The University of Nottingham

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# THE IMPACT OF 5-FLUROURACIL ON WHITE MATTER TRACTS AND POSSIBLE PROTECTION BY FLUOXETINE OR INDOMETHACIN ADMINISTRATION

# (*IN VIVO* AND *IN VITRO* STUDY)

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**By**

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## Abstract

**Background:** Long term cognitive impairment has been found in significant numbers of patients treated with chemotherapy. Both patient and animal studies have shown that white matter (WMT) integrity is affected by chemotherapy. This indicates that these changes are a possible cause of the deterioration in cognition. However very little is known around the mechanism behind the changes in WMT or the types of cells that are affected. In the CNS, proliferating precursor and stem cells appear to be particularly susceptible to chemotherapy. Reduced proliferation of oligodendrocyte precursors (OPCs), which are required for the maintenance of myelin sheaths, may lead to de-myelination within WMTs. In addition to direct effects on proliferating cells, chemotherapy induced inflammation may be involved in the long term effects of this treatment and prevent re-myelination. These affects could contribute to the cognitive decline experienced by patients after chemotherapy treatment. Recently, work in my laboratory has found that administration of antidepressant or anti-inflammatory drugs is neuroprotective against the cellular and behavioural effects of cytotoxic agents in the hippocampus. Thus, examples of these classes of drug were used to investigate their protective abilities in WMTs.

**Objectives:** The studies in the present thesis used an *in vivo* rat model to evaluate the impact of acute and chronic systemic 5-Fluorouracil (5-FU) treatment on myelin integrity and OPCs proliferation in the optic nerve (ON) and corpus callosum (CC). The project also investigated whether 5-FU induced inflammation or DNA damage in these tissues. Additionally, an *in vitro* system was used to test the direct effects of 5-FU on the proliferation and survival of MO3.13 (OPCs); C6 (astrocytes), BV-2 (microglia) and HBMECs (endothelial) cells. Interactions between OPCs and these other cell types, when treated with chemotherapy, was investigated by co-cultures either when cells were in contact or separated by a porous membrane. Further studies investigated the potential protective effects of the antidepressant fluoxetine (FLX) and the anti-inflammatory drug indomethacin (INDO) when co-administered with 5-FU.

**Methods:** Adult male rats were either acutely (single dose) or chronically (6 doses over 2 weeks) administrated 5-FU. Some groups were co-treated with either FLX or INDO administered via their drinking water prior to and during chemotherapy. Cell proliferation (Ki67), OPCs (NG2), microglia (Iba-1), inflammation (COX-2) and DNA damage (γ-H2AX) were quantified in both ON and CC by immunohistochemistry. Transmission electron microscopy (TEM) and

electrophysiology were used to measuring the density of myelinated axons, myelin thickness and the compound action potential (CAP) 7 days after treatments.

The *in vitro* study, tested cell viability after treatment with 5-FU with or without the presence of FLX or INDO. This was followed by co-cultures of OPCs with different cell types to determine if co-culture would affect OPCs viability during chemotherapy. Conditioned media, co-cultures allowing cell to cell contact and co-cultures in which the cell types were separated by a porous membrane were used. OPC viability after 5-FU exposure was tested under these conditions together with the presence of FLX or INDO. The gap junction (GJ) protein connexin47 was stained for and dye transfer was used to determine if functional GJ were present between cell types. Treatment with the GJ blocker CBX was used to determine the effect of functional GJ on viability. An ELISA was used to detect whether microglia release TNF-α when treated with 5-FU.

**Results:** Rats treated with 5-FU showed a decrease in cell proliferation and the numbers of both OPCs and microglia in both ON and CC. Both single and multiple injections of 5-FU produced a significant increase in the numbers of COX-2 positive cells 24 hours and one week after treatment. DNA damage was found one week after 5-FU injection. TEM showed no change in the density of myelinated axons but a decrease in myelin thickness in the ON and CC. However there is no significant difference in the CAP of ON with 5-FU treatment.

This study has shown that the viability of all cell types was reduced by 5-FU with the most sensitive cells being microglia followed by OPCs. FLX or INDO co-administration before, during and after chemotherapy injections reduced and counteracted the adverse effects of 5-FU treatment *in vivo*. However treatment of OPCs *in vitro* with FLX or INDO was unsuccessful in protecting OPCs. Co-culture of OPCs with either astrocytes or endothelial cells protected OPCs from 5-FU treatment if cell contact was allowed between the different cell types. GJ proteins were detected in all cell types and dye transfer showed that functional GJ existed. Blocking GJ in co-cultures reduced the protection to OPCs from 5-FU. Chemotherapy was shown to activate microglia and increase TNF-α production which in turn inhibited OPCs survival. FLX and INDO treatment inhibited microglial activity after 5-FU treatment.

**Conclusions:** Chemotherapy caused inflammation and DNA damage in WMTs, and reduced cell proliferation and decreased the number of OPCs in ON and CC. These changes were associated with a reduction in myelin thickness. The deleterious effects of chemotherapy were prevented by prior FLX or INDO

treatment. *In vitro* studies showed activation of microglia and release of TNF-α. As inflammation is one of the main effects of chemotherapy on WMTs its reduction by FLX or INDO is likely to have beneficial effects of the cognitive impairment experienced by patients. INDO has been shown to have anti-cancer effects and to synergise with existing cancer treatments and its use may benefit patients being treated with chemotherapy.

**It is a pleasure to dedicate this thesis to:**

# My parents, my wife (Asmaa) and my daughter (Jumana)

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## Abbreviations

| | |
|---|---|
| 5-FU | 5-Fluorouracil |
| 5-HT | Serotonin |
| ANOVA | Analysis of variance |
| BBB | Blood brain barrier |
| BDNF | Brain derived neurotrophic factor |
| cAMP | cyclic adenosine monophosphate |
| CAP | Compound action potential |
| CBX | carbenoxolone |
| CC | corpus callosum |
| CNS | Central nervous system |
| COX | Ciclooxygenase |
| Cx | connexin |
| DABCO | 1,4-diazabicyclo[2.2.2]octane |
| DAPI | 4',6-diamidino-2-phenylindole |
| DG | Dentate Gyrus |
| DNA | Deoxyribonucleic acid |
| FLX | Fluoxetine |
| GJ | gap junction |
| i.p. | intaperitoneal |
| INDO | Indomethacin |
| LPS | Lipopolysaccharide |
| MRI | Magnetic resonance imaging |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| MTX | Methotrexate |
| O-2A/OPCs | Oligodendrocyte-type-2 astrocyte progenitor |
| OCT | Optimal cutting temperature compound |
| ON | Optic nerve |
| OPCs | Oligodendrocyte precursor cells |
| PAP | Peroxidase-Antiperoxidase |
| PBS | Phosphate Buffered Saline |
| PFA | Paraformaldehyde |
| PG | Prostaglandin |
| RECA-1 | Rat endothelial cell antigen-1 |
| ROS | Reactive oxygen species |
| SGZ | Subgranular zone |
| SSRI | Selective serotonin reuptake inhibitor |
| SSRI | Selective serotonin reuptake inhibitor |
| SVZ | Subventricular zone |
| TEM | Transmission electron microscope |
| TNF-α | Tumour necrosis factor |
| VEGF | Vascular endothelial growth factor |

# **CHAPTER ONE:** GENERAL INTRODUCTION

## 1.1 General overview

Epidemiological studies have shown that over two million people suffer from cancer in the UK, and one in every three people will develop a form of cancer at some time in their life. This is predicted to rise by more than 3% a year (Cancer Research UK, 2011). The huge advances in diagnostic approaches and therapeutic options for cancer patients have considerably increased the survival rate of patients suffering from many cancers. Nowadays chemotherapy is considered as one of the essential modalities of cancer treatment both as palliative or as adjuvant therapy, and thus the effects of this treatment can impact on a large number of patients. Chemotherapy, given to treat non CNS cancers, has significant long term effects on the brain. These include prolonged deficits in memory and concentration associated with both a reduction in stem cell proliferation and changes in white matter tracts (WMTs) (Wigmore 2012). Although the focus of this thesis is chemotherapy treatment using 5-Fluorouracil (5-FU) which has been shown to be able to cross the blood-brain barrier and so directly impact brain tissue, the adverse effects of chemotherapy on WMTs are not restricted to this particular drug. In the present thesis a rat model was used to study the effects of 5-FU on cell proliferation and cell survival in two adult white matter tracts, the optic nerve and corpus callosum (CC). The selective serotonin reuptake inhibitor (SSRI) fluoxetine, and the nonsteroidal anti-inflammatory (NSAIDs) drug, indomethacin, were investigated to determine their ability to prevent both the cellular deficits found after chemotherapy treatment.

## 1.2 Chemotherapy

Chemotherapy is the use of cytotoxic drugs to treat different types of cancers. It is used alone or more commonly combined with other treatments such as radiotherapy, biological therapies and hormonal therapy; or after surgical operations. Chemotherapy drugs have significantly increased the survival rates of cancer patients since their introduction over 50 years ago (Walters *et al.* 2011). There are different types of chemotherapy which work in various ways but their main actions are to prevent cell division or kill dividing cells; to target metabolism, enzymes and hormones that are required for cells to grow; to stop the growth of the new blood vessels required for tumours; or by triggering apoptosis in cancer cells (Bhosle and Hall 2009, Mutsaers 2009, Waller *et al.* 2014). As well as killing cancer cells, chemotherapy affects non-cancerous cells and in particular the dividing stem or progenitor cells within different tissues. Within the brain there are a number of different stem cells including neural stem cells which produce new neurons and astrocytes in the process of adult neurogenesis and oligodendrocyte

precursors which are required for the maintanance of the myelin sheaths within white matter tracts. Both of these types of progenitor are thought to be sensitive to chemotherapy (Wigmore, Mustafa et al. 2010; Han et al 2008) and a reduction in their proliferation and survival could cause cognitive impairments (Seigers and Fardell 2011).

The sensitivity of dividing cells to anticancer drugs may depend on the type and period of the treatment. Single injections of chemotherapy lead to a transient reduction in cell proliferation which recovers in a matter of days (Yang, Kim et al. 2011). In contrast, multiple injections lead to a prolonged and progressive decrease in stem cell proliferation (Han, Yang et al. 2008; ElBeltagy, Mustafa et al.2010). This difference may be due to the sensitivity of cells in different parts of the cell cycle to DNA damage and repair. DNA damage may block cell cycle progression at the G1/S transition point in the cell cycle and initiate the expression of the transcription factor p53. Cells may then become quiescent to allow DNA repair or initiate apoptosis (Gewirtz, Holt et al. 2008). Most chemotherapy drugs cause direct damage to DNA or the process of DNA synthesis. This can be by DNA strand breaks, mitochondria dysfunction or by oxidative damage to DNA (Khansari, Shakiba et al. 2009).

### 1.3 The common adverse effects of chemotherapy

Most cancer patients are treated by adjuvant chemotherapy. This treatment is constantly improving which has led to a reduced risk of reoccurrence and an increased survival rate for patients. Although chemotherapy treatment is often effective and kills many types of cancer cell, it also has adverse effects on normal cell types in multiple organ systems (Hodgson 2008). These effects can be seen in parts of the body not directly affected by the cancer as chemotherapy is usually delivered systemically either intravenously or orally, rather than locally to the site of cancer. These side effects can occur within a few days of the administration of chemotherapy or after many weeks or years later (van Dam *et al.* 1998, Rodgers *et al.* 2008). The common adverse effects of chemotherapy are immunosuppression, gastrointestinal disorders, anaemia, fatigue, hair loss, infertility and adverse neurological effects (Keidan *et al.* 1989, Fisher and Yang 2002, Chadha and Shenoi 2003, Berger *et al.* 2010, de Ruiter *et al.* 2012, Morgan *et al.* 2012). Clinically meaningful neurological side effects can occur when systemic chemotherapies are used for non-CNS cancers (Silverman et al. 2007). Cognitive dysfunction is one of the commonest long term adverse effects of systemic chemotherapy among cancer survivors and chemotherapy-induced cognitive impairment has been called chemobrain (Argyriou et al. 2011).

## 1.4 Cognitive deficit

Cognition is a process of mental activity to acquire knowledge through thought, experience and the senses. This process including many activities such as attention, judgment and evaluation, problem-solving and decision making and production of language. One neurological side effect which has attracted increasing interest, is reports by patients of cognitive problems that involve impairments in memory and their inability to concentrate. These effects have been termed 'chemobrain' or post-chemotherapy cognitive impairment (Tannock *et al.* 2004). A number of studies, using animal models have demonstrated that chemotherapy can cause memory deficits in rodents which are associated with a reduction in hippocampal neurogenesis (Mustafa *et al.* 2008, Seigers *et al.* 2010, ElBeltagy *et al.* 2012, Lyons *et al.* 2012). *In vitro* models have shown that chemotherapy agents are especially toxic to certain cells within the CNS including oligodendrocytes and oligodendrocyte precursors (OPCs) (Cho *et al.* 2004, Dietrich *et al.* 2006, Zou *et al.* 2007, Hyrien *et al.* 2010). Relatively few studies have looked at the impact of chemotherapy on white matter tracts despite patient neuroimaging studies which have shown changes in these structures after chemotherapy (Abraham *et al.* 2008, Deprez *et al.* 2011, Koppelmans *et al.* 2014). It has been demonstrated that systemic treatment with chemotherapeutic agents causes a significant increase in apoptosis and a prolonged reduction in cell division in the CC (Soussain, et al. 2009). This investigation confirmed that the susceptibility of cells *in vivo* was similar to that observed *in vitro*. Zhao *et al.* (2005) observed that the total number of proliferating cells were reduced in the CC after exposure to chemotherapy which they attributed to a decrease in progenitor cells such as O-2A/OPCs. Previous studies have reported that the most widely used chemotherapeutic agents associated with acute and delayed CNS toxicities, including cognitive decline, are 5-fluoruracil (5-FU), cyclophosphamide (CYP) and Methotrexate (MTX) (Yang and Moon 2013). This has led to many clinical investigations focusing on the underlying causative mechanisms for this cognitive deterioration.

### 1.4.1 Chemo brain – patient studies

Epidemiological studies have shown that adjuvant chemotherapy can produce neurotoxicity leading to cognitive dysfunction or impairments and psychological distress in diverse types of cancer which have collectively been named chemo-brain or chemo-fog (Anderson-Hanley *et al.* 2003, Wefel *et al.* 2004, Hurria *et al.* 2007, Myers 2012). These post-chemotherapy cognitive deficits may appear directly after the end of the treatment period and can extend for up 10 years

(Ahles *et al.* 2002). The degree of cognitive dysfunction has been found to significantly depend upon the chemotherapeutic dose and treatment period (van Dam *et al.* 1998, Ahles *et al.* 2005, Reid-Arndt *et al.* 2010, Yamada *et al.* 2010). Cancer in other parts of the body may affect the brain and the diagnosis and treatment may cause depression and anxiety. As these effects may cause cognitive decline they must be taken into account when looking for the effects of chemotherapy (Shilling *et al.* 2006). The decline in cognition negatively impacts on patients surviving cancer by reducing their quality of life, career; educational decisions, and treatment regimen (Ahles *et al.* 2002).

### 1.4.2 The incidence of chemobrain in patient studies

Once "chemobrain" was identified as an effect of chemotherapy, it was important to try to determine the proportion of patients' affected. An early study reported that between 5 and 86% of patients suffered from neuropsychological deficits as a result of chemotherapy treatment (Peterson and Popkin 1980). This wide range of patient reports could be attributed to the different chemotherapeutic drugs used, the number of other drugs co-administered with the chemotherapy, generalized toxic effects on the CNS and differences in the way changes in cognition were investigated. Subsequently it was found that 75% of patients suffered from a deterioration in concentration, verbal, visual and visuospatial memories after six months treatment with cyclophosphamide, methotrexate and 5-FU (Wieneke and Dienst 1995). Another study showed that 17% of patients taking a standard dose of chemotherapy still had cognitive impairments two years after the end of treatment; while 32% of patients suffered from the same clinical findings after they had taken a high dose of chemotherapy (van Dam *et al.* 1998).

Longitudinal studies, in which patients are given neuropsychological tests before and after chemotherapy, have found that that up to 20% of patients prior to the start of treatment have cognitive dysfunction (reviewed in Wigmore, 2013), as compared with population norms (Wigmore 2013). The cause of this is unclear and may indicate that the cancer itself can cause cognitive decline. Patients showing cognitive dysfunction before treatment are particularly vulnerable to further decline after chemotherapy. Overall longitudinal studies have found up to 45% of patients suffer from cognitive decline associated with chemotherapy administration (Ahles *et al.* 2010, Hess *et al.* 2010, Deprez *et al.* 2011, Biglia *et al.* 2012, Hedayati *et al.* 2012). MRI studies have found structural and functional changes in brain volume and blood flow in patients after treatment with chemotherapy (Inagaki *et al.* 2007, Koppelmans *et al.* 2012). A longitudinal study by McDonald *et al.* (2010) showed reductions in temporal lobe, frontal cortex and

cerebral grey matter of brain in patients scanned one year after chemotherapy. They, however, found that there was a recovery in most affected areas within four years of treatment.

Chemotherapy also impacts negatively on the white matter tracts (WMTs) of the brain. Using MRI and diffusion tensor imaging has shown increasing brightness in WMTs after treatment with chemotherapy. This indicates that the chemotherapy has caused an increase in the water content of white matter tracts and is interpreted as demyelination and damage to axons (Abraham *et al.* 2008, Akitake *et al.* 2011, de Ruiter *et al.* 2012, Deprez *et al.* 2012). Imaging studies on breast cancer patients have shown that chemotherapy is associated with a focal loss of microstructural WM integrity up to 10 years post-treatment (Koppelmans *et al.* 2014). Additionally standard-dose adjuvant chemotherapy is associated with a decrease in gray matter and an increase in WM volumes in subjects surviving for more two decades post-treatment (Koppelmans *et al.* 2012). In the same group of patients, neuropsychological testing revealed worse cognitive impairments in the domains of verbal memory, processing speed, executive functioning as compared to women without a history of cancer. Chemotherapy can cause long term effects with delayed damage associated with 5-FU, however short term exposure to 5-FU can also cause damage to white matter tracts. This damage can lead to impairments in impulse conduction by neurons; and indeed may contribute to cognitive dysfunction and to patients having a slower processing speed (Abraham *et al.* 2008).

Furthermore, 5-FU treatment can induce demyelination of CNS tracts and alterations in blood flow and metabolism during cognitive tasks (Ferguson *et al.* 2007, Silverman *et al.* 2007, Kesler *et al.* 2009). In many cases there is a post treatment delay in manifesting the damaging effects indicating that these drugs can produce long-term as well as acute effects (Abraham *et al.* 2008, Han *et al.* 2008, de Ruiter *et al.* 2012, Koppelmans *et al.* 2012). Human studies are limited as direct access to the brain is generally not possible and they have often been carried out using small number of patients. In addition, it is difficult to find appropriate controls to compare with patients treated by chemotherapy.

### 1.4.3 Animal models of chemobrain

Treating rodents with chemotherapy produced many of the cognitive impairments described in patient studies. Behavioural studies showed impairment after chemotherapy including spatial learning tasks (Morris water maze); place, object and context recognition tasks, T maze, eye blink conditioning and non-matching

to sample tests (Tannock *et al.* 2004, Wigmore *et al.* 2010, Seigers and Fardell 2011, Winocur *et al.* 2014, Winocur *et al.* 2015). These changes in behaviour have been attributed to deficits in hippocampal and frontal cortex functions and provide information on the neurological basis for cognitive dysfunction after drug administration (Seigers and Fardell 2011). Animal models have also found a reduction in the density of the corpus callosum after treatment with methotrexate (Seigers *et al.* 2009). Animal studies have summarized in the table 1-1

***Table 1:1*** *Summary of in vivo and in vitro studies examined the effects of chemotherapeutic agents on behavioural impairment and neurotoxicity in the CNS. Adapted and modified from (yang and Moon 2013).*

| **Anticancer drug** | **Animal or cell type** | **Cognition &histological and biochemical evaluations** | **Comment** | **Reference** |
|---|---|---|---|---|
| 5-FU | Lister-hooded rat | Spatial working memory impairment (OLR) BDNF, DCX level ↓ | No change of hippocampal proliferation | (Mustafa *et al.* 2008) |
| 5-FU, CYP | Fischer-344 rat (7 or 18 months) | No memory impairment (MWM, Stone 14-unit T-maze); no induced LTP (acute) | Transient memory & LTP ↑ (recovery) | (Lee *et al.* 2006) |
| 5-FU&CYP &MTX | Wistar rat (4 months) | Hippocampal cell proliferation ↓; memory impairment (MWM) | Histone acetylation ↑; histone deacetylase activity ↓ | (Briones and Woods 2011) |
| 5-FU&MTX | BALB/C mouse (2 months) | Memory impairment (MWM, NMTS, delayed NMTS) | No impairment (cued memory, discrimination learning) | (Winocur *et al.* 2006) |
| CIS, CYP, MTX, Vinblastin, thiotepa, Ifosfamide | Primary cortical cells (7 DIV) Wistar rat (7 days) | Dose-dependent cell death Apoptosis ↑; neuronal degeneration–dendritic swelling | Excitotoxic mechanism, caspase-mediated cell death; age-dependent neurotoxicity | (Rzeski *et al.* 2004) |
| CIS, MTX, Taxol | Primary cortical neurons (7, 10, or 14 DIV) | Neuronal degeneration–neurite degeneration; changes in the actin cytoskeleton | Rho signaling pathway | (James *et al.* 2008) |
| CYP | Male ICR mouse (8–10 weeks) | Hippocampal neurogenesis ↓; no induced apoptosis | Cognitive impairment (PA, ORT) | (Yang *et al.* 2010) |
| CYP | Cortical neurons (4 DIV) | Prevention of cell death after serum deprivation | PI3K/Akt&MAPK/ERK activation ↑; Bcl-2&BDNF ↑ | (Kitazawa *et al.* 2010) |
| CYP, DOX | Athymic nude rats (2 months) | Memory impairment (NPR, CFC); hippocampal | Microglia activation (CYP) ↑ | (Christie *et al.* 2012) |

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|---|---|---|---|---|
| | | neurogenesis (DCX) ↓ | | |
| DOX | Primary cortical cells (8 DIV) | Neuronal cell death, cytotoxicity ↑ (under 0.5 µmol/L); no effect (higher concentration) | Different pathway (caspase-dependent/-independent apoptosis) | (Lopes *et al.* 2008) |
| DOX | Wistar rat (2–3 months) | Memory impairment (IA)–acquisition (not consolidation) | Exploratory behavior ↓ | (Liedke *et al.* 2009) |
| Ifosfamide | Mature cortical neurons (12 or 20 DIV) | Cellular acidification | AMPA/kainite receptor activation ↑ | (Chatton *et al.* 2001) |
| MTX | Wistar rat (4 months) | Brain amines concentration ↓; memory impairment (dark-bright arena test, conditioned avoidance task) | No effect on anxiety behavior | (Madhyastha *et al.* 2002) |
| MTX | Primary cortical cells (12 DIV) | Tau phosphorylation ↑; PP2A methylation ↓ | Folate deficiency | (Yoon *et al.* 2007) |
| MTX | C57BL/6 mouse (10–18 weeks) | Memory impairment (CFC) | Ability to gate incoming auditory stimuli ↓; no memory impairment (ORT) | (Gandal *et al.* 2008) |
| MTX | Wistar rat (3 months) | Hippocampal cell proliferation ↓; white matter density ↓; memory impairment (MWM, CFC) | No induced hippocampal cell death | (Seigers *et al.* 2009) |
| MTX | Long Evans rat (10 weeks) | Spatial memory deficit; no induced deficits (ORT) | Serum and CSF folate ↓ | (Li *et al.* 2010) |
| MTX | Wistar rat (3 months) | Hippocampal blood vessel density ↓; Microglia activation | No change of hippocampal cytokine levels | (Seigers *et al.* 2010) |
| MTX | Buffalo rat (Hepatoma 7777 - 3 months) | Hippocampal cell proliferation ↓ | No differences between animals with or without a tumor | (Seigers *et al.* 2010) |
| MTX | C57BL/6 mice (8–9 weeks) Primary hippocampal neurons (0 DIV) | Cognitive impairment (ORT), depressive behavior (TST), neurogenesis ↓ Cytotoxicity ↑; cell viability ↓ | Folate deficiency Caspase-dependent cell death | (Yang *et al.* 2011) |
| MTX | C57BL/6 mouse (8–9 weeks) | Changes in synaptic plasticity-related signals (biphasic-early and late phase ↑); changes in neurotrophic | Related to neuronal survival and plasticity-related cellular remodeling | (Yang *et al.* 2012) |

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|---|---|---|---|---|
| | | factors (late phase ↑) | | |
| MTX | C3H/HeN mouse (FM3A breast cancer cell–6 weeks) | Cognitive impairment (PA); depressive behavior (TST), hippocampal neurogenesis (DCX)↓; proinflammatory enzymes (COX2, iNOS) ↑ | Tumor formation; depressive behavior ↑; no effect on cognition; trend of neurogenesis ↓; trend of proinflammatory enzymes ↑ | (Yang *et al.* 2012) |
| 5-FU, CYP, MTX | Female Spragne-Dawley Rat (12 months old) | Unimpaired NOR<br>Impaired NOL<br>Temporal order memory – affected and improved by inhibitor | Immuno<br>Olig precusors O4 - reduced<br>Myelin basic p – reduced<br>Cox-2 increased<br>TEM- reduced number myelinated cell indicating myelin loss, loss of myelin thickness, reduced by inhibitor. | (Briones and Woods, 2014) |
| 5-FU<br>Oxaliplatin<br>Glucose | Male C57BL/6J RJ mice (younger: 6 week, older: 19.5 month) | Behaviour testing 24 days after drug treatment.<br>Elevated plus maze-no effect<br>Forced swim test-no effect<br>Spontaneous activity-no effect.<br>Morris water maze- no effect<br>Object recognition-hyper interest in novel object with 5FU in young but not old animals. | 75mg/kg 5FU caused sig wt loss<br>BrdU, given at end of behaviour testing (50days post drug) for 1day, put down 3hrs after last inject. Decrease with 5FU in young but not old. High numbers BrdU cells in dentate outside SGZ.<br>BrdU given for 4 days, now non sig decrease in 5FU+glucose compared with controls in SGZ | (Dubois et al., 2014) |
| 5-FU | Male, Swiss-Webster mice | Behavioural changes 24hrs after end of drugs.<br>Comet assay shows DNA damage. | Small non sig wt loss.<br>Comet assay shows DNA damage.<br>No major health effects | (Krynetskiy et al., 2013) |
| ThioTepa | male C57BL/6J mice | Diminished sucrose pref further diminished by stress. Increased cortico steroid response. Chemo may increase depression related effect | Reduced cell proliferation dentate by TT. | (Wilson and Weber, 2013) |
| MTX, 5-FU | Rat | 2wks running; water maze, cued and spatial memory, non-matching to sample | DCX counted in hippocampus. Exercise prevented cognitive Exercise prevented cognitive and | (Winocur et al., 2014) |

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|---|---|---|---|---|
| | | | neurogenic decline of chemotherapy | |
| CYP, MTX, DOX, 5-FU | C57Bl/6J mice | the effect on neurogenesis, blood vessel density, and neuroinflammation was analyzed using immunohistochemistry | The observed decrease in microglia cells is indicative of inflammation that occurred after treatment. | (Seigers et al.,2016) |
| 5-FU, MTX | Rats | Chemotherapy was severely impaired in the hippocampus-sensitive, high-interference memory test. | reduced neurogenesis in the hippocampus | (Winocur et al., 2015) |
| Paclitaxel (PTX) or Oxaliplatin | Male C57BL/6J mice | PTX and OXA cause distinct pathological changes in the periphery and nervous system, which may contribute to chemotherapy-induced neuropathic pain. | PTX and OXA significantly increased circulating CD4+ and CD8+ T-cell populations. increase in the percentage of interleukin-4+ lymphocytes no measurable changes in IBA-1+ microglia/macrophages in the dorsal and ventral horns | (Makker et al., 2017) |

**5-FU**: 5-Fluorouracil; **OLR**: object location recognition; **BDNF**: brain-derived eurotrophic factor; **DCX**: doublecortin; **CYP**: cyclophosphamide; **MWM**: Morris water maze task; **LTP**: long-term potentiation; **MTX**: methotrexate; **NMTS**: non-matching to sample learning test; **CIS**: cisplatin; DIV: days in vitro; **PA**: passive avoidance; **ORT**: object recognition memory test; **DOX**: doxorubicin; PI3K/Akt: phosphoinositide 3-kinase/Akt; **MAPK/ERK**: mitogen-activated protein kinase/extracellular signal-regulated kinase; **NPR**: novel place recognition task; **CFC**: contextual fear conditioning task; **IA**: inhibitory avoidance conditioning; **ROS/RNS**: reactive oxygen species/reactive nitrogen species; **NF-kB**: neclear factor-kappa B; **GSH**: glutathione; **TST**: tail suspension test; **COX2**: cyclooxygenase-2; **iNOS**: inducible nitric oxide synthase.

### 1.5 Mechanisms by which chemotherapy may affect cognition

To cause cognitive changes and memory deficits, most chemotherapy drugs must cross the blood-brain barrier. This is achieved in the case of 5-FU by simple diffusion leading to cerebro-spinal fluid concentrations of approximately 20% of that found in plasma (Kerr *et al.* 1984). Treatment with some anti-cancer drugs including cipsplatin, carmustine and 5-FU leads to an increase in blood-brain barrier permeability. This allows increased concentrations of chemotherapy drugs to enter the brain and increases CNS toxicity and is a particular issue where multiple chemotherapy drugs are given and could explain the toxic effects of low concentrations chemotherapeutic agents when drug combinations have been used (Soussain *et al.* 2009). An exception to the requirement for chemotherapy agents

to access the brain is doxorubicin which appears not to cross the BBB but to produce a peripheral inflammatory reaction which indirectly affects the brain (Zheng *et al.* 2011, Makker *et al.* 2017).

### 1.5.1 Chemotherapy may damage blood vessels within the CNS

A recent review (Nelson *et al.* 2007) suggested that cognitive decline may be due to direct damage to the endothelial lining of blood vessels by chemotherapy agents. High-dose chemotherapy causes cortical stromal blood vessel damage and decreases the growth of capillaries (Meirow *et al.* 2007). In support of this hypothesis, animal studies have found a reduction in blood vessel density and a transient increase in endothelial cell apoptosis within blood vessels (Han *et al.* 2008, Seigers *et al.* 2010). Damage to the blood supply to different regions of the brain could lead to functional changes and cognitive decline.

### 1.5.2 Chemotherapy may cause inflammation within the brain

Chemotherapy drugs are known to produce inflammation in peripheral tissues, particularly gut and liver (Cleeland *et al.* 2003, Janelsins *et al.* 2012, Vyas *et al.* 2014, Al-Asmari *et al.* 2016, Ribeiro *et al.* 2016, Sommer *et al.* 2017). This can lead to elevated levels of peripheral cytokines which may cross the blood brain barrier (BBB) where they may activate microglia within the brain. As noted above, many chemotherapy drugs can pass through the BBB and enter the brain itself and inflammatory markers have been associated with cognitive decline after chemotherapy (Kesler *et al.* 2013, Pomykala *et al.* 2013, Wang *et al.* 2017). Animal studies have found an increase in activated microglia after methotrexate treatment (Seigers *et al.* 2010) while cyclophosphamide, methotrexate, and 5-fluorouracil treatment produced elevations in the inflammatory cytokines COX-2, TNF- α and IL-1ß and a decrease in the anti-inflammatory cytokine, IL-10 (Briones and Woods 2014). High levels of inflammatory cytokines such as TNF-α cause mitochondrial impairment, while oxidative stress induces chronic inflammation and finally neuronal death (Aluise *et al.* 2011, Janelsins *et al.* 2012). Inflammation whether produced by chemotherapy or infection is known to produce cognitive impairments (Briones and Woods 2011, Czerniawski *et al.* 2015). Thus, one of the hypothesis of this study is that neuroinflammation, induced by chemotherapy, leads to damage to WMTs and neurological impairment.

### 1.5.3 Chemotherapy may reduce hippocampal neurogenesis

Many of the cognitive deficits described by patients after chemotherapy involve cognitive domains requiring hippocampal functions and are dependent upon hippocampal neurogenesis (Gandal *et al.* 2008, Han *et al.* 2008, Garthe *et al.*

2009, Wigmore 2013). Animal studies have shown that many chemotherapy agents including 5-FU, cause a decrease in hippocampal neurogenesis as well as cognitive declines (Mustafa *et al.* 2008, ElBeltagy *et al.* 2012, Lyons *et al.* 2012, Winocur *et al.* 2015, Seigers *et al.* 2016). These results have led to the belief that one of the mechanisms behind chemotherapy induced cognitive deterioration, is a reduction in hippocampal neurogenesis. The effects of chemotherapy on neurogenesis could be by direct killing of dividing neural stem cells or indirect by inducing the production of pro-inflammatory cytokines that are also known to affect hippocampal neurogenesis (Wu *et al.* 2012). Moreover, the effects of chemotherapy on neurogenesis are different depending on the length and type of the treatment. It is found that a single injection of methotrexate or cyclophosphamide reduced cell proliferation in the SGZ, but this returned to normal 10 days after treatment (Seigers *et al.* 2008). It seems that acute treatment leads to an abrupt decrease in cell proliferation followed by a recovery to normal proliferation levels after a short period of time (Mondie *et al.* 2010, Yang *et al.* 2010). 5-FU is a metabolic substance which disrupts nucleic acid synthesis or nucleotide synthesis, leading to an arrest in the cell cycle (Perry 2008). 5-FU was selected for use in this study as it is commonly used in breast cancer patients and has been identified as being associated with cognitive impairments posts chemotherapy (Choi *et al.* 2001, Pusztai *et al.* 2004, Menning *et al.* 2017)

### 1.5.4 Chemotherapy may produce oxidative stress

Anticancer drugs such as Anthracyclines produce high levels of free radicals which induce oxidative stress (Conklin 2004, Gaman *et al.* 2016, Tabaczar *et al.* 2017). Free radicals can damage DNA, kill neurons and induce inflammation, all of which may impact on cognition. Many studies have found that chemotherapy can cause cell senescence which is accelerated by the expression of p53 and p21 growth arresting genes which are expressed in response to DNA damage (Panda *et al.* 2008, Dabrowska *et al.* 2009). Increasing production of reactive oxygen species (ROS) and a decline in DNA repair leads to an increase in oxidative stress and initiates cellular quiescence or senescence (Chen *et al.* 2007, Liu *et al.* 2009). This can subsequently alter normal cell metabolism and can lead to the induction of apoptosis and cell death (Dabrowska *et al.* 2009, Dabrowska *et al.* 2011).

### 1.5.5 Genetic factors

Contributing elements include genetic susceptibility, inflammatory responses, stress and more recently, epigenetic induced reduction in cell proliferation (Wigmore 2013). Thus, genetic factors are a well-established risk factor for

cognitive impairment. Some patients react particularly badly to chemotherapy and genetic susceptibility has been associated with cognitive impairment (Janelsins *et al.* 2011). Apolipoproteins (APO) are involved in the formation of lipoproteins and APOE comes in three isoforms (APOE2, 3 and 4). The presence of APOE4 is associated with a number of neurodegenerative diseases and BBB breakdown. The presence of this isoform allows toxic substances to access the brain leading to an increase in the effects of chemotherapy in susceptible individuals (Ahles *et al.* 2003, Bell *et al.* 2012).

### 1.5.6 Changes in neurotransmitters and growth factors by chemotherapy

Many studies have shown that different growth factors such as brain-derived neurotrphic factor (BDNF), vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF1) that are secreted by endothelial cells are important in supporting neural cells (Palmer *et al.* 2000, Guo *et al.* 2008, Lieberwirth *et al.* 2016). Furthermore, differentiation of oligodendrocyte progenitors (OPCs) to mature oligodendrocytes is regulated by a number of growth factors including platelet derived growth factor (PDGF) which induces rapid proliferation and fibroblast growth factor 2 (FGF2) which prevents OPC differentiation (Butt (Butt and Berry 2000) and Berry 2000). Animal studies have shown that treatment with methotrexate reduced levels of dopamine, norepinephrine and serotonin in the mouse hippocampus, a change which was associated with cognitive impairments (Madhyastha *et al.* 2002). In addition, both central and peripheral inflammatory changes and changes in hormonal or growth factor levels have been proposed as possible causes for the changes seen after chemotherapy (Wigmore 2013). Similarly 5-FU has been shown to reduce the levels of BDNF in the hippocampus of treated rats. BDNF is required for many aspects of brain plasticity including neurogenesis and a reduction in the level of this growth factor is likely to cause cognitive impairment (Mustafa *et al.* 2008).

This thesis uses *in vivo* animal and *in vitro* models to study the effects of systemic chemotherapy on white matter integrity. This has received relatively little attention despite the evidence that patients treated with chemotherapy show significant changes to white matter areas of the brain (Inagaki *et al.* 2007, Andryszak *et al.* 2017). The following section will review the literature on the impact of chemotherapy on white matter tracts.

## 1.6 Chemotherapy impacts on white matter tracts

Patient neuroimaging studies, using structural MRI and diffusion tensor imaging, have found changes in white matter tracts after chemotherapy (Abraham *et al.*

2008, Akitake *et al.* 2011, de Ruiter *et al.* 2011, Deprez *et al.* 2011, de Ruiter *et al.* 2012, Deprez *et al.* 2012, Koppelmans *et al.* 2014, Menning *et al.* 2017). Biopsies of the focal lesions shown by MRI after 5-FU chemotherapy, found that these changes consisted of areas of demyelination which had left the axons intact (Israel *et al.* 2000). These results are similar to an earlier study after chronic dosing of dogs with 5-FU (Okeda *et al.* 1984).

*In vitro* studies, using doses of 5-FU equal to the minimum concentration observed in the CSF of patients, have shown that both dividing oligodendrocyte precursors and non-dividing oligodendrocytes are more sensitive to 5-FU than neural stem cells and many types of cancer cell (Han *et al.* 2008). This will mean that clinically relevant doses are likely to impact on the cells within white matter tracts. *In vivo* rodent studies have shown that 5-FU causes an increase in the number of apoptotic cells in the corpus callosum and a reduction in cell division in this tissue. This was associated with reduced myelin staining and evidence of demyelination in corpus callosum (Han et al 2008; Seigers et al 2009; Briones & Wood 2014) and optic nerve (Weng *et al.* 2014).

Chemotherapy could cause demyelination in several ways. It has been suggested that 5-FU may interfere with the control of transcription of the myelin basic protein gene by reducing the expression of the transcription factor TCF7L2 (Weng *et al.* 2014). Other studies have demonstrated that oligodendrocytes and their precursors are particularly sensitive to chemotherapy and are killed or stop dividing after treatment (Han et al. 2008). A third possibility is that the inflammation induced by chemotherapy causes myelin destruction. Treatment of rats with a commonly used combination of methotrexate, cyclophosphamide and 5-FU, (CMF) produced elevations in the brain of the pro inflammatory cytokines IL1-β and TNF-α and increased expression of the prostaglandin producing enzyme COX2 (Briones & Wood 2014). This was associated with reduced myelin thickness and cognitive impairments. Treatment with a COX2 inhibitor reduced inflammatory cytokine expression and the effects of chemotherapy on myelin thickness and cognition. Previous studies have however not found changes in plasma cytokine levels after methotrexate administration but have demonstrated an increase in the numbers of activated microglia in the brain (Seigers et al. 2010).

Investigations of the mouse auditory system, after 5-FU treatment, found reduced conduction between the cochlea and the brain stem and impaired auditory gating (Gandal *et al.* 2008, Han *et al.* 2008). These changes are consistent with the

hypothesis that 5-FU can cause demyelination. Demyelination would reduce the conduction speed of action potentials and alter the behaviour of neuronal circuits (Gutierrez *et al.* 1995).

From the previously discussed literature, the demyelinating effect of chemotherapy has been identified as one of the major contributing factors for the decline in the network speed and one of the probable causes of the cognitive impairments found in patients (Deprez *et al.* 2011). The present project will look at the role of chemotherapy in reducing OPC proliferation which is required to maintain myelin integrity. The major adverse effects of chemotherapy on cells is that cell division can be delayed, differentiation accelerated, an increase in the cell cycle length of progenitor cells or the cells may take on a different fate (Hyrien *et al.* 2010). Further investigation revealed that the systemic treatment with chemotherapy caused a prolonged reduction in cell division in the CC and caused a significant increase in apoptosis (Soussain *et al.* 2009). This study confirmed that the susceptibility of cells *in vivo* was similar to that observed *in vitro*. Furthermore, Fancy *et al.* (2004) observed that the total number of Bromodeoxy uridine (BrdU) positive cells which were reduced in the CC after exposure to the chemotherapy, was due to a reduction in the division of progenitor cells. In this study, in addition to looking at the effects of chemotherapy on OPCs, the extent of inflammation induced by chemotherapy will be quantified both *in vivo* and *in vitro* as well as the role of activated microglia in killing OPCs.

### 1.7 5-Flurouracil (5-FU)

5-FU is a pyrimidine analogue and belongs to the antimetabolite family of chemotherapy drugs which was developed in 1957 as a toxic analogue of the RNA base uracil (Longley *et al.* 2003). Clinically, it has been widely used for over four decades in treating various cancers including colorectal, breast, head and neck, ovarian cancers and oesophageal carcinoma (Adachi *et al.* 1999, Shirasaka and Taguchi 2006, Wang *et al.* 2013). Structurally, 5-FU is similar to the pyrimidine base, Uracil used in the synthesis of RNA but contains a fluorine atom which makes it toxic (Figure 1.1). It has previously been shown that standard doses of 5-FU are highly permeable to the CNS from the blood to the CSF by simple diffusion through the BBB (Bourke *et al.* 1973). This is due to its low molecular weight which enables it to have a direct effect on the brain (Kerr *et al.* 1984, Peterson *et al.* 2008) (Peterson and Popkin, 1980, Hussain et al., 1993). However, 5-FU has a short half-life of 10 to 25 minutes in serum for both humans (Diasio and Harris 1989) and rats (Celio et al. 1983). It has been found that the concentration of 5-FU in the CSF is about 48% relative to the plasma concentration when given as a

bolus dose. Thus, 5-FU is a greater effect on the brain than other cytotoxic drugs (Grochow and Ames, 1998).

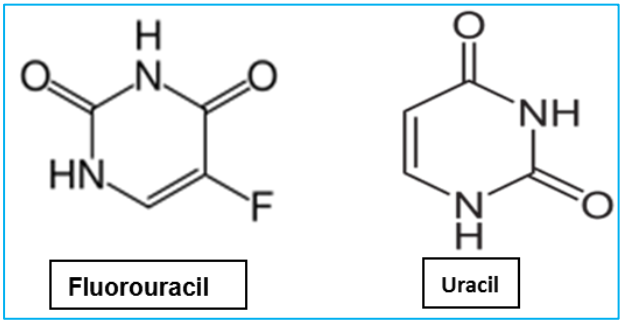



***Figure 1.1*** *Chemical structure of Uracil and 5-FU.*

### 1.7.1 Mechanism of action of 5-FU

The main mechanism of action of 5-FU in the treatment of cancer is by blocking the synthesis of the DNA base thymidine which is required in DNA replication (Longley *et al.* 2003). Intracellularly, 5-FU is converted into one of its active metabolites (fluorodeoxyuridine monophosphate (FdUMP) (Figure 1.2). FdUMP plays a major role by irreversibly inhibiting the enzyme thymidylate synthase (TS) which is required to convert deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) which is necessary for the synthesis of the pyrimidine base of DND 'thymidine' (Longley *et al.* 2003).

In addition, 5-FU is converted into cytotoxic active metabolites, including fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). During the S-phase of the cell cycle, FdUMP binds to TS blocking its dUMP binding site. Whereas, FdUTP can be mis incorporate with dUMP into DNA and RNA. Moreover, the 5,10-methylene tetra-hydropholate ($CH_2THF$) facilitates the binding action of dUMP to TS (Parker and Cheng 1990). This leads to DNA damage (Mader *et al.* 1998) which can cause cell death (Longley *et al.* 2003, Wyatt and Wilson 2009) (Figure 1.2). To maximize the effects on inhibition of thymidylate synthase, Calcium folate (leucovorin) is co-administered with 5-FU to potentiate its cytotoxic effects by increasing the tight binding between FdUMP and the thymidylate synthase enzyme (Murakami *et al.* 1998).

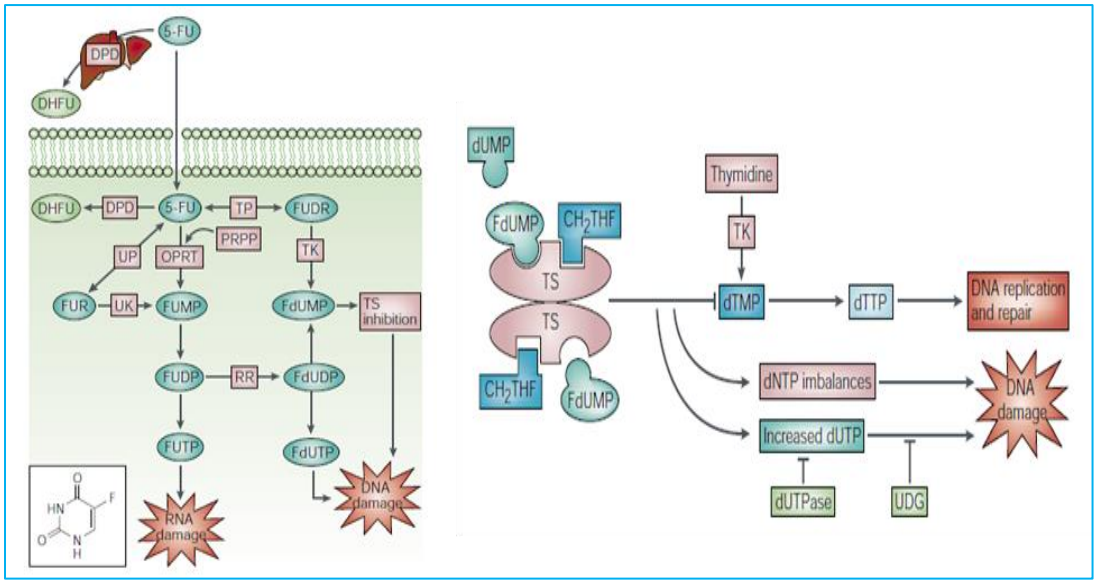



***Figure 1.2*** *Shows 5-FU metabolism and the mechanism of thymidylate synthase inhibition. Under normal conditions deoxyuridine monophosphate (dUMP) is converted to deoxythymidine monophosphate (dTMP) which is essential for the synthesis of the DNA pyrimidine base, thymidine. This is catalysed by thymidine synthetase (TS) and facilitated by the binding of 5,10-methylenetetrahydrofolate (CH2THF) to TS. The major active metabolite of 5-FU, fluorodeoxyuridine monophosphate (FdUMP), competitively binds to TS (also facilitated by the binding of CH2THF) inhibiting the production of dTMP, leading to DNA damage. This disruption also causes a build-up of dUMP. Both dUMP and fluorodeoxyuridine triphosphate (FdUTP) get incorporated into DNA causing lethal damage. The third active metabolite of 5-FU, fluorouridine triphosphate (FUTP), is misincorporated into RNA affecting cellular metabolism and viability. Adapted from (Longley et al. 2003).*

### 1.7.2 5-FU neurotoxicity

As with other chemotherapeutic drugs; 5-FU has many general adverse effects such as myelosuppression, cardiotoxicity, dermatitis, mucositis, nausea and vomiting (Huang *et al.* 2002, Saif *et al.* 2009). Within the CNS a number of adverse side effects such as neurological toxicity, cerebral ataxia (Perry 2008), sub-acute encephalopathy with severe cognitive dysfunction such as confusion, disorientation, lethargy and seizures can occur (Moore *et al.* 1990, Bygrave *et al.* 1998, Wigmore *et al.* 2010). As described previously 5-FU can produce cerebral demyelinating syndrome which is reminiscent of multifocal leukoencephalopathy (Fassas *et al.* 1994, Savarese *et al.* 1996, Choi *et al.* 2001) and a decrease in neurogenesis within the CNS, by negatively affecting the proliferation of neural progenitor and stem cells (ElBeltagy *et al.* 2010, Wigmore *et al.* 2010, Wigmore 2013). 5-FU can also act on the cell membrane to change its structure and alter the transmembrane potential and impair glycoprotein synthesis (Diasio and Harris 1989, Parker and Cheng 1990).

## 1.8 White Matter Tracts: Structure and Cells

White matter accounts for 50% of the volume of the central nervous system (CNS) and is composed of bundles of myelinated and unmyelinated axons which connect different areas of grey matter (Zhang and Sejnowski 2000). Loss of WM integrity is a major factor in many pathological diseases and causes a significant proportion of the functional deficits found (Agartz *et al.* 2001, Goldberg and Ransom 2003). Advances in understanding white matter functions are being helped by increasing knowledge of the major cellular components of its structure. White matter contains myelinated and unmyelinated axons, glial cells, and blood vessels. Glial cell types include astrocytes, microglial and OPCs as well as mature oligodendrocytes all of which are important in the response of white matter to damage and in providing maintenance and regeneration (Nishiyama *et al.* 2002, Goldberg and Ransom 2003).

### 1.8.1 Oligodendroglia

In mammals, oligodendrocytes are derived from Oligodendrocyte Precursor Cells (OPCs) (Young *et al.* 2013). These cells arise from neural stem cells during embryogenesis and the early postnatal stages of life, from restricted periventricular germinal areas (Vallstedt *et al.* 2005). The generation of OPCs is controlled by local signals from axons acting on oligodendrocyte specific transcription factors (Yang *et al.* 2011).

Oligodendrocytes play a specialist role in the formation and maintenance of the myelin sheath of CNS axons (Schoemans *et al.* 2010, Mangas *et al.* 2013). They also have an important function in axogenesis and participate in the development and maintenance of axons. Oligodendrocytes contribute to the regulation of axonal calibre and can also inhibit axonal growth and regeneration (Sherman and Brophy 2005). Typically adult oligodendrocytes each provide support for about 30 separate myelin sheaths on different axons which are connected to the cell body by fragile processes (Butt and Berry 2000). Butt *et al.* (1998) describe the myelin sheath unit which includes the internodal lengths and the number of lamellae of the myelin sheaths. Depending upon the diameter of the fibre and the number of myelin sheaths per unit, oligodendrocytes are subdivided into four types (Bakiri, Karadottir et al. 2011). Morphologically, oligodendrocyte **types I** and **II** are similar (Bakiri *et al.* 2011) as they envelope many small diameter axons and have short intermodal lengths with few lamellae within the myelin sheath. In contrast, **type III** oligodendrocytes, myelinate only a few large diameter axons. Type III cells have abundant lamellae within the myelin sheath and long internodal lengths.

Finally, **type IV** oligodendrocytes are identical to the type III; however the cell body is attached directly to the myelinated large diameter axon. Hildebrand *et al.* (1993) describe how most optic nerve oligodendrocytes are type I or type II. A further cell type which does not produce myelin but regulates the microenvironment around the neurons are called satellite oligodendrocytes (Baumann and Pham-Dinh 2001). In addition, oligodendrocytes can be divided according to the density of their cytoplasm into light, medium and dark oligodendrocytes (Mori and Leblond 1970). It seems that the light cells are more active and divide more. As these cells mature they become darker.

#### 1.8.1.1 Oligodendrocyte progenitor cells (OPCs)

Oligodendrocytes are derived from precursors which have unfortunately been given multiple names including: oligodendrocyte, type 2A astrocyte (O-2A) progenitors; oligodendrocyte progenitors (OPCs); polydendrocytes and NG2 cells (Kato *et al.* 2017). These progenitors are derived from the neural epithelium during development and are highly migratory and proliferative and continually self-renew throughout life (Noble *et al.* 2003). Phenotypically oligodendrocyte precursors are identified by their expression of the neuron-glial antigen 2 (NG2) protein and NG2+ cells give rise to all types of oligodendrocytes (Butt and Berry 2000). NG2 is a chondroitin sulphate proteoglycan which is expressed by progenitor cells both in developing and adult CNS. Differentiation of OPCs to mature oligodendrocytes is regulated by a number of growth factors including platelet derived growth factor (PDGF) which induces rapid proliferation, fibroblast growth factor 2 (FGF2) which prevents OPC differentiation (Butt and Berry 2000). NG2+ cells form around 3-9% of the glial cell population in the adult CNS (Wolswijk and Noble 1989, Dawson 2003). Pringle *et al.* (1992) found that these cells have extensive processes which arborize to form direct contacts with the axolemma at the nodes of Ranvier. After CNS damage, NG2 positive cells migrate within the CNS to the site of injury around demyelinated axons and destroyed myelin sheaths and differentiate into mature oligodendrocytes (Smith *et al.* 1979, Franklin and Ffrench-Constant 2008). This is illustrated in the adult rat in which axons can be remyelinated but with thinner and shorter myelin sheaths (Ffrench-Constant and Raff 1986). Furthermore, many studies have shown that the migration and proliferation stages of OPCs are the initial steps required in the remyelinating process after which these cells differentiate into oligodendrocytes. On the other hand, remyelination is not always successful in cases such as multiple sclerosis due to the failure of OPCs to differentiate and these cells can become quiescent in areas of chronic demyelination (Wolswijk 1998). These cells may

therefore play an important role in the remyelination of the CNS after chemotherapy.

#### 1.8.1.2 Developmental lineage and differentiation of oligodendroglia

Although the cortical neurons of most mammalian species attain their full complement of oligodendrocytes during the first half of gestation, the maturation of most oligodendroglia happens during early postnatal life. 0.5 million oligodendrocyte progenitor cells are found in the brain of the neonatal rat which give rise to about 60 million mature oligodendrocytes (Pfeiffer *et al.* 1993). The germinal region of the sub ventricular zone (SVZ), located deep to ventricular zone, gives rise to the glial lineages (the source of astrocytes and oligodendrocytes) (Parnavelas 1999). In the SVZ of the mammalian brain there is a mosaic of lineage precursors from which the multiple types of cells are derived (Levison and Goldman 1993). The progress in the differentiation of the oligodendroglial lineage can be schematically divided into four stages (Zhang 2001). These stages start with bipolar progenitors (early OPCs), followed by pro-oligodendrocytes (late oligodendrocyte progenitor cells OPCs), immature oligodendrocyte (pre-myelinating) and finally, mature (myelinating) oligodendrocytes. During development, oligodendrocytes at each stage can be identified by their morphological features and specific markers (Lee *et al.* 2000, Buchet and Baron-Van Evercooren 2009).

Cells in the first stage of differentiation are called early OPCs and these are small round bipolar cells (10-15 μm) which can proliferate rapidly and have the potential for migration. They can be identified by expression of the transcription factors Olig1/2 and Sox10 (Figure 1.3). OPCs are the direct ancestors of oligodendrocytes and astrocytes (Dietrich *et al.* 2006). They were originally called oligodendrocyte-type-2 astrocyte progenitor (O-2A) or (O-2A/OPCs) after their discovery in cultures of optic nerve by Raff and his colleagues. The second stage of oligodendroglial differentiation produces promyelinating oligodendrocytes or pro-oligodendroblasts (Pro-OL) which possess multipolar short processes (Deng and Poretz 2003). At this stage, the cells lose expression of PDGFR- α before expressing myelin basic protein (MBP) and at this phenotypic stage the cells are characterized by expression of the transcription factor Sox17 (Sommer and Schachner 1981). This expression persists until the pre-myelinating stage. The pre-myelinating oligodendrocyte is the third progression stage where the cells are also called immature oligodendrocytes and which is a transient developmental stage. At this stage there is a loss of the A2B5 and NG2 markers and these now

post-mitotic cells are characterized by long ramified extensions (Yu *et al.* 1994) and the expression of GalC. The cells stain positive for CNPase, DM20/PLP but not with the monoclonal antibody O1 or for MBP (Bhat *et al.* 1996). Finally, Pre-OLs convert to mature oligodendrocytes when axonal contact occurs and they extend myelin membranes around the axons to form compact enwrapping sheaths (Buchet and Baron-Van Evercooren 2009, Payne *et al.* 2012). At this stage, mature oligodendrocytes have dark cell bodies with numerous "Web like" processes (Figure 1.3) that support the multilamellar, spirally wrapped sheaths of myelin membrane around the axons (Zhao *et al.* 2005). The number of mature myelinating oligodendrocytes depends upon the balance between the proliferative rate of their progenitors and their loss by apoptosis during development or by exposure to chemical or physical agents (Barres *et al.* 1994, Dietrich *et al.* 2006).

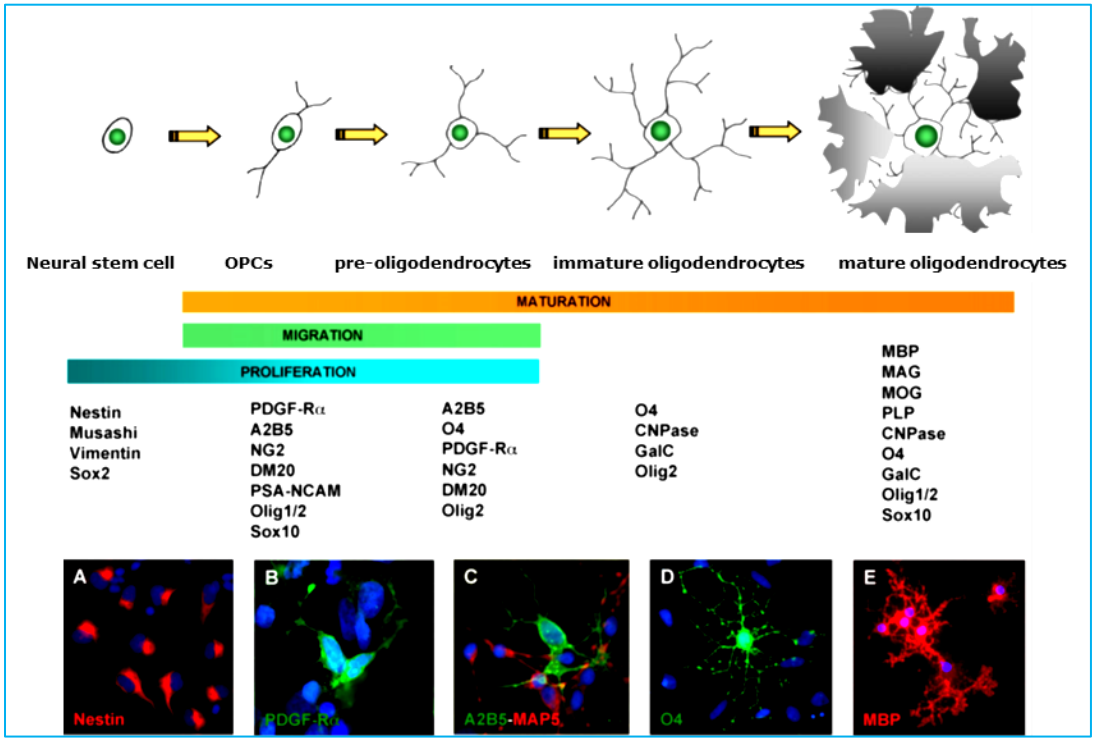



***Figure 1.3*** *shows the oligodendroglial lineage and the specific markers for each stage. Oligodendrocyte progenitor cells (OPCs), pre-oligodendrocytes, immature oligodendrocytes and mature (myelinating) oligodendrocytes (Buchet and Baron-Van Evercooren 2009).*

### 1.8.1.3 Myelin and Myelination

Myelin is a dielectric sheath that wraps around large axons in the central and peripheral nervous systems. It has a vital function in axons by potentiating conduction (Patel and Klein 2011). The commonest proteins in myelin are Myelin Basic Protein (MBP), Proteolipid Protein (PLP), Myelin associated glycoprotein

(MAG) and Myelin oligodendrocyte glycoprotein (MOG) (Butt *et al.* 1998). The axons form a complex junction with the oligodendrocytes by the formation of axoglial adhesions at the paranodal junctions of Ranvier. These contacts assist the action of sodium channels in the axolemma and are concentrated at the nodes of Ranvier (Rasband *et al.* 1999). Myelin is the second factor after the diameter of axon, in increasing conduction velocity. The optimum thickness of myelin is not the same for all axons, and varies with the diameter of nerve fibres (Bakiri *et al.* 2011). The appearance of myelinating cells in the CNS during development helps in the growing of the nervous system and allows salutatory conduction of the nerve impulse (Richardson *et al.* 1997).

Neurons and oligodendrocytes have a reciprocal association in which neurons modulate the proliferation and migration of OPCs by supplying mitogenic cues and trophic factors while oligodendrocytes support the wellbeing of neurons (Nave 2010, Lee *et al.* 2012). Data from previous studies has shown that the diameter of the axon plays an important role in myelin regulation and large diameter axons tend to be myelinated whereas small diameter axons remain unmyelinated (Voyvodic 1989). Myelin sheaths in the CNS are made from plasma membrane extensions of oligodendrocytes which wrap around the axon with a periodicity of about 12 nm for each lamellae while the length of each myelin segment, between two nodes, is about 150-200μm (Butt and Ransom 1989). The axonal membrane is separated from the myelin sheath by the periaxonal space which is a narrow extracellular cleft (Agrawal *et al.* 2009).

On postnatal day 12, in the rat corpus callosum, the average myelinated axon diameter was found to be 0.36 μm; while the thickness of compact myelin around the axon of unfixed optic nerve was about 0.06 μm made up of four to five myelin lamellae (Bjartmar *et al.* 1994). In the healthy adult rodent it has been estimated that about 99% of optic nerve axons are myelinated (Dangata *et al.* 1996, Bartsch *et al.* 1997). As most axons are myelinated we might not expect the generation of new oligodendrocytes to be taking place in the adult optic nerve. However, Young et al. (2013) found that significant numbers of new oligodendrocytes (6.5%) were generated in the adult optic nerve (Young *et al.* 2013). This allows myelination of the 1% of axons which remain unmyelinated at that age and the addition of new nodes to existing myelinated axons.

Myelination happens by interactions between axons and oligodendrocytes (Sherman and Brophy 2005). Axon contact is called **phase I** and occurs after phosphorylation and radial growth of axonal neurofilaments to a critical diameter.

There is induction of presumptive nodes of Ranvier, and oligodendrocytes express CNP and MBP during their migration. **Phase II** is called axon ensheathment, it starts when the processes of oligodendrocytes adhere to the axon. In this phase oligodendrocytes express MAG and PLP and the establishment of nodes occurs between internodal myelin sheaths. The spiralling of the oligodendrocyte plasma membrane around the axon and maturation, is **phase III** of myelination. This phase involves myelin compaction, radial growth of myelinated fibres, redistribution of PLP and MBP; and finally, ion channel aggregation in the axolemma at nodes and juxtaparanodes (Butt *et al.* 1998).

#### 1.8.1.4 Turnover of oligodendroglia in CNS

OPCs form about 5% of the total cells in the adult CNS. These cells have the ability to migrate for long distances within the brain and spinal cord to reach their final destination. During development the migration of OPCs, in the optic nerve, occurs from the optic chiasm to the retina (Sugimoto *et al.* 2001). Ultimately, the number of surviving oligodendrocytes should directly correspond to the number of axons that require myelination (Burne *et al.* 1996). The number of myelinated axons may be change during adult life due to myelination of unmyelinated axons and growth of new axons which may be depend on motor tasks and learning (Bengtsson *et al.* 2005). This suggests that the environment has a strong effect on the rate of OPC turnover, a process which occurs throughout life. For example in the normal human brain; myelination continues into the third decade of life (Fields 2008). Also, it has been reported that 70% of axons in some white matter tracts are unmyelinated in the adult mammal, suggesting that newly formed oligodendrocyte are required to continue to produce myelin in the adulthood to myelinate the previously unmyelinated axons in those regions (Sturrock 1980).

Zhu *et al.* (2011) describe how OPCs present in the adult CNS can divide asymmetrically into a cell which remains as an OPC, and a cell which differentiates into a mature oligodendrocyte. In addition, they describe how OPCs can also divide symmetrically to give two new NG2 expressing OPCs. These two types of division depend on the polarisation and segregation of NG2 on the surface of the cell such that the daughter cell which retains NG2, remains an OPC. Adult OPCs playing a vital role in CNS maintenance by replacing oligodendrocytes which die due to injury, disease or aging (Dawson 2003). Loss of oligodendrocytes and their precursors may lead to a susceptibility to cognitive impairments. In response to myelin damage and destruction of oligodendrocytes, NG2 cells migrate to the site of injury and differentiate to produce new mature oligodendrocytes. Adult OPCs have a slower migration, and differentiation and a longer cell cycle time than

embryonic cells (Psachoulia *et al.* 2009). They also generate thinner and smaller myelin sheath segments.

Recently, it has been determined that the rate of production of new oligodendrocytes depends on their location, age and region in the CNS. For example, the cell division of OPCs in grey matter is slower than in white matter; and it is lower in optic nerve than corpus callosum (Young *et al.* 2013). As nearly all axons are myelinated in the adult optic nerve it has been concluded that OPCs in the optic nerve are engaged in myelin remodelling and maintenance rather than de novo myelination of unmyelinated axons (Fields 2008). In adult fully myelinated white matter tracts, OPCs can intercalate into myelinated axons to generate shorter inter nodes, a feature which has been reported in adult axons when compared with that found in early postnatal life. After damage oligodendrocytes die leaving many axons partly naked, reducing impulse transmission and electrical conductivity. A number of studies have claimed that both oligodendrocyte progenitors and ependymal cells can generate myelinating oligodendrocytes (Dimou *et al.* 2008, Zawadzka *et al.* 2010). However it has been demonstrated that in the injured spinal cord, the majority of new oligodendrocytes are derived from oligodendrocyte progenitors suggesting that they form a specific lineage (Barnabe-Heider *et al.* 2010).

#### 1.8.1.5 The oligo-vascular niche

The term "Oligo-Vascular niche" or "Oligo-Vascular signalling" has been used to describe the association of OPCs and vascular cells which produces conditions in these locations which are favourable for supporting OPCs, sustaining oligodendrocyte renewal and homeostasis in mammalian CNS. It has been found that the number of maturing oligodendrocytes, in different brain regions, is related to blood vessel density after stroke (Jiang *et al.* 2011). OPCs are essential for repairing the loss of myelin after damage but do not work in isolation and receive support and signals from neighbouring cells to proliferate and differentiate into mature oligodendrocytes which can remyelinate white matter tracts after damage (Itoh *et al.* 2015). OPCs located close to endothelial cells in the CC, may communicate with each other in both normal and pathological conditions via secreted soluble factors which participate in the remodelling and repair of white matter (Pham *et al.* 2012).

Many studies have shown that endothelial cells are important in supporting neural cells by the secretion of different growth factors such as brain-derived neurotrphic factor (BDNF), vascular endothelial growth factor (VEGF) and insulin-like growth

factor-1 (IGF1) (Palmer *et al.* 2000, Guo *et al.* 2008, Lieberwirth *et al.* 2016). It is likely that similar interactions occur in supporting OPCs in the white matter as well. Endothelial-oligodendrocyte trophic coupling via secreted soluble factors, is important in maintaining brain functions,especially in white matter (Miyamoto *et al.* 2014). Previous studies have shown that BDNF and fibroblast growth factor (FGF-2) are the main trophic factors released from cerebral endothelial cells and that these promote the proliferation and survival of OPCs (Arai and Lo 2009, Pallast *et al.* 2009). Downregulation of BDNF and FGF production by noncytotoxic levels of oxidative stress, blocks the ability of endothelial cells to support OPCs. In addition to growth factors, it has been found that endothelial-conditioned media could amplify OPC proliferation via activation of the Akt and Src signaling pathways (Arai and Lo 2009) by blocking the cell death pathways after oligodendrocyte damage. The protein kinase Akt has been found to sustain OPC proliferation and also may mediate myelin synthesis in mature oligodendrocytes (Flores *et al.* 2000). In addition, the growth factor VEGF-A, secreted from cerebral endothelial cells, promotes cell-cell signaling between OPCs and stimulates OPC migration by reorganising the actin cytoskeleton in the leading-edge processes of OPCs (Hayakawa *et al.* 2012).

OPCs can in turn support blood-brain barrier (BBB) integrity and endothelial function via OPC-derived trophic factors. The BBB is essential in maintaining the highly specialized microenvironment and homeostasis of the brain, OPCs together with endothelial cells and astrocytes contribute to the formation and maturation of the BBB by trophic coupling (Ronaldson and Davis 2012, Obermeier *et al.* 2013). For example, OPCs are a critical source of trophic factors which help in regulation of the BBB integrity in WMTs of CNS (Miyamoto *et al.* 2014). In a related study, Seo *et al.* (2014) found that OPCs contacting the basal lamina of cerebral endothelial cells release the soluble factor transforming growth factor (TGF-β) which can support BBB integrity. TGF-β increases the expression of tight-junction proteins and the activation of TGB-β-receptor-MEK/ERK signaling pathway leads to a decrease in BBB permeability. TGB-β1 also upregulates cadherin-2 in cerebral endothelial cells which stimulates pericytes to produce the extracellular matrix. This induces astrocytes to secreate Angiopoietin 1 which increases tight junction protein expression and enhance BBB tightness. These findings indicate that OPCs play a crucial role in maintaing BBB function and promoting BBB integrity and maturation (Seo *et al.* 2014). Therefore, a part of the present *in vitro* study (chapter three) was designed to determine the effect of co-culture of OPCs with

endothelial cells to replicate aspects of the oligo-vascular niche and its response to chemotherapy.

### 1.8.2 Astrocytes

Astrocytes are the most common cell type in the brain and are estimated to consist of 20% to 40% of the total number of glia cells which varies in different regions of the CNS (Verkhratsky and Butt 2013). Astrocytes form the subclass of macroglia together with the oligodendrocytes that are derived from stem cell precursors arising from the sub ventricular zones of the developing CNS. Morphologically, astrocytes form two categories. Protoplasmic astrocytes are found in the grey matter and extend their long processes to contact neurones and their end-feet surround blood vessels. The second type of astrocytes are called fibrous astrocytes and are found in WMT in association with the nodes of Ranvier and so are in contact with axons. These, have thinner processes which project further than those found in grey matter (Butt *et al.* 1994). In recent years, astrocytes have been shown to serve many functions beyond the traditional idea that they simply contribute to the structural support of the BBB and the maintenance of the extracellular environment and the stabilization of cell-cell communications in the CNS. As noted above astrocyte foot processes make contact with the brain endothelial cells of capillaries in an arrangement which forms the BBB. In addition, astrocytes are metabolically coupled with neurones, cerebral blood flow and the modulation of excitatory synaptic transmission. As such these cells have a vital role in nutrient, amino acid and ion metabolism in the brain (Maragakis and Rothstein 2006). Several studies have shown that astrocytes act as an energy reserve providing fuel for axons in the absence of glucose. This mechanism occurs by using the glycogen that is stored in astrocytes to support axon conduction when there is a lack of glucose (Ransom and Fern 1997, Wender *et al.* 2000, Brown *et al.* 2003). In white matter, lactate which is a product of glycogen metabolism, is released to be taken up by axons to sustain their energy demands. Lactate released into the extracellular space is converted into pyruvate and oxidised by neurons to support axonal conduction during neural activity (Bouzier-Sore *et al.* 2002, Tekkok *et al.* 2005, Brown and Ransom 2007).

In addition to communication between astrocytes and neurons, astrocytes also release factors which can control myelination. Dreyfus *et al.* (1999) demonstrated that, in addition to the cytokines, astrocyte can be release many trophic factors such as brain-derived neurotrophic factor (BDNF) and NT-3 that support neuronal and other glial survival. The release of these factors depends upon the activation phenotype of the astrocytes. Astrocytes release leukaemia inhibitory factor (LIF)

which promotes the formation of myelin by mature oligodendrocyte as a response to ATP secretion from active axons (Ishibashi *et al.* 2006). More interestingly, in terms of this project, astrocytes help in the survival of oligodendrocytes by secreting Platelet-derived growth factor (PDGF). Fibroblasts produce neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) that stimulate OPCs proliferation, migration and increase oligodendrocyte differentiation from progenitors (Noble and Murray 1984, Moore *et al.* 2011). These factors also promote the myelination of regenerating axons (McTigue et al. 1998) (Figure 1.4).

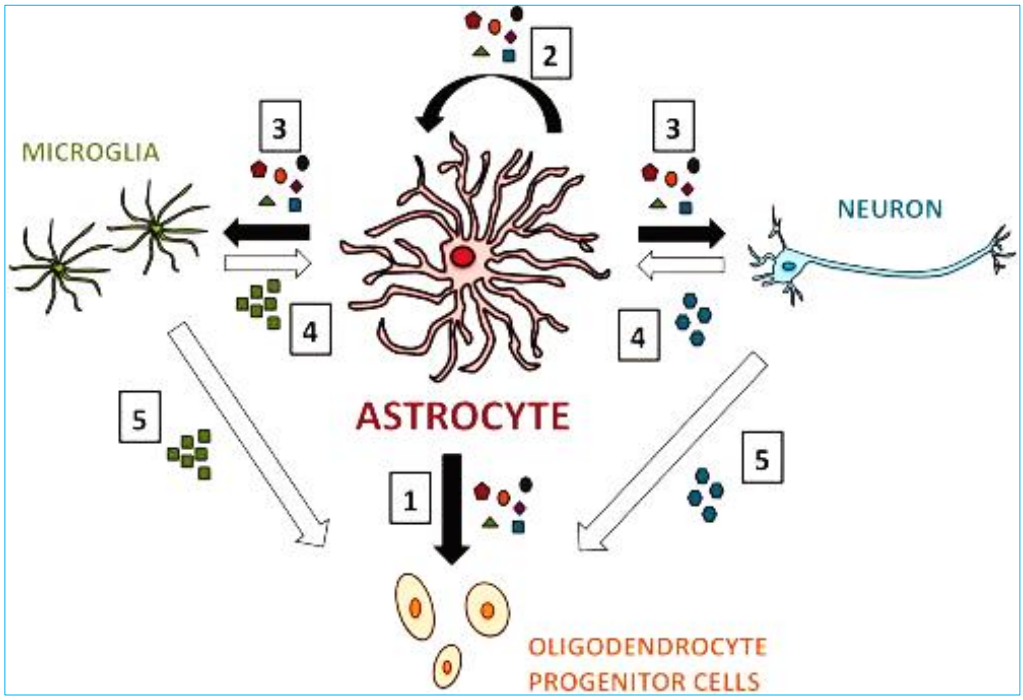



***Figure 1.4*** *The role of trophic factors, secreted from astrocytes, on OPCs and myelin repair. (1) The direct effect of secreted factors from astrocytes on OPCs. (2) Releasing factors that affect astrocytes themselves which promote the further release of factors. (3) Positive or negative stimulating factors from astrocytes on other cell types in the CNS (neurons and microglia). (4) Releasing factors or signals from different types of CNS cells that affect astrocyte action on OPCs. (5) Direct positive or negative actions of factors secreted by neurones and microglia on OPCs (Moore et al. 2011).*

In addition to soluble growth factors, astrocytes contribute to the structural support of the CNS and function as a source of cytokines. Under normal conditions, astrocytes may be involved in regulating the inflammatory response within the CNS and support the cerebral immune defence response (Farina *et al.* 2007). It has been demonstrated that the cytokine ciliary neurotrophic factor (CNTF)

secreted by activated astrocytes, promotes remyelination in active lesions (Albrecht *et al.* 2007, Nash *et al.* 2011). In response to CNTF, reactive astrocytes secrete a soluble factor that promotes OPC survival and increases myelination (Franklin *et al.* 1991). Other studies have found that astrocytes can induce chronic neuroinflammation by the release of tumour necrosis factor α (TNF- α) in response to LPS stimulation. This response also increases expression of glial fibrillary acidic protein (GFAP) and decreases expression of myelin basic protein (Lee *et al.* 1993, Cai *et al.* 2000). Furthermore, in vitro studies have shown that stimulation of γ-interferon (IFNγ) is able to increase the expression of major histocompatibility complex molecules (MHC) class I and II antigens in astrocytes (Wong *et al.* 1984) which may be involved in the immunological response and is able to activate T lymphocytes *in vitro* (Nair *et al.* 2008).

### 1.8.3 Microglia

Microglia, are generally thought to be specialized macrophages. They are the resident immune cells within the brain and play an integral part of the immune defence in the CNS. It is estimated that microglia comprise approximately 20% of the total glial population within the CNS (Kreutzberg 1995) (Kreutzberg 1995), and consist of 5% of all cells within the white matter (Lawson *et al.* 1990). Microglia are broadly classified into two categories, resting (ramified) and activated (non ramified). Under normal conditions in the healthy CNS, resting microglia, with a ramified structure and long processes actively survey of the local microenvironment to detect any changes. Accordingly they act as the first line of immune defence in CNS (Gao and Hong 2008). Activated microglia respond to any changes in the environment of CNS such as infection, inflammation, neurodegeneration or response to injury, acting as pathological sensors within the CNS. Moreover, microglia have the ability to migrate to an injury site, where they proliferate and change to a macrophage morphology. In this state they interact with the immune system by antigen presentation and phagocytoses (Vela *et al.* 2002, Kettenmann and Verkhratsky 2011).

Importantly, activated microglia change their shape by losing their processes and developing an amoeboid shape with enlargement cell body with short cellular processes. In this state, they are able to phagocytose cellular debris or foreign bodies after immunogenic stimuli or injury. This state is called innate activation with a phagocytic phenotype. At the same time, microglia produce chemical factors or cytokines that promote their proliferation, and this state is called adaptive activation or an antigen presenting phenotype (Gehrmann *et al.* 1995, Town *et al.* 2005).

Interestingly, there are two main sub categories of activated microglia that can distinguished by the type of cytokines produced in neuroinflammation (Cherry *et al.* 2014). Classical activation (M1) is characterised by the secretion of inflammatory cytokines such as tumour necrosis factor-α TNFα, IL-6, IL-1β, interferon-γ (IFNγ) (Boche *et al.* 2013) together with reactive oxygen species including cytotoxic factors such as superoxide (O2-), and nitric oxide (NO) (Block *et al.* 2007). M1 has been shown to cause neurotoxicity which can contribute to neuronal damage in neurodegenerative diseases (Figure 1.5). Chemotherapy agents which can cross the BBB, can cause injury to the CNS which activates microglia which may subsequently increase neuronal damage and oligodendrocyte death (Ahles and Saykin 2007, Han *et al.* 2008). Other studies, have shown that activated microglia act as antigen presenting cells during chronic inflammation and up-regulate a myriad of cell surface antigens such as MHC type I and II to initiate an immune response via T-cells and increased expression of inflammatory markers such as cluster of differentiation (CD) CD4, CD11B, CD18 and ectodermal dysplasia (ED) 1 (Schroeter *et al.* 1999, Dimayuga *et al.* 2005, Taylor *et al.* 2005).

On the other hand, activated microglia can take on an anti-inflammatory phenotype which is involved in inhibiting inflammation and restoring homeostasis in the CNS, this state is called Alternative activation (M2) (Gordon 2003). M2 activated cells are potentially key players in providing a neuroprotective effect by the production of cytokines such as IL-4 which have potent anti-inflammatory functions. M2 cells also produce different factors including IGF1, PDGFα and TGFβ which are upregulated in microglia during the recovery phases of disease (Figure 1.5). The production of neurotropic mediators by M2 activated cells has been shown to stimulate myelin repair and to eliminate toxic proteins that prevent remyelination and regeneration as well as preventing neurodegeneration. Expression of cytokines from M2 activated microglial cells delivers trophic support during remyelination and recruits endogenous OPCs to the injury site (Gao and Hong 2008, Olah *et al.* 2012). Therefore activated microglial are thought to exert both protective and damaging effects to the CNS after injury.

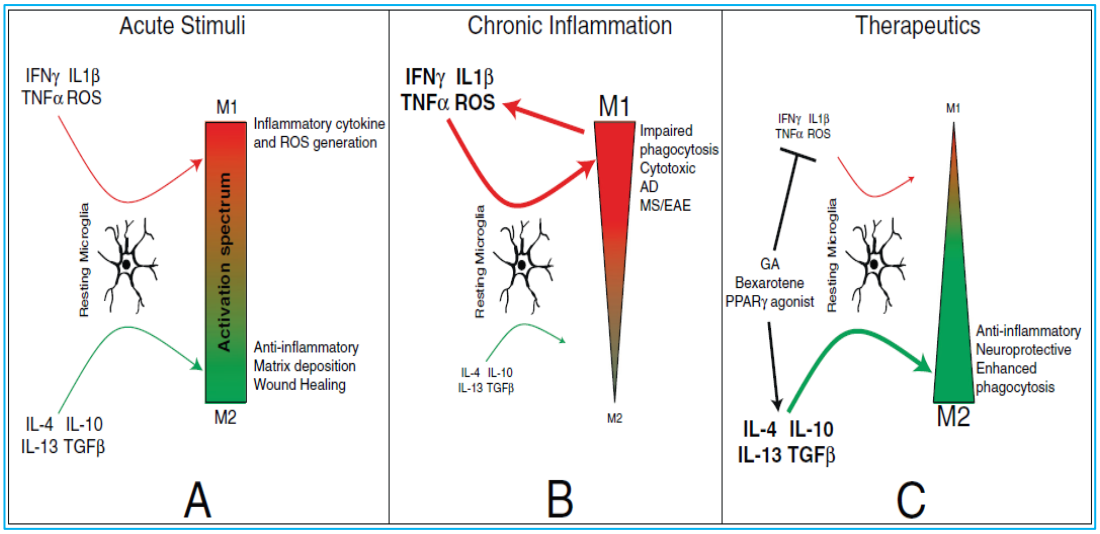



***Figure 1.5*** *The effect of the two main states of activated microglia in response to neuroinflammation. (**A**) Different cytokines can polarise microglia to M1 or M2 states. Stimulus type can shift the microglia towards one or other end of the spectrum. (**B**) The impact of prolonged or chronic inflammation on microglial polarisation towards the M1 phenotype. M1 microglia produce inflammatory cytokines that cause neurotoxicity which contributes to neuronal damage in neurodegenerative diseases. (**C**) Effects anti-inflammatory agents which inhibit neuroinflammation as well as enhancing M2 activation of microglia to act as phagocytes and release anti-inflammatory cytokines. Adapted from Cherry et al. (2014).*

### 1.8.4 Blood vessels and endothelial cells

In addition to glial cells, WMTs also contain blood vessels which are necessary for the delivery of nutrients. Several studies have shown that blood vessels do not just deliver oxygen and glucose to cells, but the endothelial cells that line blood vessels also act to release a variety of mediators that can regulate both local blood flow and signal to glial cells to sustain ongoing angiogenesis/ oligodendrogenesis (Faraci and Heistad 1998, Park *et al.* 2003, Miyamoto *et al.* 2014). The blood-brain barrier (BBB) which consists of endothelial cells and other supportive cells, including pericytes, glial cells and the end-feet of astrocytes plays an important role in maintaining CNS homoeostasis and regulating the microenvironment of neurons (Abbott *et al.* 2006).

Adult angiogenesis is the process of the growth and development of new blood vessels from pre-existing vessels (DeWitt 2005, Madeddu 2005). It is occurs as a response to pathological and physiological conditions such as tumour formation, brain ischemia and during wound healing(Folkman 2007). It is normally controlled by growth factors and inhibitors but it may exhibit uncontrolled growth due to the mechanical or chemical stimulation of pre-existing endothelial cells. Vascular

endothelial growth factor (VEGF) is an endothelial secreted growth factor which induces blood vessels to provide a matrix to support neurogenesis (Mackenzie and Ruhrberg 2012). Angiogenesis proceeds in a set of steps involving the proliferation of activated endothelial cells, their migration and sprouting in situ (Madeddu 2005). Many studies have shown that there is an interdependent relationship between neurogenesis and angiogenesis in the CNS (Park *et al.* 2003, Heine *et al.* 2005). Miyamoto *et al.* (2014) and Palmer *et al.* (2000) have proposed the existence of an 'Angiogenic niche' which provides the inter related conditions for angiogenesis, oligodendrogenesis and neurogenesis. They observed that glial and neural cells often form cell-cell interactions or clusters associated with the branching at the ends of delicate capillaries within the WMT and sub granular zone (SGZ), the neurogenic region of the hippocamapus. Importantly, VEGF-A is one of the growth factors involved in the vascularisation of CNS which also helps in supporting neural progenitors (Louissaint *et al.* 2002, Mackenzie and Ruhrberg 2012). Most cancer cells induce angiogenesis by altering gene expression to produce uncontrolled release of VEGF. For this reason, some anticancer drugs work as anti-angiogenic agents by blocking the VEGF signalling pathway and causing endothelial cell retraction (Nicolson and Custead 1985), an action which can also affect normal cells of the CNS.

### 1.9 Gap junctions (GJ)

Gap junctions (GJ) are specialized intercellular channels connecting the cytoplasm of two adjacent cells. These membrane channels mediate direct cell to cell communication and allow the passage of various small molecular weight solutes, including some metabolites, between the adjacent cells (Kumar and Gilula 1996, Simon and Goodenough 1998, Simon 1999). GJs can allow ions and electrical impulses to directly pass through a regulated gate and coordinate metabolic coupling between cells. These channels are formed by two hemichannels (the connexon) which are themselves composed of hexameric assemblies of proteins called connexions (Cx) (Bruzzone *et al.* 1996, Kumar and Gilula 1996). GJs can consist of few to about $10^5$ channels which allow the diffusion of substances smaller than 1000-1200 Dalton such as glutamate, ATP, ADP, cyclic adenosine monophosphate (cAMP), glucose and glucose derivatives but prevent the movement of proteins or nucleic acids (Balice-Gordon *et al.* 1998, Sohl *et al.* 2005, Retamal *et al.* 2007, Goodenough and Paul 2009).

In the rodent, the connexins protein family consists of 20 members and in the nervous system at least 8 connexins have been identified which are named based on their molecular weight Cx26, Cx29, Cx30, Cx31, Cx32, Cx36, Cx43, Cx45 and

Cx47 (Dermietzel and Spray 1993, Nagy *et al.* 2003, Nagy *et al.* 2004). These Cxs are expressed in most cells including neurons, oligodendrocytes, astrocytes and microglia and more than one type of Cxs can be present in the gap junctions between different cell types. Interestingly, Cx47, is abundant on the somata of oligodendrocytes, and can form junctions with Cx32 found on the surface of astrocytes to form astrocyte/oligodendrocyte gap junctions (Wasseff and Scherer 2011). Axonal loss and structural abnormalities in the myelin sheaths have been found in Cx32 or Cx47 knockout mice indicating a vital role for these connexins in maintaining and enhancing the functions of myelinated fibres and myelination (Menichella *et al.* 2003, Odermatt *et al.* 2003).

With regards to injury and pathological changes in CNS, it is not yet clear whether GJs are neuroprotective or neurodestructive. GJ have been identified as major contributing factors that play an important role in intercellular communication in both neuronal and astroglial cells. This communication via GJs which regulates the passage of small signalling molecules passing between cells has a crucial role in controlling the proliferation and differentiation of these cells during development of the CNS (Worsdorfer *et al.* 2008, Belousov *et al.* 2017). Blocking these channels using the specific GJ blocker, carbenoxolone (CBX), leads to a reduction in both neurogenesis and gliogenesis (Bani-Yaghoub *et al.* 1999). Glutamate can induce cytotoxic effects in neural cells due to depolarization of the cells after activation of ionotrophic receptors which leads to swelling and subsequent apoptosis and necrosis (Formigli *et al.* 2000). Ozog *et al.* (2002) found that co-culture of astrocytes with neurons protected the neurons from glutamate cytotoxicity. Blocking GJ coupling in these cultures with 25μM CBX, lead to increased glutamate cytotoxicity and caused neuronal cell death. This increased susceptibility to glutamate toxicity produced by GJ junction blockers only occurred when GJ formed between neurons and astrocytes, and not between neurons and neurons. The neuroprotective effect of GJs between astrocytes and neurons it thought to be due to the buffering of cytotoxic levels of metabolites and ions like glutamate and $K^+$ in neurons by movement of these substances into the GJ coupled astrocytes (Goldberg *et al.* 1999, Ozog *et al.* 2002).

Endothelial cells also secreted soluble factors that promote the proliferation and differentiation of neural stem cells are also thought to be involved in direct cell-cell interactions mediated by cell surface proteins. These interactions are critical players in maintaining quiescent neural stem cells (Ottone *et al.* 2014). Using green fluorescently tagged Cx43, it was found that GJs interacted with zonula occluden-1 to regulate F-actin filaments during endothelial cell spreading and

migration (Chen *et al.* 2015). It has been shown that GJ coupling allows activity-dependent intercellular trafficking of glucose and its metabolites from blood vessels to neurons via an astroglial network (Rouach *et al.* 2008). Importantly for this project, it has been demonstrated that contact with astrocytes can protect both melanoma and glioma cells from cell death induced by various chemotherapeutic drugs (Lin *et al.* 2010, Chen *et al.* 2015). This protection is thought to be by the removal of toxic levels of intracellular calcium from the melanoma or glioma cells through GJs to the astrocytes. Treatment with GJ blockers significantly reduces the protection provided by astrocytes. Chemotherapy is known to induce apoptosis by causing high cytoplasmic calcium concentrations in cells and reducing this effect prevents apoptosis (Roderick and Cook 2008, Chen *et al.* 2015).

Although, little is known about the role of GJs and Cxs in oligodendrocytes, transgenic mice with Cx genes knocked out show myelination and remyelination defects (Li *et al.* 2014). However the exact pathways by which glial networks contribute to the regulation and development of adult OPCs remains unknown, but high levels of Cx47 are found in proliferating OPCs (**Figure 1.6**) and reduced expression of Cx47 leads to a failure in the maturation of oligodendrocytes, myelin formation and myelin vacuolation (Grinspan *et al.* 1998). The presence of GJs formed from Cx47 allows trafficking of glucose through GJs necessary for the synthesis of fatty acid and myelin lipids from acetyl-CoA. Ablation of Cx47 prevents oligodendrocyte-astrocyte coupling, and decreases oligodendrocyte-oligodendrocyte coupling by 80% (Maglione *et al.* 2010). Apoptosis by astrocytes causes a downregulation of Cx43 and Cx47 induced by a rapid osmotic changes (Li *et al.* 2014); This suggests that, demyelination could be a secondary consequence of astrocyte dysfunction leading to a disruption of Cxs-mediated glial networks. Low expression of Cx47 is found in the CC after demyelination induced by cuprizone (Parenti *et al.* 2010). Cx47 was however expressed in astrocytes located close to the demyelinated areas and its expression in newly formed myelin was associated with complete remyelination after cuprizone administration stopped. From the above results Cx-mediate GJ channels and in particular Cx47, have an important role in the regulation of intercellular calcium, myelination and cytoskeleton remodelling required for recruitment of adult OPCs and their migration to dysmyelinated areas (Scemes *et al.* 2003, Li *et al.* 2014).

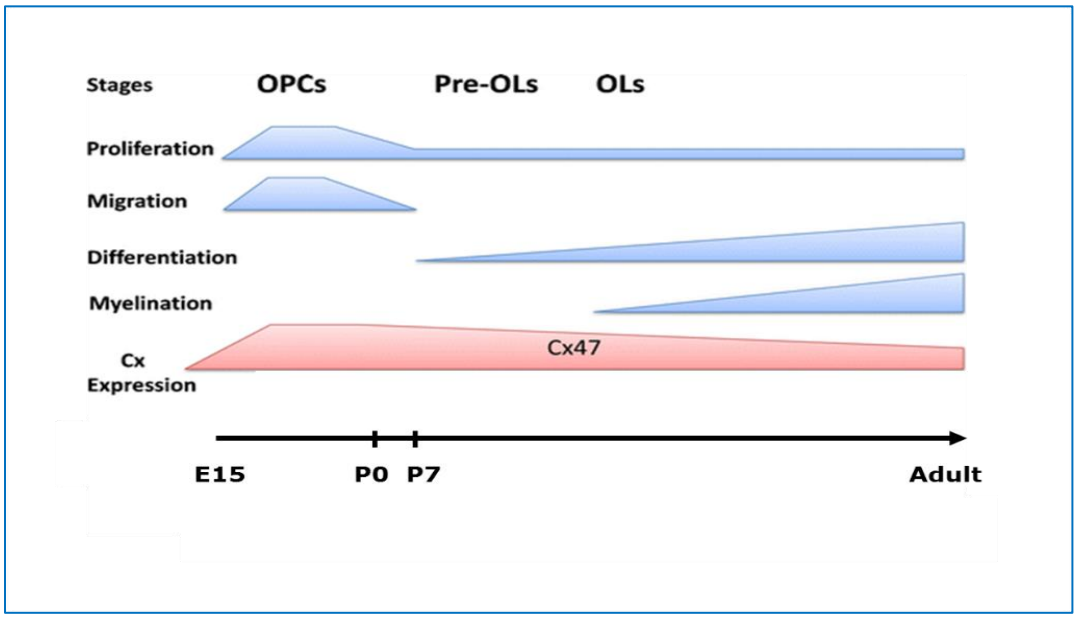



***Figure 1.6*** *Cx47 expression throughout brain developmental stages. (Red signs) Cx47 is detected at early embryonic stages (E15) and its increased expression is correlated with OPCs proliferation and migration (blue signs). Adapted from(Li et al. 2014).*

In contrast to the above literature, many studies have found that GJs can also play a neurodestructive role by allowing the movement of toxic metabolites between connected cells (Ozog *et al.* 2002). Additionally the response of microglia to brain injury or disease can lead to the secretion of pro-inflammatory cytokines such as TNF-α and IL-1β. The release of these cytokines can impair astrocytic GJ coupling leading to increased neuronal injury (Retamal *et al.* 2007). Blocking GJs containing Cx36 by pharmacological or genetic means prevents N-methyl-D-aspartate receptor (NMDAR)-mediated glutamate excitotoxicity after ischemia, traumatic brain injury or epilepsy in animal models and leads to a dramatic reduction in neuronal death (Wang *et al.* 2010, Paschon *et al.* 2012, Wang *et al.* 2012, Fontes *et al.* 2015). Therefore, one of the aims in this thesis is to evaluate the potential protective or damaging effects of GJ on OPCs by co-culturing OPCs with different cell types when treated with 5-FU. This will enable a better understanding of the mechanisms involved in chemotherapy neurotoxicity and whether it is mediated by cell-to- cell contact involving GJ or is indirect involving the secretion of cytokines or other factors.

## 1.10 Optic nerve

The optic nerve was used in experiments in this thesis because it is a fully myelinated white matter tract which lacks neuronal cell bodies. This enables white matter and compound action potentials to be studied without any confounding effects from neurons.

### 1.10.1Anatomy

The optic nerve is the second cranial nerve and consists of the axons of second order retinal ganglion neurons which transmit visual signals from the retina of eye to the lateral geniculate nuclei in the brain. It leaves the orbit through the optic canal and joins the brain after passing through the optic chiasma beyond which it forms the optic tract (Glaser 1999). Conveniently, the optic nerve can be divided into four segments; the optic nerve head which is also subdivided 'according to its relationship with the lamina cribrosa in the scleral canal' into prelaminar, laminar and retrolaminar portions (Figure 1.7). The intraorbital part is the second part of the optic nerve which as an outgrowth of the brain, is surrounded by the meninges. The intracanalicular or intraosseous region is the third part of optic nerve and this part passes through the optic canal. The fourth portion of the optic nerve is the intracranial optic nerve which extends to the optic chiasma (Sadun 1986). The axons of the optic nerve originate from retinal ganglion cells which are located peripherally to the optic disc and project to the margin of the optic nerve head. Provis *et al.* (1985) found that, in the human, about one million axons are present in each optic nerve.

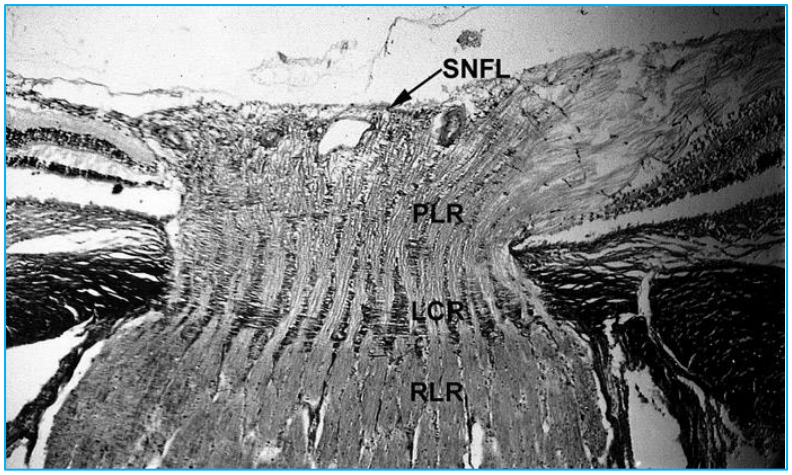



***Figure 1.7*** *A longitudinal section of a normal optic nerve head and the retrolaminar optic nerve. LCR Lamina cribrosa region, PLR Prelaminar region, RLR Retrolaminar region, SNFL Surface nerve fiber layer (Hayreh 2011).*

The nerve fiber layer of the most superficial parts, especially at the disc and prelaminar portion of the optic nerve head is supplied by small capillaries derived from direct branches of the central retinal artery. The prelaminar, laminar and retrolaminar portions are nourished by prepapillary choroidal arterioles from the anastomotic circle of Zinn-Haller. This circle of arterioles is formed via branches of the posterior choroidal arteries (Hayreh 1995). Whereas, the venous drainage of optic nerve mainly flows into the central retinal vein and inconsistent peripapillary optociliary collaterals (Onda *et al.* 1995).

### 1.10.2Histological organization of optic nerve

The optic nerve is composed of axons and glia together with connective tissue which supports and provides nourishment. This supporting tissue is made up of fibroblasts, meningothelial cells and blood capillaries (Goldberg and Hirschi 2009). The anterior part of the optic nerve contains less connective tissue and conversely there is a greater glial presence. While posterior to the lamina cribrosa region, the optic nerve abruptly increases in diameter and the axons are ensheathed with myelin (Magoon and Robb 1981). In this region oligodendrocytes form about 70% of interstitial cells. The presence of oligodendrocytes demonstrates that the optic nerve is similar to the white matter tracts of the CNS rather than the PNS. Astrocytes form the glial framework that covers the surface of the nerve fiber layer and extends over the whole head of optic nerve to form more than 50% of the optic nerve volume. The orbital and intracanalicular parts of the optic nerve have a well-developed septal system (Hayreh 1984). The septa extend from the pia mater and divide the nerve fibers into parallel columns of variable shape and size.

Astrocytes are closely related to the septa and play an important function in the support and nutrition of axons. They also form parts of the blood-brain barrier elements.

The densely stained cytoplasm and short longitudinal protoplasmic processes of oligodendroglia can be seen as angular elements within the nerve fiber bundles. In addition thin branched microglial cells are dispersed between the nerve fibers (Miron *et al.* 2011). These cells participate in the reticuloendothelial system. The optic nerve fibers are arranged in 800-1200 bundles in the retrolaminar and orbital region. The nerve fibers bundles connect with each other by an inter communicating tubular framework, and there is a continual swap of fibers between adjacent bundles to form a network accompanied by glia. This network is supported by astrocytes, oligodendrocytes and some microglial cells which could affect the conduction speed of action potential propagation (Hogan *et al.* 1971).

## 1.11 Corpus Callosum

### 1.11.1Anatomy

The corpus callosum (CC) is a major white matter tract which forms a commissure connecting the two cerebral hemispheres (Figure 1.8). This structure is the largest tract within the brain, forming about 30% of total volume of the CNS (Marner *et al.* 2003). It is mostly composed of bundles of nerve cell processes (axons) and glial cells and contains virtually no neuronal cell bodies. It plays an important role in cognition by connecting the different areas of the cerebral cortex (Fields 2008). The CC is not fixed and shows changes in volume depending upon usage, for example the learning of new motor skills (Scholz *et al.* 2009). White matter dysfunction is associated with a number of pathological conditions including dyslexia (von Plessen *et al.* 2002), Tourette's syndrome (Plessen *et al.* 2004), Down's syndrome (Teipel *et al.* 2003), depression (Lacerda *et al.* 2005), chronic schizophrenia (Agartz *et al.* 2001) and dementia (Malloy *et al.* 2007).

The main function of CC is to integrate the sensory and motor functions of the two sides of the brain (Aboitiz *et al.* 1992). Generally the CC is divided anatomically into three parts, anterior genu or knee, middle body or truncus and posterior splenium. However, while there are no clear anatomically distinct areas of the CC; Witelson's Classification subdivided the CC into five segments. The anterior third including the rostrum, genu, and rostral body which contains fibers of prefrontal, premotor and motor supplementary cortical areas; more caudally, the anterior and posterior midbody including fibers of motor cortex; the posterior third and the

posterior one fifth including isthmus and splenium transmits fibers of temporal, parietal, and occipital cortical regions (Witelson 1989).

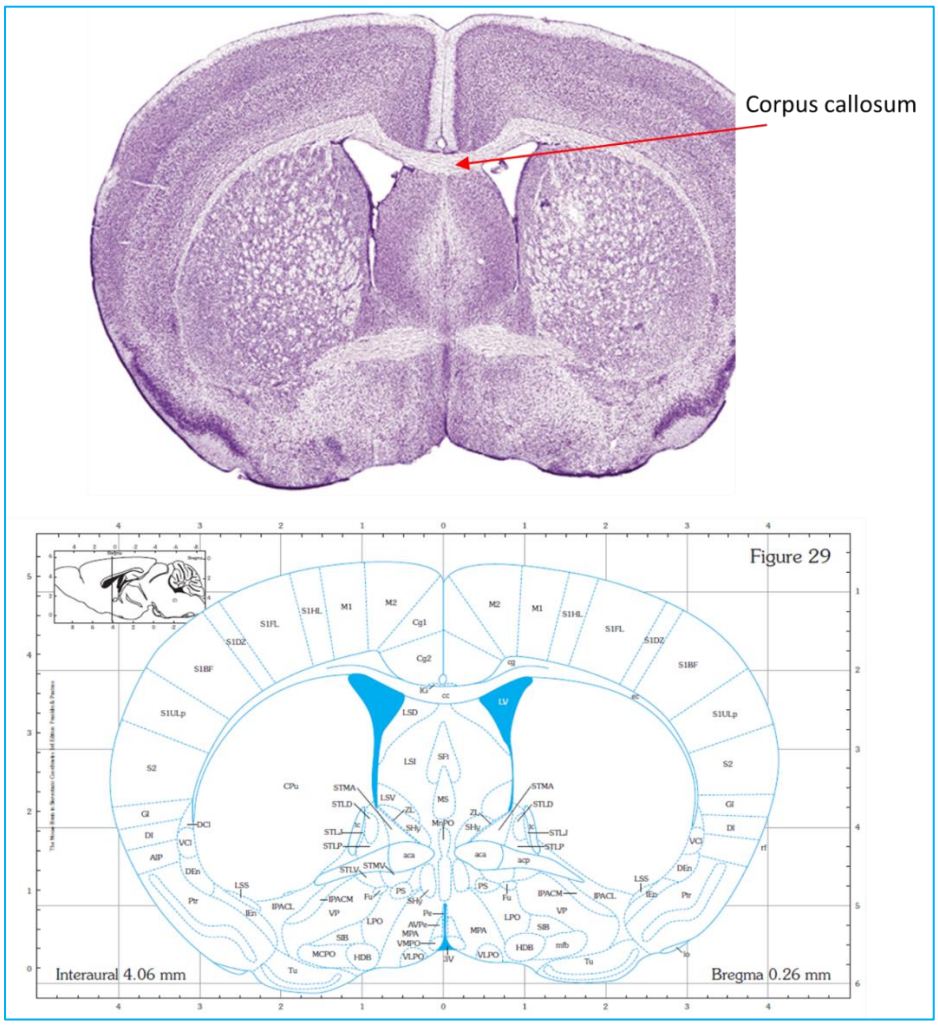



***Figure 1.8*** *A coronal section of the rat forebrain.The corpus callosum (CC) visible at center, in light gray (arrow) connects the right and the left hemispheres. The CC also connects sensory-motor regions and high cognitive performance regions in different parts of the brain. The fibers of the CC radiate and pass to the various parts of the cerebral cortex from superior and lateral sides.*

### 1.11.2Histological study of corpus callosum

As mentioned above, the CC is composed primarily of axons and glial cells while nerve cell bodies constitute less than 1% of its volume. The fibre tracts of CC are not homogeneous and show regional differences in glial cell density, the ratio between myelinated and unmyelinated fibres, the diameter of myelinated fibres and the functions of the brain regions they connect (Mack *et al.* 1995). The anterior and posterior (rostral and caudal in rodents) regions are mostly formed

from dense thin unmyelinated fibres, while the thick myelin fibres which connect the primary and secondary motor area are concentrated in the midbody of CC (Aboitiz 1992, Doron and Gazzaniga 2008). The CC contains different glial cells which show regional differences in density within the CC. For example the density of glial cells in the genu is higher than in the body or splenium (Reyes-Haro *et al.* 2013). The common glial cells in the CNS are oligodendrocytes, which produce the myelin sheath around the neuronal axons and astrocytes that contribute to the blood-brain barrier and microglia which contribute to the innate immune system in the CNS. Finally, researchers have not found any sex differences in the microscopic structure of the CC except that the overall size of the structure varies between males and females (Aboitiz 1992, Aboitiz *et al.* 1992, Olivares *et al.* 2000).

### 1.12 Treatment of chemobrain

To overcome the adverse effect of chemotherapy in causing chemobrain, a considerable number of studies have been published on possible treatments of the cognitive impairments involved. Possible non-pharmacological treatments include physical activity, cognitive behavioural therapies and anti-oxidant supplements in the diet (Fardell *et al.* 2011). While pharmacological treatments such as psychstimulants drugs, erythropoietin, donepezil and Fluoxetine are under investigation for their effects (Fardell *et al.* 2011). In recent years there has been an interest in the effects of antidepressant drugs (especially Fluoxetine) in protection against oxidative stress and in producing improvements in cognition (Nelson *et al.* 2007, ElBeltagy *et al.* 2010, Wigmore 2013). A number of studies have suggested that chemotherapy and radiation-related cognitive impairments may be associated with neuroinflammation and subsequent demyelination. While using anti-inflammatory drugs or cytokines could reverse the neuropathology and neurobehavioral deficits (Montine *et al.* 2002, Monje *et al.* 2003, Briones and Woods 2014).

### 1.13 Impact of antidepressant drugs on CNS

Anti-depressants such as selective serotonin or noradrenalin reuptake inhibitors (NSSRIs) and monoamine oxidase inhibitors are commonly used to treat the pathological condition of depression. These drugs also increase the proliferation of neural progenitor cells in the of the sub granular zone in the dentate gyrus by up to 40%, which enhances hippocampal neurogenesis and cognition (Encinas *et al.* 2006). This increase in cell division is caused by a decrease in the expression of the cyclin-dependent kinase inhibitor P21 which normally restricts cellular

proliferation and maintains cellular quiescence (Pechnick *et al.* 2011). As well as their effect on hippocampal neurogenesis, antidepressants appear to be neuroprotective and cognitive enhancers in a number of pathological situations. Patients suffering from post-traumatic stress disorder (PTSD) showed an increase in global hippocampal volume after 9-12 months treatment with an SSRI (paroxetine) (Vermetten *et al.* 2003). Post-mortem studies of patients who had suffered from major depressive disorder (MDD) and were treated with antidepressants showed a higher levels of cell proliferation in the anterior hippocampus when compared with non-medicated and non-depressed subjects. This study found that SSRI treatment increased both angiogenesis and neurogenesis in the hippocampus. A result which supports the idea that, there is a co-regulation between blood vessel angiogenesis and progenitor cell proliferation and cell survival in the CNS and that antidepressants may have neuroprotective effects (Boldrini *et al.* 2012). This has received support from animal studies which demonstrated that Fluoxetine (FLX) treatment prevented the decline in hippocampal cell proliferation produced by chemotherapy (ElBeltagy *et al.* 2012, Lyons *et al.* 2012). Though the mechanisms behind these effects are not entirely understood, these studies indicate that SSRIs are a possible treatment to prevent the reduced cell proliferation and cognitive decline produced by chemotherapy.

The antidepressant Hyperforin, an extract from St John's wort, acts as a reuptake inhibitor of monoamines including serotonin, norepinephrine and dopamine. *In vitro* study found that Hyperforin promotes the maturation of oligodendrocytes and increases mitochondrial function in both neural progenitors and oligodendrocyte precursor cell lines (Wang *et al.* 2011). Hyperforin also prevented rotenone induced mitochondrial toxicity in these cells. Rats treated with antidepressants for experimental allergic encephalomyelitis had increased survival of oligodendrocytes and reduced demyelination that occurs from this autoimmune disease (Cabrelle *et al.* 2008). It is possible that Hyperforin increases the level of cytoskeleton proteins and the expression of genes involved in energy metabolism, which are key factors in oligodendrocyte differentiation and myelination as well as in neuronal plasticity (Pennington *et al.* 2009). FLX also prevented oligodendrocyte cell death and demyelination after spinal cord injury (Yune *et al.* 2014). The effects of FLX may be region or cell type specific as in vitro treatment of cerebellar derived cells increased neuronal but not astrocyte or oligodendrocyte cell numbers (Zusso *et al.* 2008). Part of the mechanism behind some of these effects may be the anti-inflammatory properties of FLX which inhibits microglial activation (Kao *et al.* 2016, Shan *et al.* 2016).

### 1.14 Fluoxetine (FLX)

Currently, FLX is the most prescribed antidepressant drug. It is a selective serotonin reuptake inhibitor (SSRIs) (Figure 1.9) that is used widely in the treatment of moderate to severe depression (Millan 2006, Waller *et al.* 2014). It is also used to treat psychological disorders such as anxiety, obsessive compulsive disorder, aggression, panic attacks and post-traumatic stress disorder. These conditions are related by common serotonin dysfunctions (Mancini and Ameringen 1996, Wong *et al.* 2005).

The main mechanism of action of FLX involves the regulation of serotonin (5-HT) re-uptake from the synaptic cleft by blocking its uptake at the transporter and preventing its transport back into the presynaptic neuron (Wong *et al.* 2005). Under normal conditions, when a neuron is activated, 5-HT is released from the presynaptic neuron and after diffusing across the synaptic cleft binds to 5-HT receptors on the post synaptic terminal. 5-HT is then removed by reuptake transporters from the synapse. SSRIs antidepressants cause 5-HT accumulation in the synaptic cleft for a longer duration of time by decreasing the 5-HT affinity for the reuptake transporters (Stahl 1998). BDNF gene expression is stimulated by 5-HT binding to its receptors through cyclic adenosine monophosphate (cAMP), protein kinase A (PKA) and cAMP response element binding protein (CREB) pathways (Duman *et al.* 2001). The increases in CREB, in the cell nucleus, leads to increased transcription of BDNF which is associated with increased neurogenesis and neural stem cell survival (Miller *et al.* 2008).

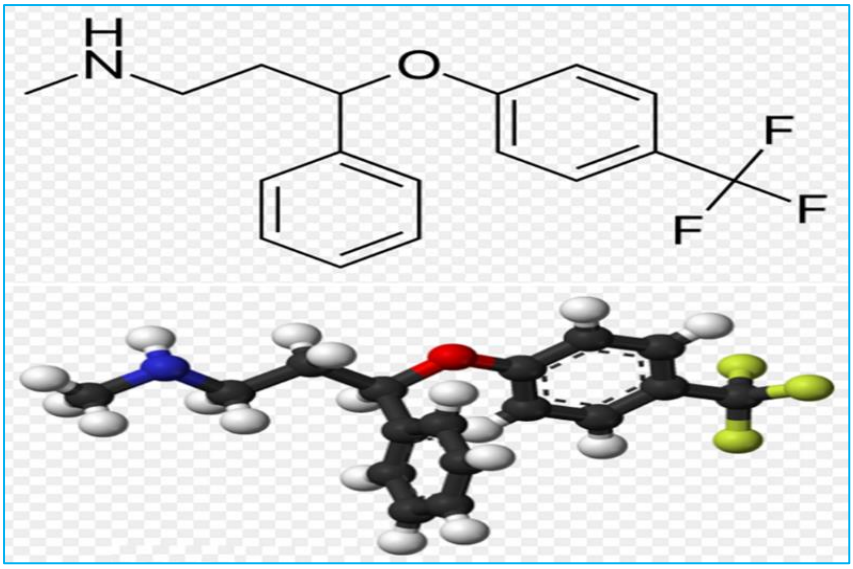



***Figure 1.9*** *Skeletal formula and Ball-and-stick models of SSRI (FLX). Carbon: C (black), Hydrogen: H (grey), Nitrogen: N (blue), Oxygen: O (red), Fluorine: F (green). From (commons.wikimedia.org).*

The therapeutic effect of FLX usually appears after 2-4 weeks of treatment (Black *et al.* 2000). Many studies have shown that FLX can improve cognitive performance especially after memory impairment (Cassano *et al.* 2002, Horsfield *et al.* 2002, Mowla *et al.* 2007). Both clinical and animal studies have shown that FLX can increase hippocampal neurogenesis (Chen *et al.* 2006, Hitoshi *et al.* 2007, Boldrini *et al.* 2012). However the mechanism of this is unknown but may be due to its pro-neurogenic effects through increasing BDNF production and its receptor TrkB and reducing p21 expression (Pechnick *et al.* 2011, Castren and Kojima 2017).

FLX administration increases neurogenesis in the SGZ and improves cognition (Chen *et al.* 2006, Marcussen *et al.* 2008). Work in our lab has shown that co-administration of FLX with chemotherapy prevents the decline in cell proliferation in the hippocampus and the reduced cognition (Mustafa *et al.* 2008, ElBeltagy *et al.* 2012, Lyons *et al.* 2012). Serotonin is one of the several neurotransmitters that influences cognitive function (Seigers and Fardell 2011), and increasing levels of serotonin with FLX could improve cognition.

As mentioned above it is now known that FLX has anti-inflammatory actions, however the mechanism behind this effect is still not fully understood (Caiaffo *et al.* 2016). For example FLX has been shown to decrease inflammation and reduce the effects of kainic acid toxicity and so prevent apoptosis in the brain (Jin *et al.*

2009). Both RNA and protein expression of inflammatory markers in microglial cells including COX-2, TNF-α, IL-1β, IL-6, were significantly reduced after FLX treatment (Chung *et al.* 2011, Liu *et al.* 2011, Zhang *et al.* 2012). It has been suggested that the transcription factor Nf-kB is essential for the creation of pro-inflammatory molecules such as TNF-α, NO and IL-6 and FLX may suppress Nf-kB (Ha *et al.* 2006, Lim *et al.* 2009, Chung *et al.* 2011). FLX may also act on microglia which when activated can secrete various pro-inflammatory cytokines and produce oxidative stress (Chung *et al.* 2010). FLX significantly reduced the lipopolysaccharide (LPS) activation of the BV-2 microglial cell line (Liu *et al.* 2011). FLX also inhibited IkB-a degradation and blocked the phosphorylation of p38 mitogen-activated protein kinase (MAPK) in the LPS-stimulated cells. This prevented the production of TNF-a, IL-6 and NO. In different study, chronic treatment with FLX increased IL-4 and decreased IL-8 and CX3CL1 expression in the hypothalamus (Alboni *et al.* 2013). These results indicate that FLX can influence the inflammatory response of microglia and that this may underlie some of its protective and antidepressant effects.

There is an unambiguous relationship between oligodendrocyte cell death and microglia activation after white matter injury. For example it has been shown that microglia are activated after spinal cord injury and contribute to secondary damage by releasing pro-inflammatory cytokines, reactive oxygen species (ROS) and nitric oxide which result in tissue destruction and the apoptotic cell death of oligodendrocytes and neurons (Popovich *et al.* 1997). Interestingly, FLX has been shown to inhibit the apoptotic cell death of oligodendrocytes and to decrease axonal and myelin losses after spinal cord injury. This was achieved by preventing the activation of microglia and p38 mitogen-activated protein kinase (p38-MAPK) (Lee *et al.* 2015).

Apoptotic processes are controlled by the balance between pro and anti-apoptotic signals (Covington *et al.* 2010). FLX appears to be able to regulate the balance between pro-apoptotic proteins such as BAD and BAX and anti-apoptotic proteins such as Bcl-2 and Bcl-XL (Reus *et al.* 2012, Shan *et al.* 2016). Both Bcl-2 and BAD control the release of cytochrome c which plays a role in the initiation of apoptosis (Li *et al.* 1997). Treatment with FLX decreases the expression of the apoptotic protein caspase-3 and also the number of annexin-positive cells and cells with mitochondrial depolarization. Interestingly FLX can reduce the effects of activated macrophages in promoting cancer (Ghosh *et al.* 2015). Taken together, these results suggest that the therapeutic effects of FLX are partially mediated through its ability to modulate microglial activation and suppress inflammation.

While a number of studies have looked at the beneficial effects of FLX on cognition and hippocampal neurogenesis after 5-FU treatment (Han *et al.* 2008, ElBeltagy *et al.* 2010, Wigmore 2013); there have been few studies on the effects of these drugs on demyelination or cellular damage in the corpus callosum and optic nerve. For this reason, the effects of FLX was studied *in vivo* and *in vitro* for its effects on white matter tracts and cells (Chapter two and three) in combination with 5-FU administration. FLX may offer neuroprotection and may possibly be employed as novel therapeutic agent against the cognitive impairments and other disorders associated with chemotherapeutic treatments. However there are contradictory reports of the interaction between FLX and cancer and its treatments (Juurlink 2016, Bielecka-Wajdman *et al.* 2017, Choi *et al.* 2017) which require further study.

### 1.15 Impact of anti-inflammatory drugs on the CNS

Neuro-inflammation and the expression of cytokines are associated with significant cognitive deficits.(Dik *et al.* 2005, Palta *et al.* 2015). A number of studies have found that TNFα is released in the CNS by microglia and astrocytes (Woodcock and Morganti-Kossmann 2013). This cytokine signals through the TNF receptors 1 or 2 and mitogen-activated protein kinases (MAPKs) and IκB kinase (Medeiros *et al.* 2007). This mechanism leads to the activation of N-methyl-D-aspartate receptors which results in excitotoxicity induced neuronal cell death. The generation of Nitric oxide (NO) via inducible nitric oxide synthase (iNOS), is one of the most intense reactions following TNF-alpha expression which leads to cellular dysfunction and death (Eberhardt *et al.* 1996). Under these conditions, NO reacts with superoxide anions to generate NO-derived reactive nitrogen species such as peroxynitrite which is cytotoxic (Medeiros *et al.* 2007). Increased levels of interleukin-6 (IL-6) lead to a decline in psychomotor performance which is thought to be due to damage to myelin sheaths (Palta *et al.* 2015). Similarly elevated levels of cyclooxygenase 2 (COX2), iNOS and pro-inflammatory cytokines produced by glia cells after an inflammatory stimuli, caused cortical neuronal apoptosis and cognitive impairment (Andreasson *et al.* 2001). COX-2 is normally expressed by microglia and neurons where it is required for synthesises of prostaglandins from arachidonic acid. COX-2 is constitutively expressed at low levels in many brain regions (Hoozemans *et al.* 2001, Sasaki *et al.* 2003). In non-inflammatory situations it may be important for adult neurogenesis as some COX-2 inhibitors reduce neurogenesis possibly by inhibiting microglia (Gonclaves et al 2010). Increased expression of COX-2 after infection or damage leads to a reduction in hippocampal neurogenesis and causes an increase in apoptosis

(Bastos *et al.* 2008, Ahmad *et al.* 2009, Goncalves *et al.* 2010). These changes are associated with behavioural deficits (Andreasson *et al.* 2001).

Anti-inflammatory treatments such as nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the activity of COX-2 which blocks the biosynthesis of prostaglandins (PGs) which are involved in the development of inflammation (Dannhardt and Kiefer 2001). NSAIDs have been used to reduce inflammation in Alzheimer's disease; stroke; cranial irradiation and adult cerebral injury (Netland *et al.* 1998, Montine *et al.* 2002, Monje *et al.* 2003, Sieber *et al.* 2011). In this way NSAIDs may have a protective role and reduce the onset and progression of dementia in patients suffering from mild to moderate Alzheimer's disease (Rogers *et al.* 1993). However, administration of anti-inflammatory agents in non-inflammatory situations had no effect on increasing hippocampal neurogenesis (Boehme *et al.* 2014), but it may have an effect in modulating neurogenesis which has been disrupted by inflammation (Monje *et al.* 2003). This effect is seen in the suppression of inflammation which occurs after irradiation and is similar to the inflammation reducing effects of NSAIDs after endotoxin challenge. In another study, it was found that treatment with NSAIDs, in the animal model, lead to a reduction in hippocampal inflammatory markers and an increase in the secretion of glucocorticoid hormone and PGE-2 and subsequently prevented cognitive dysfunction (Casolini *et al.* 2002). However it is unclear what the effect of anti-inflammatory drugs is on the turnover of cells in white matter tracts. The *in vivo* and *in vitro* experiments in this study were designed to determine if decreasing inflammation using NSAIDs such as indomethacin (INDO) could protect CNS white matter tract cell proliferation after chemotherapy. INDO treatment may prevent some of the long-term behavioural effects of long term diabetes by increasing hippocampal cell proliferation (Ho *et al.* 2015). Only one study has looked at white matter after co-administration of a COX-2 inhibitor with chemotherapy (Briones and Woods 2014). This found that inflammation was reduced and myelin damage and cognitive decline prevented. Therefore, inhibition of microglial activation, using NSAIDs such as INDO, could provide a potential protective and/or therapeutic strategy for the treatment of inflammation-related neurological and myelination disorders after chemotherapy treatments.

The interaction between NSAIDs and cancer therapies is complex. Touhey *et al.* (2002) showed that some anti-inflammatory agents such as INDO and its analogues enhanced the toxicity of different chemotherapeutic agents to cancer cells and so potentiated the effect of treatment in patients treated with anti-mitotic drugs. Studies on cell lines have shown that NSAIDs significantly increase the

cytotoxic effect of vincristine and etoposide on human glioma cells (Benyahia *et al.* 2004). The increased chemosensitization produced by INDO may be due to competition between NSAIDs and chemotherapy agents at the transport sites of the drug efflux proteins involved in multidrug resistance or may be due to inhibition of cyclooxygenase and glutathione-S-transferase enzymes (Draper *et al.* 1997). However these studies looked at the interaction between anti-inflammatory drugs and chemotherapeutic agents in terms of their effect on cancer cells, but have not looked at white matter tracts.

### 1.16 Indomethacin (INDO)

Indomethacin (1-(P-chlorobezoyl)-5-methoxy-2-methyllindole-3-acetic acid) (Figure 1.10), is a common non-steroidal anti-inflammatory drug (NSAID) widely used for its analgesic and anti-inflammatory properties (Waller 1983). It is a non-selective inhibitor of the prostanoid-forming enzymes cyclooxygenase-1/2 (COX-1 and COX-2) and so reduces the synthesis of prostaglandin endoperoxide synthase or cyclooxygenase COX that are involved in the development of inflammation, pain and fever (Vane 1971, Netland *et al.* 1998, Monje *et al.* 2003).

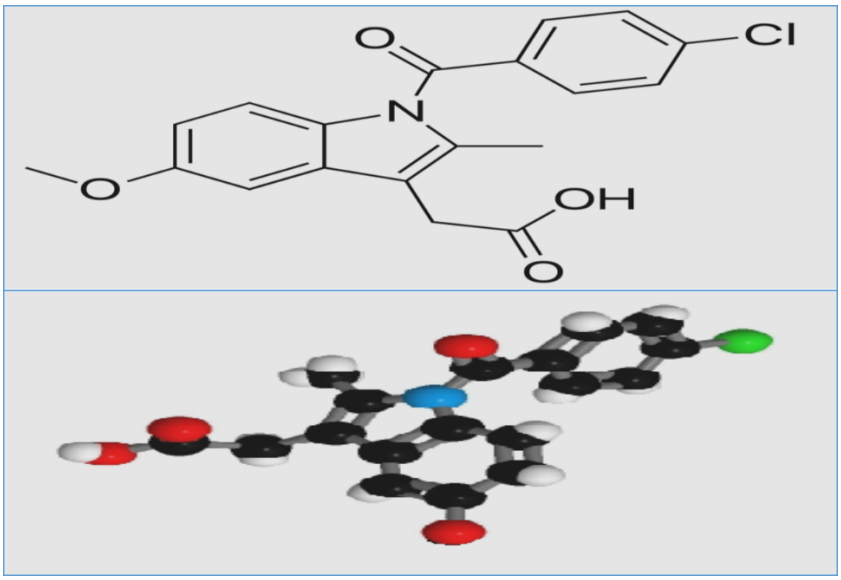



***Figure 1.10*** *Skeletal formula and ball-and-stick models of Indomethacin (INDO). Carbon: C (black), Hydrogen: H (grey), Nitrogen: N (blue), Oxygen: O (red), chloride: Cl (green). From (https://en.wikipedia.org/ and https://www.indigo.com/).*

Generally, COX-1, also called Prostaglandin-endoperoxide synthase-1 (PTGS-1) is expressed constitutively in different cell types and is involved in regulating normal cellular processes including cytoprotection, platelet and kidney function and vascular homeostasis. COX-1 also is expressed at any site of inflammation. COX-

1 is involved in prostaglandin synthesis from arachidonic acid by converting prostaglandin (PG) G2 (PGG2) to prostaglandin (PG) H2 (PGH2) (Figure 1.11) as well as the synthesis of prostacyclin (PGI2) and thromboxane A2 (TXA2) (Smith *et al.* 1996, Vane *et al.* 1998).

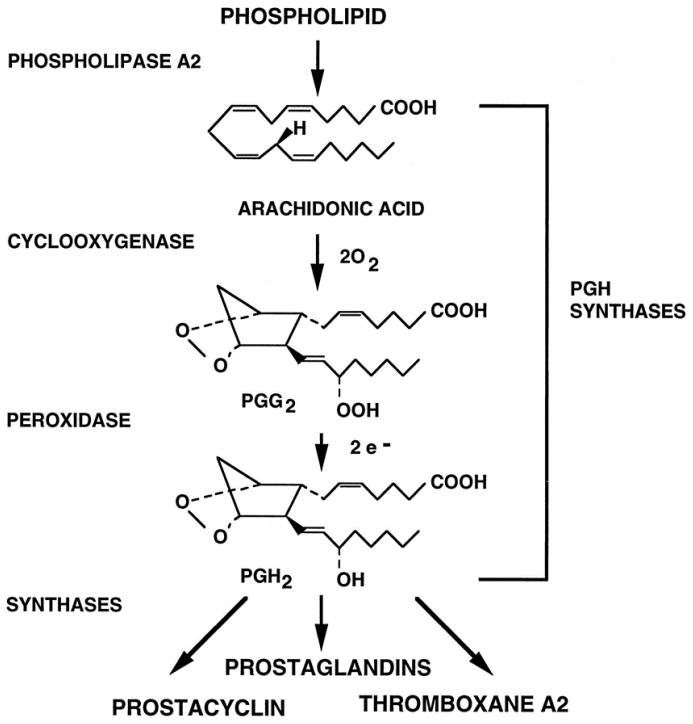



***Figure 1.11*** *Prostaglandins biosynthetic pathway. After stimulation arachidonic acid is hydrolysed from the cell membrane phospholipids by phospholipase $A_2$ activation. The following steps transform Type II phospholipase $A_2$, which is secreted and acts on phospholipids on the extracellular surface of the plasma membrane by cyclooxygenase (COX). Next the unstable PGG2 product is immediately converted into the more stable PGH2 by a peroxidase enzyme reaction, then PGH2 is isomerized to the final biologically active prostanoid products (PGs, thromboxane and prostacyclin)(Smith et al. 1996).*

COX-2 also called Prostaglandin-endoperoxide synthase 2 (PTGS2), is an inducible synthase which can be detected in most normal tissues. However, its expression is increased dramatically when cells are exposed to growth factors, hypoxia, cytokines and lipopolysaccharides (LPS) and activated monocytes and macrophages (Bolten 1998). Upregulation of COX-2 by inflammatory stimuli, such as cytokines can reduce cell proliferation and survival in adult neurogenesis (Bastos *et al.* 2008). Its expression also leads to behavioural deficits and neuronal

loss in aged animals (Andreasson *et al.* 2001). This can be attributed to the ability of COX-2 to generate PGs such as (PG) E2 and PG (D2). These factors are able to induce apoptosis and impairs the survival of newly generated cells derived from neural stem cells after LPS treatment (Bastos *et al.* 2008). Administration of 5-FU increased expression of COX-2 and induced inflammation in peripheral tissues in animal models of chemotherapy (Lopes *et al.* 2009). It seems likely that 5-FU chemotherapy could induce COX-2 expression and inflammation in the CNS either by direct effects on neural tissue or an indirect action by peripheral pro-inflammatory cytokines crossing the BBB and induce inflammatory changes in CNS. For this reason, in the current study, it was decided to assess the effect of chronic 5-FU treatment on the number of cells expressing COX-2 in the WMTs of the CNS.

Previous studies have reported that INDO can penetrate the BBB readily allowing it to have a direct effect on neuroinflammation (Parepally *et al.* 2006). This study also showed that the INDO treatment abolished the cognitive and memory deficits observed in LPS treated animals. However, the role of INDO in other kinds of cognitive impairment and the mechanism of protection have not been elucidated yet. It has been reported that INDO decreased the levels of pro-inflammatory cytokines secreted by activated microglia but did not change the number of proliferating cells or immature neurones in the hippocampus (Ji *et al.* 2013). Similarly in a mouse model of intracerebral haemorrhage which reduced hippocampal neurogenesis and spatial memory, INDO treatment improved spatial memory, reduced microglia activation and enhanced hippocampal neurogenesis (Yang *et al.* 2015). In an organotypic study using hippocampal slices, INDO reduced the expression of the pro-inflammatory cytokines IL-1B and TNF-a and inhibited glial activation and iNOS expression after exposure to oxygen-glucose deprivation (Bernardi *et al.* 2010).

Interestingly, combining INDO with adjuvant chemotherapy reduced tumour progression and improved outcome and quality of life (Lundholm *et al.* 1994, Lonnroth *et al.* 2014). This appears to be due to the upregulation of the CDKN2A/p16 tumour suppressor gene by INDO. INDO has also been reported to increases the stem cell master regulator SOX2 and the tumour suppressor miR-630 and reduce the aggressiveness of colorectal tumours (Lonnroth *et al.* 2014). It has also been demonstrated that pre-treatment with INDO, when given in cases of neuronal dysfunction within the hippocampus prevented both the increase of TNF-α and tumour necrosis factor receptor 1 (TNFR1) protein in activated microglia (Goncalves *et al.* 2010). Also it has been shown that INDO is able to

prevent Methamphetamine (METH)-induced neuroinflammation in the mouse hippocampus by downregulation of GFAP which prevents astrogliosis (Goncalves *et al.* 2010). Correspondingly INDO prevented the decrease in Tuj-1 protein levels which is a specific immature neuron marker and also considered a general marker of neurons. Indeed, the results in this study concluded that anti-inflammatory administration prevents activation of astrocytes and microglia in the mouse hippocampus by modulating TNF-α levels and consequently contributes to the homeostatic activity-dependent organising of synaptic connectivity (Goncalves *et al.* 2010). Furthermore, in recent study, Lopes *et al.* (2016) found that inflammation within the striatum or sub ventricular zone (SVZ) in adult rats, produced by microglia/macrophage activation, was associated with focal ischaemia and lead to neuronal loss. It has been shown that treatment with INDO reduced microglia activation and increased the number of neuroblasts in both the SVZ and striatum. Also INDO was able to regulate and improve the migration of neuroblasts to the ischaemic striatum two weeks after stroke (Lopes *et al.* 2016). Moreover, it was found peripheral administration of INDO induced a 3-fold upregulation of VEGF and a reduction in the expression of COX-2 in the dentate gyrus, CA1 and CA3 layers of the hippocamus (Sathyanesan *et al.* 2015).

On the basis of the literature reviewed, in this thesis, INDO was chosen as an anti-inflammatory drug to test for its ability to protect WMTs from the effects of chemotherapy.

### 1.17 Aims and objectives

The studies presented in this thesis were carried out to investigate the acute and chronic effects of chemotherapy on adult white matter tracts (WMTs). It was hypothesised that treatment with the chemotherapy drug 5-FU would decrease the numbers of oligodendrocyte precursor cells (OPCs) and their proliferation which would lead to a disruption of the structural integrity of myelinated sheaths in both optic nerve and corpus callosum. These changes may contribute to the cognitive impairments that are found in patients and animal models after chemotherapy treatment. To further examine the effects of chemotherapy on WMT, compound action potentials and transmission electron microscopy of optic nerve were carried out. To treat or prevent chemotherapy induced WMT damage, the antidepressant Fluoxetine (FLX) or the anti-inflammatory drug Indomethacin (INDO) were co-administrated with 5-FU.

To further investigate the effects of 5-FU, FLX and INDO on the different cell types found in WMTs, these drugs were tested on individual cell types and in co-cultures with OPCs to determine the relative sensitivity of each cell type and whether co-culture was protective or harmful to OPCs.

**Specific aims:**

1. To use a rat model to investigate the toxic effect of 5-FU on OPCs and microglial cells in ON and CC. This was investigated using immunohistochemical markers for cell division (Ki67), OPCs (NG2) and microglia (Iba-1) and the detection of DNA damage (pH2AX).
2. To measure the CAP of optic nerves and to use transmission electron microscopy of optic nerve and corpus callosum to determine damage to the myelin sheath and its effect on conduction under different drug regimens.
3. To examine the ability of 5-FU to induce inflammation in WMTs as determined by COX-2 expression.
4. Create an *in vitro* model to observe the direct effects of 5-FU on the viability and proliferation of different cell types found within WMTs.
5. Establish co-cultures of OPCs with other cell types and investigate their response to 5-FU when in contact or separated by a porous membrane.
6. Examine the inflammatory response that may be induced by treating microglial with 5-FU and a possible cytokine release that could affect oligogenesis and OPCs survival.
7. To determine the effect of antidepressant (FLX) and anti-inflammatory (INDO) treatments with or without 5-FU on progenitor cells, neuro-inflammation and DNA damage in adult WMTs.
8. To determine whether FLX or INDO could prevent changes in the ultra-structure of WMTs.

# **CHAPTER TWO:** THE NEUROPROTECTIVE EFFECTS OF FLUOXETINE AND INDOMETHACIN ON ADULT RAT OPTIC NERVE AND CORPUS CALLOSUM AFTER ACUTE AND CHRONIC 5-FLUOROURACIL TREATMENT

## 2.1 Introduction

The changes in brain structure and function which are found after chemotherapy treatment are likely to depend on a number of different mechanisms. These mechanisms appear to include a reduction in stem cell proliferation. This may occur both to neural stem cells in the hippocampal neurogenic region and to oligodendrocyte precursors found in white matter tracts. In the latter case this could lead to demyelination and changes in conduction within these tracts. In addition, both central and peripheral inflammatory changes and changes in hormonal or growth factor levels have been proposed as possible causes for the changes seen after chemotherapy (Wigmore 2013). Frequently, chemotherapy is well known to have a number of side effects that may depend on the type of drug, the route of administration and the duration of treatment. The side effects can occur after a few hours or days from administration or chronically after many weeks or years (Rodgers *et al.* 2008). Cognitive dysfunction is a common adverse effect of chemotherapy which significantly depends upon the chemotherapeutic dose and the treatment period (van Dam *et al.* 1998, Ahles *et al.* 2005, Reid-Arndt *et al.* 2010, Yamada *et al.* 2010). Although chemotherapy kills cancer cells, it also has adverse effects on normal cell types in multiple organ systems (Hodgson 2008). This is because most chemotherapies are delivered systemically either intravenously or orally, rather than locally to the site of the cancer. A significant number of studies have investigated the effects of chemotherapy on cognition in both human patients and animal models. However cognitive impairment has been found in most studies, but the mechanisms behind this effect are still unclear.

### 2.1.1 Effect of chemotherapy on cell proliferation:

Many studies show that chemotherapy agents are designed to act as cytostatic or cytotoxic drugs. This action occurs by blocking the proliferation of dividing cells in tumours but also non-cancerous stem and progenitor cells. Chemotherapy induces the expression of the transcription factor p53 which in turn may lead to apoptosis or expression of the cyclin-dependent kinase inhibitor p21, which can lead to cell cycle arrest at the G1/S point in cell division (Gewirtz *et al.* 2008).

Relatively few studies have looked at the impact of chemotherapy on cell survival and proliferation in WMTs. Additionally, administered 5-FU to rats observed a reduction in OPCs proliferation, and non-dividing mature oligodendrocytes were also sensitive to chemotherapy both *in vitro* and *in vivo* studies (Han *et al.* 2008).

These effects could lead a progressive loss of oligodendrocytes with resulting demyelination in white matter tracts. Systemic administration of different types of chemotherapeutic agents such as 5-FU, methotrexate and cisplatin resulted in significant decreases in cell division in the CC, SGZ and SVZ (Lee *et al.* 2005, Mustafa *et al.* 2008, Seigers *et al.* 2009, Yang *et al.* 2011, ElBeltagy *et al.* 2012, Lyons *et al.* 2012). Importantly, this reduction in cell division can persist for at least two months after the end of chemotherapy exposure (Dietrich *et al.* 2006, Han *et al.* 2008). In another study using electrophysiology, Gandal *et al.* (2008) found that treatments of adult male mice with a combination of methotrexate and 5-Fluorouracil for 6-7 weeks led to substantial impairment in the gating of auditory stimuli. They suggested that these effects, which were found 5 weeks after treatment, may underlie the associated cognitive impairments. A decline in auditory gating indicates a reduction in the activity of inhibitory circuits and in the ability of brain to modulate its response to incoming stimuli. From these results a reduction in the proliferation and survival of oligodendrocytes and their precursors could be one of the causes of cognitive impairment (Seigers and Fardell 2011, Wigmore 2013). Furthermore, OPCs are continually proliferating and differentiating to maintain the integrity of white matter tracts (Nishiyama *et al.* 2002, Nishiyama 2007, Zhu *et al.* 2011) and this is particularly important in response to white matter damage where they play a critical role in repairing after injury. Thus, the numbers of OPCs, as detected by NG2 immunoreactivity, increases two days after cerebral injury to restore the number of mature oligodendrocytes in the following weeks (McIver *et al.* 2010). In conclusion, the number and rate of proliferation of OPCs is important in maintaining white matter tracts and enabling them to respond to injury to the CNS in order to recover white matter damage.

The sensitivity of dividing cells to chemotherapy may depend on the type and duration of the treatment. Single injections of anticancer drug have been shown to produce transient changes in cell proliferation in regions of the brain. For example Yang *et al.* (2011) observed a transient drop in cell proliferation within the SGZ after a single injection of methotrexate. In contrast, Han *et al.* (2008) and ElBeltagy *et al.* (2010) demonstrated that multiple doses or a single high dose, produces a progressive and long lasting reduction in the proliferation of proliferating cells in the SGZ of the hippocampus. Moreover, it appears that short term exposure to 5-FU can also cause irreversible damage to white matter tracts. This damage can lead to impairments in impulse conduction by neurons; and indeed may contribute to cognitive dysfunction and to patients having a slower processing speed (Abraham *et al.* 2008). Studies have shown that exposure to

systemic cisplatin or cytarabine lead to decreases in BrdU labelling in the dentate gyrus of mice 24 hours after treatment (Dietrich et al., 2006). Another study showed that mice exposed to three i.p. injections of 5-FU, did not have an initial drop in cell proliferation but this was found after two weeks and showed a large drop after six months which was associated with apoptosis in both the SVZ and SGZ (Han *et al.* 2008). Other researchers found that no changes in cell division after 24 hours of 5-FU injection in the rat hippocampus, while they found a significant decrease in the BrdU staining cells in the dentate gyrus after 48 hours of 5-FU exposure. Moreover, they observed a significant suppression in cell division in the dentate gyrus after exposure to other chemotherapeutic agents including cyclophosphamide, paclitaxel, and doxorubicin (Mignone and Weber 2006, Mustafa *et al.* 2008, Janelsins *et al.* 2011). Winocur in 2012 found severe effects of chemotherapy on cognition after short and long term MTX and 5-FU treatment in a mouse model. He used three weekly injections and then looked for cognitive changes especially on hippocampal-sensitive memory and discrimination learning tests (Winocur *et al.* 2012). Interestingly, a single injection of chemotherapy caused an initial reduction in cell proliferation and increased cell death, then it was followed by a marked rebound in cell proliferation. This indicates that limited repair is possible following cytotoxic insult. While, chronic exposure to cytotoxic agents lead to long-term suppression of cell division and continued cell death in the hippocampus and major white matter tracts (Dietrich *et al.* 2008).

As well as preventing cell division, chemotherapy can cause cells to delay division and accelerate differentiation and cells may take on a different fate (Hyrien *et al.* 2010). Transient exposure to chemotherapeutic agents such as carmustine or BCNU, increased the cell cycle time of oligodendrocyte progenitor cells and decreased their time to differentiation rather than causing cell death or apoptosis. Similarly, an *in vivo* study by Dietrich *et al.* (2006) found that systemic treatment of mice with three injections of the chemotherapy drug AraC, caused cell death and reduced cell division in neuronal and oligodendrocyte precursors. Treatment with MTX caused a rapid reduction in the number of glial progenitor cells in gray and white matter (approximately 45% of control values) and an increase in the numbers of microglial cells two days after treatment. This was followed by a full recovery in numbers by day 5. These studies have suggested that glial progenitor cells might be particularly vulnerable to cytotoxic agents (Morris *et al.* 1995, Han *et al.* 2008).

It would be very beneficial to patients if the negative effects of chemotherapy on the CNS could be prevented without interfering with the anti-cancer actions of these drugs. A number of treatments have been tried in both human and animal studies with mixed results (Wigmore 2013). Anti-depressants enhance hippocampal neurogenesis producing a 30-40% increase in the proliferation of early neural progenitor cells in the sub granular zone of the dentate gyrus (Encinas *et al.* 2006). Post-mortem studies of patients who had suffered from major depressive disorder and who had received antidepressants showed a higher cell proliferation and a higher blood vessel density in the hippocampus compared to non-medicated subjects (Boldrini *et al.* 2012). Studies in our lab have demonstrated that if the antidepressant fluoxetine is given before and during 5-FU treatment this prevented the decrease in cell proliferation in the SGZ of the dentate gyrus and reduction in spatial memory found in 5-FU alone treated animals (ElBeltagy *et al.* 2012, Lyons *et al.* 2012). These results on neural stem cells in the hippocampus suggest that these drugs could have a protective effect on glial precursors. Support for this idea has come from reports that chronic administration of fluoxetine or the atypical antipsychotic Olanzapine can increase the proliferation of new-born glial cells in the frontal cortex of adult rats (Kodama *et al.* 2004). Additionally, rats with experimental allergic encephalomyelitis showed increased survival of oligodendrocytes and reduced demyelination when given antidepressant treatment (Cabrelle *et al.* 2008, Pennington *et al.* 2009). These promising results on the effects of antidepressants on glial cell precursors indicate that these drugs may have beneficial or protective effects on this cell type in addition to the neural stem cells of the hippocampus.

Seigers *et al.* (2016) observed a decrease in the number of microglial cells but an increase in the number of activated microglia in the prefrontal cortex after short term treatment with cyclophosphamide, docetaxel and 5-FU in adult mice. Activated microglial release inflammatory cytokines which could affect both microglial and other surrounding cell types. Chemotherapy may induce cytokine release in the body which can cross the BBB (Kerr *et al.* 1984, Ahles and Saykin 2007); or it lead to cytokine release in the brain (Cai *et al.* 2000). The consequence of this result is sickness associated with cognitive impairment including memory impairment (Meyers 2008). In a different studies, Christie *et al.* (2012) found an increase in microglia activation one week after multiple injections of cyclophosphamide. Whereas, Seigers *et al.* (2010) found an increase 1 to 3 weeks after a single injection of methotrexate (MTX). The reduction in the number of microglia after activation is attributed to apoptosis that occurs due to lack of growth factors to support the proliferation response (Reid *et al.* 1993) or

to the cytokines secreted by activated microglia which also underwent cell death themselves (Miller *et al.* 2007). This mechanism is important to control the number of microglia in the CNS after activation (White *et al.* 1998). Therefore, Microglia act as part of the immune system which can promote or down regulate cell proliferation by releasing either pro or anti-inflammatory substances (Butovsky *et al.* 2005).

Based on the literature, the hypothesis of this study is that acute chemotherapy, consisting of a single injection of 5-FU, will produce a transitory decrease in cell proliferation in ON and CC but that normal levels will return after a recovery period. In contrast after chronic chemotherapy, consisting of multiple injections, there will be a continued reduction in the numbers of dividing cells into the recovery period. Co-administration of antidepressant, FLX, or NSAID, INDO, may prevent these adverse effects of 5-FU.

### 2.1.2 The role of DNA damage in the actions of chemotherapy:

DNA damage, which includes altering bases and single- and double-strand breaks, is known to be neurotoxic and to be involved in the pathogenesis of many neurological disorders. DNA damage is considered to be one of the main actions of 5-FU in killing cancer cells (Li *et al.* 2009, Wyatt and Wilson 2009). This probably occurs by several routes including misincorporatation of uracil (dUTP) or fluorinated uracil (FdUTP) in the place of thymidine during DNA synthesis. In addition 5-FU disrupts the action of thymidylate synthase which is required for the synthesis of the DNA base thymidine, resulting in a deficiency of thymidine. The net result of these actions is to cause damage to the DNA structure which is detected and leads to the phosphorylation of histone H2AX (γH2AX) adjacent to the damage sites (MacPhail *et al.* 2003). 5-FU inhibits Chk1 kinase and increases Chk1 phosphorylation the result of which is nuclear foci of phosphorylated histone H2AX (γH2AX) which are associated with DNA double-strand breaks (Fujinaka *et al.* 2012). Therefore, γH2AX is a prominent marker of DNA damage resulting from chemotherapy or radiotherapy treatment (Bourton *et al.* 2011, Garcia-Villa *et al.* 2012).

In addition, activation of caspase 3 and caspase 8 by 5-FU chemotherapy is a key mediator of apoptosis and is involved in DNA fragmentation and associated morphological changes (Janicke *et al.* 1998, Adachi *et al.* 1999). DNA can also be damaged by mitochondrial dysfunction or oxidative damage (Parker and Cheng 1990, Peters *et al.* 2002, Khansari *et al.* 2009), while the mechanisms of

apoptotic pathways and cell cycle arrest are differs depending to the type of causative agents.

It has been demonstrated that systemic treatment with chemotherapy causes a significant increase in apoptosis and prolonged reduction in cell division in the corpus callosum (CC) and this is associated with a reduction in the numbers of OPCs (Fancy *et al.* 2004, Soussain *et al.* 2009). Loss of these cells is associated with a delayed white matter tract degeneration (Okeda *et al.* 1990, Akiba *et al.* 1996, Han *et al.* 2008). In a rodent study by Briones and Woods (2011) which examined the effects of cyclophosphamide, methotrexate, and 5-Fluorouracil (CMF) drug combination on cell proliferation and histone modifications in the CNS. They found that treatment reduced the self-renewal potential of neural progenitor cells in the hippocampus. There was also chromatin remodelling in the hippocampus by increased histone acetylation and decreased the deacetylase activity of histone, both of these might be potential mechanisms in explaining chemotherapy-induced cognitive chemobrain.

### 2.1.3 The impact of Indomethacin and Fluoxetine on DNA damage

As both FLX and INDO will be used in this project to determine if they are protective against some of the effects of chemotherapy on the brain it is useful to review the possible effects of these drugs on cancer and DNA damage. INDO is a nonsteroidal anti-inflammatory drug (NSAID). This drug increases the expression of the transcription factor SOX2, a marker for stem cells while down regulating BMP7, a marker for poor prognosis in patients with colorectal cancer (Lonnroth *et al.* 2014). Treatment of cancer patients with NSAIDs, with or without adjuvant chemotherapy, is associated with reduced tumour progression and improved quality of life. This can attributed to the effect of INDO in the upregulation of the CDKN2A/p16 tumour suppressor gene and a decrease in DNA methylation enzymes. These actions lead to an increase in the activity of the immune system (Lundholm *et al.* 1994). Recently, NSAIDs has been given as an adjunct in the treatment of cancer. Overall INDO appears to have anticancer properties and does not interfere with the anticancer effects of chemotherapy, this can be attributed to their effect on inflammation (Grivennikov *et al.* 2010). Moreover, preclinical studies have shown that INDO prevents the spread of lung cancer (Cai *et al.* 2011) and breast cancer (Ackerstaff *et al.* 2007). Touhey *et al.* (2002) showed that INDO and its analogues enhanced the toxicity of chemotherapeutic agents and so potentiated the treatment. Similarly in human glioma cells, INDO significantly increases the cytotoxic effect of vincristine and etoposide (Benyahia *et al.* 2004). The increased chemosensitization produced by

INDO may be due to competition between INDO and chemotherapy agents at the transport site for drug efflux produced by multidrug resistance proteins or may be due to inhibition of cyclooxygenase and glutathione-S-transferase enzymes (Draper *et al.* 1997). Due to this talented evidence, more research is required to understand the role of INDO in the protection and treatment of cancer and the turnover of cells in WMTs. The *in vivo* experiments in this study were designed to determine if decreasing inflammation, using INDO, could protect WMT cell proliferation from chemotherapy.

FLX is often taken by cancer patients suffering from depression, however its impact on the cancer itself and the treatment of cancer are unclear. FLX can have an anti-inflammatory role by inhibiting the release of pro-inflammatory cytokines, this drug also increases the production of antioxidant enzymes, and the reducing cell apoptosis and the expression of pro-oxidant enzymes and harmful free radicals (Caiaffo *et al.* 2016). FLX can reduces lipid peroxidation in the brain which helps to reverse damage (Moretti *et al.* 2012) and reduces oxidative stress and oxidative cell damage (Novio *et al.* 2011).

It has recently been found that FLX protects against IL-1b-induced DNA damage and neuronal apoptosis via downregulation of p53 dependent pathways (Shan *et al.* 2016). Furthermore, FLX treatment inhibits the release of lactate dehydrogenase (LDH), chromatic agglutination and karyopyknosis induced by IL-1b stimulation which causes nuclear fragmentation and cell damage (Shan *et al.* 2016). Yune *et al.* (2014) demonstrated that FLX (10mg/kg, i/p) prevented oligodendrocyte cell death and demyelination after spinal cord injury and inhibited microglial activation. While van den Berg *et al.* (2014) have found both *in vivo* and *in vitro*, that FLX has an anti-inflammatory effect on the brain, and significantly reduced microglial activation and inflammatory markers. One hypothesis for the mechanism of action of FLX is suppression of NF-κB a transcription factor required for the production of pro-inflammatory factors like TNF-α, NO and IL-6 (Ha *et al.* 2006, Roumestan *et al.* 2007, Tynan *et al.* 2012). This has led to the hypothesis in this thesis that chronic treatment with FLX or INDO may be able to prevent DNA damage, induced by chemotherapy and maintain gliogenesis in white matter tracts.

### 2.1.4 The role of COX-2 in inflammation and chemotherapy:

COX-2 is the inducible isoform of the enzyme Cyclooxygenase, which converts arachidonic acid to prostaglandin H2. Under non pathological or non-inflammatory conditions, it is produced by sub populations of neurons in response

to synaptic activity and is thought to be involved in synaptic plasticity (Breder *et al.* 1995, Minghetti 2004). Recent investigations have suggested that low levels of COX-2 expression are important for hippocampal neuorogenesis and LTP (Minghetti 2004, Goncalves *et al.* 2010). Cox-2 expression is significantly upregulated by cytokines and other stimulators of inflammation including LPS or Aβ peptides where it is expressed by neurons, glia and microglia (Chen *et al.* 2005). Elevated COX-2 levels are found in the brain in a number of neurodegenerative diseases including Alzheimer's and Parkinson's diseases and inflammatory induced over expression of COX-2 is thought to mediate a reduction in hippocampal neurogenesis (Minghetti 2004, Ekdahl *et al.* 2009). However, the involvement in COX-2 in reducing cell proliferation is still under investigation, it was also suggested that COX-2 may modify neurogenesis in the dentate gyrus by generation prostaglandin (PG) E2 and PGD2 which are able to induce apoptosis in a variety types of cells in CNS (Bastos *et al.* 2008). An *in vivo* study has shown that constitutively over expressing COX 2 transgenic mice, have increased cortical neuronal apoptosis and glial activation which lead to cognitive impairment in aged animals (Andreasson *et al.* 2001).

Recent studies have shown that cancer itself and chemotherapy causes an elevation in the levels of inflammatory markers in both patients and animal models. Lopes *et al.* (2009) found that administration of 5-FU induced inflammation in peripheral tissues in animal models and increased the expression of COX-2. It has also been observed that the neuroinflammation associated with ageing or chemotherapy lead to increased microglial activation, neurodegeneration and neurological impairment (Lucin and Wyss-Coray 2009, Christie *et al.* 2012). It was hypothesized that chemotherapy induced inflammation could indirectly affect normal cell proliferation in the brain and reduce neurogenesis. A recent study (Briones and Woods 2014) showed that neuroinflammation may play a role in causing chemotherapy induced demyelination which in turn may lead to cognitive impairment. This paper showed that a combination chemotherapy treatment consisting of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) produced cognitive impairment and damage to WMT microstructure in rats. This was associated with a decrease in the level of the anti-inflammatory cytokine IL-10, and an increase in the levels of the inflammatory mediators COX-2, TNF-α and IL-1ß which were in turn associated with a decrease in myelin basic protein. Cytokines play a role in the regulation of certain neuronal processes, including glial function, repair, and the metabolism of certain neurotransmitters (Ahles and Saykin 2007). However,

uncontrolled or prolonged elevation of cytokine levels is associated with cognitive impairment due to their neurotoxicity (Ahles and Saykin 2007). This suggests that the effect of 5-FU on microglia and other glial cells may be an important in producing neurotoxicity *in vivo* as Wilson and colleagues have shown that these cells can release inflammatory cytokines and produce neurodegenerative effects (Wilson *et al.* 2002).

COX-2 is blocked by NSAIDs including INDO. Interestingly COX-2 is overexpressed in many tumours where it is associated with cancer induced inflammation. This has led to the use of NSAIDs in the treatment and prevention of cancer (Sobolewski *et al.* 2010). Treatment with the NSAID (NS-398) reduced expression of inflammatory markers and prevented demyelination in animals treated with chemotherapy (Briones and Woods 2014). Administration of anti-inflammatory drugs can enhance neurogenesis by restoring the interaction between neural stem cells and the microvasculature which is disrupted by inflammation. INDO protects hippocampal neurogenesis after cranial irradiation or LPS induced inflammation but has no effect in non-inflammatory situations such as ageing (Monje *et al.* 2003, Boehme *et al.* 2014). In a parallel study, Parepally *et al.* (2006) found that in the rats, INDO penetrates the BBB readily, thus the anti-inflammatory agent can act directly on the CNS. Additionally, they also found that prior administration of INDO can reverse the cognitive deficits induced by the LPS. Interestingly, INDO alone does not affect cell proliferation, or the number of new neurones produced during hippocampal neurogenesis. This suggests that anti-inflammatory drugs while not affecting neurogenesis directly, can restore neurogenesis which is reduced by inflammation (Ji *et al.* 2013).

Therefore, our hypothesis is that, the impact of chemotherapy on white matter tracts may at least in part be indirect with 5-FU stimulating microglia to release inflammatory cytokines which in turn reduce cell proliferation and enhance apoptosis. The effect of on OPCs would be to reduce the maintenance and repair of myelin sheaths. The ability of FLX and INDO to reverse the neuroinflammatory process may reduce the effects of cytokines and prevent cell death. As part of the investigations into the acute and chronic effects of 5-FU treatment, it was decided to quantify the number of COX-2 expressing cells in both the ON and CC by immunohistochemistry at different time points after drug treatment and to evaluate the effects of FLX or INDO when co-administrated with or without chemotherapy.

## 2.2 Aims and objectives:

Several studies have demonstrated the impact of 5-FU on the central nervous system and the positive effects of FLX or INDO on neurogenesis in the hippocampus and its protection of this brain region. However there is little information on the impact of 5-FU on the gliogenesis within white matter tracts in general or on the ON and CC in particular. The studies presented in this chapter were carried out to investigate the impact of systemic chemotherapy on WMT and the associated precursor cells in the ON and CC.

Therefore, the objectives of these investigations were:

1. Establish an *in vivo* model to determine if acute and chronic 5-FU treatment regimens have different effects on WMT cell proliferation after 24 hours of treatment, and whether cells are able to recover after 1 week.
2. Elucidate the toxic effect of 5-FU to OPCs and microglial cells in ON and CC, using NG2 and Iba-1 as a specific markers.
3. To investigate the effect of the chemotherapy to induce inflammation in CNS by using immunohistochemistry technique to examine the COX-2 expression in WMT.
4. Examine the effect of 5-FU on DNA damage detected by phosphorylation of histone H2AX adjacent to the damage sites.
5. To study the effect of FLX and INDO with or without 5-FU treatment in WMT and to determine whether co-administration prevented or reversed the adverse effects of chemotherapy.

## 2.3 Materials and methods

### 2.3.1 Animals and design of experiments

All animals were male Hooded Lister rats, 200-280g, obtained from Charles River, UK and were housed in the Biomedical Services Unit (BSU) University of Nottingham. All procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and with approval of University of Nottingham ethical review committee and were carried out under authority of Home Office, Project Licence (PPL 40/3194) and Personal Licences (PIL 70/26438 and 70/22103).

Animals were randomly assigned to different groups of between 4 to 8 animals per treatment group, the number and grouping regimen was based on our previous studies and in accordance with a relevant pilot study. Animals housed 4 per cage (dimensions: 52 × 32 × 20 cm) under standard conditions of a 12 hour light/dark cycle (07.00: 19.00 hour); room temperature (21 ± 2°C) and humidity (60 ± 5%). Animals given free access to food and water. Animals were habituated for at least one week to the BSU prior to the start of treatments, and water bottle and animal weights were monitored daily in each cage during each experiment to monitor animal health and to adjust the concentrations of drugs administered in the drinking water. Throughout the study the number of animals used and their suffering were kept to a minimum.

Four different *in vivo* experiments are described in this chapter of the thesis. These were firstly carried out to investigate the effects of acute and chronic treatment with 5-FU and secondly to evaluate the ability of antidepressant or anti-inflammatory treatments to protect the white matter tracts from the effects of chemotherapy.

### 2.3.2 Study one: Effect of a single injection of 5-FU analysed one day after injection.

16 rats were randomly divided into control (saline injected, n=4), FLX treated (n=4), 5-FU treated (n=4) and combined FLX and 5-FU treated (n=4) groups. Animals allocated to FLX and 5-FU/FLX groups received 10mg/kg/day FLX (Fluoxetine Hydrochloride F-132, National Institute of Mental Health NIMH, USA) via their drinking water for 21 days prior to chemotherapy treatment. FLX dosage was calculated from the amount of daily water intake and animal weight (Yau *et al.* 2002) to ensure, as closely as possible, that animals were receiving the appropriate dose per day as they increased in weight. The dose of FLX was chosen based upon previous studies by our group in which this dose has produced

behavioural changes in rats and cellular changes in the hippocampus (Lyons *et al.* 2012). Both 5-FU and the 5-FU combined with FLX groups received a single intraperitoneal (i.p) injection of 5-FU at a concentration of 35mg/kg (stock solution 50 mg/ml, Medac, Germany) immediately after treatment for 21 days with FLX. 5-FU was diluted in 0.9% w/v saline (Baxter Healthcare, UK) and the volumes were calculated for individual animals based on their weights. Control and FLX alone groups received a single injection of 0.9% vehicle saline i.p of an equal volume to those receiving 5-FU. All animals were monitored for 6 hours after injection in case of adverse reactions. All rats were killed 24 hour after administration of 5-FU or saline by a schedule 1 method. The duration of acute treatment experiment was 22 days (Figure 2.1).

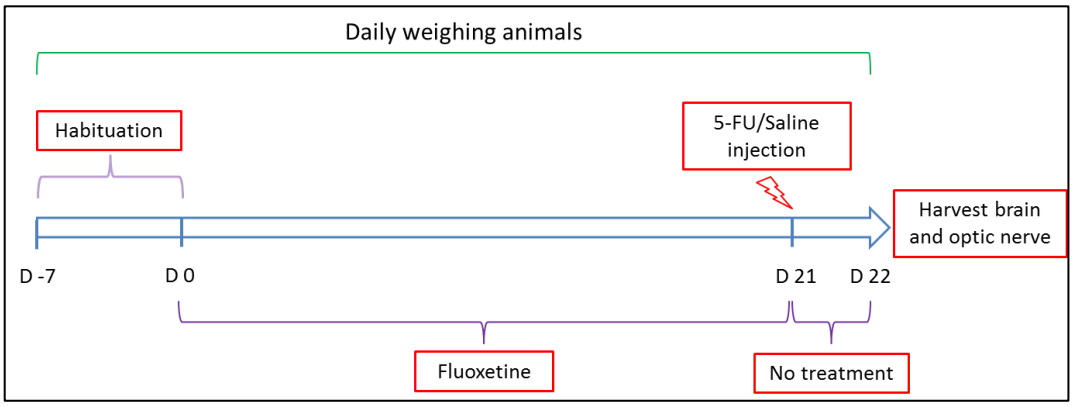



***Figure 2.1*** *Timeline showing the experimental design of study one. Acute treatment by a single injection i.p. of 5-FU or saline after 21 days of FLX treatment. Animals were killed 1 day after injection.*

### 2.3.3 Study 2: Effect of a single injection of 5-FU analysed one week after injection.

16 male Lister-hooded rats were divided randomly into control (saline), FLX, 5-FU and FLX+ 5-FU treated groups (each, n=4). Animals receiving FLX were given the drug in their drinking water at a dose of 10mg/kg/day for 21 days prior to chemotherapy injection. 5-FU treated animals received a single i.p injection of 5-FU at a dose of 35mg/kg. Non 5-FU treated animals received an identical sequence of normal 0.9% saline injections. Animals had no drugs for a week before being killed (Figure 2.2). The period of the acute experiment with one week of recovery was 28 days from beginning of FLX administration.

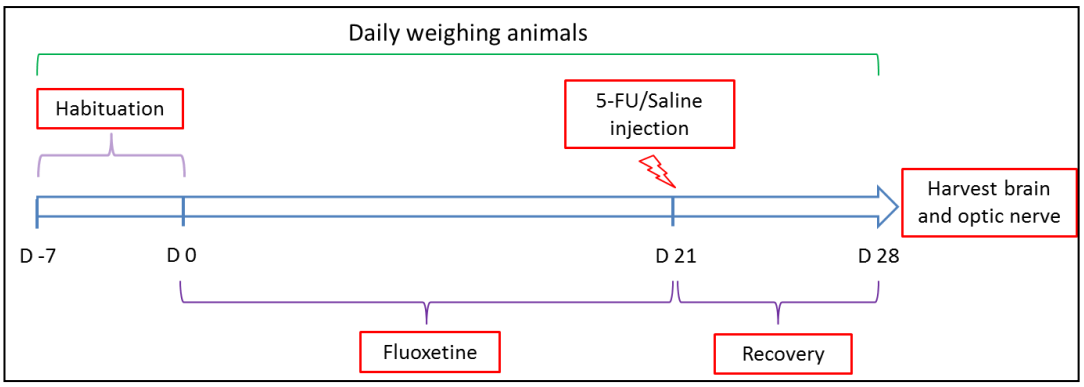



***Figure 2.2*** *Timeline showing experimental design of study two. Acute treatment by a single i.p. injection of 5-FU or saline after 21 days of FLX treatment. Rats were then maintained for 1week to allow recovery. D0 Experiment starting day;* ↯ *represents single i.p. injections of 5-FU.*

### 2.3.4 Study three: Effect of multiple injections of 5-FU analysed one week after the last injection.

32 rats were assigned to control, FLX treated, 5-FU treated and combined FLX and 5-FU treated groups (each, n=8). 5-FU treated groups received 6 i.p injections of 5-FU 35mg/kg/day each 3 days apart for 18 days. Non 5-FU treated animals received an identical sequence of i.p. injections of 0.9% saline. Animals receiving FLX were given the drug in their drinking water at a dose of 10mg/kg/day for 21 days prior to and during chemotherapy treatment such that they received FLX for a total of 36 days. All animals then had a week of no treatment starting on the day of the last injection (Figure 2.3).

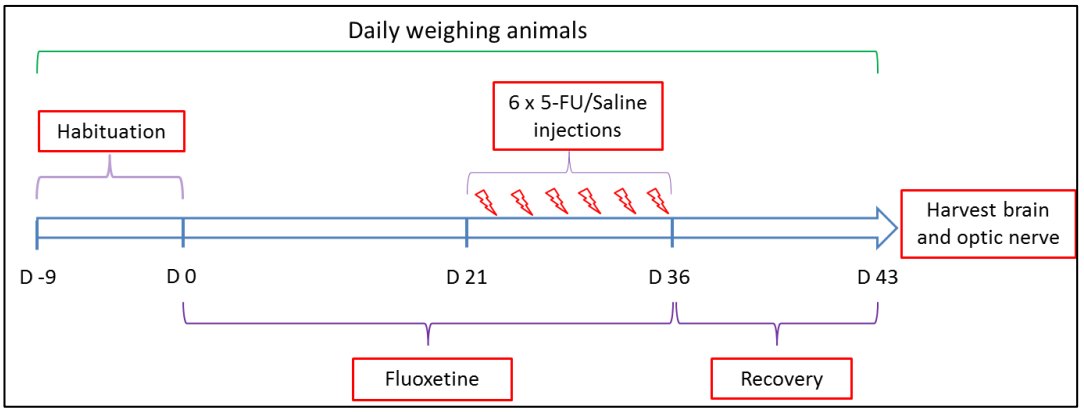



***Figure 2.3*** *Timeline showing the experimental design of study three which Involved chronic treatment with chemotherapy. Rats received six i.p. injections of 5-FU or saline every other day over 18 days after 21 days with one group having received FLX administration. Rats were then maintained for one week after the final injection.*

### 2.3.5 Study four: Effect of multiple injections of 5-FU analysed one week after the last injection. Some groups receiving FLX or INDO throughout all the experimental period.

Rats (n=24) were randomly allocated to control saline injected, FLX treated, indomethacin (INDO) treated, 5-FU treated, FLX+5-FU treated, and INDO+5-FU treated groups. They were housed 4 per group. Drinking water containing FLX or INDO was administered to the FLX, FLX+5-FU, INDO and INDO+5-FU groups starting 21 days before 5-FU injection. 5-FU treated animals received six i.p injections of 5-FU 35mg/kg/day each 3 days apart over 18 days. Non 5-FU treated animals received 0.9% sterile saline of equivalent volume injections. Animals receiving FLX, 10mg/kg/day or INDO, 1mg/kg/day (17378-SIGMA, CHINA) were given the drugs in their drinking water throughout all the experimental period for 43 days in total. FLX and INDO dosages were calculated from the amount of daily water intake and adjusted accordingly. All animals were killed one week after the last 5-FU or saline injection (Figure 2.4).

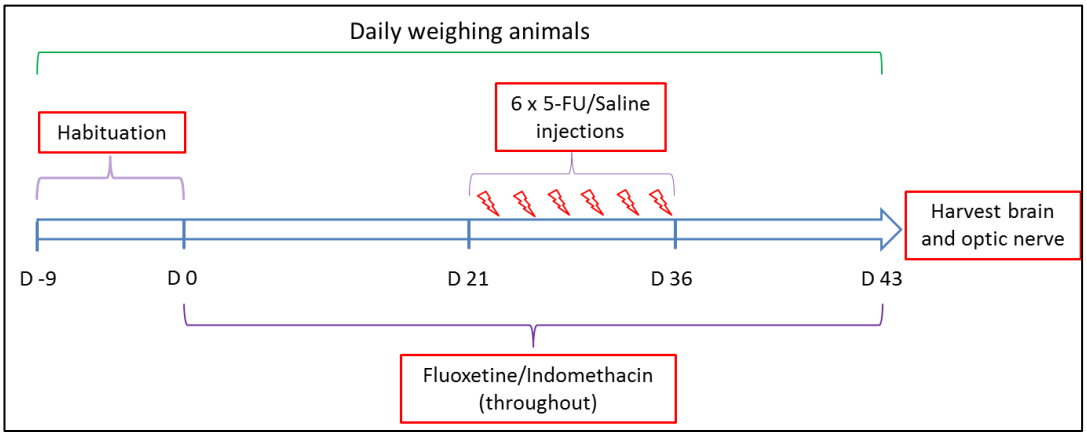



***Figure 2.4*** *Timeline showing the experimental design of study four. 5-FU treated rats received 6 i.p. injections of 5-FU while non 5-FU treated animals received equivalent injections of saline every 3 days. This was given after 21 days of FLX or INDO administration in groups receiving these drugs. FLX and INDO administration were continuous throughout the experimental period until animals were killed and their optic nerves and brains removed.*

### 2.3.6 Tissue preparation

At the end of the experiment, rats were killed by rapid concussion followed by cervical dislocation and decapitation. The optic nerves (ON) were cut with fine scissors as they entered the posterior surface of the eye ball. The cranium was then opened and the brain elevated without removal to expose the optic chiasma

and the ON. After cutting other cranial nerves, the ONs were then gently pulled from the optic canal and each ON cut immediately rostral to the optic chiasma. Each nerve was approximately 6-8 mm in length. Then 1.5-2 mm of ON in the region adjacent to the eye, was excised for processing for electron microscopy. The remaining part of the right ON was immersed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.5) for 6 hours at 4°C followed by 30% sucrose/phosphate buffer at 4°C for 4 hours. After which the rostral end of the nerve was dipped in toluidine blue (Sigma Aldrich, UK) to facilitate identifying the nerve end before embedding in Optimal Cutting Temperature Compound (OCT) (VWR International Ltd, Lutterworth, UK). Nerves were then snap frozen in isopentane cooled in liquid nitrogen and stored at -80°C as fixed material until sectioning. The left ONs were dehydrated in 30% sucrose solution for 4 hours at 4°C then the rostral end of the nerve was dipped in toluidine blue and submerged in OCT and snap frozen in liquid nitrogen-cooled isopentane and stored at -80°C for obtaining fresh frozen sections.

Brains were removed, cut sagittally and one half was cryopreserved in 30% sucrose for 4-6 hours at 4°C, then submerged in OCT before flash freezing in isopentane cooled by liquid nitrogen. Brains were stored at -80°C until required for sectioning as fresh frozen samples. The other half of each brain was immersed rapidly in 4% PFA and stored at 4°C overnight. The following day brains were remove from PFA and stored in a 30% sucrose solution at 4°C until required for slicing as a post fixed free-floating samples.

### 2.3.7 Tissue sectioning

Both post-fixed and fresh frozen ON samples were removed from -80°C storage, left to warm to -20°C then serially sectioned transversely at 20 microns (µm) on a HM505E cryostat (Midwest Lab Equipment LLC; Iowa, US). Five sections per slide were collected on slides coated with 3-Aminopropyltriethoxysilane (APES). Sections were air dried and stored at -20°C until required for staining. In total 20 slides were made per nerve. The remaining part of the ON embedded in OCT, was wrapped in cling film and stored at -80°C for subsequent use.

The fresh frozen brains were removed from the -80°C freezer and left to warm to -20°C, before 20 µm coronal sections were cut using the HM505E cryostat at -20°C starting at the anterior of the cortex. Sections were thaw mounted on in sequence, four to a slide coated with APES and air dried before being stored at -20°C until used for immunohistochemistry. The post fixed half brains were sectioned on a sliding freezing microtome (Anglia Scientific, UK) by attaching

them to a cooling plate with the rostral end facing up, and fixed to the plate using OCT before coronal sectioning at 60 µm. Free floating sections were collected and stored in anti-freeze filled wells of WHO plates (Ace Ltd, Berkshire) at -20°C until used for immunohistochemistry. Coronal sections were cut at the fornix region of the corpus callosum (CC) which was identified from toluidine blue stained sections compared with a rat brain atlas (Paxinos and Watson 2006).

### 2.3.8 Immunohistochemistry

For fresh frozen samples, at least 8 coronal sections from each brain and 10 cross sections from ON were selected using a systemic random sampling technique representative of the entire ON and CC (Mayhew 1991). Immunocytochemistry was performed on fresh frozen sections using antibodies against Ki67, NG2, COX2 and H2AX antigens. Whereas the markers Iba1, and also Ki67 were stained using post fixed sections.

All primary antibodies were incubated at 4°C in a light-proof humidity chamber. While the secondary antibodies were incubated in a light proof humidity chamber at room temperature (RT). All dilutions and washes (each performed thrice, 5 minutes each) were carried out with phosphate buffered saline (PBS). For each rat brain and ON stained with this method, control slides were stained with an identical protocol with either, no primary, no secondary or no antibodies at all. These sections were checked to detect auto fluorescence and to test the specificity of each antibody.

#### 2.3.8.1 Fresh frozen sections immunostaining

Selected slides were removed from the -20°C freezer and left to thaw at RT for 10 minutes in a humidity chamber. A permanent marker pen was used to draw around each nerve section on the underside of the slide to identify the sections. The sections were circled with a Peroxidase-Antiperoxidase (PAP) pen (Vector Laboratories H-4000, Canada). ON and brain sections were fixed with 2% paraformaldehyde (PFA) for 5 and 10 minutes respectively at RT before being washed. Non-specific binding sites were blocked using blocking solution B (0.2% (v/v) goat serum, 1% (w/v) bovine serum albumin (BSA), 0.3% (v/v) Triton X-100, PBS) for 1 hour at RT. To detect proliferating cells, sections were incubated overnight at 4ºC in monoclonal mouse anti-Ki67 (1:100; Vector laboratories, UK) diluted in blocking solution B. The following day, sections were washed. Secondary antibodies; Alexa Fluor 488 rabbit anti-mouse IgG (1:1000; Invitrogen, UK) diluted in blocking solution C (1% (w/v) Bovine serum albumin, 0.1% (v/v) Triton X-100, PBS) were applied for 1 hour in the dark at RT. Sections

were then counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (1.5µg/ml) (1:1500; Vector laboratories, Inc, USA) and all slides cover slipped (22×64mm, Scientific Laboratory Supplies), and stored in the dark at 4°C.

To detect other markers, sections were fixed in 2% PFA for 5 minutes and washed. Sections were preincubated in 5% (w/v) Bovine serum albumin, 0.3% (v/v) Triton X-100, PBS) (day one buffer) for 1 hour at RT before being incubated overnight at 4ºC in diluted antibody to optimize the signal to background ratio. The antibodies used were monoclonal mouse anti-NG2 (1:250; Abcam laboratories, UK) a marker of OPCs, polyclonal rabbit anti-COX2 (1:500; Abcam laboratories, UK) an inflammatory marker, monoclonal mouse anti-pH2AX (1:1000; Millipore, USA) a DNA damage marker. The following day, sections were washed in day two buffer (1% (w/v) Bovine serum albumin, 0.1% (v/v) Triton X-100, PBS) as usual washing. Secondary antibodies generated in goat (anti-rabbit, and anti-mouse) were coupled to Alexa 488, Alexa 594 or Alexa 568 were used according to the species of the primary antibody. Sections were incubated for 1 hour in day two buffer containing the secondary antibodies (dilution 1:1000) in the dark at RT. Sections were washed then slides were mounted in a Vectashield DAPI and coverslipped.

#### 2.3.8.2 Post fixed sections immunostaining

The selected free-floating sections were first washed and blocked in (5% (w/v) Bovine serum albumin, 0.3% (v/v) Triton X-100, PBS) for 1 hour at RT. sections were incubated in 1% (w/v) Bovine serum albumin, 0.2% (v/v) Triton X-100 and PBS overnight at 4°C with primary antibodies; polyclonal rabbit anti-Iba-1 (1:2000; Wako Chemicals Inc., USA) raised against microglia, and polyclonal rabbit anti-Ki67 (1:200; Novus Biologicals, Littleton, USA) which detects proliferating cells. Sections were washed in buffer solution (1% BSA, 0.1% Triton X-100 and PBS). The sections were then incubated with the relevant secondary antibody (1:1000; Alexa Fluor 594 goat anti-rabbit, and Alexa Fluor 568 goat anti mouse). Sections were washed and mounted on gelatine coated slides, followed by mounting and counterstaining with Vectashield and DAPI, and sealed with nail varnish.

### 2.3.9 Fluorescence microscopy imaging and cells quantification

Cross-sections of ON and coronal sections of CC were examined with a fluorescence microscope (Nikon Optiphot-2, Japan) coupled to a SPOT insight QE digital camera running SPOT for Windows (version 3.5.0). 20x and 40x

magnifications were used for photographing the whole optic nerve and CC to visualise positive cells in these areas.

For cell counting, antibody labelled cells and the total number of DAPI labelled cells were counted for each ON section and the average from 10 sections per animal recorded. In the CC, cells were counted from 4-8 nonadjacent sections per rat and averaged; cell counts are presented as an average from at least 4 rats per group. The positive cell density under different condition of treatment where performed by normalising cell density to both area and total cell number, and the relevant statistics performed (Figure 2.5). Antibody positive cells were quantified in three CC regions: the medial or midline, lateral and regional or the end of CC at the level of the fornix. Some analyses were also restricted to the midline CC as described previously (Arnett *et al.* 2001). The analysis was limited within the boundaries of the CC, as detected by DAPI distribution. Manually cell quantification was done blind in 5 consecutive, non-overlapping fields beginning with the medial aspect of the CC zone and proceeding laterally. Total and relative numbers of cells expressing different antigens were estimated by scoring the number of cells labelled with the markers. Once the counting was complete, counts from all sections of each ON and CC were averaged to give a mean positive cell count per section for each animal in each treatment group.

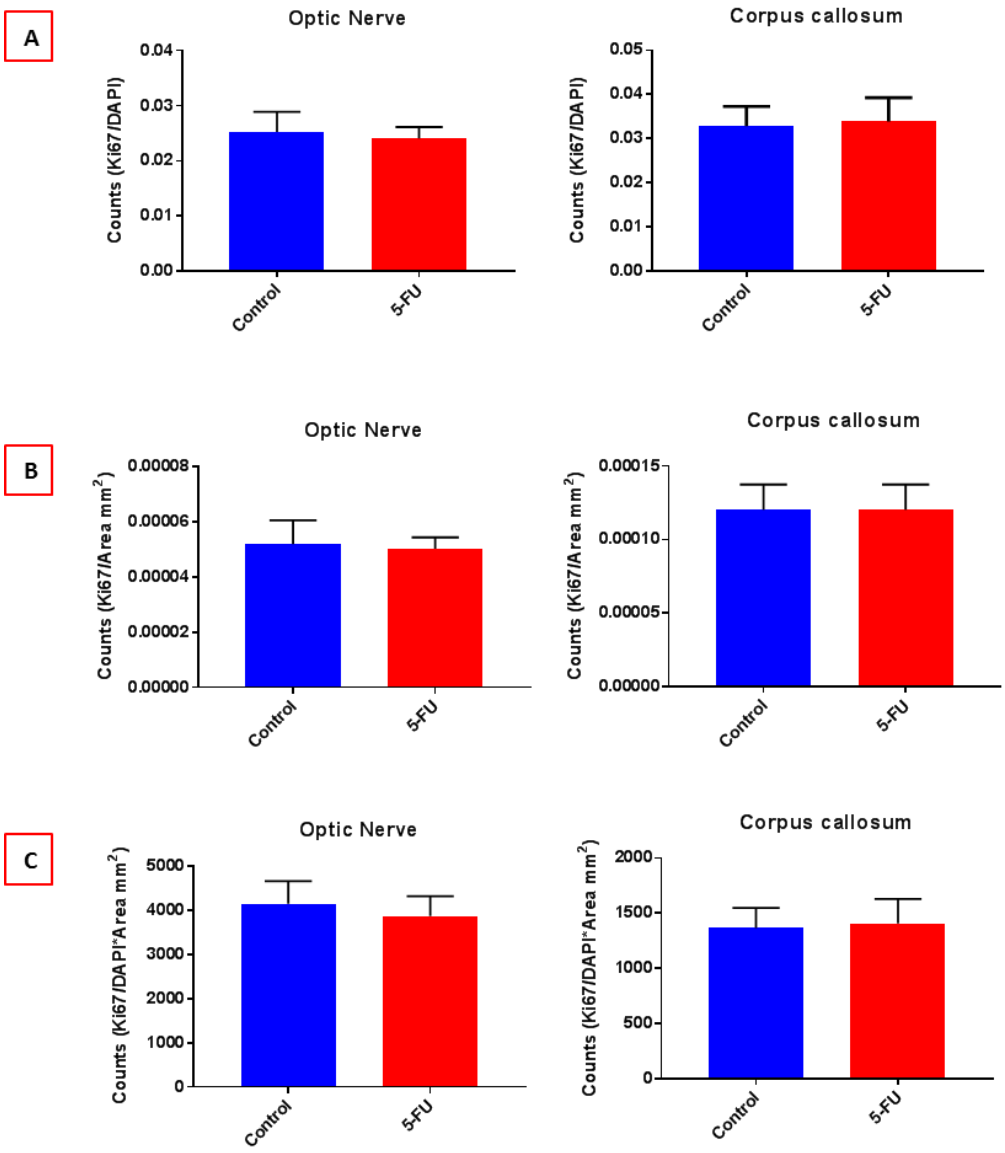



***Figure 2.5*** *Analysis of positive cells density between treated and untreated groups. (A) Data normalised to Ki67 positive cells and total cells number in ON and CC. (B) Graphs showing cells density normalised to area of measurement from a smaller region of interest in each field of ON and CC tissue. (C) Data showing Ki67 positive cells density normalised to area and to area plus total cell density in both ON and CC. Data analysis showed that both the entire population of Ki67+ cells and total cells density that normalised to area of counting were not affected by acute 5-FU treatment when compared with controls. The data are represented by means± SEM (n=4) for each group and analysed by Unpaired Student t-test (two-tailed) to compare controls and 5-FU treated groups (significance was regarded as p< 0.05).*

### 2.3.10 Statistical analysis

The statistical analysis and graphs were performed in GraphPad Prism v7.0 Ink software (GraphPad, San Diego, USA). All data are expressed as mean ± standard error mean (Mean ± SEM). Unpaired Student t-test (two-tailed) was used in normalising positive cell density to both area and total cells number when comparing between controls and 5-FU treated groups in ON and CC. Repeated measures of two-way ANOVA with Bonferroni's post hoc test were used to analyse the body weights for individual animals in each group at different time points. Analysis of variance (one-way ANOVA) was used to analyse water consumption with treatment given in drinking water for each cage and the effects of injected drugs. This was done to compare the interactions between 5FU, FLX, INDO and controls. When results for each marker compared between all groups were significant, Bonferroni post-hoc multiple comparison two-way ANOVA analysis was then carried out to determine specific groups with significant difference. For all statistical tests, $p \leq 0.05$ was regarded as statistically significant.

## 2.4 Results

### 2.4.1 Study one (Effect of a single injection of 5-FU, analysed one day after injection).

#### 2.4.1.1 The effect of FLX and 5-FU treatment on animal weight:

To investigate the effect of chronic FLX and acute 5-FU treatment on weight gain, animals were weighed daily throughout the experiment. All animal weights significantly increased over the time ($F_{(18, 216)} = 670.8$, $P<0.0001$). However during FLX treatment, FLX groups showed a significant reduction in weight gain when compared to the control group ($F_{(54, 216)} = 6.843$, $P<0.0001$). Bonferroni post-hoc analysis showed that this reduction in weight gain started on day 12 after FLX administration (p=0.0090) (Figure 2.6), but the weight difference caused by FLX was non-significant after 5-FU administration (P=0.3575) on day 22. Two way ANOVA showed that animals receiving a single injection of 5-FU had significantly reduced weight, compared to controls the day after injection producing a difference of approximately 26 grams (p = 0.0463) (Figure 2.7).

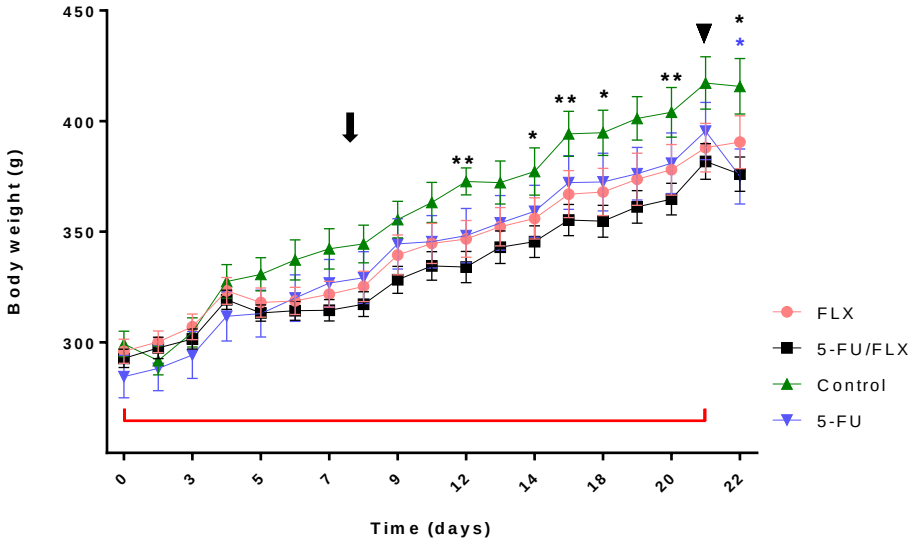



***Figure 2.6*** *The mean body weight for each experimental group recorded in study one (Mean± SEM, n=4 per group). Animals were weighed daily throughout the course of FLX treatment from day 0 to day 21 (red line) and a single injection of 5-FU at day 21 (arrow head). Two-way ANOVA analysis showed an overall weight gain over the period (P<0.0001), and post-hoc test shows the interaction between time and treatment was highly significant (P<0.0001). Both FLX and FLX+5-FU animals shows a significant reduction in the gain of mean body weight compared to controls and the 5-FU group shows a significant reduction in body weight compared to controls after the 5-FU injection (P = 0.0055).*

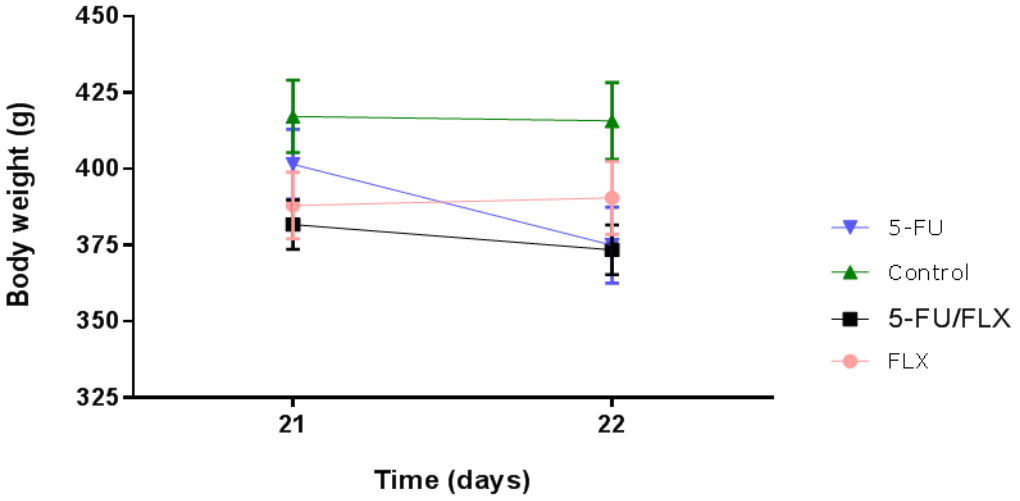



***Figure 2.7*** *Shows the difference in body weight for each experimental group in study one (Mean± SEM, n=4 per group) between day 21 and 22. Animals of 5-FU and FLX + 5-FU groups received a single injection of 5-FU (35mg/kg) on day 21 (arrow), while the animals in control and FLX groups received a saline injection at same time. An acute single 5-FU injection caused a significant reduction in weight in 5-FU and FLX+5-FU groups at day 22 compared to vehicle treated controls (P=0.0463 and P=0.0372 respectively). The FLX group showed no significant difference from control group after saline injection (P=0.3575).*

#### 2.4.1.2 Effects of FLX and 5-FU treatments in Water consumption:

The water intake per cage was calculated for each experimental group by daily weighing of water bottles. Drinking water treated with FLX was administered to the FLX and the 5-FU+FLX groups throughout 21 days before injection of 5-FU. The dose of FLX was recalculated every other day to keep the concentration of FLX consumed unchanged over the course of treatment. In general there was a highly significant decline in water intake for those groups receiving FLX in their water ($F_{(1.667, 25.01)} = 27.11$, $P<0.0001$) especially in days 1-3 (Figure 2.8A). This is probably due to an aversion to the distinct flavour of fluoxetine which caused animals to reduce their water intake. One way ANOVA with post hoc tests showed a significant reduction in mean water consumption in FLX and 5-FU+FLX groups ($P=0.0002$ and $P<0.0001$ respectively), but no significant difference in the 5-FU group ($P=0.0928$) when compared to controls (Figure 2.8B).

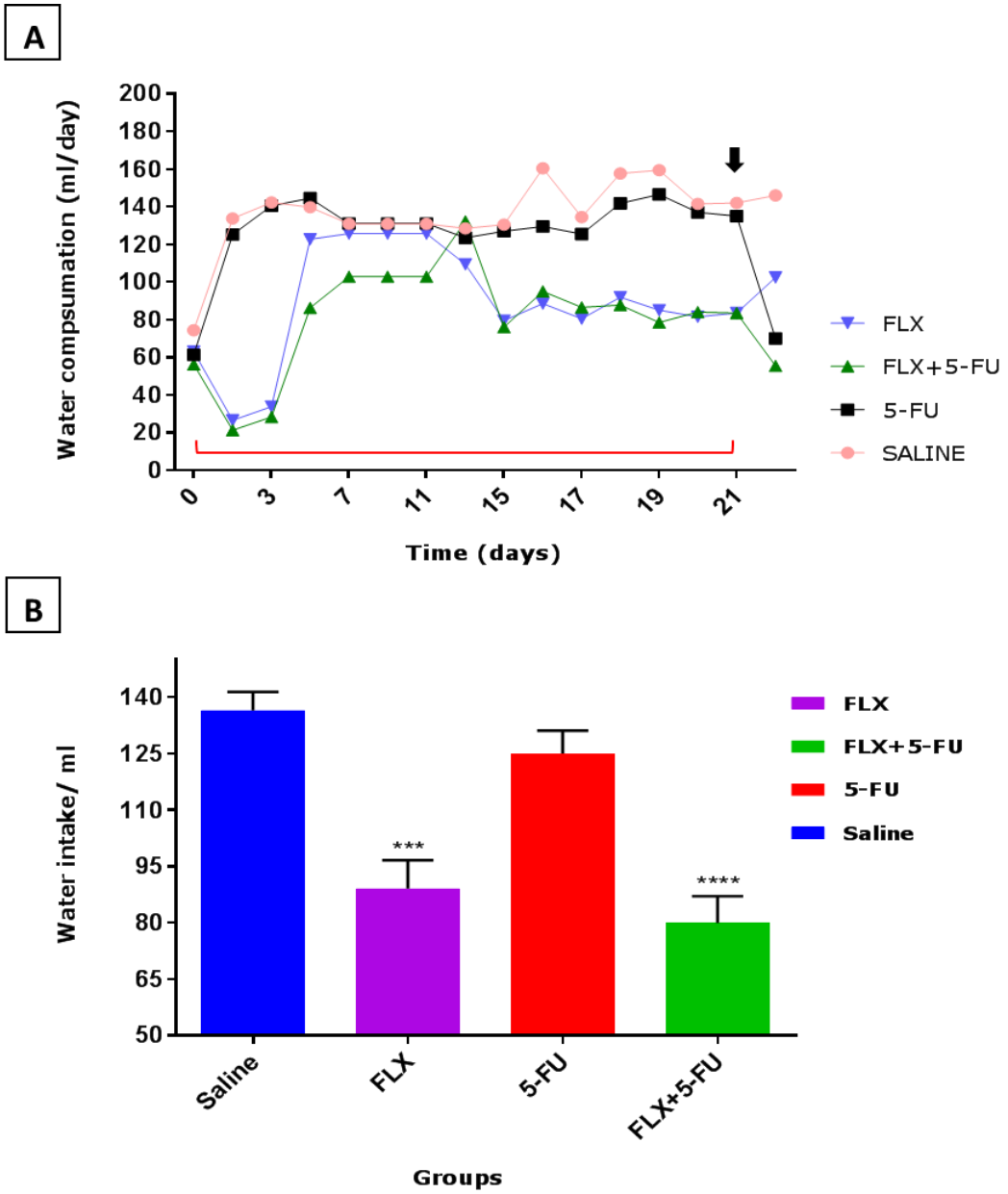



***Figure 2.8*** *shows the volume of water intake for each experimental group recorded in study one.* ***(A)*** *Daily water consumption from day 0 of FLX administration represented as ml/group. The red line indicates the period of FLX administration in the drinking water of the FLX treated groups, and the arrow indicates the day of 5-FU injection. FLX treatment caused a significant decline in water intake (P<0.0001). Although there was no significant difference in the mean volume of water compared to controls and 5-FU treated groups (P>0.05), there was also a clear reduction in water consumption after 5-FU injection on day 21.* ***(B)*** *The difference in mean water intake between treatment groups. Rats of FLX and FLX+ 5-FU groups treated with FLX for 21 days produced a significant water reduction compared to the other groups ($F_{(1.667, 25.01)} = 27.11$, P<0.0001; one-way ANOVA). In contrast, no significant difference was found between the 5-FU and the control groups in water intake during the treatment period (P= 0.0928).*

#### 2.4.1.3 The effect of a single injection of 5-FU on cell proliferation in the ON and CC one day after injection (study one)

The number of Ki67+ cells in the two white matter tracts (ON and CC) was counted to determine the effect of chronic treatment with FLX and a single injection of 5-FU on cell proliferation 24 hours after injection of 5-FU. In the ON, no significant difference was found in the number of Ki67+ cells between the groups ($F_{(1, 60)}$ = 0.3711, P=0.5447) after 5-FU injection. Further analysis with A multiple comparison test with a Bonferroni post-hoc test showed that animals treated with FLX alone also showed no significant change in the proliferating cell number when compared with the control group ($F_{(1, 60)}$ = 0.2226, P=0.6388) (Figure 2.9). This indicated that a single dose of F-FU and chronic treatment with FLX alone does not change cell proliferation in the ON.

In the CC, a two-way ANOVA test showed no significant interaction between FLX groups and 5-FU treatments ($F_{(1, 12)}$ = 0.2673, P=0.6146). However as seen in the ON, 5-FU treatment reduced cell proliferation compared with controls (P= 0.0667) and this difference reached significance when compared with the FLX alone treated group. Bonferroni post-hoc multiple comparison two-way ANOVA tests showed a higher Ki67+ cell number in animals treated with FLX alone, compared to 5-FU and FLX-5-FU groups (P=0.0022 and P= 0.0347 respectively) (Figure 2.10).

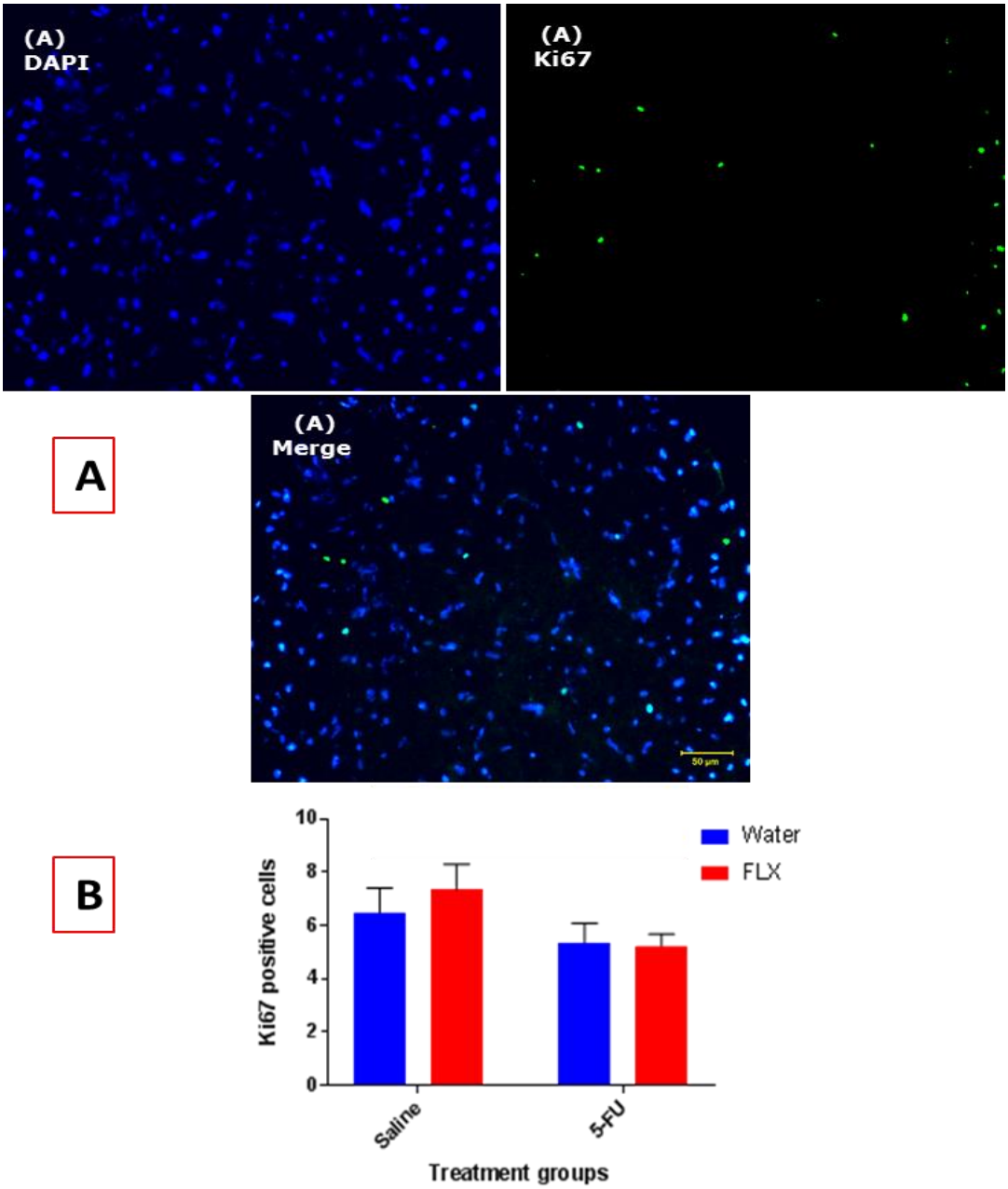



***Figure 2.9*** *Ki67+ cells in ON after 5-FU, FLX or a combination treatment, one day after 5-FU injection (study one). (**A**) A representative image of rat ON. Proliferating cells are immunostained for Ki67 (green) and counterstained with DAPI (blue) and merged. Scale Bar=50µm. (**B**) The total numbers of Ki67+ cells per section in different treatment groups (mean ± SEM, n=4). There was no significant difference between the FLX, 5-FU, FLX+5-FU, and saline treated groups (p>0.05, two-way ANOVA).*

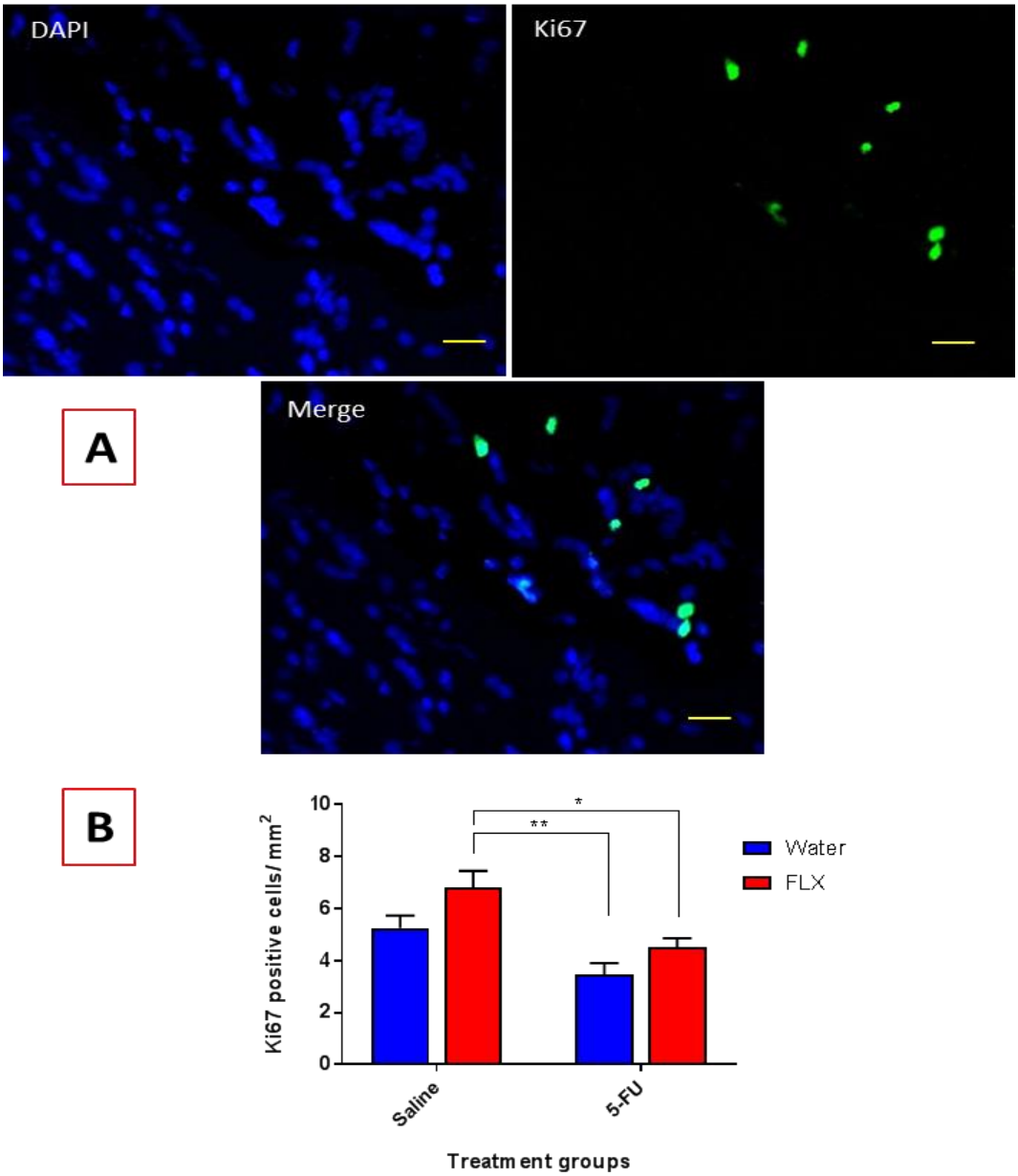



***Figure 2.10*** *Ki67 proliferating cells in CC after 5-FU, FLX or a combination treatment one day after treatment (study one). (**A**) A representative image of the CC from a control animal. Proliferating cells immunostained for Ki67 (green) and counterstained with DAPI (blue) and merged. Scale Bar=25µm. (**B**) There was no significant difference in the number of proliferating cells between treatment groups and the control group (p >0.05) (mean±SEM, n=4). Rats receiving FLX only had significantly higher Ki67+ cells than 5-FU and FLX+5-FU groups (**P=0.0022 and *P= 0.0347 respectively, Two-way ANOVA with Bonferroni post-hoc test).*

#### 2.4.1.4 The effect of a single injection of 5-FU and chronic FLX treatment on the number of oligodendrocyte precursor cells (OPCs) in the ON and CC one day after 5-FU injection.

OPCs, phenotypically are recognized by their expression of neuron-glial antigen 2 (NG2) and are termed 'NG2 cells'. It was hypothesized that treatment with chemotherapy would lead to a loss of OPCs. To determine the effect of single injection of 5-FU (35mg/kg) with or without administration of FLX on adult OPCs populations, immunohistochemistry was carried out using an antibody against the NG2 protein and the numbers of NG2 positive cells were counted in the ON and CC.

In the ON, no significant difference was observed in the NG2+ populations in all treatment groups compared with the control group ($F_{(1, 12)} = 1.143$, P=0.3060; two-way ANOVA). Post-hoc multiple comparison showed that there was significant effects of chemotherapy treatment in all rats receiving 5-FU comparing with the FLX only treated group ($F_{(1, 12)} = 17.01$, P=0.0014) (Figure 2.11).

In the CC, staining for NG2 found a significant difference between controls and FLX alone groups. More OPCs were found in FLX treated animals compared with the 5-FU treated group (P=0.0293) and co-treatment with FLX and 5-FU, prevented the reduction in NG2+ found with 5-FU alone (P=0.0079), and the pre-treatment with FLX ameliorates this effect in FLX+5-FU group (P=0.6584) (Figure 2.12). Post-hoc analysis indicated that FLX treated groups had significantly more OPCs than the control and 5-FU treated groups ($F_{(1, 12)} = 15.12$, P=0.0022; Bonferroni's multiple comparisons test).

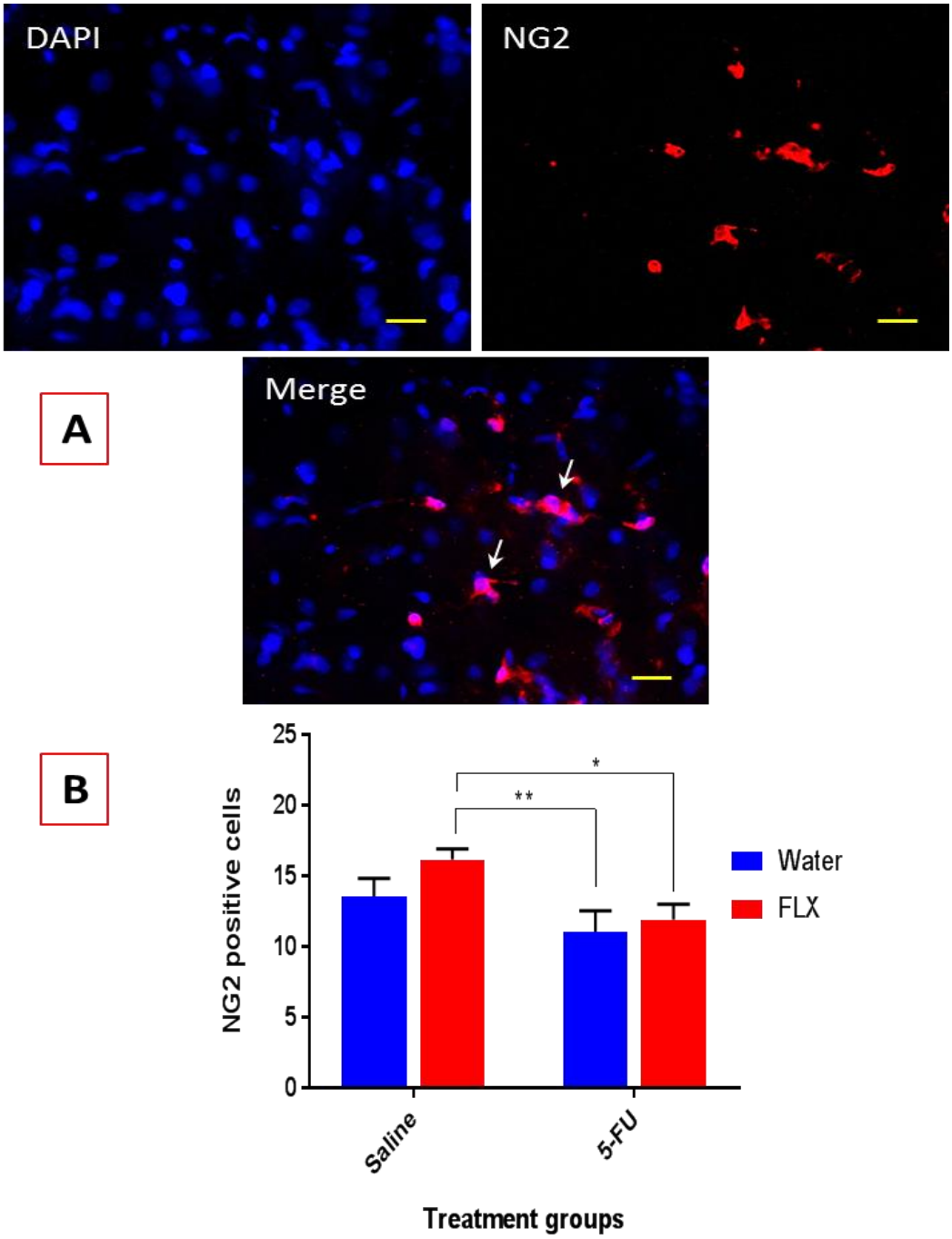



***Figure 2.11*** *NG2+ cells in the ON one day after 5-FU injection (study one).(**A**) Representative image of rat ON. OPCs are immunostained for NG2+ (red) and counterstained with DAPI (blue) and merged. NG2+ cells are identified by the arrows. Scale Bar=20µm. (**B**) Shows the average number of NG2+ cells per section (mean ± SEM, n=4) in adult rat ON in different treatment groups one day after a single injection of chemotherapy. Analysis showed a non-significant difference of OPCs in the 5-FU treatment group (p=0.1897, two-way ANOVA) compared with the saline-control group. The groups receiving 5-FU show a significant difference from the FLX group (**P=0.0041, Two-way ANOVA with Bonferroni post-hoc test).*

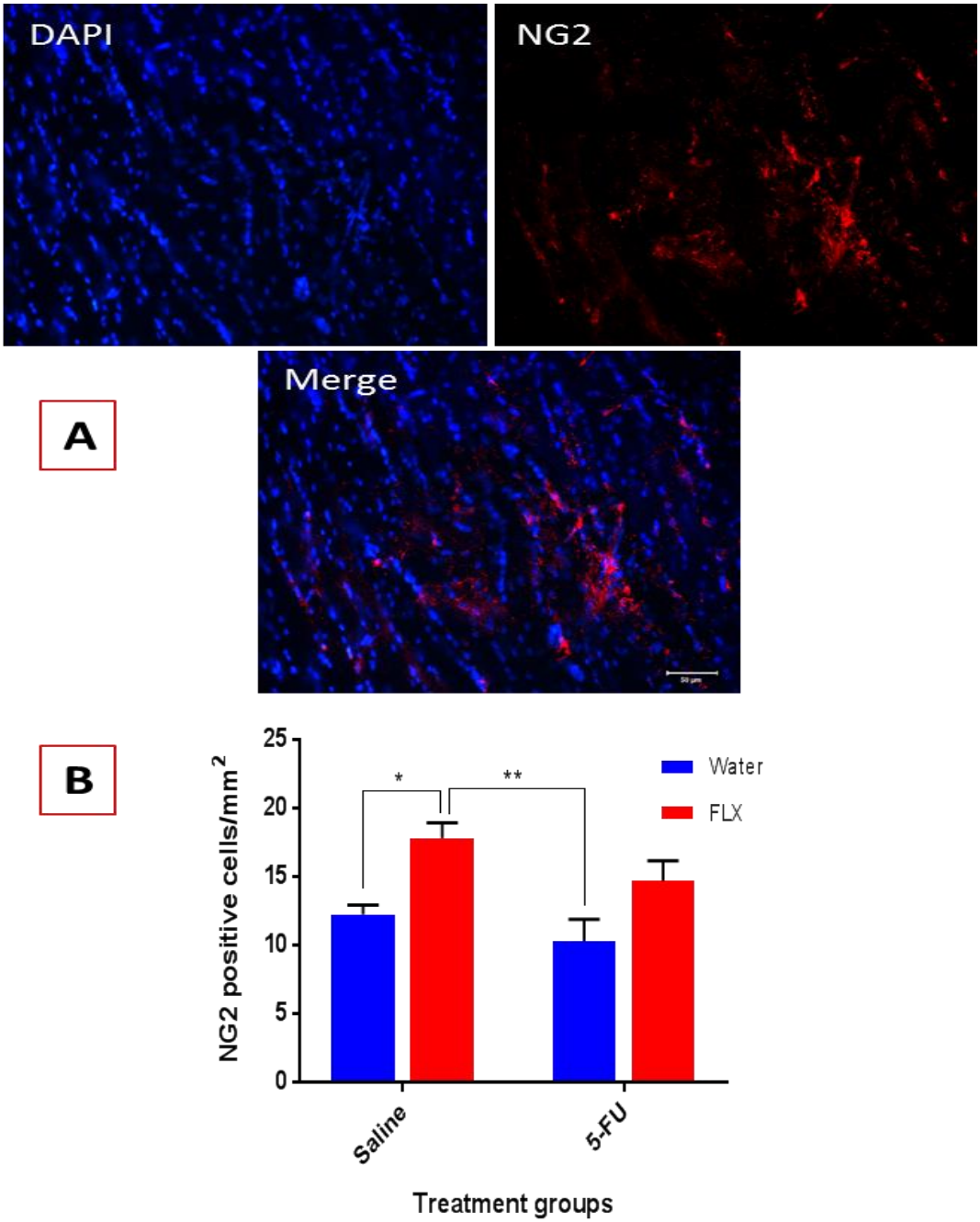



***Figure 2.12*** *NG2+ cells in the CC one day after 5-FU injection (study one). (**A**) Representative images of rat CC immunostained with NG2+ (red) and counterstained with DAPI (blue) and merged. Scale Bar=50µm. (**B**) The mean number of NG2+ cells per mm2 (mean ± SEM, n=4) in adult rat CC in different treatment groups one day after a single injection of 5-FU. Analysis showed a significant increase of NG2+ cells in the FLX treated group (*p=0.0293, two-way ANOVA) compared with controls. The groups receiving 5-FU did not significantly differ from the controls but had significantly fewer NG2+ cells than the FLX group (**P=0.0079, Two-way ANOVA with Bonferroni post-hoc test). The group receiving 5-FU and FLX did not show this reduction and had no significant difference from the FLX alone group (p>0.05).*

#### 2.4.1.5 A single injection of 5-FU induces the expression of COX-2 in the ON and CC (study one).

COX-2 is an inducible enzyme which is produced under inflammatory conditions. The number of COX-2 expressing cells in both ON and CC was quantified by immunohistochemistry after three weeks of FLX and 24 after a single injection of 5-FU (Figure 2.13). One day after the end of treatment the number of COX-2 positive cells was significantly greater in the ON of 5-FU treated animals compared to controls ($F_{(1, 12)}$ = 63.92, P<0.0001; two-way ANOVA). It was also observed that there was no significant difference in COX-2 positive cells with FLX treatment (P=0.9714). Post-hoc multiple comparison tests revealed that there was a significant difference in COX-2 positive cells in animals treated with FLX+5-FU group compared to 5-FU alone (P=0.0032). Indicating that the increase in the number of COX-2 positive cells, was prevented by prior chronic treatment with FLX.

In the CC, 5-FU also significantly increased the number of COX-2+ cells in the 5-FU alone treated group (P=0.0073). However co-treatment with FLX (FLX+5-FU) did not significantly reduce the number of COX-2+ cells compared to the saline group (P=0.0389). In contrast, treatment with FLX alone showed no significant difference from the control group (P=0.9206). Moreover, multiple comparison tests revealed that no significant difference between FLX+5-FU group and 5-FU group (P=0.7735, two-way ANOVA) (Figure 2.14). Overall, Bonferroni post-hoc analysis shows that 5-FU treatment significantly affected all treated groups ($F_{(1, 12)}$ =30.57, P=0.0001).

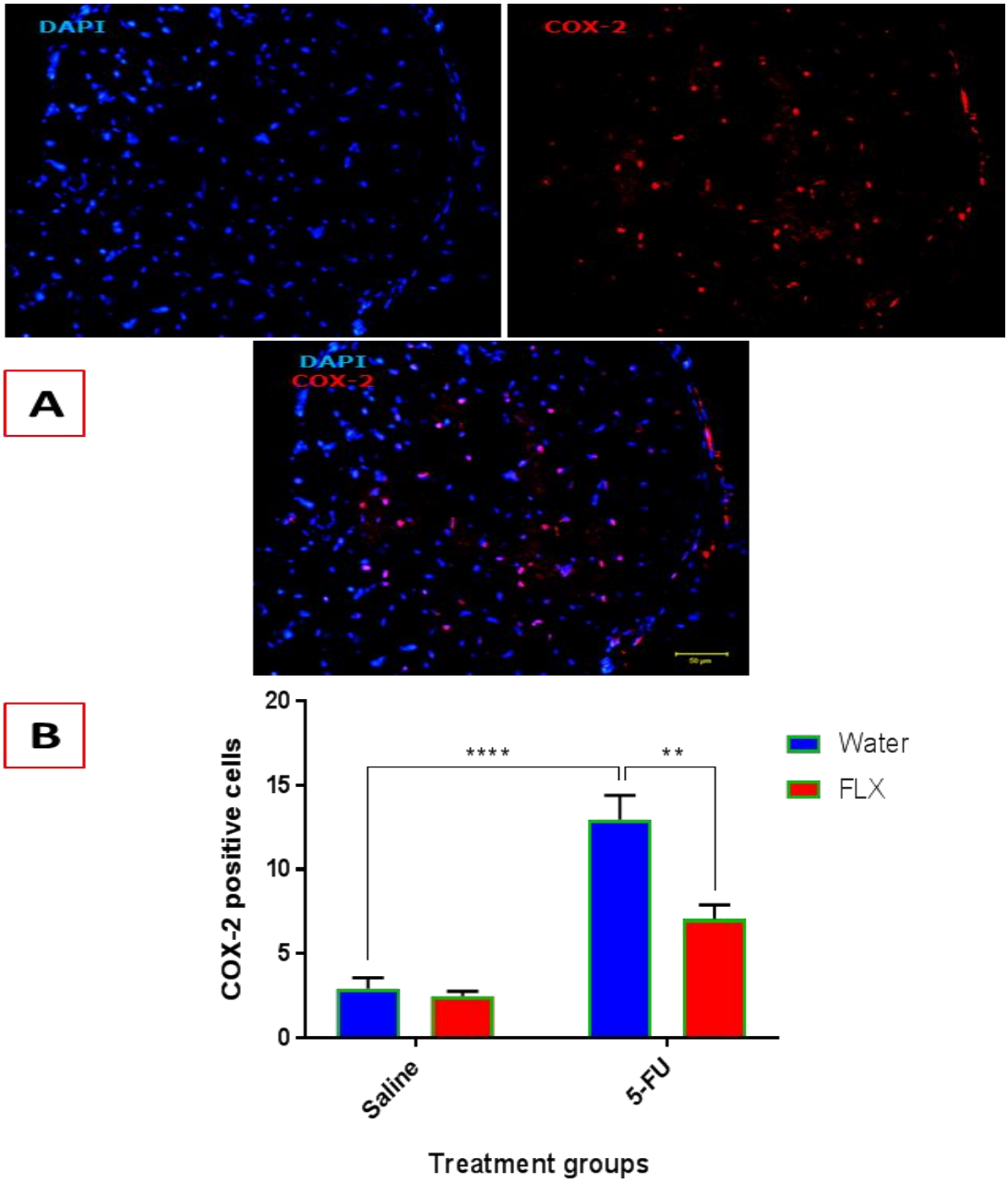



***Figure 2.13*** *Quantification of COX-2+ cell numbers in the ON (study one). (A) Representative image of rat ON immunostained with COX-2 (red) and counterstained with DAPI (blue) in and merged. Scale Bar=50μm. (B) Shows the average number of COX-2+ cells per section (mean ± SEM, n=4) in adult rat ON in different treated groups sacrificed one day after a single injection of 5-FU. Analysis showed a significant increase in COX-2 expressing cells in the 5-FU treated group (****P<0.0001, two-way ANOVA) compared with the saline-control group. The groups receiving FLX did not significantly differ from the controls and had significantly fewer COX-2+ cells than the 5-FU group (****P<0.0001). Co-treatment of 5-FU and FLX prevented the significant increase in positive cells found in the 5-FU group (**P=0.0032, Bonferroni's multiple comparisons test).*

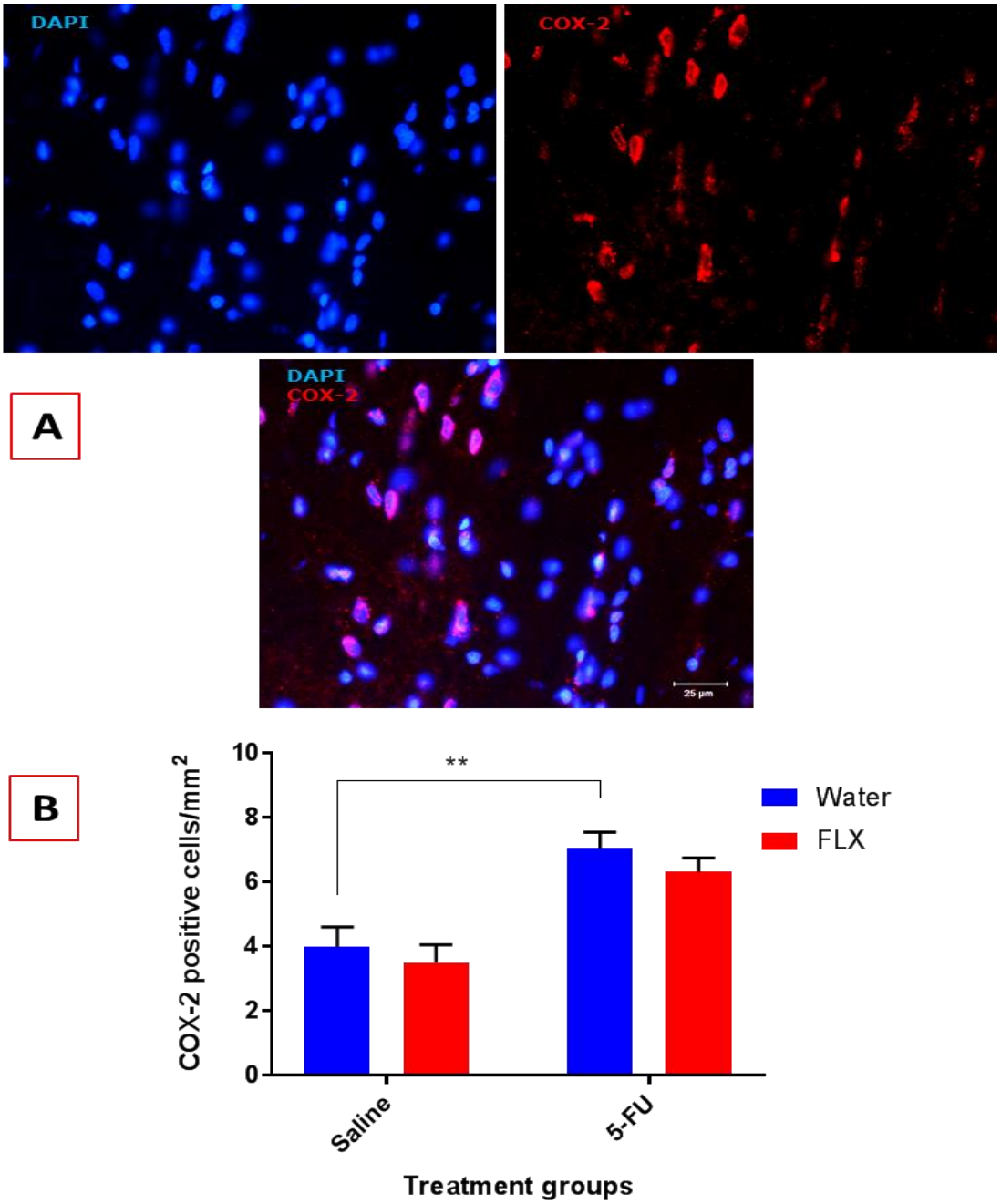



***Figure 2.14*** *Quantification of COX-2+ cell numbers in the CC (study one). (A) Representative image of rat CC immunostained with COX-2 (red) and counterstained with DAPI (blue) in and merged. Scale Bar=25µm. (B) Shows the average number of COX-2+ cells per mm$^2$ (mean ± SEM, n=4) in adult rat CC in different treated groups one day after a single injection of 5-FU. Analysis showed a significant increase of COX-2 expressing cells in the 5-FU treated group (**P=0.0073, Two-way ANOVA with Bonferroni post-hoc test) compared with the control group. The group receiving FLX alone did not significantly differ from the controls but had significantly less COX-2+ cells than the 5-FU group (**P=0.0025). Co-treatment with 5-FU and FLX did not prevent the increase in COX-2+ cell numbers found with 5-FU alone (P=0.7735) which remained significantly higher than controls (*P=0.0389).*

#### 2.4.1.6 The effect of a single injection of 5-FU and chronic FLX treatment on the total number of microglia in the ON and CC (study one).

Microglia are the resident immune cells of the CNS. Changes in these cells can indicate altered inflammatory states which are found in many cases of brain pathology, in response to injury or invasion of a foreign material into the CNS. To investigate the effect of chronic treatment with FLX and the acute effect of chemotherapy on the total number microglia in white matter tracts after 24 hour of single injection of 5-FU, the total number of Iba-1 positive cells in sections was counted. In the ON, data analysis showed a significant increase in microglial number in the 5-FU treated group as compared to FLX-treated animals (P=0.0022, two-way ANOVA with Bonferroni post-hoc test). The 5-FU group had a non-significant increase in Iba-1+ cells compared to the control group (P=0.0680). No significant differences were observed between the vehicle-treated group and FLX or FLX+5-FU groups (Figure 2.15).

In the CC, data analysis showed a significant increase in Iba-1 positive cell number in the 5-FU treated group as compared to controls (p= 0.0378, two-way ANOVA). Treatment with FLX on its own had no effect on Iba-1 numbers but co-treatment of FLX and 5-FU prevented the increase in Iba-1 numbers found with 5-FU alone (P=0.0004, multiple comparison two-way ANOVA with Bonferroni post-hoc test), as illustrated in Figure 2.16. Bonferroni post-hoc analysis shows that there was a significant interaction between FLX and 5-FU treatments in all treated groups ($F_{(1, 12)} = 12.99$, P=0.0036).

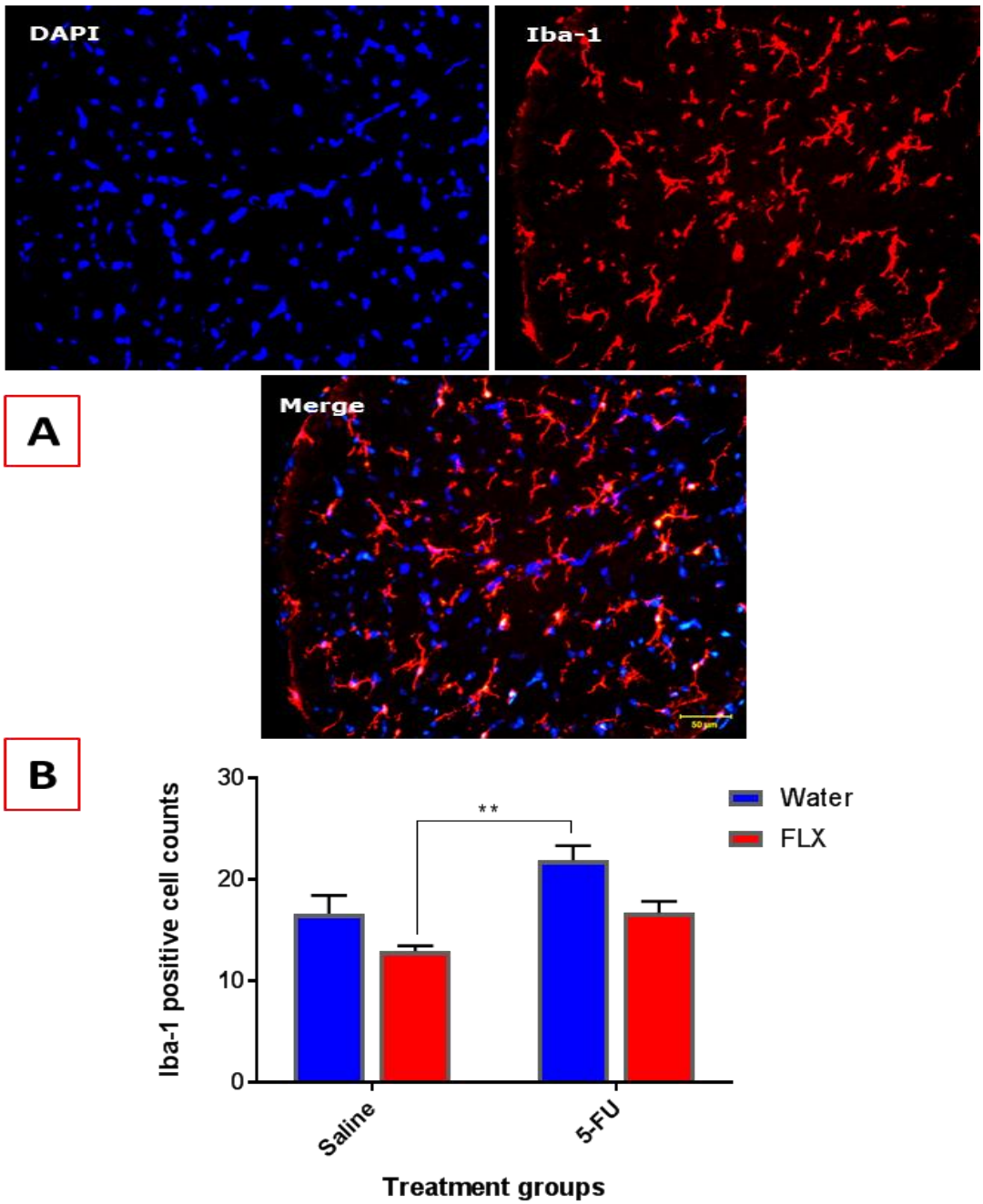



***Figure 2.15*** *Quantification of Iba-1+ cells in the ON one day after a single 5-FU injection (study one). (A) Representative image of rat ON. Microglia were immunostained with Iba-1 marker (red) and counterstained with DAPI (blue) and merged. Scale Bar=50µm. (B) Shows the average number of Iba-1+ cells per section (mean ± SEM, n=4) in adult rat ON. Analysis showed a non-significant increase in the mean number of microglia in the 5-FU treatment group compared with control (P=0.0680, two-way ANOVA). The groups receiving 5-FU showed a significant difference from the FLX group (**P=0.0022 two-way ANOVA with Bonferroni post-hoc test). Whereas the group receiving FLX or 5-FU and FLX had no significant differences in the number of positive cells compared to the control group (p>0.05).*

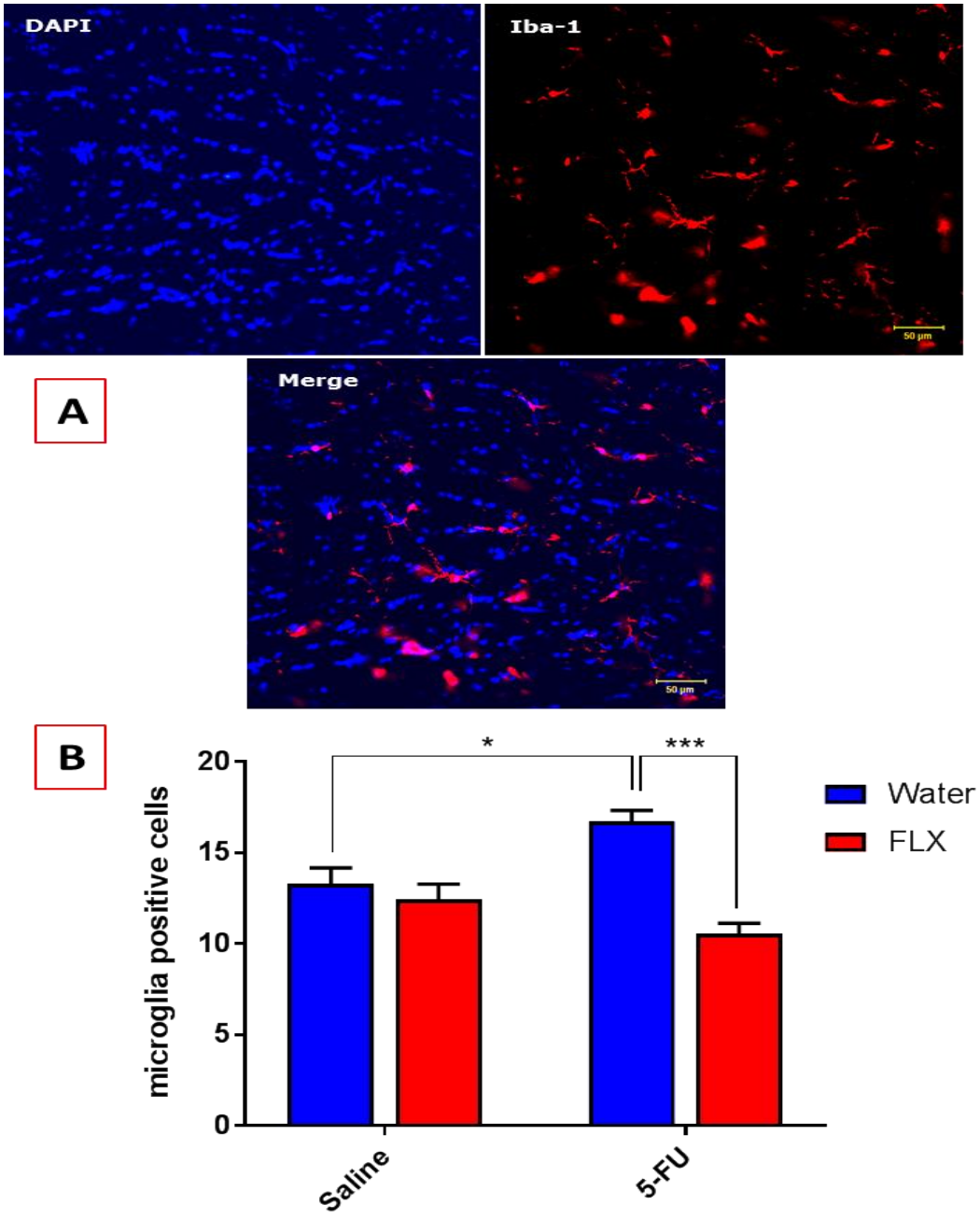



***Figure 2.16*** *Quantification of Iba-1+ cells after a single 5-FU injection and FLX treatment in the CC one day after injection (study one). (A) Representative image of rat CC, immunostained with Iba-1 (red) and counterstained with DAPI (blue) and merged. Scale Bar=50µm. (B) Shows the average number of Iba-1+ cells per mm2 (mean ± SEM, n=4) in adult rat CC in different treated groups one day after a single injection of 5-FU. Analysis showed a significant increase of Iba-1 expression cells in the 5-FU treated group (*p=0.0378, two-way ANOVA with Bonferroni post-hoc test) compared with the saline-control group. The groups receiving FLX did not significantly differ from the controls (P=0.9689) but had significantly less Iba-1 positive cells than the 5-FU group (**P=0.0089). Data also shows the group receiving 5-FU and FLX had significantly fewer positive cells than the 5-FU group (***P=0.0004). No change in the number of microglia was observed in co-administrated FLX+5-FU group compared with controls (P=0.1309).*

#### 2.4.1.7 Effect of 5-FU on the number of cells expressing a marker for DNA damage in the ON and CC after 1 day of injection (study one)

In order to assess an aspect of the cytotoxic effect of 5-FU, immunohistochemistry was used to detect the phosphorylated form of the histone H2AX (pH2AX) that is found after DNA double strand breaks. In addition this study was designed to determine whether FLX can prevent DNA damage after a single injection of chemotherapy. PH2AX is a very sensitive marker of DNA damage and is efficient at marking cells with double strand DNA breaks within hours of chemotherapy treatment.

In the ON, no significant difference was found in the number of H2AX+ cells between the groups treated with 5-FU and controls ($p>0.05$, two-way ANOVA) 24 hours after 5-FU treatment (Figure 2.17). While, multiple comparisons tests showed that animals with FLX treatment only, had significantly less DNA damage than 5-FU and FLX+5-FU groups ($P=0.0116$ and $P=0.0408$ respectively). This suggests that FLX has had no effect on the levels of DNA damage. It also suggests that 5-FU does not begin to cause visible increases in DNA damage in ON one day after a single injection.

Similarly, in the CC, statistical analysis showed that there was no significant interaction between 5-FU and FLX treatments in all study groups ($F_{(1, 12)} =1.509$, $P=0.2428$; two-way ANOVA). Regarding the effect of acute 5-FU after 24 hours of treatment on DNA damage, it seems that the numbers of pH2AX+ cells is not different between the groups or from controls ($p>0.05$, two-way ANOVA). Additionally, FLX appeared to have no significant effect either on FLX group, ($P=0.8207$) or FLX+5-FU group ($P=0.9440$), when compared with the control group (Figure 2.18).

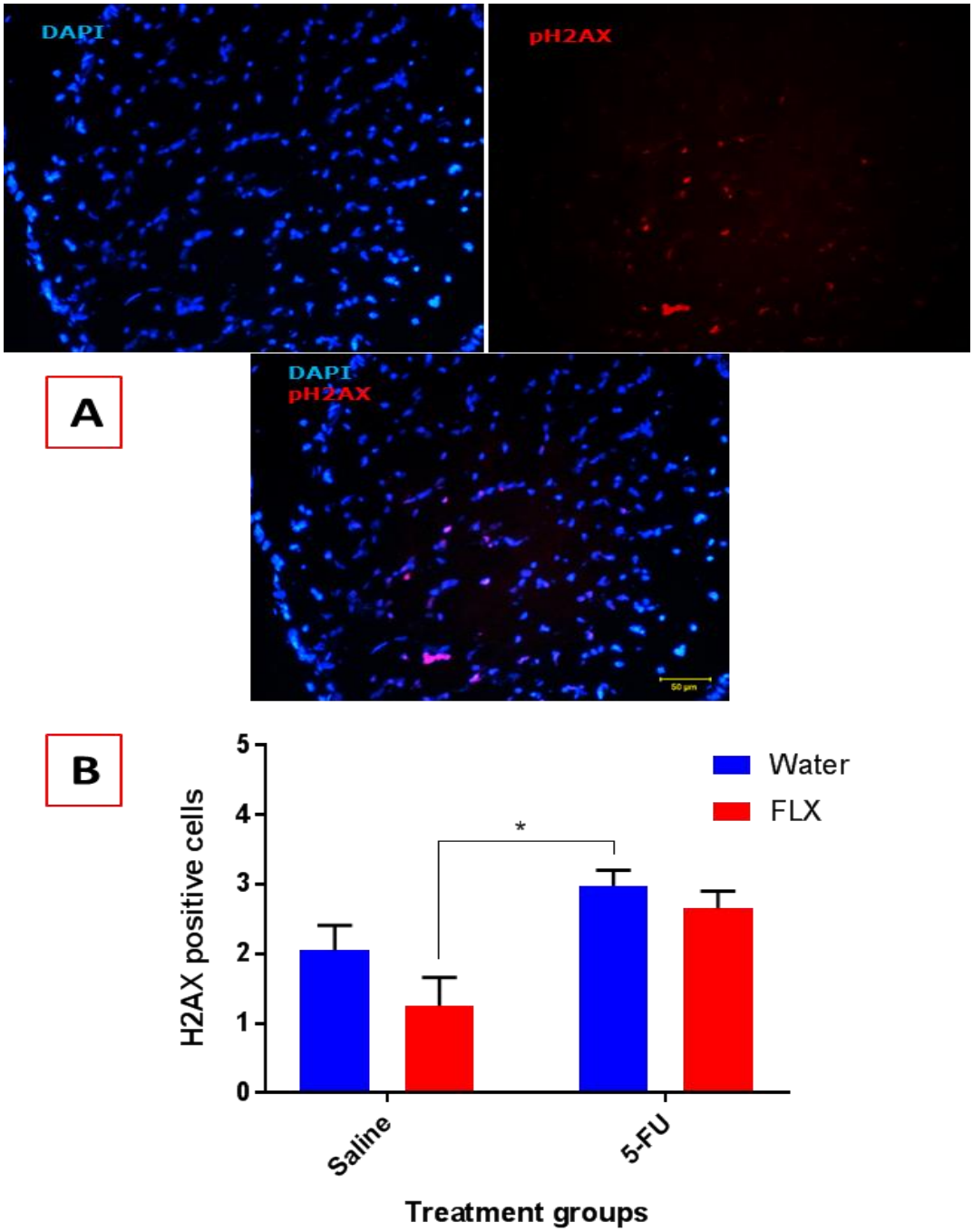



***Figure 2.17*** *The numbers of pH2AX cells in the ON after a single injection of 5-FU and chronic FLX treatment (study one). (A) A representative image of rat ON, cells containing DNA damages are visualized by immunostaining for pH2AX (red) and counterstained with DAPI (blue) and merged. Scale Bar=50µm. (B) The total number of H2AX+ cells per section in different treatment groups (mean± SEM, n=4) were estimated. There was no significant difference between the FLX, 5-FU, FLX+5-FU, and saline treated groups (p>0.05). The groups receiving 5-FU show a significant difference from the FLX group (*P=0.0116). Also, the group receiving FLX+5-FU had significantly higher numbers of H2AX+ cells than the FLX group (*p=0.0408, Bonferroni post-hoc multiple comparison two-way ANOVA).*

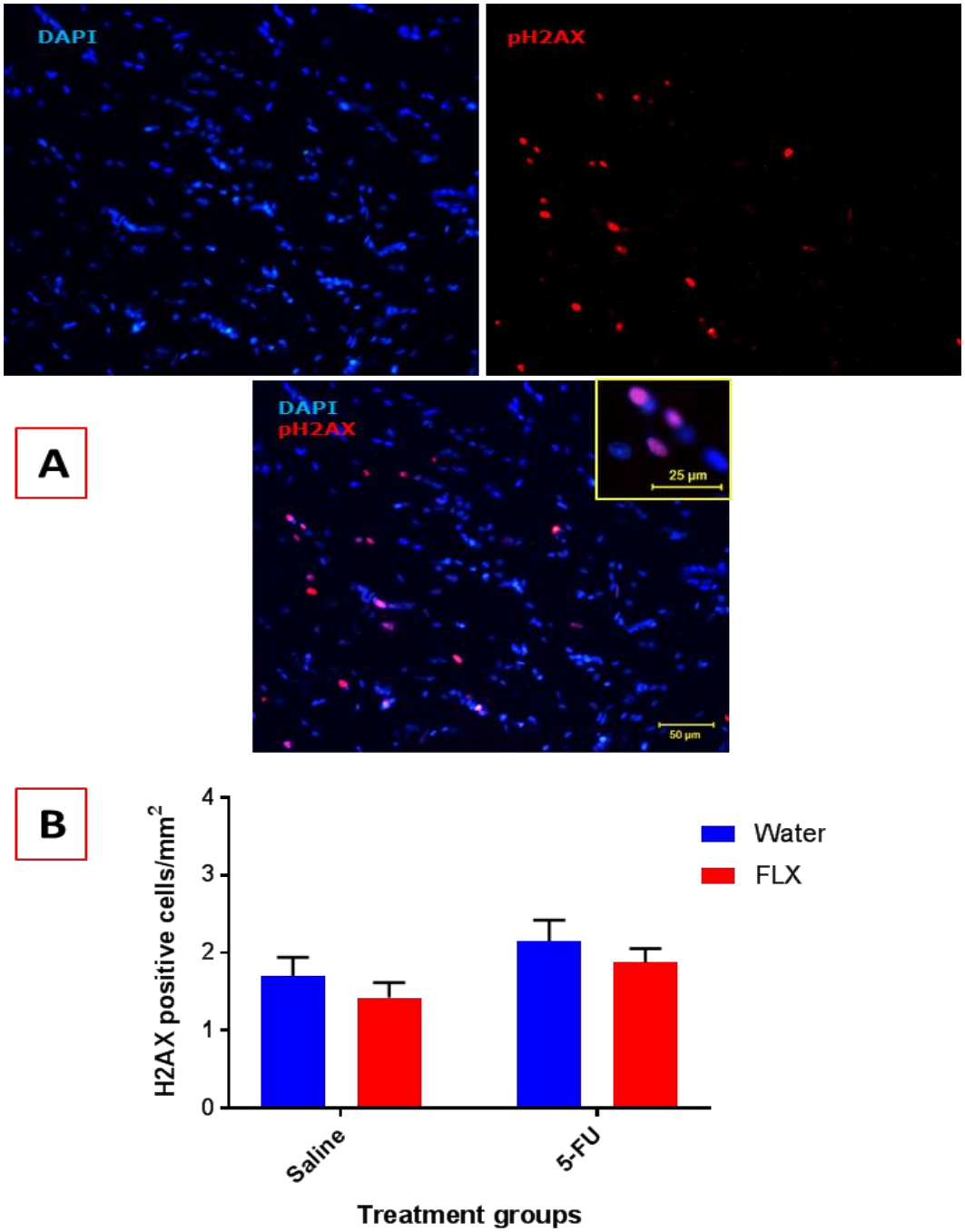



***Figure 2.18*** *The numbers of gamma H2AX cells in the CC after a single injection of 5-FU and chronic FLX treatment (study one). (A) A representative image of rat CC, DNA damaged cells are immunostained for pH2AX (red) and counterstained with DAPI (blue) and merged. Scale Bar=50µm. (B) The average number of H2AX+ cells per mm2 in different treatment groups (mean ± SEM, n=4) were estimated from positive cell count. Multiple comparison two-way ANOVA revealed no significant difference between the FLX, 5-FU, FLX+5-FU, and saline treated groups (p>0.05).*

### 2.4.2 Study two (Effect of a single injection of 5-FU analysed one week recovery after injection).

#### 2.4.2.1 FLX and 5-FU treatments reduce in weight gain during experiment period

To investigate the effect of FLX and 5-FU on weight gain, the weight of animals was monitored daily throughout the treatment. During the administration, the FLX treatment rats showed a significant decrease in weight gain when compared to those drinking only tap water ($F_{(3, 28)}$ =4.299, P=0.0129, two-way ANOVA). Overall animal weights significantly increased over time for all groups ($F_{(20, 560)}$ =897.9, P<0.0001; two-way ANOVA). The interaction between the time and the treatment was significant ($F_{(60, 560)}$ = 3.181, P<0.0001) (Figure 2.19). Animals treated with a single injection of 5-FU showed, in the 24 hours after the injection, a drop in weight which recovered over the following days but remained significantly below control weights (P=0.0423). Animals co-administrated FLX and 5-FU showed a highly significant decrease in their weight compared to controls by the end of the study (P=0.0001, repeated measures of two-way ANOVA) (Figure 2.20).

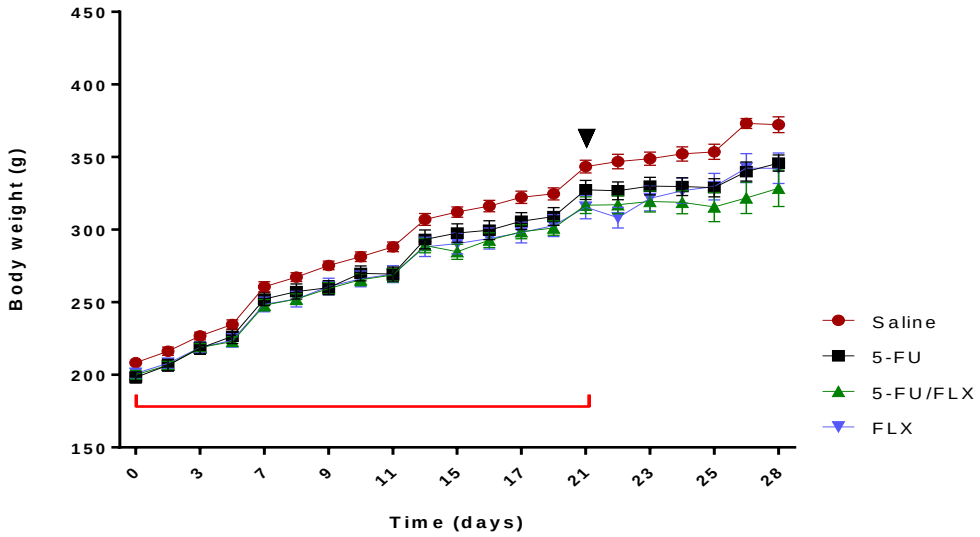



***Figure 2.19*** *Body weight for each experimental group in study two (Mean± S.E.M, n=8 each group). Animals were weighed daily throughout the experimental period (day 0-28). FLX treatment was from day 0 to day 21 (red line) and a single injection of 5-FU was given on day 21 (arrow head). Animals then had a one week recovery period from day 22 to day 28. Two-way ANOVA analysis showed an overall weight gain over the period of the experiment (****P<0.0001), and Bonferroni post-hoc multiple comparison testing showed an interaction between time and treatment which was highly significant (****P<0.0001). Both FLX and FLX+5-FU animals showed a significantly lower weight gain compared to controls during the treatment period of FLX. Also the 5-FU group showed a significant reduction in body weight comparing to control group after the 5-FU injection (*P=0.0423).*

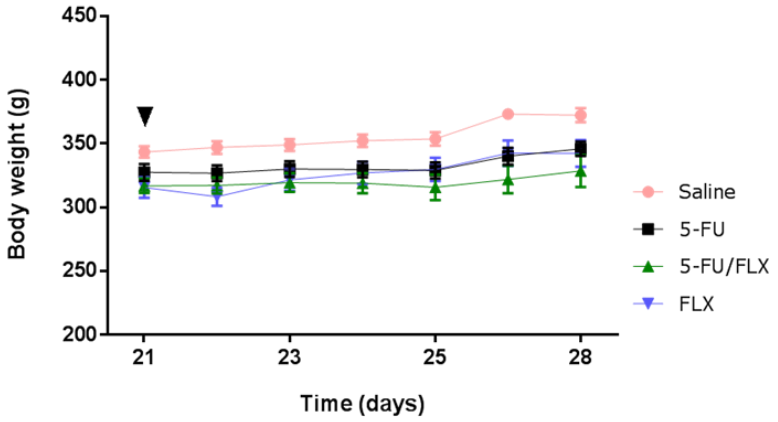



***Figure 2.20*** *Body weight changes in the week after 5-FU injection in study two (Mean± SEM, n=8 each group) between day 21 and 28. Animals in 5-FU and FLX + 5-FU groups received a single injection of 5-FU (35mg/kg) on day 21 (arrow), while the animals of control and FLX received the saline injection at same time. Animals then had a recovery period for one week (day 22 to day 28). FLX caused significant reductions in body weight compared to the saline group by the end of the experiment (*P=0.0142). The single 5-FU injection caused a significant reduction in weight in 5-FU group at day 22 (*P=0.0423) and day 28 (**P=0.0040). The FLX+5-FU group showed a significant drop in weight 24 hours after the injection (***P=0.0009) and after one week of recovery (****P<0.0001, two-way repeated measures multiple comparison two-way ANOVA).*

#### 2.4.2.2 The effects of FLX and 5-FU treatments on water consumption in study two

As in study one, water intake was measured for each experimental group in study two by weighing water bottles daily. Drinking water containing FLX was administered to the FLX and the 5-FU+FLX groups for 21 days before injection of 5-FU. All groups after 5-FU or saline injections received tap water during the one week recovery period (day 21 to day 28). Although water intake fluctuated within and between groups, there was a highly significant decline in water intake for those groups receiving FLX in their water ($F_{(1.606, 33.72)} = 14.57$, $P<0.0001$; one-way ANOVA). This decline in water intake extended throughout the period of FLX administration but was particularly prominent from day 5 and continued to the recovery period (Figure 2.21A). The Bonferroni post hoc test showed a significant reduction in mean water consumption in FLX group (P=0.0003) and in FLX-5-FU group (P=0.0024) during the recovery period compared to controls (Figure 2.21B). There was no significant difference in the water consumption between controls and 5-FU treated groups, although there was a tendency for a progressive reduction in the mean of water intake in the 5-FU treated group during the recovery period (P=0.0587).

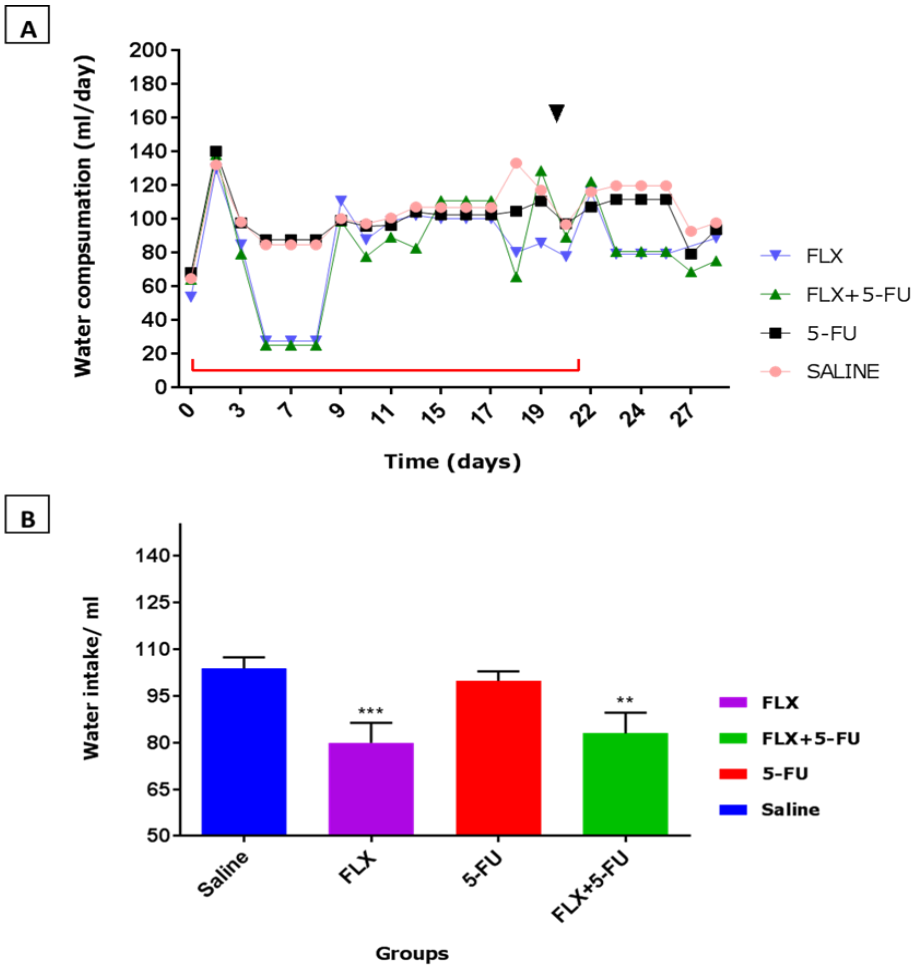



***Figure 2.21*** *Water intake for each group recorded in study two.* ***A****. Daily water consumption from day 0 shown in ml/day. The red line indicates the period of FLX administration in the drinking water of the FLX treated groups, and the arrow indicates the day of 5-FU or saline injection. Between day 22 and day 28 is the recovery period (no treatments). Treatment and time significantly affected the amount of water consumption (P<0.0001, one-way repeated measures multiple comparisons two-way ANOVA).* ***B****. The difference in mean water intake between treatment groups. Rats treated with FLX throughout experiment period showed a significantly lower water consumption compared to the other groups (F (1.606, 33.72) = 14.57, P<0.0001; one-way ANOVA). Both FLX and FLX+ 5-FU groups showed a significant reduction in the mean volume of water intake compared to controls (***P=0.0003 and **P=0.0024 respectively). In contrast, no significant difference was found between the 5-FU and the saline treated groups (P=0.0587).*

#### 2.4.2.3 The effects of 5-FU and FLX treatments on cell Proliferation in the ON and CC after one week of recovery (study two)

The recovery period extended for one week after pre-treatment with FLX and a single injection of 5-FU. As shown in Figure 2.22, there was no significant difference in cell proliferation between 5-FU treated groups and controls ($F_{(1, 28)} = 0.02846$, P=0.8672). The data showed a significant decrease in Ki67+ cells in the FLX treated group as compared to control animals (p=0.0167, two-way ANOVA).

Similarly in the CC there was no significant interaction between 5-FU and FLX treatments across all study groups ($F_{(1, 28)} =0.2362$, P= 0.6308; two-way ANOVA). After one week of recovery, cell proliferation was the same as controls (P=0.6727, two-way ANOVA). Additionally, FLX appeared to have no significant effect either on the FLX group (P=0.5038) or FLX+5-FU group, (P=0.1401), when compared with the control group (Figure 2.23).

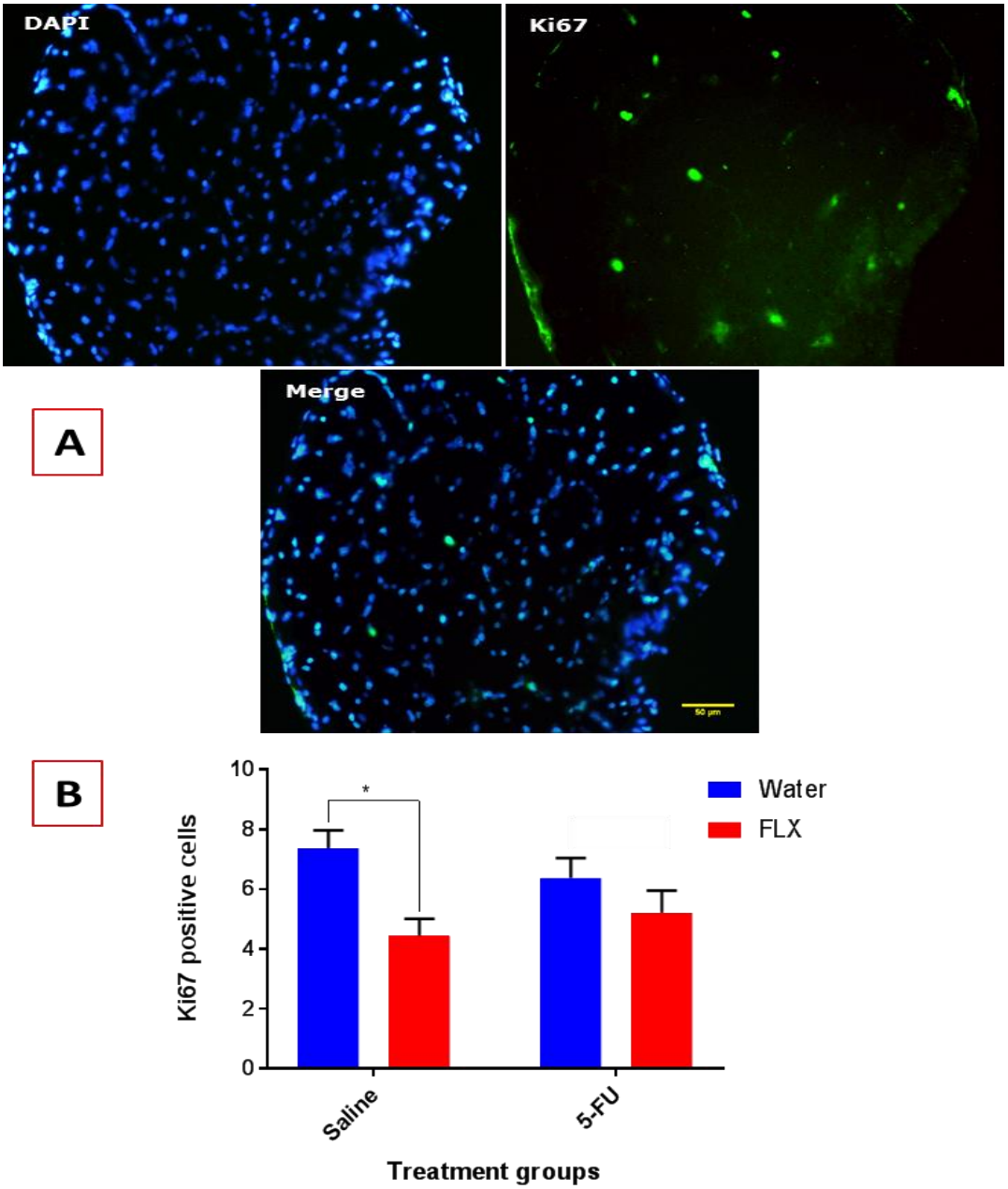



***Figure 2.22*** *Cell proliferation in the ON one week after 5-FU injection (study two). (A) A representative image of rat ON proliferating cells are immunostained for Ki67 (green) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50μm. (B) The total number of Ki67+ cells per section in different treatment groups (mean ± SEM, n=8). There was no significant difference between the 5-FU or FLX+5-FU groups and saline treated groups (p>0.05, two-way ANOVA). Bonferroni post-hoc analysis showed a significant decrease of Ki67+ cells in the FLX treated group (*p=0.0167) compared with the saline-control group.*

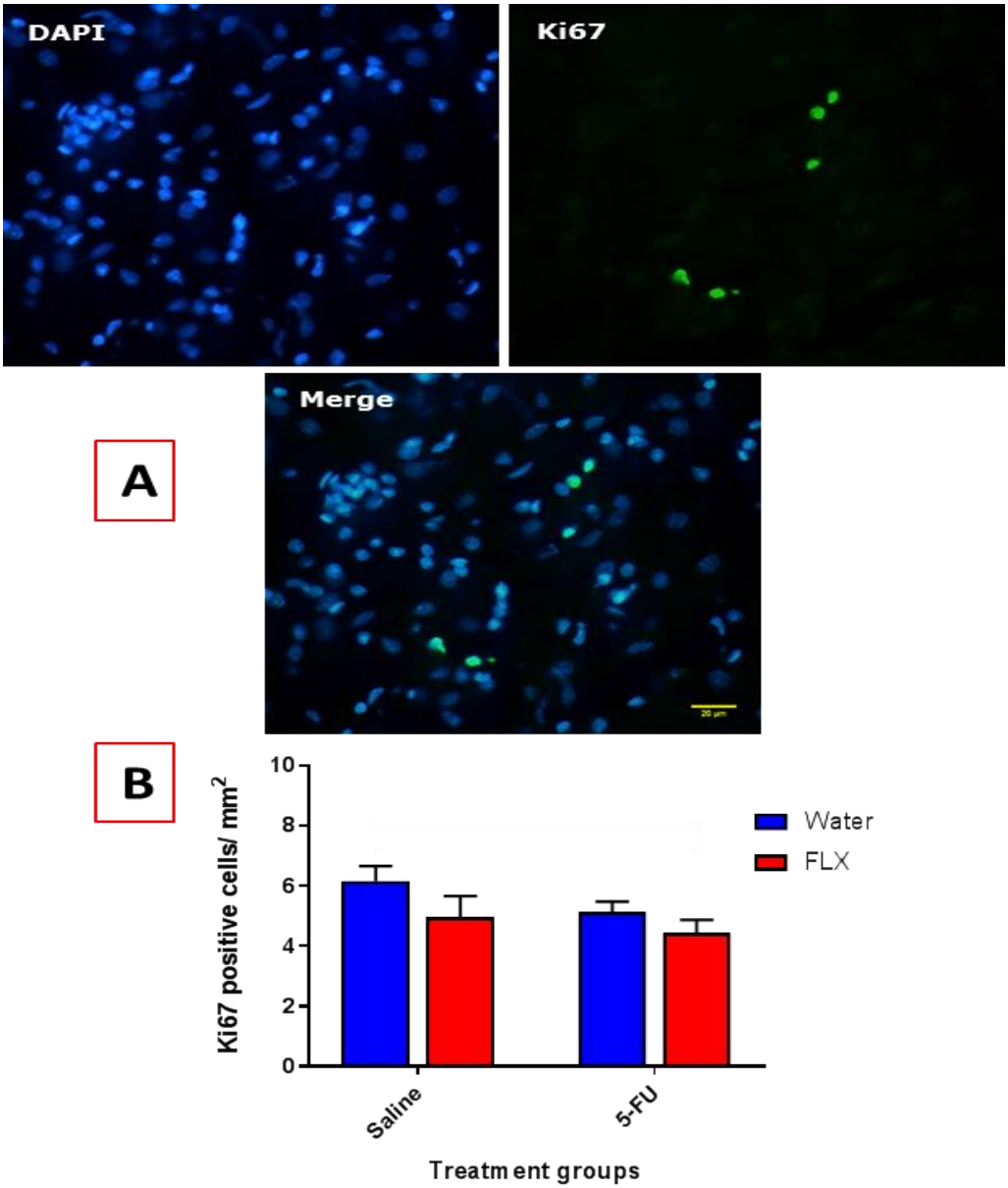



***Figure 2.23*** *Cell proliferation in the CC one week after 5-FU injection (study two). (A) A representative fluorescent image of rat CC. Proliferating cells are immunostained for Ki67 (green) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=20μm. (B) The average number of proliferating cells per mm2 in different treatment groups were estimated from Ki67+ cell counts (mean ± SEM, n=8). There was no significant difference between the FLX, 5-FU, FLX+5-FU and saline treated groups (p>0.05, two-way ANOVA).*

#### 2.4.2.4 The effect of acute 5-FU and chronic FLX treatments on the number of OPCs in the ON and CC after one week of recovery after 5-FU injection (study two)

The ON and CC are regions of white matter tracts which contain proliferating oligodendrocyte progenitor cell populations. Both FLX pre-treatment and the acute effect of 5-FU treatment were studied after a recovery period using an unbiased counting method which showed significant differences with treatment in the number of OPCs in both regions as detected by NG2 immunolabeling. In the ON, both FLX and 5-FU treatment showed significant effects in all treated groups ($F_{(1, 28)}$ =17.75, P=0.0002 and $F_{(1, 28)}$ =5.18, P=0.0307 respectively). Compared to control animals, there was a significant increase in the mean number of cells expressing NG2 positive per section in animals treated with 10 mg/kg of FLX for three weeks (P=0.0301, two-way ANOVA). This suggests that FLX administration and one week of recovery stimulates the proliferation of OPCs in the ON. Although there was no significant difference between the 5-FU treated group and the saline treated control group (P=0.3773), multiple comparisons two-way ANOVA showed a significant decrease in NG2+ cell numbers compared to the FLX treated group (P=0.0005). This suggests that the acute 5-FU regimen had little effect on the numbers of OPCs in the ON one week after injection. However FLX appears to stimulate OPC proliferation, both when given on its own and in combination with 5-FU (P=0.0270), a result similar to that found one day after 5-FU injection (Figure 2.24).

When the CC was analyzed, Bonferroni post-hoc analysis showed that there was a significant effect of 5-FU treatment in all treated groups ($F_{(1, 27)}$ = 55.58, P<0.0001) (Figure 2.25). 5-FU significantly reduced the number of NG2+ cells in 5-FU group compared to the saline control group (P<0.0001, two-way ANOVA). Chronic pre-administration of FLX in the FLX+5-FU group significantly increased OPC numbers compared with the 5-FU alone group but did not restore OPC numbers to control levels (P=0.1161) after one week of recovery. Treatment with FLX alone showed a significant increase compared with the control group (P= 0.0245). Overall, Bonferroni post-hoc analysis showed that FLX treatment significantly affected all FLX treated groups ($F_{(1, 27)}$ =16.84, P=0.0003).

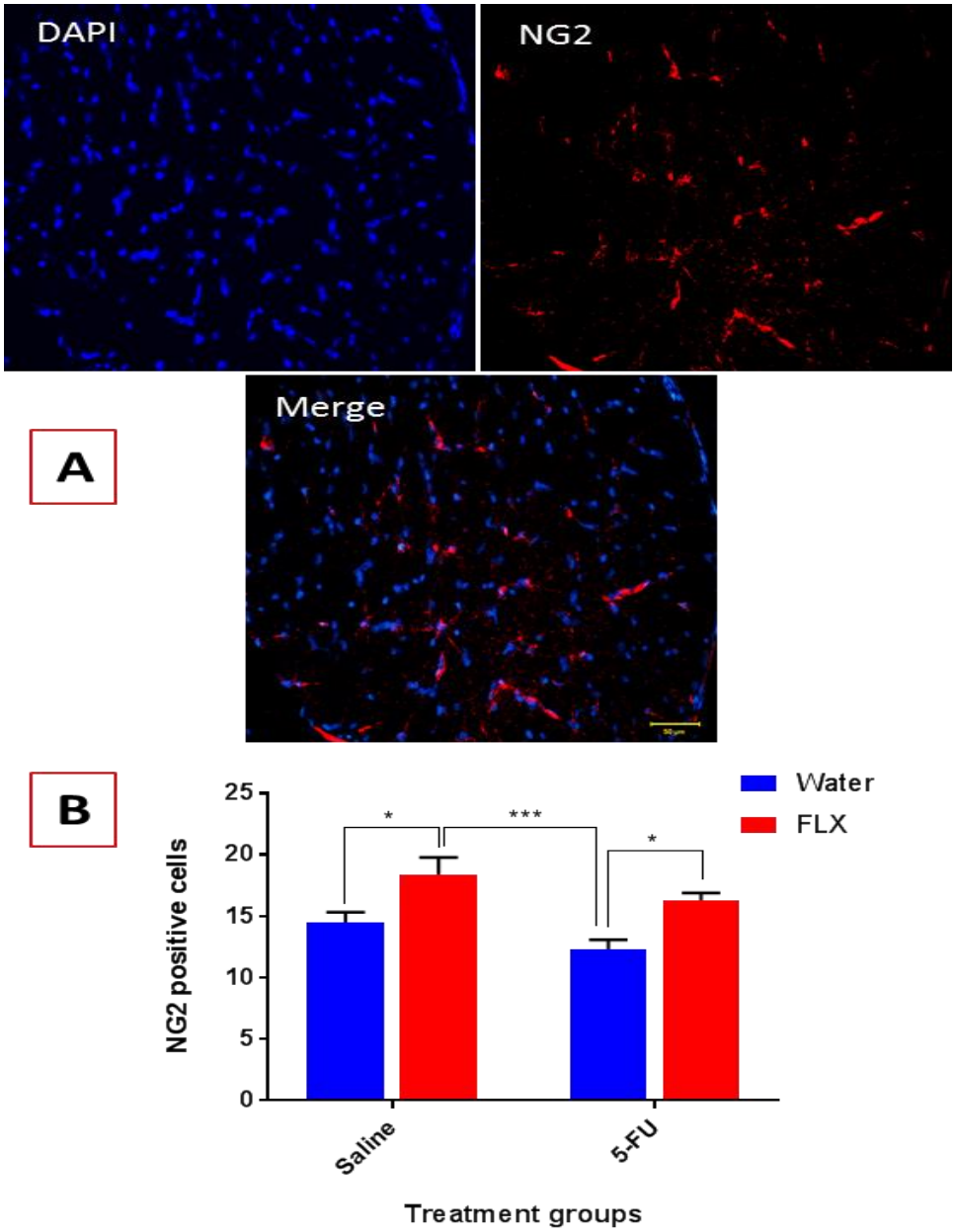



***Figure 2.24*** *NG2+ cells in the ON one week after 5-FU injection (study two). (**A**) A representative image of rat ON, NG2+ cells were immunostained in red and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) The total number of NG2+ cells per section in different treatment groups (mean ± SEM, n=8) was estimated from cell counts. The groups receiving FLX showed a significant increase in NG2+ cells from the control group (*P= 0.0301, two-way ANOVA). There was no significant difference between the 5-FU and saline treated groups (p>0.05). Rats receiving FLX+5-FU had significantly higher NG2+ cells than the 5-FU group (*P=0.0270, Bonferroni post-hoc multiple comparison two-way ANOVA). There was a significantly lower number of OPCs in the 5-FU treated animals compared to the FLX group (***P=0.0005, Bonferroni post-hoc test).*

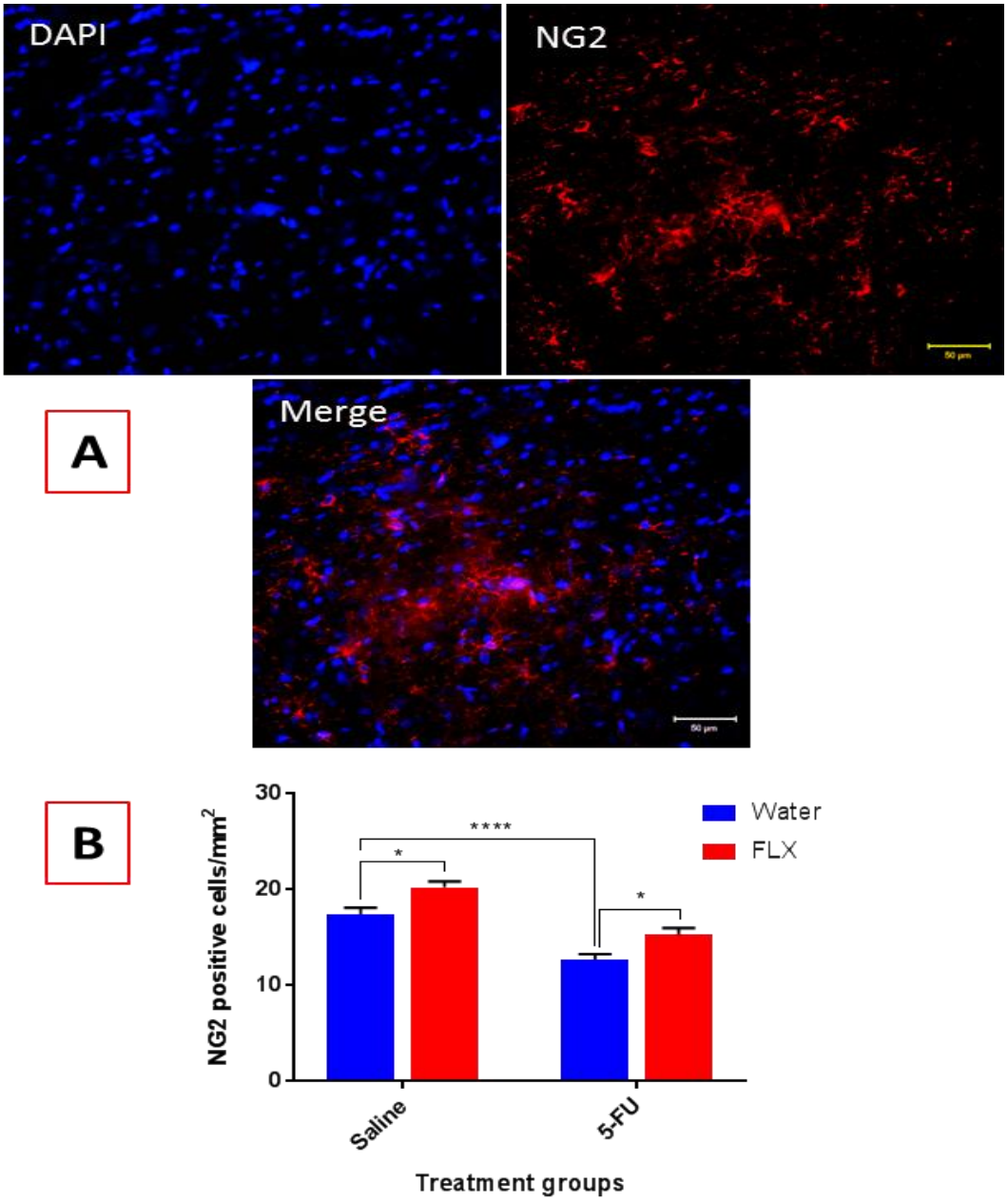



***Figure 2.25*** *NG2+ cells in the CC one week after 5-FU injection (study two). (**A**) A representative images of rat CC; progenitor cells are immunostained for NG2 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) The average number of OPCs per mm2 in different treatment groups (mean ± SEM, n=8). Rats receiving 5-FU had significantly fewer NG2+ cells (****p<0.0001, two-way ANOVA) in the CC and rats receiving FLX had significantly more (*P= 0.0245, two-way ANOVA) than the saline-treated control group. Rats treated with both FLX and 5-FU did not significantly differ from the controls but had significantly more NG2+ cells than the 5-FU alone group (*P=0.0478, Bonferroni post-hoc multiple comparison two-way ANOVA).*

#### 2.4.2.5 The effect of a single injection of 5-FU and chronic FLX treatment on the total number of microglia in the ON and CC one week after injection (study two)

The effect of 5-FU with or without FLX on the numbers of microglia was quantified from sections. Iba-1 staining of the ON showed no difference between all treated groups and controls ($p > 0.05$, two-way ANOVA). After multiple comparison analysis, two-way ANOVA indicated a significant difference between the groups treated with FLX ($F_{(1, 28)} = 10.23$, $P=0.0034$). Further analysis with a Bonferroni post-hoc test revealed that rats treated with 5-FU had a significantly increased number of Iba-1+ cells comparing to rats treated only with FLX group ($p=0.0039$). In contrast, rats treated with FLX+5-FU had less Iba-1+ cells compared with the rats treated with only 5-FU ($P=0.0348$) (Figure 2.26).

As shown in Figure 2.27, Iba-1 staining of the CC. There was a significant reduction in the total number of Iba-1- positive cells in the 5-FU treated group compared to saline-treated rats ($P= 0.0018$, two-way ANOVA). This difference was abolished by co-treatment with FLX ($P= 0.7638$, two-way ANOVA). While FLX alone significantly increased the total number of Iba-1- positive cells compared to controls ($P= 0.0396$, two-way ANOVA). These results indicate that the number of microglia in the CC was decreased one week after a single injection of 5-FU. Co-administration of FLX with 5-FU prevented this decrease. The reduction in microglial number in the CC suggests that 5-FU treatment increases cell death not just in the OPCs population but also in microglial cell populations.

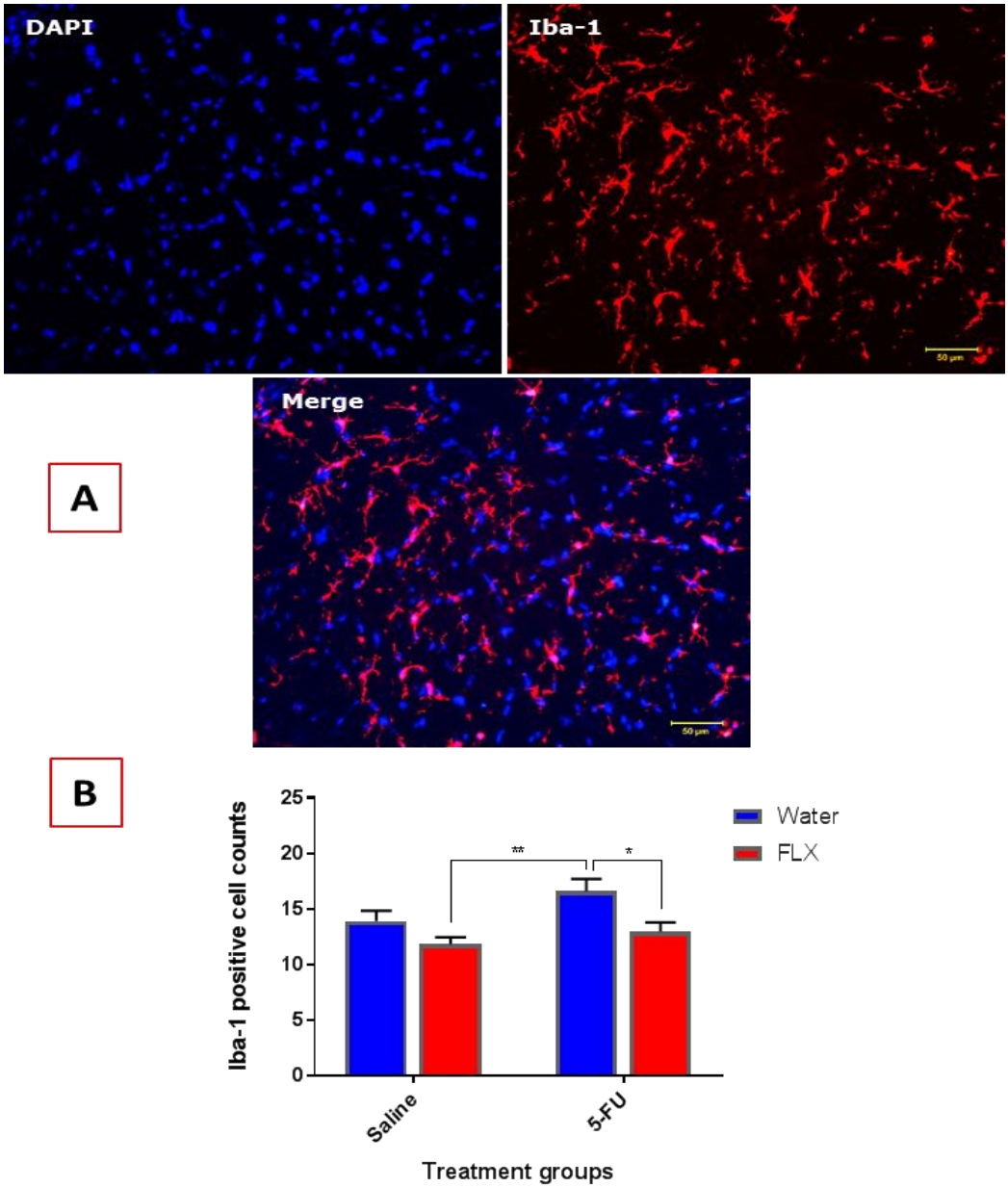



***Figure 2.26*** *Iba-1+ cells in the ON one week after a single 5-FU injection (study two). (**A**) Representative image of rat ON. Microglia are immunostained with Iba-1 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) Shows the average number of Iba-1 cells per section (mean ± SEM, n=8) in adult rat ON in different treatment groups one week after a single injection of chemotherapy. Analysis showed a non-significant difference in microglia number in the 5-FU treatment group (p= 0.1603, two-way ANOVA) and in FLX group (P= 0.3787, two-way ANOVA) compared with the saline-control group. The groups receiving 5-FU show a significant difference from the FLX group (**P=0.0039, Bonferroni post-hoc multiple comparison two-way ANOVA).*

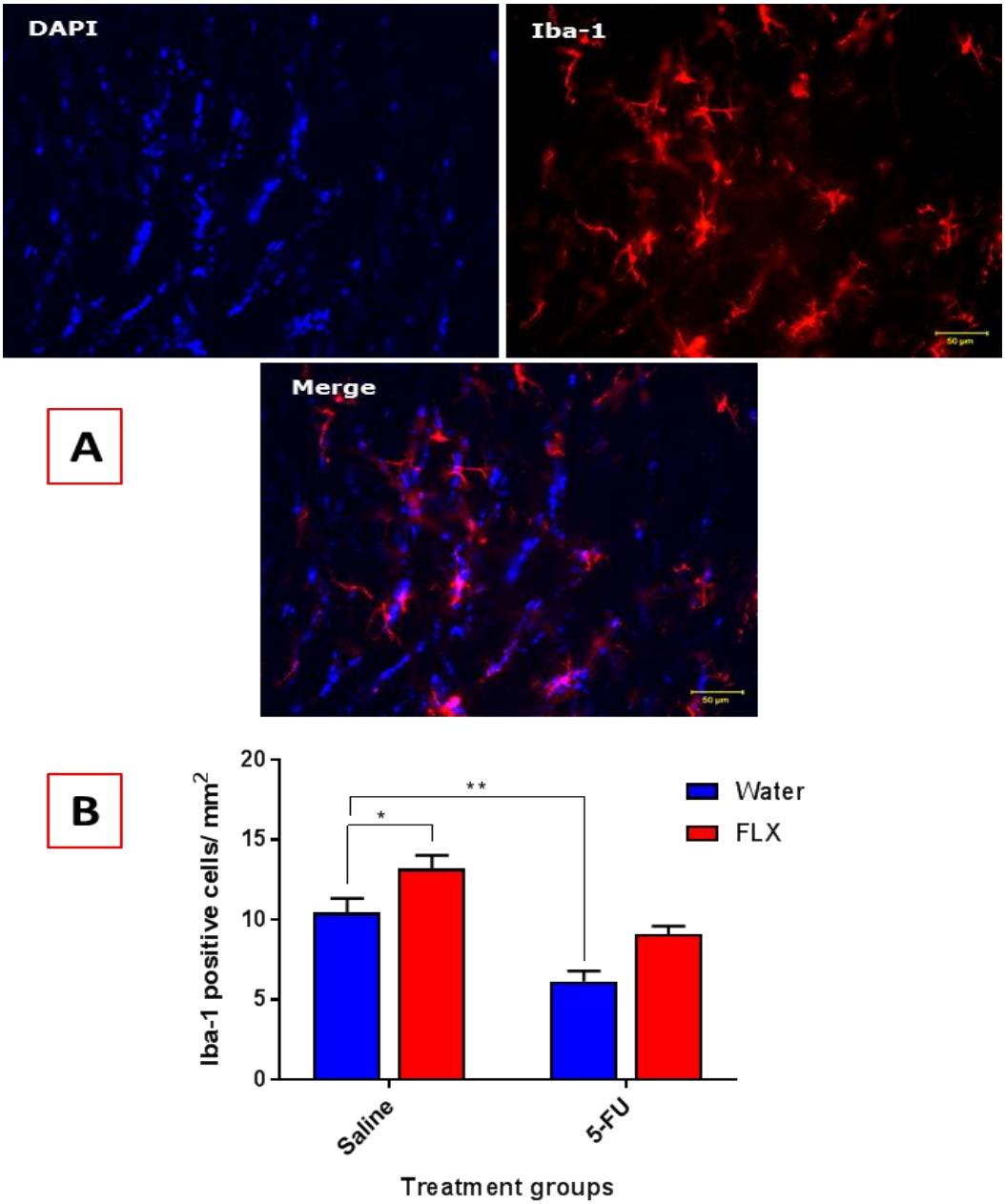



***Figure 2.27*** *Iba-1+ cells in the CC one week after a single 5-FU injection (study two). (**A**) Representative image of rat CC after FLX and/or 5-FU treatment, immunostained with Iba-1 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) Shows the average number of Iba-1+ cells per mm2 (mean ± SEM, n=8) in adult rat CC in different treatment groups one week after a single injection of 5-FU. Analysis showed a significant decrease of Iba-1 expression cells in the 5-FU treated group (**p=0.0018, two-way ANOVA) compared with the saline-control group. The group receiving FLX alone significantly differed from the controls (*P=0.0396). Data also shows the group receiving 5-FU and FLX did not experience the same decline in Iba-1+ cell numbers as the 5=FU alone group and were not significantly different from controls (p>0.05, two-way ANOVA).*

#### 2.4.2.6 The effect of 5-FU and FLX treatments on the inflammatory response (Cox-2 expression) in the ON and CC one week after a single injection of 5-FU (study two)

To determine if 5-FU was inducing inflammation within the WMTs, the numbers of Cox-2 positive cells was quantified in the ON and CC one week after single injection of 5-FU with or without FLX. The number of COX-2 positive cells was significantly elevated in the ON of the 5-FU treated group compared to the control group (P=0.0004, two-way ANOVA). There was no significant difference in COX-2 positive cells with FLX treatment (P=0.9714). Analysis showed that there was no significant difference in the number of COX-2 positive cells in animals treated with FLX+5-FU compared to the saline group (P= 0.2160, two-way ANOVA) (Figure 2.28). This result suggests that pretreatment with FLX reduced the inflammatory effect of 5-FU one week after injection.

In the CC, 5-FU also significantly increased the number of COX-2+ cells compared with the saline group (P=0.0087, two-way ANOVA). Co-administration of FLX with 5-FU showed no significant difference in Cox-2+ cells from control group ($p > 0.05$, two-way ANOVA). Figure 2.29 shows that treatment with FLX alone did not produce any significant difference from the control group (P=0.9123). Bonferroni post-hoc analysis showed that FLX and 5-FU treatments significantly affected all treated groups one week after 5-FU injection ($F_{(1, 28)} = 6.19$, P=0.0191 and $F_{(1, 28)} = 11.23$, P=0.0023 respectively). This indicates that 5-FU chemotherapy elevated the inflammatory response in the CC while co-administration with FLX, before injection of 5-FU, prevented this change.

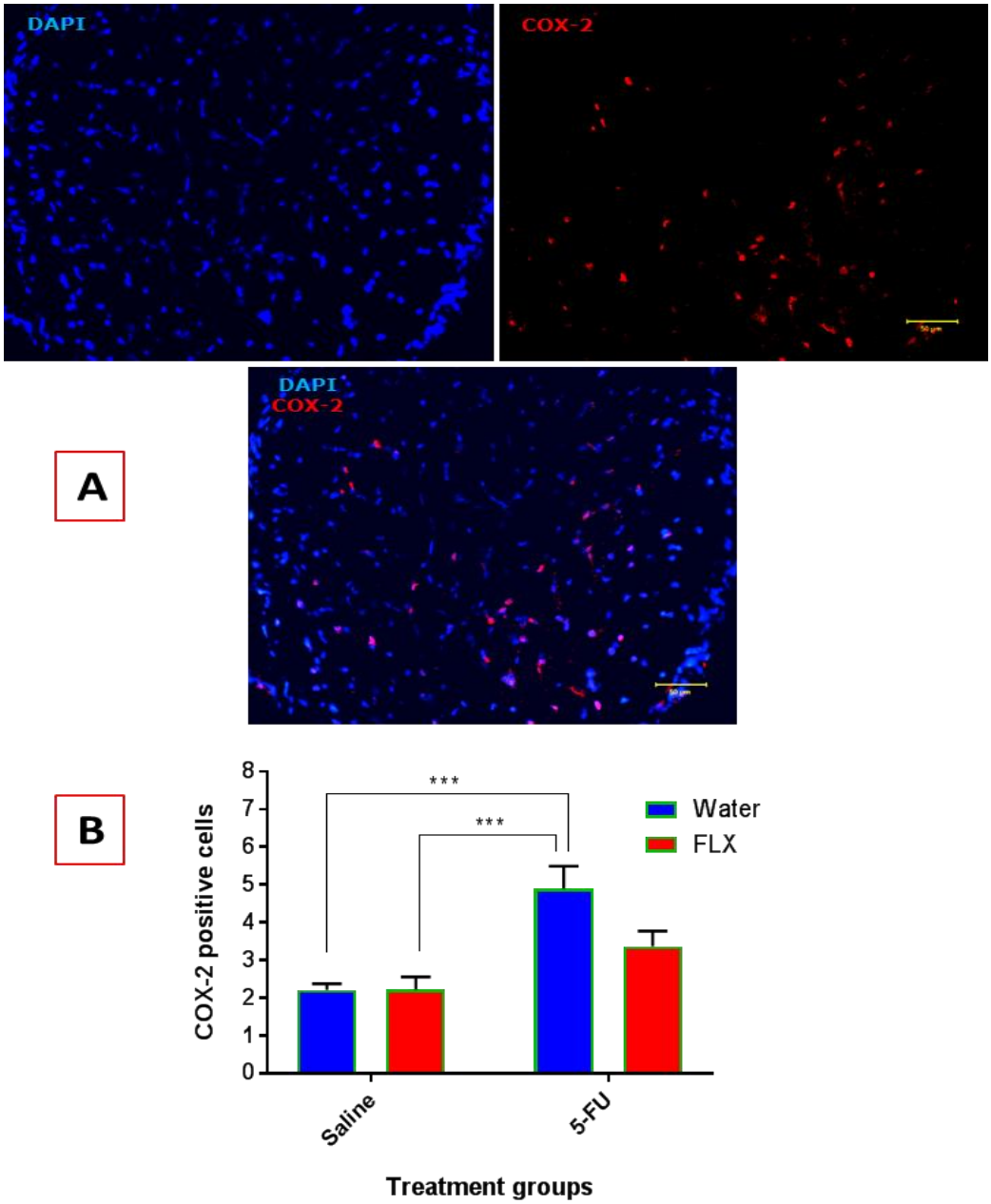



***Figure 2.28*** *Cox-2+ cells in the ON one week after a single 5-FU injection (study two). (**A**) Representative image of rat ON immunostained with COX-2 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) Shows the average number of COX-2+ cells per section (mean ± SEM, n=8) in adult rat ON in different treatment groups one week after a single injection of 5-FU. Analysis showed a significant increase in the number of COX-2 expressing cells in the 5-FU treated group (***P=0.0004, two-way ANOVA) compared with the saline-control group. The groups receiving FLX did not significantly differ from the controls and had significantly fewer COX-2+ cells than the 5-FU group (***P=0.0004; Bonferroni post-hoc multiple comparison analysis). The group receiving 5-FU and FLX had no significant difference in the number of positive cells compared with controls (p>0.05, two-way ANOVA).*

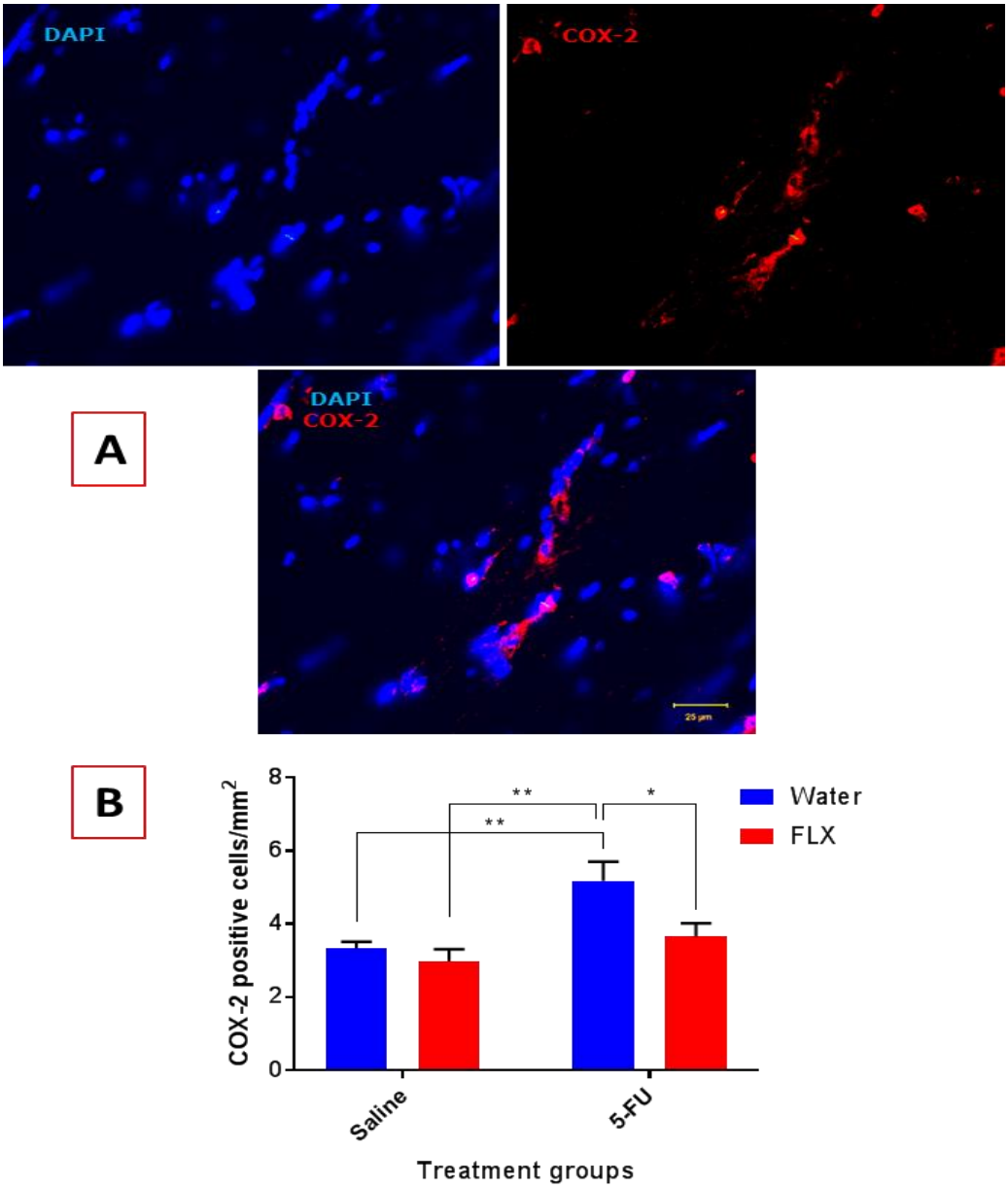



***Figure 2.29*** *Cox-2+ cells in the CC one week after a single 5-FU injection (study two). (A) Representative image of rat CC immunostained for Cox-2 with COX-2 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=25µm. (B) Shows the average number of COX-2 + cells per mm2 (mean ± SEM, n=8) in adult rat CC in different treatment groups one week after a single injection of 5-FU. Analysis showed a significant increase in the number of COX-2 expressing cells in the 5-FU treated group (**p=0.0087, two-way ANOVA) compared with the saline-control group. The groups receiving FLX did not significantly differ from the controls (p>0.05) but had significantly fewer COX-2 + cells than the 5-FU group (**P=0.0016, Bonferroni post-hoc multiple comparison two-way ANOVA). Data also shows no change in the number of COX-2 with co-administration of FLX+5-FU compared with controls (p>0.05, two-way ANOVA). The group having co-administration FLX+5-FU had significantly fewer numbers of positive cells than the 5-FU group (*P=0.0373, multiple comparison two-way ANOVA).*

#### 2.4.2.7 The effect of 5-FU in causing DNA damage in the ON and CC after one week of injection (study two)

As described previously, staining for pH2AX was used as a marker to detect the phosphorylation of histone 2 which indicates DNA damage. In the ON, 5-FU treatment caused a significant increase in DNA damage which persisted for at least one week after 5-FU injection ($F_{(1, 28)} = 26.37$, $P<0.0001$; two-way ANOVA with Bonferroni post-hoc test). The statistical analysis shows that there was no significant difference between the FLX alone treatment group and the saline control group ($p>0.05$, two-way ANOVA). The 5-FU alone group showed a significant increase in the mean pH2X positive cell count ($P=0.0020$). In contrast, rats co-administrated with FLX+5-FU showed no significant difference from controls ($P=0.4786$, two-way ANOVA) indicating that the FLX had prevented the DNA damage in the ON caused by 5-FU (Figure 2.30).

A two-way ANOVA, showed significant differences between mean pH2AX positive cell counts in CC ($F(1, 28) = 15.74$, $P=0.0005$). Further analysis with a Bonferroni post hoc test revealed that rats treated with 5-FU and FLX+5-FU had a significantly increased number of pH2AX-positive cells ($P=0.0083$ and $P=0.0147$ respectively). In contrast, rats treated only with FLX had no significant difference from control animals ($p>0.05$) (Figure 2.31). This indicates that FLX pre-treatment did not have any protective effect against DNA damage, as positive cell numbers were similar to those seen in the control group. One week of recovery after 5-FU injection, did not show any recovery in the CC.

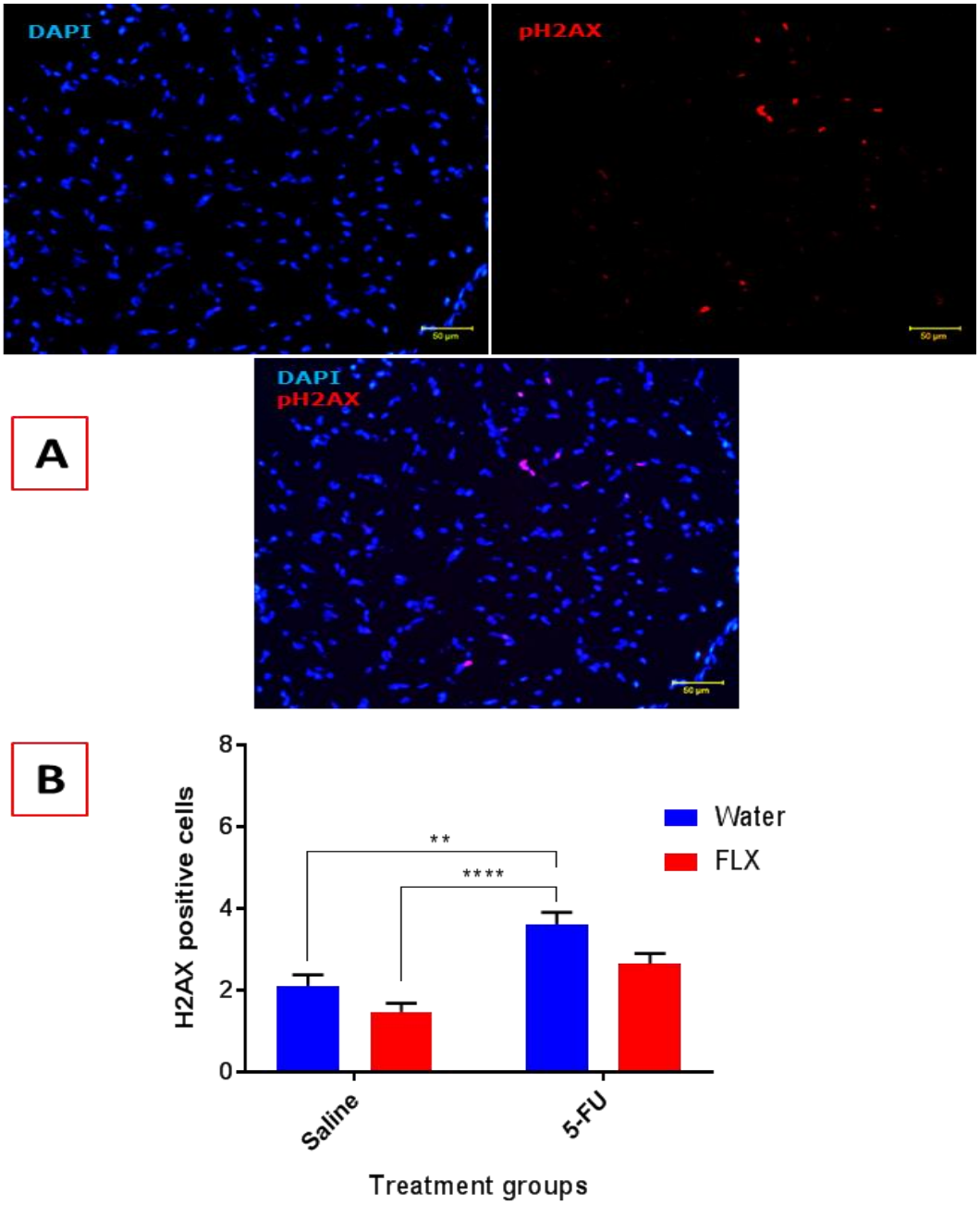



***Figure 2.30*** *pH2AX+ cells in the ON one week after a single 5-FU injection (study two). (**A**) A representative image of rat ON, cells with DNA damage are immunostained for pH2AX (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) The total number of H2AX+ cells per section in different treatment groups (mean ± SEM, n=8). There was no significant difference between the FLX and saline treated groups (p>0.05, two-way ANOVA). The groups receiving 5-FU showed a significant increase in positive staining compared with controls (**P=0.002; Bonferroni post-hoc with two-way ANOVA). Also there was a marked and significant difference between the 5-FU and FLX groups (****P<0.0001, Bonferroni post-hoc multiple comparison two-way ANOVA). The group receiving FLX+5-FU showed no difference in the number of H2AX+ cells compared with controls (p>0.05).*

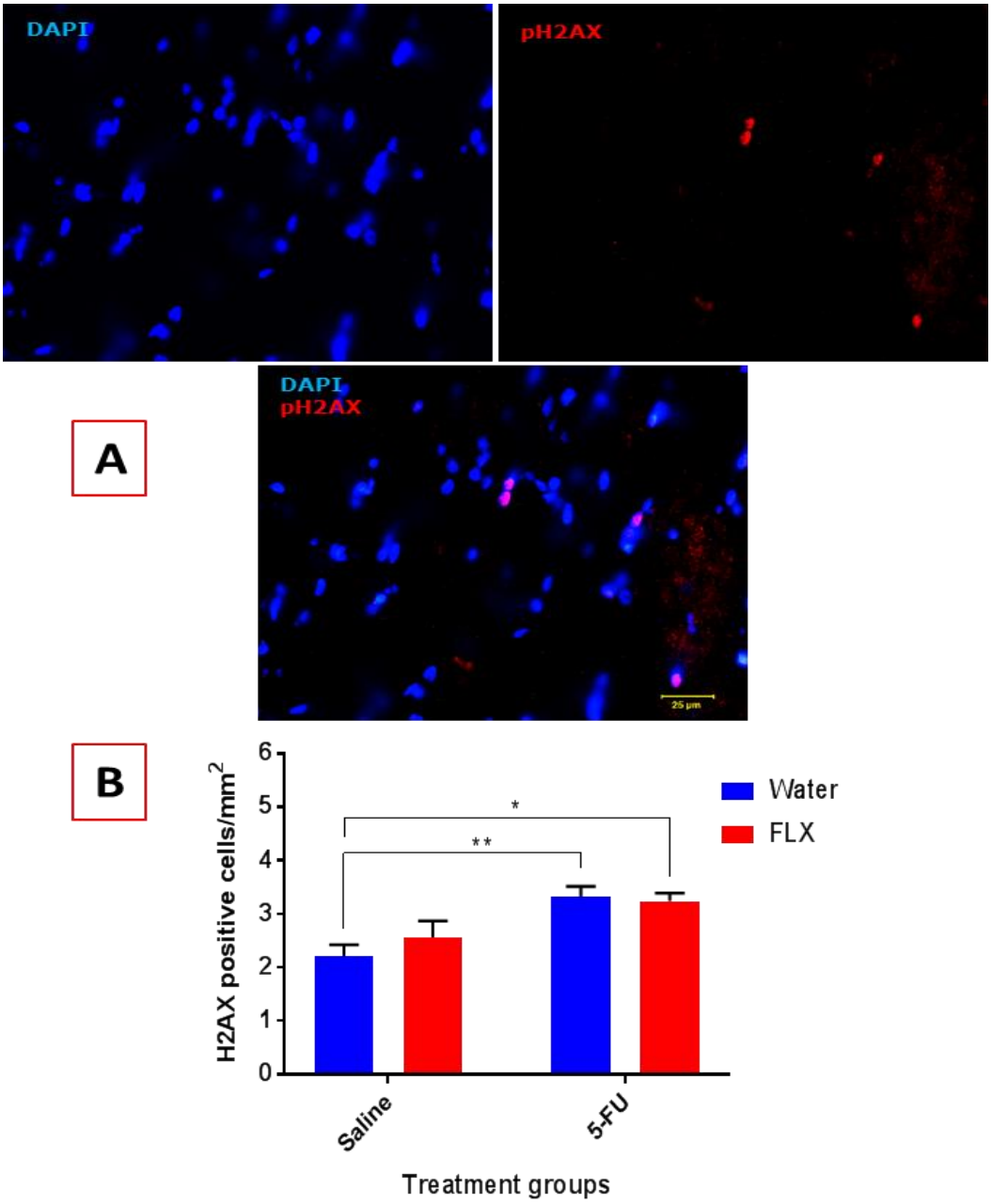



***Figure 2.31*** *pH2AX+ cells in the CC one week after a single 5-FU injection (study two). (**A**) Representative image of rat CC immunostained with pH2AX (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=25µm. (**B**) Shows the average number of pH2AX+ cells per mm2 (mean ± SEM, n=8) in adult rat CC in different treatment groups one week after a single injection of 5-FU. Analysis showed a significant increase in the number of pH2AX expressing cells in the 5-FU treated group (**P=0.0083, two-way ANOVA) compared with the saline-control group. The groups receiving FLX did not significantly differ from the controls (p >0.05). Data additionally shows the group receiving FLX+5-FU had no significant difference in positive cells compared with the 5-FU group (p>0.05). FLX did not reduce the DNA damage when co-administrated with 5-FU group comparing with control group (*P=0.0147, two- way ANOVA with Bonferroni post-hoc test).*

### 2.4.3 Study three (The effect of multiple injections of 5-FU analysed one week after the last injection).

Chemotherapy treated rats received six injections of 5-FU over a period of 16 days with FLX treated groups having had three weeks of FLX administration prior to 5-FU treatment. Animals were put down one week after the final 5-FU injection.

#### 2.4.3.1 The effect of FLX and 5-FU treatments on weight gain during the experimental period in study three.

During the period of study three, animal weights significantly increased over the time ($F_{(29, 198)} = 362.5$, $P<0.0001$, two-way repeated measures ANOVA). Moreover, the interaction between time and treatment was significant ($F_{(87, 594)} = 4.528$, $P<0.0001$) (Figure 2.32). Bonferroni post-hoc analysis showed that the reduction in weight gain after FLX administration on day 41, in those animals receiving FLX ($P=0.0182$), but the weight difference caused by FLX was non-significant throughout the remaining treatment days ($P>0.9999$). As shown in Figure 2.33, multiple 5-FU injections caused a significant weight reduction, compared to vehicle treated controls ($F_{(3, 21)} = 10.24$, $P=0.0002$). Further analysis showed that the saline and FLX treated groups were significantly different from 5-FU and FLX+ 5-FU treated groups starting one day after the start of the 5-FU injections at day 22 and through until the end of recovery period at day 43 ($p<0.0001$, two-way repeated measures ANOVA). These results indicate that FLX cessation in the week after the end of 5-FU injections, lead to the regaining of body weight in the FLX group, while the 5-FU groups also regained body weight but remained lower than the saline group.

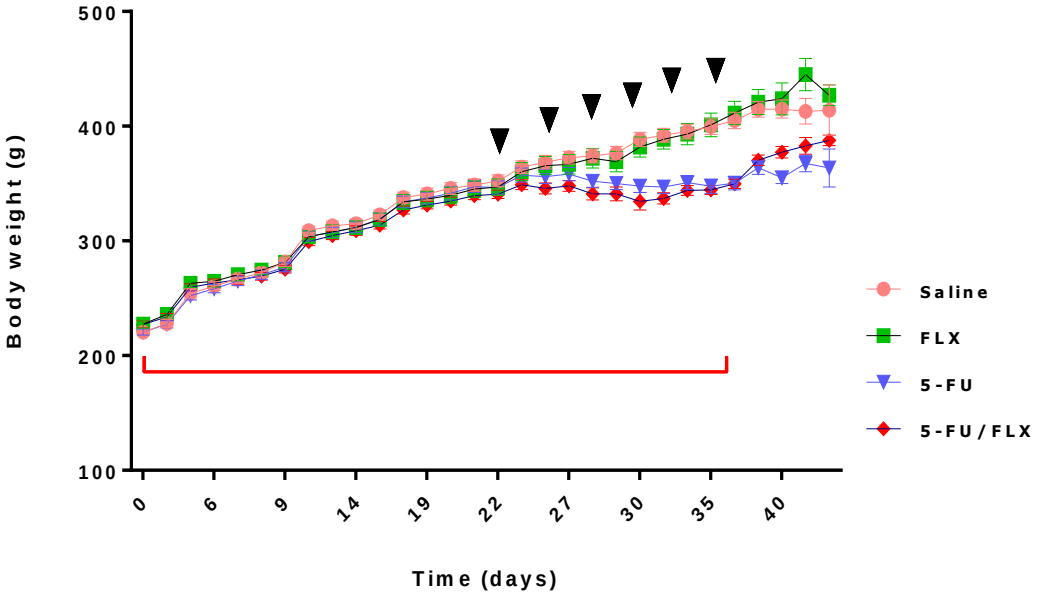



***Figure 2.32*** *Body weight for each experimental group recorded in study three (Mean± S.E.M, n=8 each group). Animals were weighed daily throughout the experimental period (days 0-43). The red line indicates the period of FLX treatment from day 0 to day 36 and the arrow heads indicate the 5 injections of 5-FU from day 21 to day 36. After the last 5-FU injection, animals had a recovery period from day 37 to day 43. Two-way ANOVA analysis shows that overall weight gain significantly increased over the experimental period (****P<0.0001), and post-hoc testing shows that the interaction between time and treatment was highly significant (****P<0.0001). There was no significant difference in weight gain between all treated groups (p>0.05, repeated measures two-way ANOVA). Both 5-FU and FLX+5-FU animals show a significant reduction in their mean body weight compared to the control and FLX groups during the treatment period of 5-FU (***P=0.0002, Bonferroni post-hoc multiple comparison two-way ANOVA).*

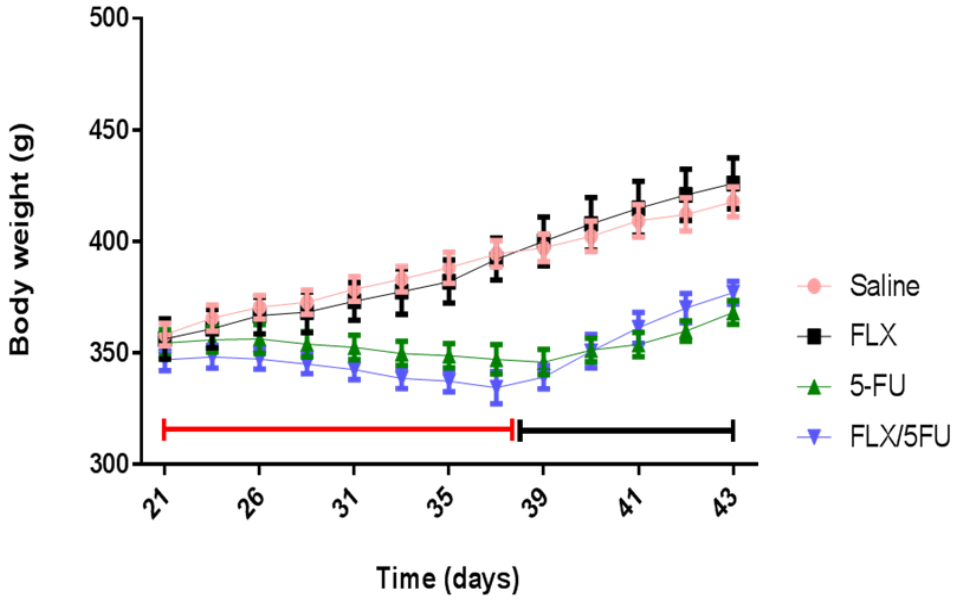



***Figure 2.33*** *The changes in body weight for each experimental group in study three (Mean± SEM, n=8 each group) between day 21 and 43. Animals of 5-FU and FLX+5-FU groups received a six injections of 5-FU (35mg/kg), while the animals of control and FLX groups received saline injections at the same time (red bar). Animals then had a recovery period for one week after the last injection (black bar). The 5-FU injections caused a significant reduction in weight gain in the 5-FU group from day 23 (**P= 0.0033) until day 43 (****P<0.0001, repeated measures two-way ANOVA). Also the FLX+5-FU group showed a significant drop in weight one day after the first injection (***P=0.0005) and throughout the recovery period (****P<0.0001). There was no significant difference between FLX and saline treated groups from day 21 o day 43 (p>0.05, repeated measures two-way ANOVA).*

### 2.4.3.2 The effect of FLX and 5-FU treatments on water consumption in study three.

The average consumption of water per cage was calculated for each group in study three. Treatment significantly affected the amount of water consumed ($F_{(1.576, 33.09)} = 35.97$, P<0.0001, one-way repeated measures ANOVA) during the period of 5-FU treatment and following one week of recovery (Figure 2.34A). The results show a significant difference between the 5-FU treated group and the saline-treated group (P=0.0001). While there was no significant difference in the water intake between FLX and saline-treated groups (P>0.9999, one-way repeated measures ANOVA). Co-administration of FLX and 5-FU showed a significant decline in consumption (P<0.0001, one-way ANOVA with Bonferroni's multiple comparisons test) (Figure 2.34B).

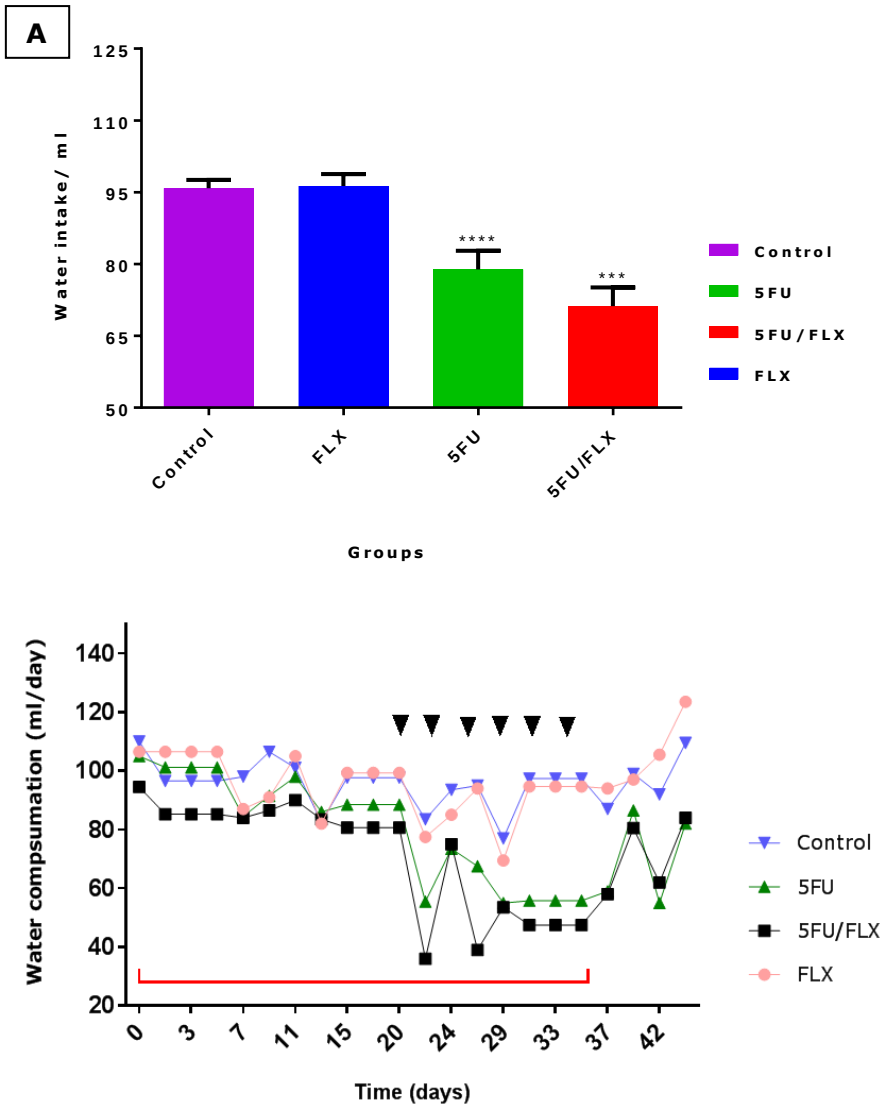



***Figure 2.34*** *The intake of water for each group recorded in study three.* ***A****. Daily water consumption from day 0 of FLX administration represented as ml/day/cage. The red line indicates the period of FLX administration in the drinking water of the FLX treated groups, and the arrows indicate the days of 5-FU or saline injections. From day 37 to day 43 is the recovery period (no treatments).* ***B****. The difference in mean water intake between treatment groups. Rats treated with 5-FU showed a significant reduction in water intake in both 5-FU and FLX+5-FU groups compared to the control group (***P=0.0001 and ****P<0.0001 respectively; one- way ANOVA with Bonferroni's multiple comparisons test). In contrast, no significant difference was found between the FLX and the control treated groups in the water intake (P>0.9999, one-way repeated measures ANOVA).*

#### 2.4.3.3 The effect of six injections of 5-FU analysed one week after the last injection on cell proliferation (study three)

To investigate possible causes of deterioration in white matter integrity, the level of cell proliferation in the ON and CC was determined by staining for the cell proliferation marker Ki67. As shown in Figure 2.35, there was no significant difference in cell proliferation between the FLX treated group and the saline group ($F_{(1, 28)}$ = 0.1467, P=0.7046). In contrast the 5-FU treated group showed a significant reduction in cell proliferation compared to controls ($F_{(1, 28)}$ = 12.47, P=0.0015 P=0.0002, two-way ANOVA). Co-treatment of 5-FU with FLX largely prevented the reduction in proliferation seen with 5-FU alone such that there was no significant difference between this group and controls.

Regarding the CC, there was a significant reduction in the total number of Ki67 positive cells of the 5-FU treated group compared to saline-treated rats (P<0.0001, two-way ANOVA). There was no significant difference between the FLX group and controls (P=0.8937), indicating that administration of FLX alone did not change cell proliferation in CC (Figure 2.36). Although there was a significant effect in the FLX+5-FU group, (P=0.0308), when compared with the control group. Multiple comparisons analysis, two-way ANOVA indicates a significant increase in cell proliferation compared with the 5-FU alone treated group (P=0.0002). FLX appears to prevent the decrease in cell proliferation when co-administered with chronic treatment of 5-FU.

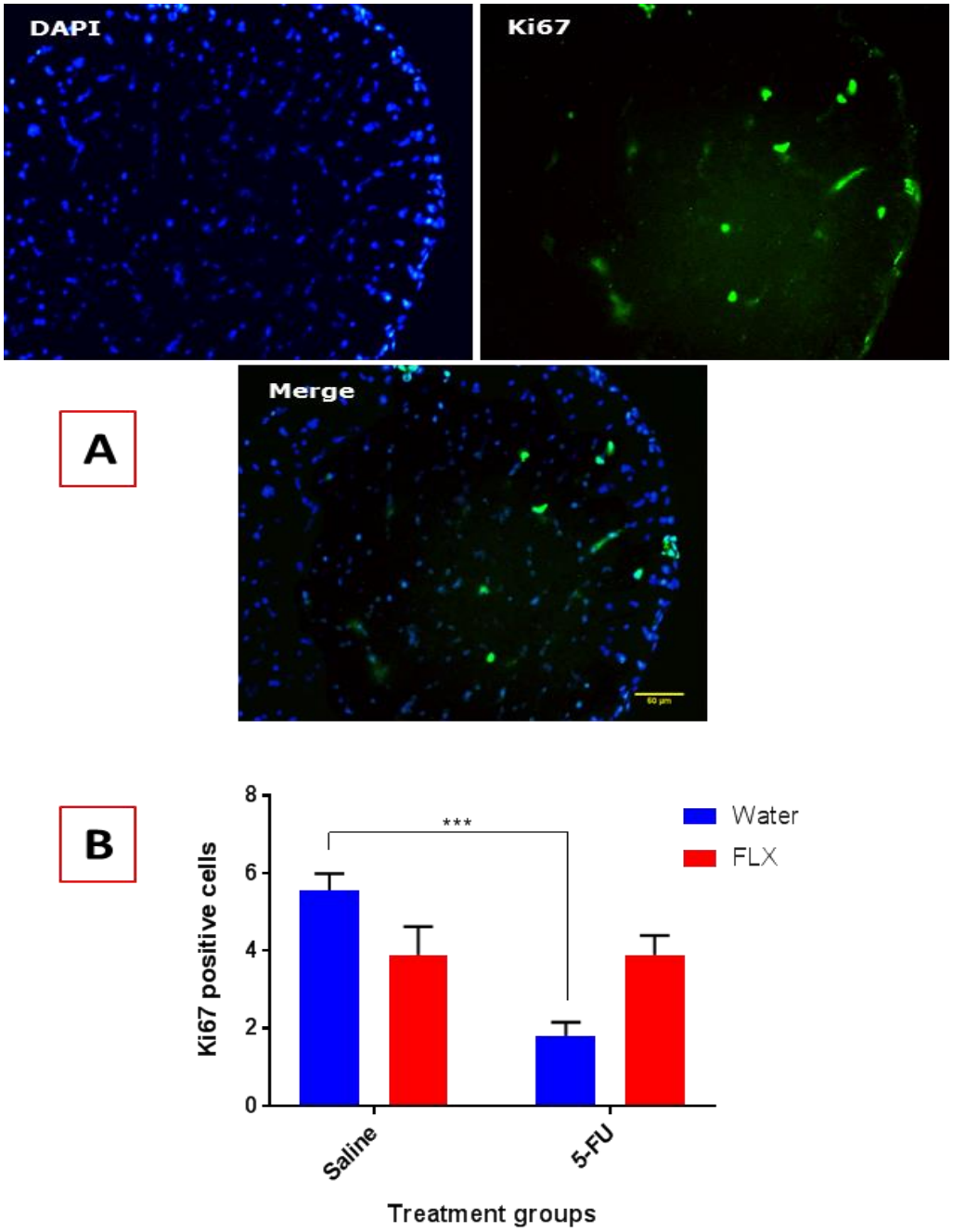



***Figure 2.35*** *The impact of 6 injections of 5-fU on cell proliferation in the ON (study three). (**A**) A representative image of rat ON, proliferating cells were immunostained for Ki67 (green) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) The total number of Ki67+ cells per section in different treatment groups (mean ± SEM, n=8). There is a highly significant difference between the 5-FU treated group and controls (***P<0.0001, two-way ANOVA with Bonferroni post-hoc test). No significant difference is present between the FLX or FLX+5-FU groups and saline treated groups (p>0.05, two-way ANOVA).*

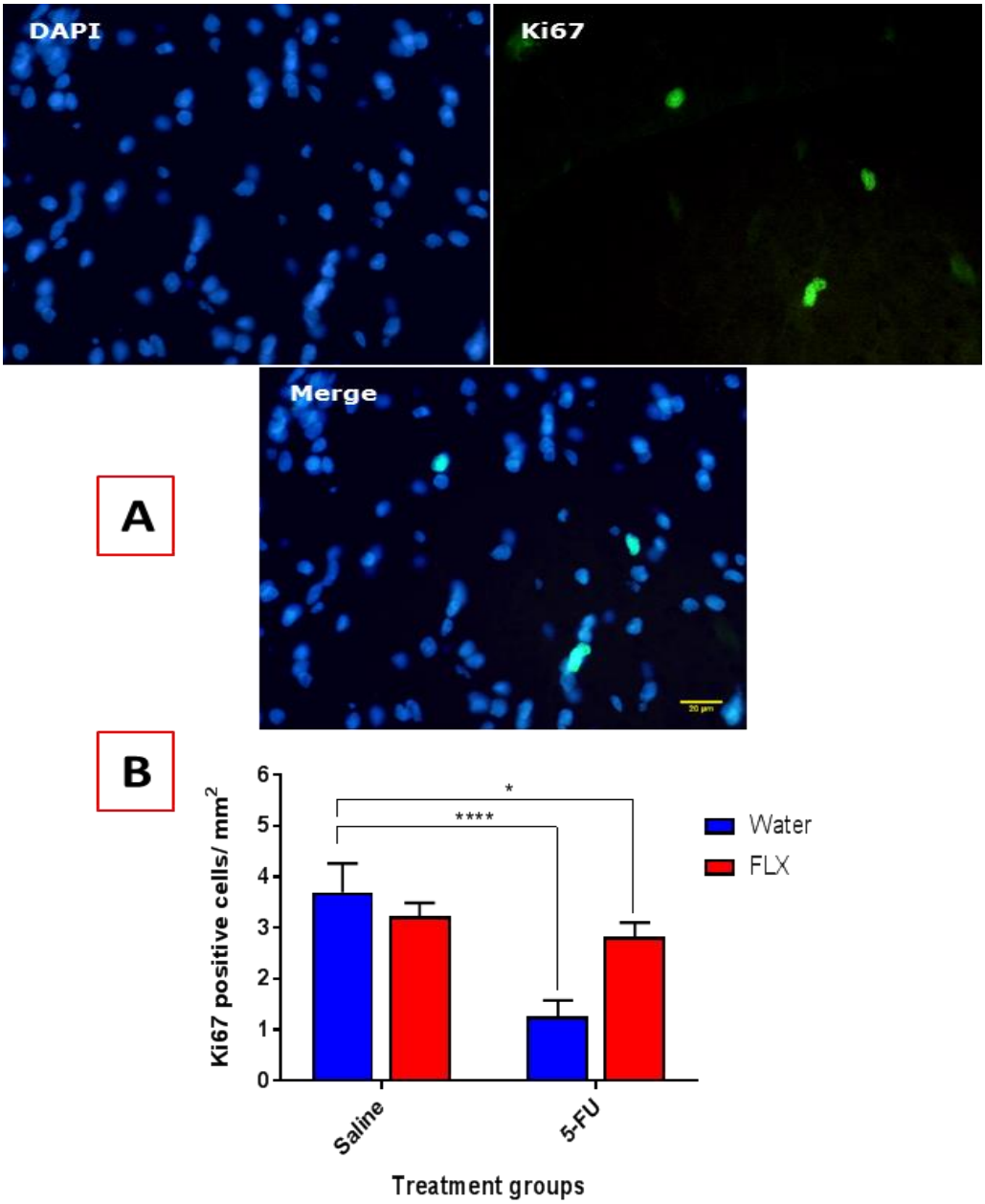



***Figure 2.36*** *The impact of multiple injections of 5-FU on cell proliferation in the CC (study three). (**A**) A representative image of rat CC proliferating cells were immunostained for Ki67 (green) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=20µm. (**B**) The average number of Ki67 + cells per mm2 in CC (mean ± SEM, n=8). There was no significant difference in the proliferating cell population between FLX group and the saline control group (p>0.05, two-way ANOVA). The group receiving 5-FU shows a marked and significant difference from the control group (****P<0.0001; Bonferroni post-hoc with two-way ANOVA). Although the FLX+5-FU group was still significantly below controls (*P=0.0308), it was significantly above the 5-FU group (***P=0.0002, two-way ANOVA with Bonferroni's multiple comparisons test).*

#### 2.4.3.4 The effect of six injections of 5-FU and chronic FLX treatments on the number of OPCs in the ON and CC after one week recovery (study three).

OPCs, identified by immuno staining for the NG2 antigen, were counted in a cross section of the ON. The number of NG2 positive cells was estimated from different groups after treatment with FLX and multiple injections with 5-FU. Compared to control animals, there was no significant difference in the mean number of cells expressing NG2 positive per section in animals treated with FLX ($p>0.05$, two-way ANOVA). Both 5-FU and FLX+5-FU groups showed a decrease in the number of OPCs cells (Figure 2.37). The decrease in NG2+ cells was significantly ($P<0.05$, two-way ANOVA) different in these groups when compared to saline treated controls. This indicates a reduction in the number of OPCs after multiple injections of 5-FU. Treatment with FLX or allowing one week of recovery after the final 5-FU injection did not prevent this reduction.

In the CC, the number of OPCs present following administration of multiple injections of 5-FU was significantly reduced compared to untreated controls ($P=0.0005$, Bonferroni post-hoc with two-way ANOVA). Interestingly, there appeared to be an increase in OPCs after FLX treatment ($P=0.0473$) (Figure 2.38). This may suggest that chronic FLX administration stimulates OPC proliferation in the CC. Although there was a significant decrease in the number of NG2+ cells in FLX+5-FU group ($P=0.0171$, two-way ANOVA), this reduction was less than with 5-FU alone. This result suggests that pre-administration of FLX although it may stimulate OPC proliferation was not sufficient to prevent the reduction caused by 5-FU.

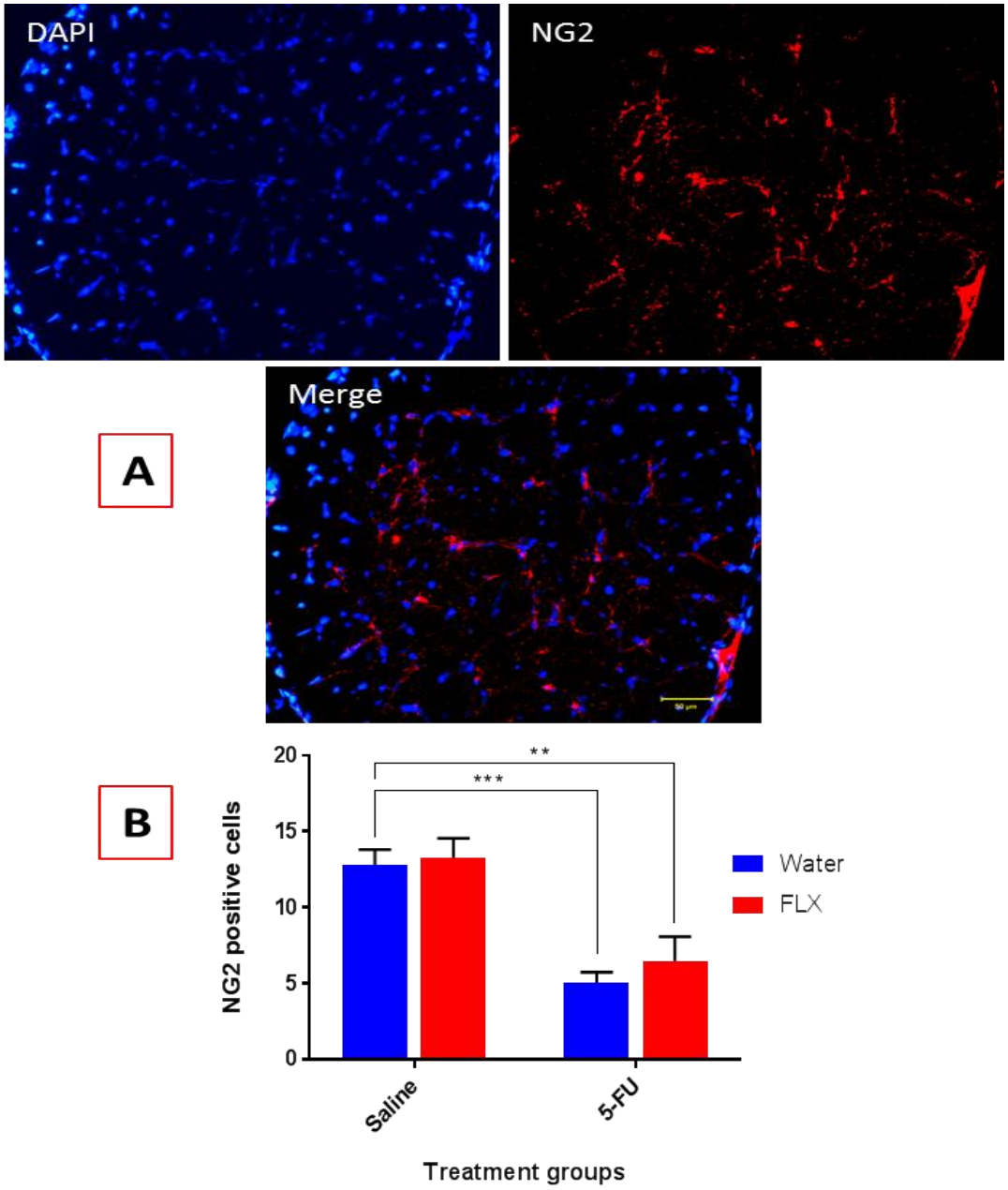



***Figure 2.37*** *NG2 positive cells in the ON one week after multiple injections of 5-FU (study three). Chemotherapy treatment reduced the number of NG2+ OPCs (A) a representative image of rat ON. Progenitor cells were immunostained for NG2 (red) and counterstained with DAPI (blue) and merged. Scale Bar=50μm. (B) The total number of NG2+ cells per section in different treatment groups (mean ± SEM, n=8) was estimated from cell counts. There was a significant difference between the 5-FU and the control group (***P=0.0005, two-way ANOVA with Bonferroni post-hoc test). No significant difference was found between the FLX and control group (p>0.05, two-way ANOVA). Multiple comparisons analysis showed no significant difference between the 5-FU and FLX+5-FU groups (p>0.05) while the FLX+5-FU group showed a significant reduction in OPCs comparing with the control group (**P=0.0049, two-way ANOVA).*

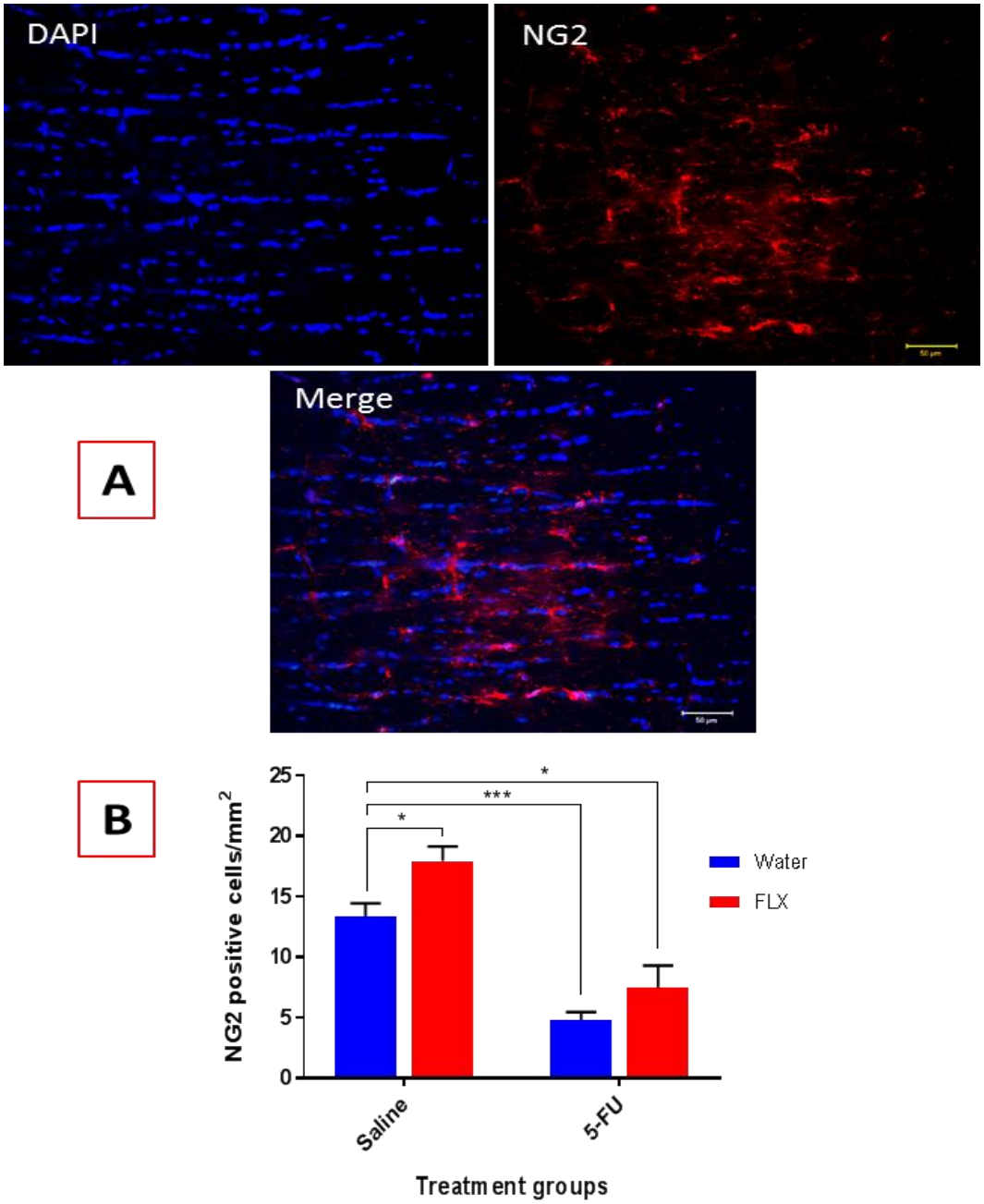



***Figure 2.38*** *NG2 positive cells in the CC one week after multiple injections of 5-FU (study three). (**A**) A representative images of rat CC. Progenitor cells were immunostained for NG2 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) The average number of NG2 + cells per mm2 in CC (mean ±SEM, n=8). Analysis showed a significant increase of NG2 expression cells in the FLX treated group (*P=0.0473, two-way ANOVA with Bonferroni post-hoc test) compared with the saline-control group. While, there was a significant decrease in 5-FU group (***P=0.0005) and FLX+5-FU group (P=0.0171) comparing to the control group.*

#### 2.4.3.5 The effect of six injections of 5-FU, analysed one week after the last injection on the total number of microglia in the ON and CC (study three)

As carried out in the previous results (studies one and two), the role of multiple injections 5-FU and FLX in microglial numbers in both ON and CC, was assessed by staining for the microglia marker Iba-1.

In the ON, treatment with multi injections 5-FU caused a significant decrease in the numbers of microglia compared to controls (P=0.0020, two-way ANOVA with Bonferroni post-hoc analysis) (Figure 2.39). Chronic treatment with FLX alone however did not significantly change microglial number from control values. Co-treatment with FLX and 5-FU raised microglial numbers such that they were no longer significantly different from controls (p>0.05, two-way ANOVA).

Similarly, in the CC, injections of 5-FU significantly reduced Iba-1+ numbers (P=0.0120, two-way ANOVA with Bonferroni post-hoc test) while FLX on its own (p>0.05, two-way ANOVA) did not affect numbers. In combination with 5-FU, FLX reduced the decrease in microglial numbers such that they were not significantly different from controls (P=0.1691) (Figure 2.40). From these results it can be concluded that the number of microglia in the ON and CC is decreased one week after the last injection of 5-FU. Pre-administration of FLX for three weeks and during the 5-FU treatment period prevented this decline.

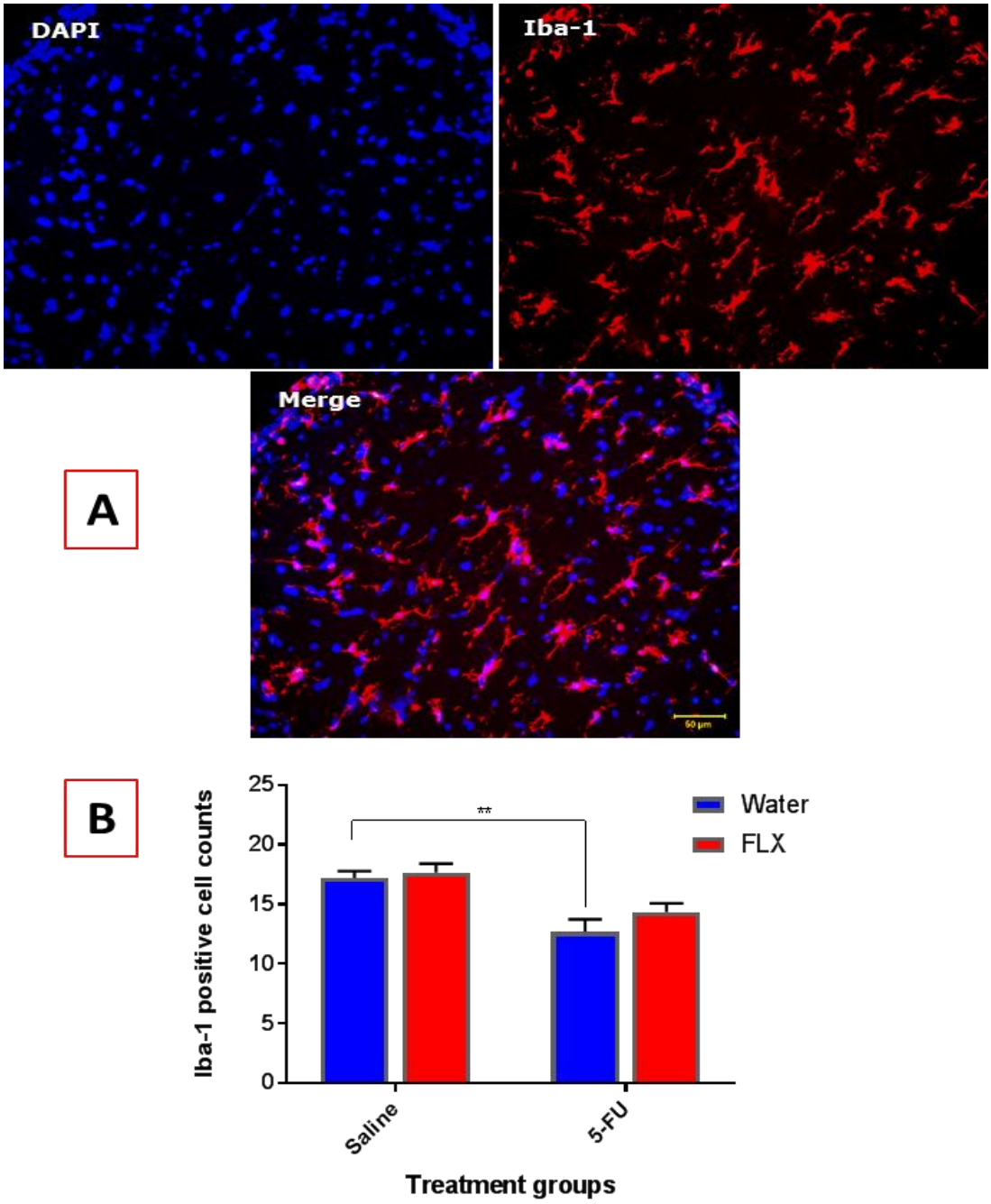



***Figure 2.39*** *The impact of six injections of 5-FU on Iba-1+ cell numbers in the ON (study three). (**A**) Representative image of rat ON. Microglia were immunostained for Iba-1 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) The total number of Iba-1 + cells per section in different treatment groups (mean ± SEM, n=8). There was a significant difference between the 5-FU and saline treated control group (**P=0.0020, two-way ANOVA with Bonferroni post-hoc test). There was no significant difference between the FLX alone group and FLX+5-FU group when compared to controls (p>0.05, two-way ANOVA).*

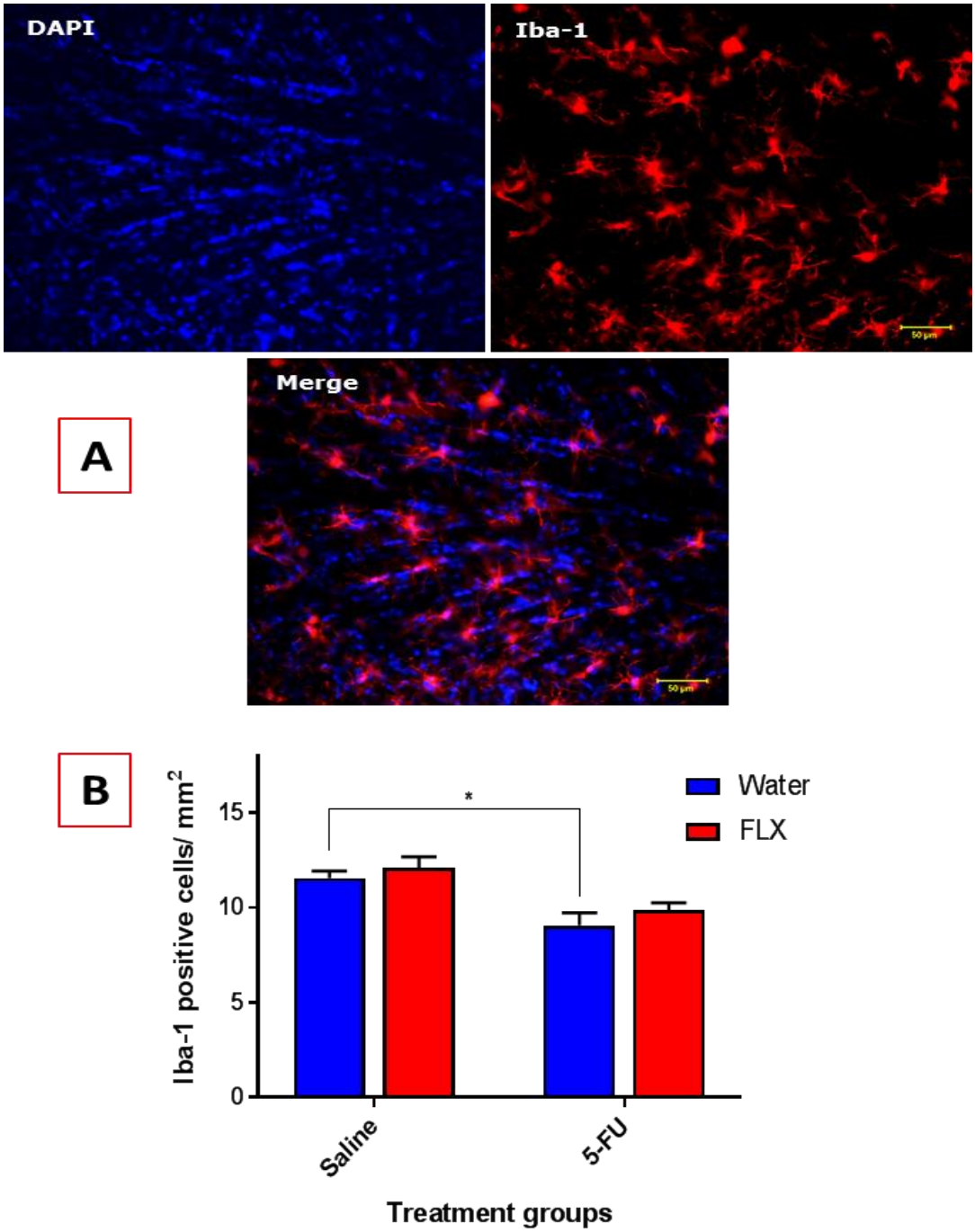



***Figure 2.40*** *The impact of six injections of 5-FU on Iba-1+ cell numbers in the CC (study three). (**A**) A representative images of rat CC. Microglial cells were immunostained for Iba-1 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar= 50µm. (**B**) The average number of Iba-1+ cells per mm2 in CC (mean ±SEM, n=8). There was a significant difference between the 5-FU and saline treated control group (*P=0.0120, two-way ANOVA with Bonferroni post-hoc test). No significant difference was found between FLX alone group and FLX+5-FU group comparing to controls (p>0.05, two-way ANOVA).*

#### 2.4.3.6 The effect of multiple injections of 5-FU and chronic FLX treatments on COX-2 positive cell numbers in ON and CC (study three)

To determine the effects of chronic 5-FU and FLX treatments on the inflammatory response by white matter tracts, the number of cells expressing COX-2 was counted in ON and CC one week after the last injection of 5-FU.

In both ON and CC, numbers of COX-2 expressing cells were low in control animals and were unaffected by FLX treatment ($P = 0.4137$, ON; $P= 0.9123$, CC). The six injections of 5-FU significantly increased numbers of COX-2 + cells in both ON and CC ($F_{(1, 28)} = 125.6$, $P<0.0001$ and $F_{(1, 28)} = 180.4$, $P<0.0001$ respectively) Bonferroni multiple comparisons post-hoc analysis in both tissues co-treatment with 5-FU and FLX significantly reduced the numbers of COX-2+ cells compared with the 5-FU group ($P=0.0031$, ON; $p<0.0001$, CC), while still remaining significantly above controls ($P<0.0001$, two-way ANOVA) (Figure 2.41 and Figure 2.42).

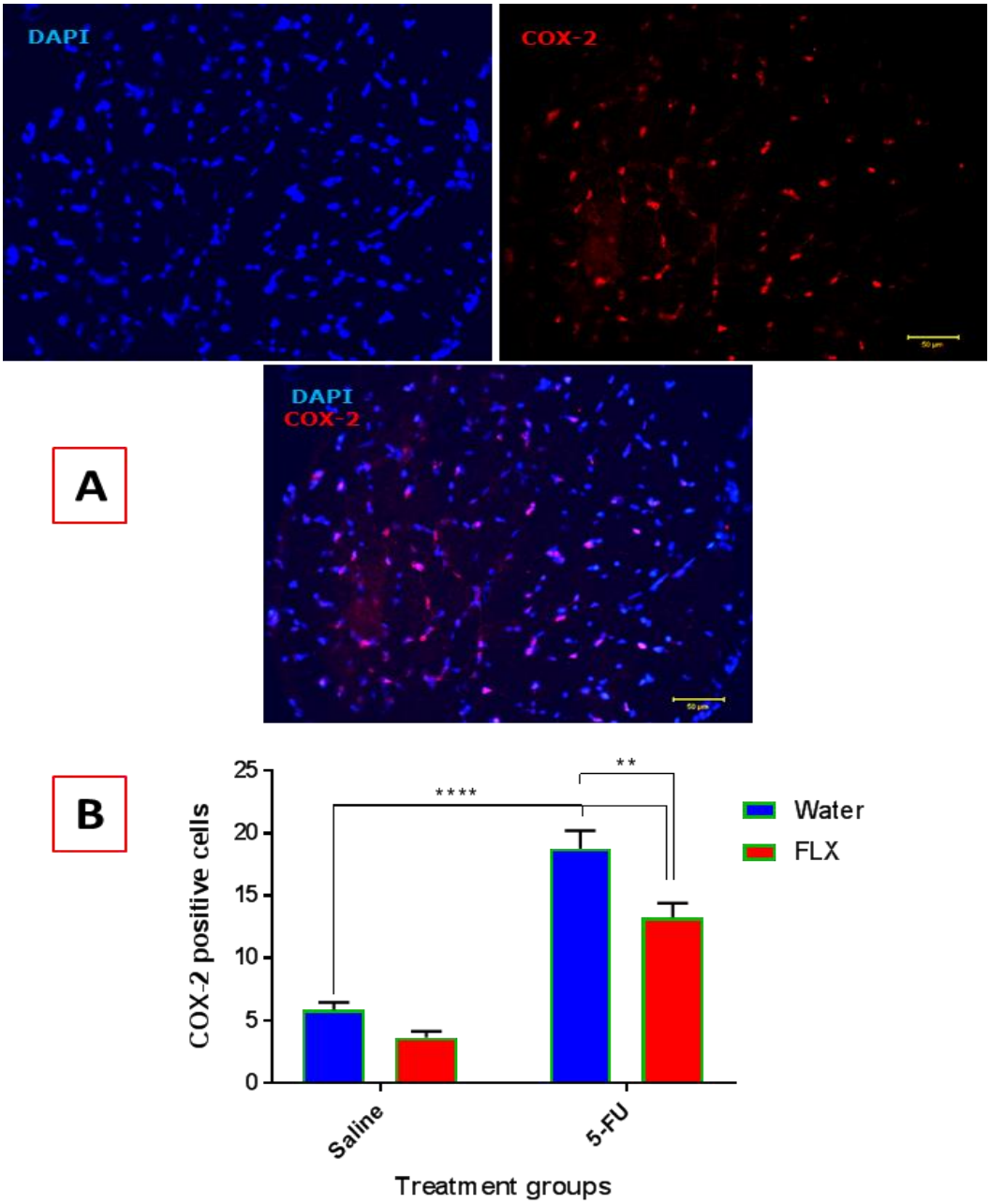



***Figure 2.41*** *The impact of 6 injections of 5-fU on COX2+ cell numbers in the ON (study three). (**A**) Representative image of rat ON immunostained with COX-2 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) The average number of COX-2+ cells per section (mean ± SEM, n=8) in adult rat ON in different treated groups sacrificed one week after the last injection of 5-FU. Analysis showed a significant increase in COX-2 expressing cells in the 5-FU and FLX+5-FU groups (****P<0.0001, two-way ANOVA) compared with the control group. The group receiving FLX did not significantly differ from controls (p>0.05, two-way ANOVA). The group receiving 5-FU and FLX together had significantly less COX-2+ cells than the 5-FU alone group (**P=0.0031; Bonferroni post-hoc multiple comparison analysis).*

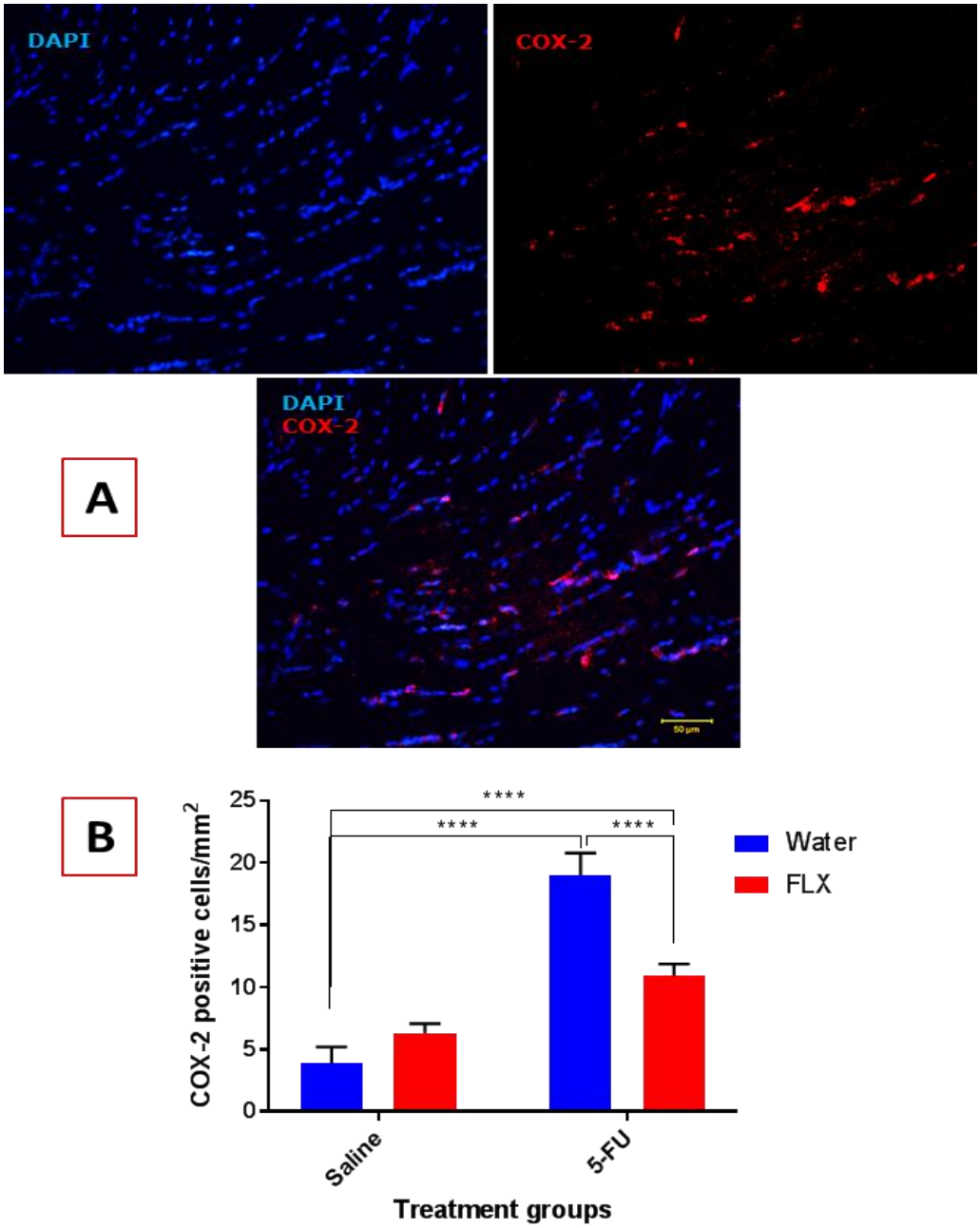



***Figure 2.42*** *The impact of 6 injections of 5-fU on COX2+ cell numbers in the CC (study three). (**A**) Representative images of rat CC after one week of 5-FU treatment, immunostained with COX-2 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) Shows the average number of COX-2+ cells per mm2 (mean ± SEM, n=8) in the CC in different treated groups. Analysis showed a significant increase in COX-2 expression in both 5-FU and 5-FU+FLX treated groups (****P<0.0001, two-way ANOVA) compared with the saline-control group. The groups receiving FLX did not significantly differ from the controls (p>0.05). The group co-administrated with FLX+5-FU had significantly fewer positive cells than the 5-FU group (****P<0.0001, Bonferroni post-hoc multiple comparison two-way ANOVA).*

#### 2.4.3.7 The effect of six injections of 5-FU, analysed one week after the last injection on DNA damage in the ON and CC (study three)

As 5-FU chemotherapy is known to induce DNA damage in dividing cells, the number of cells affected by 5-FU treatment with or without chronic FLX treatment was quantified by immunostaining for pH2AX. In both ON and CC, the number of pH2AX + cells was very low in control animals and was not affected by FLX treatment ($p>0.05$, two-way ANOVA), (Figure 2.43 and 2.44). However after 6 injections of 5-FU the number of pH2AX+ cells very significantly increased in both ON and CC. ($F_{(1, 28)} =314.7$, $P<0.0001$, ON; $F_{(1, 28)} =207.8$, $P<0.0001$, CC; two-way ANOVA with Bonferroni post-hoc test). Immunostaining for pH2AX showed positive cells scattered across both ON and CC (Figure 2.43A and 2.44A). Groups co-treated with 5-FU and FLX (5-FU+FLX) also showed highly significant increases in pH2AX in both ON and CC compared with the control group ($P<0.0001$, two-way ANOVA ON and CC). In ON, multiple comparisons analysis two-way ANOVA showed that rats co-administrated with FLX+5-FU showed a significant decrease in DNA damage compared with the 5-FU group ($P=0.0085$), indicating that the FLX had had some effect in reducing the amount of DNA damage in the ON caused by 5-FU. In the CC however co-treatment with FLX did not produce a significant reduction in the numbers of pH2AX cells ($F_{(1, 28)} =0.1874$, $P=0.6684$).

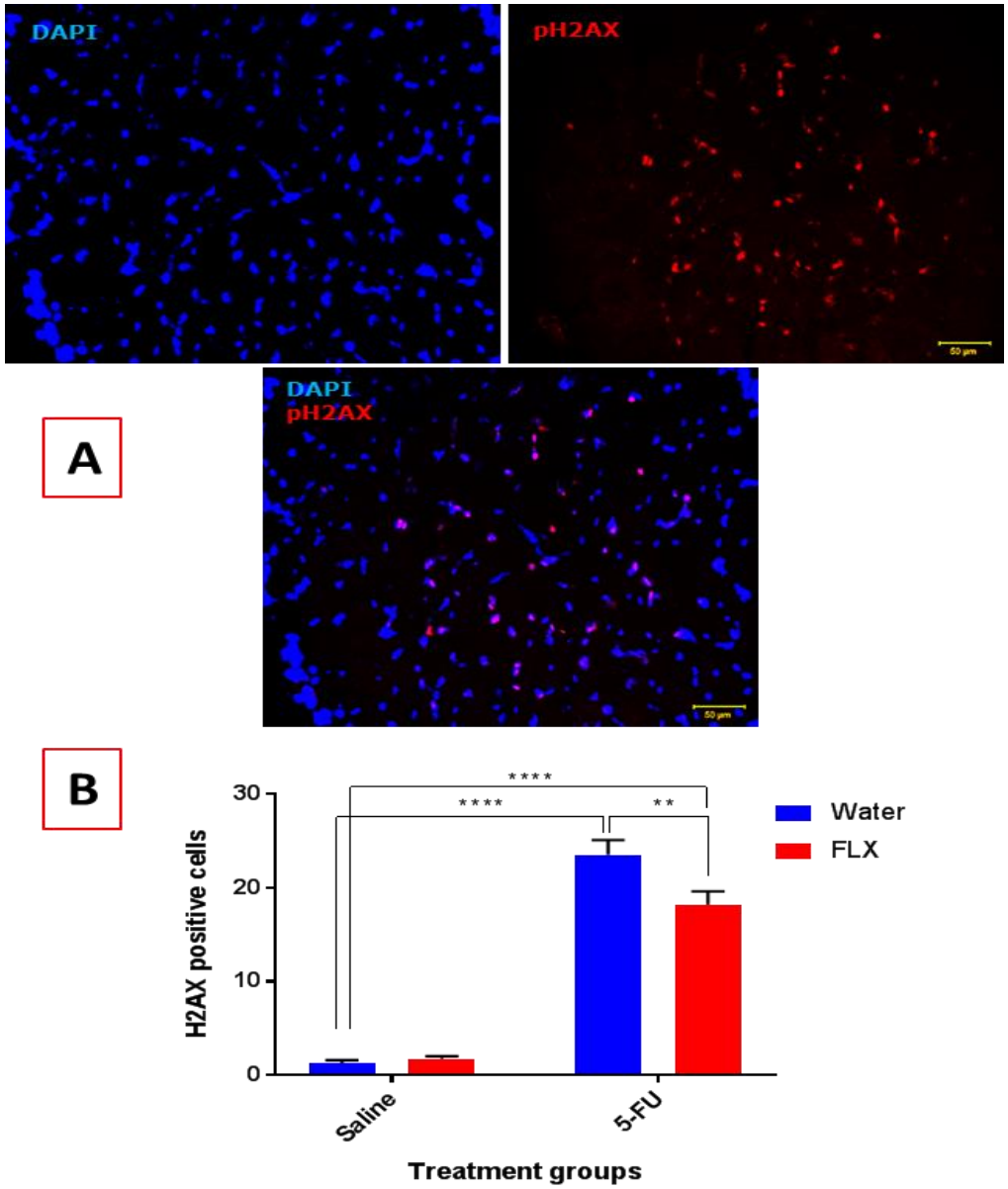



***Figure 2.43*** *The impact of 6 injections of 5-fU on pH2AX + cell numbers in the ON (study three). (**A**) Representative image of rat ON immunostained with pH2AX (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) Shows the average number of pH2AX + cells per section (mean ± SEM, n=8) in ON in different treatment groups put down one week after the last injection of 5-FU. Analysis shows a significant increase of pH2AX expression in the 5-FU and FLX+5-FU groups (****P<0.0001, two-way ANOVA) compared with the saline group. The groups receiving FLX did not significantly differ from the controls (p>0.05, two-way ANOVA). Whereas the group receiving 5-FU and FLX had significantly less pH2AX+ cells than the 5-FU group (**P=0.0085; Bonferroni post-hoc multiple comparison analysis).*

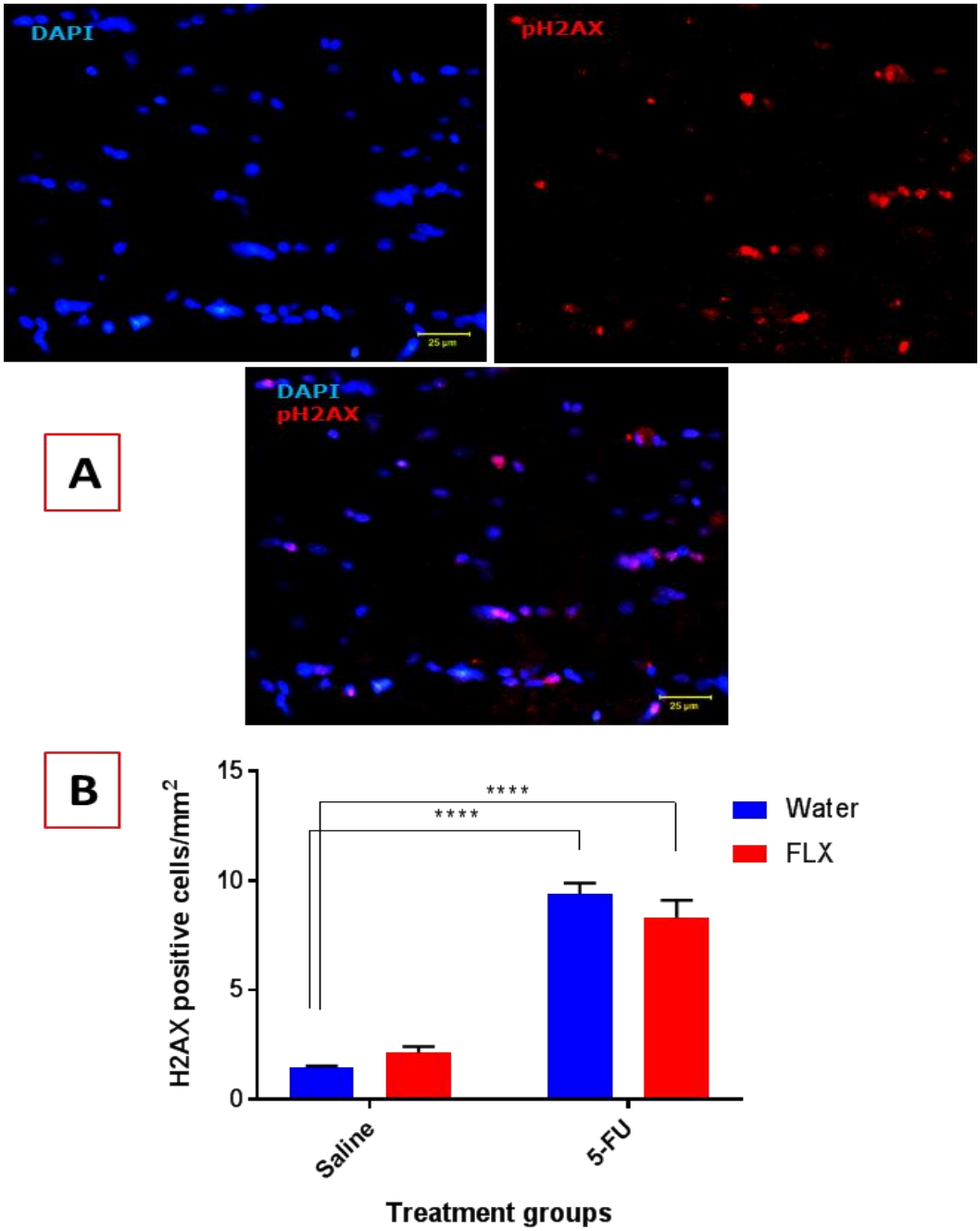



***Figure 2.44*** *The impact of 6 injections of 5-FU on pH2AX+ cell numbers in the CC (study three). (**A**) Representative image of rat CC after chronic 5-FU, immunostained with pH2AX (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=25µm. (**B**) Shows the average number of pH2AX + cells per mm2 (mean ± SEM, n=8) in the CC in different treatment groups one week after six injections of 5-FU. Analysis showed a significant increase of pH2AX expression in both 5-FU and 5-FU+FLX treated groups (****P<0.0001, Bonferroni post-hoc multiple comparison two-way ANOVA) compared with the saline-control group. The group receiving FLX alone did not significantly differ from the controls (p>0.05). Co-administration FLX+5-FU had no significant effect on DNA damage compared with the 5-FU alone group (p>0.05, multiple comparison two-way ANOVA).*

### 2.4.4 Study four: The effect of six injections of 5-FU analysed one week after the last injection with groups receiving FLX or INDO throughout the experimental period.

Chemotherapy treated rats received six injections of 5-FU over a period of 16 days after three weeks of FLX or INDO treatments. Animals receiving FLX or INDO were given the drugs in their drinking water throughout all the experimental period for 43 days in total. Animals were put down one week after the final 5-FU injection. This study aimed to compare the effects of FLX and INDO in protection of WMTs from the adverse effects of 5-FU.

#### 2.4.4.1 Effect of FLX, INDO and 5-FU treatments on weight gain during experiment period in study four

The effect of treatment and animal weights was monitored by weighing animals daily (Figure 2.45). Overall animal weights significantly increased over the time ($F_{(20, 560)} = 897.9$, $P<0.0001$; two-way ANOVA). Moreover, the interaction between the time and the treatment was significant ($F_{(19, 342)} = 1126$, $P<0.0001$; two-way repeated measures ANOVA). The period before chemotherapy treatment, the groups administrated FLX or INDO in their drinking water, showed no significant differences from those drinking only tap water ($p>0.05$, two-way ANOVA). In contrast, animals receiving six injections of 5-FU showed a significant reduction in body weight, compared to those animals receiving saline treatments throughout the injection period (16 days) ($F_{(5, 18)} = 5.188$, $P=0.0040$; two-way repeated measures ANOVA). The week after the end of 5-FU treatment, showed that animals receiving chemotherapy regained body weight in all treated groups. The FLX+5-FU group however still showed a significantly lower body weight compared with the control group by the end of study ($P=0.0007$, two-way ANOVA with Bonferroni post-hoc test) (Figure 2.46).

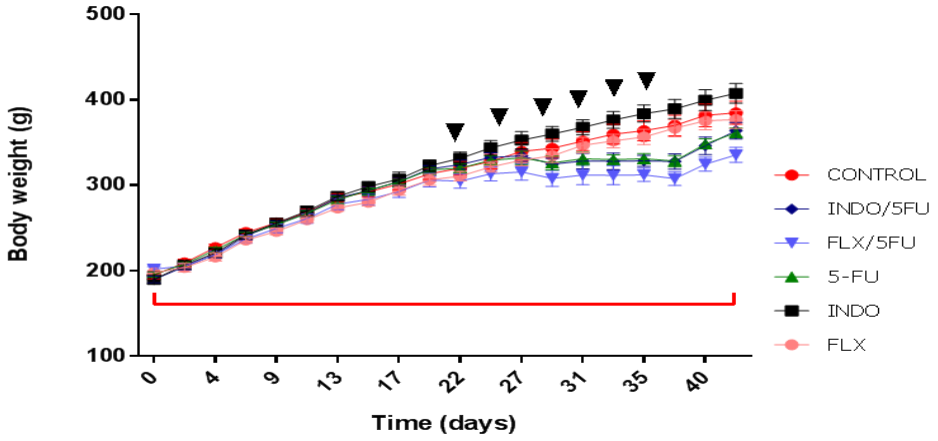



***Figure 2.45*** *Body weights for each experimental group recorded in study four (Mean± S.E.M, n=4 each group). Animals were weighed daily throughout the experimental period (day 0-43). Red line indicates the course of FLX and INDO treatments from day 0 to day 43 and the arrow heads indicate the injections of 5-FU from day 21 to day 36. Animals were put down one week after the last injection on day 43. Two-way ANOVA analysis shows a significant overall weight gain over the experimental period (****P<0.0001), and post-hoc testing shows the interaction between time and treatment was highly significant (****P<0.0001). All treated groups showed no significant differences in body weight gain (p>0.05, repeated measures two-way ANOVA) from day 0 to 27. The 5-FU, FLX+5-FU and INDO+5-FU groups showed a significant reduction in mean body weight compared to the saline groups during the 5-FU treatment period (**P=0.0040, two- way ANOVA with Bonferroni post-hoc test).*

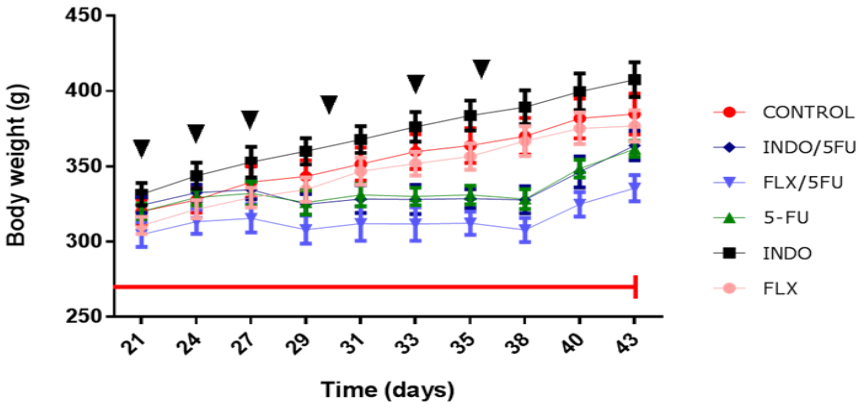



***Figure 2.46*** *The changes in body weight for each experimental group in study four (Mean± SEM, n=4 each group) between day 21 and 43. Animals in the 5-FU, FLX+5-FU and INDO+5-FU groups received six injections of 5-FU (35mg/kg), while animals in control, FLX and INDO groups received saline injections at same time (arrows). During the one week after the last injection, animals still received FLX or INDO treatments in groups treated with these drugs (red bar). Overall, the 5-FU injections caused a significant reduction in weight gain in the 5-FU treated groups from day 28 until day 43 (**P=0.0040, repeated measures two-way ANOVA). At the end of the experiment (day 43) the FLX+5-FU group had a significantly lower weight compared to controls (***P=0.0007), but there were no significant differences between other groups and controls (p>0.05, two-way ANOVA).*

#### 2.4.4.2 The effects of FLX, INDO and 5-FU treatment on water consumption in study four

In study four the water intake was calculated for each experimental group by daily weighing of water bottles. Drinking water treated with FLX or INDO was administered to the FLX, INDO, 5-FU+FLX and the INDO+5-FU groups throughout the experimental period (43 days), while injection of 5-FU took place from day 21 to day 36. The dose of FLX and INDO was recalculated every other day to keep the concentration of FLX and INDO unchanged over the course of treatment. Although water consumption fluctuated between the groups, there was a significant decline in water intake for those groups receiving FLX in their water ($F_{(3.102, 58.93)} = 25.24$, $P<0.0001$; Repeated measures one-way ANOVA). This decline in water intake extended throughout the period of FLX administration (Figure 2.47A). There was no significant difference in the intake of water between INDO and saline-treated groups throughout the treatment period ($P=0.2363$). The results also show a significant reduction in water consumption in 5-FU treated group during the inject ion period ($P=0.0077$). In the week after injections, the amount of water drunk showed no significant differences from the water consumption of the control group. Co-administration of FLX and 5-FU also showed a significant decline in consumption ($P<0.0001$, one- way ANOVA with Bonferroni's multiple comparisons test) which extend to day 43 (Figure 2.47B). Co-administration of INDO and 5-FU showed no significant reduction in water intake compared to the control group ($P=0.0943$).

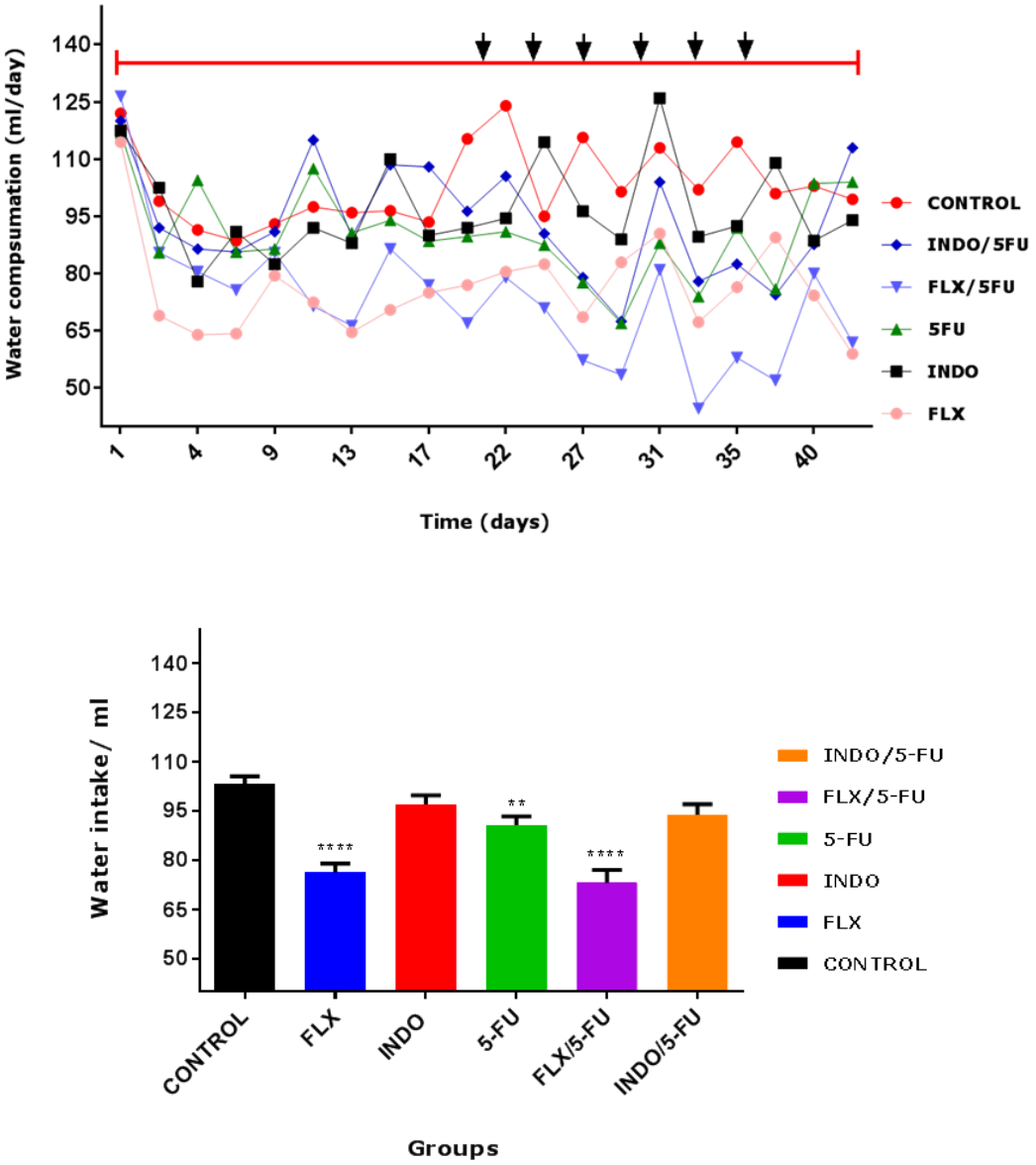



***Figure 2.47*** *The impact of 6 injections of 5-FU and treatment with FLX and INDO on water intake (study four). (**A**) Daily water consumption from day 0 of FLX and INDO administrations represented as ml/day. The red line indicates the period of FLX or INDO administrations in the drinking water of the FLX or INDO treated groups. The arrows indicate the days of 5-FU or saline injections. From day 37 to day 43 is the recovery period (no 5-FU treatment). (**B**) The difference in mean water intake between treatment groups. FLX, 5-FU and FLX+5-FU groups produced a significant water reduction compared to the control group (****P<0.0001, ** P=0.0077 and ****P<0.0001 respectively; one- way ANOVA with Bonferroni's multiple comparisons test). In contrast, no significant difference between the INDO and INDO+5-FU groups when comparing to the control treated group (p>0.05, one-way repeated measures ANOVA).*

#### 2.4.4.3 The effects of FLX, INDO and 5-FU treatments on cell proliferation in the ON and CC of study four

Cell proliferation was quantified by counting the number of Ki67+ cells in the ON and CC one week after the last 5-FU injection. In both the ON and CC, (Figures 2.48 and 2.49) neither the FLX or INDO groups showed any significant difference from the control group over the treatment period (43 days) ($P>0.9999$, two-way ANOVA).

In contrast, the number of Ki67 positive cells per section in the 5-FU-treated groups in both ON and CC were significantly decreased (~1- fold, ON; ~3.3- folds, CC) in comparison with controls ($P=0.0377$, ON; $P<0.0001$, CC two-way ANOVA).

FLX and INDO were given chronically before, during and after 5-FU treatment to see if these drugs would protect the level of cell proliferation after 5-FU treatment. This hypothesis proved to be correct and co-administration of FLX or INDO in the FLX+5-FU and INDO+5-FU groups enabled these groups to retain control levels of cell proliferation. Neither ON or CC FLX+5-FU or IND+5FU groups showed any significant differences in proliferating cell number compared with the saline control groups ($p>0.05$, two-way ANOVA).

These results indicate that, 5-FU reduced cell proliferation in both the ON and CC of adult rats. Co-treatment with FLX or INDO before, throughout and after one week following 5-FU injections prevented the decrease in cell proliferation produced by 5-FU.

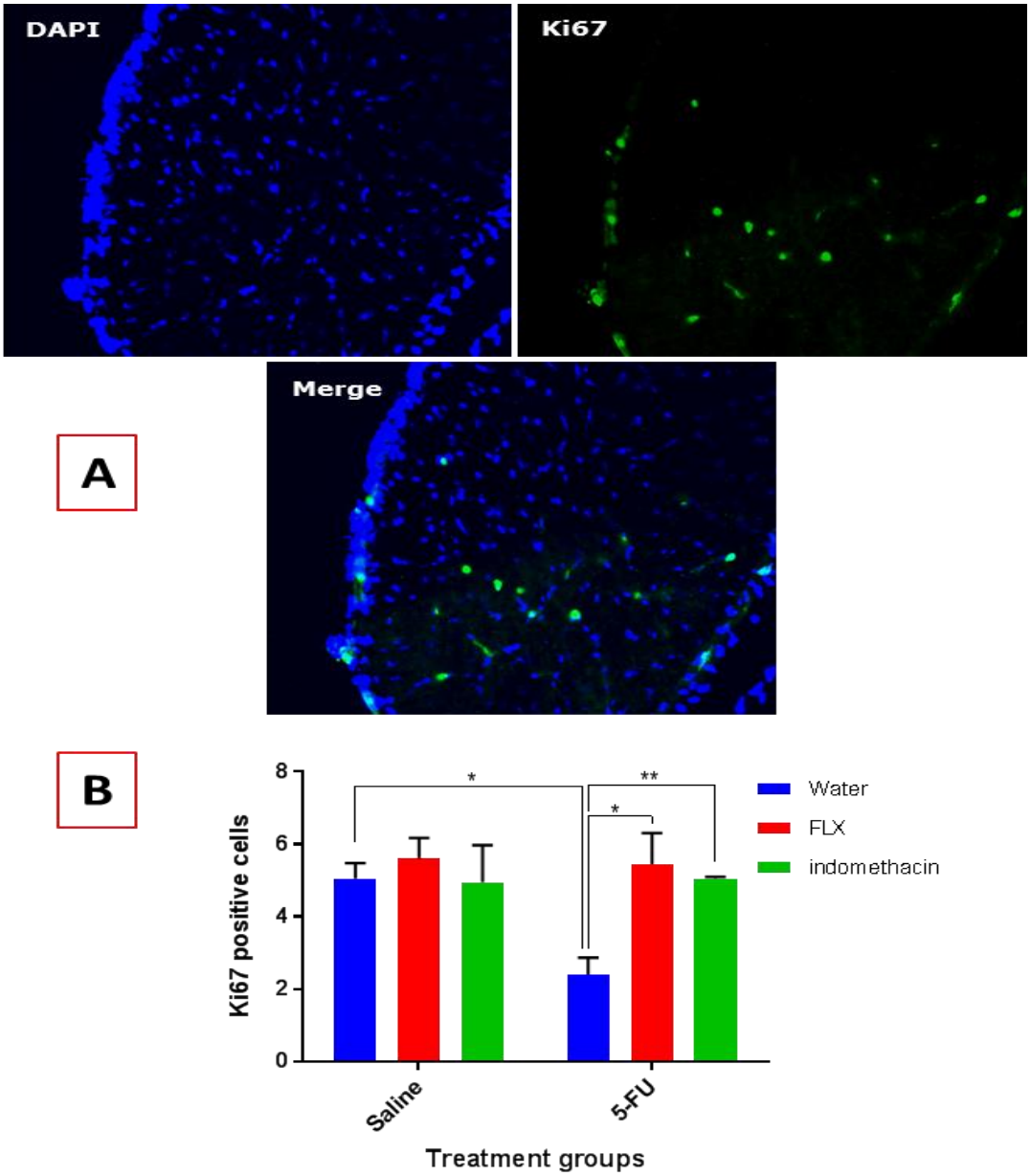



***Figure 2.48*** *The impact of 6 injections of 5-FU on ki67+ cell numbers in the ON (study four). (**A**) A representative image of ON. Proliferating cells were immunostained for Ki67 (green) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) The total number of Ki67+ cells per section in different treatment groups (mean ± SEM, n=4). There was a significant difference between the 5-FU and water saline treated group (*P=0.0377, two-way ANOVA with Bonferroni post-hoc test). No significant difference between the FLX, INDO, FLX+5-FU or INDO+5-FU groups and saline treated groups (p>0.05, two-way ANOVA).*

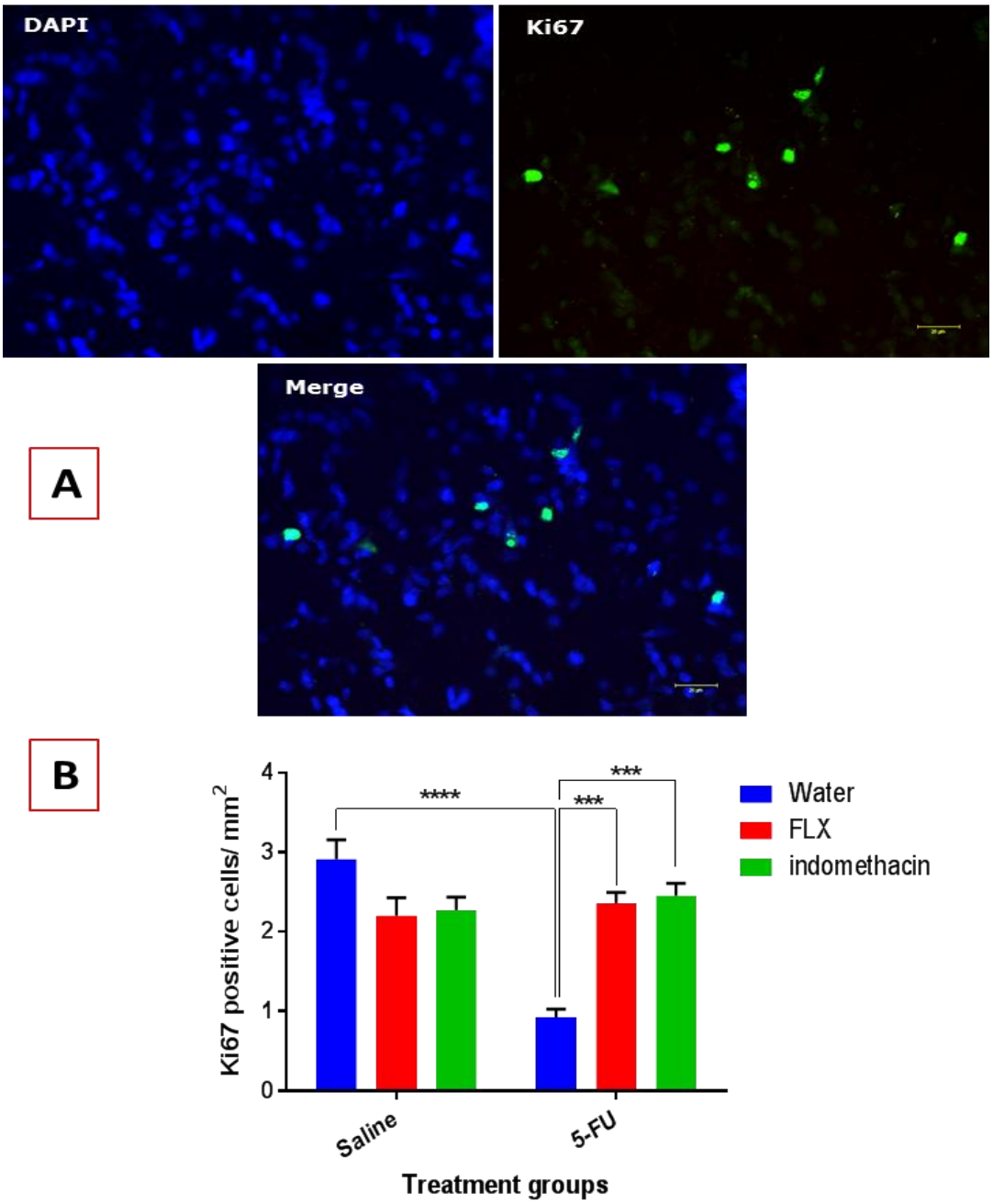



***Figure 2.49*** *The impact of 6 injections of 5-fU on Ki67+ cell numbers in the CC (study four). (**A**) A representative image of CC. Proliferating cells were immunostained for Ki67 (green) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=20µm. (**B**) The average number of Ki67 + cells per mm2 in CC (mean ± SEM, n=4). The group receiving 5-FU shows a marked and significant difference from the control group (****P<0.0001; Bonferroni post-hoc with two-way ANOVA). There was no significant differences in the proliferating cell populations between FLX, INDO, FLX+5-FU or INDO+5-FU groups and the saline control group (p>0.05, two-way ANOVA).*

#### 2.4.4.4 The effects of FLX, INDO and 5-FU treatments on OPCs in the ON and CC; study four

To determine the effect of the six injections of 5-FU, with or without administration of FLX or INDO, on adult OPCs populations, sections were immune stained for NG2 and positive cells counted in both ON and CC.

In both ON and CC, there was no significant difference in the mean number of cells expressing NG2 positive per section in FLX and INDO groups compared with controls ($p>0.05$, two-way ANOVA). Treatment with 5-FU significantly reduced the number of NG2+ cells in both ON and CC compared to the saline treated group ($P=0.0491$, ON; $P=0.0273$, CC; Bonferroni post-hoc with two-way ANOVA). In the ON, Co-administration of FLX or INDO in the FLX+5-FU and INDO+5-FU groups prevented the decrease in NG2 positive cells ($P=0.8166$ and $P=0.9986$ respectively, two-way ANOVA) (Figure 2.50). In the CC the INDO+5-FU group showed a significant decrease in NG2 positive cells ($P=0.0359$) compared with controls, there was no significant differences between FLX+5-FU treated group and controls ($P>0.9999$) (Figure 2.51).

These results indicate that co-administration of FLX before and throughout the 5-FU treatment periods can increase the survival of OPCs when measured one week after the last injection. The protective effect of INDO was less clear with maintenance of cell proliferation in the ON but not CC.

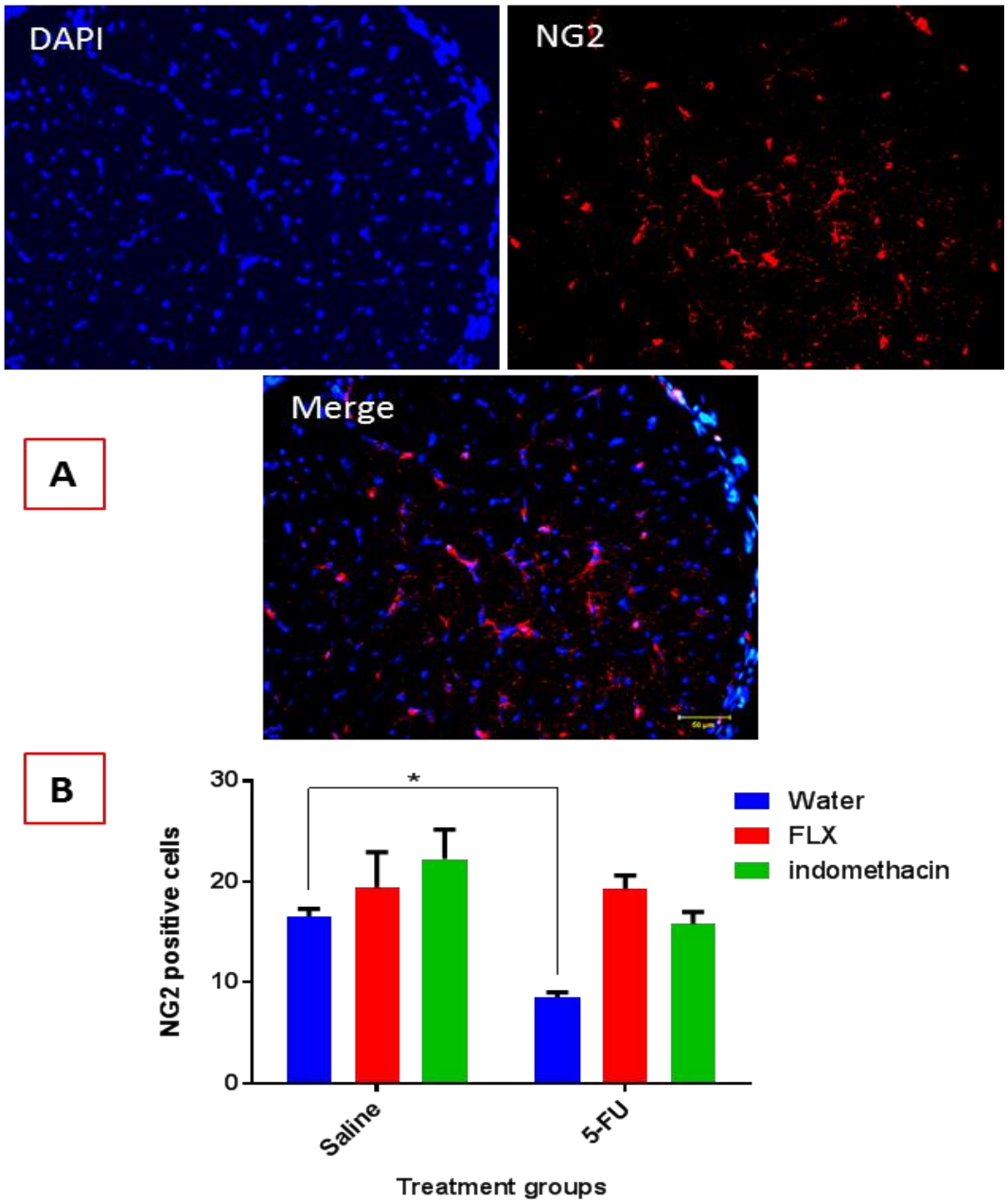



***Figure 2.50*** *The impact of 6 injections of 5-FU on NG2+ cell numbers in the ON (study four). (**A**) A representative image of the ON. Progenitor cells were immunostained for NG2 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) The total number of NG2+ cells per section in different treatment groups (mean ± SEM, n=4). There was a significant difference between the 5-FU and water treated group (*P=0.0491, two-way ANOVA with Bonferroni post-hoc test). No significant difference was found between the FLX or INDO and the saline treated groups (p>0.05, two-way ANOVA). There was no significant difference between FLX+5-FU or INDO+5-FU groups when compared with the water group (p>0.05).*

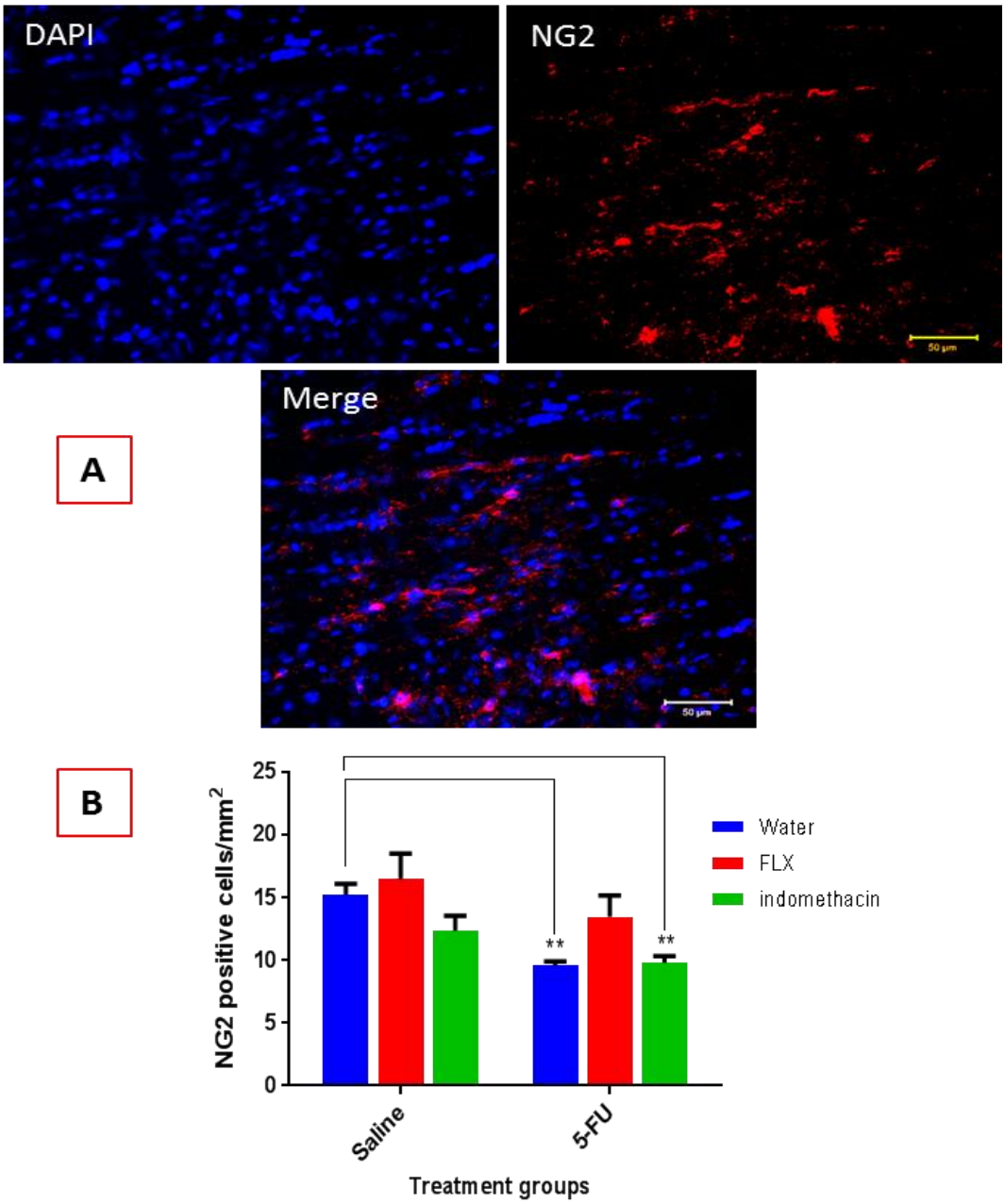



***Figure 2.51*** *The impact of 6 injections of 5-FU on NG2+ cell numbers in the CC (study four). (**A**) A representative image of the CC. Progenitor cells are immunostained for NG2 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) The average number of NG2 + cells per mm2 in the CC (mean ±SEM, n=4). Bonferroni post-hoc with two-way ANOVA analysis showed a significant decrease of NG2+ cells in the 5-FU group (**P=0.0273) and INDO+5-FU group (**P=0.0359) compared to controls. No significant difference was found between the FLX, INDO and FLX+5-FU groups when compared with controls (p>0.05, two-way ANOVA).*

#### 2.4.4.5 The effects of FLX, INDO and 5-FU treatments on the total number of microglia in ON and CC of study four

As mentioned earlier in the chapter one, microglia are the principal component of the CNS immune response. Hence, to understand the effect of chemotherapy in inducing inflammation in the white matter tracts, the effect of 5-FU on microglia was investigated. In addition to 5-FU, FLX and INDO treatments was used in study four to determine if these drugs were able to prevent or reduce the effects 5-FU has on microglia. Microglia were identified by immunostaining for the Iba-1 antigen. Analysis of both ON and CC (Figures 2.52 and 2.53), showed that treatment with FLX or INDO alone had no significant effect on microglial number ($P=0.2428$ and $P=0.8369$ respectively, ON; $P= 0.0016$, CC two-way ANOVA). 5-FU treatment on its own produced a significant decrease in microglial number compared to controls in both ON and CC ($P=0.0004$, ON; $P=0.0016$, CC two-way ANOVA).

Rats treated with FLX+5-FU or INDO+5-FU had more Iba-1 positive cells compared with the rats treated with only 5-FU and the numbers reached control values in the ON. In the CC there was no significant differences between INDO+5-FU and water group, however the FLX+5-FU group showed a significant reduction in the numbers of microglial cells compared to the water treated group ($P=0.0395$, two-way ANOVA with Bonferroni post-hoc test).

These results indicate that the systemic administration of 5-FU reduces microglial number, whereas co-administration INDO throughout 5-FU treatment may protect this cell type. Additionally, FLX was able to protect microglia in the ON but not the CC.

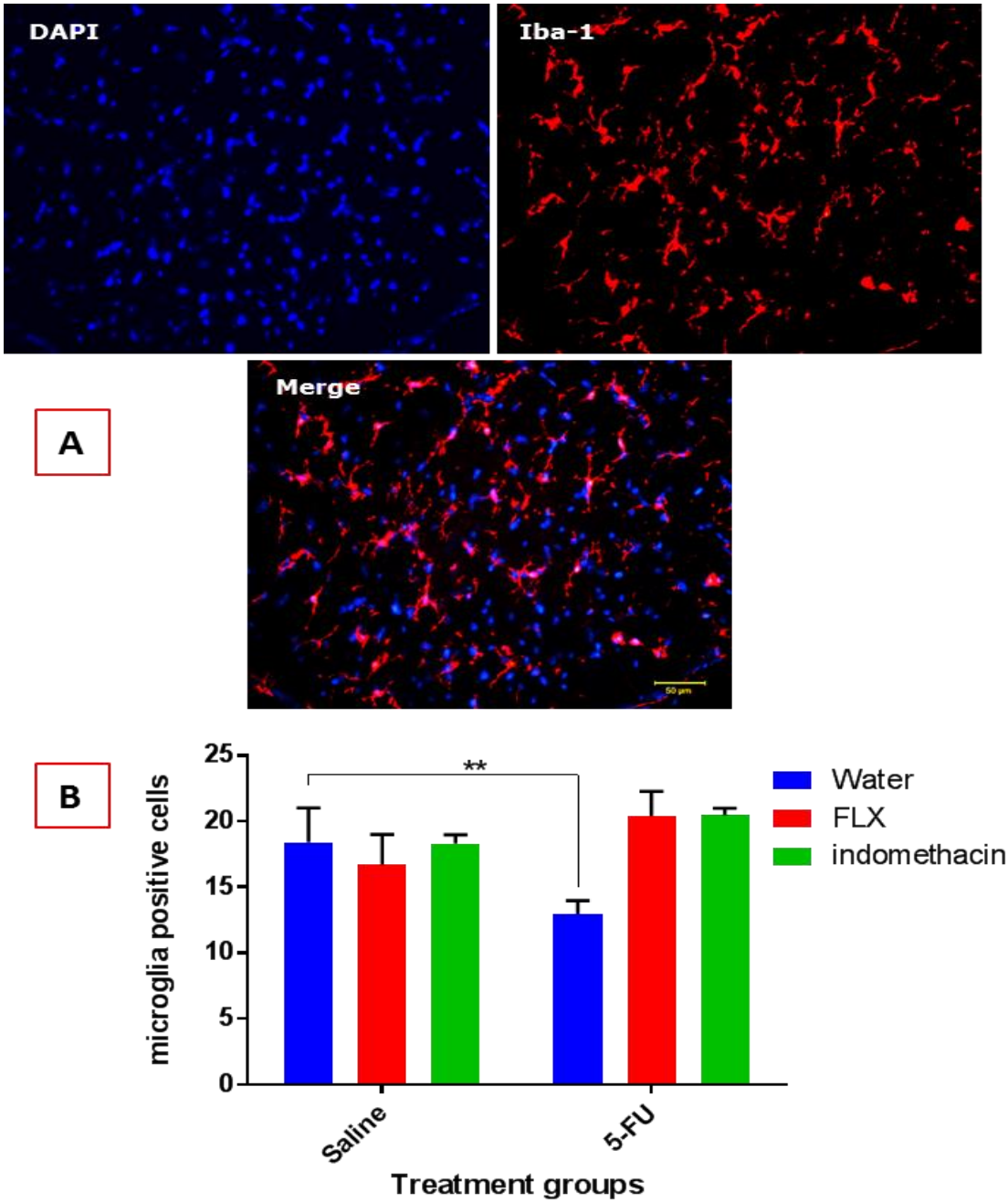



***Figure 2.52*** *The impact of 6 injections of 5-FU on Iba-1 positive cell numbers in the ON (study four). (**A**) Representative image of ON. Microglia were immunostained for Iba-1 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) The total number of Iba-1+ cells per section in different treatment groups (mean ± SEM, n=4). There was a significant difference between the 5-FU and saline treated group (**P=0.0004, two-way ANOVA with Bonferroni post-hoc test). No significant difference was found between the FLX, INDO, FLX+5-FU and INDO+5-FU groups when compared with the saline group (p>0.05, two-way ANOVA).*

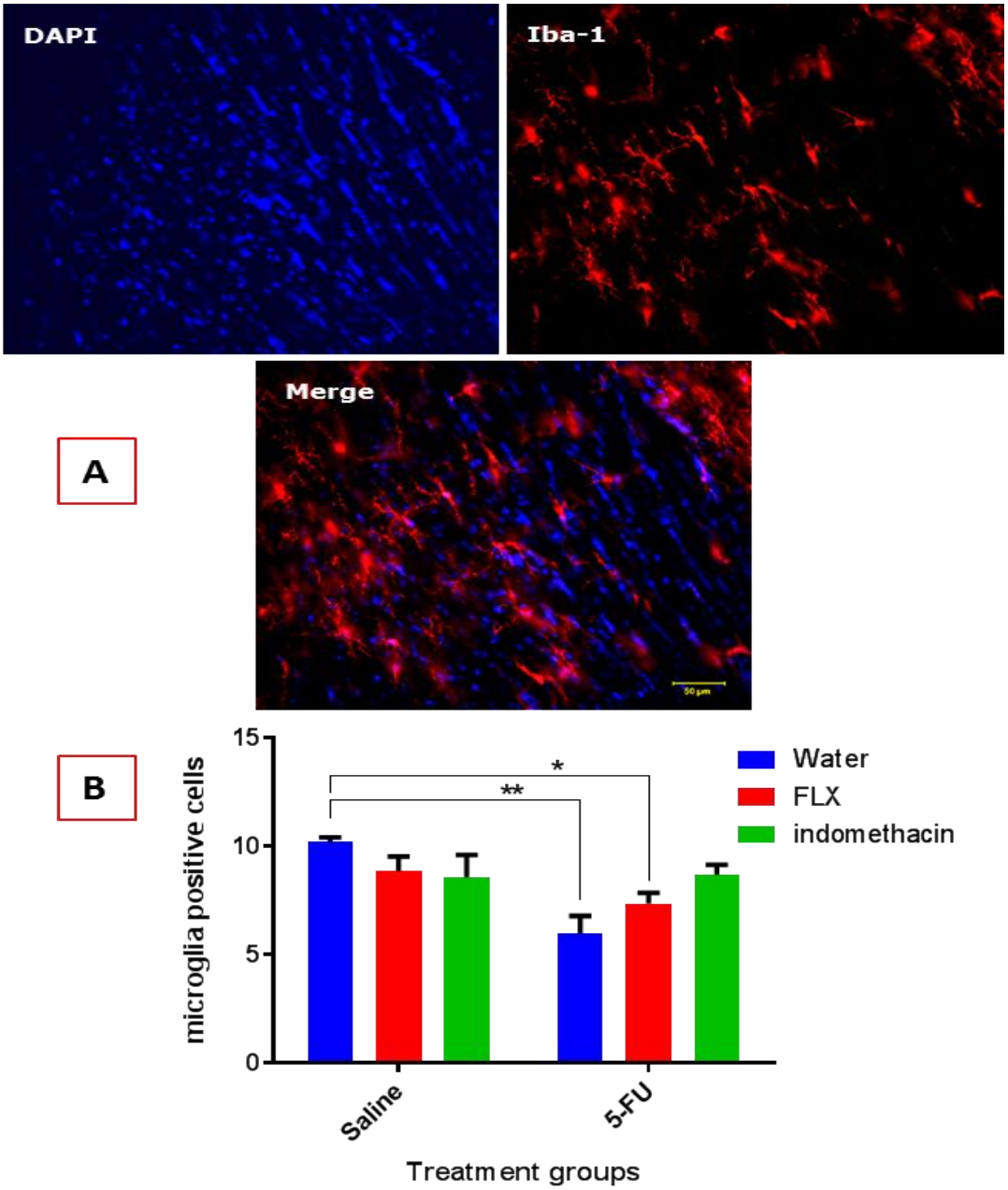



***Figure 2.53*** *The impact of 6 injections of 5-FU on Iba-1 positive cell numbers in the CC (study four). (**A**) A representative image of CC. Microglial cells were immunostained for Iba-1 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50 µm. (**B**) The average number of Iba-1+ cells per mm2 in CC (mean ±SEM, n=4). Bonferroni post-hoc with two-way ANOVA analysis showed a significant decrease in Iba-1+ cells in the 5-FU group (**P=0.0016) and FLX+5-FU group (*P=0.0395) compared to the saline group. No significant difference was found between FLX, INDO, and INDO+5-FU group and the saline group (p>0.05, two-way ANOVA).*

#### 2.4.4.6 The effects of FLX, INDO and 5-FU treatments on the number of COX-2 positive cells in the ON and CC (study four)

The inflammatory response was investigated in study four by quantifying the number of cells that expressed COX-2 in both ON and CC after long term FLX, INDO and 5-FU treatments. COX-2+ cells were easily identified in both ON and CC. In both tissues, numbers of COX-2+ cell were relatively low in the control groups. Treatment with FLX or INDO alone did not significantly change COX-2+ cell numbers in either white matter region when compared with the control groups ($p>0.05$, two-way ANOVA); (Figures 2.54 and 2.55).

Overall, treatment with 5-FU significantly increased the number of COX-2+ cells in both ON and CC (Bonferroni post-hoc analysis ($F_{(1, 18)} =28.09$, $P<0.0001$ and $F_{(1, 18)} = 42.92$, $P<0.0001$ respectively) compared with the control groups.

In the ON, co-treatment with FLX or INDO reduced the numbers of COX-2+ cells such that they were not significantly different from the control group ($p>0.05$, two-way ANOVA). In the CC, co-treatment of 5-FU with INDO maintained COX-2 numbers at control levels but after co-treatment with FLX, COX-2 levels were significantly above controls ($P=0.0024$, two-way ANOVA). While co-treatment with the INDO before and throughout all 5-FU treatment period prevented the increase in the number of COX-2+ cells such that there was no significant difference with the control group ($P=0.3340$, two-way ANOVA). This indicates that, 5-FU treatment increased COX-2+ cell numbers in both ON and CC but this was prevented by co-treatment with either FLX or INDO in the ON and INDO in the CC.

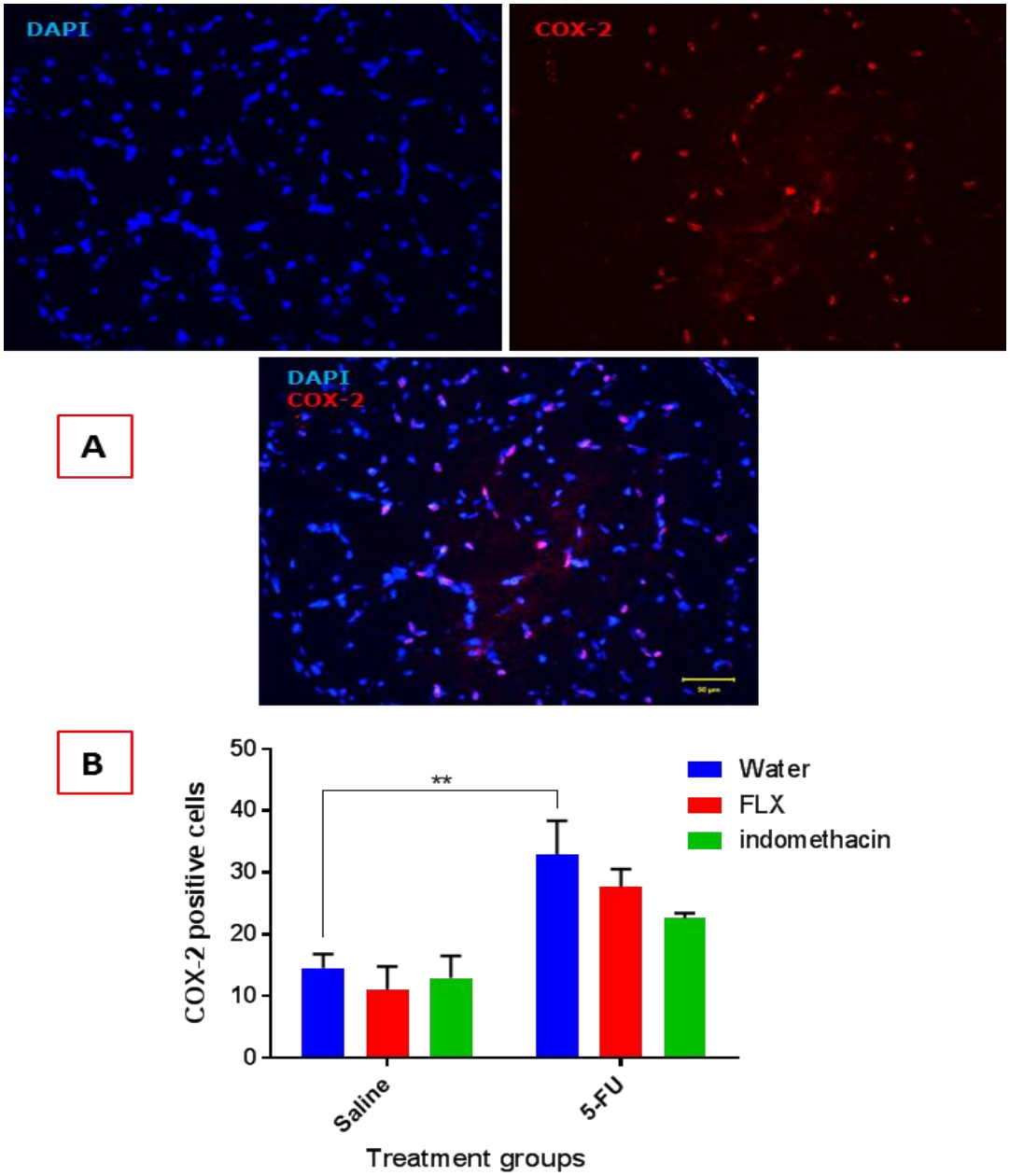



***Figure 2.54*** *The impact of 6 injections of 5-fU on COX-2+ cell numbers in the ON (study four).(**A**) Representative image of rat ON immunostained with COX-2 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50μm. (**B**) Shows the average number of COX-2+ cells per section (mean ± SEM, n=4) in adult rat ON in different treated groups. Analysis showed a significant increase of COX-2 expression cells in the 5-FU group (**P=0.0071, Bonferroni post-hoc multiple comparison analysis) compared with the saline group. No significant difference from controls was found in FLX, INDO, FLX+5-FU and INDO+5-FU groups (p>0.05, two-way ANOVA).*

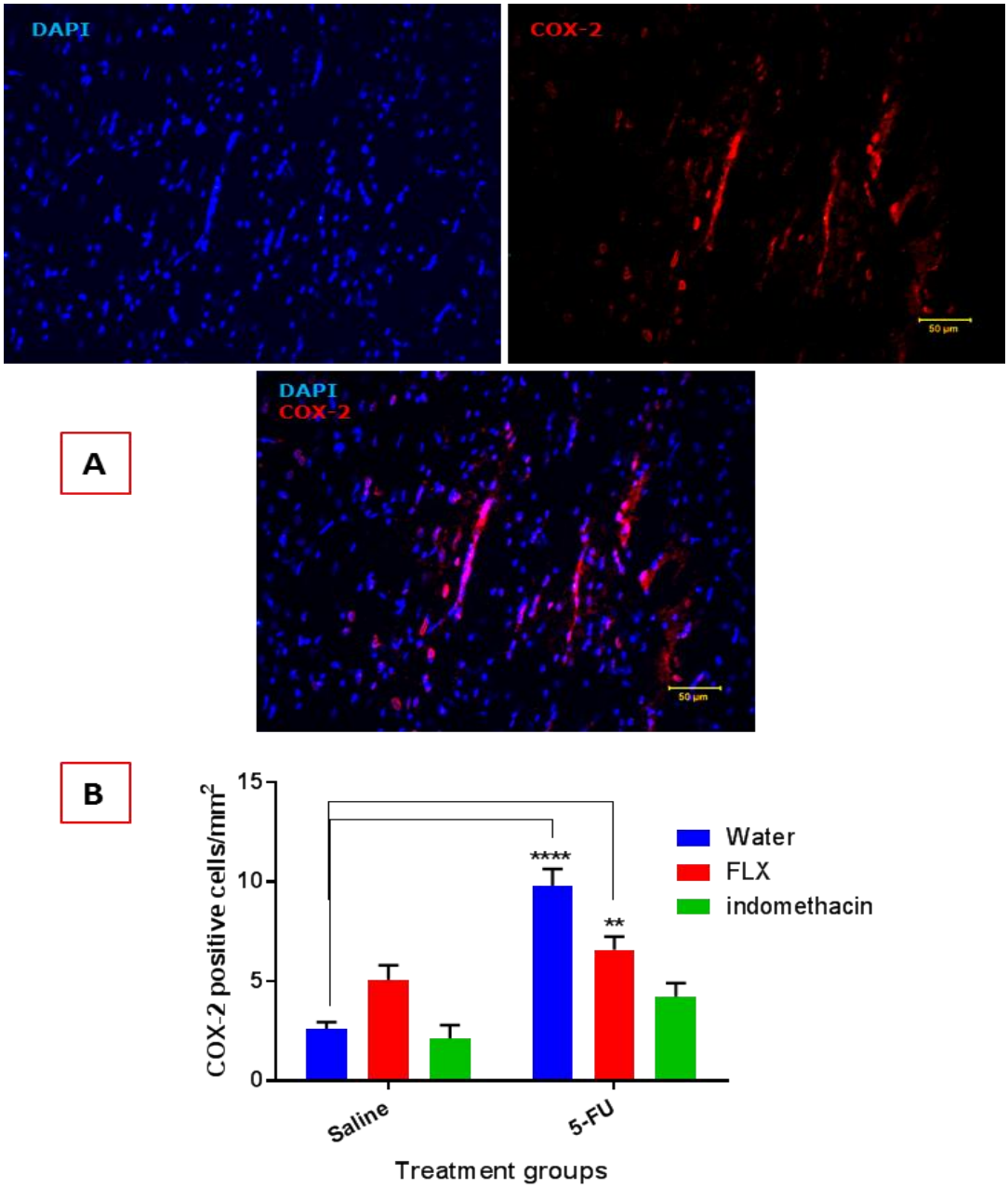



***Figure 2.55*** *The impact of 6 injections of 5-fU on COX-2+ cell numbers in the CC (study four). (**A**) Representative image of rat CC immunostained with COX-2 (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50μm. (**B**) The average number of COX-2 + cells per mm2 (mean ± SEM, n=4) in adult rat CC. Bonferroni post-hoc with two-way ANOVA analysis showed a significant increase of COX-2 expression in both the 5-FU (****P=0.0001) and 5-FU+FLX (**P=0.0024) treated groups compared with the saline-control group. No significant difference was found in FLX, INDO, and INDO+5-FU group when compared to the saline group (p>0.05, two-way ANOVA).*

#### 2.4.4.7 The effects of FLX, INDO and 5-FU treatments on DNA damage in the ON and CC of study four.

The number of pH2AX+ cells was quantified to analyze the effect of chronic 5-FU treatment on DNA damage in ON and CC. In addition, FLX and INDO were administered of different treatment groups to clarify what impact these treatments had on DNA damage. As previously found, levels of pH2AX+ cells were low in controls from both ON and CC (Figures 2.56 and 2.57 respectively). Treatment with either FLX or INDO alone did not significantly change the number of cells showing DNA damage in either tissue ($p>0.05$, two-way ANOVA).

In both the ON and CC, six injections of 5-FU produced a highly significant increase in DNA damage ($F_{(1, 18)} = 41.29$, $P<0.0001$, ON; $F_{(1, 18)} =30.16$, $P<0.0001$, CC; two-way ANOVA with Bonferroni post-hoc test). Co-treatment of 5-FU with FLX or INDO reduced pH2AX levels in both ON and CC. Levels remained significantly above controls for FLX+5-FU in the ON ($P=0.0142$), but were not significantly different from controls for the remaining 3 groups across the ON and CC (ON, INDO+5FU $P=0.0570$; CC, FLX+5-FU, INDO+5FU $p>0.05$, two-way ANOVA). In conclusion co- treatment with either FLX or INDO reduces the numbers of pH2AX cells after 5-FU treatment.

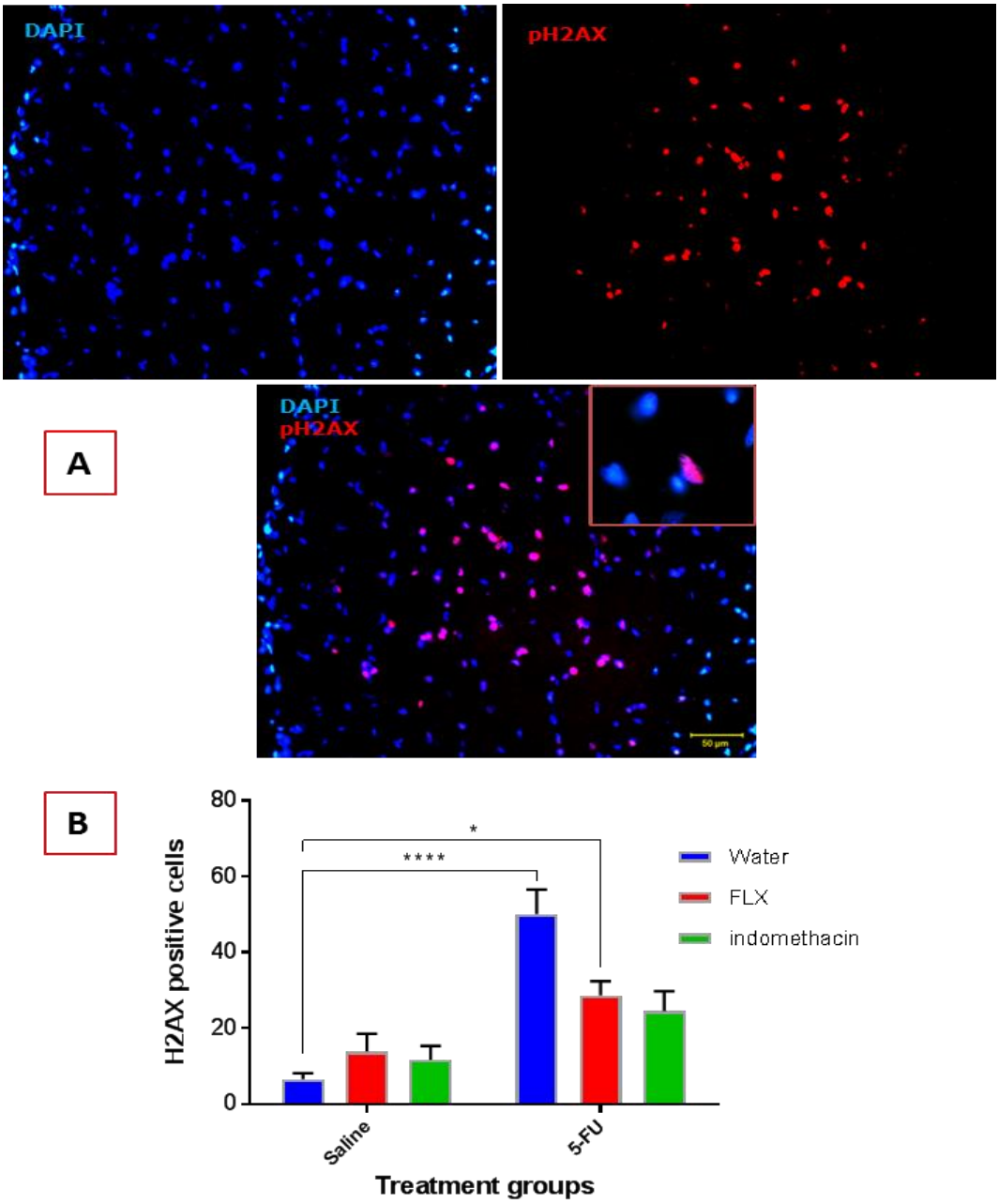



***Figure 2.56*** *The impact of 6 injections of 5-FU on pH2AX positive cell numbers in the ON (study four). (**A**) Representative image of rat ON immunostained for pH2AX (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=50µm. (**B**) Shows the average number of pH2AX + cells per section (mean ± SEM, n=4) in ON in different treatment groups. Analysis showed a significant increase in pH2AX expression in the 5-FU and FLX+5-FU groups (****P<0.0001 and *P=0.0142 respectively, Bonferroni post-hoc multiple comparison two-way ANOVA) compared with the saline group. No significant difference was found between the FLX, INDO, and INDO+5-FU groups compared to the saline group (p>0.05, two-way ANOVA).*

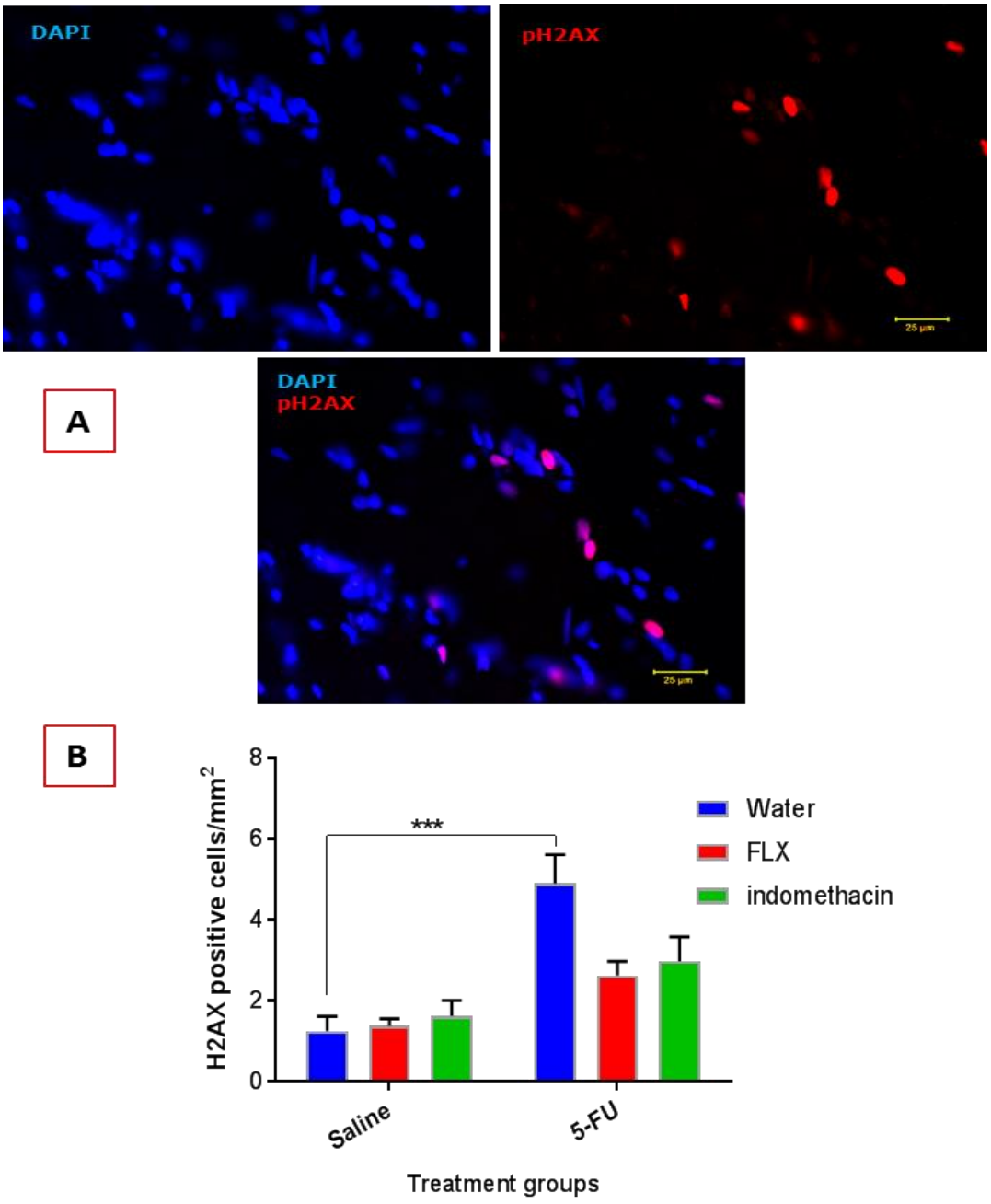



***Figure 2.57*** *The impact of 6 injections of 5-FU on pH2AX+ cell numbers in the CC (study four).(**A**) Representative image of rat CC immunostained for pH2AX (red) and counterstained with DAPI (blue) in separate channels and merged. Scale Bar=25µm. (**B**) The average number of pH2AX + cells per mm2 (mean ± SEM, n=4) in CC. Analysis showed a significant increase of pH2AX expression in 5-FU treated group (***P=0.0001, Bonferroni post-hoc with two-way ANOVA test) compared with the saline-control group. No significant difference in DNA damage was found between the FLX, INDO, FLX+5-FU and INDO+5-FU groups and controls (p>0.05, two-way ANOVA).*

## 2.5 Discussion

Despite the abundance of data regarding chemotherapy induced neurotoxicity and problems with memory, there is still considerable uncertainty apropos the pathological effects and mechanism of chemotherapy drugs on CNS WM tracts. Various anticancer drugs like 5-FU, methotrexate and Cisplatin have been shown to damage WMT structure in humans and animals by blocking the proliferation of dividing cells such as stem and progenitor cells or due to inflammatory response and DNA damage (Dietrich *et al.* 2006, Han *et al.* 2008, Soussain *et al.* 2009, Yamaguchi *et al.* 2009, Seigers *et al.* 2010, Weng *et al.* 2014). The current experiments in this chapter investigated the effects of acute and chronic treatments with chemotherapy on gliogenesis and WM integrity in the adult rat CNS. In addition, this study aimed to test the effectiveness of antidepressant 'FLX' or anti-inflammatory 'INDO' drugs at preventing the changes in the optic nerve and CC induced by 5-FU.

### 2.5.1 Impact of the treatments on body weight and water intake

From the results of all experiments, it was found that all groups of rats gained weight across each experiment with different variances in some treated groups. Generally, chemotherapy can rapidly cause weight loss in treated patients. In rodents this is seen as a reduction in the weight gain over the course of the experiment with 5-FU and FLX. In study one and two, a significant decline in weight was seen for all groups treated with FLX on day 7-21. This could be attributed to disruption of intestinal absorption by FLX (Freeman *et al.* 2006); However, this reduction had disappeared in the last day of experiment after drug cessation. Moreover, 5-FU groups showed a significant reduction in body gain which can be attributed to a number of cellular mechanisms. In agreement with the current results, Huang *et al.* (2002) observed that chemotherapy treatment was associated with a reduced ability of the gastrointestinal tract to absorb nutrients. This may be due to cytotoxic damage to proliferating cells lining the intestinal wall (Lee *et al.* 2006). Others have found that chemotherapy injures healthy tissue which can cause an elevation of cytokines levels and can be responsible for anorexia, weakness and fatigue (Ahles and Saykin 2001, Jansen *et al.* 2005). Experiments three and four, found that FLX alone did not induce a significant weight loss compared to controls. However there was a highly significant reduction in the weight of FLX+5-FU treated group for all experiments. This is likely to be due to a compound effect on weight gain of the animals when both drugs were given (ElBeltagy *et al.* 2012).

Most animals treated with FLX regained body weight in the one week recovery period. The regained weight could be attributed to improved food intake after the cessation of treatment, suggesting that weight loss was a temporary effect. At the advanced period of FLX treatment, the weight of animals did not relatively increase compared to vehicle-treated animals, however there was no reduction in weight during this time (Figures 2.7, 2.20, 2.33 and 2.46). It was seen that chronic 5-FU produced a significant reduction in weight gain starting 24 hours after the first injection and extending to the recovery period. This is likely to be due hypophagia or damage to the gut lining (Douillard *et al.* 2000, Huang *et al.* 2002, Yanez *et al.* 2003, Miyata *et al.* 2009). Based on the daily weighing data, the INDO treated group showed weight gains comparable to the other treated groups. This result is similar to other researchers' results which have found an increase in body weight in mice treated with INDO. This was attributed to increased food consumption to relieve gastric pain that occurs in long-term NSAID use (Risser *et al.* 2009, Ho *et al.* 2014).

Drinking water containing FLX or INDO was freely available to the animals. Based upon regular water intake measurements, water consumption was analyzed using one-way repeated measures ANOVA. In all four studies of this chapter, FLX treatment caused a significant reduction in water intake. This could be due to a taste aversion to FLX (Prendergast *et al.* 1996). Contrary to expectation, the single 5-FU injection in studies one and two, did not produce a significant prolonged difference in water intake, however there was a transient decrease in water consumption 24 hours after injection. It seems that a single dose of chemotherapy induced an increase in the sickness behaviour response after treatment, suggesting that the effect was temporary. The significant decline in water intake seen in 5-FU treated animals after chronic treatment with 5-FU in studies three and four has also been observed in the previous studies (Walker *et al.* 2011, Lyons *et al.* 2012). Likewise, this regimen of 5-FU may have led to stress and sickness behavior and a consequent decrease in water intake (Baskerville and Batter-Hatton 1977).

### 2.5.2 FLX and INDO prevent the reduction in proliferating cell numbers in WMTs caused by 5-FU chemotherapy

Firstly, in this study, the number of proliferating cells positive for Ki67 in the normal WMTs of CNS was looked at. Secondly, it was determined if 5-FU affected cell proliferation transiently or had a long term effect. Based on past work on 5-FU, it was expected that chemotherapy treatment would cause a significant reduction in the number of proliferating cells (Yang et al., 2012, Yang et al., 2011).

In study one and two, unexpectedly, after acute treatment there was a non-significant difference in the number of dividing cells in the ON and CC either one or seven days after a single injection by 5-FU (Figures 2.9, 2.10, 2.22, 2.23). There are several possibilities to explain this outcome which are partly supported by results from previous studies. Mustafa *et al.* (2008), showed that acute administration of 5-FU did not significantly reduce the number of Ki67 positive cells in SGZ of hippocampus. This might be due to cell cycle kinetics and the high metabolism of 5-FU in rodents which is estimated to be have a half-life of approximately 15 minutes. Moreover, the cell cycle time of progenitor cells in the dentate gyrus is about 25 hours (Cameron and McKay 2001) and as about 38% of the cell cycle time is spent in 'S' phase of cell cycle (i.e. 9.5 hours from total cell cycle time)(Dayer *et al.* 2003), single injections of 5-FU will only affect a small proportion of dividing cells. Clinically, 5-FU breakdown is slower in humans and the drug is normally given as a chronic series of injections (Au *et al.* 1983, Kutscher *et al.* 2000). Alternatively, it could be that too low dose of 5-FU (35 mg/kg) was used in these experiments. This dose is less than the chronic dose of 50mg/kg that reduced cell proliferation in mice (Mignone and Weber 2006), but is similar to the dose given in multiple injections which produced significant effects on hippocampal cell proliferation (Dietrich *et al.* 2006, Seigers *et al.* 2009, Lyons 2011). It has been shown that 5-FU slightly increased the Olig2 expression, a gene necessary for the generation of normal oligodendrocytes in CC 1 day after injection, while after 56 days of treatment the number of Olig2+ cells dramatically decreased. These results indicated that 5-FU exposure was sufficient to cause considerable delayed abnormalities in progenitor cells biology (Han *et al.* 2008).

The results of the chronic treatment with 5-FU (study 3) which involved 6 injection of 5-FU, 35m/kg given every 3 days, showed a significant reduction in cell division in both the ON and CC (Figures 2.35 and 2.36). This result provides significant support for the hypothesis that chemotherapy reduces the levels of cell proliferation in WMTs and that this could be at least in part responsible for the subtle cognitive impairments found after chemotherapy treatment in patients. This result is in agreement with other studies that have found that 5-FU is toxic for both progenitor and mature oligodendrocyte cells. These studies also suggest that these effects can be progressive and lead to delayed damage to the myelinated tracts of the CNS (Han *et al.* 2008). Previous studies have observed that chemotherapy treatment increases cell apoptosis and reduces adult neurogenesis as well as progressive damage to WM tracts (Dietrich *et al.* 2006, Mignone and Weber 2006).

Treatment with the antidepressant FLX or the anti-inflammatory drug INDO on their own did not affect levels of cell proliferation. When FLX or INDO were given in combination with 5-FU, this prevented the decrease in cell proliferation seen when 5-FU was given on its own. This result demonstrates that these drugs have a protective role in WTM tracts. This effect is similar to the effects that chronic treatment with FLX has when given prior to 5-FU on hippocampal neurogenesis where the antidepressant treatment prevented the decrease in cell proliferation in the SGZ of the dentate gyrus (Lyons *et al.* 2012). Similarly, Marcussen *et al.* (2008) showed that chronic FLX treatment increased adult neurogenesis in the hippocampus of rodents not treated with other drugs. However, this result was not confirmed by studies in this laboratory which found that 21 days of FLX treatment on its own did not increase cell proliferation of hippocampus of rats (ElBeltagy, et al. 2010). It is possible that the effects of FLX on hippocampal neurogenesis or OPCs proliferations depend on the level of neurogenesis and gliogenesis during treatment. When neurogenesis or gliogenesis is reduced, FLX can protect or augment neurogenesis and oligogenesis, but FLX treatment cannot increase the levels of neurogenesis above a certain maximum level. In the present experiment four, 43 days of FLX treatment prevented the adverse effect of chemotherapy which would normally reduce cell proliferation in WMTs. It is also likely that the period of FLX administration is important in providing the neuroprotective effect, with prior treatment being required to produce this effect as this has been found in studies of adult neurogenesis (Lyons *et al.* 2012).

Another important finding in these experiments was that INDO administration prior, throughout and after cessation of chemotherapy elevates the number of proliferating cells in CC and ON. This novel finding suggests that inflammation is a key mechanism behind the reduction in cell proliferation in WTM after chemotherapy treatment. It is unclear what the effect of INDO is on the normal cell proliferation but anti-inflammatory agents' have been shown to prevent the decline in cell proliferation in the hippocampus produced by cranial irradiation (Monje *et al.* 2003, Briones and Woods 2014).

### 2.5.3 Effects of chemotherapy on adult WMT gliogenesis

As mentioned in the literature review, NG2 is a marker for both developmental and adult oligodendrocyte progenitor cells (OPCs) which are identified by the expression of the neuron-glial antigen 2 (NG2+). Thus these cells are generally called 'OLPs or NG2' cells' (Psachoulia *et al.* 2009). Jennings and Carroll (2010) found that despite their morphological complexity, all NG2 cells in the ON are OPCs which have the ability to proliferation which has implications for efforts to

enhance and repair myelin sheaths multiple sclerosis. These cells form about 5-8% of neuroglial cells and have a ratio to oligodendrocytes of approximately 1:10. Proliferation and differentiation of NG2+ cells is thought to be important in repairing and remyelinating the CNS both in normal and pathogenic conditions (Jennings and Carroll 2010). NG2+ or as other neuroglial cells could be affected by different chemotherapeutical agents (Dietrich *et al.* 2006, Han *et al.* 2008). NG2+ cells showed a decrease 7 days after 5-FU exposure in both ON and CC (Figures 2.24, 2.25, 2.37 and 2.38). The decrease in OPCs will result in a decline in the ability of white matter to be remyelinate by the generation of new mature oligodendrocytes. As this effect of 5-FU appears to be widespread in white matter regions, it is likely that similar effects occur in patients and that these result in functional deficits which could include a reduction in conduction velocity and the ability of white matter tracts to respond to injury or pathological changes.

Although a single injection of 5-FU did not produce any significant difference in the number of NG2+ cells, multiple comparison analysis showed that OPCs in the 5-FU group declined significantly compared with the FLX group when evaluated seven days after treatment. This may indicate that the dose of 5-FU used in the acute experiment did not affect the number of precursor cells (NG2+) in the ON and CC 24 hours after injection, but may have caused a delayed effect apparent one week after treatment. Other authors have found that systemic chemotherapy treatment causes a syndrome of delayed CNS damage such that changes are not observed immediately after treatment (Han *et al.* 2008, Payne *et al.* 2013). Alternatively it is possible that the chronic treatment with FLX for 21 days induced an increase in the proliferation of progenitor cells in this group compared to cells in the chemotherapy treated group (Figure 2.24). This is a possible protective effect of FLX administration which has been reported previously in studies of hippocampal neurogenesis (Encinas *et al.* 2006, Lyons 2011, ElBeltagy *et al.* 2012). These results are largely consistent with the extant literature which shows that treatment with a low dose or single injection of 5-FU does not reduce cell proliferation (Mignone and Weber 2006, Mustafa *et al.* 2008). This might be because the total amount of 5-FU used in these two experiments was considerably lower than those used in subsequent chronic treatments; or it could be that longer periods post treatment are needed to show the impact of chemotherapy. In contrast, other studies have described how 5-FU and other chemotherapy agents given chronically or in higher doses are able to produce both short and long term defects in the cell proliferation required for neurogenesis (Dietrich *et al.* 2006, Seigers *et al.* 2009, Lyons 2011).

It is interesting to note that the CC in all four experiments of this study showed an increase in the total number of the NG2 positive cells after chronic treatment with FLX when compared with other groups. The effect of FLX on hippocampal neurogenesis has been examined by other authors and the findings of the current study are consistent with those of Surget *et al.* (2011) who found that administration of FLX and other selective serotonin reuptake inhibitor's (SSRIs) caused an increase in the levels of adult-generated neurons. These findings further support the idea that antidepressants or atypical antipsychotics which can increase neurogenesis in the adult hippocampus, also increase the proliferation of glia in white matter regions. This effect could reverse the loss of glia that has been observed in depressed patients (Kodama *et al.* 2004), and may provide treatments in other neurodegenerative diseases. A possible mechanism for this might be that chronic treatment with FLX decreases levels of endogenous cell cycle inhibitors such as p21Cip1, p18INK or p21Kip1 an effect which was associated with an increase in the proliferation of neuronal progenitors (Pechnick *et al.* 2011). Another possible explanation is that FLX is known to increase expression of growth factors such as Brain-derived neurotrophic factor (BDNF). BDNF increases neurogenesis by increasing the binding of 5-HT to its receptors, which up-regulates intracellular MAP kinase or cAMP Response Element Binding protein (CREB) (Duman *et al.* 2001, Alme *et al.* 2007, Merz *et al.* 2011). In contrast to the results in the CC, the increases in NG2+ cells were not significant except in study two.

As FLX can induce proliferation in stem cells, administering it concurrently with a chemotherapy drug may at least compensate for the decrease in oligogenesis caused by a decrease in NG2+ cells. Interestingly, this may have happened when FLX treatment reversed the reduction in gliogenesis and OPCs generation caused by 5-FU in the ON of studies one and four and also in the CC of studies one, two and four. It is possible; therefore, that FLX could potentially mitigate the impact of chemotherapy on white matter tracts if it were to counteract the anti-proliferative effects of 5-FU and maintain normal rates of oligogenesis. The protective effect of FLX on white matter tracts found in the present study further supports previous work on the protective role of this drug on hippocampal neurogenesis; and indicate that FLX may be suitable to prevent chemotherapy-induced cognitive decline. Although the mechanisms by which FLX produces its neuroprotective properties are unclear, a number of possibilities have been suggested. FLX is known to be a cognitive enhancer and to increase brain serotonin levels, a neurotransmitter which itself appears to influence cognitive function (Seigers and Fardell 2011). Huang *et al.* (2013) found that FLX has a

novel role in neuroprotection by increasing the crosstalk mechanism between the PI3K/AKT and MAPK/ERK signalling pathways. These are involved in upregulating expression of phosphorylated-AKT and ERK1/2 proteins that stimulate cell proliferation in neural stem cells (NSCs). Also, some authors (Liu *et al.* 2011, Tynan *et al.* 2012, Su *et al.* 2015) have suggested that FLX exerts its effects via an anti-inflammatory effect. They found that FLX can inhibit the microglial activation which leads to the secretion of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6 and nitric oxide (NO), by inhibiting the change from the M2 to M1 states of these cells. FLX also suppresses the expression of NF-κB which is an important transcription factor necessary for the production of pro-inflammatory factors (Roumestan *et al.* 2007). Taken together, these results suggest that FLX may modulate the immune system within the CNS to suppress inflammation, a suggestion which could lead to the use of FLX as a possible neuroprotectant against the effects of chemotherapy.

Contrary to expectations based on the previous discussion, we found that INDO treatment did not produce a significant difference in the number of OPCs compared to other treatment groups in CC or any protective effect against the reduction in NG2 positive cells when co-administrated with 5-FU (Figure 2.51). In ON, treatment with INDO alone showed a tendency to increase OPCs however this did not reach significant levels when compared with controls (Figure 2.50). Reports on the impact of INDO on cell proliferation in the brain vary. INDO given to aged mice (24 months old), did not promote hippocampal neurogenesis or prevent the age related increase in neuroinflammation (Boehme *et al.* 2014). However Ho *et al.* (2014) observed that chronic treatment with INDO reversed the decrease in cell proliferation within the hippocampus of mice, possibly by reducing the pro-inflammatory cytokine TNF-α produced after administration of streptozotocin (STZ). In agreement with Ho and his colleagues, the results presented here show that co-administration of INDO prevents significant drop in OPCs with 5-FU treatment in the ON.

### 2.5.4 Impact of different treatments on microglial cells and inflammation

The mechanisms of CNS injury after systemic chemotherapy have not been fully elucidated, but they are likely to be multifactorial with different cell types being involved, including oligodendrocytes, neurons, microglia, endothelia and astrocytes. Microglia in normal situations are chiefly responsible for monitoring the brain microenvironment to maintain homeostasis and generate an immune response within the CNS (Stoll and Jander 1999). Therefore, this study set out to

assess the number of microglia within white matter after exposure to chemotherapy.

In this study, a single injection of 5-FU (Figure 2.16) was found to cause an increase in the total number of microglia 24 hours later in the CC. This suggests that the initial effect of chemotherapy is to trigger proliferation by microglia. However one week after the single injection, the number of microglia had significantly decreased compared with controls (Figure 2.27). Similarly, multiple injections of chemotherapy resulted in a decrease the number of Iba-1 positive cells in both ON and CC (Figures 2.39, 2.40, 2.52 and 2.53) one week after the final injection.

The increase in the number of microglia 24 hours after acute treatment with 5-FU (study one) may be similar to previous reports where infection or acute injury leads to an increase in microglia presumably as part of an immune response to these insults (Wegiel *et al.* 2003, Erny *et al.* 2015). In the hippocampus these inflammatory responses can promote or prevent neurogenesis but in general inhibit neurogenesis and lead to cognitive impairment (Ekdahl *et al.* 2009).

The decrease in microglial numbers in both ON and CC one week after a single in injection or after multiple injections, is likely to be due to apoptosis of microglia due to a delayed reaction to chemotherapy or a response by microglia to the increased levels of inflammatory cytokines. It is also possible that the reduction in the number of microglia after activation is caused by the apoptosis that occurs due to lack of growth factors needed to maintain proliferating microglia (Reid *et al.* 1993, Seigers *et al.* 2016). These findings further supported by Paquet *et al.* (2016) who found that microglia are vulnerable to the direct toxicity of chemotherapy.

As mentioned in the literature review, FLX and INDO can have positive effects on adult neurogenesis in the CNS and one mechanism by which these drugs act may be by reducing the neuro-inflammatory processes produced by cytotoxic agents. One important finding in this study is that co-administration of FLX+5-FU or INDO+5-FU prevents the reduction in the number of microglia that occurs after multiple in injections of chemotherapy (Figures 2.52 and 2.53). These results could be due to FLX and INDO inhibiting the activation of microglia during which they change from the M1 state to the M2 state which adapts the immune system of CNS to stress-induced oxidative cell damage (Novio *et al.* 2011, Su *et al.* 2015, Caiaffo *et al.* 2016). Also, FLX is one of the serotonergic agonists which regulation of 5-HT uptake and enhance cell genesis (Duman *et al.* 2001, Encinas *et al.* 2006).

Another possible explanation is that FLX increases Bcl-2 (B-cell lymphoma 2) expression. Bcl-2 is an anti-apoptotic gene which prevents apoptosis and enhances proliferation (Chen *et al.* 2007, Zhang *et al.* 2014).

Administration of INDO after induction of inflammation overcomes the inhibition of hippocampal neurogenesis in mice normally produced by inflammation (Ji *et al.* 2013). It is well known that 5-FU and other cytotoxic agents induce apoptosis in different types of cells (Lee *et al.* 2005, Réti *et al.* 2008, Wigmore *et al.* 2010), and especially in microglia (Reid *et al.* 1993, Seigers *et al.* 2016). Réti *et al.* (2008) showed that 5-FU disturbs the cell cycle and enhances the rate of apoptosis. The combination of 5-FU with INDO appears to decrease the rate of apoptosis. This may be attributed to the effect of NSAIDs in inhibiting COX2 (Bastos *et al.* 2008) and also increasing the level of arachidonic acid that induces cell proliferation in stem cells via cyclin D/E and CDK2/4 (Meade *et al.* 1993, Lee *et al.* 2008, Réti *et al.* 2008).

Prior studies have noted the importance of COX-2 expression in the CNS and its association with increased inflammation which are thought to contribute to the neurodegenerative processes in several acute and chronic diseases (Minghetti 2004). In this chapter the numbers of COX-2+ cells, was quantified after single or multiple 5-FU injections. The first point of interest was to find that in all four experiments, 5-FU treatment significantly increased COX-2+ cell numbers compared to controls (Figures 2.13, 2.28, 2.41 and 2.54). This increase suggests that 5-FU induces inflammation within the WMTs of the brain which could reduce WMT gliogenesis and progenitor proliferation. This finding is in agreement with Réti *et al.* (2008) who concluded that 5-FU chemotherapy treatment of HCA-7 colon cancer cells lead to increased expression of COX-2 after 48 hours. Moreover, the current results are in agreement with Briones and Woods (2014) who showed that neuroinflammation may play a role in chemotherapy induced demyelination which in turn may lead to cognitive impairment. They showed that a combination of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) produced cognitive impairments and damage to the brain WM microstructure in rats and myelin breakdown (Briones and Woods 2014). This was associated with a decrease in the level of the anti-inflammatory cytokine IL-10, and an increase in the levels of the inflammatory mediators COX-2, TNF-α and IL-1ß. These changes led to a decrease in myelin basic protein. However, the findings of the current study do not support research which found no increase in the levels of central cytokines after methotrexate treatment in rats (Seigers *et al.* 2010). This may be explained as methotrexate can act as an anti-inflammatory (Cutolo *et al.* 2001), and the

cytokine expression in the CNS will differ from the peripheral expression in the plasma which was measured by Siegers and her colleagues. It seems likely that 5-FU chemotherapy induces an inflammatory response as shown by the upregulation COX-2 and that this may be involved in the reduced production and survival of new cells in adult neurogenesis and oligodendrogenesis.

Administration of anti-inflammatory drugs can enhance neurogenesis by restoring the interaction between neural stem cells and the microvasculature which is disrupted by inflammation (Monje *et al.* 2003, Ho *et al.* 2015). In addition, SSRI antidepressants such as FLX have an anti-inflammatory effect and significantly reduce microglial activation and inflammatory markers (Roumestan *et al.* 2007, Liu *et al.* 2011, Tynan *et al.* 2012). In the present study it was found that FLX and INDO prevented some of the adverse effects of 5-FU on the ON and CC. As previously reported FLX suppresses NF-κB which is a transcription factor for the production of pro-inflammatory factors like TNF-α, NO and IL-6 (Ha *et al.* 2006, Roumestan *et al.* 2007, Tynan *et al.* 2012). The present findings are consistent with other research which has found that administration of anti-inflammatory drugs can enhance neurogenesis by restoring the interaction between neural stem cells and the microvasculature when it is disrupted by inflammation (Monje *et al.* 2003). As shown by a previous study, INDO reduces the level of inflammation after adult cerebral injury by reducing COX-1 and COX-2 expression within the brain (Netland *et al.* 1998). This also accords with Boehme *et al.* (2014) and Monje *et al.* (2003) who found that INDO protects hippocampal neurogenesis after cranial irradiation or LPS induced inflammation but has no effect in non-inflammatory situations such as ageing. In agreement with this the present study showed that administration of FLX or INDO alone did not produce any significant difference when compared with controls. Indicating that these drugs do not have a direct effect in cell proliferation or differentiation, but prevent the adverse effects of inflammatory mediators like cytokines and interleukins, to maintain an optimum environment within the brain to enable remyelination and the trophic support afforded by glial cells (Nguyen *et al.* 2002). Importantly, blocking the neuroinflammatory reaction produced by 5-FU treatment offset the detrimental effects of this cytotoxic agent on the numbers of OPCs and subsequently myelin and myelination in WMTs.

### 2.5.5 The impact of 5-FU chemotherapy on DNA damage in adult WMT

Chemotherapy agents used to treat cancer mostly work by interfering with DNA synthesis and so prevent cell division. This is the case with 5-FU which can induce double strand DNA breaks by the incorporation of uracil instead of thymidine

during DNA synthesis, leading to cell death (Longley *et al.* 2003). In order to asses this aspect of 5-FU treatment, immunohistochemistry was used to detect the presence of phosphorylated histone H2AX (pH2AX). Phosphorylation of H2AX occurs after DNA double strand breaks as a part of the DNA damage response cascade (MacPhail *et al.* 2003). Study one which looked at the effect of a single injection of 5-FU 24 hours after treatment showed no effect of treatment in any groups compared to controls in both ON and CC. The remaining studies (two, three and four) showed a significant increase in the number of pH2X positive cells after 5-FU treatment. In agreement with existing findings, it was found that 5-FU has been shown to produce a dose dependent increase in DNA damage and apoptosis (Fujinaka *et al.* 2012, Krynetskiy *et al.* 2013); and causing cell cycle arrest at the G0/G1 stage of the cell cycle, which prevents cells from progressing through DNA replication and cell proliferation (Lee *et al.* 2005). The mechanism of this is unclear, but DNA in normal cells as well as cancer cells is affected, resulting in many of the side effects of chemotherapy.

As mentioned previously, cytokines play a role in controlling certain neuronal processes, such as glial function, repair, and certain neurotransmitter metabolism (Ahles and Saykin 2007). As part of an immune response, cytokines are signalling molecules which are released in response to many different events, including DNA damage induced by chemotherapy (Ahles and Saykin 2007). The observed increase in DNA damage after 5-FU treatment could be due to the direct effects of 5-FU on DNA synthesis but also to the elevated level of cytokines, and prolonged neuroinflammation, which my produce neurotoxic effects on dividing cells by increasing oxidative stress or by glutamate receptor-mediated damage in the WMT (Ahles and Saykin 2007, Domercq *et al.* 2007). Another possible effect is that chemotherapy can cause cell senescence which is accelerated by the actions of p53 and p21 mediated mechanisms and initiating DNA damage (Panda *et al.* 2008, Dabrowska *et al.* 2009). Or by increasing production of reactive oxygen species (ROS) and a decline in DNA repair by activation of p53 lead to increase oxidative stress and initiate cellular quiescence or senescence (Chen *et al.* 2007, Liu *et al.* 2009). This can subsequently induce apoptosis (Dabrowska *et al.* 2009, Dabrowska *et al.* 2011). So far, no previous studies have related WMT damage and dysfunction to the level of DNA damage after treatment with 5-FU.

One aim of this study was to determine whether antidepressant or anti-inflammatory treatments would modulate the effect of 5-FU on DNA damage in both the ON and CC. In study two, FLX administration, before 5-FU injection, produced a decrease in the number of pH2AX positive cells one week after

treatment. Whereas, after chronic 5-FU treatment, FLX did not modulate the effect of 5-FU in the ON. In CC only study four found that FLX ameliorated the DNA damage when it was co-administrated before, during and after chemotherapy treatment. The results of this study indicate that FLX administration could prevent the effect of 5-FU in producing DNA damage when co-administrated with low dose of chemotherapy, or when co-administrated before, during and after chemotherapy treatment. It has been in agreement with studies which have found that FLX can decrease apoptosis in adult rat dentate gyrus and in stem cell cultures derived from the adult rat hippocampus (Chen *et al.* 2007, Egeland *et al.* 2010). Chemotherapy and oxidative stress can induce cells which have minor DNA damage to enter a prolonged period of quiescence (d'Adda di Fagagna 2008). Cell quiescence requires the expression of the cylcin dependant kinase inhibitor p21 which prevents cells entering mitosis (Stivala *et al.* 2012). FLX has been reported to downregulate the p21 pathway during neurogenesis (Pechnick *et al.* 2011) and it is possible that by preventing cells entering quiescence after chemotherapy treatment, and reducing DNA damage by its anti-inflammatory actions, subsequently, FLX maintains cell proliferation. This hypothesis is supported by the work of Shan *et al.* (2016) who found that FLX protects against IL-1b-induced DNA damage and neuronal apoptosis by downregulation of p53. However in study four, FLX did not produce a protective effect against DNA damage.

INDO in combination with 5-FU produced a significant decrease in pH2AX+ cells compared to 5-FU on its own. The reason for this is not clear, but it this may be related to the effect of INDO in the upregulation of the CDKN2A/p16 tumour suppressor gene, and a decrease in DNA methylation enzymes which increases the activity of the immune system (Lundholm *et al.* 1994). These findings further support the idea that INDO "releases and protects" the progenitor cell population by increasing the survival of progenitor cells and allowing them to differentiate into NG2 and oligodendrocytes as well as increasing DCX+ neurons (Hoehn *et al.* 2005). As mentioned earlier, 5-FU increases the levels of the inflammatory mediators COX-2, TNF-α and IL-1ß (Briones and Woods 2014). COX-2 is involved in the generation of reactive oxygen species which are associated with increased histone acetylation and decreased histone deacetylase activity. Changes which can adversely affect the self-renewal of neural progenitor cells and also chromatin remodelling in the hippocampus (Briones and Woods 2011). So, INDO inhibits COX-2, which is involved in the generation of reactive oxygen species, and also modulates signalling networks involved in inflammation including nuclear factor $_{k}$B (von Knethen *et al.* 1999, Takahashi *et al.* 2004). This is consistent with Jiang *et al.* (1998) and Wada and Kamisaki (2003) who found that INDO also

antagonises the transcription factor peroxisome proliferator-activated receptor-$_{\gamma}$, which inhibits the expression of pro-inflammatory cytokines in monocytes or microglia (Hoehn *et al.* 2005). These effects may prevent DNA damage and support neural stem cell survival.

***Table 2:1*** *Summary of results from the in vivo experiments presented the effects of different treatments on optic nerve.*

| | FLX | INDO | 5-FU | FLX/5-FU | INDO/5-FU |
|---|---|---|---|---|---|
| Study 1: Acute effects of 5-FU treatment experiment | | | | | |
| Ki67 | = | N/A | = | = | N/A |
| NG2 | = | N/A | = | = | N/A |
| Iba-1 | = | N/A | = | = | N/A |
| COX-2 | = | N/A | ↑↑↑↑ | ↑ | N/A |
| pH2AX | = | N/A | = | = | N/A |
| Study 2: Acute effects of 5-FU after one week from treatment | | | | | |
| Ki67 | ↓ | N/A | = | = | N/A |
| NG2 | ↑ | N/A | = | = | N/A |
| Iba-1 | = | N/A | = | = | N/A |
| COX-2 | = | N/A | ↑↑↑ | protection | N/A |
| pH2AX | = | N/A | ↑↑ | protection | N/A |
| Study 3: Chronic effects of 5-FU after one week of recovery | | | | | |
| Ki67 | = | N/A | ↓↓↓ | protection | N/A |
| NG2 | = | N/A | ↓↓↓ | ↓ | N/A |
| Iba-1 | = | N/A | ↓↓ | Protection | N/A |
| COX-2 | = | N/A | ↑↑↑↑ | ↑↑ | N/A |
| pH2AX | = | N/A | ↑↑↑↑ | ↑↑↑ | N/A |
| Study 4: Chronic effects of 5-FU after one week of treatments | | | | | |
| Ki67 | = | = | ↓↓ | Protection | Protection |
| NG2 | = | = | ↓↓↓ | Protection | Protection |
| Iba-1 | = | = | ↓↓ | Protection | Protection |
| COX-2 | = | = | ↑↑ | Protection | Protection |
| pH2AX | = | = | ↑↑↑↑ | ↑↑ | protection |

***Table 2:2*** *Summary of results from the in vivo experiments presented the effects of different treatments on corpus callosum.*

| | FLX | INDO | 5-FU | FLX/5-FU | INDO/5-FU |
|---|---|---|---|---|---|
| Study 1: Acute effects of 5-FU treatment experiment | | | | | |
| Ki67 | = | N/A | = | = | N/A |
| NG2 | ↑ | N/A | = | = | N/A |
| Iba-1 | = | N/A | ↑ | protection | N/A |
| COX-2 | = | N/A | ↑↑ | ↑ | N/A |
| pH2AX | = | N/A | = | = | N/A |
| Study 2: Acute effects of 5-FU after one week from treatment | | | | | |
| Ki67 | = | N/A | = | = | N/A |
| NG2 | ↑ | N/A | ↓↓↓↓ | protection | N/A |
| Iba-1 | ↑ | N/A | ↓↓ | = | N/A |
| COX-2 | = | N/A | ↑↑ | = | N/A |
| pH2AX | = | N/A | ↑↑ | ↑ | N/A |
| Study 3: Chronic effects of 5-FU after one week of recovery | | | | | |
| Ki67 | = | N/A | ↓↓↓↓ | Protection | N/A |
| NG2 | ↑ | N/A | ↓↓↓ | Protection | N/A |
| Iba-1 | = | N/A | ↓ | Protection | N/A |
| COX-2 | = | N/A | ↑↑↑↑ | ↑↑ | N/A |
| pH2AX | = | N/A | ↑↑↑ | ↑↑ | N/A |
| Study 4: Chronic effects of 5-FU after one week of treatments | | | | | |
| Ki67 | = | = | ↓↓↓↓ | Protection | Protection |
| NG2 | = | = | ↓↓ | Protection | ↓↓ |
| Iba-1 | = | = | ↓↓ | ↓ | Protection |
| COX-2 | = | = | ↑↑↑↑ | ↑↑ | Protection |
| pH2AX | = | = | ↑↑↑ | Protection | protection |

## 2.6 Conclusion

Overall, one of the more significant findings to emerge from this study is that 5-FU exposure caused a decrease in proliferating, OPCs and microglial numbers in both ON and CC. Indicating that chemotherapeutic agents have cytotoxic effects on these two cell populations. The results of this investigation show that both single and multiple injections of 5-FU produce a significant increase in the inflammatory response both 24 hours and one week after treatment. DNA damage as demonstrated by pH2AX expression, was found one week after 5-FU injection. The second major finding was that FLX or INDO co-administration before, during and after chemotherapy injections could reduce and counteract these adverse effects. This could provide a novel therapeutic approach to reduce cognitive impairment after chemotherapy. Therefore, more work should be done to explore the detailed mechanisms of antidepressant and anti-inflammatories in providing protection to WMTs exposed to chemotherapy. In the next chapter the cell specific effects of 5-FU, FLX and an INDO will be investigated in vitro. This was done to see if these drugs act directly on OPCs or indirectly by changing the inflammatory environment.

# **CHAPTER THREE:** THE PROTECTIVE OR ADVERSE EFFECTS OF FLUOXETINE, INDOMETHACIN AND ENDOTHELIAL, ASTROCYTIC AND MICROGLIAL CELLS ON THE VIABILITY AND SURVIVAL OF OLIGO PROGENITOR CELL LINES TREATED WITH 5-FLUOROURACIL

## 3.1 Introduction

Many studies have found that stem and precursor cells, within white matter tracts, play a vital role in the maintenance of white matter integrity (Small *et al.* 1987, Wolswijk and Noble 1989, Zhang *et al.* 1999, Nishiyama *et al.* 2002, Nishiyama 2007). Adult oligodendrocyte progenitors are essential for remyelination after injury and are also required to replace oligodendrocytes lost in the normal turnover of these cells (Gard and Pfeiffer 1989, Nishiyama *et al.* 1996, Reynolds and Hardy 1997, Butt and Berry 2000). These cells have extensive arborisations that form direct contacts with the axolemma at the nodes of Ranvier (Pringle *et al.* 1992). Moreover, Nishiyama (2007) has described how OPCs are an additional glial population to the main glial types 'microglia, astrocytes and oligodendrocytes' and are the source of new oligodendrocytes during development.

Within the WMTs of the brain, oligodendrocyte precursor cells (OPCs) divide to produce mature oligodendrocytes throughout adulthood. The cell division required for this makes them susceptible to the effects of chemotherapy. In addition, mature oligodendrocytes are also susceptible to chemotherapy as shown by both *in vivo* animal studies and *in vitro* investigations (Han *et al.* 2008). Previous research has found that approximately 55-70% of OPCs and mature oligodendrocyte are killed after exposure to 1μM 5-FU for 24 hours *in vitro*. This concentration is equal to the low end of the concentration observed in the CSF of patients after intravenous infusion of 5-FU and is below that which affects cancer cells (Dietrich *et al.* 2006). In addition these authors showed that neural stem cells and astrocytes are less susceptible to 5-FU; but are still affected by prolonged exposure. However the mechanism that mediates the toxic effects of chemotherapy on OPCs and why they are so sensitive remains unclear. White matter defects related to chemotherapy treatment could be due to both the direct and indirect neurotoxicity of these drugs (Weiss 2008). Direct effects could be the toxicity of chemotherapy to dividing cells while indirect effects could be mediated by changes in the microenvironment of the cells due to inflammation, loss of supporting cells or oxidative stress. Microglia are macrophage-like cells which are resident within the CNS. A number of *in vitro* studies have found that activated microglia are able to produce various factors that are cytotoxic to cells (Kingham *et al.* 1999, Hooper *et al.* 2005, Carlson *et al.* 2010, Yeo *et al.* 2012). However, the exact mechanism by which these reactive glial cells induce neuronal, OPCs or oligodendrocyte death is not understood. Chronic activation of microglia with various chemical insults, including chemotherapy, leads to persistent inflammation and the release of inflammatory mediators including tumour necrosis

factor-alpha (TNF-α), interleukin1-beta (IL1-b) and the release of superoxide and NO (Block *et al.* 2007, Yeo *et al.* 2012). In addition the expression of enzyme COX-2 is upregulated in both neurons and microglia, changes that can lead to neuronal and glial cell death (Woodcock and Morganti-Kossmann 2013, Briones and Woods 2014). However the mechanisms by which systemic chemotherapy produces neurotoxicity are not well understood. *In vivo* studies have found that some chemotherapy agents including 5-FU, can penetrate the blood brain barrier and enter the CNS causing microglial activation which may subsequently lead to oligodendrocyte and neuronal damage (Ahles and Saykin 2007, Han *et al.* 2008). Even drugs which cannot directly access the brain may produce peripheral inflammation which produces inflammatory cytokines which can pass through the blood brain barrier. In either case uncontrolled release of pro-inflammatory molecules can induce an increase in cytotoxic factors which could produce significant destruction of healthy tissue. Similarly, Walder *et al.* (1997) found that activation of microglia lead to neural damage by extracellular secretion of reactive oxygen species (ROS). It has conclusively been shown that these oxidative conditions are capable of inducing irreversible damage to cell organelles and molecules such as proteins and nucleic acids (Ray *et al.* 2012). In contrast, the ability of FLX and INDO to reverse the neuroinflammatory process may abolish the effects of pro inflammatory cytokines and prevent cell death. However INDO alone did not change the number of the proliferating cells or immature neurones detected by DCX expression; it was found that INDO reduced the levels of pro inflammatory cytokines secreted by activated microglia which create a detrimental inflammatory environment (Ji *et al.* 2013). In another study, Bernardi *et al.* (2010) found that INDO markedly reduced the level of IL-1B and TNF-a levels after 48 hours in organotypic hippocampal cultures exposed to oxygen-glucose deprivation (OGD). It also prevented glial activation induced OGD by reducing iNOS activation, as well as reducing the levels of ERK1/2 and JNK phosphorylation. Selective serotonin reuptake inhibitors (SSRIs) which are often the first choice in the treatment of depression. However studies have also found that FLX has many additional important effects on the CNS which include acting as an anti-inflammatory, anti-apoptotic and antioxidant, properties which enable this drug to have neuroprotective actions (Liu *et al.* 2011, Song *et al.* 2012, Zhang *et al.* 2012, Obuchowicz *et al.* 2014, Su *et al.* 2015, Caiaffo *et al.* 2016, Shan *et al.* 2016). Su *et al.* (2015) showed that FLX and the anti-depressant S-citalopram significantly modulated the immune system by down-regulated the number of microglia showing M1 activation and cause up-regulation of M2 activation in both primary cells and a cell line.

In addition to the activated microglia, astrocytes can be activated by various stimuli. Thus, activated astrocyte produce cytokines that enhance the activation of microglia and maintain the reactive state of all glial cells (Ben Haim *et al.* 2015). In addition, Savarin *et al.* (2015) found that activated astrocytes play an important role in neurodegenerative diseases including multiple sclerosis (MS). Specifically, astrocytes release IL-6 which is a key mediator of progressive MS disease which increases myelin loss and axonal damage. Moreover, astrocytes play essential roles in the neuroinflammatory response in the brain which can lead to the loss of oligodendrocyte gap junctions and consequently enhance the progression of MS (Markoullis *et al.* 2014).

However, while activation of microglia and astrocytes can induce expression of cytokines including interleukin (IL)-1β and TNFα, astrocytes can also support neuronal and other glial cell survival by releasing trophic factors such as brain-derived neurotrophic factor (BDNF) and NT-3 (Dreyfus *et al.* 1999). In addition activated astrocytes upregulate the expression of intermediate filaments such as glial fibrillary acidic protein (GFAP), proliferation and form a dense network of hypertrophic cells that fills the gaps left by neuronal or glial degeneration after injury. This response to injury by astrocytes forms a glial scar and it called reactive gliosis (Reier and Houle 1988, Rolls *et al.* 2009). Interestingly, it was found that astrocytes secret platelet-derived growth factor (PDGF) which interacts with the PDGF receptor present on OPCs and promotes OPC proliferation, migration and differentiation (Moore *et al.* 2011). In another study, astrocytes secreted PDGF which lead to an increase in oligodendrocytes numbers in demyelination lesions produced by cuprizone. Also this growth factor promoted the remyelination of chronic lesions by supporting the generation of new oligodendrocytes and preventing oligodendrocyte apoptosis (Vana *et al.* 2007).

Astrocytes have been shown to assist in the maintenance of the CNS, including the preservation of the extracellular environment. They also secrete laminins to maintain the extracellular matrix which interact with the integrin and dystroglycan receptors on OPCs and mature oligodendrocytes (Moore *et al.* 2011). The interaction between laminins and dystroglycan receptors facilitates OPC migration and differentiation and also regulates the terminal steps of myelination including myelin formation and stabilisation (Colognato *et al.* 2007). In a different study, Talbott *et al.* (2005) found that activated astroctes contribute to the repair of demyelinated axons by creating functional nodes of Ranvier during normal development. *In vitro* studies have shown that astrocytes support the contact and alignment between oligodendrocyte processes and axons and help

oligodendroglial lamella in first spiralling around the axon (Meyer-Franke *et al.* 1999, Liberto *et al.* 2004).

Cell-cell communication is one of the crucial factors in supporting cells in the CNS. Gap junction channels are an important structure formed by direct cell-to-cell contact and play a vital role in communicating between cells by acting as diffusion channels for the exchange of ions and small molecules (Gajardo-Gomez *et al.* 2016). Moreover, gap junctions are formed by each contacting cell producing a hemi-channel called a connexon made of connexin proteins (Kumar and Gilula 1996). The presence of gap junctions can play a role in neuroprotection by regulate immune responses, cell proliferation, migration, apoptosis and carcinogenesis making them attractive therapeutic targets for treating inflammatory and neoplastic disorders in different organ systems (Belousov *et al.* 2017, Wong *et al.* 2017). In terms of neuroprotection, gap junctions could enhance the survival of energy-exhausted cells by allowing essential molecules such as glucose and ATP to pass between cells toward cells with a high demand. Additionally, in the astrocytic syncytium which is connected by gap junctions, gap junctions allow buffering extracellular space from cytotoxic levels of metabolites and some ions such as glutamate $Ca^{+}$ and $K^{+}$ (Dermietzel and Spray 1993, Goldberg *et al.* 1999). When human astrocytes are co-cultured with glioma cells *in vitro*, the glioma cells become resistant to the chemotherapy agent Temozolomide. Chen and his colleagues have shown that this resistance requires functional gap junctions between the two types of cell. Treatment of the cells with the Cx43 inhibitor carbenoxolone (CBX) inhibited calcine-AM (dye transfer) between astrocytes and glioma cells and abolished the protection conferred by astrocytes (Chen *et al.* 2015). In another study, Ozog *et al.* (2002) found that blockage of gap junctions in cortical neuron-astrocyte co-cultures caused significant neuronal cell death during exposure to a high concentration of extracellular glutamate. However, as uptake of glutamate by astrocytes was not affected, it is the transfer of glutamate between neurons and astrocytes via gap junctions which was important in producing the protective effect. Hence, a potential therapy for some neurological damage (i.e. ischemia) may lie in upregulating functional gap junctions. On the other hand, gap junctions could spread potentially toxic metabolites into the non-affected cells and in this way cause neurodegeneration (Nedergaard *et al.* 1995, Ochalski *et al.* 1995). Oligodendrocytes express a number of connexin proteins including Cx43 and Cx47. Astrocyte Cx30 and oligodendrocyte Cx47 are widely present at oligodendrocyte/astrocyte (A/O) gap junctions in mouse brain (Nagy *et al.* 2003).

The close association between oligodendrocytes and blood vessels has been called the oligovascular niche or signalling, and promotes trophic coupling between cerebral blood vessel endothelium and OPCs. In white matter this association is important for sustaining white matter homeostasis and maintaining brain functions (Arai and Lo 2009, Pham *et al.* 2012, Miyamoto *et al.* 2014). It has been found for example that white matter pathogenesis and development of lesions is due to the disruption of endothelium-OPC/OLG coupling (Arai and Lo 2009). Moreover, endothelial cells play a central role in promoting OPC proliferation and are involved in the regulation of OLG development and survival by secretion of endothelial-derived growth factors such as brain-derived growth factor (BDNF) and FGF-2 (Miyamoto *et al.* 2014). It has been suggested that endothelial-oligodendrocyte trophic coupling involving trophic factors within the oligovascular niche preserves their cellular functions and sustains continued angiogenesis and oligogenesis. Some OPCs for example in the CC, are located adjacent to cerebral endothelial cells, and these cells may communicate each other in both normal and pathological conditions by the secretion of soluble factors that may participated in remodelling and promotes repairing of oligodendrocyte processes (Pham *et al.* 2012). Interestingly, it has been shown that conditioned medium (CM) from endothelial cells can stimulate the differentiation of neural stem cells into OPCs and increase their proliferation. CM can also facilitate OPC migration due to the presence of vascular endothelial growth factor (VEGF-A) (Plane *et al.* 2010, Hayakawa *et al.* 2012). Taken together, other growth factors such as IGF-1 also protect cultured OPCs and oligodendrocyte cells from apoptosis induced by tumour necrosis factor-alpha (TNF-α), glutamate, and growth factor deprivation (Ness and Wood 2002, Cui *et al.* 2005).

## 3.2 Aims and objectives:

The studies in this chapter focus on the direct effect of 5-FU on the proliferation and survival of MO3.13 (OPCs) and outlines the various cell interactions between these cells and C6 (astrocytes), BV-2 (microglia) and endothelial cells which support OPCs by direct or indirect methods. In addition the direct effects of FLX and INDO on OPCs were investigated *in vitro*. This may provide information on the mechanisms to develop a protective treatment for WMTs against the effects of chemotherapy which are involved in causing chemobrain. The principal objectives of the present studies were as follows:

1. To establish an *in vitro* system, to test the direct effects of chemotherapy on the proliferation and viability of different cell types.
2. To investigate whether interactions between OPCs and other cell types affects their response to 5-FU by culturing these cell populations separately or together.
3. To elucidate if the adverse effects induced by 5-FU on OPCs, can be prevented or reversed by the co-administration of FLX or INDO.
4. To examine the inflammatory response that may be induced by treating microglial with 5-FU and the effect of this on OPCs survival.

## 3.3 Materials and Methods

### 3.3.1 Tissue Culture: cell lines

#### 3.3.1.1 MO3.13 Oligodendrocyte Hybrid Cell Line

Human oligodendrocyte progenitor (MO3.13) cell line (tebu-bio; cat no. CLU301-A) was formed by fusion of a human oligodendrocyte to a human Rhabdomyosarcoma (RD) cell line. It has the characteristics of primary oligodendrocytes and expresses the characteristics of OPCs (Craighead *et al.* 1999, Lily *et al.* 2004, Boscia *et al.* 2012, Accetta *et al.* 2016). After differentiation MO3.13 cells expresse Myelin basic protein (MBP), Cyclic nucleotide phosphodiesterase (CNPase) and myelin oligodendrocyte glycoprotein (MOG) (Figure 3.1A).

#### 3.3.1.2 C6 glioma cells

C6 a rat astrocyte cell line (ATCC©CRL-2199TM) was originally derived from a N-nitrosomethyl-urea-induced glial rat brain tumour; and consists of more or less differentiated astrocyte-like cells containing the S-100 protein(Benda *et al.* 1968) (Figure 3.1B).

#### 3.3.1.3 BV-2 cells

The BV-2 cell line is a murine cell line which has been widely used as a tool in microglial cell studies. To produce the BV2 line, primary mouse microglial cell cultures were infected with the v-raf/v-myc recombinant retro-virus J2 (Blasi *et al.* 1990). BV-2 cells preserve most of the functional and phenotypical properties of primary microglial cells including their phagocytic ability, and the secretion of pro-inflammatory cytokines and immune responses (Blasi *et al.* 1990, Han *et al.* 2002) (Figure 3.1C).

#### 3.3.1.4 Endothelial cells (HBMECs)

Human Brain Microvascular Endothelial Cells (HBMECs) were obtained from ScienCell Research Laboratories (Elsheikha *et al.* 2013) (Figure 3.1D). Endothelial cells line blood vessels and constitute a major cellular component of the blood brain barrier (BBB). These cells have a role in the resistance of the brain to chemotherapy by decreasing the CNS availability of anti-cancer drugs (Steeg *et al.* 2011). The close association of dividing cells with vascular micro vessels in neurogenic regions make them a candidate for the protection of OPCs against the adverse effects of 5-FU and they may also mitigate the effects of inflammation. Therefore endothelial cells were used in this chapter to investigate the role of

these cells in the protection the OPCs against the adverse effects of 5-FU when co-cultured with MO3.13 cell line.

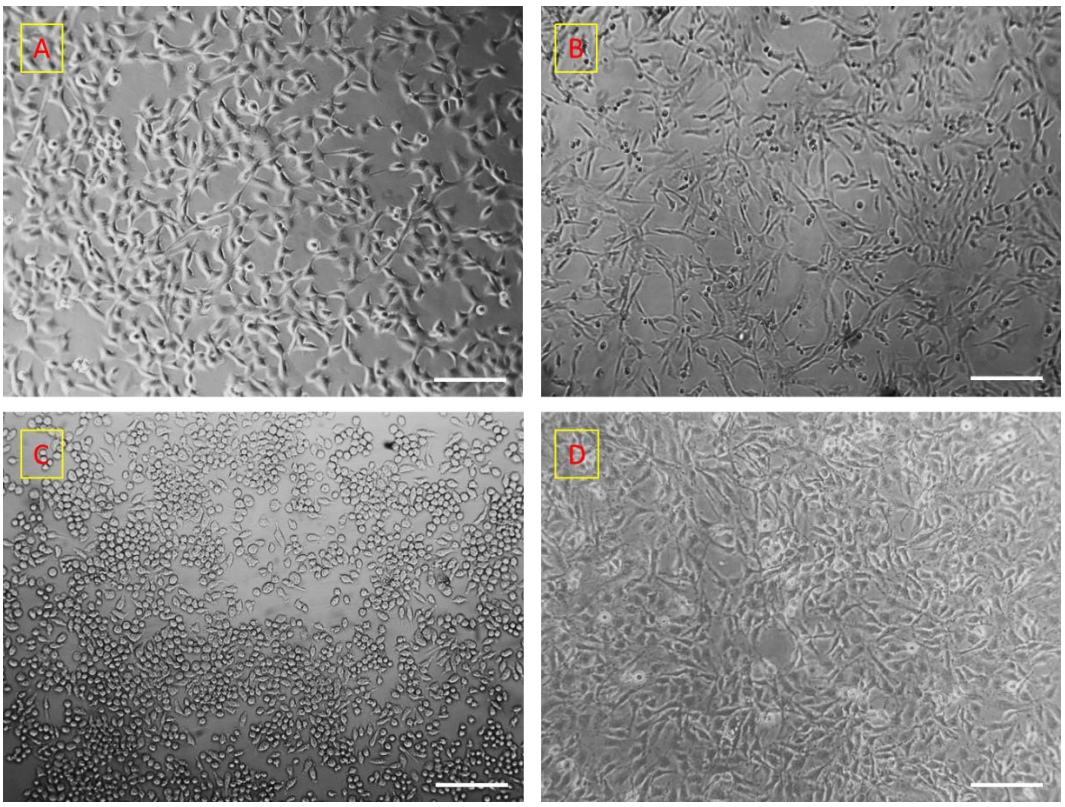



***Figure 3.1*** *photographs of different cell lines used in this study (**A**) MO3.13 (OPCs). (**B**) C6 (astrocytes). (**C**) BV-2 (Microglia). (**D**) HBMECs (Endothelial cells). (Scale bar=100µm; magnification, X100).*

### 3.3.2 Culturing and passaging of Cells

For all experiments, the different cell types were taken from liquid nitrogen storage and thawed quickly in warm water by gently agitating the vial for 1 min in a 37°C water bath. 10 ml of Dulbecco's modified Eagle's medium (DMEM) (Sigma Aldrich D5796) was added to a 20 ml Universal tube. Before opening, the outside of the vial was wiped with 70 % industrial methylated spirit (IMS). As soon as the ice crystals within the vial melted, the thawed cells were gently pipetted into the 20 ml sterile Universal tube, Steralin), before centrifuging at approximately 200 G for 5 min. After centrifugation, the supernatant was decanted without disturbing the cell pellet. 1 ml of DMEM was added and mixed with the pellet before transferring into a 75 $cm^2$ flasks (Corning, UK) containing

growth medium (GM) DMEM which contains 4.5g/L glucose supplemented with 10% fetal bovine serum (FBS) (Sigma F7524, UK), 100 U/ml penicillin, 10 mg/ml streptomycin, and 2 mmol/l L-glutamine (Sigma- Aldrich, UK). Cells were cultured in a 5% CO2-buffered incubator at 37°C. Cells were passaged when they reached 80% confluence (typically after 3 days); cells were washed with sterile Dulbecco's Phosphate Buffered saline DPBS (Sigma, UK) and incubated with 2 mls of sterile 0.25% w/v Trypsin/EDTA (Sigma-Aldrich, UK) for 2-3 minutes at 37°C to remove adhered cells. Cells were collected in medium for centrifugation at 200 G for 5 mins, the cell pellet was re-suspended in medium and diluted 1:10 in 20ml cell GM at 37°C / 5% CO2. Cells removed from frozen storage were grown for two passages before use in experimental studies.

### 3.3.3 Automated Counting of cells

To ensure the equal seeding of cells into each well or flask, the cells were counted using a TC20 automated cell counter (BioRad, Hemel Hempstead, UK). The number of cells in the suspension was counted by mixing 10 µl of cell suspension to 10 µl trypan blue (Sigma, UK), loaded onto specific Tc 20 counting slides (Bio-Rad, UK). This test also measure the percentage of living cells by using the vital dye trypan blue which is negatively charged and can only penetrate the damaged membranes of dead cells.

### 3.3.4 Drug Treatments:

MO3.13 progenitor cells were seeded onto 24 well plates (Nunclon, UK) (25000 cells/0.5 mls GM per well). Cell counts were performed using a TC20 automated cell counter. After a 24 hour growth period, media was removed and drugs (5-FU, FLX, INDO or combinations) were added at concentrations ranging from 0.1 to 10 µM for varying time periods. Post treatment cell viability was measured using a cell viability assay (MTT Assay). The drug concentration that produces A half-maximal effect ($IC_{50}$) was used to determine the dose of 5-FU given to MO3.13, BV-2, C6 and HBMEC cells.

Preliminary experiments produced a dose response curve from which the concentration of 10µM 5-FU was found to be the $IC_{50}$ for the inhibition of growth. This dose was used in further experiments (is the $IC_{50}$ value for 5-FU inhibition in MO3.13 cells). In co-treatment experiments, the FLX or INDO was added at the same time or 24 hours before 5-FU treatment. The period of drug incubations varied between 24, 48 and 72 hours, depending on the experimental design (acute, chronic and recovery experiments).

C6, BV-2 and endothelial cells were routinely cultured for 24 hours before treatment in 24 well plates (50000, 25000 and 25000 cells/ 0.5 mls GM per well respectively). Then the GM was carefully removed with an aspirator and cells were treated with different concentrations (0, 0.1, 0.5, 1, 5 and 10 μM) of treatments in fresh GM and incubated for 24 and 48 hours at 37°C with 5% CO2. All statistical analysis was done using GraphPad Prism software Version 7. $IC_{50}$ values are quoted with 95% confidence intervals. Data were tested for normal distribution with the D'Agostino & Pearson omnibus normality test and were compared using the one-way ANOVA with either Tukey's multiple comparisons or Dunnett's multiple comparisons post-tests. Curve fitting was carried out by converting values to a log scale and using the curve fitting function within Prism. Statistical significance is defined as $P<0.05$ and is flagged as * in graphics, ** when $P<0.01$, *** when $P<0.001$ or **** when $P<0.0001$.

### 3.3.5 Cellular Growth Rate

To determine the effect of time when growing different cell lines, the growth kinetics were assessed. Cells were plated on 13 mm sterilised glass coverslips in 24 well plates at a density of $25x\ 10^3$ cells/ well in 24 well plates, and the proliferation rate was measured at different time points (0, 12, 24, 48, 72 and 96 hours). At each time point, cells growing on coverslips were fixed in 2% PFA at RT after which cells were washed twice with cold phosphate-buffered saline (PBS). After 15 minutes cells were permeabilized with 0.1% Triton X-100 for 15 min, and blocked with 10% normal goat serum for 60 min. Coverslips were incubated overnight at 4°C with monoclonal mouse Ki67 primary antibody at a 1:100 dilution, washed, and then incubated for 60 minutes with secondary antibodies Alexa Fluor 488 goat anti-mouse (1:500 dilution) at RT. Following incubation, the cells were washed 3 times in PBS to remove traces of the secondary antibodies. Cells were then counterstained with DAPI (1:1200) and all coverslips mounted on a glass slides. The coverslips were carefully sealed onto the slides using nail varnish and then viewed under a fluorescence microscope or stored in the dark at 4°C. Images were captured using a SPOT insight QE digital camera running SPOT for Windows (version 3.5.0). Cells were counted in four random fields of view per coverslip and the mean percentage of Ki67 positive cells on each coverslip was then calculated for each cell line. In each experiment, three coverslips for each condition studied were used. All growth rate experiments were repeated in triplicate. Data was analysed by using two-way ANOVA tests, followed by Dunnet's multiple comparison test to check the significance of any difference. When the P

value was >0.05 it was considered to be not significant. All statistical analysis was done by using GraphPad Prism software Version 7.

### 3.3.6 Cell Viability Assay (MTT Assay)

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used to test the effects of a range of concentrations of 5-FU, FLX and INDO on the viability of oligodendrocyte progenitor, Astrocyte, Microglia and Endothelial cells. This colourimetric assay is based on the ability of viable cells to reduce a soluble yellow tetrazolium salt MTT metabolically to a purple formazan product. This reaction takes place when mitochondrial reductase enzymes are active (Mosmann 1983, Berridge *et al.* 2005).

After cells had been grown in 24-well plates and incubated with different drug regimes, 50 µl of the MTT solution (Sigma Aldrich, UK- 5mg/ml in PBS) was added to each well. 30 mins afterwards, growth media and MTT were removed and 1 ml of dimethyl sulfoxide (DMSO) (Sigma-276855, UK) was added to each well to dissolve the MTT-formazan crystals. The plate was agitated slightly, and 200 µl aliquots from each well were transferred into a 96 well. The plates were scanned using a Dynex MRX Model 96 Well Plate Reader (MTX Lab Systems Inc., USA). Each plate was then photometrically quantified at an absorbance of 570 nm. The amount of purple formazan produced by treated cells was compared with that produced by untreated control cells. All experiments were repeated at least 3 times with essentially similar results. The viability was calculated as a percentage of controls. Statistical analysis was performed using GraphPad Prism software Version 7. Data was analysed using one-way ANOVA and Dunnet's multiple comparison test. Statistical significance was taken at $p \leq 0.05$, presented as mean ± SEM.

### 3.3.7 Direct Co- Culture studies

To study physical cell–cell interactions and the supportive effects of astrocytes on oligodendrocyte precursor cells (OPCs) or to obtain mixed cultures of primary endothelial and OPCs or the impact of microglia on OPCs, co-cultures of the two cell populations were developed. This technique is relevant for indirect drug effects on specific types of cells and provides a more representative *in vivo* like tissue model in monitoring drug effects which takes into account cell to cell interactions (Wu *et al.* 2010).

### 3.3.8 MO3.13 cells Labelling with PKH26 red fluorescent dye:

In order to distinguish OPCs from other cell types in co-culture models, MO3.13 cells were labelled with the fluorescent cell permeable dye PKH26GL (Sigma Aldrich). The labelling technique was performed following the manufacturer's instructions with some modifications. Cell suspensions were acquired as described previously, placed in a conical tube containing $20\times10^6$ cells, centrifuged (200rpm 5 mins) and washed once with media without FBS. Following this, one ml of diluent C (PKH26 kit) was added to the pellet together with 1 ml of PKH26 stain (4 µl of the stain to 1 ml of diluent C) and incubated at 37°C for 5 mins with periodic mixing. After 5 mins, 2 ml of FCS was added to the suspension and incubated at 37°C for 2 mins to bind the excess dye, the cells were re-centrifuged (160 G for 5 mins) and the supernatant removed. The pellet was re-suspended in growth media, transferred to a fresh tube and gently washed with media before being centrifuged (160 G for five minutes). These steps were repeated 3 times. The pellet was re-suspended in complete DMEM growth media and was then ready to seed into 4 chamber slides in which the other cell type had formed a mono layer. Cells were grown at 37°C and 5% CO2 for 24 hour before treatment with chemotherapy. The PKH26 fluorescent cell linker dye is a membrane-intercalating agent which stably incorporates a fluorescent dye into lipid regions of the cell membrane, once the dye binds to cell membranes it is cleaved and adjacent cells cannot take up the dye

### 3.3.9 Co-culture techniques and treatments

To obtain co-cultures of (C6) astrocytes +(MO3.13) OPCs, or (VB-2) Microglia+(MO3.13) OPCs, or (HBMECs) endothelial+(MO3.13) OPCs, cells were seeded at the ratio 2:1, 3:2, and 2:1 respectively. For all co-culture experiments, on day one of the experiment, cell suspensions were prepared as described previously. C6, BV-2 and HBMEC cells were seeded at a density of ($40x10^3$), ($30x10^3$) and ($40x10^3$) respectively into chamber slides (Lab Tek- Nuna, Germany). The cells were allowed to attach and grow to confluence in DMEM medium for 24 hour at 37°C with 5% CO2. On day two, MO3.13 at passage 9, 10 or 11 were labelled with PKH26 (as described previously) before co-culturing. After 24 hours, ($20x\ 10^3$) labelled OPCs were seeded onto the confluent host cells (astrocytes, microglia and endothelial monolayer cells). Host cell monolayers without added OPCs and OPCs alone in growth media were used as controls. One day after plating OPCs, the medium was carefully removed and the cells were treated with DMEM contains 10µM of 5-FU for both C6+MO3.13 and HBMEC+MO3.13 or DMEM contains 0.5µM for VB+2-MO3.13. The cells were

incubated for a further 48 hour at 37°C and 5% CO2. The concentrations of all compounds used to treat cell lines were based upon cell viability assay results or on previous studies. At the end of incubation, growth media was removed and cells were fixed with 2% PFA for 10 minutes. Cells were then finally washed and stained with the DAPI nuclear marker before being mounted and coverslipped for fluorescent microscopy. Stained slides were stored in the dark at 4°C. All slides were viewed under a Nikon EFD-3 fluorescence microscope and images were captured using a Spot Insight QE camera. All nuclei were stained blue whereas the cell membrane and the cytoplasm of OPCs was stained red.

### 3.3.10 Functional Gap Junction and Dye Coupling Assay (parachute assay)

Gap junctions are formed by clusters of connexin proteins (Cx) that form intracellular channels between two adjacent cells. These channels allow direct cell-to-cell passage of ions and small molecules (Goodenough *et al.* 1996). Cx47 is a major gap junction protein that is required for oligodendrocyte: Astrocyte coupling (O/A junction). It was hypothesised that the presence of gap junctions would enhance the protection of OPCs. Gap junction function was assessed by dye coupling (Suzhi *et al.* 2015). To examine the role of gap junctions in cell protection, a gap junctional blocker carbenoxolone disodium salt (CBX) (C4790-Sigma, UK) was used to treat OPCs and astrocyte or endothelial co-cultures during exposure to chemotherapy. Different concentrations of CBX were tested and the lowest concentration that totally blocked gap junction channels was used in this experiment.

To determine expression of Cx47 protein in oligodendrocytes, these cells were immunostained with an antibody against this protein. After growing the cells in chamber slides, they were fixed in 2% paraformaldehyde at RT for 10 minutes. Cells were washed with PBS, permeabilized in 0.1% Triton for 15min and blocked with 5% normal goat serum for 60 min. Chamber slides were incubated overnight at 4°C with polyclonal Cx47 primary antibody (Invitrogen, USA) at a 1:1000 dilution (3% BSA, 0.1% Triton X-100, PBS), washed, and then incubated for 60 minutes with a secondary antibody Alexa Fluor 488 goat anti-rabbit (1:1000 dilution) at RT. Following incubation, the cells were washed 3 times in PBS to remove traces of the secondary antibodies. Cells were then counterstained with DAPI (1:1200) and the slide mounted with a coverslip. Slides were viewed under a fluorescence microscope.

For the parachute assay , the donor cells (C6 or endothelial) were grown to confluence and incubated with 5μM dye Acetoxymethyl (AM) called 'calcein AM'

for 20 minutes in a humidified incubator (37°C, 5% CO2) and washed 3 times in cell culture media. Calcein AM labelled cells were then seeded onto the receiver cells 'MO3.13' that had been pre-labelled with PKH26 (as mentioned previously). The labelled cells were incubated with or without the gap junction inhibitor CBX (50 µM) for 6 hours in a humidified incubator (37°C, 5% CO2), and then fixed. Cells were examined and photographed under fluorescence to detect the passage of calcein AM green dye from donor cells to receiver cells.

### 3.3.11 Indirect Co- Culture studies (in-direct co-culture)

The transwell co-culture system or in-direct co-culture, is an *in vitro* technique used to explore the effect of different cells populations that share the same medium but do not have any physical contact (Guo *et al.* 2008). This method enables the effect of free soluble molecules and secreted proteins that are produced by one population of cells on a second population which transported via the medium. This system is composed of two chambers, an upper insert and the lower culture base which are separated by a microporous membrane.

After preparation of cell suspensions, MO3.13 cells were plated in the base of 24 well plate at a density of 20000 cells/ml/well. BV-2 were either cultured on the surface of 0.4µm pore size cell culture inserts (13-mm transwell membrane) (Falcon, 3495) that were fitted into the culture wells of 24 well plates at a density of 30000 cells/ insert above MO3.13. After the cells had grown to 70-80% confluence, the medium was removed and replaced with fresh DMEM containing 0.5µM 5-FU. The cells were incubated for a further 48 hrs at 37°C and 5% CO2 and cell viability was assessed by MTT assay. The same set of experiments was repeated for MO3.13 with C6 and MO3.13 with endothelial cells. The experimental rationale is depicted in (Figure 3.2). Each experiment was done in duplicate or triplicate at one time and then repeated three to five times independently. Quantitative data were expressed as mean± SEM and analysed with two-ANOVA followed by Tukey HSD multiple comparisons. Differences of $P<0.05$ were considered significant.

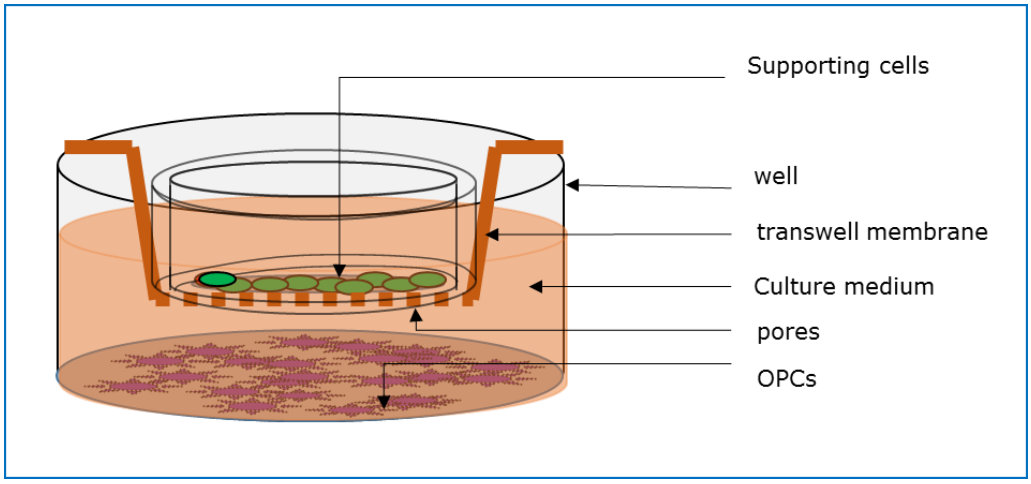



***Figure 3.2*** *Schematic diagram illustrating the transwell co-culture technique.*

### 3.3.12 Conditioned Medium (CM) technique

In order to confirm the hypothesis of this study "CM added to OPC cultures, could induce OPC death or could promotes OPC survival depending on the cell type that CM was collected from". Therefore, different CMs were collected from C6, HBMEC and BV-2 cells and investigated and compared for their effects on OPCs treated with 5-FU.

For CM transfer experiments, C6 or endothelial cells were plated into 75 cm2 Falcon flasks at 50% confluence and after one day (approximately to confluence) changed to serum-free medium. This serum-free media was collected 24 hours later and used as C6-CM or endothelial-CM (E-CM). After conditioning, the medium was aspirated from the cells, centrifuged (2000 G for 5 min), filtered through a 0.22µm-pore-diameter Millipore filter, and stored frozen at -80°C until required. For preparing the two types of microglial M-CM (treated M-CM and non-treated M-CM), BV-2 cells were seeded in 75 cm2 Falcon flasks. After one day, cells received either fresh serum-free medium or serum-free medium containing 5-FU (at 0.5 µM). Cells were incubated for 24 hours and medium was aspirated from the cells as mentioned above. MO3.13 cells were treated directly with C6-CM or E-CM or M-CM at a ratio of 1:1 in their fresh culture medium (20% FBS being added) and the cells treated with 5-FU, after 48 hours incubation with CM and chemotherapy, cells were used for cell viability assay to assess the CM protection in MO3.13 cells. Statistical analysis was performed using Graph Pad Prism software Version 7. Data obtained from three time repeats from three different passages (average 4 wells from each treatment group). Data were analysed using two-way ANOVA and Tukey's post-hoc multiple comparisons test to compare between treatment

groups. Statistical significance was taken at $p<0.05$, presented as mean ± SEM and statistical significance is defined as $P\leq0.05$ and is flagged as * in graphics, ** when $P<0.01$, *** when $P<0.001$ or **** when $P < 0.0001$.

### 3.3.13 TNF-α measurement by Enzyme-linked immunosorbent assay (ELISA)

Levels of TNF-α in the different condition media of BV-2 or co-cultured BV-2+MO3.13 with or without treatment by different treatments such as FLX, INDO and 5-FU and also (lipopolysaccharide (LPS) as a positive control) were measured by ELISA (Anti-Mouse TNF-α Immunoassay designed to measure TNF-α levels in cell culture supernatants). The selected concentrations of treatments are justified in the corresponding result sections and were based on previous studies. After 48 hours of treatment, the cell culture supernatants were collected and placed in individual 1.5ml Eppendorf tubes. Then the samples were centrifuged at 10,000 G for 15 minutes at 4°C to remove any floating cells or debris. The supernatants were transferred to new Eppendorf tubes, and quickly frozen and stored at -80°C for subsequent analysis of TNF-α.

The ELISA is a technique that allows for the quantification of protein in the different type of samples. ELISA have two main different approaches, first, by detecting the presence of antigens that can recognized with an antibody. Second, ELISA can be used to test for antibodies that recognize an antigen (Van Weemen and Schuurs 1971). To detect the cytokine TNF-α which is recognised by an antigen-specific antibody, a quantitative sandwich ELISA kit (mouse TNF-α, R&D Systems, Abingdon, UK) was used. This method measures the quantity of TNF-α released in control and treated cell culture medium samples (Xaus *et al.* 2000). The underlying mechanism of a sandwich ELISA depends on primary monoclonal antibody binding to a surface and then the unknown antigen directly binds to the bound antibody. The advantage of a sandwich ELISA is it reduces the non-specific binding of the sample onto the surface and decreases errors.

The assay was performed according to the manufacturer's instructions. Briefly, all reagents were brought to room temperature before the experiment. 96 well plates pre-coated with capture monoclonal specific mouse anti-TNF-α antibody in PBS pH 7.4 (100µl) overnight. Nonspecific binding was blocked with 300µl PBS containing 1% BSA for one hour. 50µl diluent (PBS with 0.05% Tween and 0.1% BSA) was added to each well followed by an equal amount (50µl) of experimental samples and known standards. The plate was then incubated for 2 hours at RT. The plate was then aspirated and washed for three times with the wash buffer (400µl). 100µl of anti-TNF-α conjugate was then added to each well and was incubated for 2 hours

at RT. This was followed by another three washes with the wash buffer. Then, 100µl working dilution of streptavidin horseradish peroxidase conjugate HRP was added to the wells and incubated at RT for 20 minutes in dark place. The plate was aspirated and washed again, followed by adding 100µl of K-Blue Max substrate solution for 20 minutes in the dark place. The reaction was stopped after 20 min by adding 50µl hydrochloric acid solution (stop solution) and mixed thoroughly. The optical absorbance of the wells was read at 450 nm with the reference filter set to 540 nm to correct for optical imperfections using a microplate reader (MTX Lab Systems Inc., USA). The concentration of TNF-α in the cell medium samples was expressed as pg/ml based on standard curves plotted (Figure 3.3) using protein standards supplied with the kits. Statistical analysis was performed using Graph Pad Prism software Version 7. Data were analysed using two-way ANOVA and Sidak's multiple comparisons test. Statistical significance was taken at $p<0.05$, presented as mean ± SEM. Data obtained from three time repeats from three different passages.

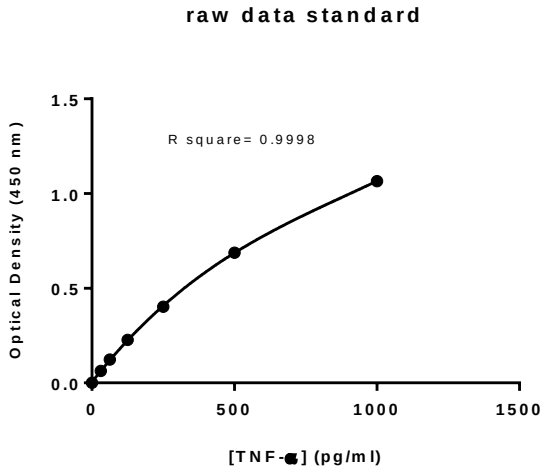



***Figure 3.3*** *Representative TNF-α standard curve (ELISA assay). Cell medium sample concentrations were expressed as pg/ml based on standard curves plotted using protein standards supplied with the kits.*

## 3.4 Results

### 3.4.1 The effect of 5-FU and FLX or INDO on the viability and proliferation of glial cells in vitro

#### 3.4.1.1 The effect of different 5-FU concentration on OPCs (MO3.13) after short and extended exposure

To assess the impact of low concentrations of chemotherapy on cell viability, MTT assays were performed for 5-FU concentrations between 0 μM and 10 μM (0, 0.1, 0.5, 1, 5 and 10 μM) and read after 48 hours incubation with the drug. Optical densities were expressed as a percentage of the control (0μM), where a higher optical density corresponded to a higher number of viable cells. There was a significant difference between treatments groups ($F_{(5, 144)}$ =56.92, P<0.0001; one-way RM ANOVA). Statistical analysis showed an increase in cell proliferation at low concentrations (0.1 and 0.5 μM) of 5-FU treatment after 48 hours (Figure 3.4 A); whereas, 5 and 10μM 5-FU caused significantly fewer viable OPC compared to controls (P=0.0103 and P=0.0001 respectively).

It appears that with increasing the dose and with increasing the duration, the inhibitory effect of this drug became more significant (Figure 3.4 B and C). At the higher concentrations, differences between the mean for each concentration of 5-FU (5, 10, 15, 20, 25 μM) were significant ($F_{(5, 179)}$ = 70.77, one-way ANOVA, p<0.0001) and post-hoc analysis found significant decreases in cell viability in cells treated with all concentrations of 5-FU after 48 hours. 5-FU therefore has a negative dose-dependent effect on the cell viability of OPCs, which seems similar to the results *in vivo* of chapter two. The half maximal inhibitory concentration ($IC_{50}$) was used as a measure of the effectiveness of 5-FU in inhibiting OPCs viability and proliferation, and 10μM 5-FU at 48 hours was used therefore in further studies (Figure 3.4 D).

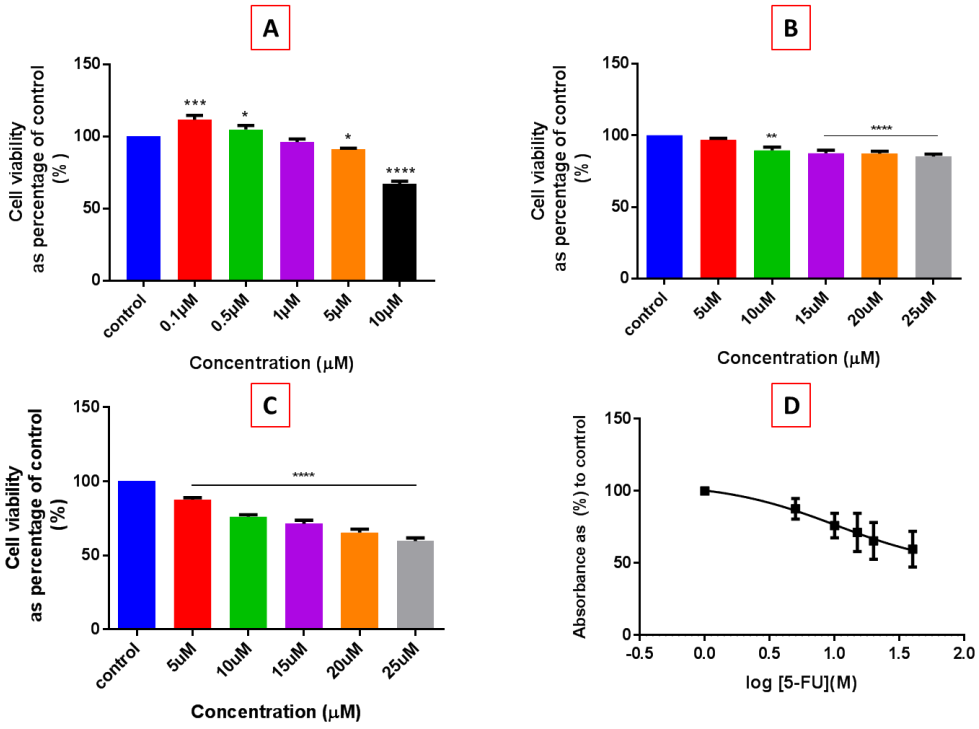



***Figure 3.4*** *Treatment and incubation of proliferating OPCs with increasing concentrations of 5-FU (MTT assay). (**A**) A one-way ANOVA showed a significant increase in cell viability in low concentration (0.1µM) of 5-FU treatment after 48 hours (***p=0.0005), and significantly lower cell viabilities in the 5 and 10uM (*P=0.0103, ****P=0.0001 respectively) treated cells. (**B**) Post hoc test revealed significantly lower cell viabilities in 10–25µM treated cells for 24 hour compared to control. (**C**) There is a significant difference in the cell viability between all treated groups compared to control for 48 hours incubation (one way ANOVA, p <0.0001). (**D**) Concentration–effect relationships for the effect of 5-FU on the reduction of MTT 5-FU inhibited viability with an $IC_{50}$ of 10.31 µM (95% confidence interval CI) for 48 hours. Data shown as means± SEM (n=4) for each time point.*

#### 3.4.1.2 The effect of different 5-FU concentration on Astroglial cells (C6) after short and extended exposure

The viability of the C6 cell line was assessed by MTT assay after treatment with different concentrations of 5-FU (0, 5, 10, 15, 20 and 25 µM). Cells were incubated with 5-FU for 24 or 48 hours. There was a significant decrease in cell proliferation with higher concentrations of 5-FU treatment after 24 hours ($F_{(5, 146)}$ = 43.14, P<0.0001; one-way ANOVA) except for 5µM 5-FU which showed no significant difference in viable C6 compared to controls (P= 0.0705) (Figure 3.5 A). For all concentrations of 5-FU, 48 hours incubation caused a highly significant effects on cell viability compared to 0µM exposure ($F_{(5, 186)}$ =502.5, P<0.0001; one-way ANOVA) (Figure 3.5 B). From these results C6 cells are much more sensitive to 5-FU after 48 hours compared to 24 hours exposure. The $IC_{50}$ of a 5-FU in inhibiting

C6 cell viability and proliferation, was ~12.43μM 5-FU at 48 hours (95% CI) (Figure 3.5 C). From these results MO3.13 cells are more sensitive to 5-FU compared to C6 cells. For co-culture experiments, a concentration of 10μM of 5-FU was used which causes a significant reduction in MO3.13 viability but does not much affect C6 cell viability.

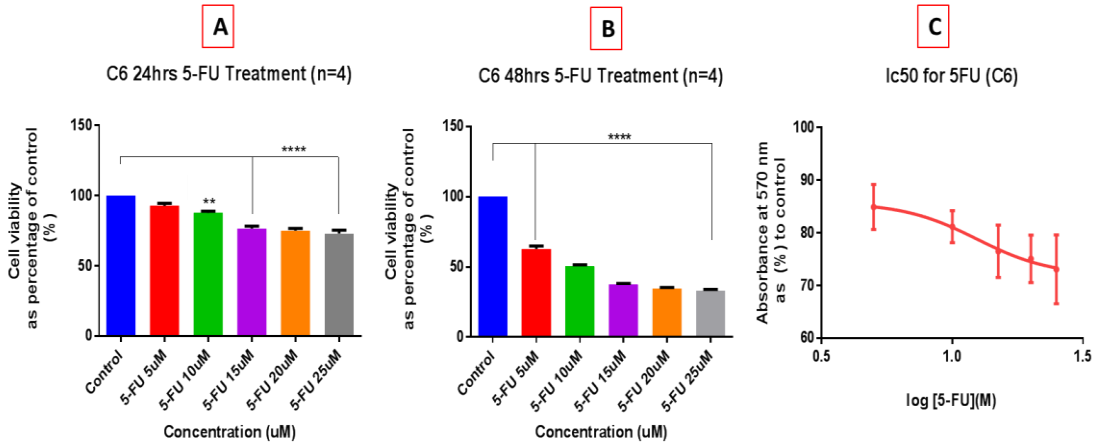



***Figure 3.5*** *Treatment and incubation of proliferating astrocytes with increasing concentrations of 5-FU. (**A**) Post hoc multiple comparison tests revealed significantly lower cell viabilities in the 10-25uM 5-FU treated cells for 24 hours (P<0.05; one-way ANOVA) 5μM 5-FU showed no significant difference in viable C6 compared to controls (P= 0.0705). (**B**) There is a highly significant reduction in the cell viability between the groups after 48 hours incubation (p<0.0001, one-way ANOVA,). (**C**) Concentration–effect relationships for the effect of 5-FU on the reduction of MTT 5-FU inhibited viability with an IC50 of ~12μM (95% CI) for 48 hours. Data shown as means± SEM (n=4) for each time point.*

#### 3.4.1.3 The effect of different 5-FU concentration on Microglial cells (BV-2) after short and extended exposure

MTT assays were performed to assess the viability of BV-2 cells after treatment with a range of concentrations of 5-FU (0, 0.1, 0.5, 1, 5 or 10μM) following incubation for 24 and 48 hours. One-way ANOVA analysis indicated a significant difference between the means of each concentration group ($F_{(5, 186)}$ = 131.8, P<0.0001). Only at a dose, of 0.1μM 5-FU, was there an increase in viability when the cells are incubated for 24 hours (P=0.0318) (Figure 3.6 A). Whereas, at doses of 0.5μM or above, 5-FU reduces cell viability in a dose-dependent manner (P<0.0001, one-way ANOVA). 48 hours exposure to 5-FU effect on cell viability caused a significant decrease in viability at 0.5, 1, 5 and 10μM when compared with the control ($F_{(5, 90)}$ = 2422, P<0.0001; post-hoc one-way ANOVA). However, 0.1μM had a significant effect on microglial viability after 48 hours (P= 0.0002) (Figure 3.6 B). Results showed that 5-FU appeared to be the most cytotoxic, and

have an $IC_{50}$ of 0.484μM after 48 hours exposure (Figure 3.6 C), whereas MO3.13 and C6 appear to be the more resistant. For co-culture experiments with BV-2 and MO3.13, a concentration of 0.5μM of 5-FU was used to explore a protective effect of these cells against the adverse effect of 5-FU in mixed culture.

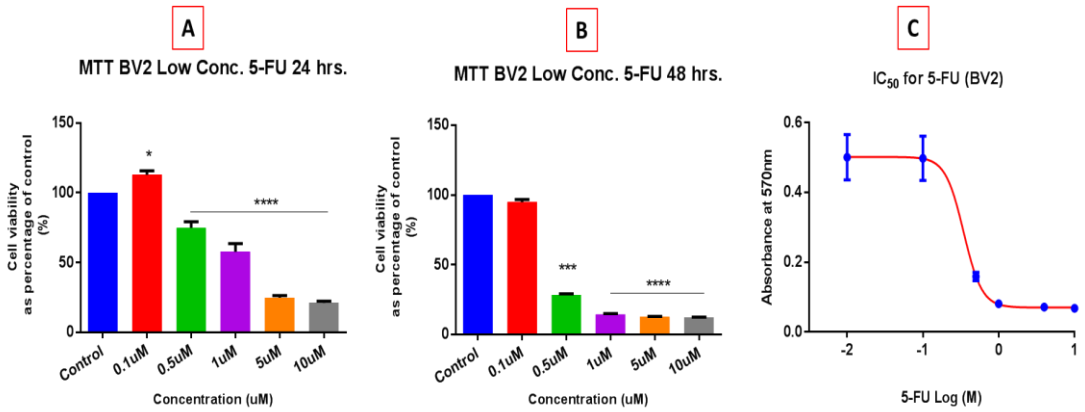



***Figure 3.6*** *Treatment and incubation of microglial cells with increasing doses of 5-FU. Data analysed with one way ANOVA. (**A**) Treatment significantly increased the cell viability at low concentration (0.1μM) after 24 hours (*P=0.031), and significantly lower cell viability in the rest of concentrations (****P=0.0001). (**B**) There is a significant reduction in cell viability among the groups after 48 hours of incubation (p<0.05). (**C**) Concentration–effects of 5-FU on the reduction of cell viability in BV-2, 5-FU inhibited cell viability ~0.484μM at IC50 of (95% CI) after 48 hours incubation. Data shown as means± SEM (n=4) for each time point.*

#### 3.4.1.4 The effect of different 5-FU concentration on Endothelial cells after short and extended exposure

The *in vitro* study found that a high dose of 5-FU (20 and 25uM) caused significant reduction in the viability of endothelial cells after one day incubation (P=0.0015 and P=0.0001 respectively, one way ANOVA). While concentrations of 5, 10 and 15μM showed no significant difference when compared with the control group (P>0.05, one-way ANOVA) (Figure 3.7 A). When the cells were incubated for two days there was no significant effect on cell viability when cells were treated with 5 and 10uM concentrations of 5-FU (P>0.05), while exposure of cells to 15, 20 and 25uM caused reduction in viability as compared to controls ($F_{(5, 138)}$ = 43.96, P<0.0001; one-way ANOVA) (Figure 3.7 B). In brief, it was noted that endothelial cells are much more resistant to 5-FU as compared to MO3.13 cells. For co-culture experiments, a concentration of 10μM of 5-FU was used which causes a significant reduction in MO3.13 viability but does not significantly affect endothelial cell viability.

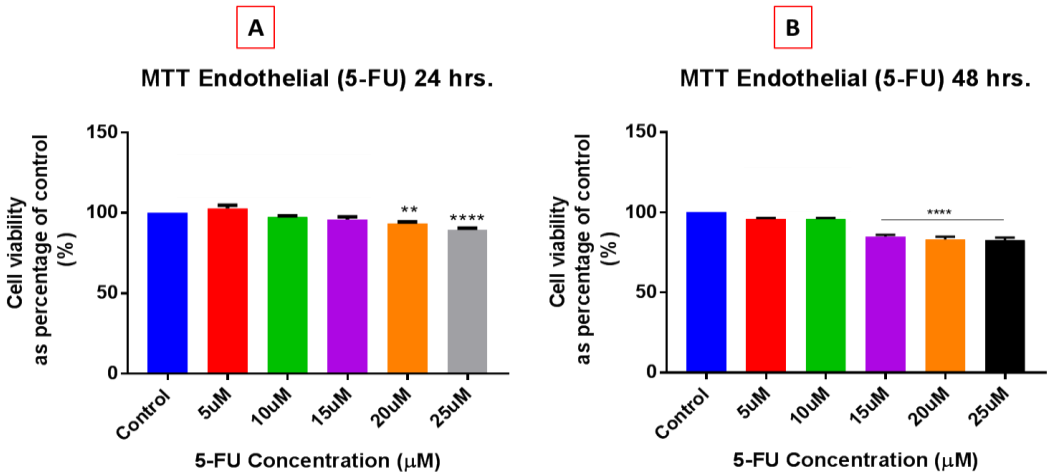



***Figure 3.7*** *Treatment of endothelial cells with increasing concentrations of 5-FU as measured with MTT assay. (**A**) Effect of treatment after 24 hours (**B**) Effect of treatment after 48 hours. The data here is represented by mean± SEM, n=3 (average 4 wells from each experiment). One-way ANOVA analysis showed no significant difference of 5, 10, 15 μM after 24 hours (P>0.05). The dose 15, 20, and 25 μM produce a highly significant reduction in the cell viability compared to controls after 48 hours (****p<0.0001, one-way ANOVA,).*

### 3.4.2 The effect of a combined treatment of 5-FU with FLX or INDO on the cell viability of OPCs

As FLX and INDO have been suggested as protective in some points for to progenitor cells against the effects of 5-FU *in vivo*; their ability to protect OPCs in vitro was tested.

#### 3.4.2.1 The effect of a combined treatment of 5-FU and FLX on the cell viability of OPCs after 24 and 48 hours incubation.

There was a significant reduction in cell viability after treatment with 10μM 5-FU alone for 24 hours (p=0.0205, one-way ANOVA). In addition, incubation with FLX alone produced no significant effects on cell viability (P>0.05, one-way ANOVA) however there was tendency in increasing cell viability with different concentrations (Figure 3.8 A). Co-treating M03.13 with both 5-FU+FLX produced a significant reduction in cell viability compared to controls (p<0.05, one-way ANOVA) indicating that the FLX did not provide any protection for this cell type.

Similarly, when cells were incubated for 48 hours, there was no significant effect on cell viability with increasing concentrations of FLX on its own compared with controls (P>0.05). Post hoc multiple comparison tests revealed a significant reducion in cell viability when treated with 10μM 5-FU for 48 hours ($F_{(11, 253)}$ = 111.6, P<0.0001). Co-treatment with different concentrations of FLX when 5-FU

was given did not prevent the loss of cell viability (Figure 3.8 B). Overall, the results indicated that both 5-FU and co-treatment 5-FU+FLX reduce the viability of OPCs when incubated for 24 or 48 hours, and FLX is ineffective at protecting cells against 5-FU.

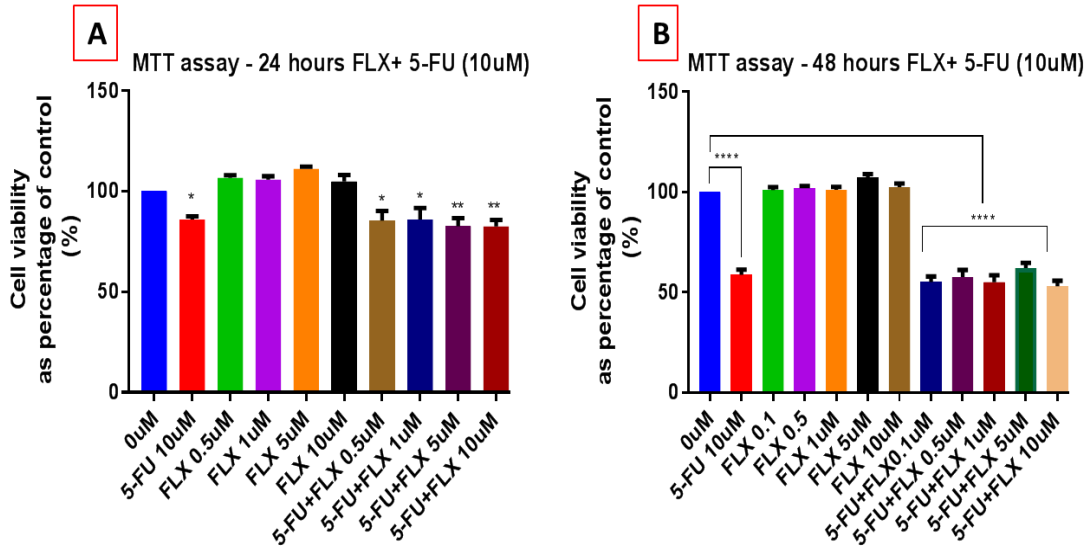



***Figure 3.8*** *Treatment of MO3.13 cell line with 10μM 5-FU and/or different doses and combination of FLX (**A**) Effect of treatments after 24 hours (**B**) Effect of treatments after 48 hours. One-way ANOVA showed significant reduction in cell viability following 5-FU treatment alone at 24 and 48 hours (*P=0.0205, ****P<0.0001 respectively). Treatment with different concentration FLX alone did not affect cell viability after 24 and 48 hours (P>0.05). The combination of 5-FU and FLX treatment showed decreased cell viability after 24 and 48 hours (*sign of significance when the P<0.05, ** when P<0.01, **** when P<0.0001).*

### 3.4.2.2 The effect of preincubation with FLX on OPC cell viability when treated with 5-FU.

As the *in vivo* protective effects of FLX require pre-treatment with FLX, cells were treated with FLX for 24 hours prior to treatment with 5-FU. After treatment with doses between 0.1 and 10μM FLX for 24 hours, MO3.13 were exposed to 10 μM 5-FU with or without FLX for 24 and 48 hours before viability was assayed by the MTT assay.

As shown in Figure 3.9 A, OPCS were significantly decreased when 5-FU alone was added for 24 hours ($F_{(11, 84)} = 43.51$, P<0.0001; one-way ANOVA). Similar effect are observed when the cells treated with 5-FU after 24 hours preincubation with FLX (p<0.0001). On the other hand incubation of cells with different concentration

FLX (48 hours) did not affected significantly (P>0.05) the cell viability comparing to the control group.

In a separate experiment, cells incubated with different concentrations of FLX alone for 72 hours showed a significant increase in viability with increasing concentrations of FLX with the highest viability when treated with 5μM FLX (P= 0.0003, one-way ANOVA). While, when 5-FU was administered for 48 hours after 24 hours preincubation with FLX, the cell viability was reduced significantly ($F_{(11, 180)}$ = 51.35, p<0.0001; one-way ANOVA) and there was no effect of increasing concentrations of FLX (Figure 3.9 B). The results indicated that 24 hours preincubation FLX is ineffective at protecting cells against 5-FU treatments.

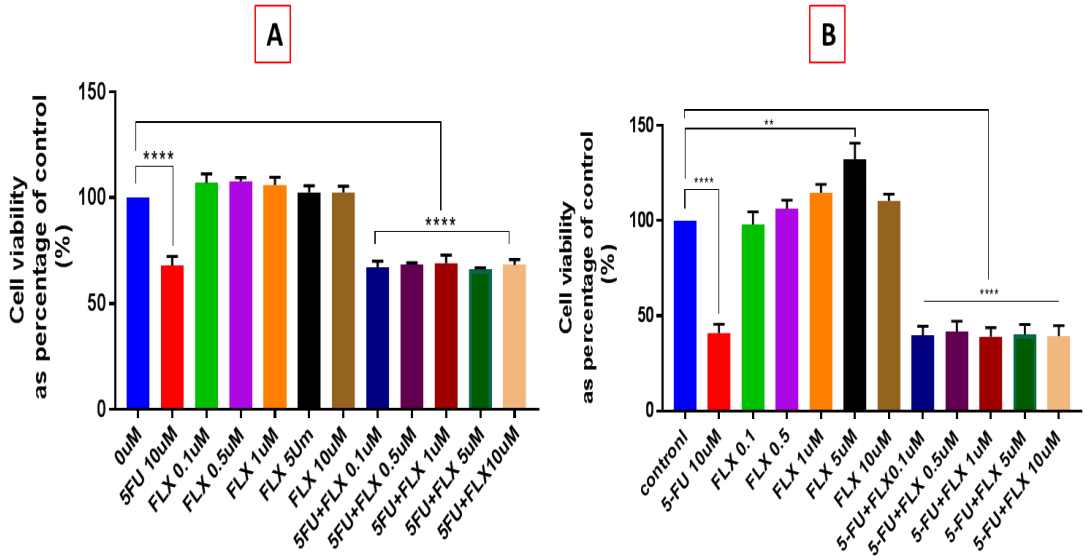



***Figure 3.9*** *Treatment of MO3.13 with 10μM 5-FU after (**A**) 24 and (**B**) 48 hours pre-incubation with and without increasing concentrations of FLX. Analysis with one-way ANOVA showed highly significant decrease (****p<0.0001) in cell viability between the 10μM 5-FU and 0μM control, and between 10μM 5-FU plus different concentration FLX and controls (****p<0.0001). Exposure to different concentrations of FLX did not significantly differ from 0 μM control after 48 hour (P>0.05). Whereas, Exposure to 5uM FLX significantly increased cell viability after 72 hours (***P= 0.0003) comparing to 0μM control.*

### 3.4.2.3 The effect of a combined treatment with 5-FU and INDO on cell viability of OPCs after 24 and 48 hours incubation.

This experiment was designed to test *in vitro,* the protective effect of an anti-inflammatory agent, indomethacin (INDO), against the effects of chemotherapy. OPCs were exposed to 0.1, 0.5, 1, 5 and 10μM of INDO with or without 10μM 5-FU for 24 or 48 hours. Optical densities were normalised as a percentage against the control (0 μM 5-FU). As shown in Figure 3.10, there was a significant decrease

in cell viability with cells treated with 10 μM 5-FU alone ($F_{(11, 331)}$ = 46.13, P<0.0001; one-way AVOVA); and this decrease was still present when cells were co-treated with INDO and 5-FU ($F_{(11,276)}$ =105.4, P<0.0001; one-way AVOVA). Treatment with INDO alone had no effect on cell viability at both time points (24 and 48 hours incubation). Based on these results, INDO alone had no effect on cell proliferation and is not protective under the conditions studied.

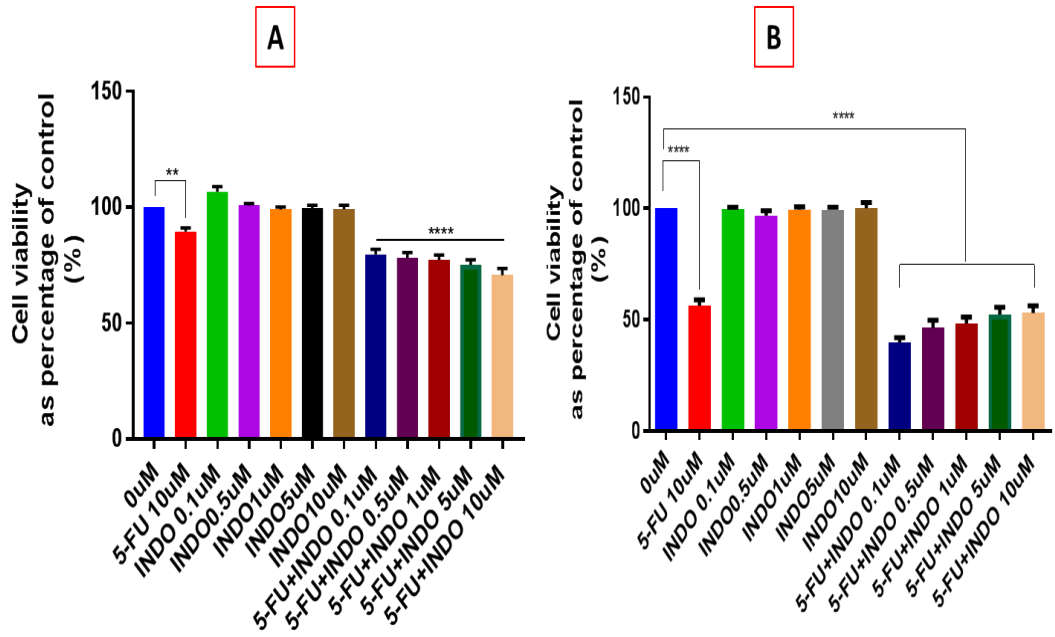



***Figure 3.10*** *Treatment of MO3.13 cells with 10μM 5-FU after (**A**) 24 and (**B**) 48 hours incubation with and without increasing concentrations of INDO. Viability measured by MTT assay. Analysis with one-way ANOVA showed a significant decrease in viability at 24 and 48 hours with 10μM 5-FU alone (**p=0.0024 and (****p=0.0001), and with 10μM 5-FU plus different concentration INDO (****p<0.0001, one-way AVOVA). Exposure to different concentrations INDO did not significantly differ from controls (P>0.05, one-way AVOVA).*

### 3.4.3 The effect of 5-FU treatment on OPC viability when co-cultured with other cell types found in white matter tracts.

Astrocytes, microglia and endothelial cells are present in white matter tracts and may influence the response of OPCs to chemotherapy. The *in vitro* co-culture technique (direct or indirect method) were selected and optimised to evaluate all three cell populations in protection of OPCs from the adverse effects of 5-FU.

#### 3.4.3.1 Optimisation of culture conditions for different cell types

The optimum incubation period for different cell types was determined. Proliferation rates were assayed by staining cells for the proliferation marker Ki67

(Figure 3.11 A). Figure 3.11 B shows that initial growth up to 24 hours is similar between all cell types ($F_{(3, 20)} = 1.2$, P=0.3351, one-way ANOVA). The cell lines showed increased rates of proliferation in culture after 48 and 72 hours (P=0.0438 and P=<0.0001 respectively) as compared to the 24 hours' time point. Although 96 hours showed an increase in growth rate, the MO3.13 and endothelial cell lines showed a decline in proliferation compared to the 72 hours' time point. However as cell proliferation increased, BV2 and C6 cells grew faster than MO3.13 and endothelial cells. Overall, the results indicate that the growth rate of BV-2 cells is faster than other cell types. Moreover, the cell viability assay showed that a low dose of 5-FU reduced cell viability after 48 hours incubation. It might be predicted that the fastest growing cell line should be most sensitive to chemotherapy which targets dividing cells. Therefore, other cellular differences besides growth rate are governing the sensitivity to 5-FU. Thus, 72 hours was chosen as the duration for future experiments.

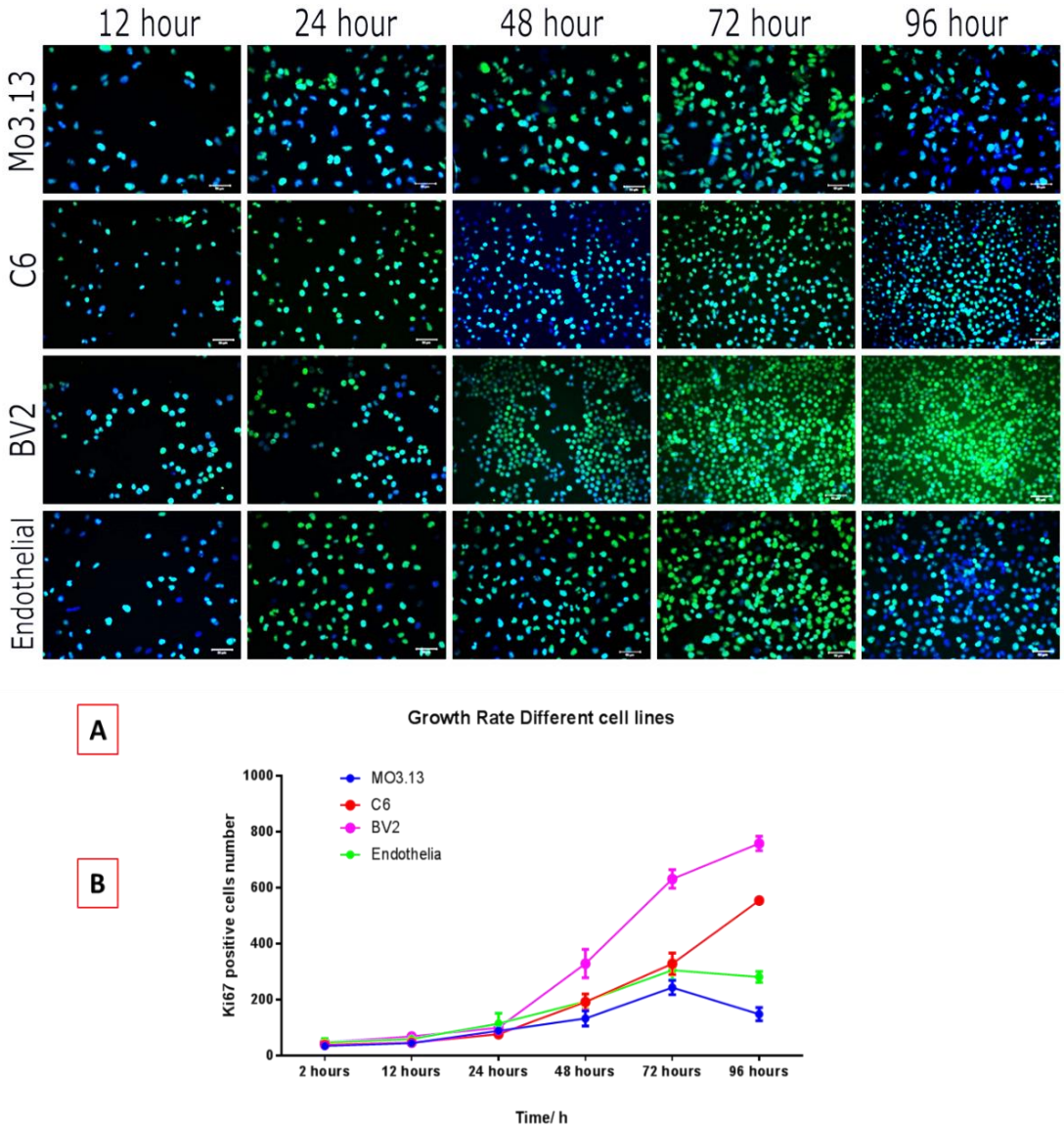



***Figure 3.11*** *Growth rate and proliferation of different cell lines. (**A**) Representative images of cells proliferation in different time points immunostained with ki67 (green) and counterstained with DAPI (blue). Scale Bar=50µm. (**B**) Growth curves for four untreated cell lines in vitro (mean ± SEM, n=3). Cell numbers were assessed at 2, 12, 24, 48, 72 and 96 hours (performed in triplicate). BV-2 and C6 demonstrated a significantly higher growth rates at 48, 72 and 96 hours as compared with MO3.13 and endothelial cells.*

### 3.4.4 Assessment of the effect of chemotherapy on OPCs in co-culture with other cells

To study changes in the impact of chemotherapy on OPCs when co-cultured with C6 or endothelial or BV-2, these cells were co-cultured either allowing contact or in the same media but with contact prevented by a membrane.

#### 3.4.4.1 Contact co-culture MO3.13 cells with C6 cells

As shown in Figure 3.12, treatment of M03.13 cells on their own with 10μM 5-FU caused a significant decrease in cell number (P=0.0017). Co-culture of MO3.13 cells in contact with C6 cells but in the absence of chemotherapy had no effect on the numbers of M03.13 cells (P=0.9845, two-way ANOVA). Treatment of co-cultures of M03.13 and C6 cells with 5-FU showed no significant difference between the treated and untreated co-cultures (P=0.8439), a result demonstrating that contact with C6 cells can be protective to MO3.13 from treatment with 5-FU.

#### 3.4.4.2 Contact co-culture of MO3.13 cells with endothelial cells

Contact co-cultures of MO3.13 and endothelial cells were established in the same way as MO3.13/C6 co-cultures and the two types of cell distinguished by staining the M03.13 cells red with PKH26 (Figure 3.12). After 48 hours incubation with 10μM 5-FU, two-way ANOVA showed significant differences between the groups ($F_{(1, 76)}$ = 63.21, P<0.0001; two-way ANOVA) and the number of MO3.13 cells cultured alone was significantly reduced by treatment with 5-FU (p<0.001). When M03.13 cells were co-cultured in contact with endothelial cells, there was no significant decrease in their numbers in both treated and non-treated groups compared to control (P=0.9889 and P=0.0672 respectively; multiple compression two-way ANOVA). As with co-culture with C6 cells, direct contact with endothelial cells appears to protect M03.13 cells from chemotherapy.

#### 3.4.4.3 Contact co-culture of MO3.13 cells with BV-2 cells

To study of the interactions between microglia and OPCs after treatment with 5-FU, these two cell types were co-cultured with the cells in contact (Figure 3.12). From the initial experiments in section **3.4.1.3**, it was observed that BV-2 cells are particularly sensitive to chemotherapy and that 0.5μM 5-FU had a significant cytotoxic effect on BV-2 cells when incubated for 48 hours. Therefore, this concentration was used in the co-cultures of BV-2 with MO3.13 cells. 0.5μM 5-FU had no significant effect on the number of MO3.13 cells when cultured alone (P= 0.9138, two-way ANOVA). Co-culture of M03.13 with BV-2 cells without chemotherapy also had no effect on M03.13 cell numbers (P=0.0890, two-way ANOVA). In contrast treatment of co-cultures of M03.13 and BV-2 cells with 0.5μM 5-FU significantly decreased the number of MO3.13 cells (P=0.0001, multiple compression two-way ANOVA). It appears that treatment with low concentrations of 5-FU makes microglia cytotoxic to OPCs.

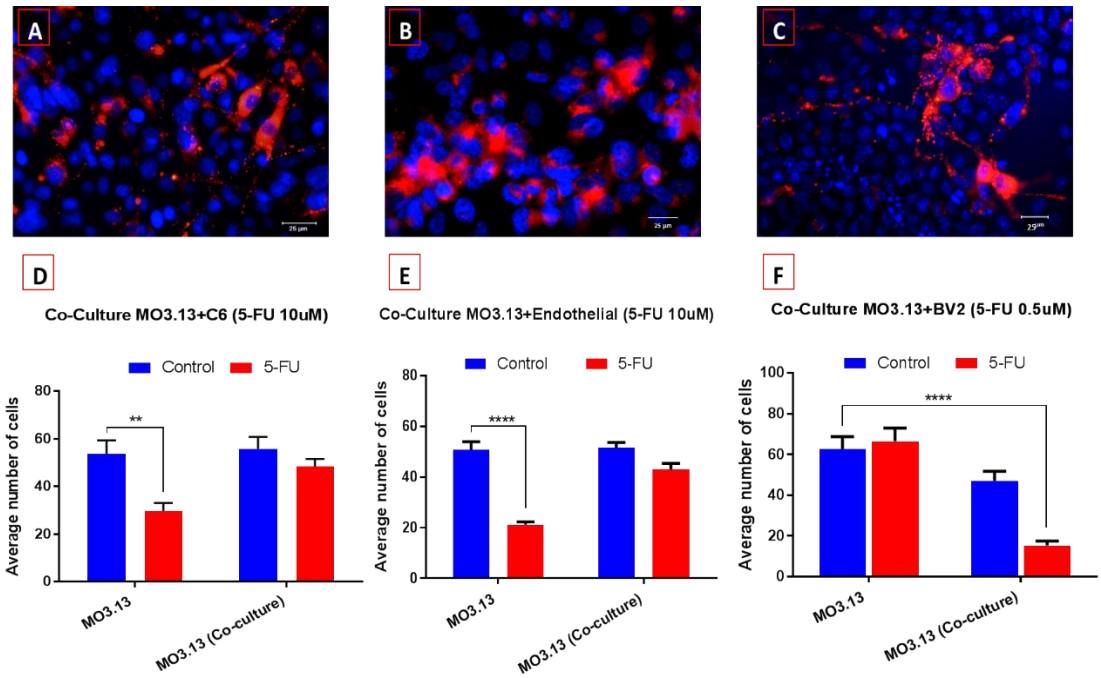



***Figure 3.12*** *Co-culture of MO3.13 with different cell lines with or without 48 hours 5-FU treatment (contact method). (**A**), (**B**) and (**C**) Representative immunofluorescent images of co-culture MO3.13 with C6, HBMECs and BV-2 cells respectively. MO3.13 cells were in red (PKH26 stain) and counterstained with DAPI (blue), other cells stained with DAPI only. (**D**) Effect of 10μM 5-FU on the number of MO3.13 cells alone or in co-culture with C6 cells. (**E**) Effect of 10μM 5-FU on the number of MO3.13 cells alone or in co-culture with endothelial cells. (**F**) Effect of 0.5μM 5-FU on the number of MO3.13 cells alone or in co-culture with BV-2 cells. The data are represented by mean± SEM, n=3 or 4 and analysed by two-way ANOVA and Dunnett's Post-hoc multiple tests to compare the mean of the control group with other groups. (ns, when the P>0.05), (** when P<0.01), (**** when P<0.0001).*

### 3.4.5 Assessment of the effect of CBX treatment on the numbers of OPCs in co-culture with different cell types with or without chemotherapy.

One of the main results of the above experiments was that co-culture of OPCs with other cells in *in vitro* induced chemoresistance and that this required direct cell to cell contact. The mechanism behind these results could be the formation of gap junctions (GJ) between the cell types.

To demonstrate that GJs were present, immunofluorescence detection of the presence of Cx47 in MO3.13 cells was undertaken and showed numerous labelled puncta around oligodendrocyte cell bodies and their processes (Figure 3.13). To demonstrate that GJs were functional between C6 and M03.13 cells, MO3.13 cells were labelled with the red dye PKH26, and co-cultured with C6 cells which had

previously been loaded with the green dye calcein-AM. Dye transfer of the green calcein-AM into the red labelled MO3.13 cells would indicate the presence of GJs. Dye transfer was observed, confirming the presence of functional GJs (Figure 3.14).

Have demonstrated the presence and functionality of GJs, the GJ inhibitor CBX was used to see if this would abolish the protection provided by astrocytes and endothelial cells. Treatment of the labelled co-cultures described above with CBX prevented dye transfer between OPCs and astrocytes (Figure 3.15). These results indicate that there is an exchange of molecules through GJs between astrocytes and OPC cells, and this exchange can be blocked by CBX. From the results obtained, 50μM CBX effectively blocked the passage of calcein-AM and this concentration of CBX was chosen for the next experiments to investigate the role of gap junctions in the protection against chemotherapy provided by co-culture of OPCs with different cell types. In these experiments cells were treated with CBX for one hour prior to further treatments which included 48 hours treatment with 10μ 5-FU.

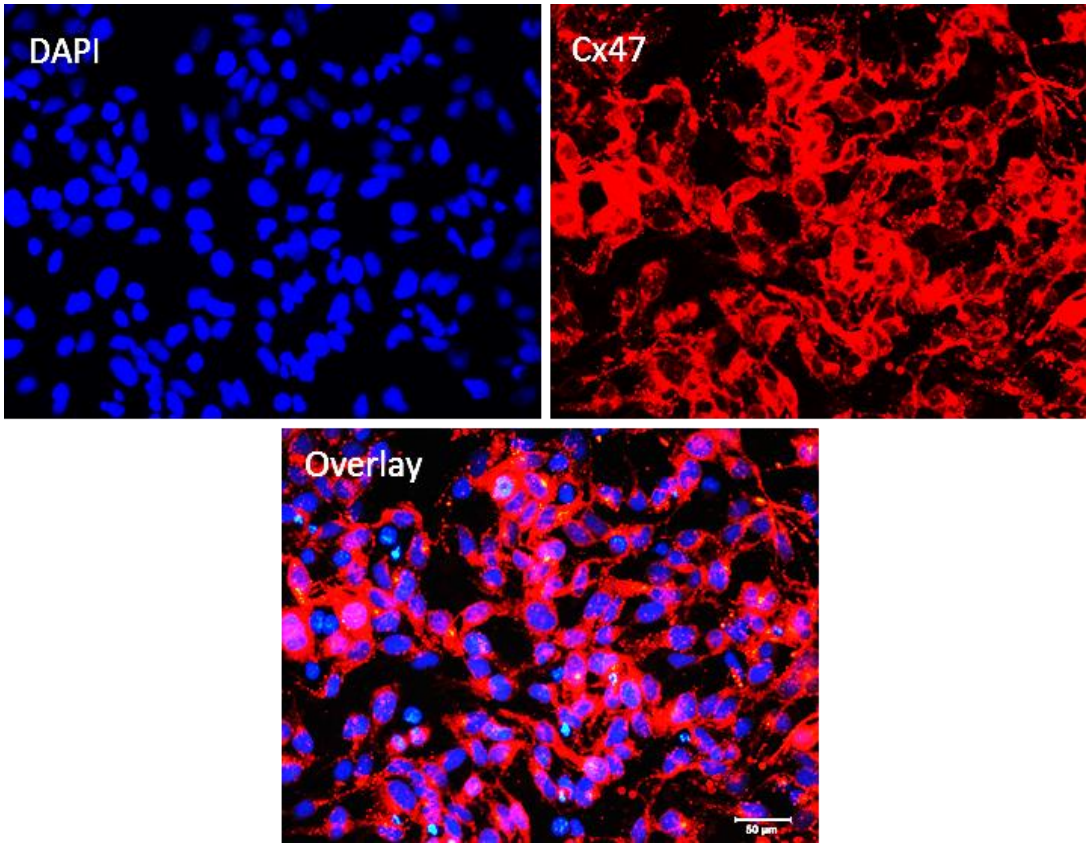



***Figure 3.13*** *Cx47 expression of MO3.13 cells identified by immunofluorescence assay. Expression of Cx47 protein (red) on cell bodies and their processes, the nuclei stained by DAPI (blue). Scale Bar=50µm.*

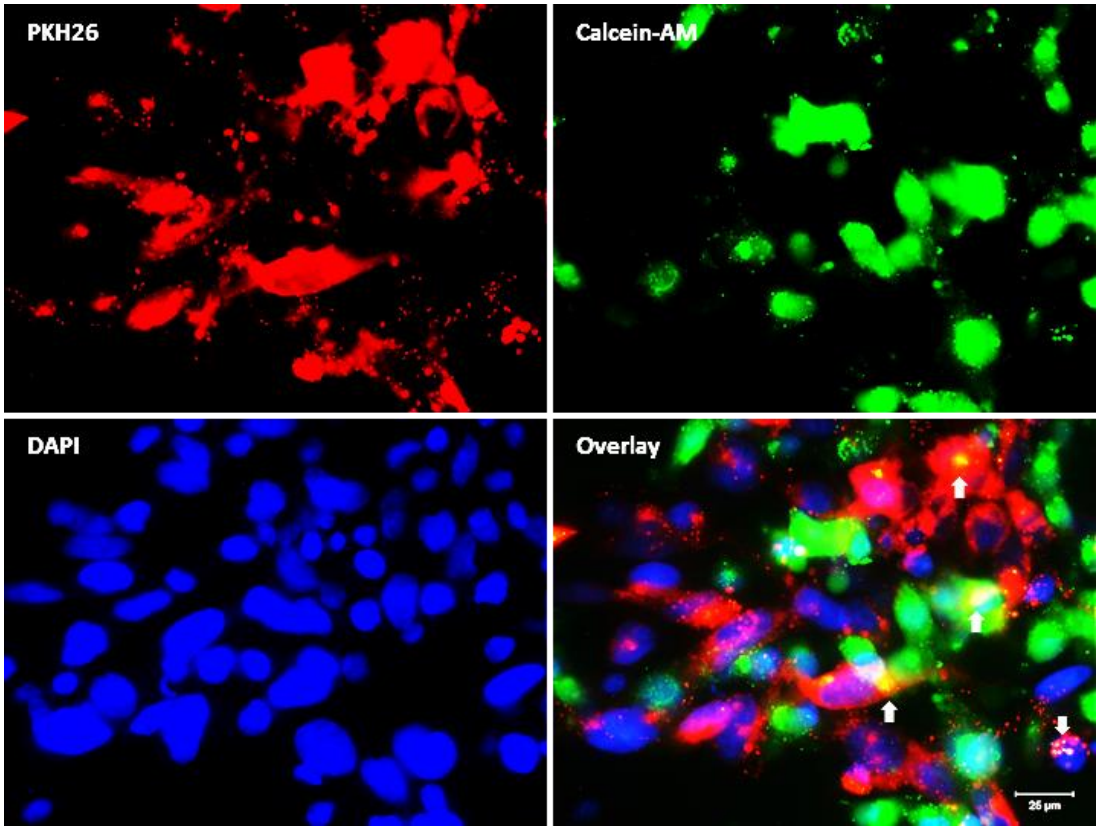



***Figure 3.14*** *Representative images of immunofluorescence dye transfer assay between C6 and MO3.13 in direct co-culture system. The passage of calcein AM green dye from donor cells (C6) into PKH26 red labelled receiver cells (MO3.13) through gap junction coupling channels (arrows). Confirming the presence of functional GJs coupling. Scale Bar=25µm.*

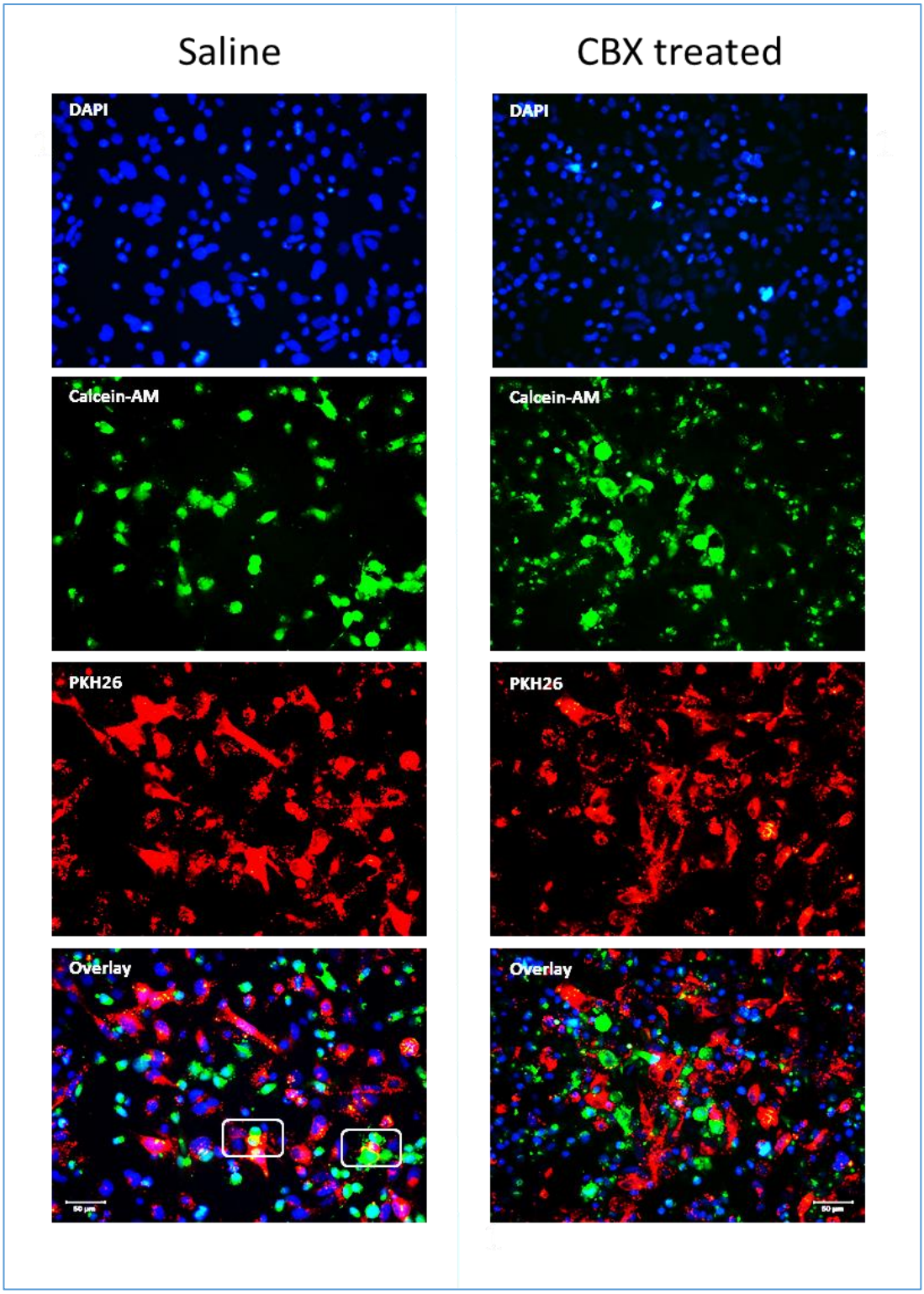



***Figure 3.15*** *Dye transfer in co-culture systems with or without CBX treatment. Representative immunofluorescent images of OPCs (red), Astrocyte (green) and the nuclei were stained with DAPI. The white squares indicate that OPCs formed GJCs with astrocytes in numerous Cx47 puncta on the surface of these cells as seen by yellow in corresponding overlay images (Saline panel). CBX treatment did not affected the growth of OPCs and astrocyte, but it decreased the dye transfer between the two types of cell (depicted in CBX treated panel). Scale Bar=50μm.*

**Figure 3.16 A,** shows the results from different treatments of MO3.13+ C6 co-cultures. Controls consisted of MO3.13 cells cultured either alone or in co-culture with C6 astrocytes but receiving no drug treatment. Co-culture with astrocytes had no effect on MO3.13 numbers ($P>0.05$, multiple comparison two-way ANOVA).

The next part of the graph shows treatment with 5-FU of M03.13 cells either in mono-culture or co-culture with astrocytes. Treatment of MO3.13 cells on their own with 5-FU reduced MO3.13 cell numbers by over 50% ($P= 0.0001$). As found previously, co-culture of MO3.13 with C6 astrocytes protected the MO3.13 cells so that their numbers were not significantly different from controls ($P=0.0841$).

Treatment of mono-cultures of MO3.13 or co-cultures of MO3.13+C6 cells with CBX had no effect on M03.13 cell numbers.

The final part of the graph shows treatment of either mono cultures of MO3.13 or co-cultures of MO3.13+C6 cells treated with both CBX and 5-FU. In both mono and co-cultures, 5-FU treatment caused a significant decrease ($P<0.0001$) in MO3.13 cell numbers similar to that found when MO3.13 cells are treated on their own. In general, the addition of 10μM 5-FU to the cultures had a significant effect ($F_{(1, 72)} = 25.21$, $P<0.0001$). The interaction of the two factors was also significant $F_{(3, 72)} = 71.57$, $P<0.0001$).

Data in **Figure 3.16 B** shows a similar set of experiments where MO3.13 cells are co-cultured with endothelial cells. As found with co-cultures with astrocytes, co-culture with endothelial cells had no effect on MO3.13 cell numbers ($P>0.05$, multiple compression two-way ANOVA). Treatment of mono cultures with 5-FU caused a highly significant reduction in MO3.13 cells to less than half control values. Treatment of co-cultures of M03.13+endothelial cells with 5-FU still significantly reduced MO3.13 cell numbers but to only 80% of control values; indicating the protective effect of endothelial cells against the adverse effect of 5-FU on a MO3.13 cells. Likewise, treatment with CBX on its own had no effect on MO3.13 number in mono or co-cultures ($P>0.05$). while, treatment of mono or co-cultures with both CBX and 5-FU cause a significant reduction in M03.13 cells in both groups; however, Post hoc multiple compression two-way ANOVA showed an increase in the number of MO3.13 in co-culture (MO3.13+endothelial) group incubated with 5-FU+CBX compared with the MO3.13 single culture group treated with 5-FU+CBX ($P= 0.0065$).

These results indicate that GJs play a significant role in protecting OPCs from chemotherapy when co-cultured with astrocytes, whereas, GJs partially

participated in endothelial-mediated protection of OPCs. Additionally, it suggested that there are other factors could have contributed in protecting of OPCs against the side effects of 5-FU.

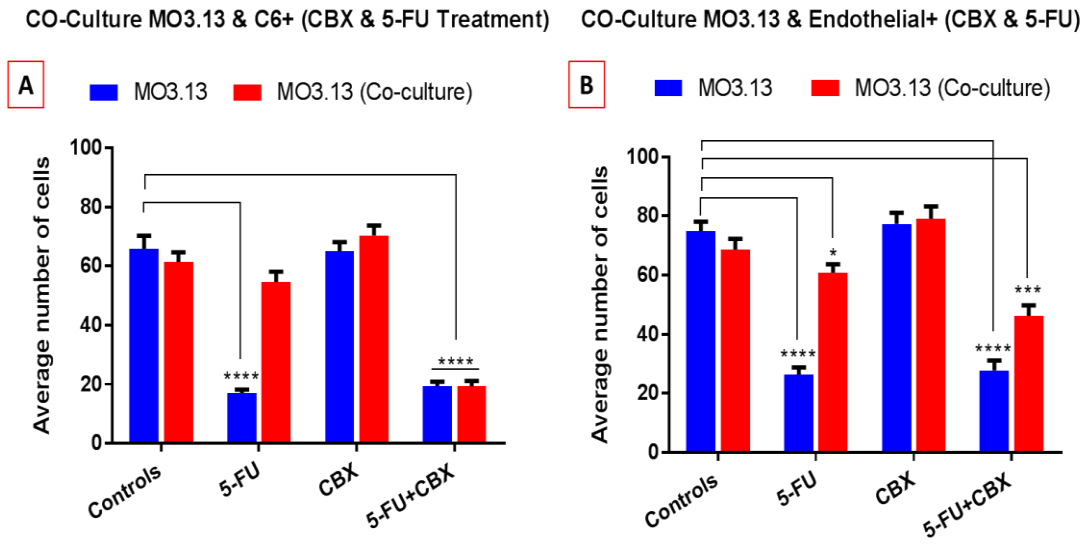



***Figure 3.16*** *Treatment of mono cultures or co-cultures with CBX, 5-FU or both. (**A**) MO3.13+C6 co-culture and (**B**) MO3.13+endothelial co-culture. 5-FU treated groups were exposed to 10µ 5-FU or 48 hours while 50µM CBX treated groups were treated with this drug for 1hour prior to any further treatment. The data are shown as the mean ± SEM of three independent experiments, where five fields of each chamber were counted. The data was analysed by using two-way ANOVA and Tukey's Post-hoc multiple test was performed to compare the mean of the control group with other groups. Levels of significance were (ns, when the P>0.05), (* when P<0.05) (*** when P<0.001), (**** when P<0.0001).*

### 3.4.6 Co-culture of OPCs with astrocytes, endothelial cells or microglia where the cell types are separated by a membrane (Transwell co-culture)

The transwell co-culture system was used in this study to test whether direct cell to cell contact between MO3.13 cells and other cell types was required for the co-culture to provide protection from chemotherapy. The transwell co-cultures system enables different cell types to grow in close proximity but be separated by a porous membrane. In this way factors secreted by cells can pass through the membrane but direct contact is prevented. In this experiment 0.4µm pore cell inserts were used to separate C6, BV-2 or endothelial cells (in inserts) from MO3.13 cells (in the bottom of the well). After adding 5-FU for 48 hours, the cell viability of the MO3.13 cells was determined by MTT assay.

The results from co-culture of MO3.13 cells with C6 cells are shown in **Figure 3.17 A** These show that 10μM 5-FU exposure significantly reduced the viability of MO3.13 cells when incubated alone or in a membrane separated co-culture with C6 ($F_{(1,60)}$ = 618.5, P<0.0001, two-way ANOVA). While, the viability of MO3.13 cells was not affected by the presence of C6 cells in cultures not treated with 5-FU (P=0.3636, multiple comparisons two-way ANOVA). These results indicate that direct cell to cell contact is required to give protection from 5-FU exposure.

Data in **Figure 3.17 B** shows the results when MO3.13 cells were co-cultured with endothelial cells. 48 hours of incubation with 5-FU significantly reduced MO3.13 viability when cultured alone (P<0.0001, two-way ANOVA with Tukey's post-hoc test). A significant reduction was also found when endothelial cells were present on the opposing side of the membrane (P=0.0001, multiple comparisons test two-way ANOVA) but the decline in M03.13 viability was reduced by the presence of the endothelial cells. These results again indicate that direct cell to cell contact is required to protect M03.13 cells but some trophic factors from endothelial cells may help in their survival when they were sharing the same media.

**Figure 3.17 C** shows the results from membrane separated co-cultures between MO3.13 cells and B-V2 cells. The results show that 0.5μM 5-FU had no effect on MO3.13 cell viability when these cells are cultured alone (P= 0.0924, two-way ANOVA). Similarly when MO3.13 cells are co-cultured with BV-2 cells, but not treated with 5-FU, there is no effect on MO3.13 cell viability (P=0.6844). In contrast, when MO3.13 cells are co-cultured with BV-2 cells and treated for 48 hours with 0.5μM 5-FU, the viability of MO3.13 cells was reduced by the presence of the BV-2 cells (P<0.0001). From this data, it can be concluded that 5-FU treatment activates microglia to secrete soluble factors that can be transmitted through the porous membrane and reduce the viability of OPCs.

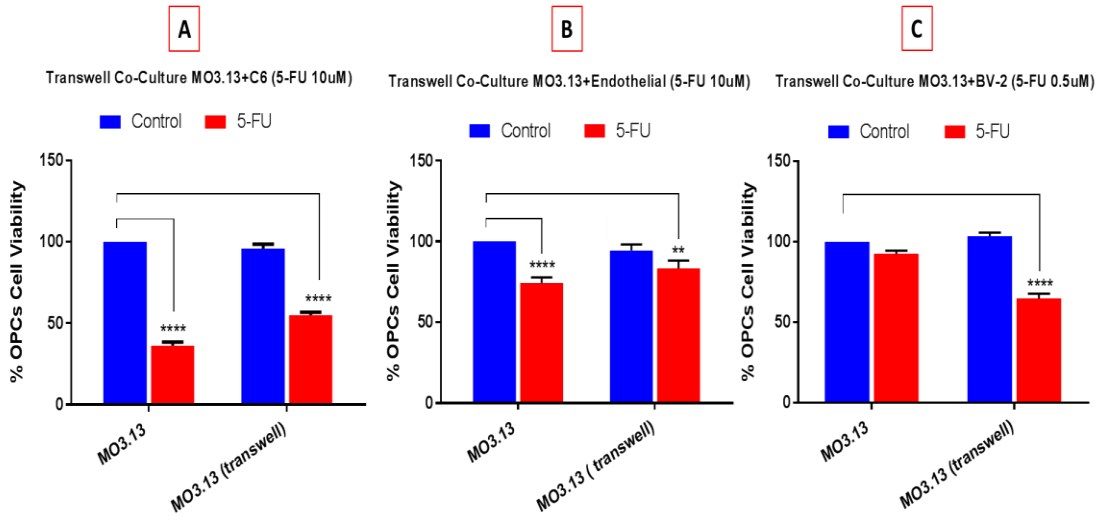



***Figure 3.17*** *Membrane separated co-culture of MO3.13 with different cell lines with or without 48 hours 5-FU treatment (transwell method 0.4µm pore inserts). (**A**) Effect of 10µM 5-FU on the viability of MO3.13 cells when cultured alone or co-cultured with C6 cells. (**B**) Effect of 10µM 5-FU on the cell viability of MO3.13 when cultured alone or co-cultured with endothelial cells. (**C**) Effect of 0.5µM 5-FU on the cell viability of MO3.13 when cultured alone or co-cultured with BV-2 cells. The data are represented by mean± SEM, n=3 (average 2 wells from each treatment group). The data was analysed by using two-way ANOVA and Tukey's post-hoc multiple comparisons test was performed to compare between treatment groups. (ns, when the P>0.05), (** when P<0.01), (**** when P<0.0001).*

### 3.4.7 The effect of conditioned medium (CM) on OPCs survival

To further extend the observations showing protection of OPCs from chemotherapy by different cell types present in white matter tracts, the effectiveness of trophic substances present in CM was tested.

MO3.13 cells were incubated in a 1:1 ratio of C6 conditioned medium (**C6-CM**) and fresh media with or without 5-FU treatment. The results presented in Figure 3.18 A showed that there was no significant effect of C6-CM on MO3.13 viability after 48 hours incubation compared with the control group ($F_{(1, 60)} = 1.586$, P=0.2128; two-way ANOVA). 5-FU treatment had a significant effect on cell viability when incubated with or without C6-CM ($F_{(1, 60)} = 53.85$, P<0.0001). The results indicate that C6-CM had no protective effect on 5-FU treated OPCs, indicating that cell–cell contact is required for protection.

CM from endothelial cells (**E-CM**) was used in the same way as C6-CM by adding it to MO3.13 cells. The results showed that the viability of progenitors was not enhanced by the presence of E-CM compared to control fresh medium (P=0.0924). 5-FU inhibited MO3.13 viability when incubated with or without E-CM (P<0.0001 and P=0.0015 respectively), Post-hoc multiple comparison tests revealed that

there was a significant increase in cells viability in 5-FU group incubated with E-CM compared to group treated with 5-FU alone ($P<0.0001$, two-way ANOVA) (Figure 3.18 B). The data indicates that the decrease in the OPCs viability was partially prevented by E-CM that may contain some soluble factor(s) which enhance cell survival.

Furthermore, two types of microglial conditioned medium (**M-CM**) (untreated and pre-treated CMs) were added to the MO3.13 cells at a 1:1 ratio with fresh medium and cell viability was assessed. Figure 3.18 C shows the results of incubation MO3.13 cells with 50% untreated M-CM for 48 hours. The most striking result to emerge from the data is that cells incubated with **untreated M-CM** showed a significant increase in viability compared to controls ($P=0.0414$, two-way ANOVA). It was also shown that treatment with 0.5μM did not affect the viability of M03.13 cells when incubated with fresh media ($P= 0.7406$). On the other hand, when MO3.13 cells were incubated with **pre-treated M-CM** (see **section 3.3.9**) or 0.5μM 5-FU for 48hours, they showed a significant reduction in cell viability compared to controls ($F_{(1, 60)} = 119.8$, $P<0.0001$) (Figure 3.18 D). Also, there was a significant interactions between 5-FU treatment and pre-treated M-CM ($F_{(1, 60)} = 7.763$, $P=0.0071$; multiple comparison two-way ANOVA). The results indicate that untreated M-CM can enhance OPCs viability indicating that soluble factors responsible for OPC survival could be secreted from non-treated BV-2 cells. Whereas B-V2 cells pre-treated with chemotherapy which could activate the microglia to secrete soluble factors as part of an inflammatory response which can negatively affect the survival of OPCs.

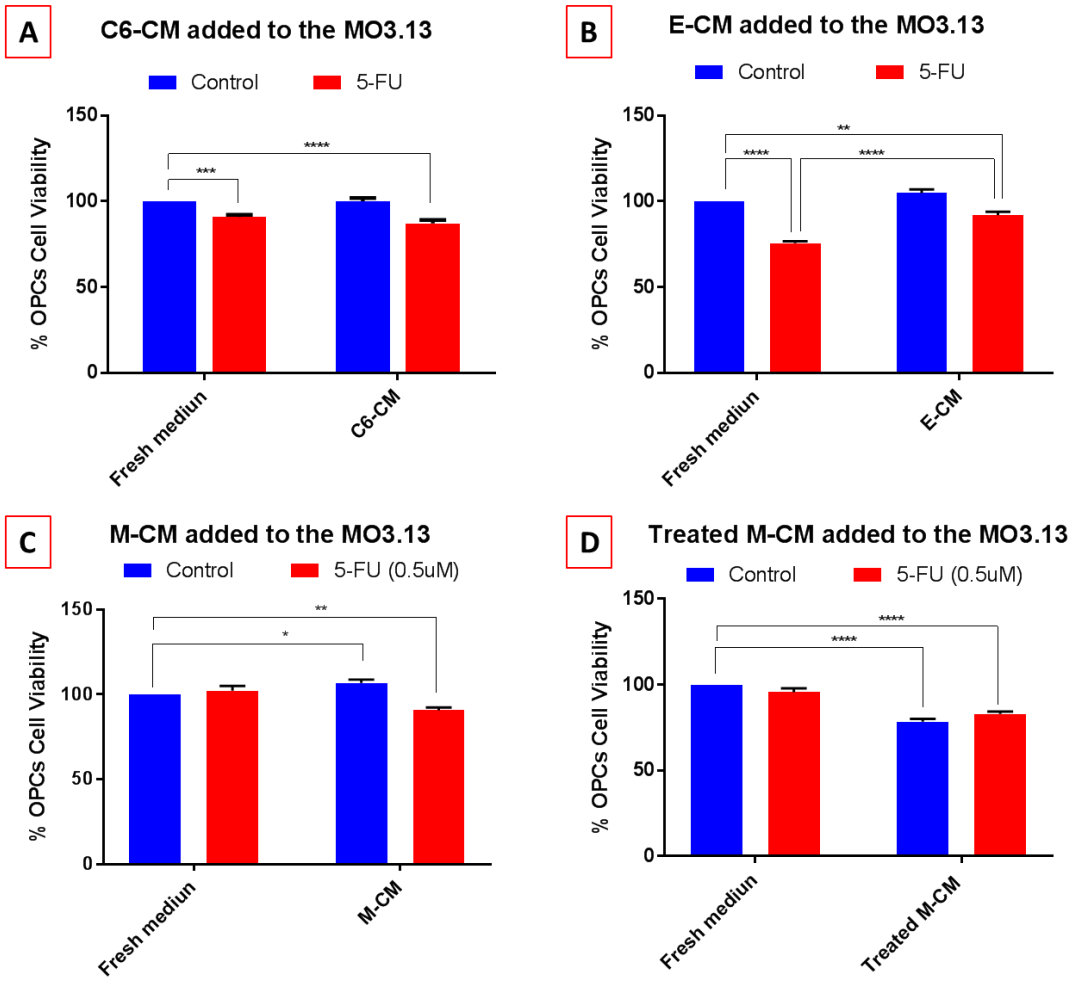



***Figure 3.18*** *Effects of different CMs on the viability of OPCs with or without 48 hours 5-FU treatment. (**A**) The cell viability of MO3.13 incubated in 50% astrocyte CM (C6-CM) with or without 10μM 5-FU treatment. (**B**) The cell viability of MO3.13 incubated in 50% endothelial CM (E-CM) with or without 10μM 5-FU treatment. (**C**) The cell viability of MO3.13 incubated in 50% untreated microglia CM (untreated M-CM) with or without 0.5μM 5-FU treatment. (**D**) The cell viability of MO3.13 incubated in 50% microglia CM exposed to 0.5μM 5-FU for 24 hours (pre-treated M-CM) with or without 0.5uM 5-FU treatment. The data is represented by mean± SEM, n=3 (average 4 wells from each treatment group) and analysed by two-way ANOVA and Tukey's post-hoc multiple comparisons test to compare between treatment groups. (ns, when the P>0.05), (* when P<0.05), (** when P<0.01), (*** when P<0.001), (****when P<0.0001).*

### 3.4.8 Does addition of FLX or INDO attenuate microglial secretion of TNF-α after treatment with 5-FU.

The signalling mechanisms by which microglial cells treated with 5-FU were toxic to OPCs were investigated. As mentioned earlier in this study, TNF-α has been suggested as a potential factor released from treated BV-2 cells that can inhibit MO3.13 cell viability or mediate MO3.13 cell death. As *in vivo* treatment with FLX or INDO has been found to be protective against the effects of chemotherapy on WMTs gliogenesis, it was investigated whether treatment with these drugs could reduce directly the negative effects produced by 5-FU treated BV-2 cells in MO3.13/ BV-2 co-cultures. If FLX or INDO could prevent the activation of microglia by 5-FU, the viability of M03.13 cells in co-culture with BV-2 cells might be improved. TNF-α released by BV-2 in all the experiment settings was measured using a highly-sensitive enzymelinked immunosorbent assay ELISA kit (see **section 3.3.13**).

Results in Figure 3.19 clearly show that 5-FU markedly induced the production of TNF-α ($P=0.0001$, two-way ANOVA). Moreover, there was a significant interaction between all treatments groups ($F_{(5, 60)} = 5.02$, $P=0.0007$) after 48 hours of incubation. When FLX or INDO was added to 5-FU treated BV-2 or treated co-culture cells ($P>0.05$, multiple comparisons two-way ANOVA test), TNF-α release was attenuated by inhibiting tumour necrosis factor α synthesis in microglia, this data is in agreement with the co-culture experiments. The results indicate that 5-FU stimulated microglial cells to release TNF-α which in turn affected the cell viability of OPCs. This induced microglial toxicity could be abolish by co-treatment of FLX or INDO.

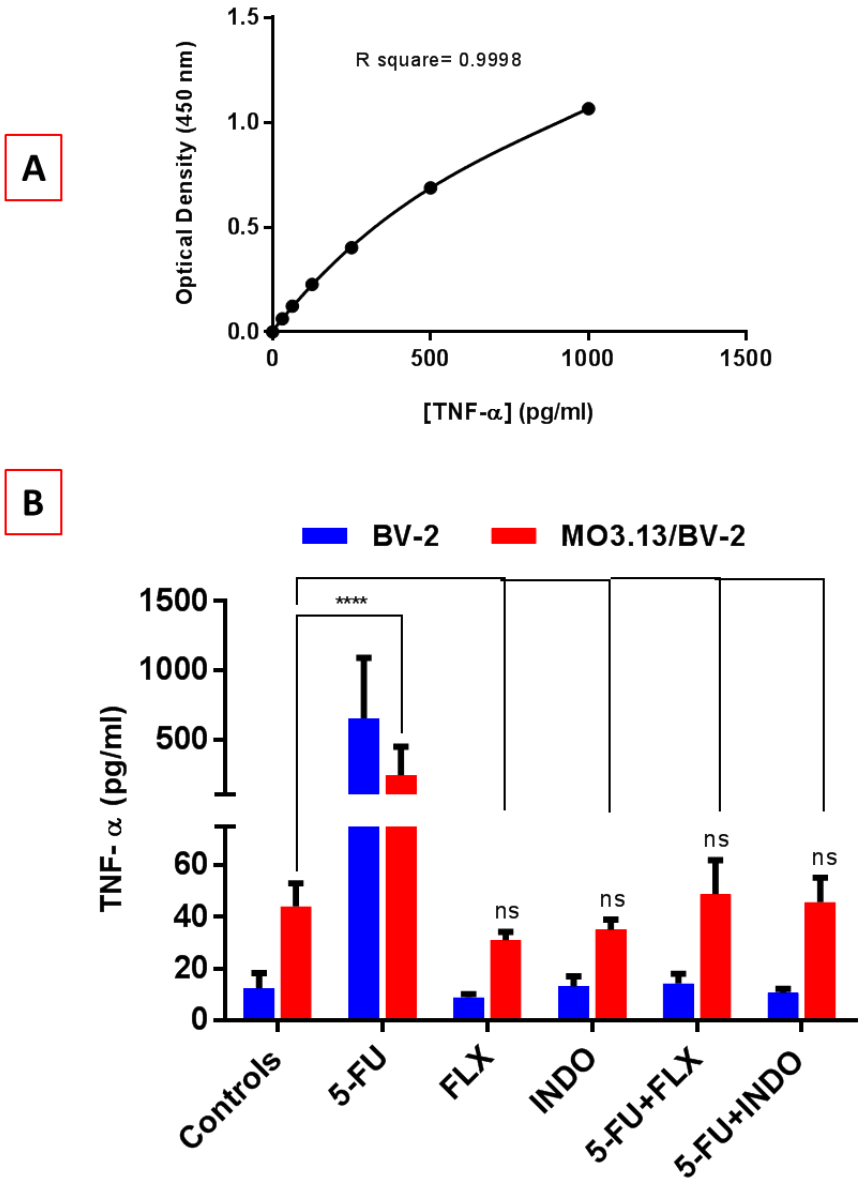



***Figure 3.19*** *Histogram showing results from the ELISA assay to detect TNF-α concentration in different treatments groups. (**A**) Representative TNF-α standard curve made up on the day of the experiment. Sample concentrations were expressed as pg/ml based on standard curves plotted using protein standards supplied with the kits. After 48 hours of incubation, supernatants were used for ELISA assay (**B**) TNF-α released from BV-2 and mixed glial cultures treated with 0.5μM 5-FU for 48 hours. Treatment with 5μM FLX or 10uM INDO attenuated BV-2 to release TNF-α. Co-treatment FLX or INDO attenuated TNF-α release when added to 5-FU treated BV-2 in contact with MO3.13. Data is expressed as a mean± SEM of three separated experiments and duplicates were run within the same experiment. Two-way ANOVA with Tukey's post-test was performed. Level of significance was (ns, when the P>0.05) and (****when P<0.0001).*

## 3.5 Discussion

This in vitro study looked at the direct impact of chemotherapy on the viability of OPCs and their interactions with astrocytes and endothelial cells. This study assessed the impact of a range of different 5-FU concentrations together with possible treatments which may protect progenitor cells against the adverse effects of chemotherapy.

### 3.5.1 Effect of different 5-FU concentrations on cultures of single cell types:

The viability of M03.13 (OPCs); C6 (astrocytes), BV-2 (microglia) and endothelial cells were tested for their sensitivity to different concentrations of 5-FU over 24 and 48 hours. Although cell culture lacks many systemic factors such as hormones, cellular proteins, different cell types, cellular pathways and signalling found *in vivo*, it remains the ideal method to determine the direct mechanisms of actions and effects of different treatments in the OPCs.

The results showed that 5-FU treatment reduced the cell viability of all cell types used in these experiments. Viability was never reduced to zero and block of cells with chemotherapy was incomplete, probably because cells are at different stages in the cell cycle and so vary in their susceptibility to 5-FU while other cells may enter quiescence stage. In addition, some of the cells become more resistant to chemotherapy which starts to upregulate multidrug resistance proteins (MDR proteins) in the cell wall that pumps harmful substances out of a cell. Moreover, the exposure period to the 5-FU is another factor that prevents 100% blocking of cell viability. Thus longer incubation times might also have further reduced cell viability.

#### 3.5.1.1 Effect of different 5-FU concentrations on OPCs (MO3.13) cell line

The viability of MO3.13 cells was not affected by 5-FU exposure at low concentrations (0.1, 0.5 and 1μM) given for 24 or 48 hours. However, 24 or 48 hours exposure to higher concentrations of 5-FU (5, 10, 15, 20 and 25μM) reduced the viability of these cells. These results demonstrate that 5-FU has dose and time dependent effects on OPC survival. Zou *et al.* (2007) found that 5-FU at concentrations between 192μM and 284 μM given for 24 hours inhibited Eca109 and Ls-174-t cancer cells in a dose dependent manner. As a cytotoxic compound, it was expected that 5-FU would cause significant reductions in cell viability as MO3.13 cells are a highly proliferative cell line, which should be vulnerable to

chemotherapy. One unanticipated finding was that at low concentrations (0.1 and 0.5μM), 48hours of 5-FU treatment significantly increased cell viability. The reason for this is not clear, but it could be that low doses of cytotoxic agents, given for short periods may lead to an increase in cell differentiation and a reduction in proliferation. Alternatively transient exposure to some chemotherapies can prolong the cell cycle period of progenitor cells and delay their differentiation. Importantly, chemotherapeutic agents may alter the differentiation decisions of neural progenitor cells as well as targeting the proliferation of these cells. This was investigated by Hyrien *et al.* (2010) who analysed the effect of BCNU on OPC cultures and found an absence of drug-induced apoptosis; however no studies have found any increase in neurogenesis or gliogenesis after chemotherapy treatment.

#### 3.5.1.2 Effect of different 5-FU concentrations on the astrocyte cell line (C6)

The viability of the C6 astrocytic cell line was also assessed by MTT assay after treatment with the same range of concentrations of 5-FU used to treat MO3.13 cells (0, 5, 10, 15, 20 and 25μM) given for 24 or 48 hours. Treatment was found to induce dose and time dependent effects (Figure 3.5). From these results, at all doses between 5μM and 25μM, cells were more sensitive to 5-FU after 48 hours compared to 24 hours exposure. Preliminary experiments produced a dose response curve from which the half maximal inhibitory concentration (IC50) for C6 cells was at a concentration of 12.43μM 5-FU for 48 hours. For MO3.13 cells the IC50 was ~10μM 5-FU at same time point. These results indicated that 48 hours in 5-FU was a more effective incubation time to demonstrate its cytotoxic effect. Moreover, MO3.13 cells appear to be more sensitive to 5-FU compared with C6 cells. The present findings are consistent with previous research which found that approximately 55-70% of OPCs and mature oligodendrocyte are killed after exposure to 1μM 5-FU for 24 hours in vitro. This concentration is equal to the low end of the concentration observed in the CSF of patients after intravenous infusion of 5-FU and is below that which affects cancer cells (Dietrich *et al.* 2006). In addition these authors showed that astrocytes are less susceptible to 5-FU; but are still affected by prolonged exposure. The current result is in agreement with Cho *et al.* (2004) who found that astrocytes were relatively more resistant to 5-FU, but oligodendrocyte were selectively vulnerable to 5-FU and led to cell death by way of the apoptotic process. They found that exposure of 5-FU at increasing concentrations (10, 30, and 100μM) for 24 hours showed that astrocytes were still resistant to 5-FU toxicity, even up to 100μM concentration.

#### 3.5.1.3 Effect of different 5-FU concentrations on microglia (BV-2) cell line

The BV-2 microglial cell line was faster growing than other cell lines. It is likely that as the fastest growing cell line, it should be more sensitive to chemotherapy which targets dividing cells. Similar to OPCs a low concentration of 5FU (0.1μM) increased the viability of BV-2 cells when incubated for 24 hours (Figure 3.6 A). Whereas, 5-FU doses of 0.5μM or above, reduced cell viability in a dose-dependent manner after both 24 and 48 hours. These results indicated that 5-FU appears to be more cytotoxic to microglial cells which an IC50 of ~0.484μM at 48 hours. The results presented here, for BV-2 cells, are similar to those found *in vitro* for primary cultures of rat cortical microglial cells (Vairano *et al.* 2004); after the cells were treated with the chemotherapy agents MTX or TMZ. While, higher levels of chemotherapy reduce microglial viability they also stimulate the surviving microglia to become activated and release cytokines (Vairano *et al.* 2004). In addition, it seems possible that that the reduction in the number of microglia after activation is due to apoptosis induced by chemotherapy and an inhibition of growth factors such as transforming growth factor β (TGF-β) that enhance toleration by proliferating microglia (Reid *et al.* 1993, Battista *et al.* 2006, Seigers *et al.* 2016). Taking these observations together, it is possible say that chemotherapy has three main effects in microglia; first these cells are sensitive to cytotoxic agents and many are killed. Secondly, the surviving cells, after exposure to the chemotherapeutic agents, are activated and secret inflammatory cytokines, and finally, the cytotoxic agents reduce the growth factors that stimulate cells to proliferate and differentiation. Based upon these results, a concentration of 0.5μM 5-FU was used in co-cultures of MO3.13 and BV-2 cells. These cultures were used to determine the impact of chemotherapy in OPCs when these cells were in direct contact or separated from microglia in co-culture.

#### 3.5.1.4 Effect of different 5-FU concentrations on endothelial (HBMECs) cell line

From the results in current chapter, endothelial cells were more resistant to 5-FU at concentrations between 5-15uM when incubated for 24 hours compared with the other cell types used in this experiment. There was no significant effect of 5-FU treatment at concentrations between 5 and 10μM for 48 hours. The resistance of endothelial cells to 5-FU treatment *in vitro* may relate to *in vivo* results in our lab. Rats treated with 5-FU showed significantly reduced non-endothelial cell proliferation in the SGZ of the dentate gyrus but no decrease in micro-vessel density (Maqbool 2015). From these initial experiments, a 5-FU dose of 10μM was

chosen as this significantly reduced OPCs number but did not significantly affect endothelial cell viability.

### 3.5.2 Direct effects of co-treatments FLX or INDO with 5-FU on MO3.13 cell line

In the previous chapter presented in this thesis, it was found that chronic 5-FU treatment reduced cell proliferation and survival in the ON and CC of the adult rat. Co-administration of FLX or INDO had the protective effect of promoting the proliferation and survival of progenitor cells against the negative impact of 5-FU.

In order to investigate the effects of chemotherapy and the protective effects of antidepressant or anti-inflammatory treatments in vitro, OPCs were cultured and exposed to 5-FU with or without FLX or INDO at different concentrations and for different periods. Incubation with 10μM 5-FU for 48 hours produced a 50% decline in OPC survival. In contrast, treatment with FLX or INDO alone did not affect cell viability except at the highest dose (data not shown) which produced a reduction in cell viability. This suggests that FLX and INDO, at low concentrations, have no cytotoxic effects on progenitor cells. The data concluded that FLX or INDO has maintained OPCs viability; whereas the highest concentrations are toxic to these cells. This also accords with earlier observations, which showed that 1-2 μM FLX stimulated cell proliferation of primary cerebral granular cells, whereas, 20 μM produced the opposite effect and inhibited cell proliferation (Manev *et al.* 2001, Zusso *et al.* 2008).

Exposure of MO3.13 to 5-FU (10 μM) plus FLX or INDO at various concentrations (0.1, 0.5,1, 5 and 10 μM) for 24 or 48 hours did not show any protection against the adverse effect of 5-FU. Moreover, 24 hours preincubation with FLX or INDO before exposure to the 5-FU for 24 hours also failed to protect cells from 5-FU. These results indicate that FLX may have a weak direct effect on the MO3.13 cell line, indicating that the protective effect of FLX *in vivo* is indirect. Thus it could be that FLX has effects via other cell types (Chen *et al.* 2007). This finding is in agreement with Nguyen *et al.* (2002) who showed that these drugs do not directly increase cell proliferation or differentiation, but act to prevent the adverse effects of interleukins and cytokines, and in doing so maintain an optimum environment for adult neurogenesis (Nguyen et al., 2002). Moreover, these results may throw some light on results produced by other researchers who have shown that FLX increases the rate of neurogenesis of adult rats (Chen *et al.* 2007, Zusso *et al.* 2008). It is interesting to note the *in vivo*, FLX requires several weeks of administration to increase hippocampal neurogenesis and cortical gliogenesis (Kodama *et al.* 2004, Conley and Hutson 2007). This is in contrast to the *in vitro*

studies presented here where 24 hours treatment was given and may be too short an exposure time to produce an effect.

There were also no significant effects of INDO treatment on its own on cell proliferation rather it was found that INDO enhanced the negative effects of 5-FU and further reduced cell proliferation. The present findings are however consistent with other research which has found that INDO and its analogues enhance the toxicity of different chemotherapeutic agents (Draper *et al.* 1997, Touhey *et al.* 2002, Benyahia *et al.* 2004). However Ho *et al.* (2015) observed that chronic treatment with INDO reversed the decrease in cell proliferation in the hippocampus, possibly by reducing the pro-inflammatory cytokine TNF-α caused by administration of streptozotocin. Accordingly, it was found that INDO reduced the levels of pro-inflammatory cytokines secreted by activated microglia which created a detrimental inflammatory environment, however INDO alone did not change the number of the proliferating cells, or immature neurones (Ji *et al.* 2013). A summary of main results of direct effects of different treatments on OPCs highlighted in the **table 3.1**.

***Table 3:1*** *Summary of results from an* in vitro *experiment represent the direct effects of different treatments on OPCs cell line.*

| Drugs | Effect on viability of MO3.13 (OPCs) |
|---|---|
| **5-FU** | Dose and time dependent reduction |
| **FLX** | Dose and time dependent effect |
| **INDO** | Dose and time dependent effect |
| **5-FU+FLX** | No protection |
| **5-FU+INDO** | No protection |

### 3.5.3 Effect of 5-FU on MO3.13 cell viability and proliferation when co-cultured with supporting cells

In co-culture experiments where two types of cell are grown together both types of cell were cultured in DMEM and FBS. Treatment was with 10μM 5-FU for co-cultures of OPCs with astrocytes or endothelial cells, and 0.5μM 5-FU for co-cultures with microglia. The normal growth rate was assessed by counting proliferating cells immunostained for Ki67 (methodology section in this chapter) for each cell line used in the co-culture experiments. The data demonstrated that

the growth media (DMEM), was sufficient for all cell lines which showed the highest number of dividing cells between 48 and 72 hours. Consequently, other cellular differences besides growth rate are governing the sensitivity to 5-FU. Therefore, the protocol for further experiments was chosen as 72 hours during the period of maximum growth rate.

#### 3.5.3.1 Direct co-culture interactions between OPCs and other cells

Cell-cell communication is one of the essential factors in the maintenance and support of cells in the CNS. Recently, intercellular interactions between OPCs and supporting cells have been reported to influence the proliferation and survival of progenitor cells (Li *et al.* 2008, John 2012, Chen *et al.* 2015, Belousov *et al.* 2017).

In this study, cells were treated with 5-FU using a concentration chosen from earlier experiments, and the protective effects of **astrocytes**, against 5-FU, was assessed in direct co-culture with OPCs. OPCs were identified in co-culture by staining them with a red fluorescent dye. The current study found that treatment of mono cultures of OPCs with 5-FU significantly reduced their viability, while treatment of co-cultures of OPCs and astrocytes maintained OPC numbers. Although, both astrocyte and OPCs are affected by 5-FU treatment in mono cultures, while the presence of astrocytes in co-culture system appears to protect OPCs from the cytotoxic effects of chemotherapy. The mechanism of this protection is not obvious yet and need further elucidation. Resent researchers found that astrocyte can protect some of cancer cells from apoptosis that induced by chemotherapy (Lin et al. 2010, Kim et al. 2011, Chen et al. 2015). However they mentioned that the protective effect of astrocytes resulted from their sequestering calcium from the cytoplasm of tumour cells, but reactive astrocytes for their own survival still an unrecognized mechanism. Astrocytes are a unique group of glial cells that play a number of roles in the maintenance and support of oligodendrocytes. Astrocytes have high antioxidative capacities which protect against peroxynitrite toxicity and can counteract the production of free radicals which may damage oligodendrocytes. Moreover, astrocyte have been shown to release trophic factors including brain-derived neurotrophic factor (BDNF) and NT-3 that protect neurons and other glial cells, particularly after injury (Dreyfus *et al.* 1999).

An *in vivo* study has shown that activated astrocytes, in affected lesions, can express the growth promoting factor (CNTF), a factor that supports OPC survival (Albrecht *et al.* 2007) and increases the remyelination of adult rat spinal cord (Franklin and Ffrench-Constant 2008). Meyer-Franke *et al.* (1999) have described

how astrocytes promote contact and alignment between oligodendroglial processes and axons in vitro, while astrocytes are responsible for generating the nodes of Ranvier (Waxman 1998, Talbott *et al.* 2005), further indicating their supporting role for oligodendrocytes. Astrocytes have high antioxidative capacities which protect against peroxynitrite toxicity and can counteract the production of free radicals which may damage oligodendrocytes (Peuchen *et al.* 1997). These interactions between astrocytes and oligodendrocytes could contribute to the protective effects of co-culturing astrocytes with oligodendrocytes. Some interactions require direct cell to cell contact while others depend on the secretion of trophic factors by astrocytes. If direct cell contact is required this may indicate that gap junctions between astrocytes and oligodendrocytes may play a part in astrocyte protection. Astrocytes have been found to form gap junctions with cancer cells and in doing so provide resistance to chemotherapy (Chen *et al.* 2015). Gap junctions facilitate the passage of small molecules between cells including glucose, ATP and ions (Gajardo-Gomez *et al.* 2016). In this way they can maintain the metabolism of connected cells and buffer the concentration of ions such as Ca2+ where excess levels can be toxic. To test whether gap junctions were involved in astrocyte protection of oligodendrocytes from chemotherapy, a number of investigations were carried out. These were co-culture of astrocytes with OPCs where the cells were separated by a porous membrane (transwell co-culture), detection of gap junction proteins and dye transfer between cells when astrocytes and OPCs were co-cultured, testing the protective ability of astrocyte conditioned media to see if diffusible substances were involved and blocking gap junctions with the gap junction blocker CBX. Dye transfer using the fluorescent dye calcein, has been regularly used to demonstrate GJs (Chen and Lee 2010). The calcein AM dye is non polar and readily diffuses through the cell membrane. Once inside a cell, esterases remove the AM (acetyl methyl) group from the molecule which makes if fluorescent and ionically charged. This later effect makes the molecule charged and unable to diffuse across the cell membrane effectively trapping the dye within the cell. Thus, any spread in fluorescence is restricted to lateral transmission through GJs (Figures 3.14 and 3.15). A limitation of this technique is that if labelled cells are phagocytosed, the dye could pass to adjacent cells and give the impression of dye transfer. This is unlikely to happen in the present experiments due to the short time of exposure.

It is known that **endothelial cells** play a crucial role in supporting OPC proliferation and are involved in regulating the development and survival of OLG by the secretion of endothelial-derived growth factors such as brain-derived growth factor (BDNF) and FGF-2 (Miyamoto *et al.* 2014). Therefore, as part of the

present *in vitro* study endothelial cells were tested to determine their effect on OPCs after exposure to chemotherapy. The results showed that co-culturing endothelial cells with OPCs during exposure to chemotherapy protected the OPCs. These findings are relevant to *in vivo* studies which have found that some OPCs are located close to cerebral microvascular endothelial cells in white matter tracts of CNS. This association may enable communication between OPCs and endothelial cells (oligo-vascular niche) in both normal and pathological conditions. Communication which may be via secreted soluble factors which are involved in the remodelling and repair of oligodendrocyte processes (Pham *et al.* 2012).

Due to the possible role of **microglia** in the response of OPCs to chemotherapy, direct cell-cell contact co-cultures of these two cell types were developed. The action of microglia in the central nervous system is complex, they can stimulate myelin repair, remove toxic proteins and prevent neurodegeneration but also produce chronic inflammatory conditions which lead to a loss of cells and functions (Gao and Hong (2008). A concentration of 0.5μM 5-FU was used in these experiments due to the sensitivity of the BV-2 microglial cell line to chemotherapy. When co-cultures of OPCs (MO3.13 cells) and microglia were treated with 5-FU, significantly fewer MO3.13 cells survived as compared to the number surviving in the absence of microglia. The 5-FU concentration of 0.5μM did not affected cell growth when OPCs were treated in single culture. These results are consistent with other studies that have found that microglia activated with different immune system stimulants, are toxic to both OPCs and mature oligodendrocytes in co-culture (Zajicek *et al.* 1992, McLaurin *et al.* 1995, Lehnardt *et al.* 2002, Li *et al.* 2005). It is likely that 5-FU treatment of microglia leads to their activation to the pro-inflammatory M1 state in which they produce cytotoxic substances like NO, TNFα and IL-1β (Block *et al.* 2007, Tang and Le 2016) which can lead to excessive and uncontrolled inflammation which damages neighbouring cells and healthy tissue. Work in our lab has shown that treatment of rats with 5-FU leads to the presence of activated microglia in the hippocampus (Lasio 2017).

#### 3.5.3.2 Indirect co-culture interactions between OPCs and other cells

Indirect contact cultures, using the transwell system, were used to explore the interactions between two different cell population that share the same medium but are not in physical contact. This model allows molecular signals secreted from different cells to pass into the OPCs culture medium. In this approach, the target cells (MO3.13) were grown in the well of the plate, and another cell type in the inserts with a porous membrane (0.4μm) between them.

The results obtained from the analysis of **MO3.13 co-cultured with C6** in transwell showed no protection from chemotherapy treatment. This strongly indicates that direct cell to cell contact between the OPCs and the astrocytes is required to obtain protection from 5-FU. The present findings are consistent with other research which has found that oligodendrocyte death in vitro is reduced in the presence of astrocytes (Orthmann-Murphy *et al.* 2008, Arai and Lo 2010). Protection was not obtained when OPCs were treated with astrocyte conditioned medium indicating that diffusible trophic factors are unlikely to be the cause of astrocyte protection of OPCs from 5-FU treatment. This result is similar to Corley *et al.* (2001) who found that protection of oligodendrocyte death by astrocytes was not reproduced by its conditioned medium and was not accounted for by several neurotrophic factors, but did occur when direct contact with the oligodendrocyte membrane was allowed. Similarly in another study conducted by Li and his collaborators, found that microglia, activated in response to LPS, did not cause toxicity to developing oligodendrocytes when the oligodendrocytes were in contact with astrocytes (Li *et al.* 2008). In general, therefore, it seems that direct cell-to-cell contact between MO3.13 and C6 is required for the OPCs to be protected from chemotherapy. This led to an investigation of the presence and function of GJs between astrocytes and OPCs.

Similar results were found when **MO3.13 cells were cultured on transwell with endothelial cells**. 48 hours incubation with 10μM of 5-FU significantly reduced MO3.13 viability despite the presence of the endothelial cells on the opposite surface of the porous membrane. However the decrease in MO3.13 cell numbers was less than that found when these cells were cultured on their own and treated with 5-FU. This result indicates that, in addition to direct cell to cell contact, non-contact co-culture between endothelial cells and OPCs can give some protection to the OPCs against the adverse effects of 5-FU when they were sharing the same media. This result may be explained by the secretion of endothelial-derived growth factors such as BDNF and FGF-2 which are involved in the regulation of oligodendrocyte development and survival and promote OPCs proliferation and migration (D'Souza *et al.* 1996, Han *et al.* 2000, Clemente *et al.* 2011, Miyamoto *et al.* 2014). Other studies have shown that the growth factor IGF-1, secreted from endothelial cells, protects cultured OPCs and oligodendrocytes from apoptosis induced by TNF-α, glutamate or lack of growth factors (Ness and Wood 2002, Cui *et al.* 2005). Moreover, Hayakawa *et al.* (2012) found that vascular endothelial growth factor (VEGF-A) secreted by cerebral endothelial cells, can support oligodendrocyte lineage cells and may participate in endothelium-OPC cell-cell signaling which increases their proliferation and also

facilitates OPC migration. In conclusion endothelial cells secrete a number of growth factors which have been shown to promote OPC survival and growth. In this way endothelial cells are able to give some protection to OPCs after treatment with 5-FU, however better protection is provided if the two cell types are in contact. This may enable short distance diffusion of trophic substances or may indicate cell contact provides additional mechanisms to protect OPCs.

When **BV-2 microglial cells were cultured with MO3.13** in transwells with 0.4μm pore inserts and treated with 0.5μM 5-FU, this induced MO3.13 death although not to the same extent as when microglial cells were in direct contact with the MO3.13 cells. From this result, it can be concluded that 5-FU treatment could be activating microglia which are then secreting soluble factors that are transmitted via the culture media and negatively affect OPC growth. However, the exact mechanism by which chemotherapy induces reactive glial cells is not entirely understood. Some authors have found that chronic activation of microglia in different ways including chemotherapy leads to the release of inflammatory mediators including TNF-α, IL1-b and induction of COX-2 expression which in turn produce neuronal and glial cell damage (Woodcock and Morganti-Kossmann 2013, Briones and Woods 2014). This also accords with other *in vivo* studies, which have shown that some anticancer drugs including 5-FU, can cross blood brain barrier of the CNS and initiate microglial activation leading to damage of oligodendrocyte and neuronal cells (Ahles and Saykin 2007, Han *et al.* 2008). It is possible that activated microglia produce large amounts of soluble cytotoxic factors such as superoxide, NO and TNFα that induces apoptosis in oligodendrocytes. Additionally, activated microglia stimulate CD137L signaling (a member of TNF receptor family) which may be a mediator of neuroinflammatory and neurodegenerative disease that in turn kill oligodendrocytes (Block *et al.* 2007, Yeo *et al.* 2012).

#### 3.5.3.3 Effect of conditioned media from different cells on OPCs treated with 5-FU

Conditioned medium (CM) transfer experiments were carried out to test for protective soluble factors from other cells on OPCs survival after treatment with chemotherapy.

**CM from the C6 astrocyte** cell line, showed no protective effect on MO3.13 viability after 48 hours incubation with 5-FU compared with control cells not treated with CM. This result differs from the observations of Gard *et al.* (1995) and Barres *et al.* (1993) which showed that astrocyte CM can promote OPCs survival through the action of PDGF, NT-3, IL-4, CNTF, leukemia inhibitory factor

and insulin-like growth factors. This difference could be due to different activation of the astrocytes or the activator substances and concentrations used. The results in this chapter are broadly consistent with earlier results by Pang *et al.* (2000) which found that CM from astrocytes, activated with LPS, had no direct effect on the survival and differentiation of OPCs. In addition, it was found that the viability of NE-4C neural progenitor cells was not enhanced by the presence of astroglial CM which did not induce neuronal differentiation of these cells (Kornyei *et al.* 2005). Finally, the involvement of astrocytes in the immune response to chemotherapy treatment, as well as their contribution in supporting progenitor cells during CNS regeneration are areas that could be further investigated.

**Endothelial-CM** (E-CM) stimulated the differentiation of neural stem cells into OPCs, increased their proliferation and also facilitated OPC migration via vascular endothelial growth factor (VEGF-A) (Plane *et al.* 2010, Hayakawa *et al.* 2012). The results in this chapter showed a significant increase in MO3.13 cell viability when treated with 5-FU incubated with E-CM compared to the group treated with 5-FU alone. However, this increase was less than the viability of the control group (Figure 3.18). These differences can be explained in part, by presence of trophic factors in E-CM produced by endothelial cells and released into the cell culture media during the 24 hour incubation on these cells before collecting and adding it to OPC cultures treated with 5-FU. Endothelial-derived growth factors such as brain-derived growth factor (BDNF) and FGF-2 promote OPCs proliferation and are involved in the regulation of OLG development and survival (Miyamoto *et al.* 2014). Moreover, Plane *et al.* (2010) and Hayakawa *et al.* (2012) found that E-CM stimulated the differentiation of neural stem cells into OPCs and increased their proliferation and facilitated OPC migration via VEGF-A. The present findings are consistent with those of Ness and Wood (2002) and Cui *et al.* (2005) who found that the growth factor IGF-1 can protect cultured OPCs and oligodendrocyte cells from apoptosis induced by TNF-α, glutamate, and growth factor deficiency. Hence, it can be hypothesized that the decrease in OPC viability was partially prevented by E-CM that could contain some soluble factor(s) which attenuate the adverse effect of chemotherapy and subsequently enhance cells survival and proliferation. Ongoing work should determine the specific type of soluble factors in E-CM which are responsible for protection.

After looking at the effects of 5-FU and the protective effects of C6-CM and E-CM on the MO3.13, two types of **microglial conditioned medium** (M-CM) (untreated and pre-treated CMs) were added to MO3.13 cells at a 1:1 ratio with fresh medium and cell viability was assessed. The most interesting finding was

that untreated M-CM enhanced OPCs proliferation when compared to control cultures grown in fresh media. This indicates that soluble factors which promote OPC growth are secreted from untreated BV-2 cells (Figure 3.18). The present findings are consistent with other research which has found that CM from non-activated microglia (M-CM) promotes OPC proliferation and enhances mature oligodendrocyte survival (Pang *et al.* 2000, Nicholas *et al.* 2001). It seems that non-activated M-CM inhibits the apoptosis of OPCs and stimulates their maturation to galactocerebroside (GalC) oligodendrocytes leading to an increase in the number of surviving GalC (+) oligodendrocytes in vitro at 48 hours (Nicholas *et al.* 2001).

M-CM, which came from microglia treated with 0.5uM 5-FU for 48 hours produced a significant reduction in OPC proliferation compared to control fresh media after incubation for 48 hours. Treating B-V2 cells with chemotherapy activates these cells to secrete soluble factors involved in the inflammatory response which negatively affected the viability of OPCs. These results indicate that the cytotoxic effect of 5-FU on OPCs is at least in part mediated by other glial cells such as microglia. It is still however unclear how chemotherapy activates microglia to produce mediators such as TNF-α, IL1-b or COX-2 that may lead to neuronal and glial cell damage (Woodcock and Morganti-Kossmann 2013, Briones and Woods 2014). These findings support results showing that conditioned media from microglia activated by treatment with LPS kills OPCs (Pang *et al.* 2000). It has also been suggested that activated microglia are responsible for the decreased mitochondrial activity observed in glial cells (Pasquini *et al.* 2007) and can induce mitochondrial membrane depolarization and cell death in oligodendrocytes (Domercq *et al.* 2007). These effects could be brought about by the release of TNF-α from activated microglia which binds to the TNF-R1 receptor expressed in developing and mature oligodendrocytes. Activation of this receptor can induce apoptosis via the MAP kinase signalling pathway (Pang *et al.* 2007, Deng *et al.* 2008). The results in this section are similar to *in vivo* results which have found that some chemotherapy agents can penetrate the CNS and cause microglial activation which subsequently leads to oligodendrocyte and neuronal damage (Ahles and Saykin 2007, Han *et al.* 2008). A summary of main results of co-cultured OPCs with supporting cells highlighted in the **table 3.2**.

***Table 3:2*** *Summary of results from an in vitro experiments represent the effect of 5-FU on OPCs cell viability and proliferation when co-cultured with supporting cells or their conditioned media.*

| Technique (Experiment) | Effect on OPCs |
|---|---|
| Direct OPCs/ Astrocyte co-culture | protection |
| Direct OPCs/ Endothelial co-culture | protection |
| Direct OPCs/ Microglial co-culture | Affected negatively |
| Indirect OPCs/ Astrocyte co-culture | Not affected |
| Indirect OPCs/ Endothelial co-culture | Slightly protection |
| Indirect OPCs/ Microglial co-culture | Affected negatively |
| Astrocyte conditioned medium | Not affected |
| Endothelial conditioned medium | Slightly protection |
| Pre-treated microglial conditioned medium | Affected negatively |
| untreated microglial conditioned medium | Slightly protection |

### 3.5.4 5-FU induce TNF-α in co-cultures containing microglia

In the present study, one of the main hypothesis was that 5-FU can activate microglia to produce cytotoxic factors including TNF-α. The importance of microglial cells in the immune response of brain and in initiating neuroinflammatory processes in response to various insults including chemotherapy is well established (Zheng *et al.* 2011, Christie *et al.* 2012). Persistent neuroinflammation results in chronically activated microglia which release inflammatory mediators such as TNF-α and IL1-b that may lead to neuronal and glial cell damage (Meyers 2008, Woodcock and Morganti-Kossmann 2013, Briones and Woods 2014). The study in this project set out to assess the role of chemotherapy in inducing TNF-α expression by microglia. It was followed by testing whether FLX or INDO could protect OPCs from the adverse effects of 5-FU in the co-cultured system. To investigate this, TNF-α released by BV-2 was measured using a high-sensitivity ELISA. The results clearly showed that 5-FU induced the production of TNF-α in single and mixed cultures. It is interesting to note that TNF-α release was attenuated when FLX or INDO was added to 5-FU treated BV-2 cells grown alone or co-cultured with OPCs. The results show that 0.5μM 5-FU stimulated microglial cells to release TNF-α which in turn affected the viability of OPCs. However the concentration used in this experiment is less than the dose of 5-FU in clinical usage (Yoshida *et al.* 1990, Findlay *et al.* 1996). The

present findings are consistent with other research which has found that TNF-α signalling in reactive microglia induces the selective killing of developing oligodendrocytes in an environment containing all major glial cell types (Zajicek *et al.* 1992, Zhang *et al.* 2003, Pang *et al.* 2007, Liu *et al.* 2011, Yeo *et al.* 2012). Moreover, disrupting the genes encoding TNF- α or its receptors TNFR1/2 prevents the toxic effect of LPS (Li *et al.* 2008). Zhang *et al.* (2003) found that 5-FU treatment prompted apoptosis by inducing the specific genes in the TNF pathway including TNF receptor 2 (TNFR2) as a response to the expression of the p53 gene.

FLX can significantly reduce inflammation and down-regulate M1 activation of microglia and up-regulate their M2 state (Su *et al.* 2015). The findings of the current study are consistent with those of Roumestan *et al.* (2007) who found that FLX, dose-dependently suppressed the number of inflammation-related cells and TNF-α release from monocytes in some clinical diseases such as septic shock and allergic asthma. In recent years it has been shown that FLX inhibits apoptosis by downregulating the p38-p53 pathways and upregulating the expression of the anti-apoptotic protein Bcl-2 (Shan *et al.* 2016). In addition, Obuchowicz *et al.* (2014) found that the anti-inflammatory drug Imipramine and FLX decreased the concentration of TNF-α and IL-1β in culture media of activated microglia. Generally, this study produced results which corroborate the findings of previous works in this field which have documented the anti-inflammatory effect of FLX (Liu *et al.* 2011, Song *et al.* 2012, Tynan *et al.* 2012, Zhang *et al.* 2012, Caiaffo *et al.* 2016).

Consistent with previous studies, it has been shown that INDO treatment reduces the levels of pro inflammatory cytokines secreted by activated microglia which create a detrimental inflammatory environment (Ji *et al.* 2013). It seems possible that chemotherapy induced inflammation may impair gliogenesis and its suppression by anti-inflammatory drugs may restore OPC viability. This is in agreement with Hoehn *et al.* (2005) who showed that INDO modulates inflammation and increases the survival of the progenitor population and neural stem cells. They showed that INDO promotes the differentiation of neural stem cells into OPCs (as evidenced by NG2 expression) and neurones (as shown by DCX expression). INDO also reduced the number of activated ED-1+ inflammatory cells and consequently blocking the activation enzymes (Hoehn *et al.* 2005). Bernardi *et al.* (2010) found that INDO treatment markedly inhibited the level of IL-1B and TNF-α levels after 48 hours in organotypic hippocampal cultures exposed to oxygen-glucose deprivation (OGD). Additionally, it is encouraging to compare my results with results by Ho *et al.* (2015) who found that INDO normalised the level

of plasma TNF-α after low-grade inflammation in diabetic mice, however the levels of IL-1, IL-6, IL-10 and IFN-gamma were not affected by INDO treatment. It is interesting to note that anti-inflammatory drugs by reducing microglia activation act to control the behavior of progenitors in the stem cell niche (Liu *et al.* 2007). Therefore, these treatments do not directly increase proliferation or differentiation of progenitors, but can prevent the adverse effects of cytokines and interleukins to maintain an optimum environment for progenitors in adult hippocampus (Nguyen *et al.* 2002). These findings support the premise that 5-FU stimulates microglial cells to release TNF-α which in turn reduces the viability and proliferation of OPCs. Additionally, microglial activation can be reduced by co-treatment with FLX or INDO.

## 3.6 Conclusions

*In vitro* studies using the MO3.13 cell line are a useful model to investigate the impact of 5-FU on OPC cell proliferation within WMTs. This study set out to determine the adverse effects of 5-FU on OPCs survival, and whether these cells could be protected by co-culture with other cell types or treatment with FLX or INDO. This study has shown that astrocytes and endothelial cells can provide different levels of protection for OPCs to 5-FU treatment which may be mediated by diffusible trophic factors in the case of endothelial cells but is enhanced by cell to cell contact for both astrocytes and endothelial cells. Treatment with FLX or INDO was unsuccessful in protecting OPCs, probably because they do not act directly on OPCs but may reduce microglial activation. Moreover, chemotherapy was shown to activate microglia and increase TNF-α production which in turn inhibited OPCs survival. TNF-α was directly toxic to OPCs or to microglia itself. While the presence of FLX or INDO inhibited microglial activity with 5-FU treatment, and had diverse effects on OPCs or microglial viability. These results further suggest that the gap junction blocker (CBX) could increase the 5-FU–induced OPCs death in vitro. These findings are important in understanding WMTs damage after chemotherapy. Future experimental studies are needed to explore the detailed mechanisms of antidepressant and anti-inflammatory action in promoting cell survival and function and eventually create new therapeutic options to prevent WMTs damage. The following chapter examines the structural changes to white matter tracts after 5-FU treatment using electron microscopy and the possible functional changes using electrophysiology.

# **CHAPTER FOUR:** THE ROLE OF FLUOXETINE AND INDOMETHACIN IN PROTECTING WHITE MATTER TRACTS AGAINST THE ADVERSE EFFECTS OF CHRONIC TREATMENT WITH 5-FLUOROURACIL

## 4.1 Introduction

White matter tracts of the CNS are mainly made up of myelinated axons and associated glial cells. The integrity of these structures is essential for learning and other brain functions as they act as relays and coordinate communication between different brain regions (Fields 2008). Structural changes in the brain including the disruption or damage to white matter tracts is likely to be associated with cognitive impairments (Ahles and Saykin 2007). The rational basis for such study is the fact that the optic nerve constitutes from the nervous tissue that is connected to the brain, and basically the visual system is part of the CNS. Therefore, it is reasonable to ask if adjuvant chemotherapeutic drugs could produce some of the deleterious effects in visual-spatial function or visual memory. Interestingly, by using different measurements to detect memory deficits, it was found that the visual memory was significantly impaired after chemotherapy more than the verbal memory (Raffa and Tallarida 2010). Chemotherapy has been suggested to cause visual deficits in patients which may contribute to their overall cognitive decline (Raffa and Tallarida 2010). Clinical and animal studies have found persistent alterations in the conduction properties of white matter tracts after chemotherapy indicative of white matter damage (Stemmer *et al.* 1994, Han *et al.* 2008, de Ruiter *et al.* 2012, Briones and Woods 2014, Koppelmans *et al.* 2014). The injury to the parenchyma occurs due to damage of myelin sheaths around axons which will reduce axonal conduction and alters the WM integrity within the brain (Beaulieu 2002, Dietrich *et al.* 2006, de Ruiter *et al.* 2012). The conduction velocity of the action potential, along myelinated axons, is proportional to the axonal thickness and the thickness of myelin around them (Gutierrez *et al.* 1995). As a consequence any demyelination produced by chemotherapy will reduce network speed and cause cognitive impairments (Deprez *et al.* 2011). There is a constant turnover of oligodendrocytes which are required for myelination and loss of these cells or their precursors (OPCs) is likely to cause demyelination in white matter tracts (Wigmore 2013). Keime-Guibert *et al.* (1998) found that short term systemic chemotherapy led to delayed CNS damage 'such as encephalopathy' that presented from four months to many years after treatment completion, however the pathogenesis of early delayed complications is still unknown. These delayed complication have been reported in three main types of disorder which included cognitive dysfunction, mineralizing microangiopathy and necrotizing leukoencephalopathy or dementia.

To directly cause damage to WMTS and cognitive changes, chemotherapy must cross the blood-brain barrier (BBB). In case of 5-FU cross the BBB occurs by simple diffusion which leads to increase the concentration of 5-FU in CSF for about 11% after few hours of injection and cause CNS direct neurotoxicity (Kerr *et al.* 1984). Treatment with a number of chemotherapy drugs such as cipsplatin, carmustine and 5-FU leads to an increase in BBB permeability which could elevate CNS toxicity, especially in the case of multidrug treatments. This process could explain the toxic effects of low concentrations of chemotherapeutic agents when drug combinations have been used (Soussain *et al.* 2009). Many clinical studies have identified a decline in mental status after chemotherapy which correlates with changes in WM integrity detectable by MRI (de Ruiter *et al.* 2012, Koppelmans *et al.* 2012). In many cases there is a post treatment delay in manifesting the damaging effects indicating that these drugs can produce long-term as well as acute effects (Abraham *et al.* 2008). A clinical study by de Ruiter *et al.* (2012) investigated the late effects of high dose adjuvant chemotherapy on WM tracts using Diffusion Tensor Imaging (DTI) and proton MR Spectroscopy (1H-MRS), which showed a reduction in cortical WM volume thought to be due to axonal degeneration and demyelination.

Further investigations using animal models, has shown that systemic 5-FU treatment causes demyelination of WM tracts (Han *et al.* 2008, de Ruiter *et al.* 2012). This destruction of myelin sheaths includes both partial and complete loss of myelin and an increase in the intra-laminar splitting of myelin sheaths (Akiba *et al.* 1996). These effects are associated with a reduction in the number of mature oligodendrocytes and their progenitors after exposure to the chemotherapy (Dietrich *et al.* 2006). 5-FU treatment has been shown to cause optic nerve, corpus callosum (CC) and spinal cord demyelination which was associated with oligodendrocyte apoptosis (Fancy *et al.* 2004, Soussain *et al.* 2009, Weng *et al.* 2014). Additionally, 5-FU after 1, 7, 14 or 56 days of treatment has been shown to exhibit a progressive decremental change in auditory brainstem response as indicated by increases in inter-peak latency values (Han *et al.* 2008). Functional analysis of these changes demonstrated that particular cancer chemotherapeutic agents may alter sensory processing and could potentially impact cognitive functioning. These findings indicated that chemotherapy produces myelin damage or loss of myelin which decreases action potential conduction in the auditory system (Han *et al.* 2008).

It is likely that 5-FU decreases the oligodendrocyte-specific transcription factor TCF7L2. This leads to disruption of the TCF7L2/HDAC1/ HDAC2 complex, which

acts on myelin-promoting factors, which plays a crucial role in the myelination of CNS (Weng *et al.* 2014). In another in vivo study, it was found that OPCs transplanted into rat spinal cord suffering from myelin destruction promoted survival and myelination. This process was facilitated when the microglia were not activated and suppressed by inhibiting microglial p38 mitogen-activated protein kinase (MAPK) phosphorylation with an antibiotic (Zhang *et al.* 2003).

A study by Briones and Woods (2014) showed that neuroinflammation may play a role in causing demyelination after chemotherapy which in turn may lead to cognitive deficit. They showed that 4 i/p injections of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) produced cognitive impairment and damage to the brain white matter microstructure in rats. This was associated with a decrease in the level of the anti-inflammatory cytokine IL-10, and an increase in the levels of the inflammatory mediators COX-2, TNF-α and IL-1ß and a decrease in myelin basic protein (Briones and Woods 2014). Treatment with the NSAID (NS-398) reduced expression of inflammatory markers and prevented demyelination. The demyelination found by Briones et al could be due to an inhibition of OPC proliferation which is required to maintain myelin integrity. This in turn would impact on the speed of information processing and have a cognitive effect. Inflammation within the brain has been implicated in a number of neurological conditions and INDO has been used to reduce inflammation in Alzheimer's disease and after stroke. However, it is unclear what the effect of INDO is on the turnover of cells in white matter tracts. It has been suggested that treatment with NSAIDs can decrease the risk of Alzheimer's disease and have a protective role by inhibiting the activity of cyclooxygenases whose levels in the brain increase with advancing age. INDO effectively reduces the level of inflammation after adult cerebral injury by reducing COX-1 and COX-2 expression within the brain (Netland *et al.* 1998, Monje *et al.* 2003).

Recently, Yune *et al.* (2014) demonstrated that FLX (10mg/kg, i/p) prevented oligodendrocyte cell death and demyelination after spinal cord injury and inhibited microglial activation. In contrast an *in vitro* study on cells from rat postnatal cerebellum found that FLX increased neuronal progenitor proliferation but did not affect glial or oligodendrocyte numbers. In another study, it was found that long-term treatment with FLX significantly upregulated genes involved in myelination in both adult and neonatal rat brains. It has been shown that chronic FLX exposure increased ciliary neurotrophic factor (Cntf) and Transferrin (Tf) expression in the hippocampus. These factors are involved in myelination and could thus contribute in the treatment of the symptoms of psychiatric disorders, like anxiety (Kroeze *et*

*al.* 2016). Furthermore, Fan et al (2012) showed that 12-weeks of SSRI treatment reduced abnormalities in white matter microstructure, particularly in terms of myelin integrity in several regions including the left striatum, right frontal lobe and midbrain (Fan *et al.* 2012). So far, however, there has been little exploration of the potential pharmacological roles of antidepressants or anti-inflammatory on myelination and whether they can protect white matter tracts from the effects of chemotherapy.

## 4.2 Aims and objectives:

In this project it was hypothesized that chemotherapy would reduce the proliferation and number of oligodendrocyte progenitors and the decrease in the number of these cells would lead to demyelination.

To test this hypothesis the effects of multiple injections of 5-FU on the structural integrity of WMTs was investigated. Secondly, this study aimed to test the effectiveness of FLX and INDO in combination, at preventing chemotherapy-induced changes in the myelin integrity of ON and CC.

The principal objectives of the present studies were as follows:

1. To determine the myelin integrity and its effectiveness in optic nerve by electrophysiologicaly measuring the compound action potential (CAP) after chemotherapy treatment.
2. To investigate the effects of the FLX and INDO treatments in myelin sheaths and to determine whether these treatments affect the conduction velocity of electrical signals in CAPs recordings.
3. To investigate potential changes in the ultra-structure of optic nerve and corpus callosum myelin using transmission electron micrograph (TEM).
4. To quantify the number of myelinated axons, and to calculate the G-ratio of axon and myelin thickness.

## 4.3 Materials and Methods:

This chapter explains the general methods used for electrophysiology and electron microscopy. Further details of the specific methods used are provided in each methods section.

### 4.3.1 Animals and treatments

All animals were male Hooded Lister rats, 200-250g and were obtained from Charles River, UK and housed in the BSU, University of Nottingham. All procedures were conducted in accordance with Home Office guidelines, licencing and local ethical approval as detailed in the UK Animals (Scientific Procedures) Act 1986 and with approval of University of Nottingham (UoN) ethical review committee and were carried out under authority of Home Office, Project Licence (PPL 40/3194) and Personal Licences (PIL 70/26438 and 70/22103). Rats were housed as described in chapter two (section 2.3.1) and allowed to habituate for 1 week prior to any drug administration.

Rats were randomly allocated to control saline injection (n=4), FLX treated (n=4), indomethacin (INDO) treated (n=4), 5-FU treated (n=4), 5-FU+ FLX treated (n=4), and 5-FU+INDO treated (n=4) groups. Groups of 5-FU, 5-FU+FLX and 5-FU+ INDO received a total of 6 i.p. injections of 5-FU 35mg/kg/day each injection given 3 days apart. Non drug treated groups received an identical sequence of saline (0.9%) injections. Animals receiving FLX or INDO were given the drugs in their drinking water at a dose of 10 and 1mg/kg/day respectively throughout the period of the experiment for a total 43 days. FLX and INDO dosages were calculated from the amount of daily water intake. Water intake changes during FLX and INDO treatments, but dosages were adjusted every day depending on water consumption and animal weights. All animals were killed one week after the last 5-FU or saline injection.

### 4.3.2 Electrophysiology technique

#### 4.3.2.1 Tissue preparation

Rats were killed by stunning followed by cervical dislocation and decapitation. The distal ends of optic nerves (ONs) were cut behind the orbit after exposure of the eye ball. The skull was then opened and the brain reflected to expose the optic chiasm and the ONs which were cut with fine scissors and gently removed. Each nerve was approximately 6-8 millimetre (mm) in length. 1.5-2 mm of the ON immediately proximal to the eye was excised for processing for electron microscopy. The remaining parts of the nerves were then immediately put into bicarbonate buffered artificial cerebral spinal fluid (ACSF) containing 126mM NaCl,

3mM KCl, 1.25mM $NaH_2PO_4$, 2mM $MgSO_4$, 26mM $NaHCO_3$, 2mM $CaCl_2$ and 10mM glucose with an osmolality of 310 mOsm. Tissue was maintained at a pH of 7.45 and bubbled with 95% $O_2$/5% $CO_2$) to provide the ONs with sufficient energy substrates for normal function (Allen *et al.* 2006).

#### 4.3.2.2 Recording of Compound Action Potentials (CAPs)

ONs were immediately placed in a laminar flow interface perfusion chamber (Harvard Apparatus) (Medical Systems Corporation, Greenvale, New York) supplied with a constant flow (2 ml/min) of ACSF maintained at 37°C to prevent desiccation as well as to maintain surface tension. ONs were allowed to equilibrate in the ACSF for 15 minutes before recording the compound action potential (CAP). Stimulating and recording suction electrodes were used to record the stimulus-evoked CAP in a manner similar to that described by Stys *et al.* (1991). The recording electrodes were back-filled with the appropriate ACSF. CAPs were recorded from ONs by firstly drawing both ends of the ON into the two suction electrodes, by applying slight negative pressure using syringes attached to the other end of each glass capillary. The rostral end of the RON was attached to the stimulating electrode, and the caudal end was attached to the recording electrode (Figure 4.1) such that all recording were orthodromic.

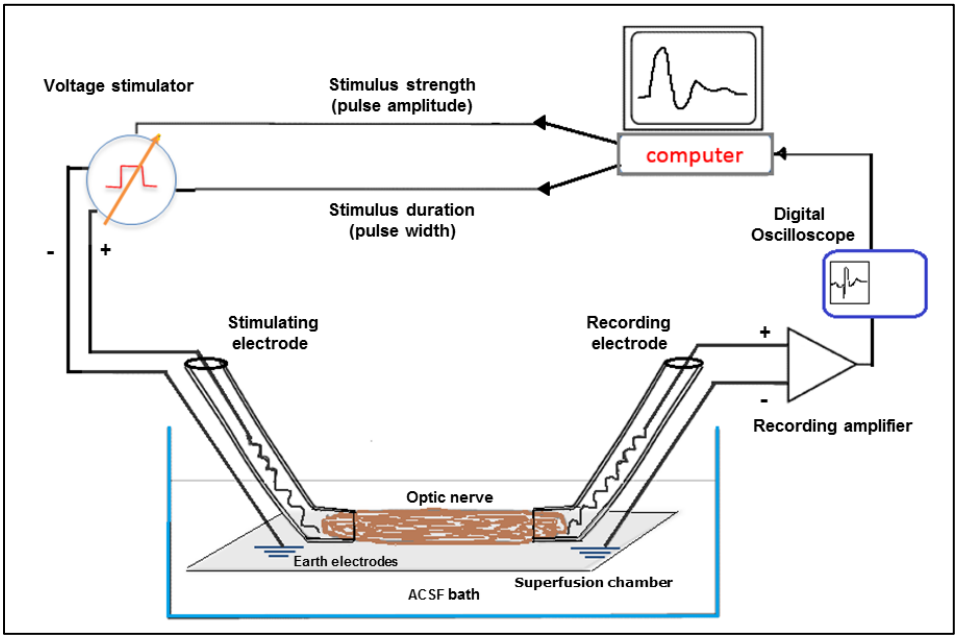



***Figure 4.1*** *The electrophysiology experimental setup for optic nerve. The compound action potentials were measured using suction electrodes. ONs were drawn into recording and stimulating electrodes. The diagram modified from Stys et al. (1991).*

To record the ON CAP, the nerve was stimulated using a constant voltage pulse of 30 μs duration delivered by a Grass S88 double pulse stimulator (WPI, Stevenage, UK), and the strength adjusted to evoke the maximum CAP possible and then increased another 25%. During an experiment, the supramaximal CAP was elicited every 10 seconds. The recording electrode was attached to an Axoprobe 1A multipurpose micro-electrode differential amplifier. This subtracted the extracellular electrode reading represented by bath potential from the internal electrode reading represented by nerve potential, in order to remove stimulus artefacts. It also amplified the CAP by x10. The signal was then further amplified by a Stanford Research Systems SR560 pre-amplifier by x100 and filtered at 20 kHz. The signal was then fed into an A/D interface and acquired at 10 kHz. (Digidata 1322A with Clampex 9.2, Molecular Devices, Wokingham, UK).

#### 4.3.2.3 Reading and Analysing Electrophysiology Data

The CAPs were recorded every ten seconds, as the stimulus was increased from 1 V to 15 V in 1 V steps (Figure 4.2). A CAP represents the summation of activity of each single axon action potential in the fully myelinated tract. Each action potential is comprised of three peaks based on conduction velocity. Differences in conduction velocity are likely to be due to differences in axon thickness. The first CAP peak represents the fastest conducting axons and are considered to be associated with the largest axons that have the lowest threshold for recruitment. The second CAP peaks usually has the largest area under the curve and amplitude and represents axons of mid-sized diameter and velocity, whereas the third peak represented the smallest and slowest conducting axon which have a higher threshold and are recruited latter to form the last peak (Stuart *et al.* 1997, Allen *et al.* 2006).

The area under the CAP is used to quantify the number of active axons recruited for the given peak. CAPs were analysed using Clampfit 10.3.0.2 (Axon Instruments, Molecular Devices, Inc). The traces were averaged and the area determined using the following relationship Allen *et al.* (2006) and Evans *et al.* (2010):

$$CAP = \sum_{i=1}^{n} \frac{A_i}{w_i\sqrt{\pi/2}} e^{-(\frac{1}{2})(\frac{(time-c_i)^2}{w_i^2})}$$

Where the parameters included, A is the area of peak, W is the width of the peak at half the maximal amplitude, and C is a latency to the centre of the peak. The

areas of the all peaks were compared between treatment groups with a decrease in latency and area taken as an indication of myelin damage.

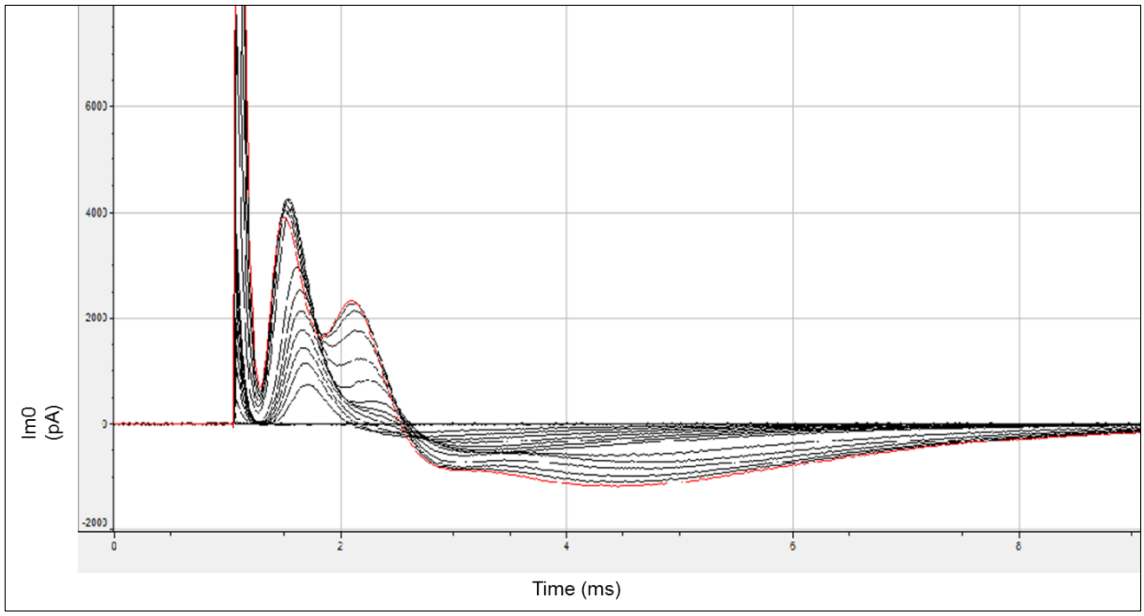



***Figure 4.2*** *Example of CAPs from the ON in a control group recruited in response to electrical stimulation. The nerve responded to stimulation from 1 to 15 V. The CAP area increased with stimulation.*

#### 4.3.2.4 Statistical analysis:

The averaged responses for 10 stimulus repetitions at an inter-stimulus interval of 2 second were used for analysis of CAPs. Excel® 2013 for Windows was used to plot the CAP area against stimulus intensity. All relevant data are represented as mean ± standard deviation (SD). The differences between control and other treatment groups were calculated using one-way ANOVA when comparing area of CAP peaks. $p<0.05$ was considered statistically significant.

### 4.3.3 Electron Microscopy

ON tissue for electron microscopy was collected from the region proximal to the eye and a 2mm thick section containing the anterior third of the CC from the right hemisphere of the brain.

Following primary fixation with 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 (Agar Scientific Ltd, Stansted, Essex, UK), ONs and CC were postfixed in 1% aqueous (w/v) osmium tetroxide for 2 hours with agitation. Tissue samples were

then rinsed with distilled water and dehydrated in ascending alcohols, each for 15 minutes at 50%, 70%, 90% and 100% ethanol (twice).

Samples were passed through two 15 minute changes of 100% propylene oxide (Agar Scientific Ltd, Stansted, Essex, UK) followed by an overnight immersion in 1:1 mixture of propylene oxide and resin with agitation. Resin was prepared by adding 20ml araldite CY212 resin to 25ml Dodecenyl Succinic Anhydride (DDSA) (Agar Scientific Ltd, Stansted, Essex, UK) and 0.8ml DMP30 (tri-dimethylamnomethyl phenol) (Agar Scientific Ltd, Stansted, Essex, UK). The following day, samples were removed and placed in 100% resin. After 4 hours samples were transferred into moulds with full resin and placed in the oven for 24-48 hours at 60°C to polymerise. Resin blocks were trimmed with a sharp blade and roughly cut with a glass knife. Semi-thin sections were cut at 0.5µm thickness using a 4mm Histo diamond knife (DiATOME Ltd, Switzerland) on an ultra-Microtome (LEICA EM VC6, UK) and placed on microscope slides (RA. Lamb, Eastbourne, UK). Ultra-thin sections were cut between 80nm with an ultra-diamond knife (DiATOME Ltd, Switzerland) on an Ultra-Microtome (LEICA EM VC6, UK) and placed on copper grids.

Semi-thin sections were allowed to dry for 3-4 minutes on a hotplate and were stained with 1-2 drops of 1% toluidine blue for 20-60 seconds and rinsed with distilled water. Slides were left to dry then mounted in DPX (dibutyl-phthalate-xylene) (Merck, Leicester, UK) and coverslipped (RA. Lamb, Eastbourne, UK). An outline of ON and CC region was determined using a light microscope as a guide for each sample resin block.

Ultra-thin sections were stained with Reynold's Lead Citrate prepared by dissolving 1.33g lead nitrate and 2.67g sodium citrate in 20ml distilled water and agitating for 30 minutes. 8ml of 0.1M NaOH was added and the volume adjusted to 50ml with distilled water. To stain samples, drops of lead citrate were placed on a square of Para-film in a petri dish. Several sodium hydroxide pellets were added to the petri dish, moistened with distilled water and left for 5 minutes to absorb the $CO_2$ from the dish. Copper grids with samples were inverted on the droplets of lead citrate inside the petri dish for approximately 5 minutes. Grids were quickly rinsed in distilled water and dried by blotting on clean filter paper.

#### 4.3.3.1 Axonal quantification and Myelin thickness morphometric analysis

Electron micrographs were taken from cross sections of the optic nerve, and the CC at different magnifications. For quantification of myelinated and unmyelinated

axons, a minimum of 5 electron micrographs per slide were taken of randomly selected fields. The G-ratio was calculated as axonal diameter/ whole fibre diameter to measure the thickness of myelin sheath (Figure 4.3). All measurements were made on scanned images using Image-J software.

**G-Ratio**, Myelin sheaths in the CNS are made from plasma membrane extensions of oligodendrocytes. The optimum thickness of myelin is inconstant, and varies with the diameter of nerve fibres (Bakiri *et al.* 2011). The optimal theoretical estimation of myelin thickness is calculated depending to the ratio between axon diameter d and fibre diameter D which called (G-ratio). Therefore fibre diameter is the sum of the axon diameter (or calibre) and the thickness of the myelin sheath (Chomiak and Hu 2009, Paus and Toro 2009). So, the higher G-ratio of myelinated axons refers to the lower thickness of the myelin sheath.

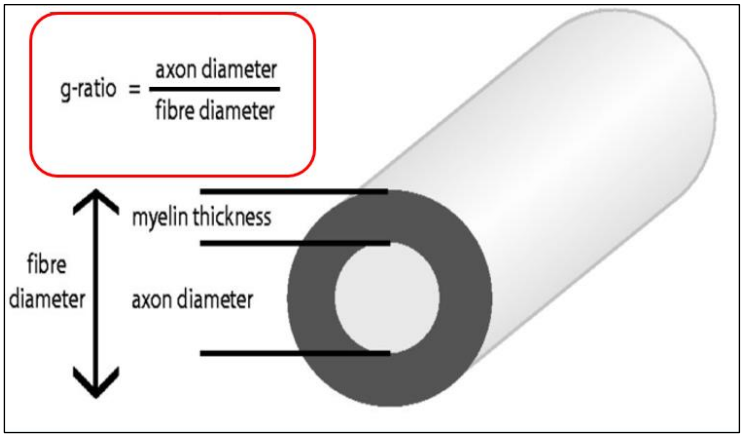



***Figure 4.3*** *Shows the schematic diagram of the optic nerve fibre. The diagram representation of an axon and its myelin sheath representing the G-ratio equation.*

#### 4.3.3.2 Statistical analysis

Statistical analyses were performed and graphs created using GraphPad Prism 7 (GraphPad software, Inc) Software. Two-way ANOVA (non-parametric) was used to analyse data of axonal and myelin thickness counting in regards to the difference between saline control and different treated groups in the ON and CC. All data within this study is expressed as mean values ± standard error mean (Mean ± SEM). The level of significance was set to 5% and is with one (*), if $p \leq 0.05$.

## 4.4 Results

Previous studies have suggested that the chemotherapy regimens common in the treatment of many cancers can produce prolonged demyelination of axons which has an effect on white matter integrity of the CNS. This chapter will quantify the effect of chronic chemotherapy on WM ultrastructure especially the effects on the myelin sheath by using electrophysiology and electron microscopy.

### 4.4.1 Electrophysiology results:

#### 4.4.1.1 Electrophysiological profile of the stimulus-evoked CAP and normalisation of CAP peak areas

The aim of this section was to determine if there is a difference in the action potentials of the optic nerves in different treatment groups. This was done by evaluating the area of CAP in a treatment group compared to the control group. I was also interested in determining whether FLX or INDO treatment affected the area of the CAP or the number of peaks when given alone or when co-administrated with 5-FU.

The majority of CAPs recorded from the ON in different groups comprised of two main peaks. The areas under the CAP were averaged in each nerve and the data was compared for every CAP recorded at all stimulation strengths applied in all groups. Differences were observed in the total peak areas for all recordings of treatment groups (Figure 4.4A). As normal biological variation exists between the treatment groups, data was normalised at 50V by setting the CAP area evoked at this voltage to 1, and comparing all other CAP areas to this value (Figure 4.4B). Generally, the statistical analysis showed that there was no significant difference in the conduction velocity of CAPs between control and other groups ($F_{(5, 25)}$ = 1.206, P=0.3350; two-way ANOVA). Whereas, a Dunnett's post-hoc two-way ANOVA test showed that there was a significant decrease in the total CAP area in the 5-FU group at 9V (P=0.0267), 10V (P=0.00780, 11V (P=0.005), 12 (P=0.0280), 13V (P=0.0487). In addition, the 5-FU+FLX group showed a significantly lower CAP area between 10V and 13V when compared to the control group (P=0.0294, P=0.0038, P=0.0056 and P=0.0051 respectively), while, there was no significant effect at stimulus voltages up to 9V.

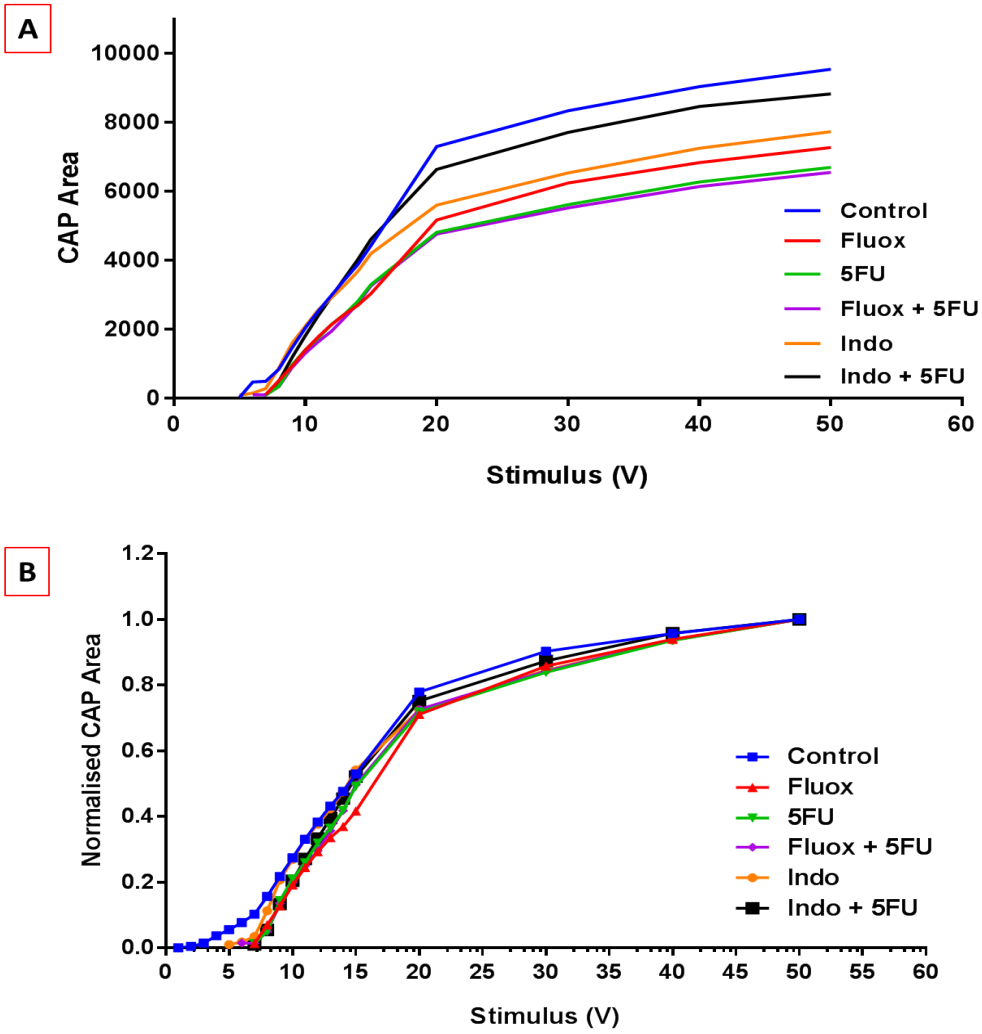



***Figure 4.4*** *The average area under CAP at each voltage recorded in rat ON for all treatment groups. (**A**) Shows the average for the CAP in all six groups with different stimulus points (n= 6-8 in each group). Increasing the stimulus voltage lead to sequentially increase the total CAP area. (**B**) The normalised data area at 50V for each group in different stimulation intensities. Standard error bars were removed due to their extensive overlapping.*

#### 4.4.1.2 Analysis of different CAP peak areas recorded from different treatment groups

The area under the CAP was used to estimate the number of active axons recruited for each peak. The average CAPs in each nerve within an individual group at each of the separate peaks were statistically analysed. For peak one area, the results indicate that there were no significant differences between all treatment groups compared with the control group ($F_{(5, 34)} = 0.6353$, P=0.6742; one-way ANOVA), indicating that all available large axons are recruited at similar voltages within different treatments groups (Figure 4.5A). Furthermore, the area of CAP peak two which followed peak one, maintained the largest area at maximum stimulation

(50V), however, there is no significant difference in the CAP peak two area values between the five treatment groups compared to the control group ($F_{(5, 34)}$ = 0.5021, P=0.7725; one-way ANOVA) (Figure 4.5B). This data suggests that peak two had the largest amplitude and represented the second fastest group of conducting axons. In addition the data indicated that the first and second peaks were unaffected by different drug administrations. Unfortunately, it was not possible to record a third CAP peak in these experiments, hence there is no third peak analysis. It can be concluded that most axons were recruited by the stimulus and that 5-FU or FLX and INDO have not affected the CAP of the rat ON, and being fastest conducting after increasing the stimulation strength.

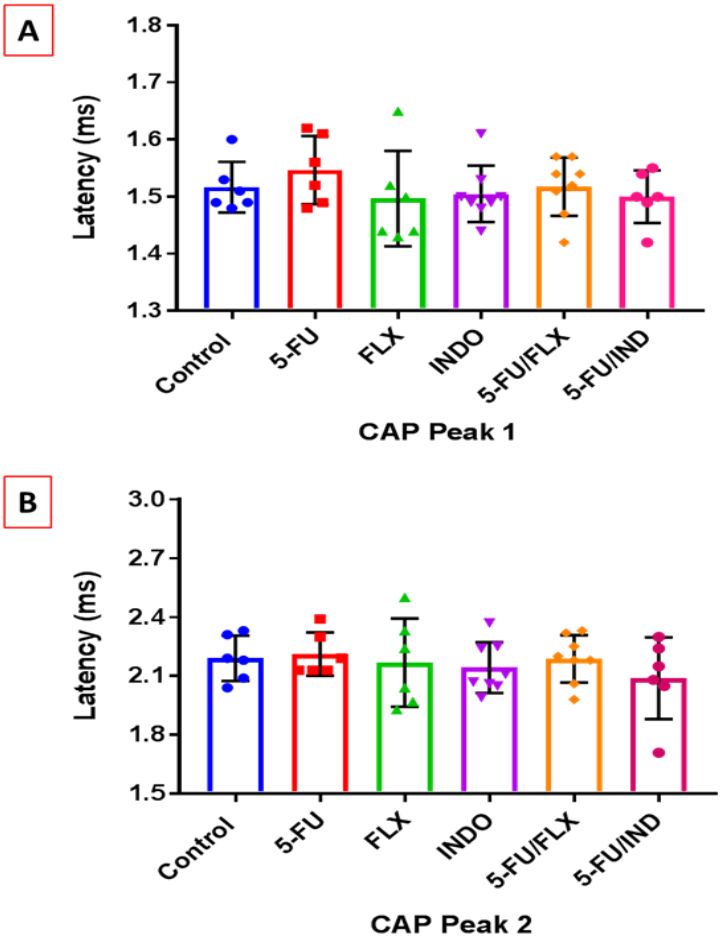



***Figure 4.5*** *The effect of different treatments on the 1st and 2nd CAP peaks at 50 V stimulus voltage. (**A**) Comparison of average CAP in peak one area recorded from the ONs stimulated at 50V for all experiment groups (mean ± SEM, n=6-8 per group). There was no significant difference between the treatments and saline treated groups. (**B**) Comparison of average CAP in peak two area stimulated at 50 V. There is no significant difference comparing to control group (p>0.05, two-way ANOVA).*

### 4.4.2 Transmission Electron Microscopy Results (TEM):

#### 4.4.2.1 The effect of chronic 5-FU treatment, with or without FLX or INDO treatment on the number of myelinated fibres and myelin thickness

To gain an understanding of how chemotherapy treatment affected WM integrity and whether FLX or INDO could protect WM, the average number of myelinated nerve fibres number per unit area and the thickness of myelin and axonal diameters were investigated in the different groups of rats. The results in chapter two (*in vivo*) showed that 5-FU treatment decreased the number of dividing cells and so the number of OPCs available for myelination. In this chapter, we wanted to investigate whether this affected the thickness of the myelin sheath or any pathological changes. Myelinated axons of ON and CC were readily observed in both control and treated groups by electron microscopy.

Quantification of the density of myelinated axons showed that there was no significant difference in the number of myelinated axons ($F_{(2, 18)} = 0.7771$, $P=0.4746$) between treated and the control groups. The results showed an increase in the number of myelinated axons in groups of antidepressant and anti-inflammatory, (62.00±8.370 and 54.75±4.250, respectively) and also in groups of 5-FU co-treated with FLX and INDO (54.00±4.50 and 62.75±7.50 respectively) (Figure 4.6).

Strikingly, assessment of the G-ratio (Figure 4.7) showed that the axons in the 5-FU group had considerably thinner sheaths compared with axons in the non-treated group ($p<0.0001$). Whereas the FLX or INDO treated groups showed no significant difference in myelin thickness compared to the control group ($P>0.9999$ and $P=0.3752$, respectively). Bonferroni post-hoc analysis shows that there was a significant interaction between different treatments in all five treated groups compared to the control group ($F_{(5, 144)} = 30.31$, $P<0.0001$; two-way ANOVA). Co-administration of FLX or INDO with 5-FU treatment did not significantly prevent the decrease in myelin thickness found in the 5-FU only group. The results suggests that 5-FU treatment decreased the ability of the ON to maintain myelin sheath thickness.

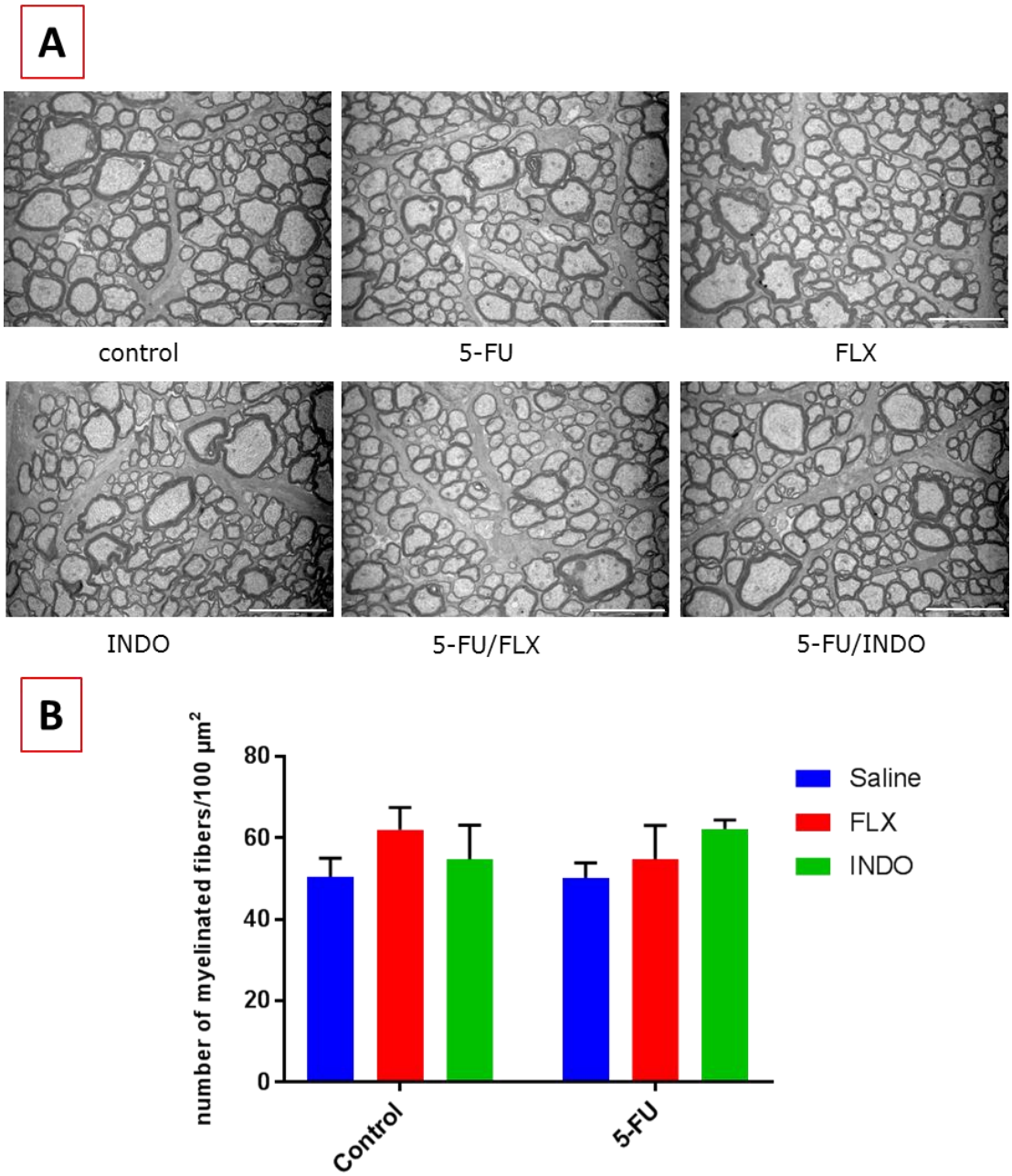



***Figure 4.6*** *The number of myelinated axons in ON after 5-FU, FLX and INDO or a combination of treatment,one week after treatment ends. (**A**) Representative electron micrographs showing cross sections of the ON in different treatment groups (4,200x magnification) Scale bars=5µm. (**B**) Number of myelinated axons per 100 µm² in optic nerve transverse sections from control and treated groups (n=4 each group). The results are expressed as mean ± SEM of at least five measurements in each experimental group. No significant differences were found when comparing the six groups (two-way ANOVA, P>0.05).*

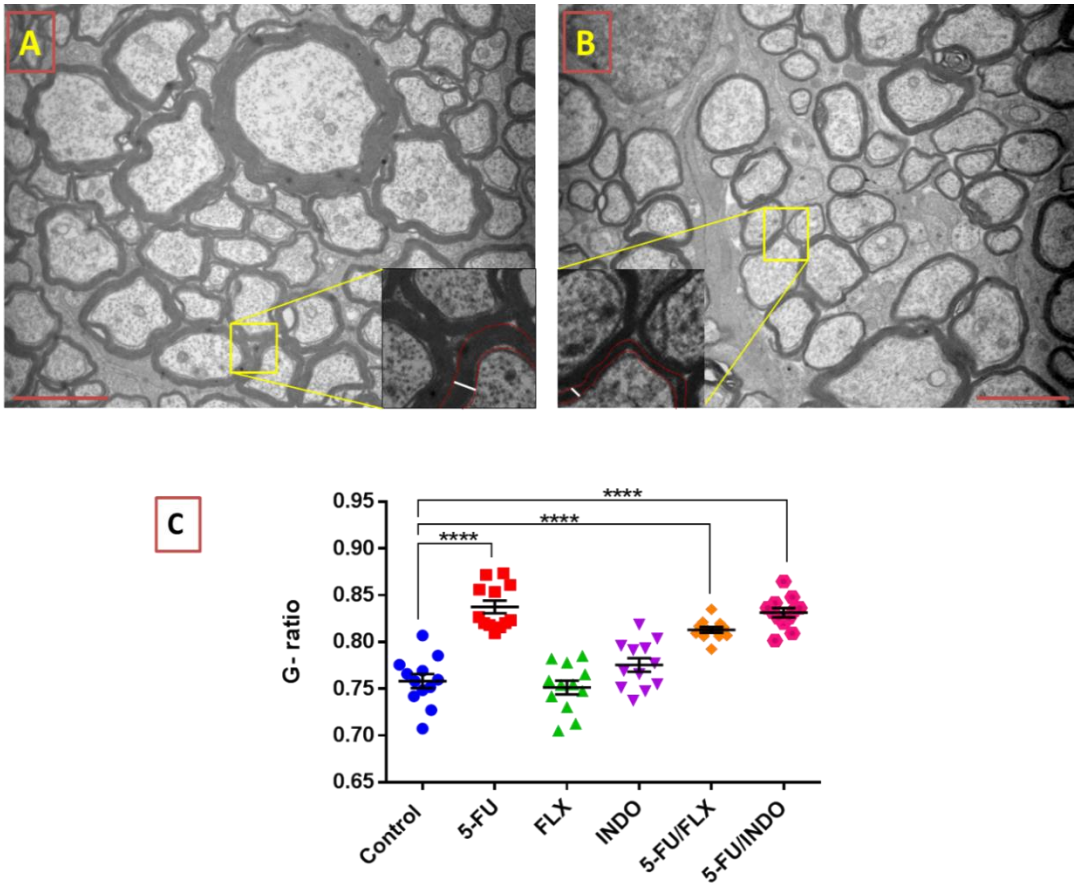



***Figure 4.7*** *Representative electron micrographs of ON showing myelin thickness in (**A**) control, and (**B**) 5-FU treatment (8,200x magnification; Scale bars=2μm) and high magnification (16,200x magnification; Scale bars=1μm). (**C**) G-ratio of myelin thickness of axons from optic nerve sections quantified in control, 5-FU, FLX, INDO, 5-FU/FLX and 5-FU/INDO treated groups. Results are expressed as minimum to maximum of at least 30 measurements in each experimental group. There is significant increase in the G-ratio of groups receiving 5-FU (****p<0.05, two-way ANOVA).*

In the CC, however the treatment with 5-FU did not significantly change the density of myelinated fibre in the treated group compared with controls (Figure 4.8A), although there was a trend for the 5-FU group to show a decrease in myelinated fibres but this did not reach significance (P= 0.1514, two-way ANOVA). Post-hoc analysis indicated that INDO treated groups had a significantly higher density of myelinated fibres than the control and 5-FU treated groups (P=0.0252). No significant differences were observed between the vehicle-treated group and FLX or FLX+5-FU groups (Figure 4.8B).

Investigation of chronic 5-FU one week after completion of treatment showed that the density of un-myelinated axons significantly increased in both 5-FU and 5-

FU+FLX treated groups when compared with the control group (P=0.0184 and P=0.0101 respectively). In contrast, co-treatment of 5-FU with INDO, prevented the increase in the density of un-myelinated fibres seen with 5-FU alone ($p>0.05$, two-way ANOVA). This result indicates that INDO treatment prevents the increase in un-myelinated axons density when co-administered during chronic treatment of 5-FU (Figure 4.8C).

The G-ratio of myelin thickness in the CC of different treatment groups was measured to evaluate the adverse effect of chemotherapy on WMT myelination and the putative protective role of antidepressant or anti-inflammatory treatments when co-administered with the chemotherapeutic agent. As shown in Figure 4.8D, the 5-FU group showed a significantly higher G-ratio than the other treatment groups (P= 0.0042, two-way ANOVA), showing that 5-FU treatment decreased myelin thickness. Whereas, administration of FLX or INDO alone is not have a significant effect on the G-ratio when compared with controls ($p>0.05$). Additionally, co-treatment of FLX or INDO with 5-FU maintained the G-ratio at the level of controls. This indicates that FLX or INDO prevented the decrease in the myelin sheaths thickness produced by 5-FU when co-administered with chronic treatment of 5-FU.

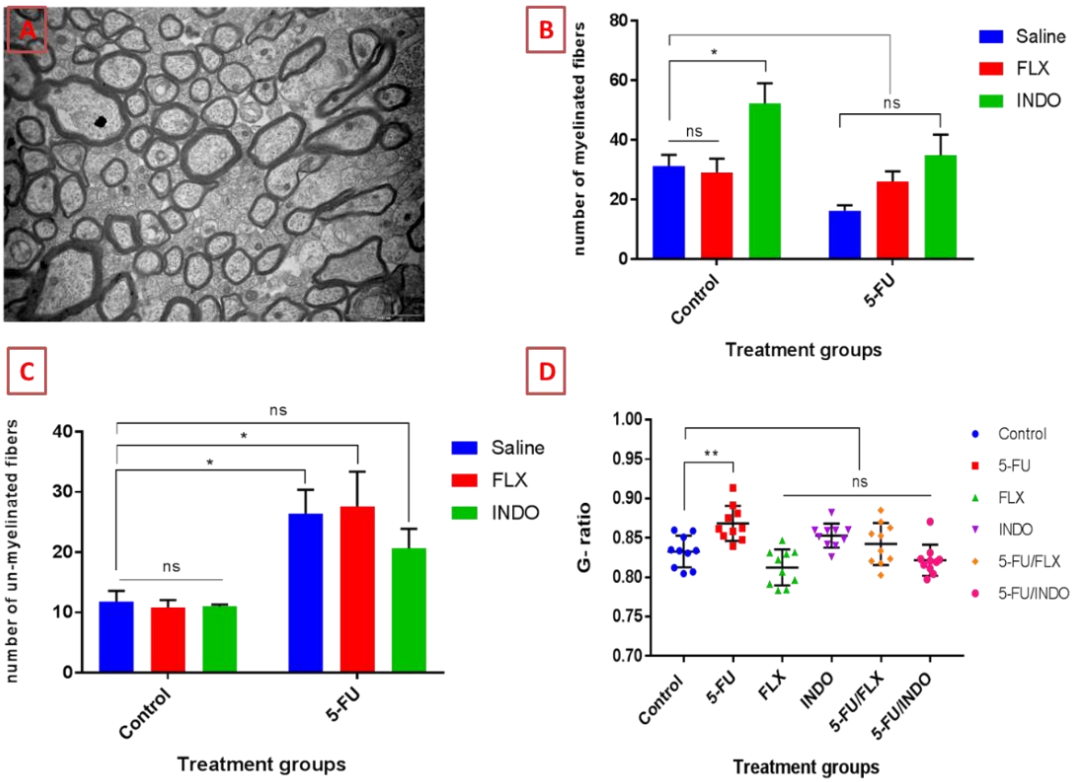



***Figure 4.8*** *The impact of 5-FU, FLX and INDO treatments on myelinated fibres in the CC. (**A**) Representative electron micrograph showing a cross section of adult rat CC (8,200x magnification) Scale bars=2µm. (**B**) The average number of myelinated axons (mean ± SEM, n=4) in all experiment groups. (**C**) The average number of un-myelinated axons present (mean ± SEM, n=4) in all groups. (**D**) G-ratio from CC sections, quantified in control, 5-FU, FLX, INDO, 5-FU/FLX and 5-FU/INDO groups. Results are expressed as minimum to maximum of at least 40 measurements in each group. The data was analysed using two-way ANOVA and Bonferroni's post-hoc multiple comparisons test to compare between groups. (ns, when the P>0.05), (* when P<0.05), (**when P<0.01).*

#### 4.4.2.2 Morphological and pathological changes in myelin sheaths:

Investigations have shown that systemic chemotherapy is sufficient to cause damage to the ultrastructure of myelin sheaths which can lead to the demyelination of WM tracts. Using transmission electron microscopy TEM, the experiments in this chapter have shown that multiple injections of 5-FU produces a number of visible pathological effects. Figure 4.9A, a cross section in ON, shows complete loss of myelin sheaths and axonal demyelination. These effects reduce the myelin volume in both the ON and CC of animals treated with chemotherapy. Analysis of transverse sections of CC provided further evidence of myelin damage with rarefaction of the axoplasm and axons with a shrunken axoplasmic compartment (Figure 4.9 B and C). Furthermore, Figure 4.9 D shows inter-laminar splitting of the myelin and the axons detaching from the myelin sheaths.

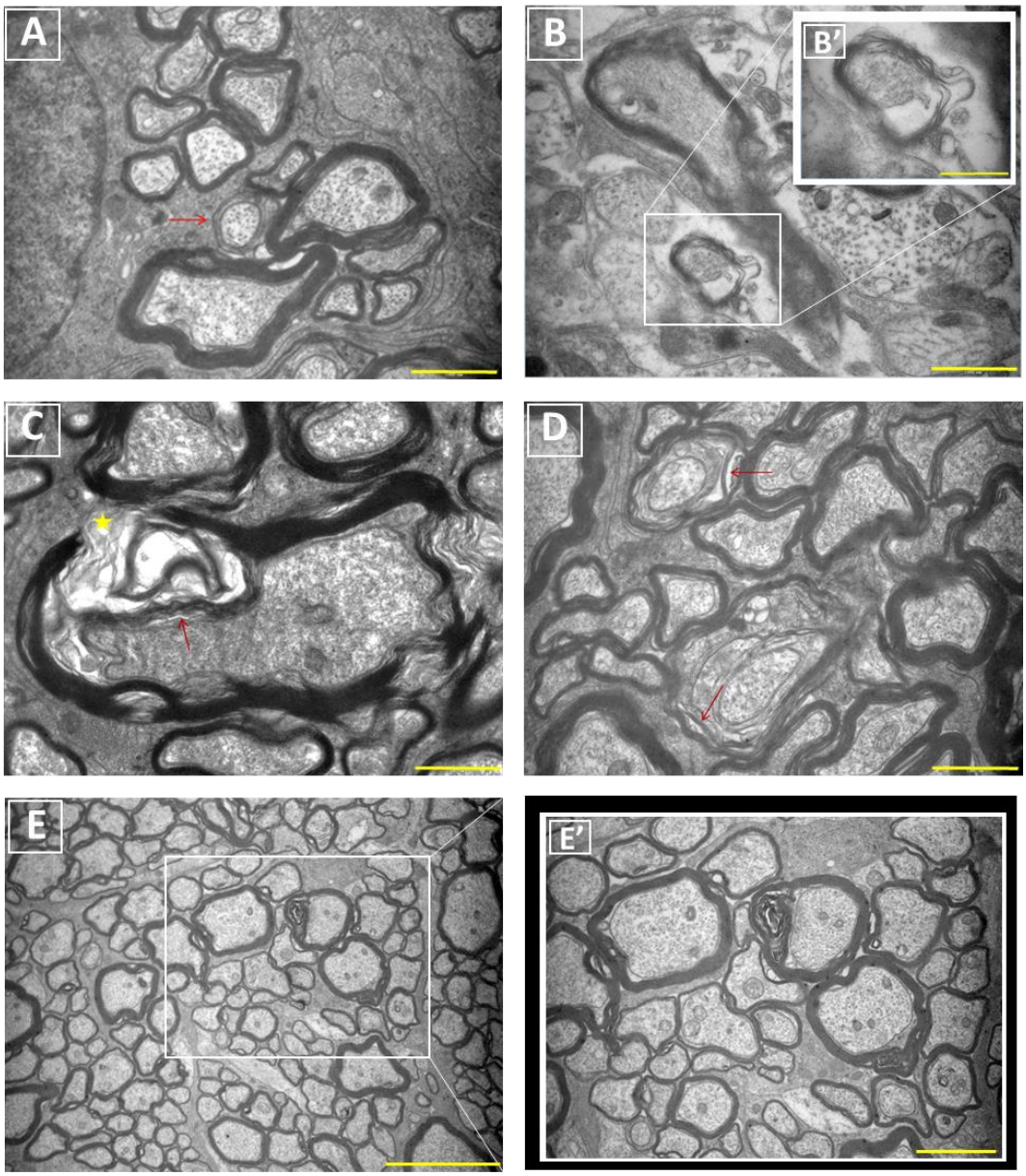



***Figure 4.9*** *Electron micrographs showing some of the pathological changes found in myelin sheaths after treatment with 5-FU. (**A**) A representative image of cross section in rat ON showing a demyelinated axon (arrow) (8,200x magnification) Scale bars=1μm. (**B**) A cross section in rat CC showing myelin damage with rarefaction of the axoplasm (8,200x magnification) Scale bars=1μm. (**B'**) enlargement of the square area in B (60,000x magnification) Scale bars=500nm. (**C**) ON showing partial destruction of the myelin sheath (asterisk) with interlaminar splitting of the myelin sheath (arrow) (20,500x magnification) Scale bars=1μm. (**D**) Interlaminar splitting of the myelin sheath in ON (arrows) (16,500x magnification) Scale bars=1μm. (**E**) ON with possible myelin debris from a degenerating axon (4,200x magnification) Scale bars=5μm. (**E'**) enlargement of the square area in E (8,220x magnification) Scale bars=2μm.*

## 4.5 Discussion

In recent years, there has been increasing interest in trying to understand the role of chemotherapeutic agents in producing cognitive impairments. A number of researchers have demonstrated that systemic chemotherapy causes a decrease in cell proliferation and consequently neurogenesis in the SGZ of the DG of the hippocampus (ElBeltagy *et al.* 2010, Wigmore *et al.* 2010, Lyons 2011, Wigmore 2013, Yang and Moon 2013). Other studies have shown that chemotherapy also affects WM integrity. These effects produce demyelination and an altered water content which affects the myelin sheaths and causes axonal changes which have been associated with cognitive deficits (Abraham *et al.* 2008, Han *et al.* 2008, Deprez *et al.* 2011, de Ruiter *et al.* 2012, Deprez *et al.* 2012). The present study was designed to determine the effect of chronic treatment with 5-FU on WM integrity in ON and CC and to see if this was associated with changes in conduction in the ON of adult rats. The former was studied by TEM to quantify the density and thickness of myelinated nerves while conduction was determined by recording CAPs. In addition, this study aimed to test the effectiveness of an antidepressant (FLX) or anti-inflammatory drug (INDO) at preventing the changes in the ON and CC induced by chemotherapy.

Contrary to our expectations, this study did not find a significant difference in the total conduction velocity of CAPs between control and 5-FU treated groups. However there was a significant decrease in the area of the CAP in 5-FU group at low stimulus voltages compared with controls. There do not appear to have been any previous studies of CAPs after chemotherapy so it is unclear how typical these results are. There are however two *in vivo* studies of the auditory system which are relevant. The acoustic brain stem response (ABR), is related to the conduction velocity from ear to brain stem. Treatment with 5-FU chemotherapy in progressively increased the latency of ABR up to 56 days after treatment suggesting myelin damage (Han et al. 2008). An *in vivo* study in mice after MTX and 5-FU chemotherapy found impaired auditory gating and a reduced response to conditioned emotional conditioning (Gandal *et al.* 2008). In auditory gating, paired auditory stimuli are given and the whole brain electrophysiological response is measured. In controls the response to the second stimuli is reduced due to inhibitory circuits which are activated by the initial stimulus. This response was reduced in chemotherapy treated animals 5 weeks after treatment, indicating reduced activation of this inhibitory circuit and possibly reduced conduction within this system.

In this section, none of the drugs tested (5-FU, FLX and INDO) produced any change in the CAP of rat ON. Most axons were recruited after increasing the stimulation strength. There are several possible reasons why the total CAP area recording did not changes in any of the treated groups. Firstly, as electrophysiological testing took place one week after the last 5-FU injection, it might be that activated OPCs generated new oligodendrocytes which repaired the damaged myelin in the week between drug administration and testing so producing a normal CAP. Previous studies have found that adult OPCs are activated and proliferate following CNS damage such as physical injury, cytotoxicity and microbial infection and repair the tissue (Levine *et al.* 2001). This indicates that the presence of NG2+ cells are essential for myelin repair (Zawadzka *et al.* 2010, Kucharova *et al.* 2011). Secondly, as described earlier, two publications have found that systemic administration of chemotherapy in animal models produces delayed damage to WM which was detected several months after treatment is completed (Gandal *et al.* 2008, Han *et al.* 2008). These data must be interpreted with caution because the above studies showed that this damage was associated with alterations in the cell cycle and extensive myelin pathology which occur due to increased cell death and decreased cell proliferation in SVZ of dentate gyrus and CC. The failure to detect a third peak in recordings from the optic nerve CAP may indicate that there were technical problems with the electrophysiology set up and further studies will be needed to understand how chemotherapy alters conduction in WT.

In the current study, TEM was used to compare the number and the thickness of myelinated axons in the rat ON. The results showed that the number of myelinated axons was not affected by any treatment (Figure 4. 6), whereas in the CC, the number of myelinated axons in groups treated with FLX or INDO, with or without 5-FU, had a greater density of myelinated axons when compared with control and 5-FU treated groups (Figure 4. 8). This data indicated that FLX and INDO may be effective at enhancing myelin formation in the CC. FLX treatment of the transgenic MBP1-hαsyn tg mouse, which has been used as a model of neurodegeneration, ameliorated the loss of myelin in the CC (Ubhi *et al.* 2012). In this study FLX treatment increased the levels of BDNF and GDNF which may have contributed to its beneficial effect. In addition, FLX increases neural stem cell proliferation within the hippocampus (Marcussen *et al.* 2008, Fardell *et al.* 2011, Boldrini *et al.* 2012), and administration of it with a chemotherapy drug may compensate for the decrease in oligogenesis.

Chronic 5-FU treatment produced significant effects on WM integrity in both ON and CC when looked at 7 days after completion of treatment. Animals receiving 5-FU had a reduced myelin thickness (i.e. increased G-ratio). This is similar to previous observations, which found myelin damage in the ON and CC following chemotherapy treatment (Han *et al.* 2008, Briones and Woods 2014, Weng *et al.* 2014). These researchers identified a reduction not only in cell proliferation within WMT, but also in the number of mature functional oligodendrocytes. This result could be explained by the fact that 5-FU treatment is likely to cause a significant reduction in the different cell types in WMTs including mature oligodendrocytes and OPCs possibly by increasing apoptosis leading to progressive damage to WM tract (Dietrich *et al.* 2006, Mignone and Weber 2006) which subsequently affected myelin formation.

However, myelin thickness affected by a treatment with chemotherapy, the total conduction velocity of CAPs did not find a significant difference between control and 5-FU treated groups. There are several possibilities to explain this outcome which is partly supported by a result from the current study which showed that the number of myelinated axons in ON was not affected by 5-FU treatment (Figure 4.6). Also, there is an incomplete loss of myelin sheaths that surround the axons; however the thickness of myelin sheaths is affected by treatment. Therefore, all axons stimulated and contributed to action potential which make the measurement sensitivity insufficient to determine small changes in the total of conduction velocity.

In ON, co-treatment with either FLX or INDO was not effective at preventing detrimental effects on myelin reduction induced by 5-FU. In CC, the number of unmyelinated fibers increased after 5-FU administration. On the other hand, groups co-treated with FLX or INDO together with 5-FU showed no significant difference in the G-ratio from controls. This result shows that FLX or INDO could prevent the decrease in the myelin sheath thickness produced by 5-FU. It is interesting to note that FLX could potentially counteract the anti-proliferative or apoptotic effects of 5-FU on cells in WT and in this way maintain the process of myelination and myelin thickness in the CC. This study confirms that chronic treatment with FLX induced neurogenesis and a faster cell proliferation in the adult brain (Kodama *et al.* 2004, Encinas *et al.* 2006). Furthermore, the results of this study showed that INDO elevated the number of myelinated fibres and also decrease the adverse effect of 5-FU on myelin thickness. The reason for this is not clear but it seems possible that INDO could promote the differentiation and maturation of oligodendrocytes and maintained myelin membranes around the

axons to form compact enwrapping sheaths that insulates axons, subsequently increasing the conduction velocity of nerve impulses (Patel and Klein 2011). However, this result has not previously been described, but it seems to be consistent with other research which found INDO positively enhanced normal cell growth and prevented the decline in cell proliferation after cranial irradiation (Monje *et al.* 2003, Briones and Woods 2014). Additionally, the current result may be explained by the fact that inflammation is known to cause demyelination, and both FLX and INDO are anti-inflammatory agents (Bastos *et al.* 2008, Jin *et al.* 2009, Bernardi *et al.* 2010, Caiaffo *et al.* 2016). Importantly, blocking the neuroinflammatory reaction to a 5-FU treatment offsets the detrimental effects of cytotoxic agents on myelin and myelination in the WMTs, indicating the direct links between neuroinflammation caused by a 5-FU and myelin breakdown (Briones and Woods, 2014). In general, therefore, it seems that the CC is more vulnerable to the 5-FU treatment than ON.

**Impact of chemotherapy on cognition**

It is likely that a number of mechanisms are behind the adverse effects of 5-FU on cognition. One mechanism is probably the effect of chemotherapy in causing myelin damage in the CNS (Han *et al.* 2008, Briones and Woods 2014, Weng *et al.* 2014). This study has demonstrated that 5-FU treatment produced damage to the myelin sheaths in both ON and CC of adult rats. It is interesting to note that in some animals, 5-FU caused non-invasive demyelination in some of the axons. These results are consistent with those described in chapter two which observed a significant reduction in the number and proliferation of OPCs after injections with 5-FU. This will consequently lead to a reduction in the number of mature oligodendrocytes and their ability to maintain myelin sheaths. Therefore, it seems that an imbalance between the proliferative rate of progenitor cells and their loss by exposure to chemical or physical agents led to an overall decrease the number of mature myelinating oligodendrocytes and increase in unmyelinated fibres (Barres and Raff 1994, Dietrich *et al.* 2006, Hyrien *et al.* 2010).

Furthermore, the ultrastructural damage produced by 5-FU could be a direct effect of chemotherapy on the myelin producing cells of the WMT which could be mediated by killing or preventing the proliferation of OPCs; or could be an indirect effect caused by the induction of an inflammatory response which then affects OPCs and oligodendrocytes in these tracts. These effects such as inter-laminar splitting of the myelin and the axons detaching from the myelin sheaths could be attributed to 5-FU producing a progressive change in the myelinated tracts that is associated with altered transcriptional regulation in oligodendrocytes and myelin

pathology. This is interpreted as indicating that the chemotherapy has caused an increased water content in white matter tracts visible in patient MRI scans which is indicative of demyelination or damage to nerve axons (Abraham *et al.* 2008, Akitake *et al.* 2011, de Ruiter *et al.* 2012). These changes in the ultrastructure of WM are indicative of destruction in myelin sheaths and can lead to both partial and complete loss of myelin and an increase in intra-laminar splitting of myelin sheaths (Akiba *et al.* 1996). As mentioned above, the protective role of FLX and INDO on the ON and CC may be due to their anti-inflammatory effects.

### 4.6 Conclusions

The present study was designed to determine the possible effects of chemotherapy on white matter damage to the CNS. It also aimed to identify whether chronic treatment with 5-FU and FLX or INDO affected the ON and CC of adult rats. Electrophysiology was used to record CAPs and transmission electron microscopy was used to investigate the changes in the morphology of myelin sheaths and ultra-structure of ON and CC. The study observed a non-significant difference in the conduction velocity between control and 5-FU treated groups, however there was a significant decrease in the CAP area in 5-FU group at discrete stimulus voltages. In addition the data indicated that the first and second peaks were unaffected by different types of drug administration. It was also shown that there was no significant difference in the number of myelinated axons in ON. The most obvious finding to emerge from this study is that chronic treatment with 5-FU showed a significant reduction myelin thickness in both ON and CC; whereas, co-treatment of FLX or INDO with 5-FU prevented the decrease in the myelin sheaths thickness. The present study confirms previous findings and contributes additional evidence that suggests that FLX and INDO could be employed to counteract the adverse effects of 5-FU on WMT integrity when it was co–administrated with chemotherapy treatment. Future experimental studies are required to explore the detailed mechanisms of antidepressant and anti-inflammatory action in promoting myelin wellbeing and reducing potential CNS damage after chemotherapy.

# **CHAPTER FIVE:** GENERAL DISCUSSION

The importance of chemotherapy, in treating a considerable number of cancer types is well established. 5-Fluorouracil (5-FU) is a widely used anticancer drug which has been in use for over four decades in treating various cancers including colorectal, breast, ovarian and oesophageal carcinoma. Chemotherapy has a number of adverse side effects, many of which are relatively transitory however in recent years it has become increasingly apparent that a significant number of patients suffer from prolonged cognitive impairments after treatment. These have been termed "chemobrain" which refers to the impairments in memory and concentration experienced after chemotherapy treatment (Tannock *et al.* 2004). Cognitive impairment associated with systemic chemotherapy treatment, is likely to have several causes including changes in brain metabolism and a reduction in hippocampal neurogenesis, however, clinical and animal studies have demonstrated changes in the conducting properties and degeneration of white matter tracts after exposure to chemotherapy. These changes may be brought about by a direct action of chemotherapy on dividing glial cells or may be damaging white matter tracts indirectly by the induction of an inflammatory response to chemotherapy treatment. Both mechanisms could produce a reduction in the cell proliferation of glial cells and their precursors within the CNS. These effects may contribute to white matter disruption and subsequent demyelination, which is a possible cause of cognitive deficits (Dietrich *et al.* 2006, Han *et al.* 2008, Wigmore 2013).

The work presented in this thesis has built on previous studies using both *in vivo* and *in vitro* models. In this project adult male rats were given doses of 5-FU which in earlier work produced cognitive deficits (ElBeltagy et al., 2010, Lyons et al., 2012) to investigate the impact of acute and chronic systemic treatment with 5-FU on cell proliferation, survival and changes to the myelin sheath. As previous studies had demonstrated the ability of the SSRI antidepressant (FLX) to protect hippocampal neurogenesis and cognition from the deficits produced by chemotherapeutic agents (ElBeltagy *et al.* 2010, Lyons 2011, Lyons *et al.* 2012), this drug was tested for its ability to protect white matter tracts. Inflammation is a possible cause of chemotherapy induced damage to the nervous system, a commonly used NSAID Indomethacin (INDO) whose effects on hippocampal neurogenesis have been investigated (Goncalves *et al.* 2010, Ji *et al.* 2013, Ho *et al.* 2014) was also tested for its protective effects. The effects of chemotherapy and both protective agents were investigated on the optic nerve and corpus callosum.

In parallel with the *in vivo* studies the effects of 5-FU on the different cell types in vitro were tested together with the effects of co-administration with FLX or INDO. As previous work had indicated that dividing neural progenitor cells in the SGZ of the hippocampus were protected from 5-FU if they were associated with blood vessels, co-culture of OPCs with endothelial cells and astrocytes was investigated. One aspect of the project has focused on the role of microglia in producing pro-inflammatory cytokines which may be a cause of white matter damage.

## 5.1 The effects of chemotherapy and protective treatments on body weight and water intake

Chemotherapy can rapidly cause weight loss in patients and animal weights were monitored to assess health and compliance with Home Office regulations. 5-FU treatments reduced body weight gain, an effect which is likely to be due to inhibition of cell proliferation in the lining of the gastrointestinal tract and reduced nutrient absorption (Huang *et al.* 2002, Lee *et al.* 2006, Song *et al.* 2013). This effect has been found by previous studies in this and other labs (ElBeltagy *et al.* 2012, Cao *et al.* 2014). It was noted that chronic 5-FU administration can induce a significant reduction in weight gain 24 hours after the initial injection and effects can extend beyond the period of chemotherapy administration. Co-treatment with FLX+5-FU in the current study, also produced a significant reduction in the weights of treated animals. This has been seen before (ElBeltagy *et al.* 2012) and may be explained by the anorexic effect of antidepressants or disruption of the neuro endocrine system of the gut (Aggarwal *et al.* 2016). In contrast treatment with the anti-inflammatory INDO produced weight gains compared with other treatment groups. This may be due to increased food consumption taken to relieve gastric pain after long-term use of NSAID (Risser *et al.* 2009, Ho *et al.* 2014). It is interesting to note that most animals, treated with FLX, regained weight after the end of treatment, but did not reach the level of vehicle-treated animals.

FLX was administered in drinking water to avoid the stress of repeated injections. There was a reduction in water intake in FLX treated animals which could be due to a taste aversion to FLX (Prendergast *et al.* 1996) and has been found in previous studies in this lab (Lyons *et al.* 2012). The results presented in (Chapter two) showed that, single injections of 5-FU caused a temporary decline in water intake 24 hours after injection but water intake was at control levels within one week. Multiple injections of 5-FU caused a significant decline in water intake, consistent with other studies (Walker *et al.* 2011, Lyons *et al.* 2012). In contrast, animals co-treated with 5-FU+INDO showed no difference in water consumption from controls which is consistent with the literature which has found that INDO can

increase water intake due to a decrease in the production of prostaglandins which alter kidney function (Kim 2008).

### 5.2 Cellular Effects of 5-FU treatment on WMTs cell proliferation:

The chemotherapy drug 5-FU, used in the present thesis, has been commonly associated with cognitive symptoms particularly in breast cancer survivors (Ahles *et al.* 2012, de Ruiter *et al.* 2012) and has been shown to cause focal loss of microstructural white matter integrity in patient studies (Koppelmans *et al.* 2014). Previous studies from our laboratory have shown that the dosage of 5-FU used in the present study, caused memory impairments and decreased hippocampal neurogenesis in adult male rats (Mustafa *et al.* 2008, ElBeltagy *et al.* 2012, Lyons *et al.* 2012). This indicates that the animal model used in this thesis is should provide clinically relevant information into the causes of patient cognitive decline after chemotherapy.

Due to the cytotoxic nature of chemotherapy, a reduction in the normal cell proliferation and gliogenesis occurring in adult WMTs, provides a possible mechanism for WMT damage and the cognitive impairments experienced by patients treated with chemotherapy. Therefore, the present study was designed to determine the effect of 5-FU on cell proliferation in the normal WMTs of the adult rat CNS. This study was also carried out to determine whether these effects are transient or more permanent after single or multiple injections of chemotherapy.

Rats given a single injection of 5-FU, showed no significant reduction in the number of dividing cells and oligodendrocyte progenitor cells (OPCs) in the ON and CC either one or seven days after treatment. 5-FU has been reported to have delayed effects on WMTs (Han et al 2008) and it is possible that allowing the animals to survive for longer would show greater effects but comparison with studies in the literature are difficult due to differences in dosage. In the hippocampus, single in injections of 5-FU cause a transient reduction in the number of dividing cells in the SGZ (Dietrich *et al.* 2006, Seigers *et al.* 2009, Lyons 2011). It may however be that the OPCs dividing in WMTs are more resistant to 5-FU than the neural stem cells dividing in the SGZ. My *in vitro* studies in chapter three on the (MO3.13) oligodendrocyte precursor cell line showed that their viability was not affected by 5-FU exposure at low concentrations given for 24 hours. However, 24 hours exposure to higher concentrations of 5-FU reduced the viability of these cells. Alternatively as reported in a previous study by our group, a single injection of 5-FU might not be sufficient to elicit cytotoxic effects

on the majority of dividing cells (Mustafa *et al.* 2008). This might be due to cell cycle kinetics and the high metabolism of 5-FU in rodents, which is estimated to be less than 1 hour. The cell cycle time of progenitor cells in the dentate gyrus is more than 24 hours (Cameron and McKay 2001) and about two-thirds of this time is spent in 'S' phase of which is when cells are most sensitive to cytotoxic agents (Au *et al.* 1983, Kutscher *et al.* 2000, Dayer *et al.* 2003).

In contrast to single injections, 6 injections of 5-FU given over two weeks, a protocol which more closely mimics clinical practice, reduced the number of proliferating cells and also the number of OPCs (NG2+ cells) in both the CC and ON. Leaving the animals for one week after the end of chemotherapy injections is not enough time to allow the number of NG2+ cells to return to control levels. Chemotherapy by targeting dividing cells, will deplete the rapidly dividing OPCs. Loss of these cells may activate quiescent progenitors which in turn are hit by subsequent injections of chemotherapy a process which could deplete the reservoir of progenitor cells and make a recovery much slower or more difficult. These results agree with the findings of other studies, using different anti-cancer agents including cyclophosphamide, MTX and 5-FU which found a significant and prolonged decrease in cell proliferation in both WTMs (Han *et al.* 2008) and the SGZ within the hippocampus after a series of chemotherapy injections (Han *et al.* 2008, ElBeltagy *et al.* 2010, Lyons 2011, Christie *et al.* 2012, ElBeltagy *et al.* 2012, Lyons *et al.* 2012).

The mechanism for the reduction in cell proliferation and the failure of dividing cells to return to control levels after systemic chemotherapy have not been fully elucidated. One possibility is that 5-FU causes misincorporation of uracil instead of thymidine during DNA synthesis which halts cell division while DNA repair is attempted (Krokan *et al.* 2002). This could result in a prolonged reduction in cell division and apoptosis of cells which entered division without repair. Alternatively chemotherapy induced inflammation may persist and inhibit cell division (Soussain *et al.* 2009). The impact of both single and multiple injections of 5-FU in producing DNA damage in WMTs was detected by immunostaining for phosphorylated histone H2AX ($\gamma$-H2AX), a marker of DNA double strand breaks. Single injections of 5-FU, investigated one day after treatment, did not show evidence of DNA damage in either ON or CC. However there was a significant increase in the number of $\gamma$-H2AX+ cells one week after a single injection. This indicates that this dose of 5-FU is causing significant DNA damage but that this takes at least several days to be detected. Multiple injections also caused significand DNA damage. These results are similar to Fujinaka *et al.* (2012) and Krynetskiy *et al.* (2013)

who also found that 5-FU treatment caused DNA damage and apoptosis in normal cells. This is likely to be due to the direct effects of 5-FU on DNA synthesis and miss-incorporation of uracil into RNA and DNA (Li *et al.* 2009). In addition oxidative stress (Kaiser *et al.* 2014) or mitochondrial damage and reactive oxygen species (ROS) may cause DNA damage (Wigmore *et al.* 2010). The inflammatory reaction produced by chemotherapy may also contribute to DNA damage (Ahles and Saykin 2007, Domercq *et al.* 2007).

Inflammation produced by 5-FU treatment is likely to activate microglia and cause the release of inflammatory cytokines. In chapter two, a marker for microglia, Iba-1 and inflammation, COX-2, was quantified 24 hours and one week after single and multiple 5-FU injections. A single injection caused an increase in the total number of microglia 24 hours later in the CC. This suggests that the initial effect of chemotherapy is to trigger proliferation by microglia. This is similar to the effects of infection or acute injury which lead to an increase in microglia, as part of an immune response to these insults (Wegiel *et al.* 2003, Erny *et al.* 2015). However both single and multiple injections of 5-FU reduced the number of Iba-1+ cells in both ON and CC one week after treatment. A reduction in microglial number has been found several weeks after 5-FU treatment (Reid *et al.* 1993, Seigers *et al.* 2016) Paquet *et al.* (2016) and the *in vitro* work presented in this thesis showed that microglia are particularly sensitive to chemotherapy compared with astrocytes and endothelial cells (Chapter three). Inflammatory activation of microglia by chemotherapy will lead to the secretion of pro-inflammatory cytokines. These have been shown to kill OPCs but not mature oligodendrocytes (Miller *et al.* 2007). Persistent neuroinflammation results in chronically activated microglia which release inflammatory mediators such as TNF-α and IL1-b that may lead to neuronal and glial cell damage (Meyers 2008, Woodcock and Morganti-Kossmann 2013, Briones and Woods 2014). To investigate this, TNF-α expression by microglia was measured using an ELISA technique. This showed that 5-FU induced the production of TNF-α from microglia grown alone or co-cultured with OPCs. The present findings are consistent with other research which increased levels of TNF-α and IL-1ß and expression of COX-2 after treatment with cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) (Briones and Woods 2014). These changes were associated with cognitive impairment and damage to WMTs. TNFα and COX-2 are released in the CNS by microglia and astrocytes which promotes inflammatory signalling (Block *et al.* 2007, Yeo *et al.* 2012, Woodcock and Morganti-Kossmann 2013).

Co-culture of microglia and OPCs were used to directly test the interactions between chemotherapy and these two cell types. Using a concentration of 5-FU which did not affect OPCs when cultured alone this produced a significant decrease in the number of OPCs when they were co-cultured with microglia. Treatment of microglia with chemotherapy lead to activation of microglia and the production of TNF-α. The receptor for this cytokine is expressed by oligodendrocytes and their precursors and its activation is toxic to OPCs (Pang *et al.* 2007, Deng *et al.* 2008). This result is broadly consistent with other studies that found that activated microglia, were toxic to OPCs and mature oligodendrocytes in co-culture (Zajicek *et al.* 1992, McLaurin *et al.* 1995, Lehnardt *et al.* 2002, Li *et al.* 2005).

COX-2 is expressed constitutively in the brain but increased expression is associated with inflammation and is thought to contribute to neurodegenerative processes (Hoozemans *et al.* 2001, Minghetti 2004). The density of cells expressing COX-2 in the ON and CC was quantified, and animals treated with either single or multiple injections of 5-FU had a significant increase in the number of cells expressing COX-2. These findings are in agreement with studies showing that 5-FU can induce inflammation in peripheral tissues by increasing the expression of COX-2 *in vivo* (Lopes *et al.* 2009) and *in vitro* (Réti *et al.* 2008).

The increase in the number of COX-2 expressing cells was associated with a decrease in the total number of microglia as quantified by Iba-1 expression. Other work in our laboratory has shown that 5-FU treatment although reducing microglial numbers, increases the proportion of microglia with an activated phenotype (Lasio 2017) and microglia are particularly sensitive to apoptotic stimuli (White *et al.* 1998). The induction of an inflammatory response by 5-FU treatment, as shown by the increased numbers of COX-2 expressing cells *in vivo* and the production of TNF-a and the toxicity of activated microglia to OPCs *in vitro* provides strong evidence that inflammation is a major cause of the reduced cell division and numbers of OPCs *in vivo* after chemotherapy. This hypothesis is consistent with evidence that production of prostaglandins by COX-2 increased apoptosis in the dentate gyrus; and impaired NSCs survival after LPS treatment (Bastos *et al.* 2008).

Interactions between the different cells types found in WMTs may affect the sensitivity of cells to chemotherapy. As found by previous authors, astrocytes and endothelial cells are less susceptible to 5-FU than OPCs and microglial cells (Dietrich *et al.* 2006). Consistent with this and in agreement with a previous *in vivo* study, that showed 5-FU treatment significantly reduced non-endothelial associated cell proliferation in the SGZ but did not affect microvessel density

(Maqbool 2015). The effect of this association was tested by co-culturing OPCs with astrocytes or endothelial cells either where contact between the cell types was allowed or by separating them with a porous membrane.

As mentioned in chapter one, astrocytes release trophic factors including brain-derived neurotrophic factor (BDNF) and NT-3 that play a number of roles in the maintenance neurons and other glial cells, particularly after injury (Dreyfus *et al.* 1999). Of importance to the present study, astrocytes also express the growth promoting factor (CNTF), a factor that supports OPC survival (Albrecht *et al.* 2007). Endothelial cells play a crucial role in supporting OPC proliferation and are involved in regulating the development and survival of oligodendrocyte by secretion of endothelial-derived growth factors such as BDNF and FGF-2 (Miyamoto *et al.* 2014). As well as producing growth factors to support other cells, astrocytes and endothelial cells can make direct contact and by cell-cell communication maintain and support adjacent cells in the CNS. In this thesis, I compared the possible protective effects of astrocytes and the endothelial cells against the effects of chemotherapy on the proliferation and survival of OPC cells by co-culturing the different cell types.

The results in Chapter 3 showed that co-culture of OPCs with either astrocytes or endothelial cells provided protection from doses of 5-FU which would reduce the viability of OPCs. This protection was lost if the endothelial cells or astrocytes were separated from the OPCs and was not provided by astrocyte or endothelial cell conditioned media. These results indicate that direct cell to cell contact is required to provide protection and a candidate mechanism would be gap junctions (GJ) between the cell types. GJ use connexin proteins (Cx) to provide intracellular channels to allow ions and nutrients to pass between cells. Immunofluorescent staining demonstrated that the GJ protein Cx47 was expressed by oligodendrocyte precursor and it thought to allow ions and nutrients to pass from the somata to other cells such as astrocytes and endothelial cells, and the presence of this protein would enable junctions to form between these cell types. Loading astrocytes and endothelial cells (donor cells) with calcine AM green dye and co-culturing with labelled OPCs cells showed that the dye could pass from donor cells to OPCs; indicating that functional GJ had formed between the cell types. To test the role of GJ in providing protection, the GJ blocker (CBX) was applied to co-cultures and prevented dye transfer between cell types. CBX was used on co-cultures of OPCs with endothelial cells or astrocytes with or without exposure to the chemotherapy. The results showed that protection was reduced when the co-cultures of OPCs and either astrocytes or endothelial cells were treated with CBX

and 5-FU. Studies of chemo-resistance to temozolomide and vincristine in cancer cells has identified GJs with astrocytes as the mechanism for resistance (Chen *et al.* 2015). GJs facilitate the passage of small molecules between cells including glucose, ATP and ions that can maintain the metabolism of connected cells (Gajardo-Gomez *et al.* 2016).

In the SGZ of the hippocampus, many neural stem cells are found on the surface of small blood vessels (Palmer *et al.* 2000). Oligodendrocytes *in vivo* also have a close association with endothelial cells and this has been called the oligo-vascular niche (Arai and Lo 2009). This is thought to be similar to the neuro-vascular niche found in grey matter where vascular cells support and maintain neurons. Studies of endothelial /oligodendrocyte interactions have found that endothelial cells can promote OPC proliferation and survival through the secretion of trophic factors, particularly BDNF, rather than by direct cell to cell contact (Arai and Lo 2009). Oligodendrocytes in turn can induce vascular remodelling after inflammatory injury to WMTs (Pham *et al.* 2012). The decrease in the numbers of OPCs co-cultured with but separated from endothelial cells by a porous membrane was less than that found when these cells were cultured on their own and treated with 5-FU. This may indicate that secretion of endothelial-derived growth factors such as BDNF and FGF-2 may promote OPC survival as well as the presence of GJ.

The BBB is maintained by tight junctions between endothelial cells which prevents the uncontrolled entry of substances into the brain. Astrocyte end feet cover the external surface of small blood vessels and form GJ with the endothelial cells and are essential for the maintenance and formation of the BBB (Abbott *et al.* 2006). Astrocytes also secrete trophic factors including BDNF which aid oligodendrocyte development, survival and promote OPCs proliferation (Miyamoto *et al.* 2014). Small numbers of GJ have been described between astrocytes and oligodendrocytes but their function in normal tissue is unknown (Nagy *et al.* 2003). As with endothelial cells, co-culture of astrocytes with OPCs where the cells were in direct contact, protected the OPCs from 5-FU treatment. Treatment with CBX abolished this protection indicating that it was dependent upon GJ.

*In vivo* OPCs on the surface of blood vessels are likely to be in contact with both astrocytes and endothelial cells with which they can form GJs with. The results presented here are the first demonstration that OPCs can be protected from chemotherapy by forming GJ with endothelial cells or astrocytes. The mechanism by which cells escape damage by chemotherapy has received extensive study since the demonstration that astrocytes could protect cancer cells if they form GJ with them (Lin *et al.* 2010, Chen *et al.* 2015). Many chemotherapy drugs including

5-FU cause an increase in the levels of intracellular $Ca^{2+}$ (Yin *et al.* 2011, Can *et al.* 2013, Marchi *et al.* 2017). This can reach toxic levels and induce apoptosis (Shoshan-Barmatz *et al.* 2017). It has been shown that astrocytes can remove excess $Ca^{2+}$ from cancer cells via GJ a process which makes them less sensitive to chemotherapy (Chen *et al.* 2015). From the results presented here removal of $Ca^{2+}$ from OPCs treated with 5-FU through GJ coupling with astrocytes or endothelial cell is a likely mechanism for their protection.

### 5.3 The effectiveness of FLX as a protectant of progenitor cells.

It is now widely accepted that antidepressants can enhance neurogenesis (Kodama *et al.* 2004, Chen *et al.* 2006, Encinas *et al.* 2006, Marcussen *et al.* 2008). FLX was co administered with 5-FU to see if it could prevent the effects of chemotherapy on cell proliferation, OPCs genesis, neuroinflammation and DNA damage. FLX on its own had no effect on cell proliferation in WMT, but it did prevent the decrease in cell proliferation caused by 5-FU when it was co administered. This result is similar to those of Lyons *et al.* (2012) and ElBeltagy *et al.* (2010), who found that FLX prevented the decrease in cell proliferation in the SGZ and the cognitive decline produced by 5-FU and MTX.

It is interesting to note that the CC showed an increase in the number of the NG2 positive cells after chronic treatment with FLX when compared with other groups. This is similar to results showing that FLX lead to increasing numbers neurons (Surget *et al.* 2011). Antidepressants, increase glial proliferation in the frontal cortex of treated animals (Kodama *et al.* 2004); and their effect on the CC may provide treatments for neurodegenerative diseases involving loss of glia.

In *in vitro*, although low concentrations of FLX were not toxic to OPCs, antidepressant treatment, using a variety of concentrations over 24 or 48 hours did not show any protective effect against the adverse effects of 5-FU. These results appear to indicate that the protective effect of FLX *in vivo* may be indirect. Thus, FLX may not act on OPCs but may prevent the adverse effects of interleukins and cytokines (Nguyen *et al.* 2002) or elevate the level of the growth factors such as Brain-derived neurotrophic factor (BDNF) (Duman *et al.* 2001, Alme *et al.* 2007). Another possible reason, is that chronic treatment with the FLX decreases cell cycle inhibitors such as p21Cip1, p18INK and p21Kip1, which are associated with a decline in the proliferation of neuronal progenitor cells (Pechnick *et al.* 2011).

Previous studies have found that treatment with chemotherapeutic agents can induce inflammation which may significantly contribute to the loss of stem and

progenitor cells with subsequent cognitive decline (Lopes *et al.* 2009, Soussain *et al.* 2009). Recently, studies have found an increase in the number of COX-2 expressing cells, TNF-α and IL-1ß after combination treatment with cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) (Briones and Woods, 2014). Consequently, these changes were associated with cognitive impairments and damage to white matter microstructure in an animal models. Several studies have shown that administration of anti-inflammatory agents can improve hippocampal neurogenesis by restoring the interaction between neural stem cells and the microvasculature which is disrupted by inflammation (Monje *et al.* 2003, Ho *et al.* 2015). Prior studies that have noted the ability of FLX to decrease the inflammation caused by kainic acid and prevent apoptosis in the brain (Jin *et al.* 2009). The present study looked at the effect of FLX in reversing the neuro-inflammatory process, and in modulating the reduction in the number of microglia that occurs after chemotherapy both *in vivo* and *in vitro*. The number of COX-2 expressing cells was quantified as a marker of inflammation. It is interesting to record that 5-FU treatment significantly increased the numbers of COX-2 positive cells in WMTs one day and one week after single or multiple injections. Whereas, FLX co-administration with 5-FU protected against the adverse effect of chemotherapy in both ON and the CC. These findings mirror those of previous studies which used a similar animal model and which demonstrated a protective role of FLX against the reduction in hippocampal neural stem cells produced by 5-FU treatment (ElBeltagy *et al.* 2012, Lyons *et al.* 2012, Maqbool 2015). FLX has anti-inflammatory properties as shown by its ability to reduce both RNA and protein levels of the inflammatory markers COX-2, TNF-α, IL-1β and IL-6 produced by microglial cells (Chung *et al.* 2011, Liu *et al.* 2011, Zhang *et al.* 2012).

As shown in Chapter three of the current study, FLX was effective in reducing inflammation caused by 5-FU treatment of microglial cell lines by lowering the level of TNF-α secreted from the microglia. These results match those observed in earlier studies (Liu *et al.* 2011, Tynan *et al.* 2012, Su *et al.* 2015). These authors have speculated that the FLX exerts an anti-inflammatory effect, by inhibiting microglial activation and the secretion of pro-inflammatory cytokines like TNF-α, IL-1β, IL-6 and nitric oxide (NO). Taken in consideration, it is now thought that FLX may modulate the immune system in the CNS either by enhancing the effects of M2 or by inhibiting the M1 activation of microglia (Su *et al.* 2015). FLX also suppresses NF-κB, an important transcription factor required for the production of pro-inflammatory cytokines (Roumestan *et al.* 2007). Furthermore, FLX can inhibits IkB-a degradation and blocks the phosphorylation of p38 mitogen-

activated protein kinase (MAPK) in stimulated microglial cells which prevents the production of TNF-a, IL-6, IL-1 β and NO (Obuchowicz *et al.* 2014).

The ability of 5-FU to induce DNA damage (Fujinaka *et al.* 2012, Krynetskiy *et al.* 2013) and subsequently increase apoptosis will reduce OPCs numbers and is a likely cause of cognitive impairment. One of the objectives of the current study was to determine whether antidepressants could reduce the DNA damage produced by 5-FU in both ON and the CC. In Chapter two, FLX administration prevented DNA damage when co-administrated before, during and after chemotherapy treatment. As FLX may not act directly on OPCs and has no known DNA repair action it is likely that this effect is indirect. One of the actions of chemotherapy is to increase production of the ROS and inflammation. It is possible that the DNA damage seen after 5-FU treatment is at least in part due to these effects. Other studies have found that FLX protects against IL-1b-induced DNA damage and neuronal apoptosis by downregulation of the p53 dependent pathway (Shan *et al.* 2016). Some authors have speculated that the antioxidant effect of FLX can reduce oxidative stress and prevent cell death (Caiaffo *et al.* 2016). There is little information on the effects of FLX administration to patients on chemotherapy treatment but further studies are required to clarify the mechanism involved in this protective effect.

### 5.4 The protective effect of INDO on cell proliferation in WMTs

It was hypothesised that the anti-inflammatory drug INDO could protect against the adverse effects of chemotherapy on WMTs. The decrease in the number of proliferating OPCs caused by chemotherapy would lead to a reduction in a key population of progenitor cells within WMTs. Treatment with INDO on its own did not affect the level of cell proliferation in ON or CC. When INDO was given in combination with 5-FU, this prevented the decrease in cell proliferation seen when 5-FU was given on its own, demonstrating that this drug has a protective effect. It is not clear what effect INDO may have on normal cell proliferation, but anti-inflammatory agents block the reduction in cell proliferation in the hippocampus produced by cranial irradiation or chemotherapy (Monje *et al.* 2003, Briones and Woods 2014). These findings suggest that inflammation is a key mechanism behind the reduction in cell proliferation in WTM after chemotherapy treatment.

Contrary to expectations, this study did not find a significant difference between the number of OPCs among the INDO and other treated groups in the CC or any protective effect against the reduction in the number of NG2 positive cells when co-administrated with a 5-FU. In the ON, the INDO treated group showed a

tendency to increase the number of OPCs however this was not significant. This also accords with other observations, which have found that the administration of INDO to mice did not promote neurogenesis or decrease age-related neuroinflammation in the hippocampus (Boehme *et al.* 2014). One unanticipated finding of the *in vitro* experiments in Chapter three was that while there was no significant effect of INDO treatment on the proliferation of the MO3.13 cell line; INDO enhanced the negative effects, on cell proliferation when co-treated with 5-FU. These findings are consistent with other researches which have found that INDO, and its analogues, enhance the toxicity of different chemotherapeutic agents (Draper *et al.* 1997, Touhey *et al.* 2002, Benyahia *et al.* 2004). It is possible therefore that anti-inflammatory drugs could be used as adjuvant treatments with chemotherapy in cancer treatments. This finding supports previous preclinical studies which have found that INDO is effective against colon and breast cancers (Lundholm *et al.* 1994, Ackerstaff *et al.* 2007). A recent study by Sun and Chen (2016) found that INDO alone or in combination with chemotherapeutic drugs such as an Oxaliplatin, inhibited the growth of human lung cancer grafts in nude mice. On the other hand, INDO treatment did however prevent the drop in the total number of NG2 positive cells in the ON when it was co-administered with chemotherapy (study four in Chapter two). It is encouraging to compare this result with that found by Ho et al. (2014) who observed that chronic treatment with the INDO reversed the decrease in hippocampal neurogenesis, which is possibly mediated by the pro-inflammatory cytokine TNF-α.

The maintenance of proliferation in WMTs when INDO is co-administered with 5-FU could be mediated by a reduction in cytokine levels, COX2 expression and microglia activation. This hypothesis is consistent with the results in this thesis which found that INDO co-administration with 5-FU reduced the decline in the number of microglia that occurs after chemotherapy in ON and CC. This result is in agreement with other authors who have demonstrated that the INDO can reduce inflammation by diminishing the levels of pro inflammatory cytokines which create a detrimental inflammatory environment for adult stem cell renewal and neuronal differentiation. In this way it can prevent the reduction in hippocampal neurogenesis after inflammation (Ji *et al.* 2013). COX-2 expression increases in the CNS with inflammation and contributes to the neurodegenerative processes of several acute and chronic diseases (Minghetti 2004). Administration of INDO with 5-FU decreased the number of COX-2 positive cells. This may be due to the effect of NSAIDs in inhibiting COX2 expression (Bastos *et al.* 2008) and is in agreement with (Netland *et al.* 1998) who showed that INDO effectively alleviated the levels

of inflammation after adult cerebral injury by reducing the COX-1 and the COX-2 expression within the brain. With regards to the *in vitro* study in Chapter three, it is interesting to note that TNF-α release was attenuated when INDO was added to 5-FU treated BV-2 cells. Reducing the levels of pro inflammatory cytokines secreted by activated microglia, could lead to an increase in the survival of progenitor cell populations after exposure to chemotherapy. This study demonstrated that TNF-α release by BV-2 cell line is associated with exposure of microglia to 5-FU and is similar to those observed in earlier studies (Zhang *et al.* 2003, Pusztai *et al.* 2004, Bernardi *et al.* 2010, Song *et al.* 2013, Ho *et al.* 2015). It is possible, therefore, that INDO treatment does not directly increase the proliferation or differentiation of progenitors, but can prevent the adverse effects of cytokines and interleukins to maintain an optimum environment for the survival of OPCs.

DNA damage is considered to be one of the main actions of 5-FU in killing the normal cells and can be produced by the direct effect of chemotherapy on the process of DNA synthesis, or indirectly by other factors such as ROS formation which causes oxidative DNA damage (Li *et al.* 2009, Wigmore *et al.* 2010). The DNA damage stimulates formation of γ-H2AX. The results of Chapter two demonstrated an increase in the level of DNA damage in animals treated with 5-FU. INDO treatment was able to reduce chemotherapy induced DNA damage. The reason behind this is not clear, but previous studies have shown that INDO can upregulate the CDKN2A/p16 tumour suppressor gene and its accessory molecules, and decrease DNA methylation enzymes (Lundholm *et al.* 1994). Moreover and as mentioned earlier, 5-FU treatment could cause an elevation in the level of inflammatory mediators such as COX-2 and lead to prolonged neuroinflammation. This may produce neurotoxic effects on dividing cells by increasing oxidative stress or by glutamate receptor-mediated DNA damage in the WMT (Ahles and Saykin, 2007, Domercq et al., 2007). So, as INDO inhibits the action of COX-2, which is involved in the generation of reactive oxygen species (von Knethen *et al.* 1999, Takahashi *et al.* 2004), it may prevent DNA damage by this mechanism, although this study did not measure the level of ROS and oxidative stress. INDO also enhances the action of the transcription factor peroxisome proliferator-activated receptor-$_{\gamma}$ (Jiang *et al.* 1998, Wada and Kamisaki 2003), which inhibits the expression of the pro-inflammatory cytokines in monocytes and microglia (Hoehn *et al.* 2005). All these actions would aid cell survival. In conclusion it is possible to suggest that blocking the neuroinflammatory reaction of a 5-FU by INDO prevented the adverse effects of inflammatory mediators on WMTs and favoured remyelination and trophic support by glial cells.

## 5.5 5-FU causes damage to the myelin sheath

The integrity of myelinated axons and associated glial cells is essential for learning and other brain functions as they act as relays and coordinate communication between different brain regions (Fields 2008). Furthermore, the thickness and degree of myelination of the axons are essential factors in the speed neural signals are conducted along myelinated (Gutierrez *et al.* 1995). Damage to myelin around axons alters WM integrity within the brain and will reduce axonal conduction (Beaulieu 2002). Clinical and animal studies, have found persistent alterations in the structure and conduction properties after chemotherapy indicative of white matter damage (Han *et al.* 2008, de Ruiter *et al.* 2012). As a consequence the demyelinating effect of chemotherapy will hamper network speed and cause cognitive impairments (Deprez *et al.* 2011). There is very little literature on the mechanisms of chemotherapy-induced changes in the structural integrity of WMTs, or the potential of different treatments, particularly in terms of myelin integrity. The present study was designed to determine the effect of 5-FU on the structural integrity of WMTs and the effectiveness of FLX and INDO in preventing chemotherapy-induced changes in the myelin integrity of ON and CC (Chapter four).

The TEM results of this study showed that the number of myelinated axons in the ON is not affected by 5-FU treatment, whereas, in the CC the number of unmyelinated axons significantly increased after 5-FU treatments. Using the G-ratio to calculate the thickness of myelin sheath relative to axon diameter, it was shown that animals receiving 5-FU had reduced myelin thickness in both ON and CC. These results agree with other studies that have found that 5-FU affected WMTs (Han *et al.* 2008) and decreased myelin thickness (Weng *et al.* 2014). These results provide support for the hypothesis that the reduction in cell proliferation by chemotherapy in WMTs is affecting the maintenance of myelin sheaths which in turn could induce subtle cognitive impairments via changing cellular structure and impulse conduction. Another important finding was that in the CC, INDO on its own elevated the number of myelinated fibres, while it decreased the number of unmyelinated fibres when co-treated with 5-FU. Moreover, co-treatment of FLX or INDO with 5-FU maintained the G-ratio at the level of controls. This result suggests that FLX and INDO prevented the decrease in the myelin thickness when administered with 5-FU. This result may be due to the cytotoxic effects of 5-FU which can cause direct damage to myelin sheaths and myelin-forming oligodendrocytes and their precursor cells (Dietrich *et al.* 2006, Han *et al.* 2008). Alternatively the effects could be indirect by the induction of oxidative stress,

neuroinflammatory reactions or immune response dysregulation (Ahles and Saykin 2007). Thus, formation of reactive oxygen species due to oxidative stress lead to increased DNA damage and apoptosis (Dabrowska et al., 2011). Therefore, protection could be brought about by preventing the adverse effects of inflammatory mediators or oxidative stress to preserve an optimum environment for remyelination and the trophic support afforded by glial cells (Nguyen et al., 2002).

From the results presented here, damage to WMTs by chemotherapy involves both loss of OPCs and demyelination. These effects will reduce conduction speed as well as, changing circuit behaviour and are likely to contribute to the cognitive decline experienced by patients after chemotherapy treatment. One of the key factors behind the changes in WMTs appears to be the induction of an inflammatory reaction which contributes to DNA damage and activation of microglia. Release of cytokines in this response is one of the factors which damages the tissue. OPCs appear to be protected from the effects of chemotherapy if they form GJ with either astrocytes or endothelial cells. This is likely to allow them to buffer intracellular Ca2+ ion concentrations and prevent the induction of apoptosis.

Damage caused by chemotherapy to WMTs could be reduced or prevented if the inflammation was reduced. Both FLX and INDO have anti-inflammatory actions and the beneficial effects they have in preventing WMT damage may be associated with this mechanism. Animal models have shown that FLX can protect hippocampal neurogenesis and cognition when given before and during chemotherapy (Lyons *et al.* 2012). However although many cancer patients are on FLX for depression, studies of its effect on cancer and cancer treatments indicate that it may have both pro and anti-cancer effects (Choi *et al.* 2017). For this reason chronic dosing of cancer patients before or during chemotherapy with FLX would need further investigation. In contrast INDO and other NSAIDS are known to have anti-cancer effects (Réti *et al.* 2008) making addition of INDO to a cancer treatment regime unlikely to cause problems. In general, based on the findings in this study, it seems that the administration of INDO in combination with 5-FU, normalized the levels of inflammatory cytokines such as COX-2 expression and reduced the deficits in myelin integrity. A summary of main results of this study highlighted in the **Figure 5.1.**

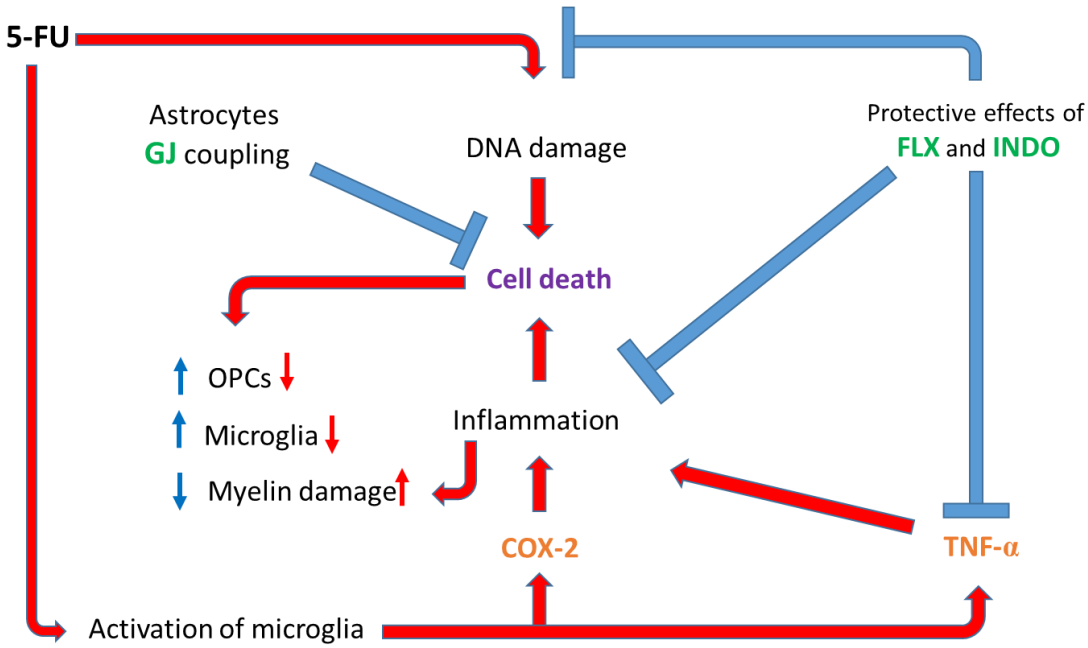



***Figure 5.1*** *Flow diagram illustrating the mechanism by which 5-FU exert the negative effects on OPCs and myelin , and the potential mechanisms that could FLX, INDO or GJ prevent the effects of 5-FU.*

## 5.6 Conclusion and future works

This study builds on previous studies in understanding the impact of systemic chemotherapy on the CNS. Therefore, the present investigation was designed to determine the effects of 5-FU on white matter. It also set out to determine whether treatment with FLX or INDO or the various cells and their interactions including astrocytes, microglia and endothelial cells in supporting OPCs could abolish the adverse effects of 5-FU on cell proliferation and myelin.

One of the findings to emerge from this study is that both single and multiple injections of 5-FU produced a significant inflammatory response which was present both at 24 hours and one week from last chemotherapy injection. It was also shown that chronic 5-FU exposure caused a decrease in proliferating, OPCs and the numbers of microglia in both ON and CC; while DNA damage was observed one week after the last injection. These results indicate that chemotherapeutic agent have cytotoxic effects on progenitor cell populations in the WMTs. While there was no change in the number of myelinated axons after 5-FU treatment, there was a significant reduction in myelin thickness in both ON and CC. Co-administration FLX or INDO before, during and after chemotherapy injections could counteract some of these adverse effects.

Using an *in vitro* model it was shown that astrocytes and endothelial cells could support OPCs after treatment with 5-FU. Investigations showed that gap junction coupling between these cells is one of the factors in supporting cell viability. Results showed that 5-FU although killing many microglia was a potent microglial activator that induced TNF-α release after 24 and 48 hours of incubation. FLX or INDO treatment inhibited microglial activity with 5-FU treatment.

This study provides a new understanding of the side effects of systemic chemotherapy on the WMTs and provides a foundation for future studies in the prevention of cognitive impairments due to cancer treatment. Further research needs to examine more closely the links between the molecular pathways of DNA damage and OPCs death induced by chemotherapy and the role of antidepressant and anti-inflammatory treatments in giving protection to WMTs. This would facilitate a better understanding of the mechanisms behind the effects of these treatments to protect cells and remyelination in damaged areas.

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