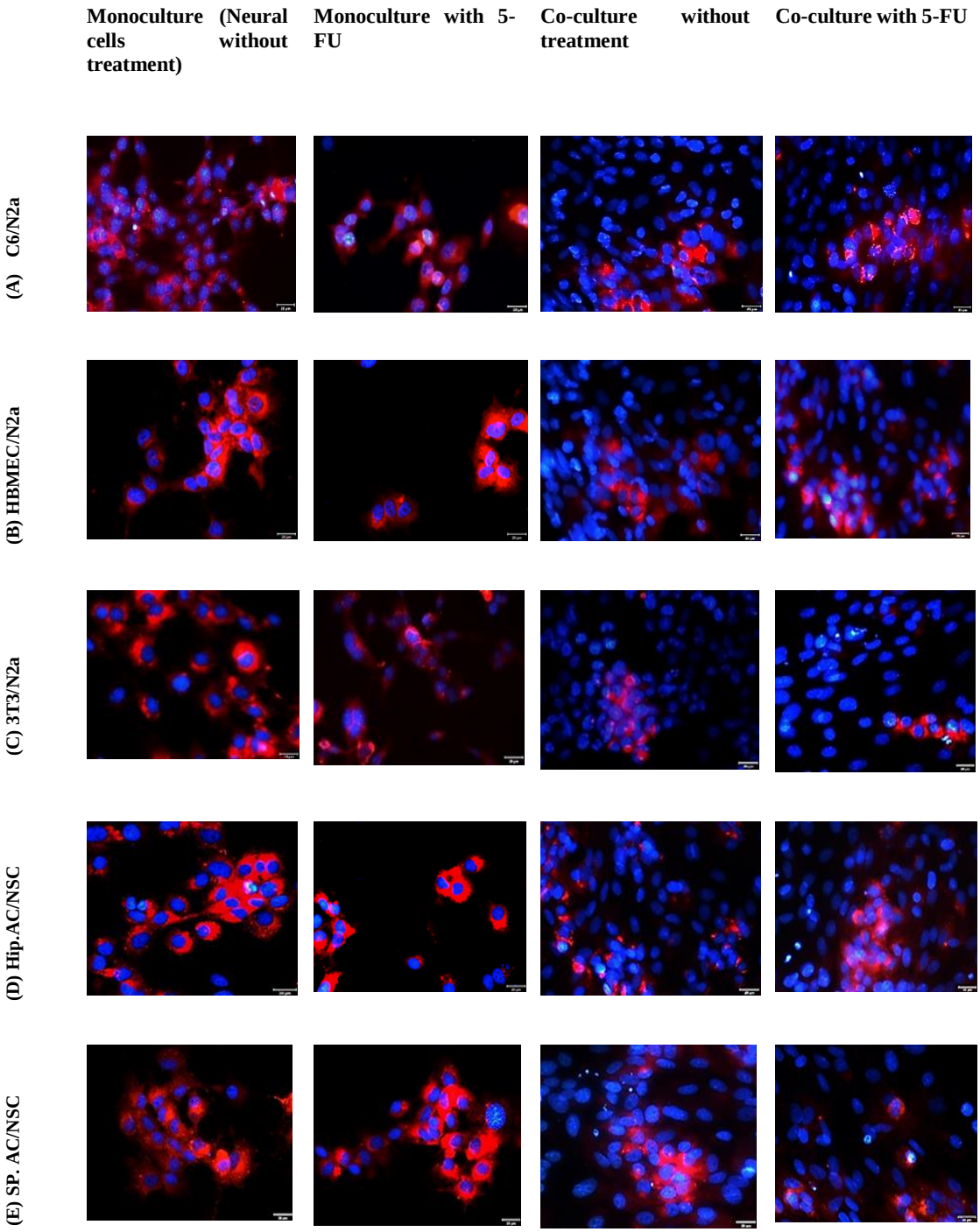
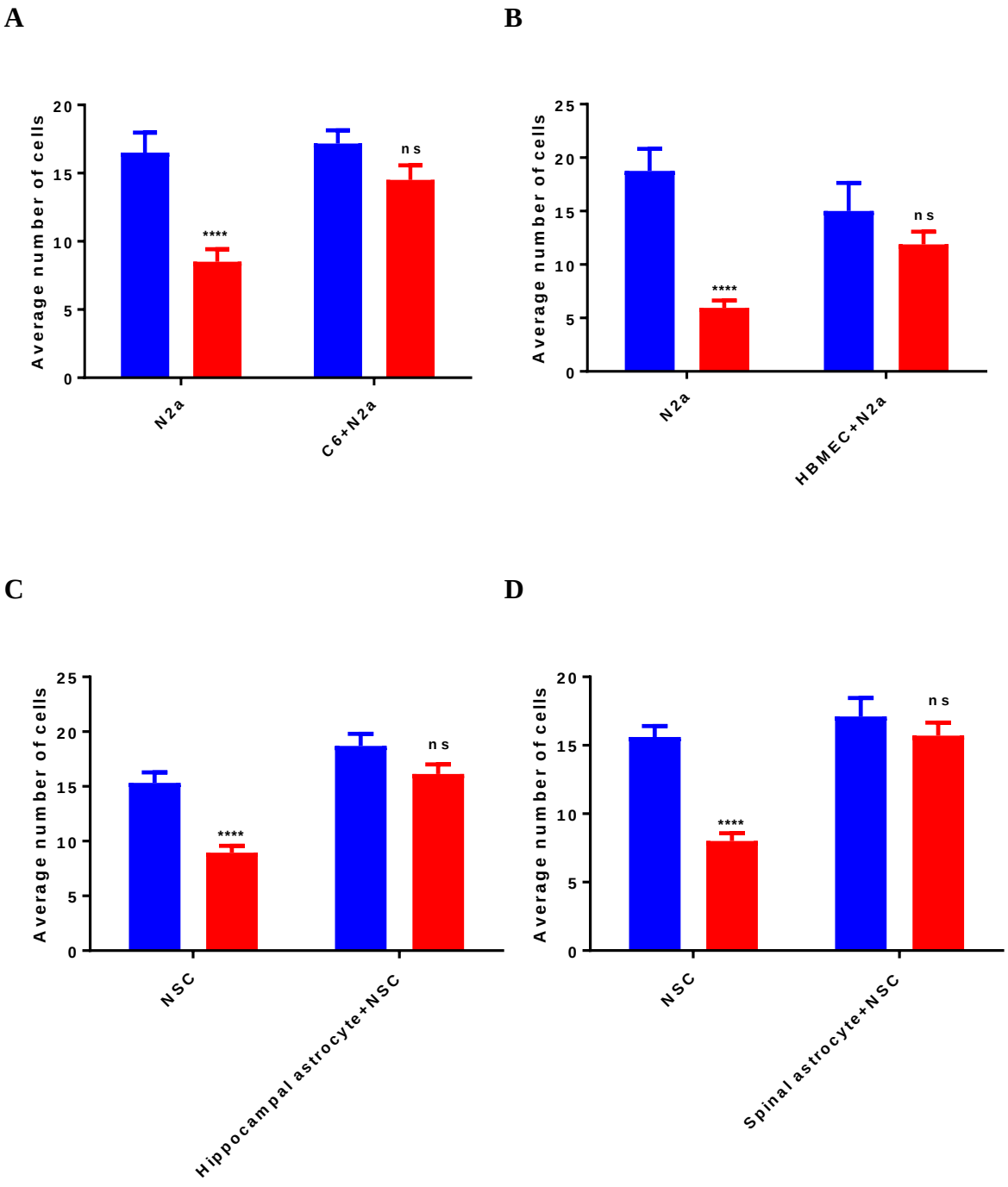


Figure 3.5 Fluorescent microscope images of different cell types co-cultured with N2a cells after 48 hours 5-FU treatment. N2a cells dyed red with PKH26 were seeded on top of confluent monolayers of the other cells types. Co-cultures were grown for 48 hours before being treated with 5-FU. Neural stained with PKH26 (red), all nuclei stained with DAPI (blue). Co-cultures of C6 with N2a (A). Co-cultures of HBMEC endothelial cell with N2a (B). Co-

cultures of 3T3 with N2a (C). Co-cultures of hippocampal astrocytes with NSCs (D). Co-cultures of spinal astrocyte with NSCs (E). Scale bar 20μm.



E

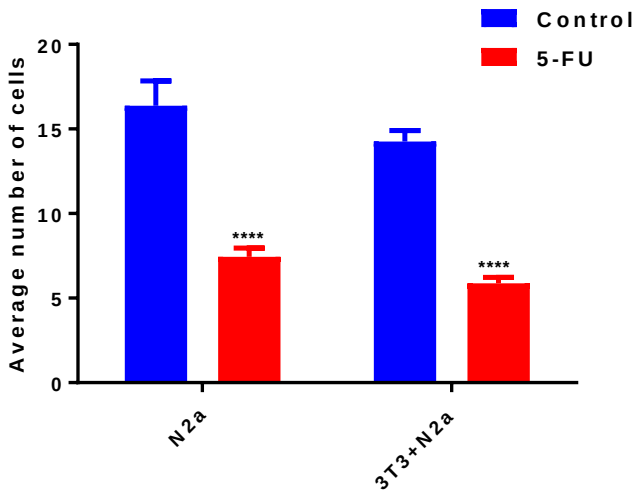


Figure 3.6 Effect of 5 µM 5-FU on the survival of N2a cells when cultured alone or co-cultured with C6 astrocytes, primary hippocampal astrocytes, primary spinal cord astrocytes, HBMECs or 3T3 cells. Blue bars indicate the number of cells per field without 5-FU treatment while red bars indicate the number of cells per field after 48hrs of 5-FU treatment. In all cases N2a cells on their own when treated with 5 µM 5-FU, showed a significant drop in cell number per field. Co-culture of N2a cells with HBMEC, astrocytes or 3T3 cells had no effect on the growth of the N2a cells in the absence of chemotherapy treatment. When N2a cells were co-cultured with astrocytes or HBMECs, treatment with 5-FU did not significantly reduce N2a viability. In contrast, no protection was observed when N2a cells were co-cultured with 3T3 cells. N2a cell number was determined by counting cells co-labelled for DAPI and PKH26 per defined area. Four optical fields were counted per group, counting was repeated in three separate experiments. Statistical significance: $p<0.005$ (Tukey Post-hoc analysis). Data shown as mean value± SEM from four repeated for each experiment. *** $p<0.0001$ indicates significant difference from the control groups.

3.3.2 The effects of CM, from different cell types within the neurogenic niche, on N2a cell number after 5-FU treatment

In the previous experiments, N2a cells were seeded directly onto monolayers of another cell type. This showed that C6 astrocytes and HBMECs provided protection from 5-FU to the N2a cells. This protection might be mediated by soluble factors secreted by the supporting cells or might require direct cell contact between the N2a cells and the underlying monolayer. To test which of these hypotheses is correct, N2a cells were grown in cell free CM from both endothelial and astrocytic cell lines and this media was tested for its protective effect on N2a cells treated with 5-FU.

Treatment of N2a cells grown in either fresh media or CM media from either HBMEC or C6 cells failed to protect N2a cells from 5-FU (Figure 3.7). This suggests that soluble factors secreted by either C6 or HBMECs do not provide the protection provided by these two cell lines when they are co-cultured with N2a cells. Based on these results, we can conclude that the protection provided by co-culture depends either on direct contact or very close association between N2a cells and astrocytes or endothelial cells. Cell-to-cell contact could allow GJs between cells or receptor-ligand binding. Alternatively, very close proximity of the protecting cells could provide high concentrations of factors released from endothelial or astrocytes to the N2a cells.

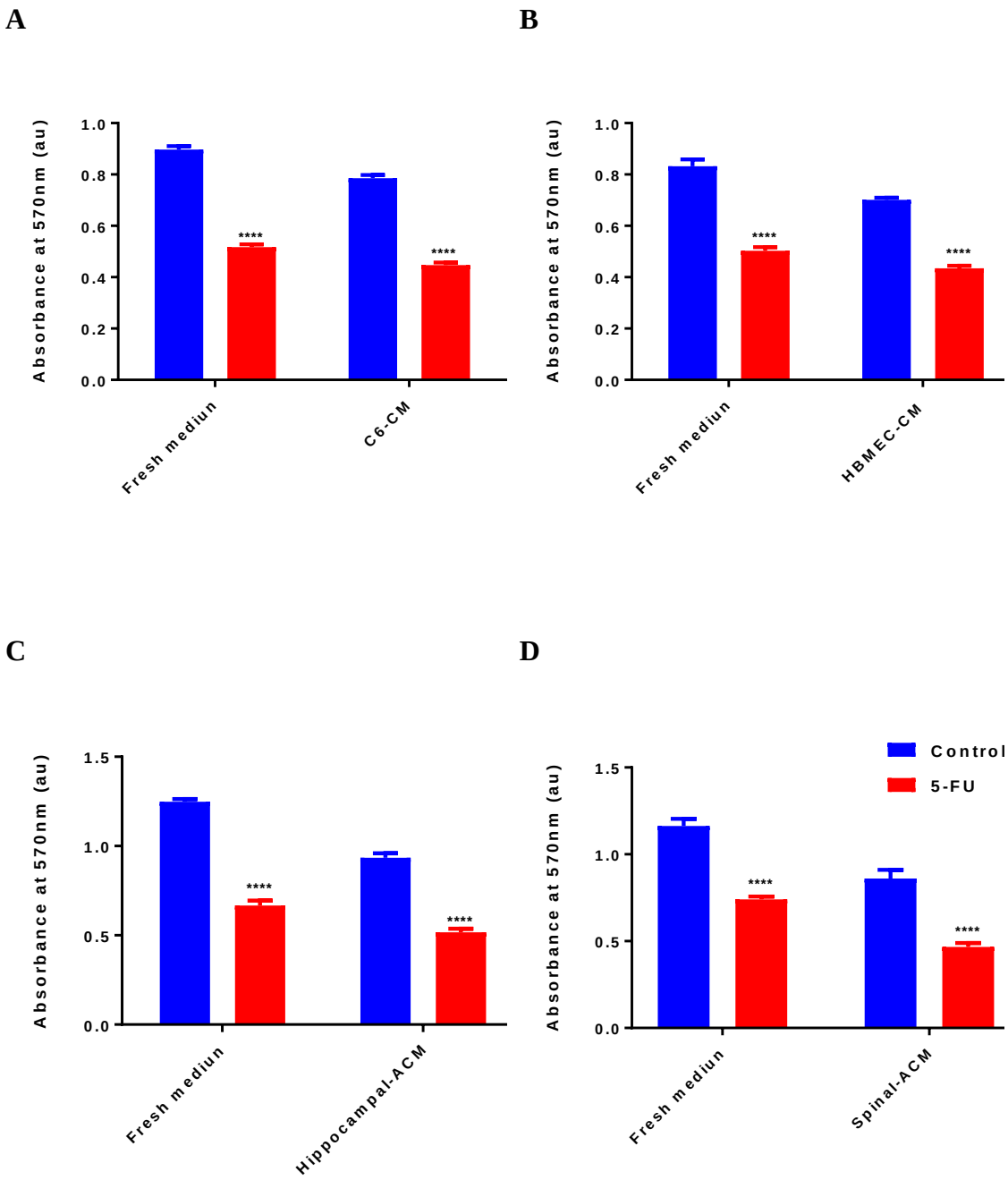


Figure 3.7 The effect of astrocyte or endothelial CM in protecting N2a cells from 5-FU. N2a monocultures were exposed to CM and 5 µM 5-FU for 48 hours. After treatment, cell viability was measured by MTT assay. The viability of neural cells treated with 5-FU and cultivated in C6-CM (A), HBMEC-CM (B), hippocampal-ACM (C) and spinal-ACM (D), were significantly reduced compared to controls cultured in fresh media without 5-FU. No protection of N2a cells was observed with CM from C6 astrocytes or HBMECs. Data are means ± SEM of three separate experiments. Two-way ANOVA with Tukey test was performed. Levels of significance were ns, **** $p<0.0001$.

3.3.3 The effect of the antidepressant FLX on N2a proliferation

Chronic FLX treatment, *in vivo*, protects proliferating NSCs in the SGZ of the dentate gyrus from the effects of 5-FU treatment (ELBeltagy et al., 2010b, Lyons et al., 2011). It is unknown if this is a direct effect of this drug, on proliferating neural cells, or whether it is an indirect effect, possibly mediated by the action of FLX on other cellular components of the stem cell niche. The effect of FLX on N2a viability was measured by MTT assay. Our results showed that FLX had no direct effect on N2a viability (Figure 3.8). Therefore, FLX may increase the proliferation and protection of neural cells *in vivo* via an unknown and possibly indirect mechanism.

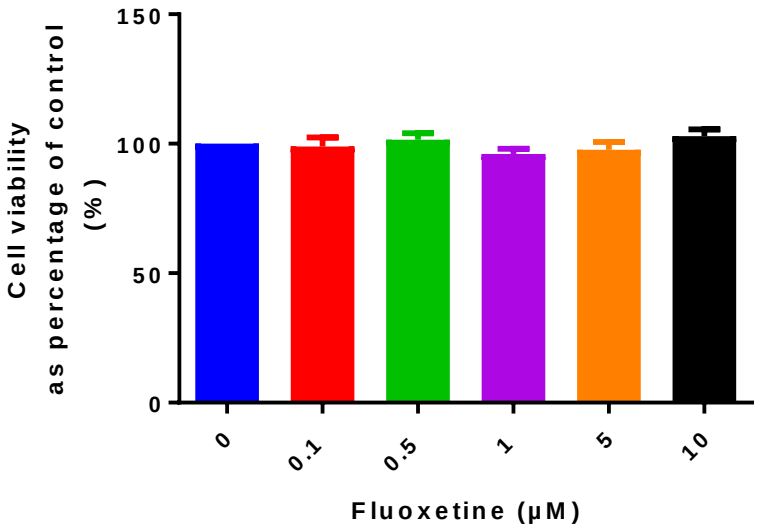


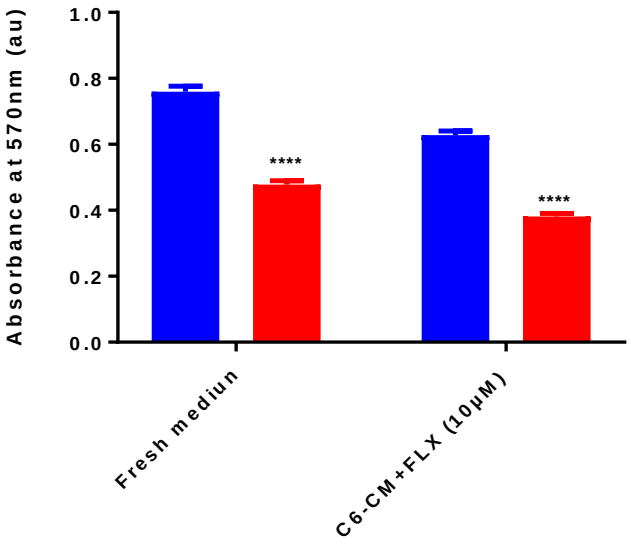
Figure 3.8 Evaluation of the cell viability by MTT assay of N2a cells in different concentrations of FLX (0.1, 0.5, 1, 5, and 10µM) for 48 hours. FLX had no effect on N2a proliferation. Data were analysed with One-way ANOVA. Significance was defined as $p < 0.0001$ as compared to control.

3.3.3.1 The effect of FLX on astrocytes and the CM produced by these cells

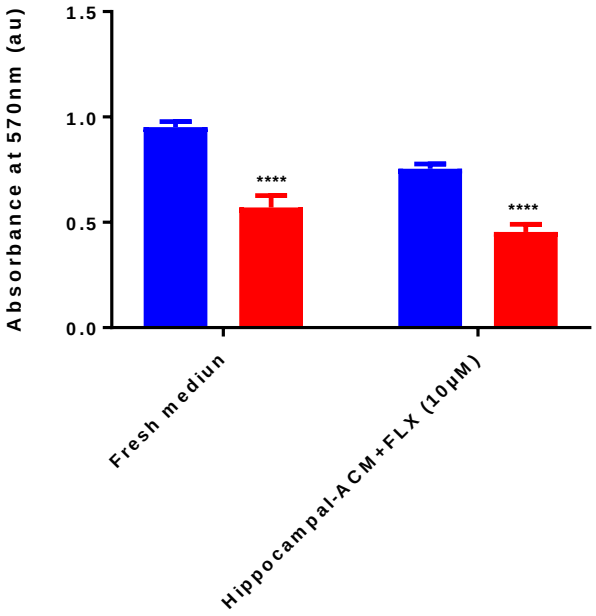
One hypothesis is that FLX-induces the release of factors from astrocytes which may increase the proliferation and survival of NSCs *in vivo*. To test this hypothesis, we tested whether CM from C6 cells treated with FLX would provide protection.

N2a cells were grown in CM from astrocytes treated with FLX in the presence or absence of 5-FU. CM+FLX was mixed with an equal volume of fresh culture medium. The survival and growth of cells were measured by MTT assay. The results showed that media from ACM which had been treated with the FLX did not contribute to the survival of N2a cells when treated with 5-FU (Figure 3.9). Therefore, FLX does not either directly protect the N2a cells from the effect of 5-FU nor via factors released from FLX treated astrocytes.

A


Absorbance at 570nm (au)
1.0
0.8
0.6
0.4
0.2
0.0
****
****
Fresh mediun
C6-CM+FLX (10μM)

B


Absorbance at 570nm (au)
1.5
1.0
0.5
0.0
****
****
Fresh mediun
Hippocampal-ACM+FLX (10μM)

C

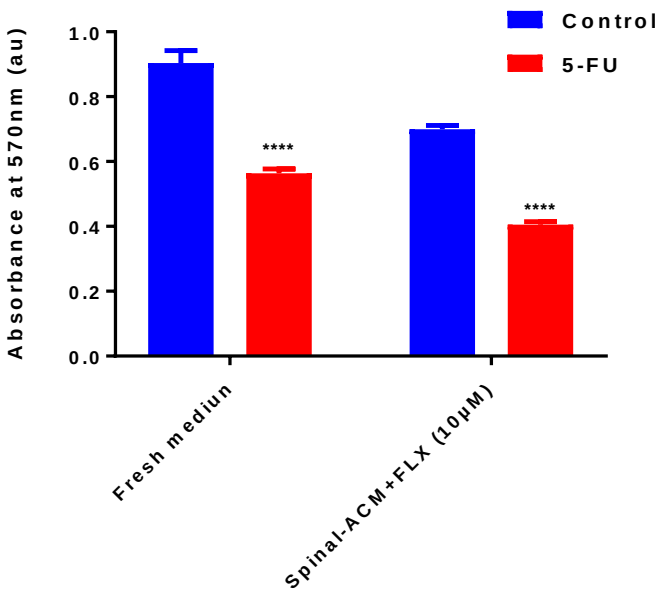


Figure 3.9 Effect of 10 µM FLX conditioned glial media on neural cells. C6 astrocytes were incubated for 24 hours in free serum-RPMI and then for a further 24hour in 10µM FLX. N2a cells were cultured in 50% FLX- CM for 48 hours 24 hours after they were serum starved. Data indicated that after 5-FU treatment there was no significant difference in the survival of cells in FLX-CM media and control media. The results are expressed as the mean±S.E.M. Statistical analysis was performed using Two-way ANOVA and Tukey post hoc test. Significance was defined as $p<0.05$, three independent experiments were carried out.

3.3.4 Co-culture of N2a cells with either astrocytes or endothelial cell in a transwell assay to test for the impact of secreted factors in protecting neural cells from 5-FU

The previous results, where cells were co-cultured and allowed to have direct cell to cell contact showed that astrocytes and endothelial cells (but not 3T3 fibroblasts) protected NSCs cells from 5-FU. To test whether C6, astrocytes; primary spinal cord astrocytes, primary hippocampal astrocytes or endothelial cells can protect N2a and

NSCs by secreting soluble factors which may only act at short range, we used a co-culture system that prevented direct cell to cell contact but cultured them in close proximity and allowed media to pass between them. However, co-culture, where there is no contact, did not provide any protection by any of the cell types. In contrast when cells were co-cultured and allowed direct cell to cell contact this provided protection (Figure 3.11). From these results we concluded that the protection provided by astrocytes requires cell-cell contact.

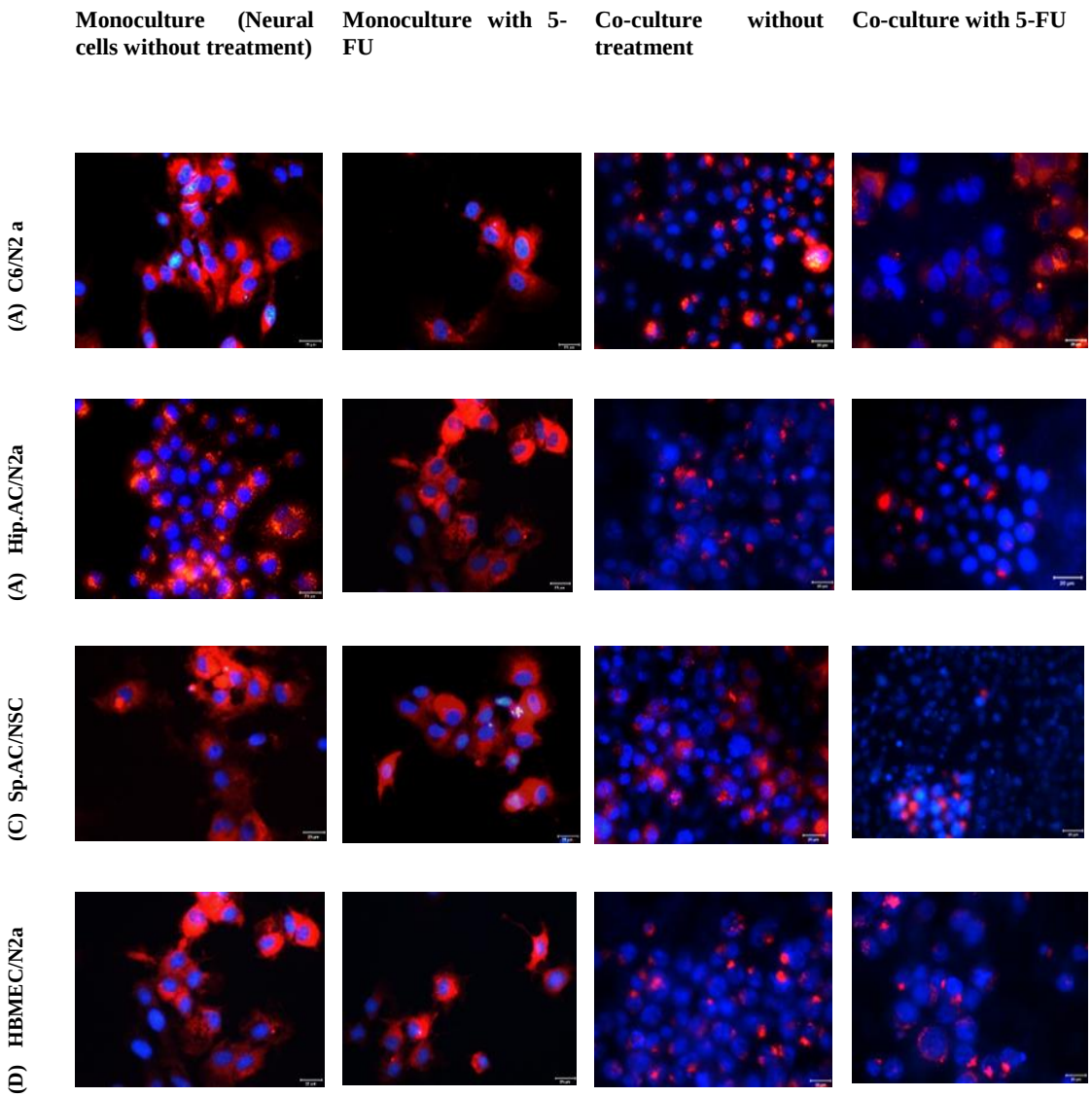


Figure 3.10 Representative fluorescence images of cells in transwell co-culture system. Neural cells were grown on the upper side of the transwell membrane filter and other cell types were grown on the lower side: C6 (A), hippocampal astrocytes (B), spinal astrocytes (C), HBMECs (D). Neural cell stained with PKH26 (red), all nuclei stained with DAPI (blue). The number of surviving neural cells in the microscope field (40X magnification) was counted. Two days after addition of 5-FU, co-cultures were fixed. Scale bar: 20μm.

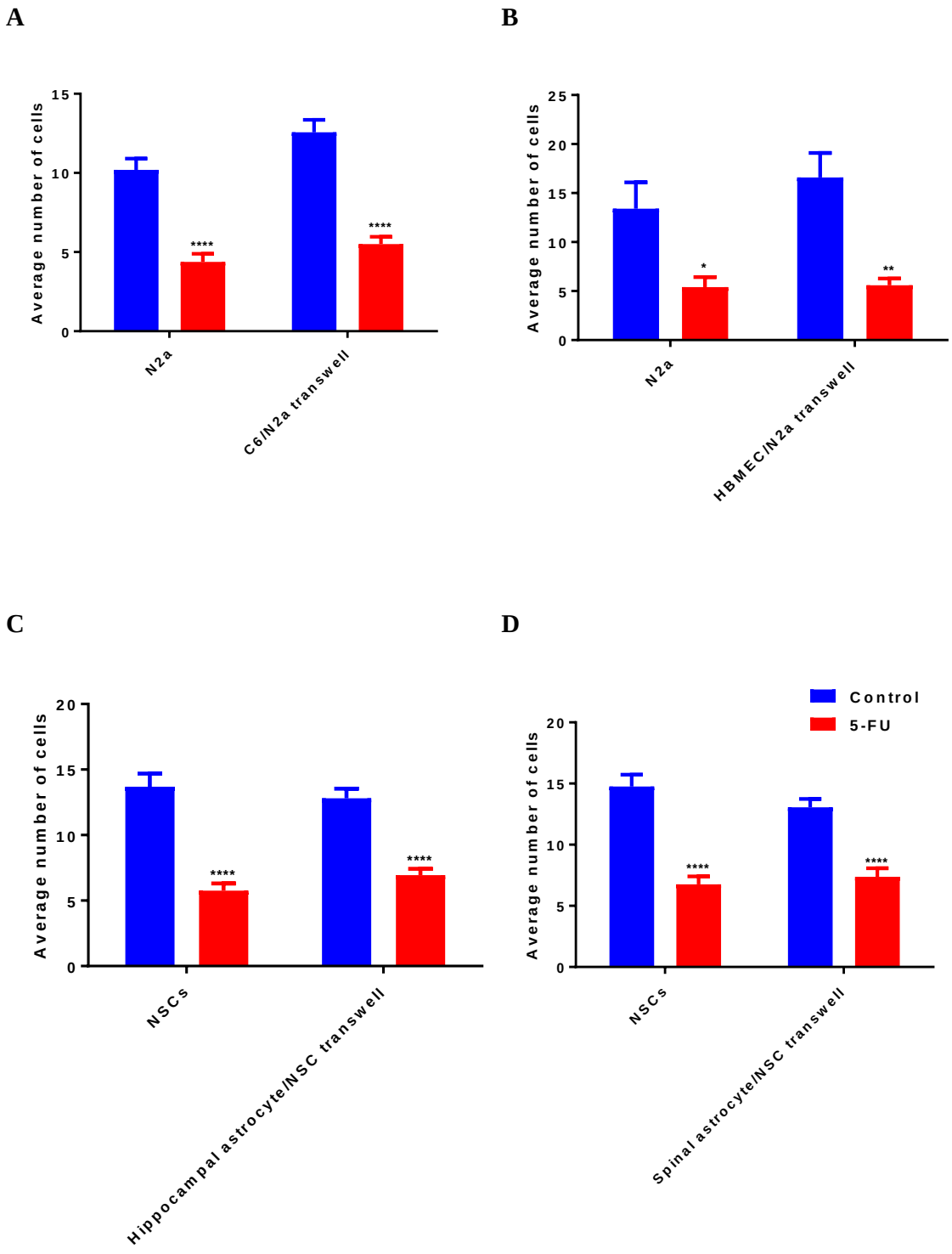


Figure 3.11 Transwell co-culture of C6 astrocytes; HBMEC; primary hippocampal astrocytes; primary spinal cord astrocytes and N2a cells. Transwell co-culture was used to test the ability of cells to protect N2a cells from the effects of 5-FU treatment. N2a cells were grown on the upper side of the transwell membrane (pore size 0.4µm) while other cell types were grown on the lower side. N2a cells and astrocytes were also plated as single cell type cultures in the well in the insert (top). Control cells were maintained in growth media. The

protection of N2a cells from 5-FU by co-culture with primary astrocytes, C6 and HBMECs was lost when they were separated by a porous membrane in transwell co-culture. The values are shown as the mean ± SEM of three independent experiments; Two way ANOVA ** $p<0.05$.

3.3.5 Identification of GJs between N2a cells and other cell types

As direct cell-cell contact appears to be required for protection from 5-FU it is possible that GJs are involved. To investigate this, cells were immunocytochemically stained using an antibody against Cx43. Positive staining for this Cx was found in astrocytes, HBMEC and N2a cells (Figure 3.12). Expression of this Cx, by all the cell types tested, would enable them to form homomeric GJs between the different cell types.

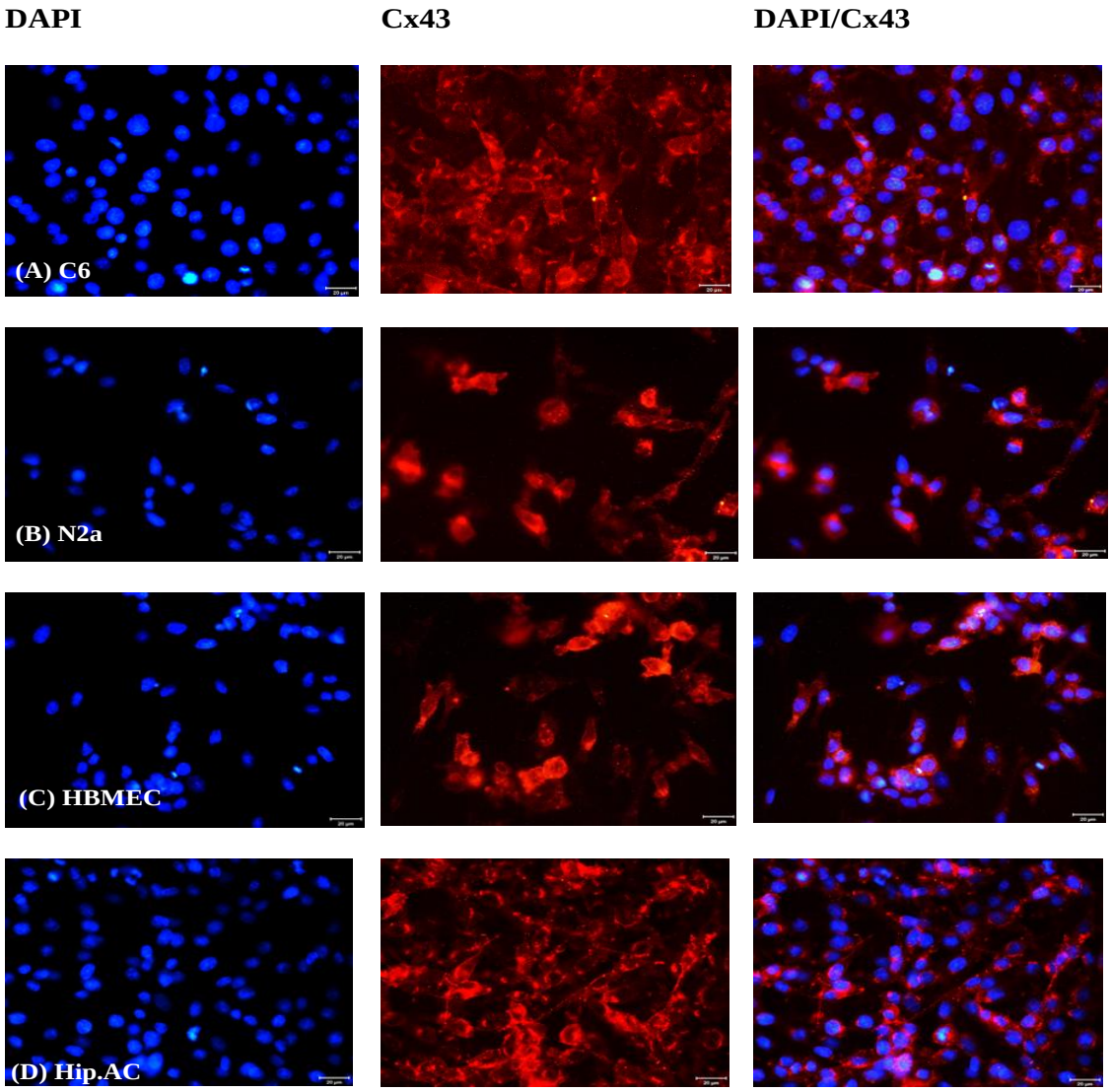


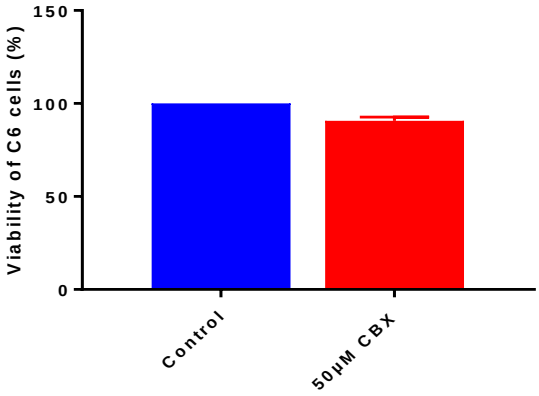
Figure 3.12 Immunofluorescence staining of for Cx43 in different cell types. Expression of Cx43 was seen in C6 (A), N2a (B), HBMEC (C) and hippocampal astrocytes cells (D). Astrocytes appeared to express the highest levels of Cx43. Cells were counter stained with DAPI (blue left hand panel), Cx43 expression (red middle panel) and merged (right hand panel). Cx43 staining is seen in the membranes of cells in contact. No staining was observed when the secondary antibody was omitted. Scale bars 20 μm.

3.3.5.1 The effect of treatment with the gap junction inhibitor CBX on the ability of astrocytes to protect N2a cells from 5-FU

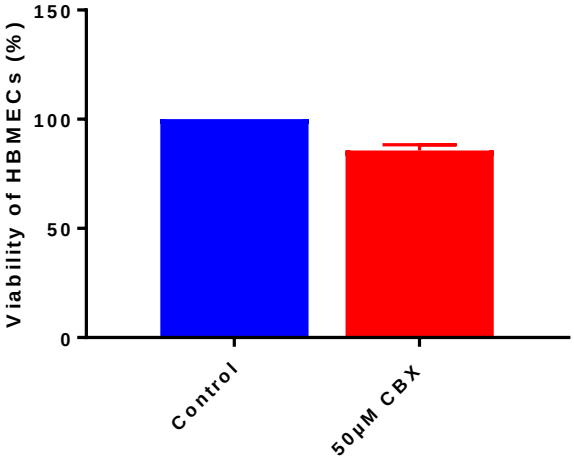
To examine whether astrocyte or endothelial mediated protection of N2a cells requires GJs, we used the GJ inhibitor CBX, on co-cultures of N2a cells grown with either

astrocytes or HBMECs exposed to 5 μM 5-FU. CBX has been reported not to effect cell viability if it used at a concentration below 100 μM (Davidson and Baumgarten, 1988). In our hands, CBX at a concentration of 50 μM was not toxic to any of the cells tested (Figure 3.13) and this concentration was used in all co-culture experiments. Treatment with CBX abolished the protection provided to N2a cells by co-culture with either astrocytes or HBMECs (Figures 3.14). Treatment with 5-FU caused a significant decrease in the number of surviving neural cells in CBX treated co-cultures with both astrocytes and HBMECs (Figures 3.15). These results support the hypothesis that the protective role of astrocytes and HBMECs requires functional GJs.

A


Viability of C6 cells (%)
150
100
50
0
Control
50μM CBX

B


Viability of HBMECs (%)
150
100
50
0
Control
50μM CBX

C

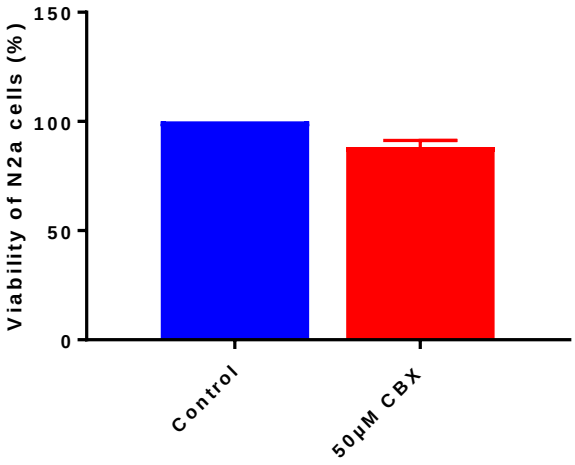
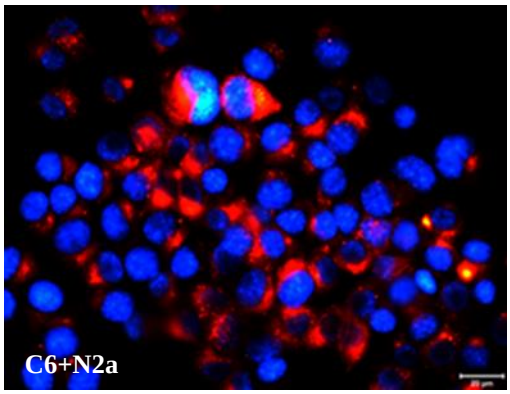
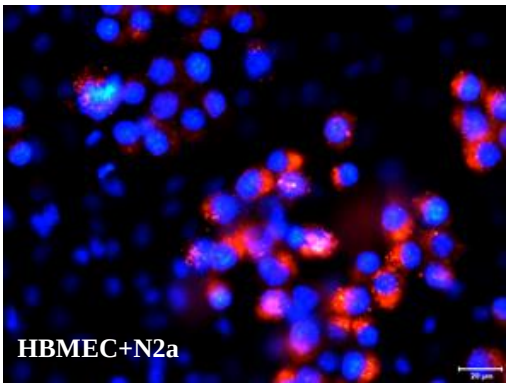
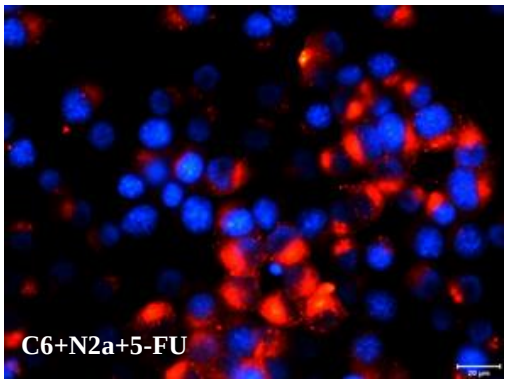
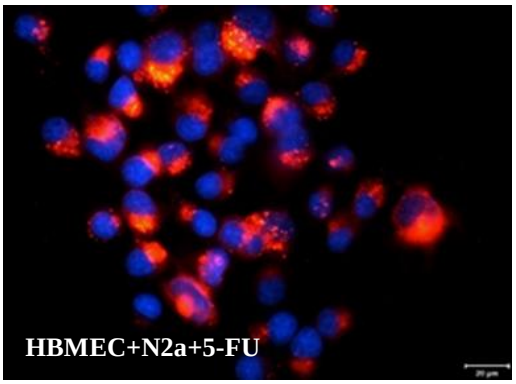


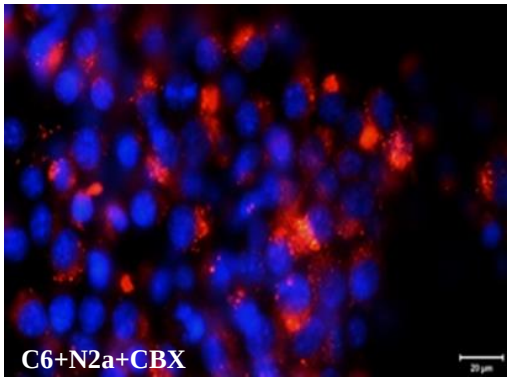
Figure 3.13 The effects of 50 μM CBX on the viability of C6, endothelial and N2a cells. Treatment with 50 μM of the GJ inhibitor CBX did not affect the cell viability of C6 astrocytes (A) endothelial cells (B) N2a cell (C). Viability was determined by MTT assay and all data normalized to number of cells at the start of the culture (5×10^4) and presented as means ± SEM. Results analysed by t-test, showed no significant difference between CBX treated and untreated controls.

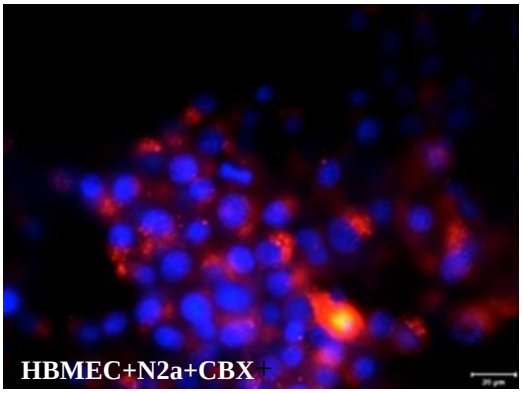

C6+N2a


HBMEC+N2a


C6+N2a+5-FU


HBMEC+N2a+5-FU


C6+N2a+CBX


HBMEC+N2a+CBX

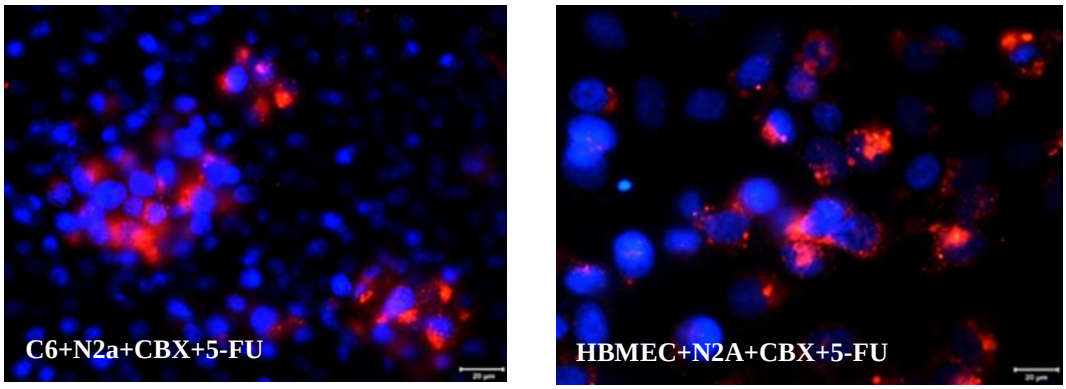


Figure 3.14 Representative fluorescence micrographs of co-cultures of N2a cells and other cell types in the absence (control) and presence of 50 µM CBX. N2a with C6 (Left panel). Co-culture of N2a with HBMECs (Right panel). N2a cells are labelled with PKH26 (red) and counter stained with DAPI (blue). The percentage of N2a cells as compared with controls which survive 5-FU treatment was significantly decreased in the CBX treated groups in comparison to co-cultures treated with 5-FU but without CBX. Data are representative of duplicates in at least three independent experiments. Images were taken with a fluorescent microscope at 40X. Scale bars 20 µm.

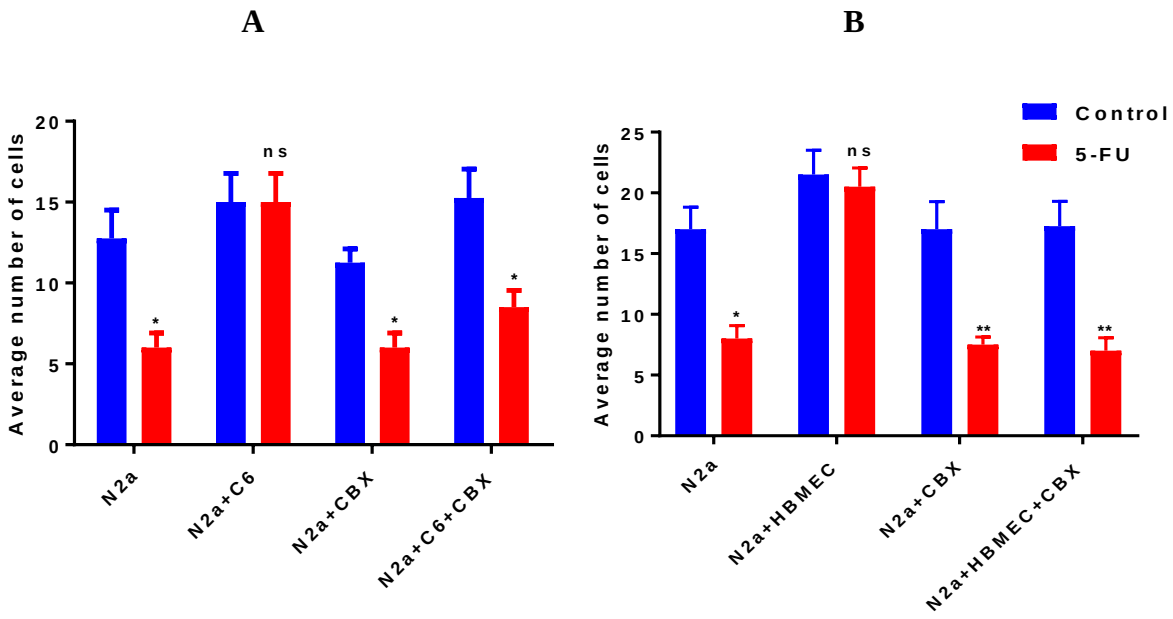


Figure 3.15 Treatment with the GJ inhibitor CBX blocks the protection from 5-FU provided by co-culture with astrocytes or endothelial cells. Co-culture of N2a cells with C6 astrocytes (A) or HBMECs (B). N2a cells on their own show a significant drop in cell number when treated with 5μM 5-FU. Co-culture in direct contact with C6 astrocytes and HBMECs protects N2a cells from 5-FU treatment. N2a cells on their own treated with CBX were not protected from 5-FU. N2a cells co-cultured with C6 astrocytes and treated with CBX (50 μM) and 5-FU (5 μM) for 48 hours were not protected from the effects of the chemotherapy. Data are means ± SEM of three different experiments. Two way ANOVA was used. Levels of significance were ns,**** $p<0.0001$.

3.3.5.2 C6 cells were functionally coupled to N2a cells via GJs as shown by dye transfer between the two cell types

To demonstrate that GJs between astrocytes or endothelial cells and N2a cells were functional, we examined whether a low molecular weight cytoplasmic dye was able to pass between these cell types. Prior to co-culture, C6 astrocytes or HBMECs were loaded with the green calcein-AM dye and N2a cells were stained red with PKH26. The cells were then co-cultured for 4 hours and double stained (red and green) cells looked

for. Double labelled cells were observed (Figure 3.16) demonstrating that the calcein dye had transferred from C6 or and HBMECs to adjacent N2a cells.

To test whether CBX was effectively blocking GJs, co-cultures of labelled cells were treated with 50 μM of CBX. Treatment with CBX appeared to cause a decrease in the number of double-labelled cells (calcein dye positive cells) but of repeat experiments was too small to do statistics his result demonstrates that CBX blocks the passage of small molecular weight molecules between astrocytes or endothelial cells to neural cells (Figure 3.17A).

To avoid the possibility of leakage of the calcein dye from C6 cells into the media and its subsequent uptake by N2a cells, we tested dye transfer between N2a and C6 cells separated by a porous membrane in transwells (Figure 3.17b). No double labelled cells were observed. In addition, no calcein transferred from the fibroblast cells to red labelled N2a (Figure 3.17C).

As shown in Figure (3.16), in direct contact co-culture between astrocytes or endothelial cells and NSC cells, transfer of calcein through GJ to adjacent cells (neural cells) was observed. This suggests that GJCs were able to transfer green calcein-AM dye from astrocytes or endothelial cells to neural cells. However, in the transwell system, which inhibited direct contact and GJ formation, dye transfer was not observed (Figure 3.17).

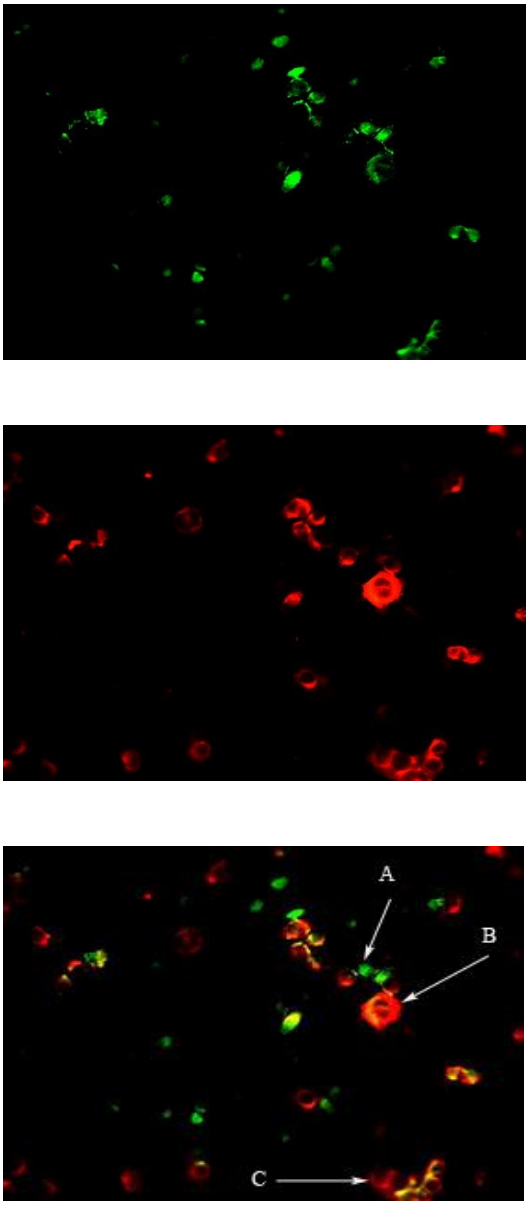


Figure 3.16 N2a cells formed functional GJCs, with C6 as shown by dye transfer. Representative images of N2a cells labelled with a non-diffusing membrane-bound red dye (PKH26) and C6 labelled with a green fluorescent gap junction-permeable dye (calcein-AM). Calcein transferred from the C6 and to red-labelled N2a cells as indicated by double labelled cells which appear yellow. Nuclei were stained with DAPI (blue). This confirms the formation of functional GJs between C6 with N2a within 4 hours. A cell containing only Calcein AM, C cell stained only with PKH26, B cell containing both Calcein AM and PKH26. Scale bar 20 μm.

(A) CBX **(B) Transwell** **(C) 3T3**

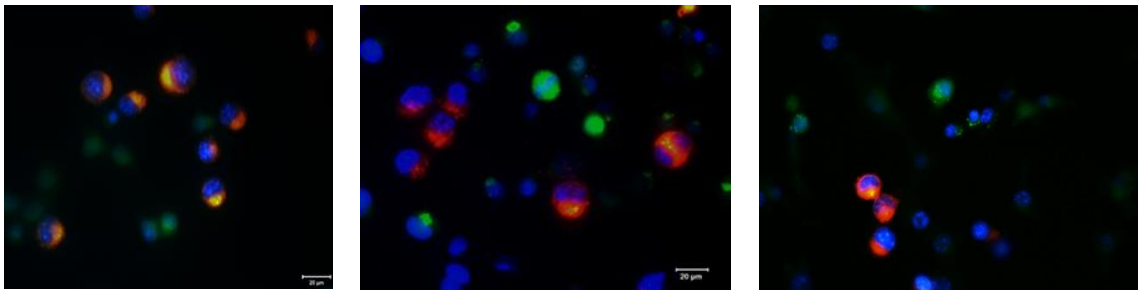


Figure 3.17 Effects of inhibition of heterocellular coupling between N2a cells and C6. Effects N2a cells labelled with PKH26 (red) and C6 cells labelled with calcein-AM (green) were co cultured for 4 hours. N2a cells were cultured together with C6 in the presence of 50 μM CBX (A). C6 cells were cultured on the lower surface of the membrane of a transwell insert while N2a cells were cultured on the upper surface (B). N2a cell were co-cultured with 3T3 (C). The inhibitor (CBX) and transwell prevent dye passage from C6 to N2a. Phenomenon of dye transfer was not observed between 3T3 and N2a. GJs were considered blocked when calcein did not pass from donor to recipient cells. When cells were cultured in Transwell system, which inhibited direct cell contact between donor and recipient cells, GJ was not established between them, and consequently, calcein AM was not transferred to the donor cells. Scale bar 20 μm.

3.4 Discussion

3.4.1 To determine whether co-culture of N2a cells with other cellular components of the neurogenic niche protects the N2a cells from the effects of 5-FU

The generation of new neurons from stem cells in the adult mammalian brain is largely restricted to two neurogenic niches. These are the SGZ in the hippocampus and the SVZ in the walls of the lateral ventricles (Parnavelas, 2000). The hippocampal neurogenic niche, found on the inner aspect of the dentate gyrus, as well as containing NSCs, also contains astrocytes, transit amplifying neural progenitor cells, mature neurons and a dense network of blood vessels. These components are thought to have a

significant influence on the survival, proliferation and differentiation of NSCs into mature neurons (Conover and Notti, 2008).

As described previously, animal studies have found that systemic chemotherapy causes a decrease in the proliferation of NSCs in the SGZ of dentate gyrus. This reduction in hippocampal neurogenesis correlates with a decline in hippocampal dependent cognition, such as spatial navigation, after chemotherapy treatment and has been found in both animal and patient studies (Ahles, 2012, Monje and Dietrich, 2012, ELBeltagy et al., 2012, Lyons et al., 2011). As the other components of the neurogenic niche may be protective to NSCs during chemotherapy, an understanding of these relationships could open up new types of treatment or prevention which may help patients suffering from cognitive decline after chemotherapy.

As first described by Palmer et al., approximately 50% of dividing NSCs are in close contact with small blood vessels in the SGZ (Palmer et al., 2000) (Figure 1.9). Recent *in vivo* experiments in our laboratory have demonstrated that those dividing cells which are in contact with capillaries are more resistant to systemic chemotherapy. The numbers of dividing cells close to blood vessels remains unchanged after chemotherapy while dividing cells some distance from blood vessels are largely lost (Maqbool PhD thesis, 2015). This relationship suggests that contact with blood vessels by NSCs confers protection from the effects of chemotherapy. NSCs on the surface of blood vessels will be in close relationship with the endothelial cells lining the blood vessels, the basement membrane on which the endothelial cells sit and astrocyte end-feet which cover the surface of blood vessels and contribute to the BBB (Obermeier et al., 2013), as shown in (Figure1.8). These vascular components of the neurogenic niche are thought to contribute to the regulation of neurogenesis by means of a number of

signalling mechanisms. These include growth factors (BDNF, VEGF, PEDGF) and other secreted signalling molecules as well as direct cell-to-cell contact whose effect may be mediated by Notch signalling (Wilhelmsson et al., 2012). Co-culture of SVZ tissue explants with endothelial cells, increased NSC differentiation and neurite outgrowth along with enhanced neuronal migration within the explants. These results together with the demonstration that NSCs respond to pro-angiogenic factors have established a role for endothelial cells in the regulation of neural cells (Leventhal et al., 1999, Cameron et al., 1998). Further investigations have demonstrated that the vascular components of the neurogenic niche promote the proliferation of NSCs, the process of neurogenesis, synaptogenesis, axonal growth and can be neuroprotective (Goldberg and Hirschi, 2009). As well as modulating normal neurogenesis, vascular and other surrounding cell types may protect cells from a number of insults. For example, it has been shown that breast cancer cells survive chemotherapy better when co-cultured in direct contact with astrocytes (Kim et al., 2011) and similar results have been found when glioma cells were co-cultured with astrocytes (Chen et al., 2015). Similarly, Rao et al 2005, found that astrocytes, co-cultured with neurons, provided protection for the neurons from the toxic effect of ammonia and prevented an increase in neuronal cell death. Further investigation of this phenomenon found that the production of free radicals increased in monoculture of neurons exposed to ammonia but not when they were co-cultured with astrocytes. It is not clear however, whether the neuroprotective effect of co-culture with astrocytes was due to their ability to scavenge free radicals or due to the production of neurotrophic factors (Rao et al., 2005).

Interestingly more recent studies have shown that co-culture of cerebral microvascular endothelial cells (CMECs) with NSCs, increased the survival and rate of neurogenesis

of the NSCs when they were exposed to oxygen-glucose deprivation (Xiong et al., 2013). These results demonstrate that a close relationship between blood vessel components and NSCs may be important for the survival of NSCs when exposed to a variety of pathological conditions and toxic insults. Understanding these processes may help to develop new therapies to protect the neural components of the CNS from damage or degeneration.

Ehret et al. recently used transwells to investigate the effects of endothelial cells, astrocytes and pericytes on hippocampal NSCs. Endothelial cells were found to have the strongest enhancement of proliferation and differentiation while astrocytes and pericytes had a more limited effect (Ehret et al., 2015). These results demonstrate that secreted substances from these neurogenic niche cell types can promote NSC growth. However, this study did not look at protection of NSCs from toxic substances such as chemotherapy.

Recent study demonstrated that treatment with the antidepressant FLX *in vivo* protects both number of dividing cells and cognition after 5-FU and MTX treatment (ELBeltagy et al., 2010c, Lyons et al., 2011). The mechanism behind this effect is unclear, although it is known that FLX can increase the rate of hippocampal neurogenesis (Lim et al., 2009), and it is possible that this is mediated by its actions on other components of the neurogenic niche. To investigate these effects, we developed several models to culture N2a cells with other cell types found in the neurogenic niche or CM derived from them. In the first approach, N2a cells were co-cultured on a monolayer of either C6, endothelial or 3T3 cells. This protocol allowed us to investigate the potential protective role of direct contact between these cell types on neural cells when exposed to 5-FU. In the second approach NSCs were cultured in CM from C6 or HBMECs while in the third

approach NSCs were cultured with C6 or HBMECs in transwells which separated the cell types by a semi-permeable membrane. These approaches were used to determine whether protection provided by supporting cells might be due to factors released by these cells into the media or required cell to cell contact. In an extension of this model, C6 astrocytes were treated with FLX to see if this stimulated the release of factors which could protect NSCs from chemotherapy.

3.4.2 To determine if direct contact with other niche cell types protects N2a cells

Experiments from the first approach demonstrated that contact between astrocytes or endothelial cells had a significant effect on the survival of N2a cells. Both C6 astrocytes and HBMECs protected N2a cells from the effects of 5-FU in contrast to co-culture with 3T3 fibroblasts which did not protect the neural cells (Figure 3.6). This indicates that cells from the neuronal niche can protect neural stem cells in a cell-cell contact dependent manner.

3.4.3 To determine if CM from endothelial or astrocytes can protect N2a cells from 5-FU

As secreted factors have been shown to improve NSC growth (Ehret et al., 2015) and CM from endothelial cells can protect neural cells from the effects of oxygen-glucose deprivation (Guo et al., 2008a), CM from both astrocytes and endothelial cells was tested for its protective ability to prevent the effects of 5-FU. Zhu et al. (Zhu et al., 2006) found that ACM protected primary hippocampal neurons from corticosterone treatment which normally leads to neuronal damage in brain regions, including hippocampus. In a somewhat different situation, Li (2006) showed that co-culture of

NSCs with brain-derived endothelial cells (BECs) either in direct contact or separated by a porous membrane induced vascular tube formation and maintenance through secretion of VEGF and BDNF and activation of vascular VEGFR2 and TrkB by NSC nitric oxide (NO) (Li et al., 2006). These results indicate substances can pass between neural cells and endothelial cells or astrocytes without the need for direct contact.

In these experiments we used viability tests to determine if N2a cells were protected from 5-FU after addition of CM from C6, astrocytes and HBMECs. The results found no protection by CM from any cell type and the decrease in neural cell number after 5-FU treatment was the same as for cells treated with fresh media (Figure 3.7).

Our results are similar to a previous study (Lim and Alvarez-Buylla, 1999), which showed that astrocyte CM alone was not sufficient to support SVZ neurogenesis, but that direct cell–cell contact between astrocytes and SVZ neuronal precursors may be necessary for the production of type A cells. These results suggest that C6, astrocyte and HBMECs could protect N2a and NSCs from 5-FU in a way that requires direct contact between these two types of cells. However, the CM used in these experiments was diluted in fresh media to maintain cell viability and astrocytes may not produce sufficient concentration of growth factors or other neuroprotective factors to protect N2a and NSCs from the effects of 5-FU.

3.4.4 CM from FLX-treated C6

Animal studies have found that FLX treatment promotes the expression of growth factors such as FGF-2, BDNF, and VEGF *in vivo*. These factors may be released from both neurons and astrocytes when stimulated by FLX (Allaman et al., 2011). Boku et al., suggest that astrocytes are involved in the mechanism of action of antidepressants

by stimulating the production of FGF2 (Boku et al., 2013). By this mechanism antidepressants could increase the proliferation of NSC in the adult dentate gyrus. *In vitro*, FLX does not seem to have a direct effect on the survival or proliferation of NSCs as treatment with FLX does not change these parameters. These results confirm those of a previous PhD student (Annabelle Chambers PhD 2016).

Our results showed no protection by CM from FLX-treated C6 astrocytes against the effects of 5-FU on N2a cells (Figure 3.9). This suggests that if FLX did increase the expression of growth factors from C6 astrocytes, this was not sufficient to protect the neural cells. Based on the above results, the C6 astrocytes and HBMECs used in our studies showed protection only when in contact with NSCs.

The transwell assay was firstly introduced by Boyden (and is therefore often called the Boyden chamber assay) to investigate the chemotactic responses of leukocytes (Boyden, 1962). The transwell co-culture system consists of a microporous insert (0.4-μm pore size) that separates the upper and the lower parts of the chamber. This membrane prevents direct contact between populations of the cells grown on either side of the membrane but permits the diffusion of substances from one side to the other. This system provides a convenient way to study the communication between two types of cells which are in close proximity but not touching (Guo et al., 2008b). In particular the model mimics the *in vivo* situation where NSCs, on the surface of blood vessels in the SGZ, are separated from endothelial cells by the basement membrane on which the endothelial cells sit.

Using these protocols, the result of the present study using indirect cell-cell communication by transwell co-culture showed that no significant protection was

provided to neural cells when hippocampal astrocytes, spinal astrocytes or HBMECs were present on the opposite side of the membrane (Figure 3.11). This suggests that direct cell contact between neural cells and other components of the vascular niche is required to protect the neural cells from the effects of chemotherapy.

Chemotherapies, including 5-FU, cause the entry of extracellular Ca^{2+} and the release of Ca^{2+} from the endoplasmic reticulum (Marchi et al., 2017, Can et al., 2013, Yin et al., 2011). This leads to Ca^{2+} overload in mitochondria, production of ROS and induction of apoptosis (Shoshan-Barmatz et al., 2017, Marchi et al., 2017). An early study showed that Ca^{2+} signalling between astrocytes and glioma cells takes place via GJ (Lal et al., 1996). Of particular relevance to the present study, Chen et al in 2015 found that glioma cells were protected from the effects of the chemotherapy drugs vincristine and temozolomide, when there was direct contact between the two cell types. This protection is thought to be brought about by the regulation of levels of cytoplasmic calcium ions in the glioma cells. Connection between cancer cells and astrocytes, via GJs, allows potentially toxic levels of Ca^{2+} ions to pass from the more sensitive neural cells to the more resistant astrocytes (Chen et al., 2015).

Ca^{2+} are one of the most significant second messengers transferred through GJs (Lin et al., 2010). Ca^{2+} have a crucial role in regulating cell proliferation and survival, as well as the activation and execution of apoptosis and death (Roderick and Cook, 2008). Thus, the ability to transfer Ca^{2+} between contacting cells is recognised to have role in astrocytes induced chemoresistance (Chen et al., 2015). To demonstrate that this is a feasible mechanism for the protection of NSCs by astrocytes it was first necessary to demonstrate that both cell types express the Cx proteins which form GJs. Different cells in the CNS express different types of Cx (Rozental et al., 2000). Endothelial cells

express Cx37 (Reed et al., 1993); Cx40 (Bruzzone et al., 1993), and also express Cx43 (Pepper et al., 1992, Bazzoni and Dejana, 2004). In addition, neurons have been reported to express Cx26, Cx32, Cx33, Cx36, Cx37, Cx40, Cx47, and possibly Cx43 (Nagy et al., 2004, Dermietzel and Spray, 1993).

Cx expression and GJC are known to be present on NSCs where they are essential for NSC differentiation into mature cells *in vivo* (Lin et al., 2003, Bruzzone and Ressot, 1997).

In the experiments given in this chapter, both astrocytes, HBMEC and neural cells expressed Cx43, at the cell surface (Figure 3.12). We were able to demonstrate that GJ were functional by observing the passage of fluorescent calcein from one cell type to the other by means of a dye transfer assay. We then showed that GJs were required for the protection of neural cells by astrocytes or HBMECs by co-culturing these cells in the presence or absence of the CBX. The presence of CBX abolished the protection provided by direct contact between astrocytes and HBMECs with neural cells in the presence of 5-FU (Figure 3.15). These findings indicate that the protection provided by astrocytes was mediated by GJC. Our findings are in line with a study by Lin et al. who showed that co-culture of human melanoma cells with astrocytes, decreased the apoptosis in the melanoma cells, and induced by different chemotherapeutic drugs including cisplatin, paclitaxel and 5-FU. This was achieved by removing calcium ions from the melanoma cells to the astrocytes through GJ, a protection which was not provided by co-culture with 3T3 fibroblasts (Lin et al., 2010). Astrocyte derived protection was lost when they were separated from the cancer cells by a membrane in transwell chambers. The ability of astrocytes to protect adjacent cells via GJ may have more general application as neurons are protected by GJ with astrocytes from the toxic

effects produced by NMDA (Zündorf et al., 2007). However, CBX treatment increased NMDA-induced cell death in co-cultures of hippocampal neurons and astrocytes (Zündorf et al., 2007). These observations may enable more effected chemotherapy treatment as well as the providing the possibility of treating some neurological injuries and diseases (Ozog et al., 2002).

Our results using neonatal astrocytes obtained from the spinal cord, showed that these cells could also enhance neural survival and produced similar results to those from neonatal astrocytes obtained from the hippocampus. These results indicate that protection of neural cells by astrocytes is not dependent on the source of the astrocytes and may be a general feature of astrocytes. However, the age of astrocytes may be significant as Song (2002), showed that when GFP^+ adult stem cells were co-cultured with astrocytes from neonatal or adult spinal cord, only the neonatal astrocytes induced neuronal differentiation. However, hippocampal astrocytes from both neonatal and adult can promote functional neurogenesis (Song et al., 2002). These support the idea that primary astrocytes obtained from regions of higher neuroplasticity such as newborn and adult hippocampus and newborn spinal cord, can stimulate the neuronal differentiation of adult NSPCs, while adult astrocytes obtained from non-neurogenic spinal cord inhibited neural differentiation (Suh et al., 2009, Barkho et al., 2006). It would be interesting to test adult spinal cord astrocytes for their ability to protect NSCs from chemotherapy.

Endothelial cells although forming GJ with neighbouring endothelial cells have not been reported to form GJ with neural stem or progenitor cells. The results presented here may indicate that they do in fact form such GJ or that other cell contact mechanisms may be involved. Membrane bound ligands are known to interact with cell

surface receptors found on endothelial and NSCs and influence adult neurogenesis (Tung and Lee, 2012). As the exact mechanism by which CBX affects GJ is unclear (Ozog et al., 2002) it is unknown if CBX also interferes with these interactions and thus disrupts cell contact dependent protection provided by endothelial cells.

3.4.5 Conclusion

In conclusion, establishing an *in vitro* model using the N2a neural cell and C6 astrocytes or HBMECs has been useful for studying the effects of 5-FU on cell survival in cells from the neurogenic niche of the hippocampus. These cells demonstrate different levels of sensitivity to chemotherapy. Protection by direct contact with astrocytes or HBMECs provides protection which is mediated by GJs.

4 Chapter Investigation of DNA damage, apoptosis and changes in the cell cycle by chemotherapy using flow cytometry

4.1 Introduction

Chemotherapeutic agents used in the treatment of cancer patients have varying mechanisms of action and different cellular targets. Most chemotherapeutic agents target the replication of DNA, often by forming covalent adducts or transferring alkyl groups to the DNA. These effects lead to cycle arrest and direct cell death, or can disturb DNA function and metabolism and inhibit DNA synthesis, prompting apoptosis later (Jamieson and Lippard, 1999). Due the targeting of the processes involved in DNA synthesis, dividing cells are more sensitive to chemotherapy than non-dividing cells (Helleday et al., 2008).

This study will look at the effect of different concentrations of the chemotherapy agent 5-FU on both primary cells and cell lines, using the technique of flow cytometry to detect changes in cell cycle stage, DNA damage and apoptotic markers.

4.1.1 Cell cycle

The cell cycle passes through a series of events in which the cellular macromolecular contents are duplicated and divided between the two daughter cells. The cell cycle is divided into four distinct phases (Figure 1.4): (1) in the G1-phase cells increase in size and prepare for DNA synthesis; (2) in the S-phase DNA is replicated; (3) in the G2-phase cells will continue to grow and double their mass of proteins and organelles (4). In the M-phase the chromosomes condense and separate and the cell divides into two daughter cells (Kastan and Bartek, 2004, Houtgraaf et al., 2006, Altieri et al., 2008). After completing the division cycle, the daughter cells can enter a new cycle, or enter the G0 phase which is a resting non dividing state (Alberts et al., 2002, Qin and Li, 2003) (Figure 4.1).

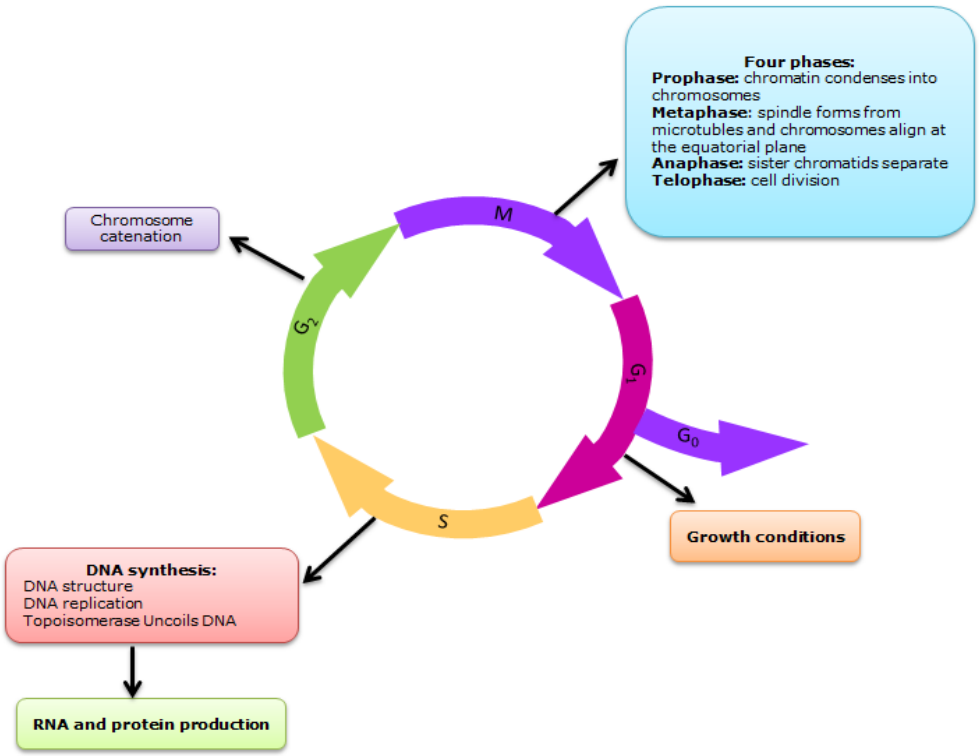


Figure 4.1 Cell cycle: potential targets for chemotherapeutic drugs (Gabrielli et al., 2012).

Many chemotherapy agents display cell cycle specificity. For example, taxanes and vinca alkaloids target M phase cells, whereas topoisomerase inhibitors and antimetabolites have an impact on S phase cells (Shah and Schwartz, 2000). The widely used antimetabolite anticancer drugs include the pyrimidine analogue 5-FU. The incorporation of purine and pyrimidine analogues into DNA during the S phase of the cell cycle prevents the addition of normal nucleobases and inhibits DNA replication and may cause double stranded breaks (DSBs) in the DNA. For example, 5-FU can be incorporated into DNA and RNA in place of thymine or uracil, respectively. Because 5-FU contains a fluoride atom at the 5-carbon position on the ring this prevents the addition of the next nucleobase in the strand, therefore terminating chain elongation which induces apoptosis (Parker and Cheng, 1990, Hwang et al., 2005). In addition, 5-FU is cell-cycle specific and affects cells only when they are in S-phase (Qin and Ng,

2002, Teicher et al., 2012). Therefore, 5-FU produces DNA base pairing problems. In addition 5FU inhibits the enzyme thymidylate synthase which produces thymidine leading to a shortage of this base during S phase. As cancerous cells have a higher metabolism than normal cells, they take up these drugs more actively than the normal cells, and therefore 5-FU is often more toxic to cancerous cells.

4.1.2 Phosphorylated histone H2AX (pH2AX)

DNA damage occurs in all cells by both endogenous and exogenous factors. Endogenous damage is caused by the cell itself and can occur by different pathways such as excision repair, oxidative damage or depurination. While, exogenous damage is caused by exposing the cells to physical damage (radiation) or chemical agents (cytotoxic drugs). The DNA damage that arises can be single stranded breaks (SSBs) or DSBs, the latter killing the cells (Kuo and Yang, 2008). Measuring DSBs in DNA allows the determination of the level of toxicity experienced by cells and the likelihood that this will lead to cell death. There are many methods used to detect DSBs including neutral elution, pulse field electrophoresis (2-D gel electrophoresis) and comet assays, but the pH2AX assay is more specific and sensitive to DSBs than the other techniques (Olive, 2004, Tanaka et al., 2009). In addition, it can detect DSBs in healthy cells and permits fluorescent visualization and the physical localization of the DSBs.

H2AX is a histone protein associated with DNA throughout the chromosome (Altaf et al., 2007). Phosphorylation of H2AX is an epigenetic modification which occurs in the presence of DNA double strand breaks and is part of the DNA damage response cascade (Chowdhury et al., 2005). It is well known that disrupting base pairing by 5-FU treatment during replication causes damage to the DNA structure. This damage causes the phosphorylation of the histone H2AX protein at serine 139 residues in the carboxyl

terminus (C-terminus) to form pH2AX (Rogakou et al., 1998, Bonner et al., 2008, Rothkamm and Horn, 2009). H2AX molecules are phosphorylated in megabase chromatin domains flanking the DSBs by the PI-3-like protein kinases, ataxia telangiectasia mutated (ATM), ATM-Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) which are all part of the Phosphoinositide-3 kinase (PI-3K) pathway and are involved in the DNA damage response (Burma et al., 2001, Cuadrado et al., 2006, Collis et al., 2005, Lavin, 2008). Following DNA repair, pH2AX is dephosphorylated.

pH2AX is often referred to as gamma H2AX and is considered an important marker of DNA damage induced by chemotherapy or radiotherapy treatment (Garcia-Villa et al., 2012a, MacPhail et al., 2003a, Olive and Banath, 2009, Bourton et al., 2011a). For example gamma-H2AX foci have been identified as an assay from which DSB can be quantified after ionizing radiation (Rogakou et al., 1998). It has also been used as a marker of the chemosensitivity of human cancers, such as leukaemia (Garcia-Villa et al., 2012a, Zhou et al., 2011). It can normally be detected within hours of chemotherapy treatment (Garcia-Villa et al., 2012a) and pH2AX proteins have been used as a sensitive and specific marker for DNA double strand breaks *in vitro* and *in vivo* (Kuo and Yang, 2008, Redon et al., 2002, Pilch et al., 2003). The present study measured DNA damage by detecting gamma-H2AX and carried out MTT assays (Chapter 2) to enable comparison between the two approaches to measuring the effect of chemotherapy.

Measuring pH2AX by flow or laser scanning cytometry offers the possibility of multiparameter analyses to correlate DNA damage with cellular DNA content and, therefore, cell cycle phase (MacPhail et al., 2003b). Determination of the cell cycle

phase affected by the drug is of importance in investigation of the mechanism of the anticancer drug activity (Ikeda et al., 2010).

Detecting pH2AX using a flow cytometry assay is a more efficient and sensitive method to detect damage levels than the comet assay (Speit and Hartmann, 1999, Kataoka et al., 2006). In addition, pH2AX has been used to detect low-levels of DNA damage as well as the pharmacodynamics of cytotoxic and tumor killing drugs (Banáth et al., 2004, Ewald et al., 2007).

4.1.3 Cell death pathways

Several pathways can lead to cell death and the type of cell death produced depends on different factors such as cell type, age of the cell, impacts from the environment, availability of nutrition etc. It is well known that cells undergoing programmed cell death die by apoptosis rather than by necrosis (Wyllie et al., 1980, Oppenheim, 1991, Vaux et al., 1994). There is now more recent evidence that other cell death pathways such as autophagy are also forms of programmed cell death. In this chapter we will shortly discuss these and some other types of cell death.

4.1.3.1 Apoptosis

Apoptosis is the main form of programmed cell death that has been evolutionarily conserved from yeast to humans. The term apoptosis is Greek word (apo: off, ptosis: falling), was first used by Kerr and his colleagues in 1972 (Kerr et al., 1972). Apoptosis is essential for tissue development and plays a crucial role in the control of cell populations, neuronal degeneration, and the homeostasis between mitosis and cell death in the adult organism (Wyllie, 1987, Bai et al., 1999, Hetts, 1998). This process is responsible for eliminating damaged, injured, infected, senescent or no longer needed

and incompetent cells from the body both quickly and efficiently (Schneider, 2014). In addition, apoptosis has a key role in several biological processes such as normal cell turnover, proper development and the functioning of the immune defence system, hormone-dependent atrophy, embryonic development and cell death caused by chemicals (Newcomb et al., 1999, Elmore, 2007).

The changes involved in apoptosis include cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation (Brown and Attardi, 2005, Nagata, 2000, Kitazumi and Tsukahara, 2011). After that, during the later stages of apoptosis, the cell fragments into several vesicles forming apoptotic bodies, which are subsequently phagocytised by macrophages or neighbouring cells, thus preventing an inflammatory response (Smale et al., 1995, Galluzzi et al., 2012) (Figure 4.2).

In addition, cell death can occur in an uncontrolled form distinguished by cell swelling and mitochondrial damage which cause the rapid depletion of energy; a breakdown of homeostatic control; cell membrane lysis and release of the intracellular contents. This causes an inflammatory response, with oedema and damage to the surrounding cells (Yeh, 1997).

Apoptosis can be induced by pro-death signalling, a lack of growth factor signalling or anti-apoptotic signalling as well as stress inducers such as radiation.

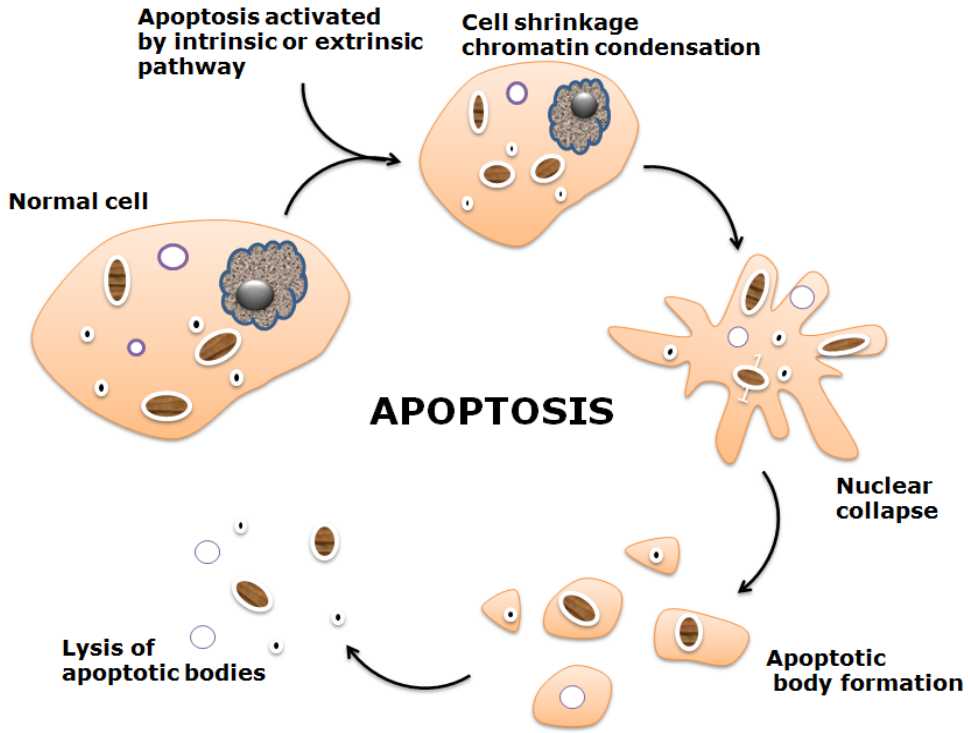


Figure 4.2 The distinct morphological steps of apoptosis. Diagram modified from (http://www.scq.ubc.ca/wp-content/uploads/2006/07/Apoptosis.gif).

The mechanism of apoptosis includes the activation of a family of cysteine proteases known as the caspases. These are synthesized as inactive zymogens requiring proteolytic cleavage to induce their activation (Kumar, 2007, Hengartner, 2000b). Caspases containing an N-terminal pro-domain of variable length and a C-terminal protease domain. Depending on the length of their pro-domains, caspases are subdivided into two categories: initiator and effector (executioner). Activation of caspase 2, 8 and caspase 9 called initiator caspases, results in consequent cleavage and activation of downstream effector caspases such as caspase 3, caspase 6 and caspase 7. The effector caspases are responsible for the induction of the biochemical and morphological changes associated with apoptosis, and are usually more abundant and

active than the initiator caspases (Gibbons and Pollman, 2000, Hengartner, 2000a). There are two main apoptotic pathways operating in mammalian cells which are activated predominantly by death receptor (extrinsic) and mitochondrial (intrinsic) pathways, respectively (Earnshaw, 1995) (Figure 4.3).

The intrinsic pathway is activated by various stimuli including irradiation or chemotherapy and DNA damage, and acts through the mitochondrial pathway which is controlled by the Bcl-2 (B-cell leukemia/lymphoma 2) family of proteins (Wang, 2001). Members of the Bcl-2 family of proteins have either proapoptotic (promoters) (Bax, Bak, Bid, Bim) or antiapoptotic function (suppressors) (Bcl-2, Bcl-XL, Bcl-W) (Iannolo et al., 2008). The anti-apoptotic Bcl-2 family maintains the integrity of mitochondria in homeostatic conditions by preventing the pro-apoptotic Bcl-2 family members such as Bax and Bak from causing mitochondrial damage. However, under stress conditions the Bcl-2-homology 3 (BH3)-only proteins are activated, counteracting the anti-apoptotic effect of Bcl-2 family members, relieving the inhibition of Bax and Bak (Martinou and Green, 2001). This causes the activation of Bax and Bak, which translocates from the cytoplasm to mitochondria, inducing the permeabilization of the outer mitochondrial membrane and the release of cytochrome C (Kluck et al., 1997). Upon release, cytochrome C binds with the adaptor protein Apaf-1 and several pro-caspase-9 proteins, forming a holoenzyme complex termed the apoptosomes (Jiang and Wang, 2000, Gupta, 2001). The activated pro-caspase-9 then cleaves and activates the executioner caspases -3, -6 and -7, which initiate DNA fragmentation and cell death (Colin et al., 2008, Li et al., 1997). As discussed in Chapter 3, influx of Ca^{2+} ions into the mitochondria primes the cell for apoptosis and is a feature of many apoptosis inducing factors.

The extrinsic pathway is mediated by external stimuli such as cytokines and growth factors, and involves the activation of TNF family receptors such as TNFR, TRAIL and Fas (Chen and Goeddel, 2002, Rich et al., 2000). Activation of these receptors leads to the formation of a death-inducing signalling complex (DISC) by recruiting an adaptor protein [FADD (Fas-associated death domain) for CD95 and TRAIL-R1/R2; TRADD (TNFR associated death domain) for TNFR1, which leads to activation of caspases-8 and 3, leading to DNA fragmentation and apoptotic cell death (Mace and Riedl, 2010, Peter and Krammer, 2003). This process takes place through the formation of two complexes. Complex I is formed at the plasma membrane and consists of TNFR1, TNFR-associated death domain (TRADD), TRAF2, RIP1, cIAP1 and cIAP2 (Wang et al., 2008).

Complex II includes TRADD, FADD, and caspase-8 and/or -10. Once the death initiator Caspase-8 and/ -10 are activated, this leads to the activation of downstream executioner caspases such as caspase-3 (Muzio et al., 1996, Eggert et al., 2001).

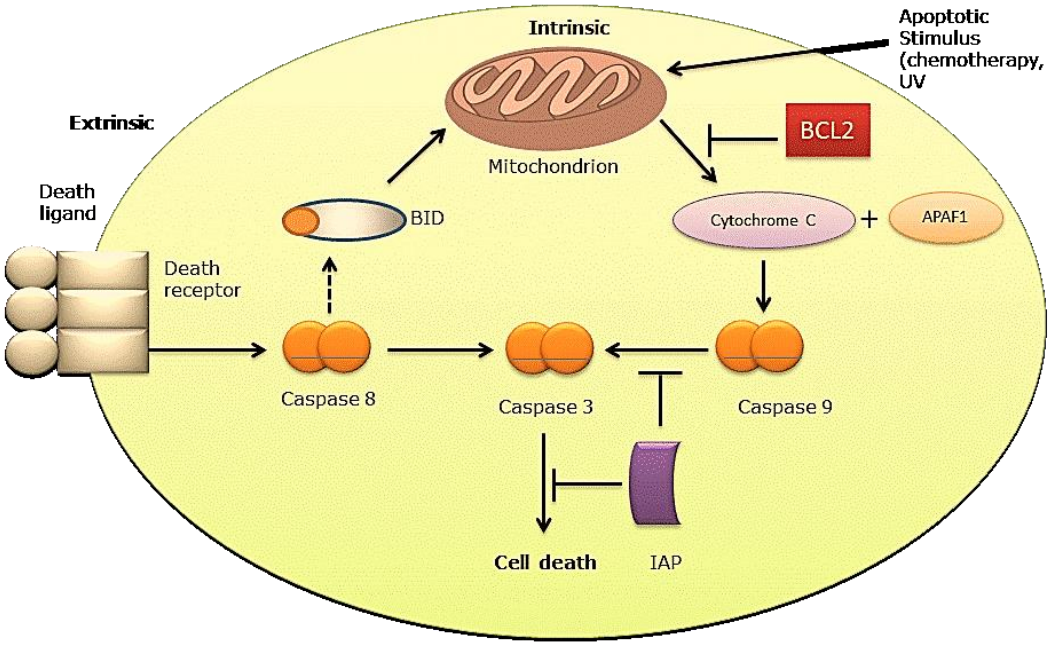


Figure 4.3 A representation of the two major apoptotic pathways; the extrinsic and the intrinsic apoptosis pathways. Diagram modified from Andersen (Andersen et al., 2005).

All pathways cause mediation of the main effector caspases including caspase-3 (casp-3), caspase-6 and caspase-7 (Taylor et al., 2008) (figure 4.3). Despite the differences in stimulus and certain upstream evens, both intrinsic and extrinsic pathways involve the mediation of caspase-3, which will further activate a series of downstream events and eventually induce cell death (Degterev et al., 2003). Therefore, caspase-3 is considered as a reliable and sensitive biomarker of apoptosis, activated caspse-3 can be measured by flow cytometry, immunohistochemical and western blot analysis to investigate the toxicity induced by chemotherapy.

Caspase-3 is involved in DNA fragmentation and the morphological changes in the cell that lead to cell death (Janicke et al., 1998). The activation of caspase 3 is dependent on the cell type, tissue type and the signalling pathways involved in cell death (Lakhani et al., 2006). A number of studies have shown that chemotherapeutic agents, including 5-

FU, cause apoptosis (Hannun, 1997, Yoneda et al., 1998, Wigmore et al., 2010, Lee et al., 2005).

Many lines of evidence have suggested that cells can be protected from programmed death by the signals or protection from adjacent cells. A study by Barde in 1989, showed that NGF secreted by the target cells of growing neurons supports the survival of neurons and protects them from apoptosis (Barde, 1989). As discussed in Chapter 3 the removal of intracellular Ca^{2+} ions through GJs to adjacent cells may be important in preventing apoptosis.

4.1.4 Alternative modes of cell death

4.1.4.1 Necrosis

Necrosis is another type of cell death, which is also associated with characteristic cell changes including increased membrane permeability and nuclear degeneration (De Bruin et al., 2002). In addition, necrosis causes the rupture of organelles as well as cells, processes which lead to the release of harmful pro-inflammatory mediators and injury to surrounding tissues. Recently, necrosis has been described as a form of programmed cell death; previously, it was considered to be more of an 'accidental' or passive response to cell injury.

Necrosis can be triggered by cytokines such as TNF and FasL, ischemia, oxidative stress or DNA damage (Vandenabeele et al., 2010). The reason that a cell enters into necrotic cell death rather than apoptosis is not well understood but is likely to be related to factors such as the energy status of the cell as well as the cell type and its environment (Crawford and Wells, 2011). Apoptosis is an ATP-dependant process, while insufficient energy or depletion of ATP could lead to the alternative necrotic

pathways. Necrosis can be distinguished from apoptosis morphologically by increased cell swelling and more membrane permeability. It is believed that chemotherapy or toxic agents are likely to cause necrosis with increasing dosage (Darzynkiewicz et al., 1992, Schrek et al., 1980). Necrosis is pathological process, which occurs due to serious physical or chemical insult caused by injury, infection, cancer, inflammation.

Unlike apoptosis, necrosis results in the loss of cell membrane integrity, while apoptosis involves no loss of membrane integrity (Elmore, 2007). Of note, physiologically, necrosis simultaneously affects a group of cells while apoptosis is triggered in each individual cell (Levin, 1998). Necrosis can be triggered by non-physiological factors, such as hypothermia (Zifman et al., 2010), hypoxia (Shan et al., 2013) or ischemica (Tudisco et al., 2015), while apoptosis can be induced by physiological stimuli (Tudisco et al., 2015). Furthermore, necrotic cells will be finally phagocytosed by macrophages, while apoptotic cells can be phagocytosed by either adjacent cells or macrophages (Erwig and Henson, 2008). Moreover, necrosis usually induces a host inflammatory response whereas inflammation responses are not observed during apoptosis process (Zong and Thompson, 2006, Chavez-Valdez et al., 2012). The difference and distinctive features of apoptosis and necrosis are given in (Table 4.1).

Apoptosis	Necrosis
Morphological features - Membrane blebbing, no loss of integrity	Loss of membrane integrity
Aggregation of chromatin at the nuclear membrane	No chromatin aggregation
Begins with shrinking of cytoplasm and nucleus condensation	Cytoplasm and mitochondrial swelling
Preservation of mitochondria	Swelling of organelles
Formation of apoptotic bodies	Total cell lysis
Biochemical features Tight regulation involving mediators and enzymes	Loss of ion homeostasis
Energy (ATP) dependent (active process)	No energy required (passive process)
Non-random inter-nucleosomal DNA fragmentation.	Random digestion of DNA
Release of some mitochondrial proteins i.e. cytochrome C	Oxidative stress, calcium overload, ATP depletion
Activation of caspase cascade	Activation of calcium depletion proteases
Loss of plasma membrane	
Physiological features Removal of damaged, transformed or infected cells	Back up mechanism of apoptosis failure
Initiates via physiological stimuli	Induced by severe physical or chemical injuries
Phagocytosis by adjacent cells or macrophages	Phagocytosis by macrophages
No inflammatory response	Extreme inflammatory response

Table 4.1 shows some distinctive features of apoptosis as compared to necrosis in terms of the morphological, biochemical and physiological features in these types of cell death.. Adapted from "Apoptosis, cell toxicity and cell proliferation", fourth edition, Roche (Rode, 2008).

4.1.4.1 Autophagy cell death

Autophagy, or autophagocytosis, is another alternative type of cell death. This mechanism is characterized by significant degradation of cellular contents, including important organelles such as mitochondria. These intracellular contents are sequestered into a double-membrane structure, known as an autophagosome, which then fuses with lysosomes to form autolysosomes for degradation. This compartment contains hydrolases that are able to degrade proteins, lipids, nucleic acids and carbohydrates (Klionsky and Emr, 2000, Sui et al., 2011, He and Klionsky, 2009). This lysosomal degradation can eliminate damaged organelles and macromolecules, providing protection against carcinogenesis (Kondo and Kondo, 2006, Janku et al., 2011). Autophagy is thought to play a key role in tumour development and growth regulation which is a cellular response to stress or nutrient deprivation.

This study will look at the impact of different concentration of 5-FU on DNA damage, cell cycle and apoptosis in different cell types were studied using flow cytometry to determine if all cell types experience the same level of effect.

4.2 Materials and Method

4.2.1 DNA damage and cell cycle analysis

4.2.1.1 Cell culture and preparation for DNA damage and cell cycle detection

In this chapter both primary cells and cell lines were used including: Hippocampal astrocytes, NSC, C6 astrocytes, endothelial, N2a and 3T3 cells. The ratio of cells at

different cell cycle phases following anticancer treatment were assessed using flow cytometry. The method of fluorescently-labelling cellular DNA content allows the estimation of the phases of the cell cycle (Nunez, 2001).

All cells were cultured in their specific growth medium on uncoated T25 flasks, except NSCs which were cultured on coated T25. Cells were seeded at a density of 5×10^5 cells at the start time and incubated overnight at 37°C after which they had reached 70% confluence. To study the concentration-dependent and duration effects of chemotherapy, cells were either refreshed as a control while treated cells had their media replaced with media containing different concentration of 5-FU (0, 5, 10. 15, 20 μM) and incubated for further 24 or 48 hours. To analyse the DNA damage and cell cycle distribution, the cells were trypsinized, harvested, transferred to flow cytometry tubes and centrifuged at 1200 rpm for 10 minutes. The supernatant was aspirated, and the pellet was washed twice with 1ml PBS. Then, the cell suspension was fixed with 500 μl of fixative containing 1% methanol free formaldehyde in PBS (Pierce #28906) and incubated at room temperature for 5 minutes, with gentle agitation. Cells were permeabilised by adding 500 μL 0.4% Triton 100X (Fisher BP151-100) in PBS to each tube and centrifuging after 1 minute.

The supernatant was carefully aspirated from each tube and 1ml 1% FBS (Sigma F7524) was added and incubated for 30 minutes at room temperature. For immunodetection 200 μL primary antibody (mouse anti pH2AX– Millipore 05-636) was added after centrifugation and incubated. After 1hour incubation, the cells were washed with 1ml 1%FBS and centrifuged.

Then, 200 µl of secondary antibody (goat anti-mouse Alexa-fluor 488– Alexa Fluor 488 (Molecular Probes A 10684)) was added to each tube and incubated for 1 hour in the dark. 1ml FBS was added to each tube and centrifuged for a final time. Once the supernatant was removed, the cells were stained with the DNA-binging dye propidium iodide (PI) (Sigma) containing RNase A (0.2 mg/ml in sterilized H_2O) and incubated at room temperature for 15 minutes in the dark. Because PI stains both DNA and RNA it is necessary to prevent RNA during staining by removing RNA with RNase A. The cell cycle was assessed by resuspending the cells in 300 µl of PI solution to stain the DNA of the cells. Then, the stained cells were analysed using a flow cytometer (Beckman Coulter FC500). A minimum of 10000 gated cells were collected.

4.2.2 Flow cytometric analysis of apoptosis

4.2.2.1 Cell culture and preparation for C3/7 apoptosis assay

To determine whether 5-FU-induced apoptosis, the activity of caspase -3/7 was examined in both primary and cell lines**.**

All cell types were cultured and passaged at above 70% confluency and plated out onto the uncoated plates except NSCs which were on coated plates and allowed to attach before treatment. In the study of the concentration-dependent effects of 5-FU, cells were treated with different 5-FU concentrations ranging from 0 to 20 µM for 24 and 48 hours. After this incubation period, the media was carefully aspirated and replaced with 5ml of media containing various concentrations of 5-FU as described earlier. In the study of the time-dependent changes of apoptosis after drug exposure, cells were incubated with this treatment for a further 24, 48 hours (N2a) or 72 hours (NSCs),

before the flow cytometry protocols were performed. These time points were chosen as apoptosis would occur after DNA damage.

On the day of the analysis, attached cells were harvested using trypsin or accutase and all the cells were collected and transferred to corresponding flow cytometry tubes. The number of cells was counted (approximately 1×10^5 cells per tube). Apoptotic and necrotic cell death was analysed by double staining with Caspase-3/7 and SYTOX. Staining was performed using the Caspase-3/7 Green Flow Cytometry Assay Kit (Thermo Fisher Scientific, USA), according to the manufacture‘s instruction, after centrifugation at 1200rpm for 10 minutes, the pellet was resuspened with 1 ml of DMEM to wash the adherent cells. Then, 1 µl primary antibody CellEvent™ Caspase-3/7 (Molecular Probes Cat. no. C10427) was added to the cell suspension and mixed gently and incubated at room temperature for 1 hour in the dark. SYTOX was used to stain cell death. During the final 5 minutes of staining, 1 µl of the 1 mM SYTOX™ AADvanced™ dead cell stain solution in DMSO was added to the appropriate samples with gentle mixing. The final labelling concentration of the stain was 1 µM. These samples were ready for analysis using the cytometer. Each sample was tested using a Beckman Coulter FC500, with 20000 cells per sample.

4.2.3 Statistical Analysis

A chi-squared test was used to analyse the data. Data plots and the percentages of cells in the subG1, G0/G1, S, and G2/M phases were determined using Weasel Software. Positive/negative regions were set based on the 0 µM control sample for DNA damage data. Cell cycle parameters were set manually based on the peak of the graph. Peak was set as G0/G1 and G2/M set as twice the x axis value. S stage was considered the area of the graph between these points. Data were analysed in Prism v7.0 (GraphPad, San

Diego, USA) and presented as % gated cell count. Gates were set so that double cell clusters and dead cells were not included in the analysis. DNA damage data and apoptosis were analysed using two-way ANOVA with Tukey's multiple comparison test, looking at cells testing positive or negative for DNA damage, comparing 5FU concentrations. Cell cycle data were analysed using two-way ANOVA with Tukey's multiple comparison test, looking at cells in each stage of the cell cycle (G0/G1, S or G2/M), and comparing 5-FU concentrations. Results are presented as mean ± SEM from replicate experiments. Results were considered significant when $p \leq 0.05$ = *; $p \leq 0.01$ = **; $p \leq 0.001$ =***.

4.3 Results

In an attempt to determine the mode of cell death induced by 5-FU in different cell lines and primary cells, DNA damage, cell cycle and apoptosis analyses were conducted. PI staining was used to determine the effect of 5-FU on the cell cycle.

Cell	Hours	DNA damage	Apoptosis	Cell cycle
3T3	24	P < 0.0001		P < 0.0001
3T3	48	P < 0.0001	P < 0.0001	P < 0.0001
Astrocyte	24	P = 0.2228		P = 0.0558
Astrocyte	48	P = 0.0037	P < 0.0001	P = 0.0122
C6	24	P = 0.0166		P = 0.003
C6	48	P < 0.0001	P < 0.0001	P < 0.0001
N2a	24	P < 0.0001		P = 0.5766
N2a	48		P < 0.0001	
NSC	24	P < 0.0001	P < 0.0001	P < 0.0001
NSC	48	P < 0.0001		P < 0.0001
HBMECs	24	P < 0.0001	P < 0.0001	P < 0.0001
HBMECs	48	P < 0.0001		

Summary of table 4.3: Shows the impact on different cell types of exposure to chemotherapy for 24 or 48 hours in terms of DNA damage; apoptosis and changes to the cell cycle. DNA damage was measured by expression of pH2AX, apoptosis by caspase 3/7 and cell cycle analysis by PI concentration. P value by Chi-square (See Appendix II).

4.3.1 DNA damage (pH2AX) flow cytometry analysis

In order to determine DNA damage induced by 5-FU treatment, pH2AX was detected by flow cytometry using immunofluorescence. The antibody used detects only phosphorylated histone2. Cell lines and primary cells are treated with different concentrations of 5-FU (0, 5, 10. 15, 20 μM) for 24 and 48 hours (see Materials and Methods) or grown under normal growth conditions (untreated).

Our results showed that 5-FU cause DNA damage in all treated cells. As expected the amount of DNA damage increases with the 5-FU concentration used in a dose-dependent manner (Chi-square, see appendix II).

Our flow cytometric DNA damage analyses by pH2AX showed a significant effect on neural cells, but not for astrocytes or endothelial cells after 24 hours 5-FU exposure (Figure 4.4). Therefore N2a were exposed to 5-FU for 24 hours as it is important to detect the pH2AX when the cells are still viable. Based on these findings, 5-FU induced more DNA damage in neural cells compared with the other cells tested (Figure 4.5). The data were first analysed in Weasel Software, to get basic statistics and numbers from the readings from the flow cytometer. The data was further analysed by chi-square (see appendix II) and Prism v7.0 (GraphPad, San Diego, USA). All data were compared to control, and expressed as % damage for pH2AX (Figure 4.5). Data from pH2AX detection suggested that 48 hours exposure to 5-FU was the optimum time to detect DNA damage in the more resistant cell types.

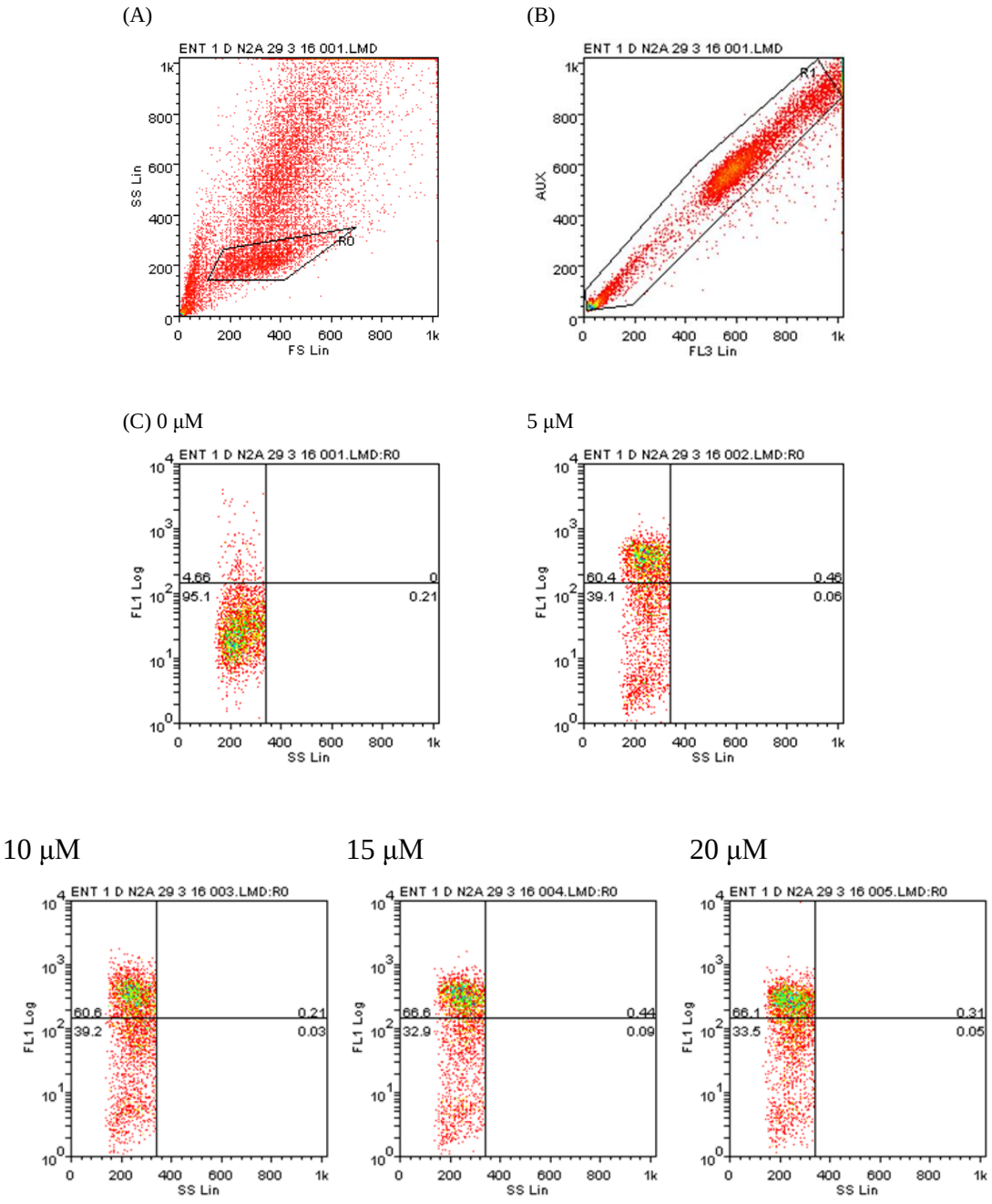
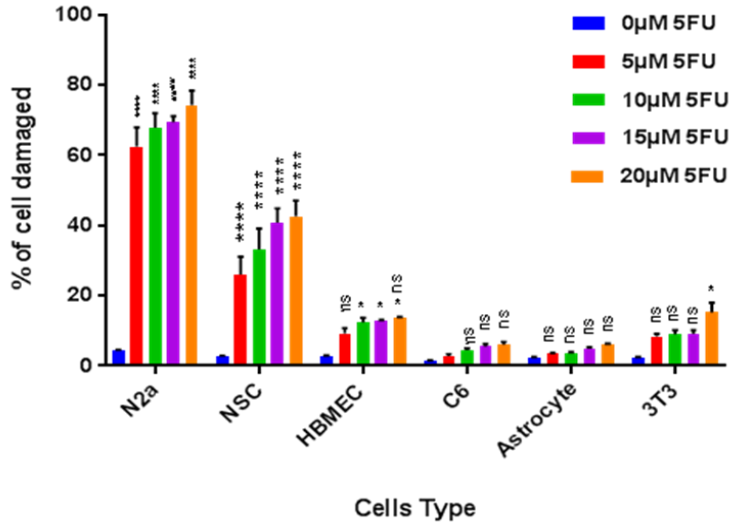


Figure 4.4 Graphs showing the different stages of Weasel Software used to analyse flow cytometry data in treated cells. This is data from N2a cells treated with/without different concentrations of 5-FU. (A) This dot plot illustrates how gates are used to select only the appropriate cells, which are viable, and not double grouped. The gates are made from control samples to make all further cell samples comparable to the controls. (B) Further gate selection to rule out dead cells or anomalies. Once the two gates were selected this encompassed the cells tested and later plotted using histograms. This histogram was drawn using data from control

cells (C). The line down the centre of the graph is a statistical marker – as control cells are not expected to have undergone any DNA damage. In the treated cells, any readings above this line are considered positively stained for pH2AX. Data from cells treated with 5, 10, 15, 20 μM to illustrate any increase in DNA damage. From these graphs, it looks 5-FU caused significant damage to N2a.

(A) DNA Damage after 24 h



(B) DNA Damage after 48 h

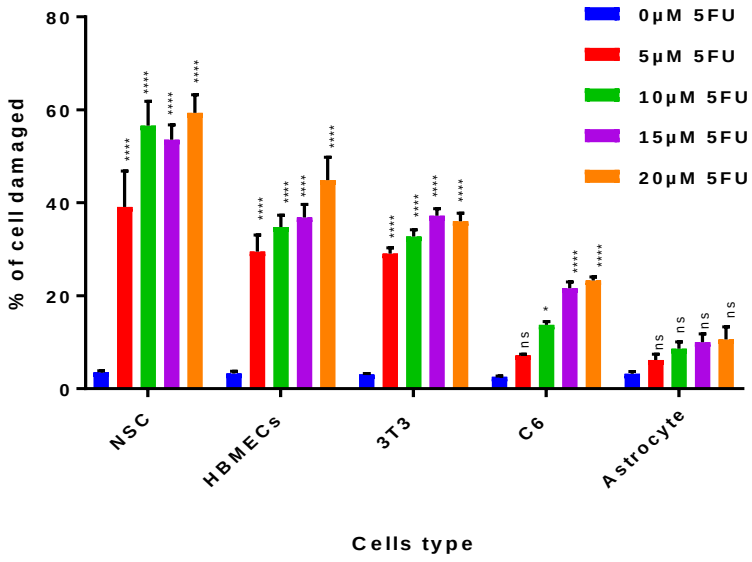


Figure 4.5 Bar chart showing the proportion of gated cells (%) stained for pH2AX (DNA damage marker), when treated with different concentrations of 5-FU (0, 5, 10, 15 or 20 µM) for 24 and 48 hours. Two way ANOVA showed a significant difference between cells. Increasing concentrations of 5-FU caused increased DNA damage levels based on pH2AX, compared to controls, and neural cells showed significantly more DNA damage, compared to other cells. The data for NSC>3T3=HBMEC>C6=astrocyte (A). NSC> HBMEC=3T3 >C6>astrocyte (B).

4.3.2 Cell cycle

The cell cycle was studied for different cell lines and primary cells with and without 5-FU treatment as shown in Figure 4.7 and appendix II. Weasel software was used for analysis, using markers for the following cell cycle stages: G0/G1, S, G2/M. Data was expressed as a % gated cell count, and therefore the total count will not equal 100%, as some cells will lie outside the cell cycle stage boundaries (Figure 4.6).

Chi^2 analysis showed a significant association between 5-FU concentration and cell cycle stage as well as a significant trend with increased concentration in all cell types except astrocytes (24 hour incubated with 5-FU), as shown in table (4.2) (see appendix II). One interesting result was that, with increasing of 5-FU concentration for 24 hours, the number of NSCs showed a decrease in the G0/G1 phase of the cell cycle as compared to the other cell types. This decrease is significant, compared to controls (two-way ANOVA) (Figure 4.7).

Astrocytes, C6 and N2a showed a decrease in the number of cells in S phase in a dose-dependent manner. A decline in this phase of the cell cycle was expected as the DNA damage caused by 5-FU prevents DNA replication.

While, NSC and 3T3 showed increasing number in S phase, whereas G2/M remains unaltered.

A decline in the G2/M phase can be explained by 5FU preventing replication of cells as well as cell growth. However, when NSCs were treated with 5-FU for 48 hours, a greater reduction in G0/G1 cells, and a greater increase in G2/M and S phase cells was observed. That there was still, the significant decline in G0/G1+ cells remains difficult

to explain. Based on this result 5-FU caused cell cycle arrest in NSCs at both the G2/M phase and S-phase (Figure 4.7 D-E-F).

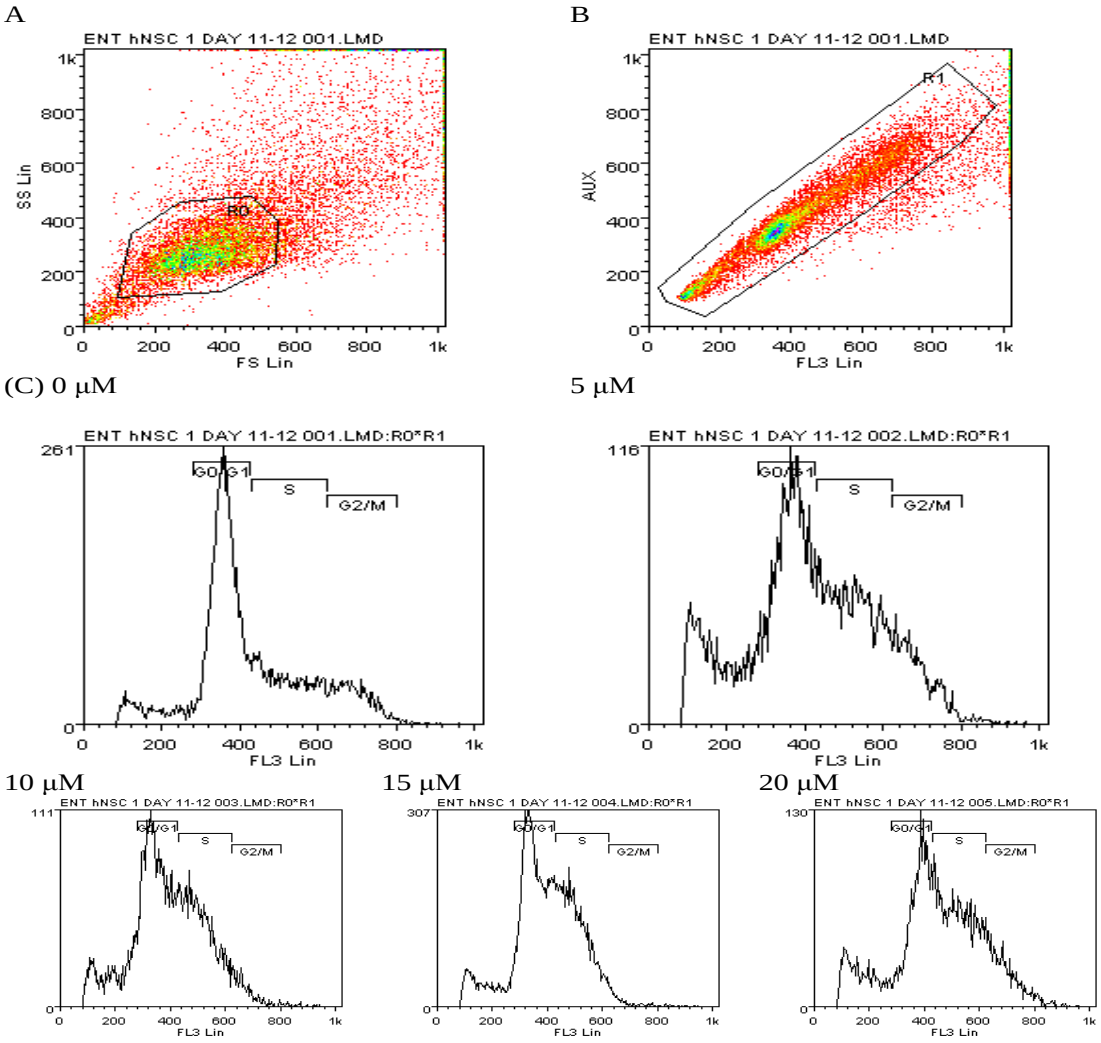
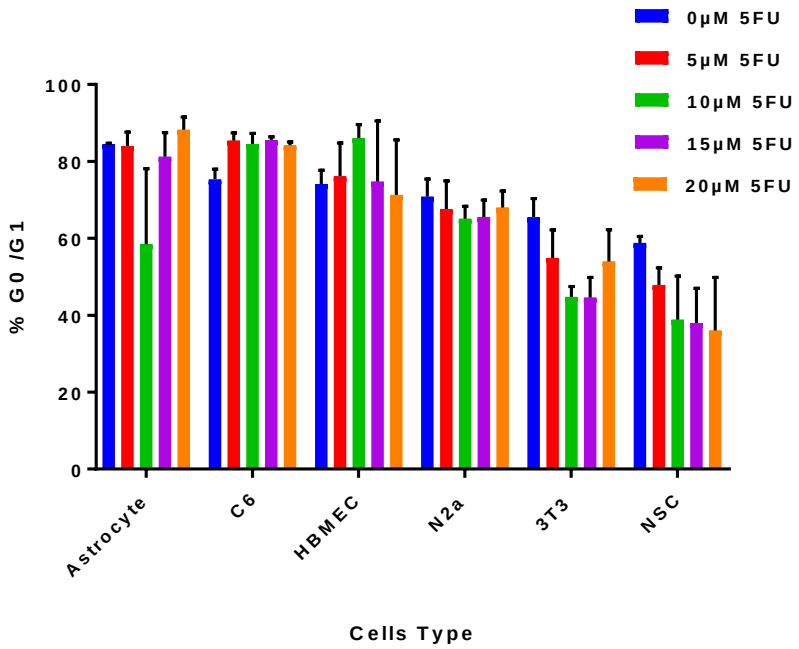
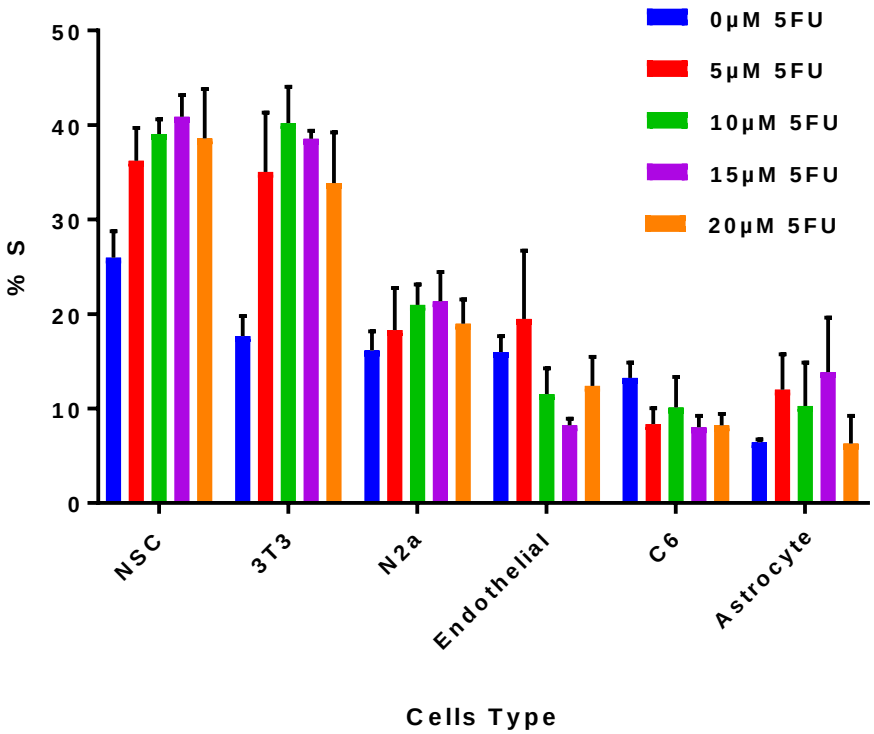


Figure 4.6 Graph readings illustrating the various stages of Weasel Software used to analyse flow cytometry data in treated cells. These are data from NSCs treated with/without different concentrations of 5-FU (0, 5, 10, 15 and 20 µM). This dot plot illustrates how gates are used to select only the appropriate cells, which are viable, and not double grouped. The gates are made from control samples to make all further cell samples comparable to the controls (A). Further gate selection to rule out dead cells or anomalies. Once the two gates were selected this encompassed the cells tested and later plotted using histograms (B). From the gated cells, a histogram can be plotted, to show the various stages of the cell cycle (C). The graph can be split into 3 sections: G0/G1, S and G2/M. This is subjective for each graph, making it difficult to determine the changes seen sometimes with different treatments. This histogram was drawn using data from control cells.

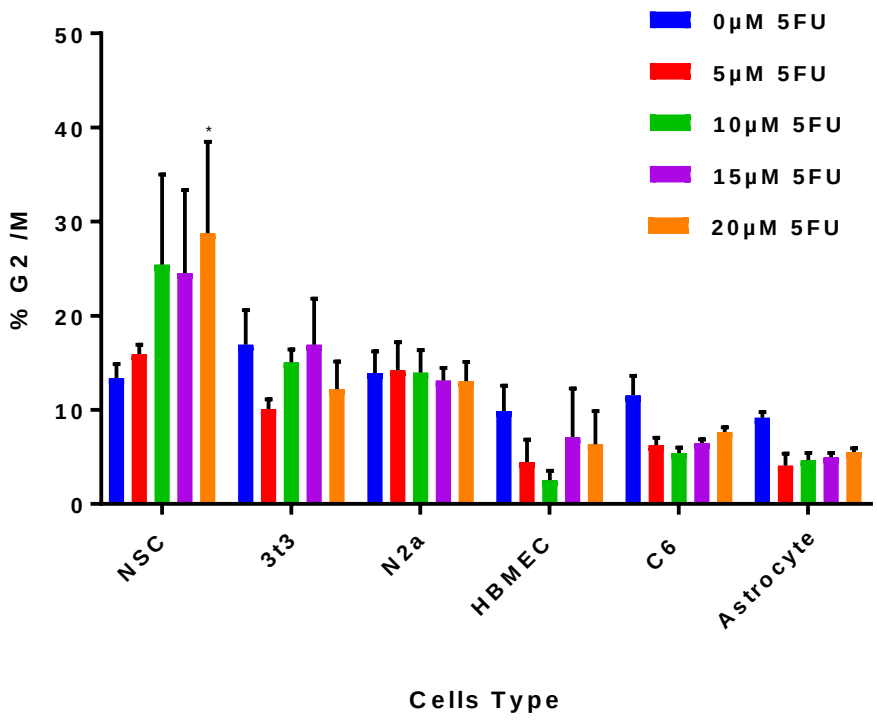
(A) 24 hr


0µM 5FU
5µM 5FU
10µM 5FU
15µM 5FU
20µM 5FU
% G0 /G1
100
80
60
40
20
0
Astrocyte
C6
HBMEC
N2a
3T3
NSC
Cells Type

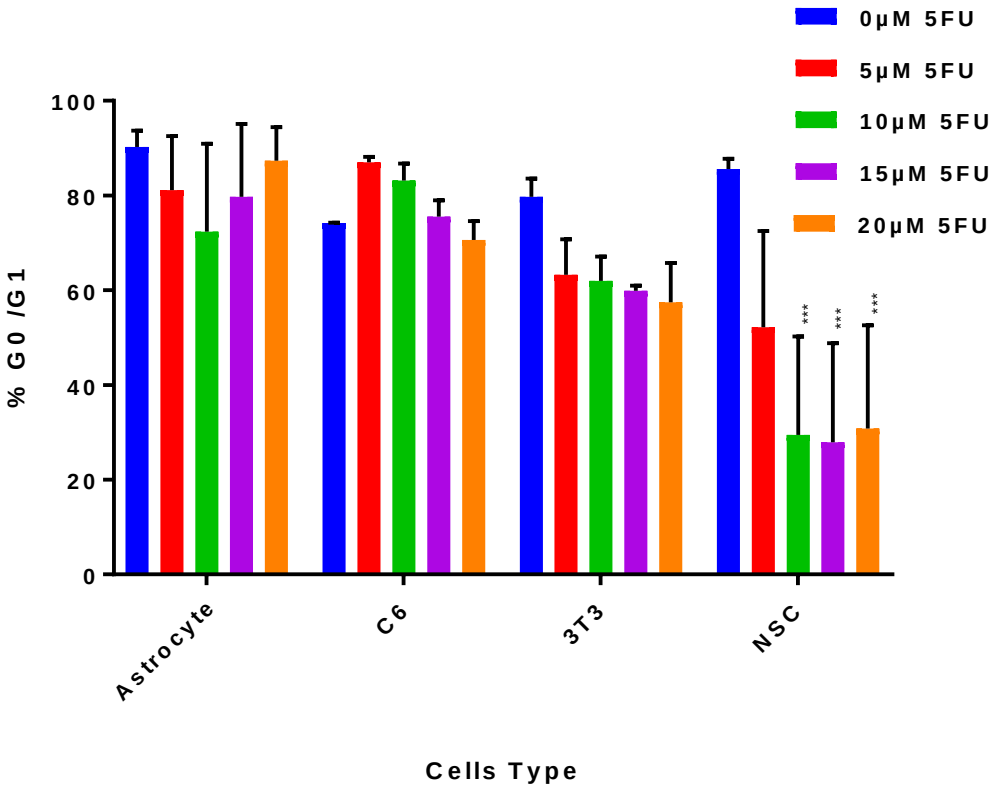
(B) 24 hr


0µM 5FU
5µM 5FU
10µM 5FU
15µM 5FU
20µM 5FU
% S
50
40
30
20
10
0
NSC
3T3
N2a
Endothelial
C6
Astrocyte
Cells Type

(C) 24 hr


0µM 5FU
5µM 5FU
10µM 5FU
15µM 5FU
20µM 5FU
% G2 /M
50
40
30
20
10
0
*
NSC
3t3
N2a
HBMEC
C6
Astrocyte
Cells Type

(D) 48 hr


0µM 5FU
5µM 5FU
10µM 5FU
15µM 5FU
20µM 5FU
% G0 /G1
100
80
60
40
20
0
***
***
***
Astrocyte
C6
3T3
NSC
Cells Type

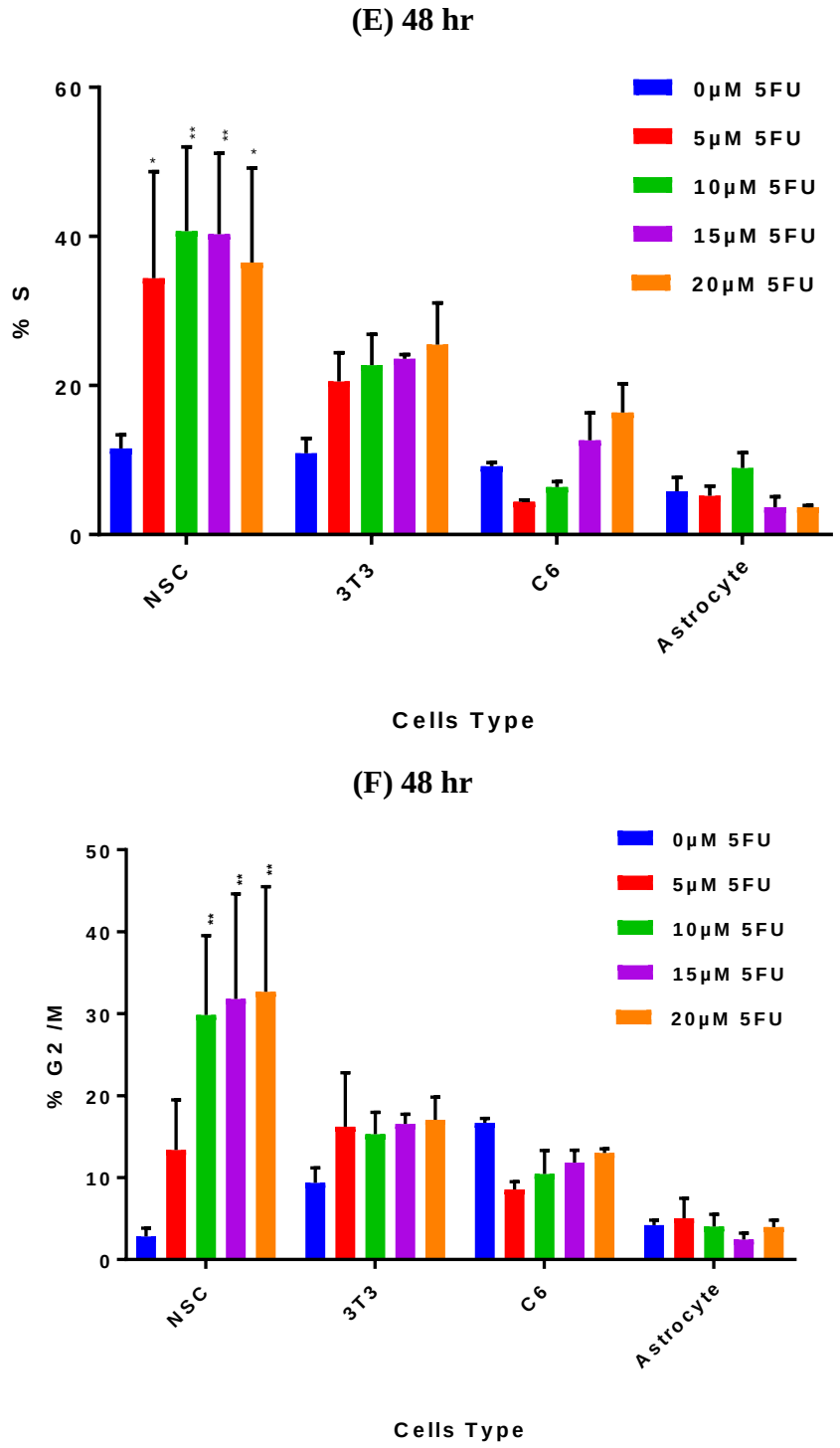


Figure 4.7 Cell cycle progression (G0/G1, S or G2/M) in C6 astrocytes, HBMEC, NSCs and N2a cells in response to 5-FU treatment (0, 5, 10, 15 or 20 μM) for 24 and 48 hours showed significant differences between the % of the cells in different stages of the cell cycle

($p<0.0001$). Increased concentrations of 5-FU caused significantly more cells to be in G0/G1 phase, and significantly fewer cells to be in S and G2/M phases, compared to control cells. cell cycle distributions showing G1/S boundary arrests at 24 hours and S phase arrests at 24 hours, showing S phase arrests at 24 hours; G1, S and G2M fractions in all cell lines at 24 hours after 5-FU addition. Cell cycle profile was determined by FACS analysis of PI-stained samples. Using two-way ANOVA analysis has been conducted on the different cell lines and primary cells. Each result represents a mean and SEM taken from 3 independent experiments.

4.3.3 Apoptosis

4.3.3.1 Caspase 3/7 – apoptosis flow cytometry analysis

Staining for caspase 3/7 and SYTOX was used to detect the effects of 5-FU treatment for 24, 48 and 72 hours. 5-FU caused apoptosis in N2a, HBMEC, C6 astrocytes, 3T3 cells and NSCs primary cells at 48 hours. High concentrations increased the proportion of late apoptotic cells (necrotic) compared to control. Result of Chi2 analysis showed that there is an association between 5-FU and apoptosis, as shown in table (4.2) (see appendix II).

In the present study, we studied whether 5-FU could induce apoptosis in cell lines and primary cells and determined the time of maximum apoptosis for each cell type. Cells were treated with different concentrations of 5-FU for 24, 48 h and 72h, and apoptotic and necrotic cells were detected by FACS analysis. The MTT assay showed that 5-FU affected the cell viability of all cells in a dose dependent manner. We attempted to investigate the mechanism underlying this effect by flow cytometry. pH2AX expression was detected at different time points as described in the materials section (See 4.2.1). Apoptosis occurs after DNA damage and pH2AX expression and is mediated of caspase 3/7. 24, 48 and 72 hour time points were tested in all cell types and 48 hours were chosen. This also made sense as it links in with the time points for pH2AX detection.

In this study, 5-FU induced apoptosis of C6, HBMEC, N2a, 3T3, astrocyte and NSC cells after 48 hours. It is demonstrated that 5-FU treatment increased the proportion of cells at the subG1 phase, which indicates apoptosis in these cells. Moreover, among these cells, a higher rate of apoptosis was observed in HBMEC and N2a cells after 48 hours, respectively as compared to other cells.

Our results, as expected, showed that all cells treated with 5-FU undergo apoptosis compared to controls, in a dose-dependent manner (two-way ANOVA, $p<0.0001$) (Figure 4.9). However, there is no apoptosis observed in 24 hours (Data don't shown).

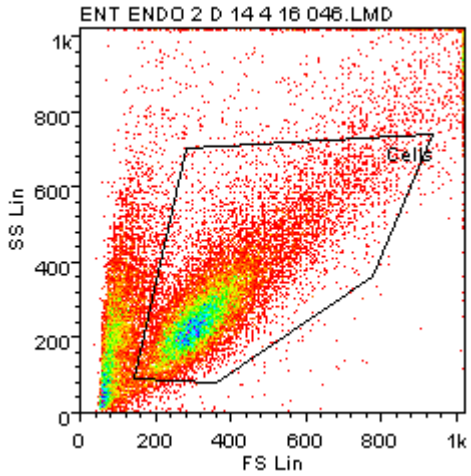
Activation of the caspase cascade, a hallmark of apoptosis, was observed within 48 hours in 5-FU treated cell lines and primary cells and continued to increase in a dose-dependent manner. Little necrosis was observed with a maximum of 2% being found in the treatments cells. Representative dot plots for caspase 3/7 and SYTOX staining in the HBMECs following 48 hours of drug treatment are shown in (Figure 4.8). The increase in caspase 3/7 and SYTOX staining following drug treatment in HBMECs in comparison to their respective controls can clearly be observed. A Two-Way ANOVA was performed as the data exhibited normality (Figure 4.9).

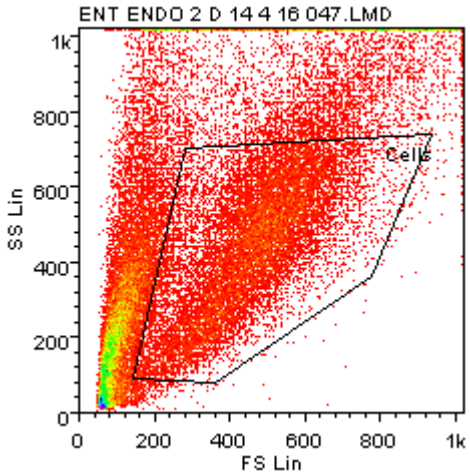
The effect of 5-FU on caspase 3/7 expression in NSC cells was less sensitive compared to the results seen in N2a cells. Increased concentrations of 5-FU cause increases in apoptosis, based on caspase 3/7, compared to controls, whereby all concentrations of 5-FU caused significantly more apoptosis, compared to controls.

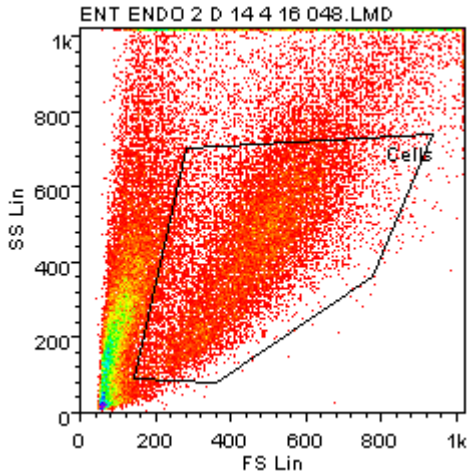
Following 72 hours, there was no substantial increase in the number of apoptotic cells. This could be because increasing numbers of cells started to die in treated group (Data not shown). As it is clear when performing the assay that there are much higher

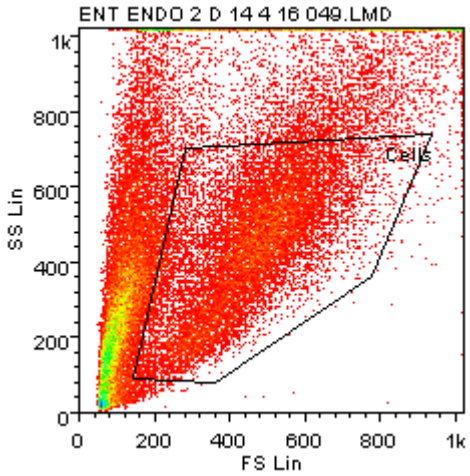
numbers of cells in the control wells, compared to those treated with 5-FU. The flow cytometry analysis however, analyses the same number of cells from each sample. Therefore, we chose just 48 hours in this experiment.

Image of NSC cells were taken to determine if any morphological changes consistent with apoptosis were observed, it can clearly be seen that at 24 and 48 hours (Figure 4.10).

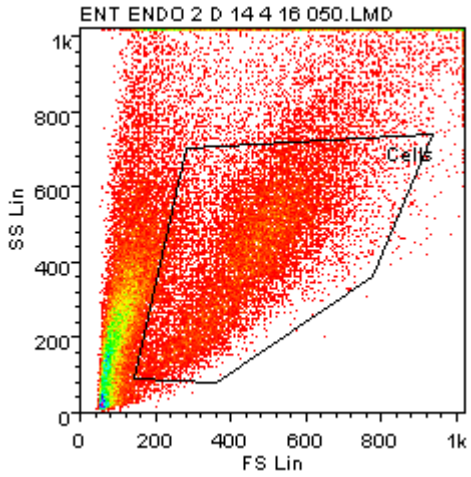

ENT ENDO 2 D 14 4 16 046.LMD
Cells
SS Lin
FS Lin
0
200
400
600
800
1k

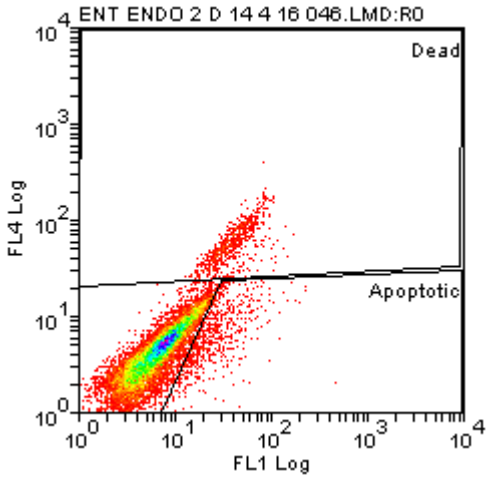

ENT ENDO 2 D 14 4 16 047.LMD
Cells
SS Lin
FS Lin
0
200
400
600
800
1k


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Cells
SS Lin
FS Lin
0
200
400
600
800
1k

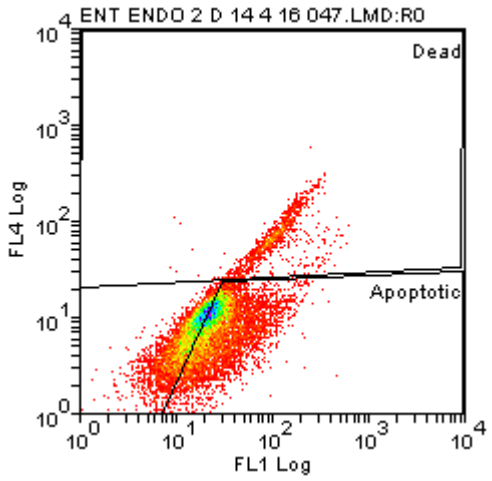

ENT ENDO 2 D 14 4 16 049.LMD
Cells
SS Lin
FS Lin
0
200
400
600
800
1k

0 μM

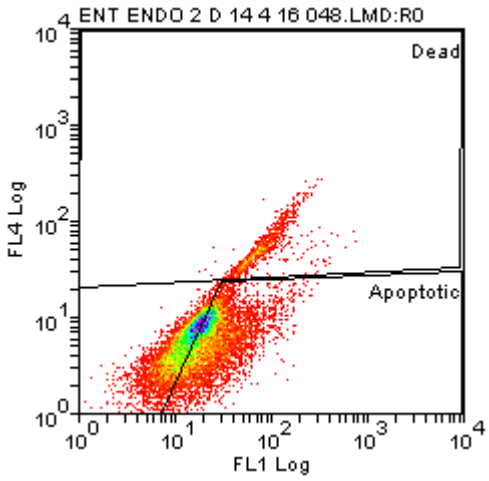

ENT ENDO 2 D 14 4 16 050.LMD
Cells
SS Lin
FS Lin
0
200
400
600
800
1k


ENT ENDO 2 D 14 4 16 046.LMD:R0
Dead
Apoptotic
FL4 Log
FL1 Log
10^0
10^1
10^2
10^3
10^4

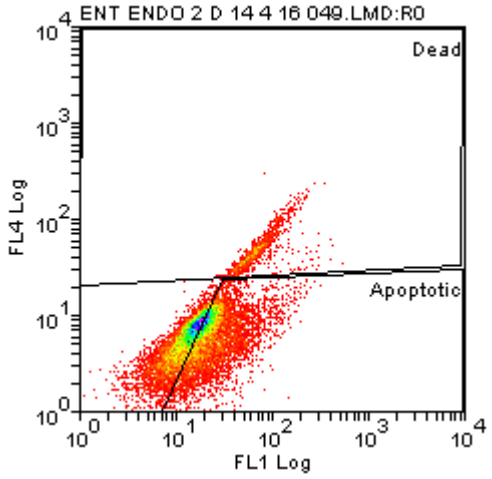
5 µM



10 µM



15 µM



20 µM

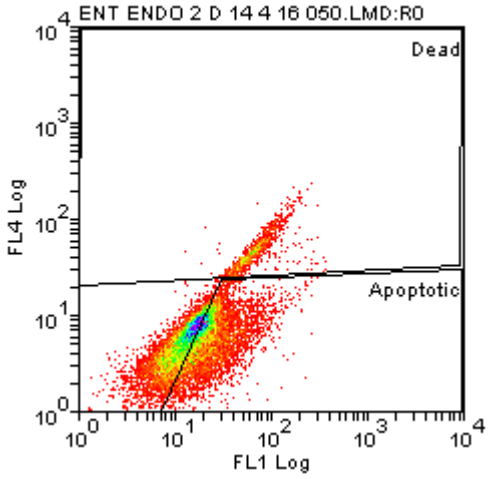


Figure 4.8 Dot-plots for apoptosis assay following 48 hours of drug treatment in HBMECs. Graph readings illustrating the various stages of Weasel Software used to analyse flow cytometry data in treated cells. These are data from HBMECs treated with different concentrations of 5-FU (0, 5, 10, 15 or 20 µM). From the gated cells, a histogram can be plotted based on the amount of staining for caspase 3/7. This histogram was drawn using data from control cells. The line down the centre of the graph is a statistical marker – as control cells are not expected to have undergone any apoptosis, this is the baseline for negative staining. In the treated cells, any readings to the right of this line are consisted positively stained for caspase 3/7. Data from cells treated with different concentration 0-20 µM 5-FU.

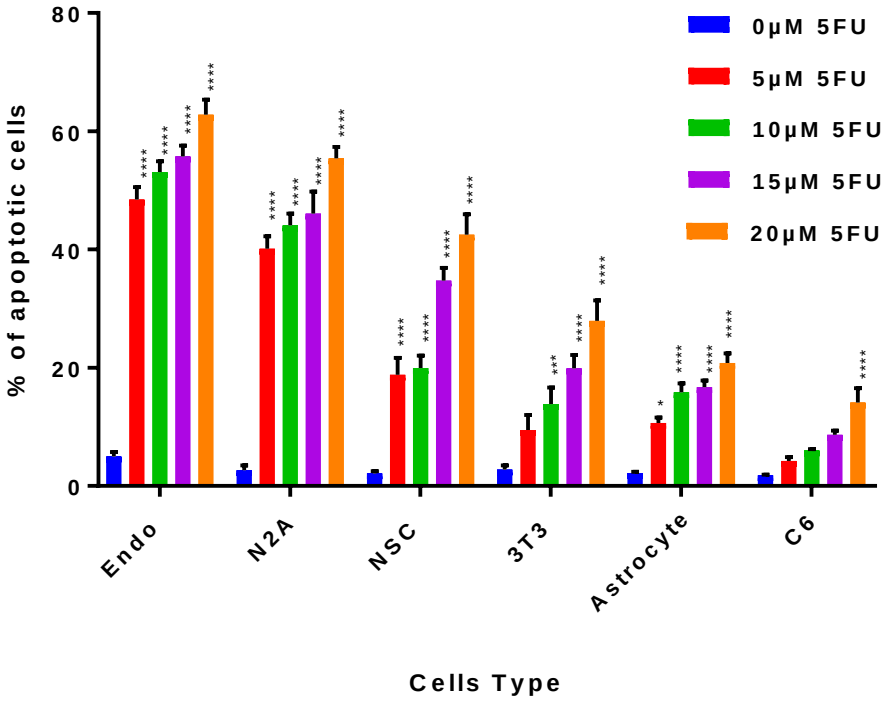


Figure 4.9 Bar chart showing the proportion of gated cells (%) stained for caspase 3/7 (apoptotic marker), when treated with different concentrations of 5-FU concentrations (0, 5, 10, 15 or 20 µM) for 48 hours. Two way ANOVA revealed that there were significant differences between different cells ($p<0.0001$). 5-FU induced more apoptotis in HBMECs. Increased concentrations of 5-FU cause an increase in apoptosis, based on caspase 3/7, compared to controls, whereby all concentrations of 5-FU caused significantly more apoptosis, compared to controls. The most sensitive cell lines were HBMEC and N2a cells. Experiments were repeated at least three times.

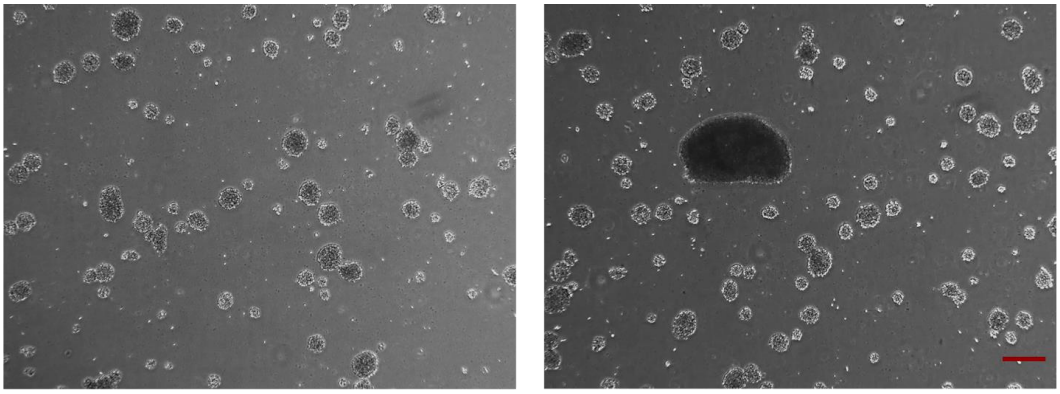


Figure 4.10 Variable sizes of neurospheres appear a few days after keeping NSCs in the medium, the larger spheres showing darker zones in the centre which denote cell necrosis, while the smaller spheres are transparent. Scale bar 50 μm.

4.4 Discussion

Previous studies from our group showed that 5-FU treatment caused a significant decrease in the number of proliferating cells in the SGZ *in vivo* (Lyons et al., 2011). Most of the dividing cells in the SGZ are NSCs and it is likely that 5-FU causes apoptosis or quiescence in these cells.

In order to determine the relative toxic effects of antimetabolite agents such as 5-FU, we exposed different cell types to different concentrations of 5-FU. The effects on cells was analysed in terms of DNA damage, cytotoxicity and apoptosis by fluorescent cell sorting (Pasha Shaik et al., 2006). It has been shown that chemotherapy and radiotherapy can cause significant DNA damage to normal as well as cancerous cells within the body (Bourton et al., 2011b). We have focused on the *in vitro* effects of chemotherapy on neural cells because there is very little literature on the *in vitro* effects of 5-FU on NSC in terms of DNA damage, apoptosis and the expression of caspase 3/7.

4.4.1 DNA damage

As described in the introduction, part of the cytotoxic action of 5-FU is to produce DNA damage in the form of DSBs (Can et al., 2013). It is known that different cell types have differing sensitivities to 5-FU and the relative levels of DNA damage produced by 5-FU in different cell types was investigated. DNA damage was measured by the expression of pH2AX, a sensitive marker of DNA damage which is rapidly produced as part of the cellular DNA damage response (Ismail et al., 2007). All cell types showed a dose dependent increase in the level of pH2AX after treatment confirming that 5-FU causes DNA damage in dividing cells. Recent studies have found DNA damaging effects of chemotherapy on cancer cells (Garcia-Villa et al., 2012b, Olive and Banáth, 2009). DNA damage produced by chemotherapy may lead to apoptosis and loss of cells or cell quiescence/senescence if DNA repair processes are initiated (Panda et al., 2008). A study by Schneider et al in 2013 suggested that DNA damage in NSCs causes cellular senescence, depriving them of their self-renewal potential and promoting astrocytic differentiation (Schneider et al., 2013). DNA damage induced senescence requires upregulation of p53 which in turn increases the expression of p21. P21 is a cyclin dependent kinase inhibitor which halts cell cycle progression (Heath, 2008)

From this investigation of the effects of DNA damage induced by 5-FU, on NSCs, the primary NSCs are less sensitive as compared to cells from the neural cell line N2a. There is evidence that N2a cells are more sensitive to cytotoxic agents than primary foetal cortical neurons and show higher levels of DNA damage due to their susceptibility to oxidative stress (Togar et al., 2015, Aydın et al., 2014). As described in Chapter 2, there is a 50% reduction in NSC viability after 48 hours with 5 μM 5-FU

concentrations. This is likely to reflect DNA damage and in the present chapter there is a significant increase in the level of DNA damage with a concentration of 5 μM of 5-FU or above. It is likely that the time between DNA damage and apoptosis is short in these cells so that there is only a matter of hours between both events.

While the DNA damaging effects of 5-FU were less in glia, there was still a significant increase in $pH2AX^+$ cells. This result would suggest that glial cells are more resistant to chemotherapy than neural cells, a result confirmed in this thesis. Chemoresistance can be due to a variety of mechanisms including increased efflux of drugs by ABC proteins and higher levels of DNA repair (Abdullah and Chow, 2013). Gliomas are known to be particularly resistant to chemotherapy, mainly due to the increased activity of ABC efflux transporters that remove chemotherapy drugs (Declèves et al., 2006). It is unclear whether this is providing the mechanism for chemoresistance in the present case but it is likely to play a role.

4.4.2 Cell cycle

Chemotherapy agents can have different effects on the cell cycle and may block proliferation or induce quiescence, differentiation or apoptosis. In this study, flow cytometry was used to investigate the position in the cell cycle of 5-FU treated cells to determine if treatment induced cell cycle arrest at particular points. All cells were treated with 5-FU using different concentration (5, 10, 15 and 20 μM) chosen from earlier experiments for 24 and 48 hours. PI staining was used to determine location in the cell cycle. The fluorescent dye- PI binds to DNA and can be used to determine the DNA content of individual cells. It has been used to distinguishing cells in the G0/G1, S, and M/G2 phases of the cell cycle (Tanaka et al., 2009).

In all cell lines and primary cells, except NSCs, 5-FU treatment caused a significant dose dependent increase in the number of cells in G0/G1 and a decrease in the number of cells in S phase. This indicates that 5-FU is causing fewer cells to enter S phase during DNA replication, and more cells remain in the resting G0/G1 phases. This consistent with cell cycle arrest at the G1 checkpoint which occurs after DNA damage (Alberts et al., 1994). Previous studies have also shown that 5-FU arrests a number of different cancer cells in G1 (Lee et al., 2005, Grem et al., 1999, Mirjolet et al., 2002, Hernández-Vargas et al., 2006). G1 arrest prevents progression through the cell cycle and can indicate quiescence and DNA repair or the initiation of apoptosis (Dabrowska et al., 2009).

In contrast to the other cell types tested there were significantly fewer NSCs in the G0/G1 phase after treatment with 5-FU for 24 hours compared to controls and as a result, the number of cells in S phase was increased. The reason for this is unclear but may indicate that cell lines react differently to 5FU in comparison to NSCs. Some types of cancer cells can pass through the G1 checkpoint, but non-cancerous cells are normally arrested at this point after chemotherapy treatment.

4.4.3 Apoptosis

After looking at the effects of 5-FU on the cell cycle and the induction of DNA damage, the final investigation in this chapter looked at the impact of different concentrations of 5-FU on inducing expression of the apoptosis associated enzymes caspase 3/7 using flow cytometry. This was done to determine whether there were changes in the level of induced apoptosis between different cell types.

It was expected that 5-FU would cause apoptosis in all cell types including NSCs and N2a cells. It has been shown that 5-FU significantly increases caspase 3 activation in cancerous cells (Adachi et al., 1999, Ohtani et al., 2000) which would support the significant decreases in viability seen in our earlier MTT assays after 5-FU treatment. Concentrations of 5 μM 5FU or higher at 48 hours caused significant increases in apoptosis in all cell types. This study has shown that 5-FU causes a significant increase in the rate of apoptosis in neural cells even at the low dose of 5 μM 5-FU after 48 hours treatment *in vitro*. Based on these results, the appropriate time point for DNA damage detection in N2a cells was after 24 hours of 5-FU exposure because by 48 hours exposure, the cells were experiencing significant cell death. However, unexpectedly, 5-FU caused significantly more apoptosis in endothelial cells compared with other cell types. C6 astrocytes and 3T3 cells exhibited low levels of apoptotic at 5, 10 and 20 μM demonstrating their resistance to this chemotherapy treatment 5-FU.

In summary chemotherapy induced DNA damage leading to apoptosis is one of the main cytotoxic mechanisms of 5FU. These experiments showed that 5-FU produced significant levels of DNA damage leading to G1 arrest and apoptosis even at 5 μM 5-FU. Different cell types from the hippocampal neurogenic niche show differences in their sensitivity to increasing concentrations of 5-FU and cell death. Endothelial and neural cells are more sensitive than astrocytes, C6 or 3T3 cells.

5 Evaluation of the cellular oxygen consumption rate in C6 and N2a

5.1 Introduction

Treatment with 5 μM 5-FU has different impacts on different cell types as measured by MTT assay. C6 astrocytes show the least effect of 5-FU while N2a cells are the most sensitive. This difference may relate to the extent to which these cell types use mitochondrial respiration.

Recent studies have shown that C6 cells have little or no mitochondrial respiration being almost entirely glycolytic (Dr Smith personal communication). It would be interesting to determine the level of mitochondrial respiration in N2a cells and whether this can be related to their sensitivity to chemotherapy. Treatment with 5-FU may create ROS within mitochondria in N2a cells but not in C6 cells. Mitochondrial respiration can be determined by measuring the oxygen consumption rate (OCR). This measurement provides an indication of the aerobic and anaerobic metabolism of the cell which may change with chemotherapy treatment and reflect the sensitivity of the cells to chemotherapy. In the present studies, the effects were measured 10mins after treatment with 5-FU. Future studies should look at longer time periods up to 48hrs.

5.1.1 Mitochondria

Mitochondria are cytoplasmic organelles found in all eukaryotic cells, and are known as the "power-house" of the cell, due to their role in energy production in the form of adenosine triphosphate (ATP) (Casley et al., 2002, Mootha et al., 2003). In addition, they have roles in signalling, cellular differentiation, cell survival, cell death and the control of cell cycle and growth (Nesti et al., 2007, Knott et al., 2008, Youle and Strasser, 2008) as well as calcium homeostasis and regulating calcium signalling which are discussed in other parts of this thesis (Taylor et al., 2003). The mitochondrion

consists of 4 compartments: the matrix, the inner mitochondrial membrane, the outer mitochondrial membrane and the intermembrane space (Martinou and Green, 2001, Bolender et al., 2008).

Mitochondria are the main producers of ROS, which are by-products of oxidative respiration under basal conditions. An increase in the generation of ROS occurs when mitochondria are damaged with the consequent dysregulation of the oxidative phosphorylation machinery (Brookes et al., 2004).

Mitochondria are involved in the way both neurons and glia handle oxygen and glucose and mitochondrial dysfunction is associated with several neurodegenerative diseases including human immunodeficiency virus (HIV)–associated neurocognitive disorders which could cause selective damage of neurons versus glial cell survival (Funes et al., 2014). Mitochondria are important for energy production and apoptotic processes in astrocytes (Beltrán et al., 2000). Mitochondrial dysfunctions can be related to either de- or hyperpolarization (Iijima, 2006) and it has been observed that mitochondrial depolarization is a crucial difference in sensitivity of astrocytes and neurons to long-term (>45 min) oxygen glucose deprivation (Almeida et al., 2001, Reichert et al., 2001).

5.1.2 Mitochondria and neuronal cells

Neuronal cells have specific characteristics that distinguish them from other cell types. For example, they are fully differentiated in the adult brain, being unable to divide and regenerate after brain injury. Neurons cannot survive in the absence of oxygen (anaerobic conditions) and die within a few minutes (Dowling, 2001). They have a high number of mitochondria per cell and a high metabolic rate and energy demand compared to other cell types (Klepinin et al., 2014, Knott et al., 2008). Neurons mainly

obtain their ATP through aerobic respiration. Mitochondria have the capacity to scavenge oxidative insult which make them important in neurons. Based on these observations, the maintenance of oxidative respiration and the antioxidant system of mitochondria within neurons plays a key role in their normal functioning and survival.

Neuronal mitochondria display a number of different enzymatic and metabolic processes compared to glial cells (Almeida and Medina, 1997). All these features may affect their vulnerability and ability to compensate for drug induced insults (Almeida et al., 2001).

Mitochondria in astrocytes are important for energy production and apoptosis (Mannella, 2006) and astrocytes can protect themselves from mitochondrial inhibition by the rapid upregulation of adenosine monophosphate (AMP)–activated protein kinase (AMPK)–mediated glycolysis. Neurons are more sensitive than astrocytes to nitric oxide mediated apoptosis, as neuron lack defense mechanism (Almeida et al., 2004).

5.1.3 The Warburg effect (aerobic glycolysis)

In the 1920s, a role for mitochondria in cancer biology was described for first time by Otto Warburg (Warburg et al., 1927). He observed that cancer cells, even in the presence of oxygen, can produce ATP via high rates of glycolysis and lactate production. This phenomenon, known as the Warburg effect, was the first indication that cancer cells have different cellular energetics and metabolism to normal cells and that cancer cells mostly depend on glycolysis (Kroemer, 2006). Cancer cells therefore, must sustain high levels of glucose uptake in order to meet their energy needs and the increased glycolytic flux in cancer cells causes them to be more sensitive to glucose deprivation (Koppenol et al., 2011, Warburg, 1956).

The purpose of the experiments described in this chapter was to investigate 5-FU has different effects on mitochondria respiration or membrane potential in glia cells (C6) and neuronal cells (N2a) which might be related to differences in their sensitivity to chemotherapy.

5.2 Materials and Methods

5.2.1 Respiration Measurements

5.2.1.1 Oxygen consumption rate

Mitochondrial oxygen consumption rate (OCR) was measured using Clark oxygen electrodes (Rank Brothers, Bottisham UK) which measure dissolved oxygen (Chance and Williams, 1955). The oxygen electrode is an electrochemical sensor for oxygen, which works by measuring the partial pressure of oxygen dissolved in solution (Figure 5.1). The electrodes are housed in a chamber and consist of a base with a platinum working electrode (typically 2 mm in diameter) surrounding which is a silver ring \ reference electrode. The oxygen electrodes were used in accordance with the manufactures instructions (Digital 20, Rank Brothers). Potassium chloride (3M) was used to provide conduction between these electrodes with a semi-permeable dual layer membrane of Teflon and tissue, which was used to separate the cell sample from the electrodes. Temperature significantly affects the operation of the oxygen electrode such that increasing temperature produces a higher signal. A constant temperature of 37°C was maintained thermostatically. In this study, oxygen consumption rates were measured for suspensions of N2a and C6 cells. The partial pressure of O_2 (PO_2) was measured at a polarographic voltage of −0.6 V with electrodes previously double

calibrated at 100% air saturation (vigorous gassing with air, ~0.25 mM) before each experiment. A zero oxygen point was achieved by adding the powerful reducing agent, sodium dithionate (addition of $Na_2S_2O_4$) which reduces the remaining oxygen inside the chamber.

A magnetic stirrer was placed inside the chamber to ensure mixing. Before each experiment, 1 ml of Hank's solution 138 mM NaCl, 4.2 mM NaHCO3, 1.2 N mM aH2PO4, 5.6 mM KCl, 1.2 mM MgCl2, 2.6 mM CaCl2, 10 mM HEPES (pH 7.4 with, NaOH)+0.1% BSA) was loaded into each chamber and left for 10 minutes for temperature equilibration. During this period, the cells were rinsed with 5ml of PBS before adding 3ml trypsin and returning the flasks to the incubator for 5 minutes. For these experiments, 1ml of cells at a density ~1 x 10^6 m1/cell were mixed with 1ml of Hank's solution+0.1% Bovine Serum Albumin (BSA) and suspended and incubated at 37ºC.

The oxygen electrode chamber was then closed from the atmosphere by a plunger to allow only the oxygen contained in the chamber to be measured. After a 10 min equilibration period, the OCR was measured first in the absence of exogenous substrate (0 glucose), Subsequently, 10mM glucose given by adding 10µl of a 1Mglucose stock solution in H_2O to the 1ml incubation volume by Hamilton syringe through the plunger. This was followed by addition of 5-FU (5 or 50µM) while the control samples had only vehicle added. Then, 1 µM of carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) was added to each chamber for 10minutes to assess maximum respiration. This compound is a protonophore, which acts as an uncoupler of oxidative phosphorylation by preferentially transporting protons across the mitochondrial membrane instead of

through the ATP synthase complex, leading to a rapid consumption of energy and oxygen without the generation of ATP.

The experiment was ended by adding 6mM of the mitochondrial inhibitor sodium azide, which is a complex IV mitochondrial inhibitor, for further 10minutes.

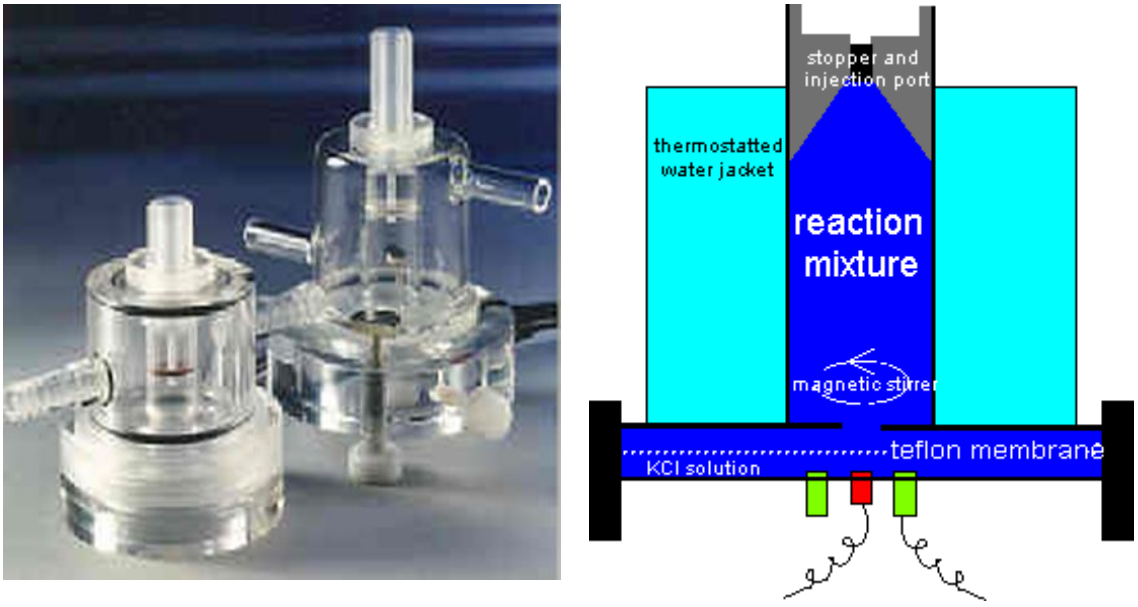


Figure 5.1 Clark oxygen electrode. The electrode membrane consisted of a layer of tissue, KCl solution concentration and a layer of Teflon.

5.2.2 Data analysis

The data on oxygen consumption were collected, (DATAQ, Inc., Ohio) and analysed using WINDAQ software, Version 2.54,). The percentage saturation of oxygen was plotted against time for each oxygen electrode.

At the end of each experiment, the basal oxygen consumption, substrate-induced oxygen consumption and the drug-induced oxygen consumption were quantified by measuring the slope of the graph. For basal O_2 consumption, the slope of the graph was measured between 2.5 and 7.5 minutes, slope measurements was also taken after the

glucose substrate was added for substrate-induced O_2 consumption and the final slope measurements after adding 5-FU for O_2 consumption on addition of drug. All values were corrected for the cell density. Treatment with azide alone completely blocks mitochondrial respiration and this treatment was used to provide the base line to compare the effects of drug treatments. The effect of the glucose substrate and drugs on O2 consumption were calculated (ESubstrate and EDrug, respectively) using the following formulae:

ESubstrate (nmol 10^7 $cell^{-1}$ min^{-1}) = [(substrate)-(basal)] *60e9*1e-3*0.0015/Col (cell)

EDrug (%) = 100* (Drug- substrate)/Drug

ESubstrate (%) =100*(Substrate-basal)/Substrate

Basal is oxygen consumption rate during 10 min without adding any things

Substrate is oxygen consumption rate after addition glucose

Drug is oxygen consumption rate after addition of chemotherapy

Lastly, the effect of chemotherapy was calculated as the percentage inhibition of glucose-induced O_2 consumption.

In addition, all data was checked for normality using the D'Agostino and Pearson omnibus normality test. Data is presented and analysed in a form appropriate to its distribution. Parametric statistical analysis using prism v7.0 (GraphPad Software, San Diego California, USA) was performed if data was normally distributed, otherwise nonparametric analyses were performed with StatsDirect (StatsDirect Ltd, Altrincham,

Cheshire, UK). Statistical significance was determined by one-way ANOVA with post-hoc Bonferroni or its non-parametric analogue; Mann-Whitney.

5.3 Results

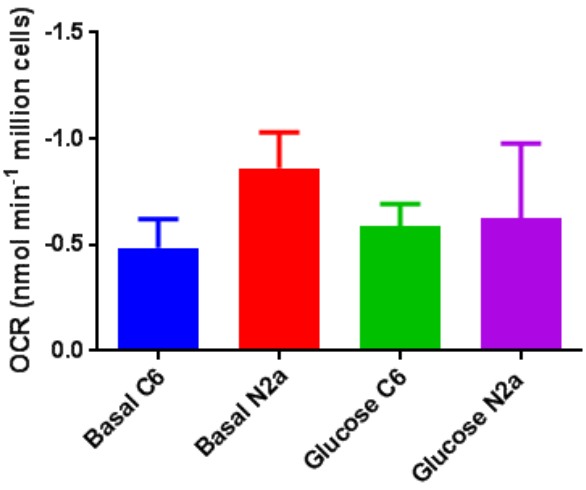
5.3.1 Comparison of oxygen consumption rates in N2a and C6 cells

Our data showed that N2a cells possessed a higher basal respiratory rate than C6 cells ($P < 0.0001$, n=6-8) (Figure 5.2A). Subsequent addition of 10mM glucose significantly inhibited ΔO_2 in N2a cells (Figure 5.2A) (p-value=0.0001, Mann-Whitney test), whereas 10mM glucose had no effect on C6 cells. As glucose failed to increase the OCR in N2a cells and in C6 glioma cells (as shown in figure 5.2A), it is difficult to determine if an increased metabolism causes an increase OCR.

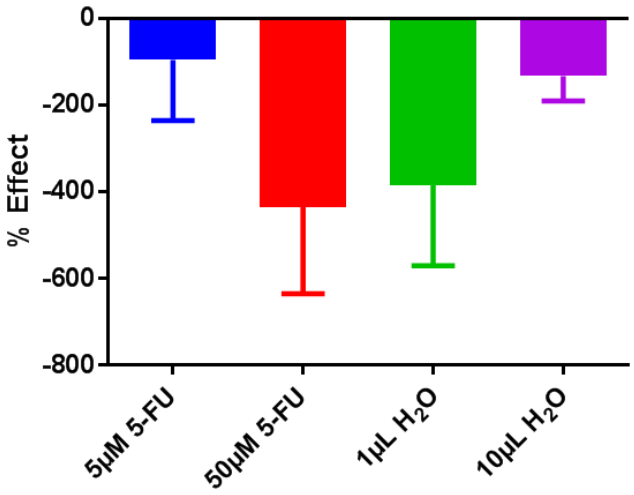
Addition of H_2O (the vehicle) or 5-FU to C6 and N2a slightly inhibited ΔO_2 (Figure 5.2 B and C). There is small effect with 5-FU in the presence glucose, but no difference can observed between the two types of cells exposed to 5-FU (5 and 50 μM) had no effect in the presence glucose (Figure 5.2B and C).

The longest period of time that the cells were treated with 5-FU was 10 minutes. These data also confirm the functional integrity of the C6 and N2a cells over time and lack of cellular damage due to stirring. Unexpectedly, the addition, FCCP had no effect on ΔO_2 (Figure 5.3). This effect is inconsistent with the action of FCCP as an uncoupler of mitochondrial oxidative phosphorylation. Subsequent addition of 6mM azide, a blocker of mitochondrial function, partly blocked O_2 consumption in both C6 and N2a cells.

A


OCR (nmol min-1 million cells)
-1.5
-1.0
-0.5
0.0
Basal C6
Basal N2a
Glucose C6
Glucose N2a

B


% Effect
0
-200
-400
-600
-800
5μM 5-FU
50μM 5-FU
1μL H2O
10μL H2O

C

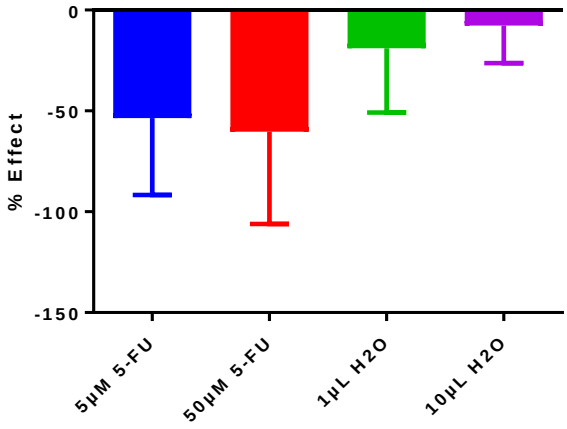
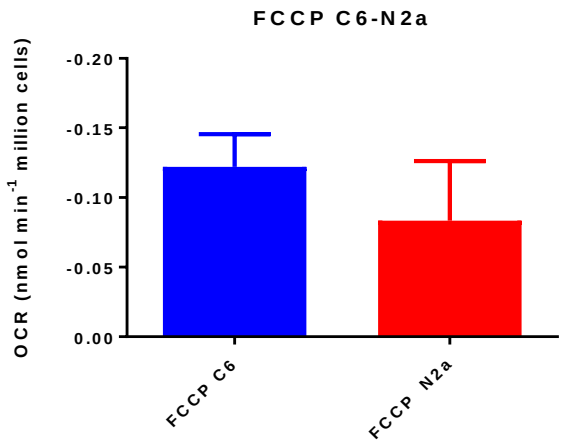
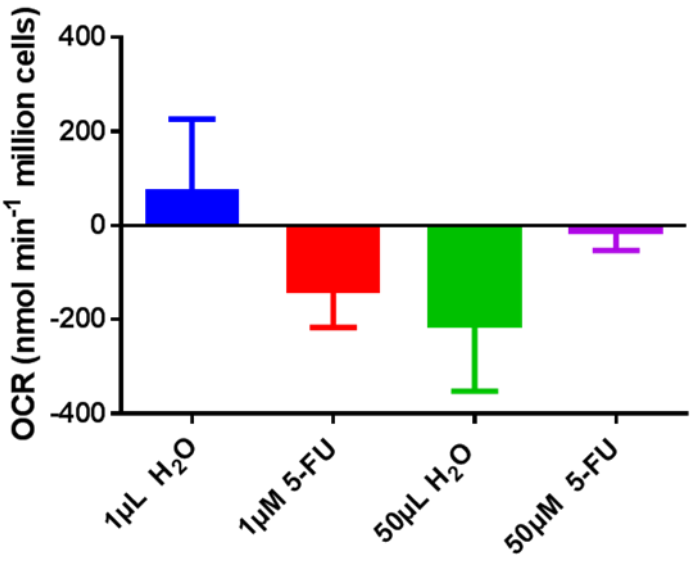


Figure 5.2 Oxygen consumption rates (OCR) of N2a and C6 cells. Comparison of the OCR for N2a cells and C6 cells with various exogenous metabolic substrates as indicated. Key: Basal, no substrate; Glu, 10 mM D-glucose; 5-FU in 10 mM glucose, 6mM azide. Mean±SEM, p-value<0.0001, n =8) ΔO_2 Vehicle control (H_2O). N2a and C6 cells under basal conditions and then in response to addition of 10mM glucose. The relationship between basal and reduction of the glucose oxygen consumption in N2a (A). Oxygen consumption rates after addition of 5-FU, C6 (B) N2a and (C).

A


FCCP C6-N2a
OCR (nmol min-1 million cells)
-0.20
-0.15
-0.10
-0.05
0.00
FCCP C6
FCCP N2a

B


OCR (nmol min-1 million cells)
400
200
0
-200
-400
1µL H2O
1µM 5-FU
50µL H2O
50µM 5-FU

C

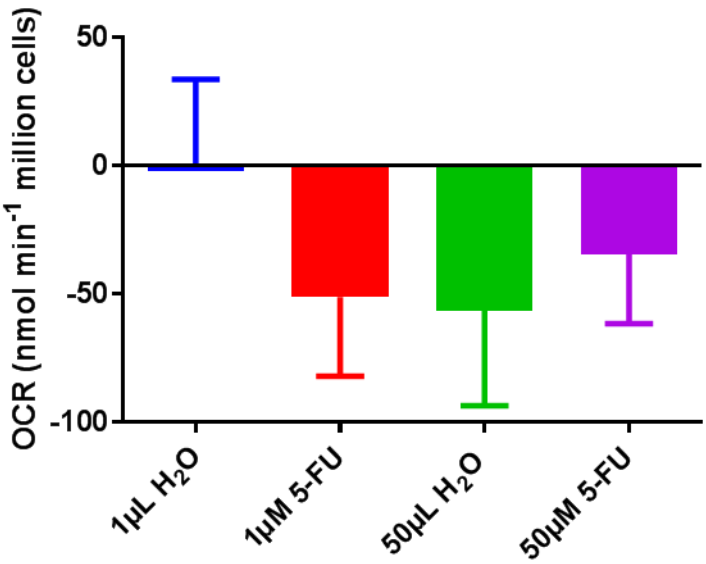


Figure 5.3 Effect of FCCP after 5-FU on OCR in C6 and N2a. Oxygen consumption rates after FCCP addition. No significant difference was observed between C6 and N2a cells regarding their response to FCCP after 5-FU by Mann-Whitney test (*p-value<0.0001) n=6 to 8.

5.4 Discussion

5.4.1 Oxygen consumption rate

The different sensitivity of C6 and N2a cells to 5-FU may relate to the degree of mitochondrial respiration that is present within these two types of cells. The ability of C6 astrocytes to use glycolytic respiration may make them more resistant to treatment with chemotherapy. This hypothesis was tested by measuring oxygen consumption under different conditions using oxygen electrodes in a sealed incubation chamber. A

study by Bolaños et al., 2010, showed substantial differences between the bioenergetics of glial cells and neurons apoptosis (Bolaños et al., 2010).

The first parameter measured was basal respiration as determined by oxygen consumption while at rest. As shown in the Figure (5.2), N2a have a higher basal respiratory rate than C6. Differences in oxygen consumption between different cell types can be due a number of reasons. Lopaschuk et al., (2010) demonstrated that the difference in basal respiratory rate between cells may be due to the fact that cells with higher oxygen consumption are metabolically more active due to the presence of lipids or fatty acids (Lopaschuk et al., 2010). An alternative reason might be that some cells are becoming apoptotic which leads to these cells consuming higher levels of oxygen. A third possibility is that increased oxygen consumption is due to a combination of both active cells and apoptotic cells. In order to prepare the cells for oxygen consumption measurements they must be trypsinised from the flasks they have been grown in and placed in suspension. This process can cause cell damage as shown by Huang et al., 2010 who found that trypsinization decreases the growth and metabolism-related protein expression levels and increases apoptosis in the breast cancer cell line (MCF-7) (Huang et al., 2010). Similarly, trypsinization encourages the overexpression of voltage-dependent anion-selective channel protein 1 and voltage-dependent anion-selective channel protein. These proteins have crucial roles in increasing mitochondrial membrane permeability which may lead to cell apoptosis (Tsujimoto and Shimizu, 2002). If these experiments are repeated it may be better to use accutase, a less vigorous protease, to remove cells from the surface of the culture flasks.

To determine the extent of mitochondrial respiration in N2a cells, 10mM glucose was added which caused a significant reduction in the rate of (ΔO_2). However, adding

glucose had no effect on C6 cells. This difference between the two cell types may indicate that N2a cells are more glycolytic or the cells have marginal oxidative capacity (Warburg effect), which leads to an increase lactate production (Warburg, 1956). Adding either 5 or 50μM 5-FU caused a slight inhibition in ΔO_2 in the presence 10 mM glucose on both N2a and C6 cells. The rate of oxygen consumption in the presence of FCCP, a compound that stimulates the metabolic activity of mitochondria, showed no difference between the two types of cell. Unfortunately, the results of these experiments were inconclusive and would need to be repeated with the addition of checking for apoptosis, for the hypothesis to be tested.

6 General discussion

Cancer survivors suffer from a number of side effects associated with their treatment (Wang et al., 2015, Soffietti et al., 2014). In this thesis we have investigated the neurological side effects of chemotherapy; effects which have been associated with a number of cognitive declines that include reduced memory and difficulties in concentration. There is increasing evidence that cancer and cancer-treatments can lead to CNS toxicity (Mitchell and Turton, 2011, Steen et al., 2001) and the decline in cognitive function found in a proportion of patients has been called 'chemobrain'. It has been found that between 16 and 75% of cancer patients treated with chemotherapy are affected by chemobrain (Myers, 2009). However, the mechanisms behind this are still not understood making it difficult to devise strategies or treatments which might prevent or protect against this effect. One likely mechanism is a decline in hippocampal neurogenesis (Wigmore, 2012). Neurogenesis in this brain region continually generates new granule cell neurons which are incorporated into the dentate gyrus within the hippocampus. New neurons are produced in a neurogenic niche which consists of a thin layer of tissue, the SGZ, found on the inside of the blades of the dentate gyrus. The generation of new neurons in this location has been shown to be required a number of aspects of memory including consolidation of short to long term memory (Kempermann et al., 2006, Squire, 2009, Rönicke et al., 2011). A decline in hippocampal neurogenesis after chemotherapy has been demonstrated in animal models by a number of authors and this has been correlated with a decline in the performance of cognitive tasks (MacLeod et al., 2007, Mustafa et al., 2008b, Liedke et al., 2009). Studies have shown that chemotherapeutic agents have cytotoxic effects on progenitor cell populations in the brain both *in vivo* and *in vitro* (Dietrich et al., 2006a, Han et al., 2008, Mustafa et al., 2008b). *In vivo* studies showed that 5-FU reduces cells proliferation in the SGZ of

the dentate gyrus and this is associated with cognitive impairments (Briones and Woods, 2011). Cell proliferation in the SGZ can recover after single injections of chemotherapy but remains reduced for long periods of time after multiple injections (ELBeltagy et al., 2012, Wang et al., 2004b).

I have studied the cell types found in the SGZ to determine their relative sensitivity to chemotherapy and possible interactions during chemotherapy treatment which might affect the impact of this treatment on neurogenesis. The cell types I have looked at were neural stem / progenitor cells, astrocytes and endothelial cells. It is unclear whether chemotherapy has a direct effect on NSCs or has effects on other components of the stem cell niche which indirectly may protect or adversely affect NSCs.

5-FU is a common therapeutic agents which has been associated with reports of chemobrain by many patients in particularly breast cancer patients (Ahles et al., 2002, Jansen et al., 2008). For this reason, 5-FU was investigated as to its effects on cells within the hippocampal neurogenic niche.

In vitro models are a useful approach enabling individual cell types and their interactions to be studied without the many confounding variables found *in vivo*. In addition, *in vitro* studies are generally cheaper, provide quicker results and do not require the use of animals. However, it is important to recognise that cells *in vitro* are in an artificial environment which will not replicate all aspects of the *in vivo* situation.

Proliferating NSCs have complex interactions with other cells and tissues in the SGZ (Shen et al., 2008a). It is thought that these other cell types play a major role in controlling the differentiation and proliferation of the NSCs within the neurogenic niche of the SGZ. Palmer et al showed the close relation between the vascular system and

neurogenesis (Palmer et al., 2000) such that approximately 50% of proliferating NSCs are closely associated with small blood vessels in the SGZ (Palmer et al., 1997). Previous work in our laboratory has shown that after *in vivo* treatment with chemotherapy, dividing cells that are in contact with capillaries are more resistant to chemotherapy compared with those some distance from blood vessels (Maqbool PhD thesis, 2015). This suggests that NSCs in contact with blood vessels, or cells associated with blood vessels, are protected from the effects of chemotherapy. Astrocytes and endothelial cells are prominent cellular components of the BBB and are closely associated on the surface of blood vessels (Song et al., 2002, Conover and Notti, 2008).

As interactions between NSCs and other components of the neurogenic niche and their response to chemotherapy may be important in determining the impact of chemotherapy on hippocampal neurogenesis, we have tried to model these interactions *in vitro* using primary NSCs, hippocampal or spinal astrocytes and C6 astrocytes, HBMEC, 3T3, N2a cells.

A number of growth factors have been identified which are produced by glia and endothelial cells including NGF, PDGF, activin, VEGF, BDNF (Engele et al., 1991, Kamei et al., 2012) that may mediate the various cellular interactions within the niche. To explore the above processes, we have tested CM from glial cell lines (C6), primary astrocytes or endothelial cells for its ability to protect neural cells. These vascular components of the neurogenic niche may also regulate neurogenesis by direct contact which can be studied by co-culture of more than one cell type.

6.1 Outline of experiments done in different chapters

In Chapter 2, the effect of 5-FU on the viability of neurogenic and non-neurogenic cells (primary NSC, N2a, C6 astrocytes and HBMECs) was studied using the MTT assay to determine their relative sensitivity to chemotherapy.

Primary astrocytes, isolated from neonatal rodents, were isolated and tested for their purity by staining for GFAP. Astrocytes from different brain regions have been described as having characteristics and for this reason primary astrocytes from hippocampus and spinal cord were isolated for testing.

In Chapter 3, the interactions between neural and astrocytes or HBMECs s was tested. This was done firstly using CM from astrocytes or HBMECs s and secondly by co-culture of neural cells with astrocytes or HBMECs s. Cell types were either grown in contact or separated by a transwell membrane. The Cx43 gap junction protein was detected by immunostaining and the functionality of GJ tested by dye transfer and using the GJ blocker CBX.

In Chapter 4, the effect of chemotherapy on DNA damage and apoptosis in different cell types were studied using flow cytometry to determine if all cell types experience the same level of DNA damage to 5-FU.

In the last part of this project (In Chapter 5), polarographic detection of O_2 consumption was used to measure the rate of respiration (ΔO_2) of N2a and C6 in order to see if 5-FU differentially affected mitochondrial function of these two cell types.

6.2 Principle discoveries

1. Different cells in the neurogenic niche have different sensitivities to 5-FU, with N2a cells and primary NSCs being significantly more sensitive than either astrocytes or endothelial cells.

In vitro experiments tested the viability of primary cells and cell lines after exposure to different concentrations of 5-FU (0, 5, 10, 15 and 20μM) for 24 and 48 hours (Chapter 2). All experiments showed that 5μM 5-FU, significantly decreased cell viability of all cell types. However, the sensitivity of different cell types to 5-FU varied. 5 μM 5-FU had a small effect on the survival of astrocyte cells, but a much larger deleterious effect on neural cells. For example, 5 μM 5-FU produced a 50% reduction in the viability of neural cells after 48 hours exposure as shown in (Figure 2.10). N2a and primary hippocampal NSCs seem to have same sensitivity to 5-FU. These results show that 5-FU was more cytotoxic to neural cells than to other cell types. As astrocytes and endothelial cells were found to be more resistant to 5-FU and it is likely that *in vivo* treatment with chemotherapy spares these cell types more than NSCs. The purpose of treating cancer cells with 5-FU is to kill proliferating cells, and we found that there is a dose-dependent decrease in cell viability, as found by others (Kim et al., 2009a).

There are a number of reasons why different cell types may show different responses to cytotoxic drugs such as chemotherapy. These include, differences in the activation or degradation of the drug by metabolic pathways within the cell; differences in the ability to exclude drugs using P-glycoprotein proteins; and differences in the ability of cells to repair DNA damage caused by cytotoxic drugs

(Luqmani, 2005). 5-FU is metabolised by the enzyme dihydropyrimidine dehydrogenase and patients deficient in this enzyme may experience severe toxicity when treated with this drug (Boisdron-Celle et al., 2017). Although it is possible that the different cell types used in this project had different levels of this enzyme this is unlikely as there are no reports in the literature of this enzyme varying between cell types. Multi drug resistance (MDR) due to upregulation of P-glycoprotein proteins which are able to remove drugs from the cytoplasm is a common mechanism by which cancer cells evade destruction and has been found in cells resistant to 5-FU (Zhou et al., 2017). Astrocytes and endothelial cells are known to have high levels of MDR proteins (Nies et al., 2004) and their presence is likely to contribute to the relative resistance of these cell types to 5-FU found in this project. DNA damage can be repaired by the DNA damage repair protein MGMT and astrocytes have higher levels of this protein than other cell types (Nutt et al., 2000). Other authors have noted the relative sensitivity of NSCs to chemotherapy as compared with other normal or cancerous cell lines (Han et al., 2008). The result of these differences is that when chemotherapy is given, dividing NSCs will be preferentially killed while proliferating astrocytes and endothelial cells remain. The majority of dividing cells in the SGZ of the dentate gyrus are thought to be NSCs or neural progenitors and previous work has shown that 5-FU treatment significantly reduces the number of dividing cells in this location (Mignone and Weber, 2006, ELBeltagy et al., 2010b). Loss of NSCs and progenitors will lead to a long-lasting reduction in hippocampal neurogenesis and associated cognitive impairment.

2. Treatment of N2a cells with FLX or CM from astrocytes treated with FlX does not protect the neural cells from 5-FU.

In vivo treatment with the antidepressant FLX increases neurogenesis (Encinas et al., 2006), protects hippocampal neurogenesis from 5-FU chemotherapy and prevents cognitive decline in animal models (ELBeltagy et al., 2010c). The mechanisms behind these results are unclear but may involve the upregulation of BDNF which is a consequence of FLX treatment. *In vitro* treatment of different cell types with FLX has been found to increase the proliferation of cerebellar granule cell precursors (Manev et al., 2001) and hippocampal NSCs (Chen et al., 2007a) but reduces the proliferation of adipose tissue derived stem cells (Sun et al., 2015a) and some cancer cell lines (Krishnan et al., 2008, Stepulak et al., 2008, Frick et al., 2008, Cloonan et al., 2010). Using the protocol described in the present project no increase in the proliferation or protection of N2a cells was found but this could be further investigated by exposing the cells to FLX for longer periods. It is possible that FLX induces neurogenesis *in vivo* indirectly by stimulating astrocytes to release BDNF. However, treatment of astrocyte cultures with FLX did not produce CM which was protective against 5-FU treatment given to N2a cells. These results are not conclusive but may indicate that the protective effects of FLX *in vivo* are indirect rather than acting on NSCs or astrocytes. One possible mechanism is the anti-inflammatory action of FLX which may contribute to its protective effects *in vivo* (Lim et al., 2009).

3. Protection is provided by co-culture of N2a cells with either astrocytes or endothelial cells if contact is allowed between the different cell types.

In Chapter 3 the effects of co-culture of different cellular components of the neurogenic niche with neural cells was investigated. Confluent cultures of astrocytes, endothelial or 3T3 cells were seeded with neural cells and then treated with 5 μM 5-FU. The presence of C6 astrocytes or HBMECs but not 3T3 cells protected both N2a cells and primary NSCs from the effects of 5-FU (Figure 3.6) with the maximum effect produced by astrocytes.

The use of co-cultures was suggested by the observation that proliferating NSCs on the surface of blood vessels *in vivo*, survive treatment with 5-FU. NSCs on blood vessels will be able to contact both endothelial cells and astrocytes and this contact or the secretion of trophic factors from these cell types may provide protection. The results of Chapter 3 demonstrated that protection could be modelled *in vitro* but did not demonstrate whether protection was contact dependent or could be provided by diffusible substances.

To distinguish between these two possibilities an indirect co-culture model where the cell types were separated by a microporous (0.4-μm pore size) membrane and CM from astrocytes or endothelial cells was tested for protective efficacy. CM was produced by growing confluent astrocytes or endothelial cells for 24 hours. The media was filtered to remove all cells and added to equal volumes of fresh media to neural cells. Our results showed that there were no protection by any of the CM for neural cells from the effect of 5-FU (Figure 3.7). A limitation of these experiments is that the CM is diluted by fresh media to maintain NSC viability and so the

concentration of any trophic factors is likely to be less than that experienced by cells growing together in co-culture. However, these results suggest that diffusible substances, produced by astrocytes and endothelial cells, are not protective against chemotherapy. Moreover, HBMEC-CM and C6-CM on their own have no harmful effects on neural cells.

The transwell culture system, where cells are grown in close proximity on either side of a porous membrane prevents direct contact but allows molecular signals and growth factors, secreted by astrocytes or endothelial cells to pass through the membrane to NSCs. NSC survival was assessed by counting the number of neural cells present after chemotherapy treatment. This protocol did not protect N2a cells from 5-FU treatment suggesting that factors secreted by astrocytes or endothelial cells were not responsible for the protection.

4. The GJ connexin protein Cx43 is present in N2a cells, astrocytes and endothelial cells. GJs between N2a cells and astrocytes or endothelial cells are functional as shown by dye transfer between the cell types.

Our co-culture experiments demonstrated that cell-cell contact between neural cells and astrocytes or endothelial cells, but not fibroblasts, provided protection to the neural cells from 5-FU. This effect could be dependent upon GJ. GJ are known to be important during neurogenesis and to be present on the surface of NSCs (Se-Ran et al., 2005). Astrocytes in particular have been shown to form GJs with cancer cells within the brain (Leite et al., 2009) and the presence of these connections has been identified as a major reason for the survival of cancer cells when treated with chemotherapy *in vivo* (Eghbali et al., 1991). The studies in this thesis confirm that

astrocytes and N2a cells express Cx43 making it possible for them to form homomeric GJ between these two types of cell in agreement with previous studies (Kang et al., 2008, Cotrina et al., 1998).

These GJ are functional as demonstrated by dye transfer through GJ between astrocytes and N2a cells. GJ would allow the passage of Ca^{2+} from N2a cells into astrocytes (Verkhratsky and Kettenmann, 1996, Charles et al., 1992). (Figure 6.1). As discussed of Chapter 3, treatment with chemotherapy can cause the release of Ca^{2+} from endoplasmic reticulum into mitochondria and the passage of extracellular Ca^{2+} into the cytoplasm. The resulting raised intra-cellular Ca^{2+} can trigger apoptosis (Marchi et al., 2017). The ability of astrocytes to buffer Ca^{2+} levels in cancer cells is thought to be a key mechanism in allowing cancer cells to avoid being killed by chemotherapy. Consistent with our findings, Lin and colleagues showed that direct co-culture of astrocytes with human melanoma cells provided protection to the cancer cells from the effect of a number of different chemotherapeutic drugs including 5-FU, cisplatin and paclitaxel (Lin et al., 2010). This protective effect was dependent on direct contact and GJs between the astrocytes and tumor cells. We found that primary astrocytes from neonatal spinal cord had similar properties to astrocytes from hippocampus in providing protection to neural cell from 5-FU. This demonstrated that the protective effects of astrocytes were not limited to those from the hippocampus.

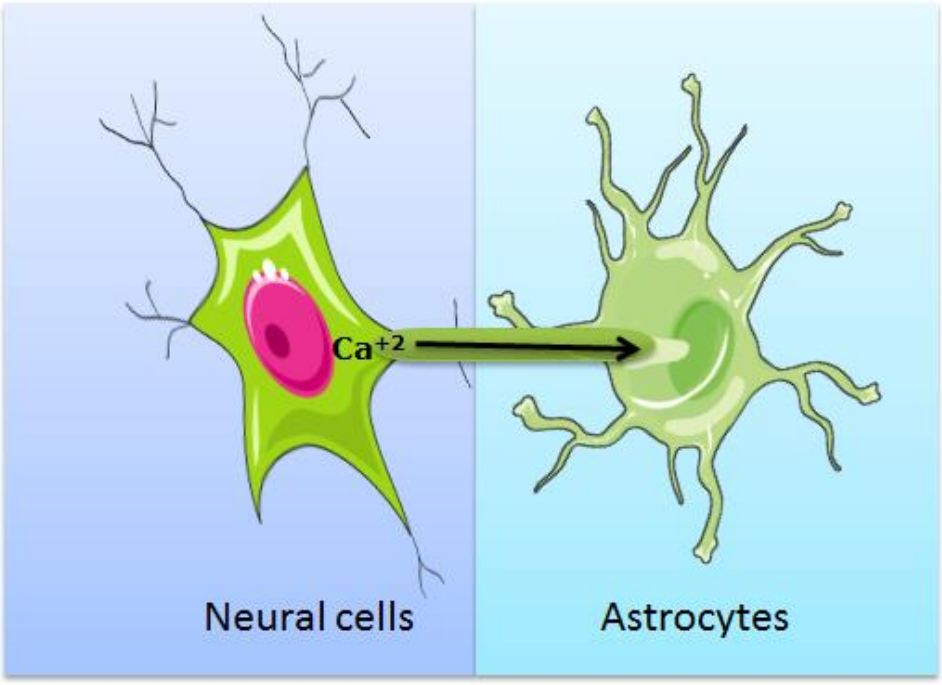


Figure 6.1 Suggested buffering of Ca^{2+} ions by astrocytes via GJ between astrocytes and neural cells.

In order to determine the importance of GJs in providing protection from chemotherapy, co-cultures of neural and astrocyte or endothelial cells were treated with the GJ inhibitor, CBX prior to chemotherapy. CBX has been widely used to block GJ (Vaney, 2002) and is believed to bind directly to Cx molecules, causing a conformation change and leading to closure of GJs. Treatment with CBX should prevent dye transfer between adjacent cells (Thomas et al., 2004). The exact mechanism by which CBX blocks GJs is still however unknown (Ozog et al., 2002). Blocking GJ between astrocytes and N2a cells with CBX prevented the astrocytes from protecting the N2a cells from 5-FU. This demonstrated the importance of GJ in the protection provided.

We also found that CBX was able to block the transfer of calcein- AM dye between adjacent cells proving that it has blocked GJ communication. Similarly, the calcein AM

dye did not pass to N2a from C6 cells when they were separated by the transwell membrane.

Co-culture with endothelial cells also protected N2a from chemotherapy and the neural cells also required direct cell to cell contact with the endothelial cells for the protection to be effective. Like astrocytes endothelial cells express Cx43 and demonstrated dye transfer between N2a cells and endothelial cells. Similarly, treatment with CBX blocked the protection provided by co-culture with endothelial cells. These results indicate that GJ between N2a cells and endothelial cells are important in the later cells providing protection. However, as mentioned earlier, unlike astrocytes, endothelial cells have not been previously described as forming GJs with neural stem or precursor cells. Further studies such as transmission electron microscopy would need to be done to prove that GJ exist between these two cell types.

Cell types	5-FU	5-FU+Neural cell (N2a or NSCs)	5FU+ Neural separate by transwell	5FU+ Neural+ CM	5FU+CBX
Sp. Astro	Reduce viability	P	N.P	N.P	
Hip. Astro	Reduce viability	P	N.P	N.P	
C6	Reduce viability	P	N.P	N.P	N.P
HBMECs	Reduce viability	P	N.P	N.P	N.P
3T3	Reduce viability	N.P			

Table 6.1 Summary of the impact of 5-FU and protection provided by different cell types or CM. Sp, Astro, spinal astrocytes; Hip Astro, hippocampal astrocytes; C6, C6 astrocyte cell line; HBMEC, endothelial cells; 3T3, 3T3 fibroblasts; P, protection; N.P, no protection.

5. Cell sorting demonstrated that 5-FU causes DNA damage with N2a cells being more sensitive than astrocytes or endothelial cells. With some treatments cells tended to arrest in the G1 phase of the cell cycle.

Results from flow cytometry (Chapter 4) showed that 5-FU had a significant effect on cell cycle progression. This is in line with evidence that anticancer treatments, such as chemotherapeutic drugs and radiation therapy kill tumor cells by causing cell cycle arrest at the G0/G1, S, or G2/M phase often in a p53 independent manner (Torres and

Horwitz, 1998, Chau and Figg, 2009). 5-FU induced apoptosis in all types of cell used in dose dependent manner. Flow cytometry also showed DNA damage at 24 and 48 hours as shown by their expression of pH2AX. Based on pH2AX expression, N2a cell had more DNA damage than other cells. 24 hours was chosen as the optimum time for DNA damage detection as apoptosis was found to be happening after 48 hours. Unexpectedly endothelial cells showed more apoptotis than other cells. A limitation in these experiments was the small number of replicates done which produced big error bars.

6. **There are likely to be significant differences in the oxidative metabolism of neural cells, astrocytes and endothelial cells but it was not possible to demonstrate that this was responsible for their different sensitivity to 5-FU.**

Measurement of OCR found no significant differences between N2a and C6 glioma cells in relation to their metabolic response to glucose and 5-FU.

6.3 Conclusion

One of the detrimental side effects of chemotherapy is neurotoxicity (Beinert et al., 2000, Yang et al., 2011a). Several studies have suggested different mechanisms involved in chemotherapy-induced cognitive decline. In this study, we have determined that 5-FU significantly reduced the viability and increased cell death in all primary cells and cell lines representing cell types present in the neurogenic SGZ of the hippocampus in a dose and time-dependent manner. Chemotherapy induced loss of NSCs from the SGZ will reduce hippocampal neurogenesis and is likely to cause prolonged cognitive

decline. Interactions between NSCs and astrocytes and endothelial cells have been suggested by *in vivo* observations of the close association between dividing cells and blood vessels. Co-culture of neural cells with astrocytes or endothelial cells demonstrated, that direct contact astrocytes and endothelial cells provided protection from chemotherapy. This protection required functional GJ between the cell types. Astrocytes from neonatal spinal cord appeared were also able to protect neural cells, suggesting that this is a general property of astrocytes.

DNA damage, changes in the proportion of cells in different parts of the cell cycle and apoptosis were all produced by 5-FU treatment. Astrocytes and endothelial cells appeared less sensitive to DNA damage or better able to repair damage as compared with neural cells. Differences in metabolism are likely to affect cell type sensitivity to chemotherapy but require further investigation.

6.4 Future directions and points to consider

Despite the difficulties in establishing *in vitro* co-culture models, they provide a useful and exciting way to study the interactions between cell types. In the present project use of this model was rewarded by the successful demonstration of significant interactions between cells involved in hippocampal neurogenesis and their response to chemotherapy. In future, the combination of other cell types such as microglia and oligodendrocytes may reveal additional interactions. It would be useful to test other chemotherapy drugs on these multi-cell type co-cultures to discover if the same or different interactions are important in the response to chemotherapy. Ca^{2+} buffering is likely to be an important cell feature of cell interactions which has implications in cancer treatment and understanding neurogenesis. Measuring, imaging and monitoring

intracellular Ca^{2+} in co-culture models under different conditions would be a useful line of investigation. An understanding of the molecular mechanism behind the protection provided by calcium ion buffering might provide alternative mechanisms to protect neural cells from chemotherapy. This understanding could come from DNA microarrays which would show which genes are up or down regulated during chemotherapy on its own and when protection is provided by calcium ion buffering.

In vivo neural stem cells are in contact with more than one other cell type and are not in two dimensional monolayers. Co-cultures of neural stem cells with more than one other cell type (microglia and astrocytes; microglia and oligodendrocytes) in 3D cultures would provide a more in vivo environment (Madl et al., 2017). In addition, the ability of neural stem cells and glial cells from different locations (hippocampus; cortex and spinal cord) should be assessed to see if the response to chemotherapy and the protection provided by supporting cells is region specific.

Flow cytometry could be used to separate stained neural cells from other cell types in co-culture to determine if co-culture has any effect on DNA damage and apoptosis. Other markers of the cell cycle could be used to determine the length and proportion of cells in G1, S, M and G2 stages of the cell cycle. This should reveal if chemotherapy treated cells arrest at particular points in the cell cycle (normally the G1/S check point) and whether other drugs such as Fluoxetine or anti-inflammatory drugs, known to protect neurogenesis in vivo, affect cell cycle progression

It is hoped that these investigations will provide useful information on the distressing phenomenon of chemobrain and in future, strategies to prevent or treat this condition.

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8 Appendices

8.1 Appendix I

Study Design	**Cancer Type (n)**	**Control group**	**Results**	**Reference**
Cross sectional 6 years post diagnosis	Breast cancer (Systemic therapy 35+ Local therapy 35) Lymphoma (Systemic therapy 35+ Local therapy 22)	Comparing local therapy (surgery or radiation) and systemic chemotherapy (standard dose chemotherapy)	Lower scores for survivors compared to local therapy in memory, psychomotor functioning, and self report of working memory.	(Ahles et al., 2002)
Longitudinal Baseline 3 weeks post chemotherapy 1 year post chemotherapy	Breast cancer (18)	Normative data	Decline in attention, processing speed, and learning	(Wefel et al., 2004)
Longitudinal Pre-treatment 1 month 6 months 18 months Post chemotherapy	Breast Cancer (123)	Chemotherapy Vs. No Chemotherapy Vs. Healthy controls	Older patients with lower baseline cognitive reserve when exposed to Chemotherapy.	(Ahles et al., 2010)
Cross Sectional	Breast Cancer (30)	Demographically matched non cancer	Worse functioning for cancer patients on global cognitive functioning, attention,	(Yamada et al., 2010)

		control (30)	psychomotor speed, and executive functioning.	
Longitudinal Pretreatment 6 months after treatment 6 months follow up	Breast Cancer (61)	Chemotherapy group to a No Chemotherapy group Both with breast cancer diagnosis	Time-by treatment interaction was significant in the motor domain..	(Tager et al., 2010b)
Prospective longitudinal Pre treatment 1 week after completing the last cycle 1 week and 6 months after the completion of all chemotherapy	Breast Cancer (71)	Comparing two chemotherapy groups and to published norms No healthy controls	Cognitive impairment was found in 23% prior to chemotherapy. Significant decreases in visuospatial skill, attention, delayed memory and motor function after treatment followed by Improvement at 6 months.	(Jansen et al., 2011)
Longitudinal 6 months and 36 months after completing treatment	Breast Cancer chemotherapy and radiotherapy group (62) Radiotherapy alone (67) Non cancer	Chemotherapy and radiotherapy to Radiotherapy alone and to No cancer history	Limited support for cognitive dysfunction post chemotherapy Non cancer controls tended to have improved performance with time on the processing speed Administration of hormonal therapy was not associated with changeover time in	(Phillips et al., 2012)

	controls (184)		cognitive performance.	

Table 1.1 A summary of some clinical studies of cognitive function post chemotherapy.

Abbreviations: Asparaginase, Asp; Bleomycin, B; Carboplatin, Cb; Cyclophosphamide, C; Cytarabine, Cy; Cytoxan, Ct; Dacarbazine, Da; Doxetaxel, D; Doxorubicin, A; Etoposide, E; Fluorouracil, F; Leucovorin, L; Mechlorethamine, Me; Mercaptopurine, Mp; Methotrexate, M; Paclitaxel, Pac; Prednisone, P; Procarbazine, Pr; Taxotere, T;Vinblastine, Vb; Vincristine, Vc

Study	**Chemotherapy agent**	**Animals**	**Findings**
(Wilson and Weber, 2013a)	thioTEPA	Mouse	Long- term increase in depressive behaviours Reduced cell proliferation in dentate gyrus
(Christie et al., 2012)	Cyclophos Doxorubi	Rat	Cognitive deficits Reduced number of DCX-positive cells Reduced neurogenesis
(ELBeltagy et al., 2012)	5-FU	Rat	Long-term reduction in cell proliferation in dentate gyrus
(Fardell et al., 2012)	5-FU Oxaliplatin	Rat	Cognitive impairments with combined drugs, prevented by wheel running
(Fremouw et al., 2012)	Doxorubi Cyclophos 5-FU	Mouse	No cognitive effects

(Lyons et al., 2012)	5-FU	Rat	Cognitive impairments; reduction dentate gyrus. Effects prevented if FLX given before and during chemotherapy
(Sharpe et al., 2012)	Oxaliplatin	Rat	Cognitive deficits
(Winocur et al., 2012)	5FU	Mouse	Persistent cognitive deficits
(Yang et al., 2012)	MTX	Mouse	Tumour and non-tumour bearing animals compared. Increase in depressive behaviours Reduced numbers of DCX positive cells Increased levels of iNOS and COX2
(Briones and Woods, 2011)	Cyclophos MTX 5-FU	Rat	Cognitive deficits Reduced cell proliferation in dentate gyrus Reduced HDAC activity
(Long et al., 2011)	Cyclophos 5-FU	Rat	No cognitive effects 2 months later
(Lyons et al., 2011)	Cyclophos	Rat	No cognitive effects. No reduction in hippocampal cell proliferation Reduced cell survival
(Lyons et al., 2011)	MTX	Rat	Cognitive deficits and reduced cell Effects prevented by co-administration of FLX

(Merzoug et al., 2011)	DOX	Rat	Increased anxiety and brain oxidative stress
(Walker et al., 2011)	MTX Tamoxifen	Mouse	Cognitive deficits which were potentiated by Tamoxifen
(Winocur et al., 2011)	MTX 5FU	Mouse	Cognitive deficits produced by chemotherapy agents which were prevented by Donepezil
(Yang et al., 2011b)	MTX	Mouse	Cognitive deficits Increase in depressive behaviours Transient declines in cell proliferation; DCX-positive cells and apoptosis

Table 1.2 Summary of recent animal models examining the effects of chemotherapy drugs. Adapted from (Wigmore, 2013a). **Abbreviations**: *Cyclophos* Cyclophosphamide.

8.2 Appendix II

3T3 cells treated for 24 hours to detect DNA damage

Chi-square test (2 by k)

	Healthy	Damaged	Total	Per cent	Score
Observed	293	7	300	97.67	1
Expected	273.6	26.4			
Observed	274	26	300	91.33	2
Expected	273.6	26.4			
Observed	273	27	300	913	
Expected	273.6	26.4			
Observed	273	27	300	914	
Expected	273.6	26.4			
Observed	255	45	300	855	
Expected	273.6	26.4			
Total	**1368**	**132**	**1500**	**91.2**	

Total Chi^2 = 30.037214 |Chi| = 5.480622 (4 DF) P < 0.0001

Chi^2 for linear trend = 24.625365 |Chi| = -4.962395 (1 DF) P < 0.0001

Remaining Chi^2 (non-linearity) = 5.411849 (3 DF) P = 0.144

Here the total chi-square test shows a statistically significant association between the classifications, i.e. between 5-FU concentration and DNA damage. We have also shown a significant linear trend which enables us to refine our conclusions to a suggestion that the proportion of damaged DNA increases with 5-FU concentration.

3T3 cells treated for 48 hours to detect DNA damage

Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	288.3	9.2	297.5	96.91	1
Expected	214.553085	82.946915			

Observed	209.9	87.2	297.1	70.65	2
Expected	214.26461	82.83539			
Observed	196.9	98.2	295.1	66.72	3
Expected	212.822236	82.277764			
Observed	190	111.7	301.7	62.98	4
Expected	217.58207	84.11793			
Observed	186.8	108.1	294.9	63.34	5
Expected	212.677999	82.222001			
Total		1071.9	414.4	1486.3	72.12

Total Chi^2 = 119.341199 |Chi| = 10.92434 (4 DF) P < 0.0001

Chi^2 for linear trend = 82.91801 |Chi| = -9.105933 (1 DF) P < 0.0001

Remaining Chi^2 (non-linearity) = 36.423189 (3 DF) P < 0.0001

Here the total chi-square test shows a statistically significant association between the classifications, i.e. between 5-FU concentration and DNA damage. We have also shown a significant linear trend: the proportion of damaged DNA increases with 5-FU concentration.

Astrocyte cells treated for 24 hours to detect DNA damage

Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	289	7.05	296.05	97.62	1
Expected	284.089188	11.960812			
Observed	286	9.93	295.93	96.64	2
Expected	283.974036	11.955964			
Observed	286	10.9	296.9	96.33	3
Expected	284.904847	11.995153			
Observed	285	14.7	299.7	95.1	4
Expected	287.591723	12.108277			
Observed	281	17.5	298.5	94.14	5
Expected	286.440205	12.059795			
Total		1427	60.08	1487.08	95.96

Total Chi^2 = 5.698617 |Chi| = 2.387178 (4 DF) P = 0.2228

Chi^2 for linear trend = 5.560628 |Chi| = -2.358098 (1 DF) P = 0.0184

Remaining Chi^2 (non-linearity) = 0.13799 (3 DF) P = 0.9869

Here the total chi-square test shows no association between 5-FU concentration and DNA damage after 1 day. However, there is a significant linear trend of increased proportion of damaged DNA with 5-FU concentration.

Astrocyte cells treated for 48 hours to detect DNA damage

Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	288	9.61	297.61	96.77	1
Expected	274.428683	23.181317			
Observed	278	18.6	296.6	93.73	2
Expected	273.497353	23.102647			

Observed	272	25.8	297.8	91.34	3
Expected	274.603883	23.196117			
Observed	267	30	297	89.9	4
Expected	273.866197	23.133803			
Observed	266	31.8	297.8	89.32	5
Expected	274.603883	23.196117			
Total		1371	115.81	1486.81	92.21

Total Chi^2 = 15.556013 |Chi| = 3.944111 (4 DF) P = 0.0037

Chi^2 for linear trend = 14.528338 |Chi| = -3.811606 (1 DF) P = 0.0001

Remaining Chi^2 (non-linearity) = 1.027675 (3 DF) P = 0.7946

Whereas after 2 days there was a significant association between 5-FU concentration and DNA damage as well as a significant trend of increased proportion of damaged DNA with 5-FU concentration.

Endothelial cells treated for 24 hours to detect DNA damage
Chi-square test (2 by k)

	Successes	Failures	Total	Percent	Score
Observed	287	8.08	295.08	97.26	1
Expected	263.612083	31.467917			
Observed	253	27.6	280.6	90.16	2
Expected	250.676259	29.923741			
Observed	247	37.1	284.1	86.94	3
Expected	253.803012	30.296988			
Observed	247	38.5	285.5	86.51	4
Expected	255.053714	30.446286			
Observed	240	40.8	280.8	85.47	5
Expected	250.854931	29.945069			
Total		1274	152.08	1426.08	89.34

Total Chi^2 = 28.158792 |Chi| = 5.306486 (4 DF) P < 0.0001

Chi^2 for linear trend = 22.75252 |Chi| = -4.76996 (1 DF) P < 0.0001

Remaining Chi^2 (non-linearity) = 5.406272 (3 DF) P = 0.1444

There was a significant association between 5-FU concentration and DNA damage as well as a significant trend of increased proportion of damaged DNA with 5-FU concentration.

Endothelial cells treated for 48 hours to detect DNA damage

Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	288	9.82	297.82	96.7	1
Expected	207.010746	90.809254			
Observed	205	88.5	293.5	69.85	2
Expected	204.007971	89.492029			
Observed	191	104	295	64.75	3
Expected	205.050601	89.949399			

Observed	181	111	292	61.99	4
Expected	202.965341	89.034659			
Observed	157	135	292	53.77	5
Expected	202.965341	89.034659			
Total		1022	448.32	1470.32	69.51

Total Chi^2 = 149.026215 |Chi| = 12.207629 (4 DF) P < 0.0001

Chi^2 for linear trend = 122.825967 |Chi| = -11.082688 (1 DF) P < 0.0001

Remaining Chi^2 (non-linearity) = 26.200248 (3 DF) P < 0.0001

There was a significant association between 5-FU concentration and DNA damage as well as a significant trend of increased proportion of damaged DNA with 5-FU concentration

C6 treated for 24 hours to detect DNA damage
Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	295	3.89	298.89	98.7	1
Expected	287.05307	11.83693			
Observed	290	7.96	297.96	97.33	2
Expected	286.159901	11.800099			
Observed	286	13	299	95.65	3
Expected	287.158714	11.841286			
Observed	281	16.2	297.2	94.55	4
Expected	285.429999	11.770001			
Observed	280	18	298	93.96	5
Expected	286.198317	11.801683			
Total		1432	59.05	1491.05	96.04

Total Chi^2 = 12.100345 |Chi| = 3.478555 (4 DF) P = 0.0166

Chi^2 for linear trend = 11.782462 |Chi| = -3.432559 (1 DF) P = 0.0006

Remaining Chi^2 (non-linearity) = 0.317882 (3 DF) P = 0.9566

There was a significant association between 5-FU concentration and DNA damage as well as a significant trend of increased proportion of damaged DNA with 5-FU concentration.

C6 treated for 48 hours to detect DNA damage

Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	385	10.4	395.4	97.37	1
Expected	336.72535	58.67465			
Observed	356	29.7	385.7	92.3	2
Expected	328.464764	57.235236			
Observed	323	58.6	381.6	84.64	3
Expected	324.973176	56.626824			

Observed	294	84.2	378.2	77.74	4
Expected	322.077712	56.122288			
Observed	277	102	379	73.09	5
Expected	322.758998	56.241002			
Total		1635	284.9	1919.9	85.16

Total Chi^2 = 122.487772 |Chi| = 11.067419 (4 DF) P < 0.0001

Chi^2 for linear trend = 121.71014 |Chi| = -11.032232 (1 DF) P < 0.0001

Remaining Chi^2 (non-linearity) = 0.777632 (3 DF) P = 0.8548

There was a significant association between 5-FU concentration and DNA damage as well as a significant trend of increased proportion of damaged DNA with 5-FU concentration

N2a treated for 24 hours to detect DNA damage

Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	287	12.9	299.9	95.7	1
Expected	132.77511	167.12489			
Observed	112	187	299	37.46	2
Expected	132.376652	166.623348			
Observed	96.3	204	300.3	32.07	3
Expected	132.952203	167.347797			
Observed	91.2	208	299.2	30.48	4
Expected	132.465198	166.734802			
Observed	76.8	223	299.8	25.62	5
Expected	132.730837	167.069163			
Total		663.3	834.9	1498.2	44.27

Total Chi^2 = 410.581024 |Chi| = 20.262799 (4 DF) P < 0.0001

Chi^2 for linear trend = 263.256675 |Chi| = -16.225186 (1 DF) P < 0.0001

Remaining Chi^2 (non-linearity) = 147.32435 (3 DF) P < 0.0001

There was a significant association between 5-FU concentration and DNA damage as well as a significant trend of increased proportion of damaged DNA with 5-FU concentration

NSC treated for 24 hours to detect DNA damage

Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	388	11.3	399.3	97.17	1
Expected	280.470984	118.829016			
Observed	284	114	398	71.36	2
Expected	279.557856	118.442144			
Observed	262	136	398	65.83	3

Expected	279.557856	118.442144			
Observed	237	160	397	59.7	4
Expected	278.855449	118.144551			
Observed	227	171	398	57.04	5
Expected	279.557856	118.442144			
Total		1398	592.3	1990.3	70.24

Total Chi^2 = 196.78543 |Chi| = 14.028023 (4 DF) $P < 0.0001$
Chi^2 for linear trend = 161.260419 |Chi| = -12.698835 (1 DF) $P < 0.0001$
Remaining Chi^2 (non-linearity) = 35.525011 (3 DF) $P < 0.0001$

There was a significant association between 5-FU concentration and DNA damage as well as a significant trend of increased proportion of damaged DNA with 5-FU concentration

NSC treated for 48 hours to detect DNA damage

Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	288	10.6	298.6	96.45	1
Expected	163.84262	134.75738			
Observed	163	117	280	58.21	2
Expected	153.63675	126.36325			
Observed	104	170	274	37.96	3
Expected	150.344534	123.655466			
Observed	117	161	278	42.09	4
Expected	152.539345	125.460655			
Observed	102	178	280	36.43	5
Expected	153.63675	126.36325			
Total		774	636.6	1410.6	54.87

Total Chi^2 = 298.198463 |Chi| = 17.268424 (4 DF) P < 0.0001

Chi^2 for linear trend = 221.098773 |Chi| = -14.86939 (1 DF) P < 0.0001

Remaining Chi^2 (non-linearity) = 77.09969 (3 DF) P < 0.0001

There was a significant association between 5-FU concentration and DNA damage as well as a significant trend of increased proportion of damaged DNA with 5-FU concentration

Apoptosis

Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	8.35	286.5	294.85	2.83	1
Expected	45.621637212869	249.228362787131			
Observed	28.5	262.3	290.8	9.8	2
Expected	44.9949876259193	245.805012374081			
Observed	41.6	245	286.6	14.52	3
Expected	44.3451287950085	242.254871204991			
Observed	59.7	224.1	283.8	21.04	4
Expected	43.9118895744013	239.888110425599			
Observed	83.8	194.6	278.4	30.1	5
Expected	43.0763567918017	235.323643208198			
Total		221.95	1212.5	1434.45	15.47

Total Chi^2 = 95.6411316999601 |Chi| = 9.77962840295888 (4 DF) 0

Chi² for linear trend = 94.5502329547973 |Chi| = 9.72369440875212 (1 DF) 0

There was an association between 5-FU and apoptosis

Astrocyte apoptosis
Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	6.49	291.55	298.04	2.18	1
Expected	41.0446419362888	256.995358063711			
Observed	32	259.42	291.42	10.98	2
Expected	40.1329672294768	251.287032770523			
Observed	47.6	240.8	288.4	16.5	3
Expected	39.7170672876986	248.682932712301			
Observed	50.1	235.8	285.9	17.52	4
Expected	39.3727792564252	246.527220743575			
Observed	62.5	216.5	279	22.4	5
Expected	38.4225442901106	240.577455709889			
Total		198.69	1244.07	1442.76	13.77

Total Chi² = 58.3500436194469 |Chi| = 7.6387200249418 (4 DF) 6.4431793234121E-12

Chi² for linear trend = 54.1027694208648 |Chi| = 7.35545847795124 (1 DF)
1.90292226420752E-13

There was an association between 5-FU and apoptosis

C6 Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	5.46	292.44	297.9	1.83	1
Expected	21.321580826179	276.578419173821			
Observed	12.7	279.62	292.32	4.34	2
Expected	20.9222037835134	271.397796216487			
Observed	18	273.99	291.99	6.16	3
Expected	20.8985847110977	271.091415288902			
Observed	26	264.98	290.98	8.94	4
Expected	20.8262960349163	270.153703965084			
Observed	42.3	244	286.3	14.77	5
Expected	20.4913346442936	265.808665355706			
Total		104.46	1355.03	1459.49	7.16

Total Chi² = 43.0071261551072 |Chi| = 6.55798186602458 (4 DF) 1.03127028028283E-08

Chi² for linear trend = 40.5854695290876 |Chi| = 6.37067261198436 (1 DF)
1.88201010331568E-10

There was an association between 5-FU and apoptosi

Endothelial cell
Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	14.9	278.6	293.5	5.08	1
Expected	142.25377589183	151.24622410817			
Observed	145	129.8	274.8	52.77	2
Expected	133.190247410817	141.609752589183			
Observed	159	119.2	278.2	57.15	3
Expected	134.838161680092	143.361838319908			
Observed	167	104.6	271.6	61.49	4
Expected	131.639269275029	139.960730724971			
Observed	188	84.3	272.3	69.04	5
Expected	131.978545742232	140.321454257768			
Total		673.9	716.5	1390.4	48.47

Total Chi^2 = 296.261682692165 |Chi| = 17.2122538527691 (4 DF) 0

Chi^2 for linear trend = 217.197542563418 |Chi| = 14.7376233688956 (1 DF) 0

There was an association between 5-FU and apoptosis

N2A Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	8.07	288.47	296.54	2.72	1
Expected	117.463583682699	179.076416317301			
Observed	120	170.67	290.67	41.28	2
Expected	115.138395727558	175.531604272442			
Observed	132	153.6	285.6	46.22	3
Expected	113.130098805486	172.469901194514			
Observed	138	140.2	278.2	49.6	4
Expected	110.198856749601	168.001143250399			
Observed	166	107	273	60.81	5
Expected	108.139065034656	164.860934965344			
Total		564.07	859.94	1424.01	39.61

Total Chi^2 = 237.136672883128 |Chi| = 15.3992426074508 (4 DF) 0

Chi^2 for linear trend = 187.775993293925 |Chi| = 13.7031380819842 (1 DF) 0

There was an association between 5-FU and apoptosis

NSC Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	6.39	289.94	296.33	2.16	1
Expected	73.370193138891	222.959806861109			
Observed	56.4	231.6	288	19.58	2
Expected	71.3077164782527	216.692283521747			

Observed	59.8	226.3	286.1	20.9	3
Expected	70.8372836264864	215.262716373514			
Observed	104	180.7	284.7	36.53	4
Expected	70.490648893606	214.209351106394			
Observed	128	149	277	46.21	5
Expected	68.5841578627639	208.415842137236			
Total		354.59	1077.54	1432.13	24.76

Total Chi^2 = 177.27938024311 |Chi| = 13.3146303081651 (4 DF) 0

Chi^2 for linear trend = 169.999796732199 |Chi| = 13.0383970154386 (1 DF) 0

There was an association between 5-FU and apoptosis

3T3 48h Cell Cycle 5FU Chi-square test (2 by k)

G0/G1 vs S+G2/M

	Successes	Failures	Total	Per cent	Score
Observed	239.2	60.79	299.99	79.74	1
Expected	193.374843	106.615157			
Observed	189.8	110.2	300	63.27	2
Expected	193.381289	106.618711			
Observed	185.9	114.1	300	61.97	3
Expected	193.381289	106.618711			
Observed	179.6	120.4	300	59.87	4
Expected	193.381289	106.618711			
Observed	172.4	127.6	300	57.47	5
Expected	193.381289	106.618711			
Total		966.9	533.09	1499.99	64.46

Total Chi^2 = 40.725678 |Chi| = 6.381667 (4 DF) P < 0.0001

Chi^2 for linear trend = 30.093646 |Chi| = -5.485768 (1 DF) P < 0.0001

Remaining Chi^2 (non-linearity) = 10.632032 (3 DF) P = 0.0139

There was a significant association between 5-FU concentration and cell cycle as well as a significant trend with concentration

3T3 Cell Cycle 5FU-24h Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	196.3	103.7	300	65.43	1
Expected	158.16	141.84			
Observed	164.7	135.3	300	54.9	2
Expected	158.16	141.84			

Observed	134.3	165.7	300	44.77	3
Expected	158.16	141.84			
Observed	133.6	166.4	300	44.53	4
Expected	158.16	141.84			
Observed	161.9	138.1	300	53.97	5
Expected	158.16	141.84			
Total		790.8	709.2	1500	52.72

Total Chi^2 = 35.89171 |Chi| = 5.990969 (4 DF) P < 0.0001

Chi^2 for linear trend = 13.346176 |Chi| = -3.653242 (1 DF) P = 0.0003

Remaining Chi^2 (non-linearity) = 22.545534 (3 DF) P < 0.0001

There was a significant association between 5-FU concentration and cell cycle as well as a significant trend with concentration

Astrocytes 24h Cell cycle

Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	253	46	299	84.62	1
Expected	252.444629	46.555371			
Observed	252	48	300	84	2
Expected	253.288926	46.711074			
Observed	244	59	303	80.53	3
Expected	255.821815	47.178185			
Observed	265	34	299	88.63	4
Expected	252.444629	46.555371			
Total		1014	187	1201	84.43

Total Chi^2 = 7.56902 |Chi| = 2.751185 (3 DF) P = 0.0558

Chi^2 for linear trend = 0.824332 |Chi| = 0.907927 (1 DF) P = 0.3639

Remaining Chi² (non-linearity) = 6.744688 (2 DF) P = 0.0343

There was a no association between 5-FU concentration and cell cycle

Astrocytes 48h Cell cycle Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	270.8	30	300.8	90.03	1
Expected	271.876838	28.923162			
Observed	243.5	21.01	264.51	92.06	2
Expected	239.076272	25.433728			
Observed	217.2	38.98	256.18	84.78	3
Expected	231.547235	24.632765			
Observed	239.3	18.37	257.67	92.87	4
Expected	232.893966	24.776034			
Observed	262.1	22.8	284.9	92	5
Expected	257.505689	27.394311			
Total		1232.9	131.16	1364.06	90.38

Total Chi² = 12.826124 |Chi| = 3.581358 (4 DF) P = 0.0122

Chi² for linear trend = 0.71333 |Chi| = 0.844589 (1 DF) P = 0.3983

Remaining Chi² (non-linearity) = 12.112794 (3 DF) P = 0.007

There was a significant association between 5-FU and cell cycle but not with concentration

C6 24h cell cycle Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	225.8	74.24	300.04	75.26	1
Expected	248.906546	51.133454			
Observed	256.1	43.86	299.96	85.38	2
Expected	248.84018	51.11982			
Observed	253.5	46.54	300.04	84.49	3
Expected	248.906546	51.133454			
Observed	256.5	43.46	299.96	85.51	4
Expected	248.84018	51.11982			
Observed	252.5	47.54	300.04	84.16	5
Expected	248.906546	51.133454			
Total		1244.4	255.64	1500.04	82.96

Total Chi^2 = 16.014753 |Chi| = 4.001844 (4 DF) P = 0.003

Chi^2 for linear trend = 6.823786 |Chi| = 2.612238 (1 DF) P = 0.009

Remaining Chi^2 (non-linearity) = 9.190967 (3 DF) P = 0.0269

There was a significant association between 5-FU and cell cycle as well as concentration

C6 48h cell cycle Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	222.5	77.51	300.01	74.16	1
Expected	234.318438	65.691562			
Observed	261.1	38.91	300.01	87.03	2
Expected	234.318438	65.691562			
Observed	249.5	50.49	299.99	83.17	3
Expected	234.302817	65.687183			
Observed	226.6	73.4	300	75.53	4
Expected	234.310628	65.689372			

Observed	211.9	88.15	300.05	70.62	5
Expected	234.349679	65.700321			
Total		1171.6	328.46	1500.06	78.1

Total Chi² = 32.183911 |Chi| = 5.673087 (4 DF) P < 0.0001

Chi² for linear trend = 6.058436 |Chi| = -2.461389 (1 DF) P = 0.0138

Remaining Chi² (non-linearity) = 26.125475 (3 DF) P < 0.0001

There was a significant association between 5-FU and cell cycle as well as concentration

Endo24h cell cycle Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	222.5	77.54	300.04	74.16	1
Expected	248.174048	51.865952			
Observed	228.3	71.73	300.03	76.09	2
Expected	248.165776	51.864224			
Observed	258	42.02	300.02	85.99	3
Expected	248.157505	51.862495			
Observed	224.1	19.01	243.11	92.18	4
Expected	201.085164	42.024836			
Observed	213.9	29.37	243.27	87.93	5
Expected	201.217506	42.052494			
Total		1146.8	239.67	1386.47	82.71

Total Chi^2 = 46.685106 |Chi| = 6.83265 (4 DF) P < 0.0001

Chi^2 for linear trend = 37.111565 |Chi| = 6.091926 (1 DF) P < 0.0001

Remaining Chi^2 (non-linearity) = 9.573541 (3 DF) P = 0.0226

There is a significant association between 5-FU and cell cycle as well as concentration

HNSC 48h Chi-square test (2 by k)

	Successes G0	Failures S G2/M	Total	Per cent	Score
Observed	256.9	43.13	300.03	85.62	1
Expected	135.648659	164.381341			
Observed	156.7	143.3	300	52.23	2
Expected	135.635096	164.364904			
Observed	88.36	211.6	299.96	29.46	3
Expected	135.617011	164.342989			

Observed	83.72	216.3	300.02	27.9	4
Expected	135.644138	164.375862			
Observed	92.5	207.5	300	30.83	5
Expected	135.635096	164.364904			
Total		678.18	821.83	1500.01	45.21

Total Chi^2 = 295.163565 |Chi| = 17.180325 (4 DF) P < 0.0001

Chi^2 for linear trend = 217.198764 |Chi| = -14.737665 (1 DF) P < 0.0001

Remaining Chi^2 (non-linearity) = 77.9648 (3 DF) P < 0.0001

There is a significant association between 5-FU and cell cycle as well as concentration

HNSC Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	305	195	500	61	1
Expected	101.901498	398.098502			
Observed	27.07	329.3	356.37	7.6	2
Expected	72.629274	283.740726			
Observed	30.99	361.7	392.69	7.89	3
Expected	80.031399	312.658601			
Observed	29.01	356.4	385.41	7.53	4
Expected	78.547713	306.862287			
Observed	21.86	374.7	396.56	5.51	5
Expected	80.820116	315.739884			
Total		413.93	1617.1	2031.03	20.38

Total Chi^2 = 675.307365 |Chi| = 25.986677 (4 DF) P < 0.0001

Chi^2 for linear trend = 398.538461 |Chi| = -19.963428 (1 DF) P < 0.0001

Remaining Chi^2 (non-linearity) = 276.768904 (3 DF) P < 0.0001

There is a significant association between 5-FU and cell cycle as well as concentration

N2A 1D Cell cycle Chi-square test (2 by k)

	Successes	Failures	Total	Per cent	Score
Observed	274.8	125.2	400	68.7	1
Expected	286.66	113.34			
Observed	289.2	110.8	400	72.3	2
Expected	286.66	113.34			
Observed	288.5	111.5	400	72.13	3

Expected	286.66	113.34			
Observed	285.1	114.9	400	71.28	4
Expected	286.66	113.34			
Observed	295.7	104.3	400	73.93	5
Expected	286.66	113.34			
Total		1433.3	566.7	2000	71.67

Total Chi^2 = 2.88891 |Chi| = 1.699679 (4 DF) P = 0.5766

Chi^2 for linear trend = 1.749816 |Chi| = 1.322806 (1 DF) P = 0.1859

Remaining Chi^2 (non-linearity) = 1.139094 (3 DF) P = 0.7676

There is no association between 5-FU and cell cycle

Table 4.3: Chi^2 analysis for DNA damage, cell cycle, and apoptosis.