IMPACT OF CHEMOTHERAPY ON HIPPOCAMPAL NEUROGENESIS AND POTENTIAL PROTECTIVE STRATEGIES

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Abstract

Background and objectives: An increasing number of cancer patients have described experiencing cognitive dysfunctions which are associated with their chemotherapy treatment. This has been called “chemobrain” in patient forums and articles. It has been shown in animal models treated with a range of chemotherapy agents that chemotherapy induces cognitive deficits and a decrease in hippocampal neurogenesis. Antidepressants, anti-inflammatory drugs and physical activity have been proposed to counteract the effect of chemotherapy and provide protection against cognitive impairment associated with cancer treatments. This thesis has used in vivo studies, in rats and Sox1-GFP transgenic mice to firstly evaluate the effect of acute and chronic 5-Fluoruracil (5-FU) treatment on different subpopulations of neural stem cells in the hippocampus. Secondly, the effect of prior treatment with the antidepressant fluoxetine and the anti-inflammatory indomethacin on the impact of chronic treatment with chemotherapy was measured in terms of their effect on cell proliferation, inflammation and DNA damage. The effect of prior physical exercise on acute treatment with 5-Fluoruracil was measured in terms of cell proliferation and differentiation.

Methods: Animals were administrated either acute (single injection) or chronic (4 to 6 injections) of 5-FU with or without fluoxetine and indomethacin or physical exercise. Cell proliferation (Ki67), early neural differentiation (DCX), neural stem cells markers (GFAP, Sox1), inflammation (Iba1, Cox2) and DNA damage (γ-H2AX) were quantified by immunostaining either 24hr or 1 week after the last injection of chemotherapy. Morphological studies, to differentiate different stages of neural stem cells development and the state of microglia activation were conducted using confocal imaging.

Results: Acute treatment of Sox1-GFP mice with 5-FU caused a decrease in cell proliferation while chronic treatment induced a depletion of the neural stem cell pool and a reduction of the number of microglia. Prior chronic treatment with indomethacin did not prevent the decrease in the number of neural stem cells but did prevent the decline in cell proliferation and microglia. Rats chronically injected with chemotherapy and allowed to recover for 1 week, showed a decreased proliferation and number of microglia, an increased microglia activation, Cox-2 expression and DNA damage. Prior chronic treatment with fluoxetine prevent the decrease in proliferation and number of microglia, the increased microglia activation and DNA damage. Prior treatment with fluoxetine and indomethacin prevented these effects. Physical exercise prior to acute treatment with 5-FU enhanced the overall amount of neurogenesis but, possibly, due to a low dose of chemotherapy it was not possible see a significant effect of the chemotherapy.

Conclusions: The chemotherapy agent 5-FU affected the neural stem cell pool and the number of proliferating cells in the hippocampus. Chronic administration with fluoxetine can prevent the decrease in the number of proliferating cells, microglia activation and DNA damage while treatment with Indomethacin protects only against the effect of 5-FU on proliferating cells and microglia activation.

Presentations and courses

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Abbreviations

5-FU 5-Fluorouracil

5-HT Serotonin

ANOVA Analysis of variance

APOE Apolipoprotein E

BBB Blood brain barrier

BDNF Brain derived neurotrophic factor

BLBP Brain Lipid binding protein

BrdU 5-Bromo-2-deoxyuridine

BSA Bovine serum albumin

CA Cornu Ammonis

CICI Chemotherapy induced cognitive impairment

CldU Clorodeoxyuridine

CNS Central nervous system

COMT Catechol-o-methyl transferase

COX Ciclooxygenase

CREB cAMP response element binding protein

DAPI 4',6-diamidin-2-fenilindolo

DCX Doublecortin

DG Dentate gyrus

DNA Deoxyribonucleic acid

ECG Electrocardiography

EEG Electroencephalography

EPO Erythropoietin

FACS Fluorescence-activated cell sorting

FGF Fibroblast growth factors

FLX Fluoxetine

GABA Gamma aminobutyric acid

GFAP Glial fibrillary acidic protein

GFP Green Fluorescent Protein

HPA Hypothalamic pituitary adrenal axis

ICCTF International Cancer and Cognition Task Force

IdU Iododeoxyuridine

IGF1 Insulin growth factor

iNOS Nitric oxide synthases

LCV Leucovorin

LPS Lipopolysaccharide

LTP Long term potentiation

MHC Major Histocompatibility complex

MRI Magnetic resonance imaging

MTX Methotrexate

NGF Neural growth factor

NMDA N-methyl-D-aspartate

NPC Neural progenitor cell

NSAID Nonsteroidal anti-inflammatory drugs

NSC Neural stem cell

PBS Phosphate buffered saline

PCNA Proliferating cell nuclear antigen

PET Positron Emission Tomography

PFA Paraformaldehyde

PG Prostaglandin

PKA Protein Kinase A

PSA-NCAM Polysialylated-neural cell adhesion molecule

RNA Ribonucleic acid

ROS Reactive oxygen species

SEM Standard error of the mean

SGZ Subgranular zone

SSRI Selective serotonin reuptake inhibitor

SVZ Subventricular zone

TLR Toll Like Receptor

TNF-α Tumour necrosis factor

VEGF Vascular Endothelial Growth factor

Chapter 1 – GENERAL INTRODUCTION

1.1What is “Chemobrain”?

Progress in cancer treatments and regimes has led to a higher survival rate in a considerable number of cancer types. This has been in part due to new chemotherapy agents and improved delivery protocols. The growing number of cancer survivors, however, has led to an increased number of patients who can experience the chronic side effects of chemotherapeutic agents. Cognitive dysfunction is one of the commonest long term adverse effects of systemic chemotherapy among cancer survivors and chemotherapy-induced cognitive impairment (CICI) has been called “chemobrain” or “chemofog” by patients. This cognitive dysfunction is characterized by the impairment of patients’ concentration, attention, memory, learning, processing speed, executive functions and visual-spatial skills (Argyriou et al., 2011b, Vardy et al., 2007, Dietrich et al., 2008, Ahles and Saykin, 2007, Wefel and Schagen, 2012). It has been described in patients treated for a variety of different types of tumour including breast, ovarian, lung, prostate and colon cancers (Sioka and Kyritsis, 2009). A summary of the clinical data from previous studies shows that CICI affects a significant proportion of cancer survivors. Estimates in the literature of the incidence varies from 16% to 75% a variation which is probably due to the different study populations, types and dosage of chemotherapy and methods used to asses CICI (Vardy and Tannock, 2007, Ahles and Saykin, 2007). Many patients who experience cognitive impairment find that it can last from 6 months to 20 years after treatment with chemotherapy (Tannock et al., 2004, Vardy et al., 2008, Joly et al., 2015). Longitudinal studies, which test the same patients before, during and after chemotherapy have reported that up to 30% of patients show cognitive dysfunctions prior any cancer treatment; up to 75% shows CICI during treatment with chemotherapy and up to 35% experience CICI months or years after the end of treatment (Janelsins et al., 2011). Symptoms of CICI significantly affect the

quality of life of patients who find difficulties when they return to work and attempt to carry out the normal processes of everyday life (Vardy et al., 2007, Collins et al., 2013, Munir et al., 2011, Boykoff et al., 2009). Qualitative interviews with breast cancer patients have indicated that these women as well as having problems when they return to work also do not feel confident in social situations. Cognitive impairment, especially deficits in memory and executive functions caused by chemotherapy treatment, have been correlated with reduced productivity and community engagement (Reid-Arndt et al., 2009). Many patients also describe difficulties in driving and reading. CICI clearly has a huge impact on work, social networks, quality of life and the activities of daily life of cancer survivors (Myers, 2012). Because of only a subgroup of patients notices post chemotherapy treatment cognitive symptoms, it is necessary to examine the risk and possible confounding factors which cause some patients, but not others, to experience this effect. A prospective longitudinal study by Ahles et al, 2010, found that age and a lower pre-treatment level of cognition, termed cognitive reserve (influenced by education, occupation and lifestyle) are risk factors for post chemotherapy cognitive decline (Ahles et al., 2010). Genetic factors are also a well-established risk factor for cognitive impairment. This has been demonstrated in an analysis by Ahles et al, 2003, where long-term survivors who were carriers of ε4 allele for APOE, which is a genetic risk factor associated with other cognitive dysfunctions like Alzheimer's disease (Wefel and Schagen, 2012) scored lower on cognitive tests after chemotherapy when compared with patients without the ε4 allele (Ahles et al., 2003). Additionally carriers of the val allele, within the catechol-o-methyl transferase (COMT) gene, who metabolize dopamine more rapidly than carriers of the met allele, performed lower on cognitive tests after treatment with chemotherapy (Small et al., 2011).

Lifestyle and medical co-morbidities as well as emotional distress, fatigue and other treatments being given could be potential confounding factors of CICI (Argyriou et al., 2011a). The emotional distress associated with the cancer diagnosis and the administration of chemotherapy may affect mood, which could in turn change sleep habits and other physiologic functions (Patrick et al., 2004). In a study of breast cancer patients who received chemotherapy or radiation treatment, pre-treatment apprehension was correlated with alterations in brain and cognitive functions in both treatment groups (Berman et al., 2014). Similarly, in several studies of women undergoing adjuvant chemotherapy for breast cancer, the authors report that distress, anxiety or poor lifestyle are correlated with self-perceived cognitive dysfunction but not with neuropsychological test results (Jansen et al., 2011, Tager et al., 2010, Biglia et al., 2012). Other confounding factors consist of fatigue, effects of the anaesthetic and surgery, effects of treatments or medications given in association with chemotherapeutic drugs for example palliative medications dispensed to mitigate the side effects of chemotherapy such as anti-emetics and anti-inflammatory drugs (O'Farrell et al., 2013). However, as I shall discuss even when these factors are taken into account a significant proportion of patients experience long term CICI which can be ascribed to their chemotherapy treatment.

1.1.1 Clinical studies

Most studies of CICI have focused on breast cancer patients as these provide a more homogenous population which has frequently reported the symptoms of CICI and comprise almost 22% of cancer survivors in the USA (DeSantis et al., 2014). A few studies have been conducted in lymphoma, colorectal cancer, ovarian cancer, testicular cancer and prostate cancer patients (Ahles et al., 2002, Vardy et al., 2015, Correa and Hess, 2012, Schagen et al., 2008, Nelson et al., 2008). However, more longitudinal

studies are necessary in other disease groups to understand if the effect of chemotherapy is the same in every group of patients or is more marked in cases of specific diseases.

One of the first studies that supported an association between cognitive functional impairment and systemic chemotherapy treatment was conducted by Ahles et al, 2002. They evaluated the cognitive impact of chemotherapy regimes in long-term (>5 years post diagnosis) survivors of breast cancer and lymphoma. They compared survivors treated with local therapy and survivors who were treated with systemic chemotherapy and found that survivors who had been treated with systemic chemotherapy scored significantly lower on neuropsychological tests compared with those treated with local therapy only, particularly in the domains of verbal memory. Moreover, survivors treated with systemic chemotherapy, self-reported problems with working memory (Ahles et al., 2002).

While earlier studies were cross sectional and looked at time points after chemotherapy a study by Wefel et al, 2004, was one of the first important prospective longitudinal studies which compared pre-treatment levels of cognition with post treatment performance. Cognitive functions of 18 patients with breast cancer were evaluated thought a battery of neuropsychological test before treatment, 6 months from the baseline and 1 year post chemotherapy which consisted of a combination of 5-Flurouracil, doxorubicin, and cyclophosphamide. After analysis, they reported that 61% of patients at the 6 months point showed cognitive declines in attention, learning, and processing speed. Approximately 50% of patients who had declines at 6 months demonstrated improvement at one year post chemotherapy, whereas 50% remained stable. However, as mentioned previously they found that 33% of patients exhibited

cognitive impairment even before starting treatment and these suffered greater cognitive declines after treatment (Wefel et al., 2004b).

A critical issue in clinical studies is the method used to assess cognitive impairment. Cognition can be assessed subjectively with a self-reported questionnaire or objectively with performance-based neuropsychological tests. Subjective measures of cognitive impairment may correlate poorly with objective tests making it necessary include both methods (Biglia et al., 2012). The International Task Cognition and Cancer Task Force (ICCTF) has now established guidelines for study methodologies and have affirmed that objective neuropsychological tests remain the best standard for the assessment of cognitive dysfunctions (Wefel et al., 2011).

Retrospective cross sectional studies are able to identify differences between those patients that receive chemotherapy and those that have not received chemotherapy or non-cancer patients but could not distinguish chemotherapy induced cognitive impairment from pre-treatment cognitive dysfunctions related for example to cancer or other diseases. These kind of studies could wrongly over or under-estimate CICI. Overestimation occurs in studies where patients are assessed once after the end of chemotherapy treatment, in fact as prospective studies has shown, up to 35% of breast cancer patients show pre-treatment cognitive impairment that could have been incorrectly attributed to chemotherapy in cross sectional studies (Cimprich et al., 2010, Schilder et al., 2010, Wefel et al., 2004a). For these reasons, in most cases, the ICCTF suggests prospective longitudinal studies and advises the inclusion of a control population of cancer patients who did not receive chemotherapy and a healthy control group (Wefel et al., 2011).

Taking into consideration the reduced pre-treatment cognitive scores, many longitudinal studies have found that 10-45% of patients exhibit cognitive impairment associated with the chemotherapy treatment (Ahles et al., 2010, Biglia et al., 2012, Jansen et al., 2011, Wefel et al., 2010, Wefel et al., 2004a). Moreover, the subgroup of patients with a pre-treatment low cognitive score, seems to show greater cognitive decline after treatment than patients with a high pre-treatment cognitive score, suggesting more resistance in this last group. (Ahles et al., 2010).

It is recognized that not all studies that have investigated CICI have found significant cognitive deficits after chemotherapy treatment. The reason of this inconsistency between studies is unclear but could be related to the type of cancer and/or at the treatment received or the method testing cognition. However, the higher proportion of longitudinal studies demonstrating a significant incidence of cognitive impairment in patients treated with chemotherapy, with respect to other studies which did not find any significant effect, confirmed that CICI is now a widely accepted side effect of chemotherapy (Wigmore, 2013).

1.1.2 Imaging studies

As described above, clinical research supports the existence of "chemobrain" in cancer patients and this has received further support from imaging studies which have shown the effect of chemotherapy on brain structure and function. In the recent past, magnetic resonance imaging (MRI) has been used to detect structural changes including a reduction in the thickness and volume of grey matter in frontal cortex and hippocampus together with changes in white matter in chemotherapy-treated patients (Inagaki et al., 2007, Abraham et al., 2008, Koppelmans et al., 2012b, de Ruiter et al., 2012, Deprez et al., 2011). Longitudinal studies have shown an alteration in grey matter density in

bilateral frontal, temporal and cerebellar regions and right thalamus after 1 month from the end of the chemotherapy treatment (McDonald et al., 2010, McDonald et al., 2012). Deprez et al, 2012, studied the white matter connectivity using diffusion tensor imaging (DTI) in breast cancer patients before treatment and 4 months after receiving chemotherapy. These patients exhibit a significantly decreased frontal, parietal and occipital white matter integrity over time when compared with controls (Deprez et al., 2012). A great number of structural imaging studies have found that chemotherapy gives rise to white and grey matter volume reductions and changes in white matter microstructure. The regional nature of the changes observed has been accepted as consistent with the cognitive deficits experienced by cancer patients after chemotherapy treatment (Kaiser et al., 2014). Cognitive dysfunctions reported by patients, including learning and memory impairment, suggest the involvement of the hippocampus. A cross-sectional study conducted in breast cancer survivors reported a reduction in the volume of the hippocampus by 8% in patients when compared with controls (Bergouignan et al., 2011). Similarly Kesler et al, 2013, found a left hippocampal volume reduction (about 7%) in chemotherapy-treated patients 1-12 months after chemotherapy (Kesler et al., 2013).

Despite the availability of fMRI for 20 years, this method has been used only recently to elucidate possible changes in functional activation patterns related to the effects of chemotherapy on cognition. Some studies on cancer patients have shown regions of reduced or over activation during the execution of cognitive tasks. In particular, McDonald et al, 2012, conducted a longitudinal study on breast cancer patients scanned with fMRI before chemotherapy, 1 month after chemotherapy and 1 year later. Compared to controls, cancer patients showed over-activation of frontal regions to support a working memory task before treatment, reduced activation of this region 1

month after chemotherapy, and a return to over-activation 1 year post-chemotherapy. The increased activation was explained in terms of compensation for chemotherapy-related cortical dysfunction (McDonald et al., 2012). Data from fMRI studies has been supported by findings from complementary techniques such as arterial spin labelling, positron emission tomography (PET) and electroencephalography (EEG). In a recent study, Nudelman et al, 2014, using pulsed arterial spin labelling MRI perfusion, demonstrated that chemotherapy treated patients, when compared with controls, show a significant increased perfusion in the right precentral gyrus post-treatment and that this was negatively correlated with the neuropsychological performance (Nudelman et al., 2014). Silverman et al, 2007, used neuropsychological tests and PET to evaluate the effect of chemotherapy 5-10 years after treatment. They found that during a short term recall task, the blood flow to specific regions of frontal cortex and cerebellum was altered in chemotherapy treated cancer survivors when compared control subjects (Silverman et al., 2007). Recently a pilot study using EEG has been applied to breast cancer survivors with high cognitive complaints, especially those relating to language and communication domains. These patients showed an increased theta and delta brain waves frequency and temporo-parietal and occipital decrease in beta waves power. These EEG alterations were associated with the overall cognitive complaints (Hunter et al., 2014). Neuroimaging studies demonstrate that localised brain structural and functional changes are associated with specific cognitive dysfunctions, but at the moment it is not possible to identify, with these techniques, which mechanisms are involved in the chemotherapy-induced cognitive impairment. Further investigations are needed to directly compare the imaging characteristics of cancer survivors with the neurobiological and molecular alterations potentially engaged in the cognitive impairment.

1.1.3 Preclinical research

Clinical research benefits from animals models of chemotherapy treatment by allowing researchers to investigate mechanisms that cannot be studied directly in patients. The effect of chemotherapy has been examined in a great number of studies utilizing healthy rodents and these have demonstrated a link between chemotherapy treatment and cognitive impairment. Adverse neurobiological effects have been identified with all classes of chemotherapy agents including antimetabolites, alkylating agents, cisplatin and its analogues, topoisomerase interactive agents and anti-microtubule agents. The cognitive domains affected by chemotherapy in healthy rodents include spatial memory, object recognition and associative tasks (Fardell et al., 2013, Reiriz et al., 2006). Cognitive deficiencies have been recognized in behavioural tasks that involve the hippocampus and the frontal cortex such as the Morris Water Maze test, contextual fear conditioning, non-matching to sample learning, novel object recognition and novel object location (Mustafa et al., 2008, Winocur et al., 2006, Mondie et al., 2010, Fardell et al., 2010, Li et al., 2010, Seigers et al., 2009). Through preclinical studies, it has been possible to link data obtained in animal models of chemotherapy induced cognitive impairment with cognitive data obtained from clinical studies in cancer patients (Seigers and Fardell, 2011, Wefel et al., 2010). For this reason animal models of chemotherapy induced neural changes allows the evaluation of a number of potential mechanisms involved in CICI. There is now a large amount of evidence that many different chemotherapy agents have a negative impact on neurogenesis and gliogenesis (Dietrich et al., 2006, Elbeltagy et al., 2012, Lyons et al., 2011a, Elbeltagy et al., 2010, Han et al., 2008, Mondie et al., 2010, Mustafa et al., 2008, Nokia et al., 2012, Seigers et al., 2010, Seigers et al., 2008a, Seigers et al., 2009). In these studies it has been shown that neural progenitor cells and oligodendrocyte precursors are the populations of cells

most affected by chemotherapy treatment. Chemotherapy induced damage to neural progenitor cells has been recognized as an important mechanism involved in the long lasting effects on cognition. Many indirect mechanism have also been investigated in animal models, such as reduced metabolism and oxidative stress, reduced myelinisation, inflammation and disruption of blood vessel density (Seigers et al., 2013). Despite the huge progress made with animal research, it is remains unknown to what degree direct and indirect mechanisms play a role in the neurotoxic effect of chemotherapy. The combination of effects on different systems are involved in the induction of cognitive impairment and for this reason it is still difficult to discriminate between different mechanisms.

1.1.4 Neurobiological processes involved in cognitive impairment.

The pathogenesis of CICI in cancer patients is still unknown. The multifactorial character of symptoms suggests the involvement of different mechanisms that cause either direct or indirect toxic effects into the brain.

1.1.4.1 DNA damage and oxidative stress

Many chemotherapy agents used to treat cancer, perform their therapeutic action by producing DNA damage, leading to the apoptosis of cancer cells (Sedletska et al., 2005). Many of the neurotoxic side effects of chemotherapy are the result of DNA damage in normal cells, especially neuronal cells (Fishel et al., 2007). DNA damage could also affect mitochondrial DNA leading to reactive oxygen species (ROS) formation and oxidative stress, moreover most of the DNA damage in neuronal cells is caused by ROS production leading to a vicious circle (Ahles and Saykin, 2007). Furthermore, high levels of free radicals and a reduced antioxidant capacity have been associated with chemotherapy treatment (Kaya et al., 2005). High levels of oxidative

stress have been found after treatment with a number of cytostatic agent such as doxorubicin (Oz and Ilhan, 2006), methotrexate (Rajamani et al., 2006) and cyclophosphamide (Oboh and Ogunruku, 2010). To confirm the involvement of oxidative stress Konat et al, in 2008, found that cognitive impairment caused by chemotherapy treatment was prevented when rodents were co-treated with anti-oxidants (Konat et al., 2008, Kaiser et al., 2014). Oxidative stress and its induction of DNA damage has been shown also in patients prior chemotherapy treatment; this factor together with genetic polymorphisms in DNA repair mechanisms seems to be involved in a delayed recovery from chemotherapy induced DNA damage (Kaiser et al., 2014).

1.1.4.2 Inflammation

Chemotherapy agents can affect cognition indirectly by their activation of the immune system. Cytokines are released by activated immune cells in response to inflammation or direct injury to neurons. Additionally, they have important roles in CNS function, including the modulation of neuronal and glial cell functioning, neuronal repair and the metabolism of dopamine and serotonin, both of which are important neurotransmitters for normal cognitive function (Wilson et al., 2002). It has been shown that chemotherapy causes injury to normal tissues which in turn could lead to the release of cytokines (Jansen et al., 2005). In some studies on cancer patients, it has been shown that a standard dose of chemotherapy, specifically, paclitaxel and docetaxel is associated with an increased level of cytokines, such as IL-6, IL-8 and IL-10 (Tsavaris et al., 2002, Pusztai et al., 2004). Cytokines are relatively large and are not likely to be able to cross the BBB, however it was demonstrated in a previous study that there is significant communication between cytokines outside the CNS and cytokines in the brain. This communication is likely to be involved in the stimulation of central cytokine release (Wilson et al., 2002). Neuronal damage secondary to cytokine exposure can be

caused by various mechanisms including excitotoxic glutamate receptor-mediated damage and oxidative stress. In particular the increased levels of cytokines in the brain causes microglia activation and subsequent neuroinflammation which has been linked with cognitive impairment (Wilson et al., 2002).

1.1.4.3 White matter damage

Many cytostatic agents, such as 5-FU are particularly toxic for oligodendrocytes which form myelin in CNS. It has been shown that 5-FU deregulates Olig2 expression, a gene necessary for the generation of normal oligodendrocytes, causing delayed myelin formation which in turn causes impaired conduction that can persist for at least 6 months after chemotherapy treatment (Han et al., 2008, Dietrich et al., 2006). Other chemotherapy drugs such as MTX are associated with damage to white matter tracks that may cause the reduced speed of information processing observed in patients treated with this kind of chemotherapy (Seigers et al., 2009).

1.1.4.4 Neurogenesis

The main aim of anti-mitotic chemotherapy drugs is to inhibit the proliferation of dividing cells. Unfortunately, both cancer and normal cells with a high rate of proliferation are affected. Neural stem cells and progenitors cells of the neurogenic niche of the hippocampus are constantly dividing and are affected by cancer treatments which are able to cross the blood brain barrier. Drugs which can cross the blood brain barrier include the frequently used chemotherapy drug 5-Fluoruracil (Diasio and Harris, 1989). The hippocampus plays a crucial role in memory formation and learning (Scoville and Milner, 1957). Throughout life new neurons are continually added to dentate gyrus of the hippocampus in a process called adult neurogenesis. Neurogenesis has been identified as one of the major mechanisms involved in brain plasticity and is

important in learning and memory. This process in adult individuals occurs mostly in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus and in the subventricular zone of the lateral ventricles, both of which provide a stem cell niche (Kempermann et al., 2004). The SGZ stem cell niche regulates the proliferation and differentiation of neural progenitor cells into either neuroblasts or astrocytes and the rate at which these cell types are produced. Most neural stem cells are in a quiescent state but their division gives rise to neural progenitor cells with a high rate of proliferation, the majority of which differentiate into dentate gyrus neurons (Lugert et al., 2010). Adult hippocampal neurogenesis is strongly influenced by external environmental changes and the behaviour of neural progenitor cells is particularly sensitive. Given the range of cognitive dysfunctions seen in cancer patients, chemotherapy induced impairment of hippocampal neurogenesis has been proposed as an important mediator of CICI (Dietrich et al., 2008, Wigmore, 2013). Many preclinical studies have reported that both neural progenitor cell proliferation and cell survival decreases in mice and rats after treatment with many chemotherapy agents, including methotrexate (MTX) (Seigers et al., 2008a, Seigers et al., 2009) , 5-flurouracil (El-Beltagy et al., 2009, Elbeltagy et al., 2012, Mustafa et al., 2008), cyclophosphamide (Lyons et al., 2011a, Yang et al., 2010) and doxorubicin (Fremouw et al., 2012). The response of hippocampal neural stem cells to cancer treatment, may be related to the regimes and duration of chemotherapy administration. Acute treatments can lead to an immediate but transient decrease in cell proliferation followed by a recovery to normal proliferation levels in a short period of time (Mondie et al., 2010, Yang et al., 2010). In contrast repeated injections of chemotherapy drugs can affect neural stem cell proliferation in a prolonged and progressive way (Elbeltagy et al., 2012, Han et al., 2008). As well as affecting rates of cell division, treatment with 5-FU also reduced

levels of brain derived neurotrophic factor (BDNF) in the hippocampus (Mustafa et al., 2008). BDNF stimulates proliferation, differentiation and modulates cell survival in the hippocampus (Chen et al., 2013) and a decrease in this important factor after chemotherapy treatment, could provide an additional mechanism behind the decrease in neurogenesis (Wigmore et al., 2010).

1.1.4.5 Cancer

Most animal studies have been performed on healthy rodents to evaluate the effects of chemotherapy drugs on cognition and to understand the biological mechanisms involved. In an animal model of cancer, Seigers et al, 2010, demonstrated that the presence of a tumor, reduces the number of dividing hippocampal cells (Seigers et al., 2010). These results indicate a possible contribution of the cancer itself to the cognitive impairments experienced by patients' prior chemotherapy treatment.

1.1.5 Potential strategies to protect and reduce the side effects of cancer treatment on cognition

While a number of researchers and clinicians are continuing to characterize CICI and the biological mechanisms involved, it would be beneficial to develop protective strategies that might alleviate or counteract the development of cognitive impairment.

1.1.5.1 Pharmacological interventions

During the last 10 years, the glycoprotein erythropoietin (EPO) has been tested as a potential candidate to prevent CICI. EPO stimulates the production of red blood cells and it has been used in anaemic patients to increase haemoglobin levels and reduce the need for blood transfusions (Milano and Collomp, 2005). It has been shown that this drug can act as a neuroprotectant after trauma, stroke and epilepsy (Ponce et al., 2013,

Subiros et al., 2012). In these conditions, EPO reduces inflammation, oxidative stress and inhibits apoptosis. It has been demonstrated, in both human subjects and animal models, that EPO improves hippocampal dependent memory and hippocampal plasticity (Adamcio et al., 2008, Miskowiak et al., 2008). There have been three main studies in cancer patients where EPO has been tested to prevent or attenuate CICI. After a couple of weeks post treatment it was found to increase haemoglobin levels and to produce a small but significant improvement in cognitive functions, suggesting that EPO may be a possible treatment (Chang et al., 2004, Iconomou et al., 2008, Mancuso et al., 2006, Massa et al., 2006). However, the use of EPO in some cancer types decreased patient survival, due to thrombolytic problems and increased growth of their tumours, making the use of EPO to protect cognition as part of their cancer treatment unlikely (Oster et al., 2012).

A number of psychopharmacologic agents may have the potential to decrease or prevent cognitive impairment in cancer patients as they have increased cognitive ability in other situations. However treatments with the psychostimulant drugs methylphenidate or modafinil have produced conflicting results. Methylphenidate (MPH) is commonly used to treat attention deficit, hyperactivity and occasionally has been used to relieve cancer related fatigue (Wagner and Cella, 2004). MPH modulates the catecolaminergic tone in the prefrontal cortex and striatum preventing the reuptake of dopamine and noradrenalin (Volz, 2008). MPH works as stimulant and enhances memory in healthy people (Repantis et al., 2010). Gagnon et al, 2005, found that treatment with MPH in cancer patients improved attention, language and psychomotor dysfunction associated with hypoactive delirium (Gagnon et al., 2005). Interestingly, it has been shown that this drug increased cell proliferation and neuroblast differentiation in the SGZ of adolescent rats. A result which is likely to be due to its action in enhancing levels of

BDNF (Lee et al., 2012). However, two randomized, placebo-controlled studies in cancer patients failed to find any beneficial effect of this drug treatment on cognition (Lower et al., 2009, Mar Fan et al., 2008). Modafinil is a drug typically used to treat narcolepsy and sleep disorders. Modafinil acts by modulating the release of noroepinephrine, dopamine and histamine (Minzenberg and Carter, 2008). In healthy individuals, modafinil improves attention while in cancer patients it improves psychomotor speed, wakefulness and reduces fatigue (Wagner and Cella, 2004, Lundorff et al., 2009). Two studies in cancer patients have shown an improvement in memory and attention but more studies are necessary to confirm these promising preliminary results (Lundorff et al., 2009, Kohli et al., 2009).

Two other drugs, donezepil and fluoxetine, which are widely used to treat psychiatric and neurological disorders have been tested to counteract chemotherapy induced cognitive impairment. Donezepil is a cholinesterase inhibitor that reduces the progression of dementia caused by Alzheimer's disease and plays a neuroprotective role in reducing inflammation, reinforcing neuroplasticity and increasing hippocampal neurogenesis (Jacobson and Sabbagh, 2008, Kotani et al., 2008). It has been shown that this drug improves cognition in patients with Alzheimer disease and in ageing individuals after brain injury (Herrmann et al., 2011, Hernandez et al., 2006, Zhang et al., 2004). Winocur et al, 2011, investigated the potential cognitive enhancing effect of donezepil in mice treated with a combination of the chemotherapy drugs MTX and 5-fluorouracil. This work showed an attenuation of the cognitive deficits in mice co-administrated with chemotherapy and donezepil (Winocur et al., 2011). However, more studies in cancer patients are necessary to evaluate the effect and the mechanisms underlying the action of this drug on cognition. Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) commonly used in the treatment of depression. It increases

levels of serotonin in the brain by inhibiting the serotonin transporter. It has been reported that fluoxetine improves memory in patients with depression and also patients affected by neurodegenerative diseases. Furthermore, animal studies have demonstrated that fluoxetine up-regulates brain-derived neurotrophic factor, BDNF and increases the rate of hippocampal neurogenesis (Mostert et al., 2008). Work in our laboratory has shown that fluoxetine administrated before and during chemotherapy treatment, with either MTX or 5-FU, prevented the memory dysfunctions and decreased cell proliferation found after chemotherapy treatment (Lyons et al., 2012, Elbeltagy et al., 2010). Both donezepil and fluoxetine are well tolerated and commonly co-administrated with chemotherapy to cancer patients to treat other conditions such as depression and fatigue (De Waele and Van Belle, 2010, Roscoe et al., 2005), suggesting that these drugs could be used to protect against CICI. However, not all antidepressants can be used to treat CICI due to their interaction with antineoplastic agents, resulting in increased side effects or reduced efficacy of the anti-cancer drug (Miguel and Albuquerque, 2011). Several animal studies have concentrated on the identification of novel pharmacological treatments to prevent CICI, these include stimulating neurogenesis with insulin like growth factor-1 (Konat et al., 2008) or glucose (Dubois et al., 2014) or treating patients with a glutamate receptor antagonist such as memantine (Cole et al., 2013). Interestingly, co-treatment with memantine in rodents intrathecally injected with MTX, resulted in protection against the development of spatial memory deficits (Cole et al., 2013).

As said above, high levels of cytokines have been associated with cognitive dysfunctions and dementia in the normal and aged population (Dik et al., 2005). Targeting cytokines with pharmacologic interventions has been shown be useful in the treatment of Alzheimer's disease in clinical studies and ameliorates cognitive

dysfunction in animal models. The TNF-alpha inhibitor, etacernet, improved cognition in Alzheimer's patients (Griffin, 2008) and treatment with non-steroidal anti-inflammatory drugs has led to improved water maze performance and reduced hippocampal inflammatory markers in animal models of aging (Casolini et al., 2002). At the moment no non-steroidal anti-inflammatory drugs or cytokines inhibitors have been tested to treat CICI, but targeting neuroinflammation could be beneficial for the prevention and treatment of cognitive dysfunctions in cancer patients.

1.1.5.2 Antioxidants and diet

As described above, several commonly used chemotherapy agents can induce oxidative stress in animal models (Seigers and Fardell, 2011). However, is has been demonstrated that consumption of antioxidant foods or supplements slows the degree of cognitive dysfunctions associated with aging or disease in humans and animal models (Joseph et al., 2009). The number of clinical studies on the potential protective effect of antioxidants against the side effects of chemotherapy is very low, but preclinical studies have found that anti-oxidant treatment prevents the onset of oxidative stress after chemotherapy treatment when administrated prior or during chemotherapy treatment. Helal et al, in 2009, found that treatment with the anti-oxidant zinc sulphate, before the administration of the chemotherapy agent carmustine, prevented memory impairment, hippocampal cell death and inflammation (Helal et al., 2009). Results from preclinical studies suggest the usefulness of anti-oxidants in preventing cognitive impairment, but it will be necessary to evaluate their action in cancer patients receiving chemotherapy. The impact of diet on cognitive functions in human and animals has been well documented. Diets low in calories and saturated fat, combined with the consumption of food high in anti-oxidants, are linked with improved cognitive functions during aging (Joseph et al., 2009). It has also been observed that a reduction in calorie intake by 30-

40% increases neurogenesis (Stangl and Thuret, 2009). In rodents, a diet rich in anti-oxidants increases hippocampal neurogenesis and levels of neurotrophic factors such as insulin growth factor 1, together with improved performance during learning and memory tasks (Casadesus et al., 2004). The absence of data in cancer patients does not allow us to predict the effect of these diets on cognition after chemotherapy treatment but it is likely to be positive in terms of overall health benefits.

1.1.5.3 Physical exercise

Physical exercise is associated with improved cognitive functions in both human and animal studies (van Praag, 2009, Hotting and Roder, 2013). Physical activity programs seem to be effective in mitigating fatigue and cognitive dysfunction in cancer patients (Myers, 2012, McNeely and Courneya, 2010). Galantino et al, in 2012, found that an 8 week yoga program, after the end of chemotherapy treatment, enhanced cognition in breast cancer patients (Galantino et al., 2012). Fardell et al, found that rats treated with 5-FU and oxaliplatin show impairment in object recognition and spatial memory. 4 weeks of free access to a running wheel after chemotherapy treatment, prevented the cognitive dysfunctions mentioned above (Winocur et al., 2013, Fardell et al., 2012a). Physical exercise acts to prevent cognitive impairment through a number of neurobiological mechanisms, increasing levels of neurotrophic factors, enhancing proliferation and survival of neural stem cells in the hippocampus, decreasing neuroinflammation and oxidative stress and stimulating changes in the vasculature (Cotman et al., 2007, Voss et al., 2013b). Many other preclinical and clinical studies are necessary to determinate the overall beneficial effects of physical activity in patients with different kind of tumours and treated with different chemotherapeutic protocols.

1.2 Adult neurogenesis

1.2.1 The anatomy of the hippocampus

The hippocampus is an important region of the brain involved in the regulation of cognition, memory and emotional processes. It has been shown that the hippocampus is one of the few parts of the brain able to produce new neurons throughout the entire life of the individual. To understand the function and regulation of hippocampal neurogenesis it is necessary look at the structure of this brain region. The human hippocampus is located in the medial temporal lobe of the brain, below the inferior horn of the lateral ventricle. This region has been described as part of the archicortex and the human hippocampus extends along a rostral-caudal axis within the temporal lobe (Amaral and Witter, 1989). In rodents, the hippocampus is found in a more superior location and extends caudally and laterally as a "banana" shaped structure, it can be divided into a rostral portion lying medially behind the septum and a ventral portion lying in the temporal region of the brain (Muller et al., 1996). The hippocampus of all mammals is divided into four internal sub regions: the dentate gyrus (DG), Cornu Ammonis (CA), the subiculum and the pre-subiculum. The dentate gyrus in the hippocampal region is where adult neurogenesis occurs (Altman and Das, 1965) while the Cornu Ammonis (CA) can be divided into three sub regions CA1, CA2 and CA3 (Amaral and Witter, 1989). The dentate gyrus, a V shaped structure in cross section, which has two blades enclosing the hilum of the dentate gyrus. It is histologically characterized by having three layers which from outside to inside, called the molecular, granular and the polymorphic layers. The dentate gyrus itself consists of the granular layer which is characterized by the presence of small granule cell neurons which send their dendrites into the molecular layer where they synapse with the axons of pyramidal

neurons whose cell bodies are located in the layer II of the overlying entorhinal cortex (perforant pathway). Granule cell neurons of the dentate gyrus in turn send their axons towards the hilum where they can synapse with neurons of the polymorphic layer on the inside of the dentate gyrus or continue and synapse with pyramidal neurons in CA3 region (Amaral et al., 2007). (See Figure 1.1)

The hippocampal network can be described as synaptically connected neurons located in the three major sub regions namely the dentate gyrus, CA3 and CA1 to form a three-synaptic circuit. Information comes from the entorhinal cortex through the medial and lateral perforant pathways to the dentate gyrus. Subsequently the dentate gyrus sends information to CA3 pyramidal neurons via the mossy fibre axons of granule cells. Pyramidal cells of CA3 send projections to CA1 pyramidal neurons via Schaffer collateral projections which in turn send axons back to layer V of the entorhinal cortex. This hippocampal three-synaptic circuit forms a loop where sensory information from specific cortical areas, is combined in the entorhinal cortex. The sensory information is then processed by the hippocampal circuitry before information is returned to the entorhinal cortex (Deng et al., 2010).

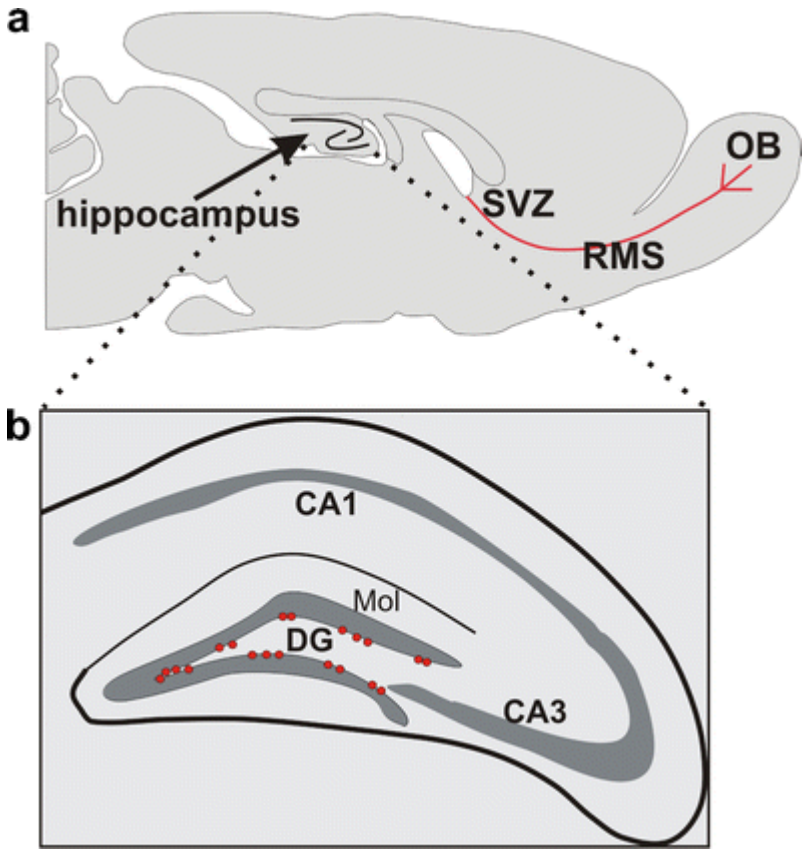


Figure 1.1 ***Adult neurogenesis and hippocampal anatomy***. ***a*** *Neurogenesis in the adult brain occurs in two main regions the subventricular zone (SVZ) in the lateral walls of the lateral ventricles and in the hippocampus.* ***b*** *Sagittal section of the adult hippocampus, the progenitor cells (red) are located in the subgranular zone (SGZ) on the inside of the blades of the dentate gyrus (DG). These cells proliferate and migrate into the granular layer of the DG and later extend their dendrites toward the molecular (Mol) layer of the DG and their axons towards the CA3 region (CA1, CA3; subdivisions of the CA region of the hippocampus). Representation of a coronal section (von Bohlen und Halbach, 2011).*

1.2.2 A brief history of adult neurogenesis

For over a century, scientists had believed that in adult mammals, no new neurons were produced and if nerve cells in the brain were damaged or died, unlike other cells in the body, they were not replaced. This dogma was first put forward by Ramon y Cajal at the start of the 20th century. The first evidence for the generation of new neurons in the adult mammalian brain came from Joseph Altman's studies in the 1960s. These used radioactive thymidine, which was incorporated into newly synthesised DNA, to label dividing cells. Examination of sections several months after the application of

radioactive thymidine demonstrated, that in rats, newly formed cells within the hippocampus appeared to differentiate into neurons (Altman and Das, 1965, Altman, 1962). A lack of neuron specific markers at this time however made identification of these cells uncertain and many scientists were still unconvinced that these new cells were neurons and suspected that the new cells might be glial cells or that the incorporation of thymidine was the result of DNA repair in cells which were not dividing. Despite these pioneering findings, Altman's results received little attention and were essentially ignored by the scientific community. Years later Kaplan and colleagues provided further evidence for adult neurogenesis from ultra-structural studies using electron microscopy. These studies demonstrated that the [^{3}H]- thymidine used by Altman, labelled cells with an ultra-structure distinctive of neurons such as dendrites and synapses (Kaplan and Hinds, 1977). Complete acceptance of adult neurogenesis arrived many years later with methodological improvements, which included the use of bromodeoxyuridine (BrdU) to label dividing cells and immunohistochemistry to identify neurons, which made it possible to prove the formation of new neurons in the adult brain (Kempermann and Gage, 1999). During 1998**,** a team of Swedish researchers under Peter Eriksson and Fred H. Gage in the USA published a study demonstrating that new neurons are generated in the dentate gyrus of the adult human brain. The study also showed that these new neurons were produced from stem cells, which then migrated through the granular layer and differentiated into mature neurons with axons and dendrites (Eriksson et al., 1998).

1.2.3 Evidence of adult neurogenesis in different brain regions

Several studies have shown that adult neurogenesis occurs mainly in two brain regions: in the subventricular zone (SVZ) of the lateral ventricle and the subgranular (SGZ) of the dentate gyrus of the hippocampus. New cells migrate from the SVZ to the olfactory

bulb via the rostral migratory stream, where they differentiate into neurons which integrate into the existing olfactory circuity replacing cells which are continually being lost. These new neurons play a role in short-term olfactory memory, long-term associative olfactory memory and olfactory fear conditioning (Ming and Song, 2011). Similarly, new born cells in the SGZ migrate the much shorter distance into the granule cell layer of the dentate gyrus of the hippocampus. Here the differentiated granule cell neurons of the dentate gyrus integrate into pre-existing circuits where they provide a cellular basis for neural plasticity (Christie and Cameron, 2006). Hippocampal neurogenesis appears to be different from that involved in olfaction in that there is little cell death of mature neurons in the dentate gyrus and the new cells add to circuits rather than replacing lost cells. It has been shown that the adult-generated neurons of the DG play a role in spatial memory, trace conditioning and contextual fear conditioning (Ming and Song, 2011). The majority of adult neurogenesis studies have focused on these two main neurogenic brain regions (olfactory and hippocampal) but there is increasing evidence suggesting that lower levels of adult neurogenesis occur in other brain regions (Migaud et al., 2010). New born neurons have been described in the neocortex (Cameron and Dayer, 2008), striatum (Inta et al., 2015) and numerous limbic structures essential to mediate social behaviour including the amygdala (Lieberwirth et al., 2016), together with the hypothalamus and medial preoptic area (Kokoeva et al., 2005, Huang et al., 1998).

1.2.4 Cognitive behaviours dependent on hippocampal function

The first evidence of the role played by the hippocampus in human learning and memory was described in studies of patients, where damage or removal of the hippocampus lead to memory dysfunction (Scoville and Milner, 1957). Later studies reported that the hippocampus plays a crucial role in different kinds of memory

including contextual association memory, episodic and declarative memory and spatial memory (Squire, 1992, Rudy and Sutherland, 1995, Wixted et al., 2014, Lee et al., 2014). The dentate gyrus of the hippocampus receives information from the entorhinal cortex and sends excitatory projections to the pyramidal neurons of the CA3 region. The dentate gyrus shows structural and functional differences along the dorso-ventral axis where the dorsal region is involved in cognition and memory processing while the ventral region plays a role in mood and stress regulation (Kheirbek et al., 2013, Tannenholz et al., 2014). Additionally, the dentate gyrus is involved in the process of pattern separation and pattern completion, two essential mechanisms necessary for declarative memory (Yassa and Stark, 2011). Pattern separation is the ability to discriminate between similar experiences and representations, processing small changes in similar inputs. Pattern completion is a mechanism of memory retrieval that enables the completion and reconstruction of an incomplete representation (Treves et al., 2008). The pattern of mossy fiber connections from DG to CA3 is involved in the regulation of pattern separation (Rolls, 1996). The integrity of the perforant pathway, that connects the dentate gyrus with the entorhinal cortex, is critical for this memory retrieval (Rolls, 2007, Lassalle et al., 2000, Lee and Kesner, 2004)

1.2.5 Function of adult hippocampal neurogenesis

After the discovery of adult hippocampal neurogenesis, Altman proposed that the new born neurons were strictly involved in memory formation (Altman, 1967). However, the significant number of new neurons (9000 per day in the rat; (Cameron and McKay, 2001)), together with the regulation of adult neurogenesis by environmental and pathological stimuli (Ma et al., 2009, Cameron and Dayer, 2008), suggests that adult neurogenesis could be required in many other brain functions. It now appears that new hippocampal neurons are involved in other hippocampal-dependent functions such as

pattern separation (Aimone et al., 2011). To evaluate the relationship between neurogenesis and cognition, many researchers have focused their attention on the effect of enhanced or ablated neurogenesis on behavioural tasks. A recent study support a role for new born granule neurons, in mediating pattern separation as the impairment of hippocampal neurogenesis leads to defective mnemonic discrimination (Sahay et al., 2011). It has been also shown, in two behavioural tasks, that the ability of mice to discriminate stimuli with little spatial separation, but not stimuli widely separated in space is impaired after ablation of neurogenesis induced by irradiation (Clelland et al., 2009). Recently it has been reported that adult hippocampal neurogenesis is implicated in other aspects of contextual and spatial memory (Deng et al., 2010). The connections of the hippocampus to other brain regions such as prefrontal cortex, suggests that adult hippocampal neurogenesis is involved in memory consolidation and the reorganization of memory traces (Kitamura et al., 2009). New born neurons show special properties such as hyper-excitability and augmented synaptic plasticity during the period of neural maturation. This, allows the newly integrated neuron to show enhanced plasticity and to take part in information elaboration and to support spatial memory development (Ming and Song, 2011, Kee et al., 2007).

1.2.6 Adult hippocampal neurogenesis

Adult neurogenesis is a complex process, composed of different phases, all of which are necessary for the generation and integration of new neurons. Adult hippocampal neurogenesis occurs only in the SGZ of the dentate gyrus in which are generated new granule cell neurons together with a small number of glial cells. Granule cell neurons are the principal excitatory neurons of the dentate gyrus and adult generated cells are indistinguishable from cells formed during development. The process of neurogenesis is controlled by a number of intrinsic and extrinsic factors which vary the number of new

cells which are produced (Kempermann et al., 2015). The process starts with large pluripotent, relatively quiescent, neural stem cells which extend an apical process across the width of the dentate gyrus but have their cell bodies adjacent to the SGZ (defined as a narrow band of tissue between the granule cell layer of the DG and the hilum). Neural stem cells are able to divide symmetrically or asymmetrically producing respectively, two new neural stem cells or a stem cell and an amplifying neural progenitor cell. Amplifying neural progenitor cells have a high rate of proliferation and form clusters of dividing cells in the SGZ. After a couple of weeks, the proliferation of this population of cells ceases and cells committed to becoming neurons start to differentiate into neuroblasts. Neuroblasts at this point are able to migrate from the SGZ into the granule cell layer of the DG where they continue their maturation. The new born granule cells during the maturation phase extend their dendrites into the molecular layer and send their axons to contact CA3 pyramidal neurons, integrating into the existing hippocampal network (Ming and Song, 2011).

Kempermann et al (Kempermann et al., 2004), have proposed a model which divides hippocampal neurogenesis into four developmental phases, identified on the basis of morphology, level of proliferative activity and expression of specific markers. The four phases suggested by Kempermann are:

- The precursor cell phase
- The early survival phase
- Postmitotic maturation phase
- Late maturation phase

1.2.6.1 The precursor cell phase

During this phase, the pool of stem cells, undergoes an expansion. The stem cells found within the SGZ show morphological and antigenic characteristics of radial glia. For this reason they are called radial glia like neural stem cells or Type-1 cells. Their round-triangular cell bodies are located in the SGZ, and as previously mentioned, they extend a single apical process through the granule cell layer which arborizes in the molecular layer (Seri et al., 2004) (See Figure 1.2). Type-1 cells have electrophysiological characteristics of astrocytes and express GFAP (glial fibrillary acidic protein), vimentin and brain lipid-binding protein (BLBP), markers typically shown by astrocytes (Filippov et al., 2003). Type-1 cells also express markers of neuro-epithelial stem cells such as nestin, Sox2 and Sox1 (Encinas and Enikolopov, 2008). These markers however are not specific for neural stem cells in the DG and it is necessary to use other tools to identify different subpopulations of neural stem cells. Type-1 cells have a low rate of proliferation, in fact only 1-2% of cells in the SGZ are in a state of cell division at any one time. Recently, a subpopulation of neural stem cells with a radial morphology has been described in the SGZ (Lugert et al., 2010). These cells are able to generate neurons, astrocytes and radial astrocytes (Suh et al., 2007) and are capable of switching between quiescence and an activated state in which they divide. The coexistence in the SGZ of quiescent and activated neural stem cells has been further investigated using a downstream target of the canonical Notch signalling pathway, the transcription factor Hes5, to label type-1 cells of the SGZ. Two subpopulations of neural stem cells were identified: the first is in a quiescent state and the second expresses the proliferating cell nuclear antigen (PCNA) marker and is believed to be in an activated state (Lugert et al., 2010). Both populations of neural stem cells are capable of self-renewal but it has been demonstrated that their response to intrinsic and

extrinsic stimuli is widely different. Quiescence has been proposed as a mechanism to protect the stem cell pool and prevent its depletion under physiological conditions. In addition, during the quiescent state the number of DNA mutations which can be accumulated during the cell cycle, can be kept low (Rolando and Taylor, 2014). Different mechanisms control the balance between quiescence and entrance into the cell cycle; the transcription factor Sox1 is believed to be a component involved in the activation of neural stem cells (Venere et al., 2012). Once the cells cease to be quiescent, type-1 cells can generate other type-1 cells, non-radial precursors, type-2 cells with a radial morphology or astrocytes. Non-radial precursors lack any radial process and elongate their extensions parallel with respect to the SGZ/granule cell boundary. These precursors express Sox2 and Sox1 but not GFAP, they are more proliferative than radial neural precursors (type-1) but they are not always in the cell cycle. This population of cells has been proposed as the major source of new neurons (Bonaguidi et al., 2016). As a result of activation, type-1 cells divide several times asymmetrically or symmetrically before terminally differentiating into astrocytes (Encinas et al., 2011). If type-1 cells divide with a mitotic cleavage plane which is horizontal (parallel to the SGZ) they generate a daughter cell located close to the type-1 cell. These cells are nestin positive and are called type-2 cells or intermediate progenitor cells (Kempermann et al., 2004); if type-1 cells divide symmetrically they produce two type-1 cells. Type-2 cells have a round cell body and extend their short processes horizontally with respect to the SGZ and have a high rate of proliferation (Filippov et al., 2003). It is possible to divide this group of cells into two subpopulations: Type-2a or early amplifying progenitor cells which still express several markers of radial glia (Steiner et al., 2006) and Type-2b or late amplifying progenitors cells which although having the same morphology, lack the expression of radial glia

markers (Wang et al., 2011) (See Figure 1.2). Type-2 cells undergo on average of 2.5 symmetrical divisions to generate further Type-2 cells before exiting from the cell cycle and starting to differentiate into type-3 neuroblasts (Kempermann et al., 2004).

1.2.6.2 The early survival phase

The exit from the cell cycle by type-3 cells marks the start of the early survival phase. Neuroblasts or type-3 cells are precursors committed to becoming neurons that express markers for migrating immature neurons such as doublecortin (DCX) and polysialic acid neural cell adhesion molecule (PSA-NCAM) as well as markers for mature neurons such as NeuN (Filippov et al., 2003, Seri et al., 2004) (See Figure 1.2). During this phase almost two third of new cells undergo apoptosis and are phagocytosed by microglia (Sierra et al., 2010). During this phase new born cells receive a GABAergic excitatory input which promotes their differentiation into neurons and synaptic integration (Tozuka et al., 2005). This GABAergic input is excitatory and only switches to being inhibitory when additional glutamatergic connections have been made (Ge et al., 2008).

1.2.6.3 Post-mitotic maturation phase

As neuroblasts start to differentiate, they undergo morphological changes involving extension of dendrites and axons. Within a week, spinal dendrites become evident and under the influence of a synaptic glutamatergic input, the new born neurons start to integrate into the existing hippocampal circuits (Encinas et al., 2013). During this phase, cells develop the morphology of hippocampal granule cells and start to extend their dendritic tree into the molecular layer on the outside of the dentate gyrus (Zhao et al., 2006).

1.2.6.4 Late maturation phase

Once glutamatergic connections have been made, new neurons show particular synaptic plasticity. New born neurons over the following 4-7 weeks develop the same electrophysiological characters as mature granule cells and become functionally indistinguishable from older granule cells (van Praag et al., 2002).

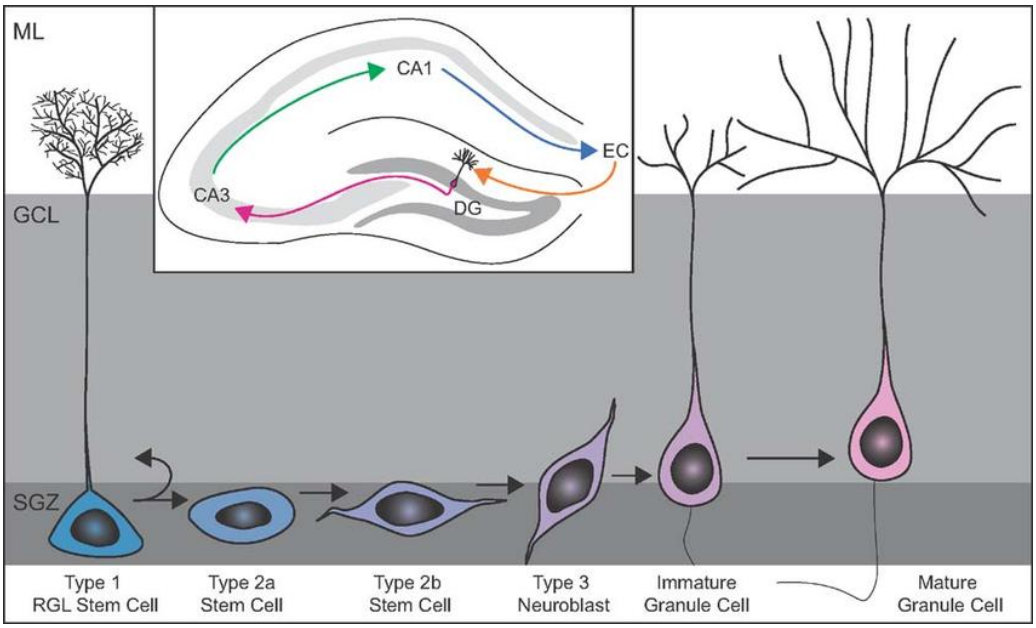


Figure 1.2. ***Neurogenesis in the adult hippocampus****. Type 1 stem cells (blue) extend a single dendritic process across the granule cell layer (GCL) that arborizes in the molecular layer (ML). Once activated, Type- 1 stem cells divide and differentiate into mature granule cells (pink) via a series of intermediate stages. Mature granule cells functionally integrate into the excitatory hippocampal circuit by receiving synaptic inputs from the entorhinal cortex (EC) and sending axonal projections to the CA3 region of the hippocampus, where they make synaptic contacts with pyramidal cells (Moss and Toni, 2013).*

1.2.7 The neurogenic niche

In the recent past, it has become clear that adult neurogenesis in the two main neurogenic regions, the subventricular zone of the lateral ventricle and the subgranular zone of the hippocampal dentate gyrus, is regulated by the local microenvironment around the cells which has been called “the neurogenic niche” (Palmer et al., 2000, Ma

et al., 2005). The neurogenic niche is able to house neural stem cells together with other cell types and extra cellular matrix which together control the behaviour of neural stem cells. The five major cellular components found in the neurogenic niche of the hippocampus are astrocytes, endothelial cells, microglia, neural stem cells and mature neurons. (Ma et al., 2005). In the SGZ, this includes the subpopulation of quiescent neural stem cells (NSCs) and their progeny, the transient amplifying progenitors and subsequently produced neuroblasts. Self-renewal, fate specification, maturation and integration of the regulatory factors and their interactions within the neurogenic niche controls stem cells functions (Ma et al., 2005).

Endothelial cells play an important role in controlling stem cells self-renewal. It has been observed that clusters of dividing cells, in the SGZ are anatomically close to blood vessels, especially capillaries. This suggests that blood vessels play a role in controlling and maintaining cell proliferation. Endothelial cells secret a number of growth factors including bFGF, IGF1, VEGF, PDGF, IL8 and BDNF which are known to be able to influence the differentiation and survival of neurons and their progenitors (Palmer et al., 2000). Thanks to these observations, it has been hypothesized that endothelial cells provide structural and trophic support to the dividing neural stem cells as well as differentiating neurons.

Astrocytes represent the most abundant cell population in the adult brain and provide structural and functional support to the surrounding cells as well as being able to modulate synaptic transmission (Horner and Palmer, 2003). As components of the neurogenic niche they are thought to be involved in the control of proliferation and fate specification of NSCs (Ma et al., 2005). In the SGZ, the secretion, by astrocytes, of regulatory factors including Wnt-3, specific cytokines and neurogenin1 has been recently described as a mechanism involved in the control of neural differentiation

(Barkho et al., 2006). Astrocytes are important also in later stages of neurogenesis such as the migration of neuroblasts into the dentate gyrus. In this phase, astrocytes have a supporting and guidance role as newly generate neurons migrate along the radial process of astrocytes to reach the granule cell layer of the dentate gyrus (Shapiro et al., 2005). Furthermore, astrocytic radial processes provide a scaffold for the axonal extension of new born neurons and supply nutrients and signalling factors (Jordan et al., 2007). However, the high levels of heterogeneity of cells with astrocytic characteristics and the absence of specific markers of neural stem cells has made it difficult to discriminate between NSCs with astrocytic characteristics and different subpopulations of astrocytes within the neurogenic niche (Horner and Palmer, 2003).

Microglia is another population of cells within the hippocampal niche which has been implicated in the regulation of neurogenesis. Microglia can have both supportive of detrimental effects on adult neurogenesis depending on their state of activation. Under healthy conditions microglia are in a resting state, in which they have a ramified morphology with a high number of fine processes and a small cell body (Glenn et al., 1992). Microglial processes are mobile and dynamic enabling microglia to scan the surrounding area to detect signs of damage or infection without disturbing neuronal networks (Nimmerjahn et al., 2005). In the adult hippocampus, resting microglia are involved in the regulation of neurogenesis through phagocytosis. New born cells which undergo apoptosis during the transition from amplifying neuroprogenitors to neuroblasts, are removed by microglia. (Sierra et al., 2010). Resting microglia show high phagocytic behaviour, suggesting that their activation it is not necessary for phagocytosis. Microglial phagocytosis, under normal physiological conditions, is essential for the maintenance of neurogenesis and prevents the transformation of apoptotic cells into necrotic cells which could lead to an inflammatory response,

potentially detrimental for neurogenesis (Sierra et al., 2010). Electron microscope imaging studies suggest that microglia may be involved in the regulation of the structure and function of dendritic spines and synapses. This is achieved by pruning processes which are important for the development of the appropriate connections of new born neurons (Ekdahl, 2012, Fortin et al., 2012, Paolicelli et al., 2011). Activation of microglia occurs after damage and in pathological states and will be dealt with in a subsequent section.

During the past few years the anatomical composition of the hippocampal neurogenic niche and the function of different types of cells has been described. However less is known about the molecular and cellular mechanisms by which every single component of the niche controls and regulates distinct phases of neurogenesis.

1.3 Regulation of adult hippocampal neurogenesis

1.3.1 Environmental factors and physical exercise

Previous studies have determined that different environmental factors can affect hippocampal neurogenesis. In particular, increased environmental complexity improved opportunities for social, cognitive, motor and sensory stimulation (van Praag et al., 2000). An enriched environment consists of various components (including social, sensory, cognitive and motor stimulation), but it has been shown by investigating separately every component of the enriched environment, that only the physical exercise component is associated with enhanced hippocampal neurogenesis (van Praag et al., 1999b). Van Praag et al, in their study showed an increase in cell proliferation and differentiation which led to a net increase in neurogenesis in mice housed with a free access to a running wheel for 2 weeks. The onset of the effect of physical exercise on neurogenesis is fast. The change in cell generation reaches a peak after three days

but after a month of running the pro-proliferative effect returns to the baseline (Kronenberg et al., 2006). The increase in cell proliferation and survival is the most observed effect found after exercise exposure (Brown et al., 2003, van Praag et al., 1999a). This effect can be produced at all ages, in young, adult and aged rodents (Marlatt et al., 2012a, van Praag et al., 2005). The effect of physical activity on neurogenesis is sustained throughout life in rodents. Indeed, in continuously exercised mice, from young to middle age, the age-related decrease of neurogenesis is less than in sedentary mice (Kronenberg et al., 2006) while in mice that start to do physical activity in the middle age or in the old age, the number of new born cells is elevated (Wu et al., 2008, van Praag et al., 2005). The enhancements of angiogenesis and neurogenesis by exercise seems to be closely related. Van der Borght and others have shown that in mice housed with a running wheel for 1, 3 or 10 days the voluntary physical exercise produced a rapid increase in blood vessel density and in the generation of new cells in the dentate gyrus of the hippocampus. After the cessation of running in these animals, the levels of both blood vessel density and cell proliferation returned to the levels seen in sedentary animals within a day. Taken together this data affirms that the hippocampus shows a rapid adaptation in response to the stimuli of running. However, the number of cells expressing neural proliferation and differentiation markers, such as Ki67 and DCX, constantly increases with a protracted running wheel access over a period of 10 days (Van der Borght et al., 2009). This suggests that a prolonged period of running is necessary for exercise-induced neurogenesis. Recent studies have demonstrated that the proliferative effect of running is transitory and that running mainly enhances neurogenesis by increasing the survival of newly dividing cells (Snyder et al., 2009, Clark et al., 2010, Fuss et al., 2010, Drapeau et al., 2007).

1.3.2 Learning

Interestingly, it had been previously reported that learning has positive effects on hippocampal neurogenesis (Gould et al., 1999b). In particular, hippocampal-dependent learning tasks, such as eye-blink conditioning, spatial navigation learning, and conditioned food preference, affect adult neurogenesis in the DG. In previous studies it has been shown that eye-blink conditioning and spatial navigation learning in the Morris water maze increase hippocampal cell proliferation (Dupret et al., 2008) and hippocampal neurogenesis (Gould et al., 1999a).

1.3.3 Antidepressant drugs

Recently, many studies have suggested that a decrease in hippocampal neurogenesis may be related to major depression (Bambico and Belzung, 2013). Many antidepressants have been shown to act as enhancers of hippocampal neurogenesis. For example, 14 or more days of treatment with the selective 5-HT reuptake inhibitor (SSRI) fluoxetine, increases the rate of neural progenitor proliferation and the survival of immature neurons in rats (Marcussen et al., 2008). The effect of these drugs on both hippocampal neurogenesis and depression is only observed after chronic but not acute administration. This time scale corresponds to the time (2-3 weeks) necessary for the differentiation of hippocampal progenitor cells into mature neurons (Encinas et al., 2006). Similar results have been shown in studies involving the SSRIs citalopram or escitalopram (Jayatissa et al., 2006, Jaako-Movits et al., 2006) but is not limited to SSRIs. Imipramine, a tricyclic antidepressant, has also been shown to increase both the proliferation and survival of new neurons (Surget et al., 2008). Many studies, have focused on the effects of fluoxetine, and have investigated if this drug acts on one particular stage of hippocampal neurogenesis. In 2006, Encinas and colleagues showed

that fluoxetine increased the proliferation of neural progenitor cells but was not able to affect the number of neuroblasts or immature neurons (Encinas et al., 2006). However, recently it has been reported that fluoxetine decreases the number of immature neurons and increases the number of mature ones, suggesting a role for this drug in the maturation of new neurons. Moreover, fluoxetine has been found to affect neural stem cell survival and serotoninergic differentiation through the modulation of Bcl-2 expression (Chen and Russo-Neustadt, 2007). Additionally, fluoxetine seems to have a role in the regulation of the functional characteristics of new hippocampal neurons by stimulating neurogenesis dependant long term potentiation (LTP) (Wang et al., 2008). The role of serotoninergic receptors in SSRI induced neurogenesis is still under investigation, but it has been shown that 5-HT1A receptors play a role in regulating the proliferation of neural progenitor cells, while 5-HT2 receptors are thought to be involved in regulating neural cells maturation (Klempin et al., 2010). Many mechanisms have been suggested for the pro-neurogenic effects of fluoxetine, including the increased production of the growth factor BDNF and its receptor TrkB (Castren and Kojima, 2017). Chronic treatment with fluoxetine seems to induce a decrease in p21 expression which is correlated with a subsequent increase in hippocampal neurogenesis. P21 is cyclin dependent kinase inhibitor, whose expression in neural progenitors in the DG is associated with a down-regulation cell division and neurogenesis (Pechnick et al., 2011).

1.3.4 Growth factors and neurotransmitters

In addition to exogenous factors, various intrinsic factors (such as morphogens, neurotrophic factors and neurotransmitters) have been reported to regulate hippocampal neurogenesis (Grote and Hannan, 2007, Lieberwirth et al., 2016). In particular, previous studies have shown that morphogens and developmentally associated receptors

including Notch, Shh, Wnts, and BMPs play a modulatory role in the maintenance, activation, and neural differentiation of neural progenitor cells (Ming and Song, 2011). Similarly neurotrophic factors, such as BDNF, insulin-like growth factor-1 (IGF-1) and VEGF seem to promote hippocampal cell proliferation (Lieberwirth et al., 2016). For example, chronic injections of BDNF into the hippocampus increases adult neurogenesis in rats (Scharfman et al., 2005). Similarly, the systemic and intracerebroventricular infusion of IGF-1 increases the proliferation and differentiation of neural progenitor cells in the DG of mice and rats (Aberg et al., 2000, Lichtenwalner et al., 2001). In addition, the central administration of VEGF upregulates hippocampal cell proliferation (Cao et al., 2004). In addition to neurotrophic factors, a number of different neurotransmitters have been found to be modulators of adult hippocampal neurogenesis. Interestingly, glutamate, the main excitatory neurotransmitter, negatively affects hippocampal neurogenesis. It has been reported that the administration of a NMDA receptor antagonist produces an increase in hippocampal cell proliferation in rats (Cameron et al., 1995). The activation of the gamma amino butyric acid (GABA), an inhibitory neurotransmitter, is able to regulate hippocampal neurogenesis in various ways. The activation of the GABAergic system has been shown to be involved in the downregulation of cell division in the hippocampus, but, also to accelerate the synaptic integration of new born hippocampal neurons (Ge et al., 2007). Similarly, it has been shown that the neurotransmitter, serotonin, is able to promote the hippocampal neurogenesis (Gould, 1999).

1.3.5 Sex hormones

It has been shown that sex hormones influence and regulate adult hippocampal neurogenesis. There is strong evidence that oestrogens are important players in this process (Duarte-Guterman et al., 2015). Females show natural fluctuations in ovarian

hormones as evident across the menstrual and oestrous cycles, but also in relation to reproductive events and age (Mahmoud et al., 2016). The rodent oestrous cycle is divided into four phases; metestrus, diestrus, proestrus, and estrus for a total duration of 4-5 days (Butcher et al., 1974). Hormonal fluctuations during the oestrous period are associated with changes in the levels of cell proliferation within the subgranular zone of the hippocampus; with the highest levels of cell proliferation occurring during the proestrous phase when there is a peak of estradiol and progesterone (Tzeng et al., 2014). Growing evidence suggests a role for androgens in the regulation of adult hippocampal neurogenesis. It has been shown that in adult male rodents, long-term exposure to androgens increases hippocampal neurogenesis by affecting the survival of new neurons (Galea et al., 2013). This evidence indicates that sex hormones are dynamic regulators of neurogenesis within the adult hippocampus of both males and females.

1.3.6 Stress

Stressors are described as events and experiences which can challenge the adaptive capacity of a subject. Release of hormones and other cellular factors stimulate the adaptation response. However, dysregulation of the stress response can lead to the activation of pathophysiological processes (Costa et al., 2015). It has been shown that stress has detrimental effects on different stages of hippocampal neurogenesis. For example, it is possible to see a significant reduction in the rate of cell proliferation in the dentate gyrus of mice exposed to intermittent social defeats (Yap et al., 2006). Furthermore chronic stress exposure reduces the survival of new born neurons (Tanti et al., 2013). Different mechanisms have been suggested to explain the effect of stress on neurogenesis; these include alterations in the hypothalamic-pituitary axis (HPA). Upon chronic stress, the HPA is upregulated with a consequent increase in the production of

glucocorticoids from the adrenal cortex (Costa et al., 2015). In a recent animal study, it was shown that chronic treatment with corticosterone reduced the proliferation of cells in the dentate gyrus of adult mice (Gourley and Taylor, 2009). Furthermore, chronic stress and corticosterone treatments seem to promote a cell fate switch by neural stem cells in the dentate gyrus. Stem cells in these situations now produce an increase in the number of oligodendrocytes and a reduced number of neurons (Chetty et al., 2014). Recently, an alteration in glutamatergic neurotransmission and the release of cytokines has been proposed as a possible mechanism involved in the mediation of the effect of stress on neurogenesis. Besides an increased level of glucocorticoids, the production of BDNF, a growth factor involved in the regulation of neurogenesis, seems to be negatively affected by chronic stress (Duman and Monteggia, 2006).

1.3.7 Aging

The decline in cognitive function with age has been correlated with a decline in hippocampal neurogenesis and many studies have shown this relationship (Ben Abdallah et al., 2010, Kempermann, 2015). Several hypotheses have been made to explain this including changes in the hippocampal stem cell niche and a reduction of growth factors expression including that of BDNF and VEGF (Hattiangady et al., 2005). The most significant change observed in the hippocampus during aging, is a reduction of neural progenitor cells proliferation (Olariu et al., 2007). The decline in neurogenesis in the aged brain has been associated either with a depletion of the neural stem cells pool (Encinas et al., 2011) or with a switch from an activated state to quiescence by neural progenitors cells (Lugert et al., 2010). The decline in neurogenesis with aging seems to be hard to reverse or prevent, and in fact some positive regulators of neurogenesis, such as the antidepressant fluoxetine, which are effective in the young brain, do not increase neurogenesis in the aged brain (Cowen et al., 2008). However,

some pro-neurogenic stimuli such as physical exercise do improve neurogenesis in aged rodents (van Praag et al., 2005).

1.4 Immune modulation of neurogenesis

Recently many studies have focused on the direct and indirect effects of the activation of immune cells (e.g microglia) and changes in the levels of inflammatory factors such as cytokines and interleukins on hippocampal neurogenesis (Borsini et al., 2015, Morrens et al., 2012). A variety of cells contribute to the immune response within the brain but many researchers have focused on microglial cells as the most important mediators of neuroinflammation. Microglia are myeloid cells derived from the embryonic yolk sac, that migrate into the brain during early development and maintain a resident population which is able to self-renew (Ajami et al., 2007, Kohman and Rhodes, 2013). Microglia are similar to peripheral macrophages as they can switch between several phenotypes in response to environmental signals, this allows them to inspect the surrounding environment, remove cellular debris or pathogens, and support regenerative processes (Kohman and Rhodes, 2013).

1.4.1 Microglia activation and hippocampal neurogenesis

Microglia can have both positive and negative effects on neurogenesis depending on their state of activation. Microglia that are activated and express a pro-inflammatory phenotype generally impair neurogenesis while microglia in a resting or anti-inflammatory state, are neuroprotective and can support the survival of new born neurons.

1.4.1.1 Resting microglia

Under non inflammatory conditions, microglia reside in the brain in a resting or quiescent state. Resting microglia have a ramified morphology, with a small cell body and several fine processes that are used to survey the surrounding environment. They down-regulate the activation of antigens such as the major histocompatibility complex (MHC) II and the expression of macrophage antigens such as complement receptor 3 and ionized calcium binding adaptor protein-1 (Kettenmann et al., 2011, Ladeby et al., 2005). As explained previously in this chapter, in the hippocampus, resting microglia contribute to adult neurogenesis by the removal of apoptotic new born cells through phagocytosis (Sierra et al., 2010). Indications that microglia, in the resting state, may regulate hippocampal neurogenesis come from a study which showed that these cells, in culture, release factors that influence the differentiation of neural cells (Walton et al., 2006). Evidence for a beneficial role of microglia in neurogenesis was provided by a study where animals exposed to an enriched environment had both increased neurogenesis and an increase in the number of hippocampal microglia (Ziv et al., 2006). Microglia in this case showed a neuroprotective phenotype characterized by the expression of MHC class II and insulin growth factor (IGF-1), both previously described as enhancing neurogenesis (Yirmiya and Goshen, 2011). A role for microglia in the control of neurogenesis has also been suggested by the finding that neural progenitor cells express toll-like receptor TLR-2, a receptor usually associated with microglia. It has been shown that signalling via this receptor is involved in the up regulation of neural progenitor cells differentiation (Rolls et al., 2007). However, further work is necessary to understand and define the precise role microglia have in supporting neurogenesis within the hippocampus.

1.4.1.2 Activated microglia

Under pathological conditions, for example after systemic infections or injury, microglial cells undergo a phenotypic transformation characterized by up-regulation of antigen presentation molecules (MHC II, CD80, CD86, and CD40) and morphological modifications which is called the M1 state or activated phenotype. During the first phase of activation, microglia develop an elongated cell body from which extend long but thick processes (Stage I). Later stages of activation (Stage II-III) are characterized by amoeboid microglia that show a round-shaped cell body with few and short processes. In the final stage of activation (Stage IV), microglial cells are rounded cells without process (Mathieu et al., 2010) (See Figure 1.3).

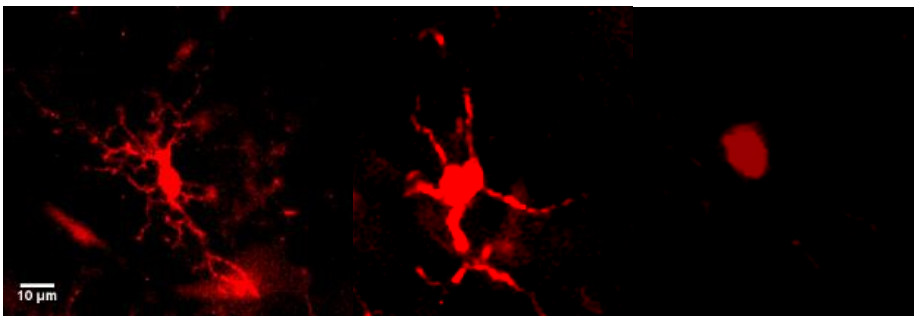


Figure 1.3 ***Morphology of resting and activated microglia identified by immunostaining for Iba-1, in adult rat brain.*** *Stage I: cells have small cell bodies with fine, ramified processes and are named resting microglia. Stage II-III: activated microglia cells have elongated cell bodies with long thick processes. Stage IV: activated microglia cells have a round shape without processes. Photographs of Iba1 positive microglia in the hippocampus of rats.*

Much evidence suggests that pro-inflammatory or activated microglia have a negative effects on hippocampal neurogenesis. The first work demonstrating this was reported by Monje and colleagues in 2003. They demonstrated that intraperitoneal injection of LPS significantly decreased new cell survival during hippocampal neurogenesis, and that the number of early neuroblast cells was negatively correlated with the number of activated

microglia. In addition, LPS administration decreased the differentiation of new cells into neurons as indicated by a reduction in the number of cells expressing doublecortin (DCX), a marker for differentiating neurons (Monje et al., 2003). Additional papers have reported that inflammation also affects proliferation during hippocampal neurogenesis. It has been reported that five hours after a single intraperitoneal injection of LPS, the expression of the proliferation marker pHH3 (serine10 phosphorylated histone H3) was decreased in the granule layer of the DG (Fujioka and Akema, 2010). Inflammation does not only affect the proliferation, survival and cell death phases of neurogenesis, but also can impact on the integration of new neurons into pre-existing neural networks and the cellular properties of the new cells. Newly generated neurons born in a chronically inflamed environment, induced by an intra-hippocampal infusion of LPS associated with microglia activation, had a different morphology and electrophysiological properties compared to neurons produced in a non-inflammatory environment (Jakubs et al., 2008). The mechanisms involved in the reduction of hippocampal neurogenesis by inflammation are still unknown, but some evidence indicates that activation of microglia and the subsequent release of pro-inflammatory molecules, creates an environment that does not support the survival of new born cells. Evidence suggests that the pro-inflammatory cytokines, IL-1β and IL-6 contribute to this process. Neural progenitor cells express receptors for pro-inflammatory cytokines, such as IL-1β and, by binding to these receptors these cytokines are able to suppress the proliferation of cells (Green et al., 2012). It has been shown with *in vitro* experiments that interleukin (IL)-6 induces expression of p21 in hippocampus-derived murine neural progenitor cells. Expression of this protein causes arrest in their proliferation (Zonis et al., 2013). Pro-inflammatory cytokines generally decrease cell survival and favour the production of astrocytes instead of new neurons, modifying the neurogenic niche and

creating an environment in which neural stem cells cannot proliferate and new neurons cannot survive. Usually, the transient activation of the immune system is beneficial and helps the body to recover after an infection or an injury, but the negative impact of a persistent neuroinflammatory response on neurogenesis could be a useful area to study (Kohman and Rhodes, 2013). Once activated, microglia secret a range of pro-inflammatory factors including chemokines, cytokines, and reactive oxygen species (ROS). The accumulation of these factors contributes to neuronal damages. Toxic soluble factors produced by the damaged neurons in turn amplify microglial activation. This association suggests the development of a vicious "self-sustaining cycle" that is likely to lead to prolonged neuroinflammation (Gao and Hong, 2008). Therefore, inhibition of microglial activation, using anti-inflammatory drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) or other drugs with a proven anti-inflammatory effect such as fluoxetine, may represent a potential protective and/or therapeutic strategy for the treatment of inflammation-related neurological disorders.

In contrast to the pro- inflammatory phenotype, microglia can acquire an alternative activated phenotype called the neuroprotective or M2 phenotype that plays an important role in regenerative processes. Neuroprotective microglia in this state show increased expression of the anti-inflammatory cytokines interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and growth factors such as insulin-like growth factor (IGF), neural growth factor (NGF), and brain derived neural growth factor (BDNF) (Colton, 2009). This kind of activated microglia is likely to be induced by the expression of anti-inflammatory cytokines, such as IL-4 and IL-13. Considering these patterns of microglia activation, it has been suggested that microglia have different effects on neurogenesis depending on the phenotype they express. Butovsky et al, in 2006 reported that neural progenitor cells cultured with microglia that were stimulated with

LPS showed low levels of neuronal differentiation, whereas NPCs cultures with the anti-inflammatory cytokine IL-4 stimulated microglia, showed an increased number of new neurons (Butovsky et al., 2006). This suggests that the final effect of microglial cell activation upon neural stem cells biology depends on the resulting profile of molecules secreted.

The evidence indicates that inflammation can suppress hippocampal neurogenesis. However, the role that microglia play, in the regulation of neurogenesis, under basal conditions (in the absence of an infection or injury) and under inflammatory conditions, is not fully understood.

1.5 Common techniques and animal models used to study neurogenesis

Over the past few decades, the use of new detection methods has allowed investigators to strengthen the evidence for adult neurogenesis in a variety of mammalian species (Gross, 2000). It is possible to group the techniques which have been used into three different approaches:

1. Use of nucleotide analogues ([^{3}H]- thymidine, BrdU, iododeoxyuridine (IdU).
2. Use of immunohistological specific markers
3. Use of genetic tools

1.5.1 Nucleotide analogues

Exogenous nuclotide analogues such as [^{3}H]- thymidine, BrdU, iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU) are often administered via intraperitoneal (i.p) injection. Nucleotide analogues are incorporated into DNA in place of endogenous thymidine during the S phase DNA replication within the cell cycle (Leuner and Gould, 2010).

Cells labelled with these analogues can be detected using autoradiography ([^{3}H]-thymidine) or immunostaining (BrdU, IdU, CldU). In the recent past BrdU has become the most used nucleotide analogue to study neurogenesis and proliferation because this technique allows the phenotypic analysis and the quantifications of new-born cells due to the capacity of BrdU to cross the blood brain barrier and its persistence in cells after division (Taupin, 2007) Pulsed administration of BrdU together with variations in the time interval between the last injection and tissue processing has allowed the study of different stages of neuoregenesis such as cell proliferation, neural differentiation and neural survival (Paizanis et al., 2007).

1.5.2 Immunohistological specific markers

Different specific markers are expressed by new born cells at distinct stages of adult neurogenesis in the DG. These molecular markers have allowed investigators to analyse the different stages of neurogenesis in more detail. Endogenous cell cycle markers like the nuclear antigens expressed during cell division can be used as proliferation markers. Ki-67 is one of the most frequently used markers to study proliferation. Ki-67 is expressed during all phases of cell cycle with the exception of the resting phase at the beginning of the G1 phase and due to its short half-life it is difficult to detect during the G0 phase (Zacchetti et al., 2003). In the dentate gyrus the number of Ki-67 positive cells is 50% higher than the number or BrdU stained cells because nucleotide analogues can be incorporated into the DNA only during S phase of DNA synthesis. In addition side effects (high mutagenesis after incorporation and stress during administration) may limit the usefulness of BrdU and for these reasons I have used Ki-67 as the most useful marker for proliferating cells (von Bohlen und Halbach, 2011). However, the use of this marker to study hippocampal neurogenesis is limited by the temporal expression of cell cycle markers. When using Ki-67 it is not possible identify new born cells after exit

from the cell cycle and distinguish between different phenotypes (glial or neural). To recognize different phenotypes during the early stage of neurogenesis there is a wide range of markers with different patterns of expression. One of these markers is glial fibrillary acidic protein (GFAP), frequently used as a marker for mature astrocytes. Recently, it has been shown that a high portion of neural stem cells in the SGZ of the hippocampus are GFAP positive. It is thought that many new born neural cells are generated from these neural stem cells which have astrocytic characteristics and express GFAP (Lugert et al., 2010). Experiments involving the ablation of this population of cells leads to the suppression of the formation of neuroblasts and new neurons (von Bohlen und Halbach, 2011). However GFAP labelling is not the best choice of marker for neural stem cells due to the extensive GFAP expression present in mature astrocytes. As well as GFAP, the HMG (high mobility group) transcription factor Sox2 is expressed by neural stem cells and early proliferating precursors (proved by co-labelling with BrdU) in the DG. Sox2 seems to play a role in the self-renewal of neural stem cells and in neural precursor differentiation (Episkopou, 2005). Due to its pattern of expression, it has been established as a marker of neural stem cells during adult neurogenesis. Sox2 marks both radial astrocytes (also known as type 1 cells) and early progenitor cells (also known as type 2a cells) (Steiner et al., 2006). Neuroblasts and young neurons, which are maturing, can be identified using the marker doublecortin (DCX). The function of this protein is still unknown but it may be involved in microtubule polarization and act as a stabilizer of this structure. DCX is also present in neurites suggesting a role in the growth of neural processes. Due to its expression in both the soma and processes of young neurons, it is possible use this marker to show the detailed morphology of new born neurons (von Bohlen und Halbach, 2011).

1.5.3 Genetic tools

Adult neurogenesis can be studied using a variety of genetic methods or reporter mouse lines. One technique that has changed the study of adult neurogenesis in animal models is germ line transgenesis. With this technique it is possible to label all cells endogenously expressing the knocked-in gene associated with a reporter gene such as the green fluorescent protein (GFP). This genetic tool has led to a greater understanding of adult neurogenesis through the expression of GFP in defined subpopulations of neural stem cells or neural progenitor cells. Cytoplasmic and nuclear expression of this protein has allowed the study of cell morphology and has enabled researchers to quantify the number of GFP-labelled cells (Enikolopov et al., 2015). Some of the experiments in this project have been conducted using the Sox1-GFP transgenic mouse line. Sox1-GFP is a reporter mouse line in which the gene coding for green fluorescent protein (GFP) is inserted into the Sox1 locus (Aubert et al., 2003). GFP knock-in allows the visualization of Sox1 expression by fluorescence microscopy, both in fixed tissue and in live cells. Sox1 is expressed widely during the development of the central nervous system with the exception of the region that contains the precursors for the dopaminergic neurons. GFP expression almost completely mimics that of endogenous Sox1 in the forebrain of Sox1–GFP knock-in mice (Barraud et al., 2005). After embryonic development ceases, Sox1 is absent from most brain areas but expression persists in neurogenic regions. From this expression pattern, Sox1 has been used as a marker to identify stem and progenitor cells actively involved in neurogenesis. The Sox transcription factors are central regulators of neurodevelopment (Favaro et al., 2009, Pevny and Placzek, 2005). Sox1 is the earliest known specific marker of the neuroectoderm lineage, being activated during gastrulation. The only non-neural role described for Sox1 is in the developing eye lens, where Sox1 has been shown to

regulate the γ-crystallin genes (Nishiguchi et al., 1998). During adult neurogenesis it is likely that Sox1 plays an initial role in the maintenance of cell division in the progenitor pool, but, its later expression, leads to neuronal differentiation (Kan et al., 2007). Gain-of-function studies in vivo have indicated that the expression of Sox1 is sufficient to modestly expand the progenitor pool at a rate consistent with one extra round of cell cycle (Kan et al., 2007). Interestingly, previous in vitro studies have also found that constitutive overexpression of *Sox1* in neural progenitor cells seems to cause one extra cell division before differentiation (Kan et al., 2004). It has been shown that during adulthood, Sox1 is expressed in the neurogenic regions as well as in a stem cell pool in the postnatal cerebellum (Aubert et al., 2003, Barraud et al., 2005, Sottile et al., 2006). These studies, however, did not fully characterize the cell type(s) positive for Sox1 within these adult germinal regions. Sox1 is expressed in only a subset of stem/early progenitor cells. Lineage tracing shows that these cells are responsible for producing a majority of the neuroblasts (Venere et al., 2012). As previously shown by Venere et al, in the dentate gyrus, Sox1-GFP did not co-stain with markers of late progenitors/neuroblasts (PSA-NCAM or DCX) or neurons (Tuj1 or NeuN). Co-staining with GFAP and Sox2 has enabled the identification of two independent Sox1 populations within the dentate gyrus. These are:

(1) GFAP-positive, Sox2-positive radial neural stem cells with a radial morphology which are likely to be in an activated state

(2) Sox2 positive non-radial neural precursors

(3) Sox1-GFP positive cells that did not co-localize with any of these markers (Sox2, DCX, NeuN) and may represent Sox1 positivity during a transition between cell types or a distinct, unidentified, population of cells within the SGZ.

The subpopulation of radial neural stem cells and non-radial progenitors cells that express Sox1 do not all differentiate into neurons. Lineage tracing has shown that a fraction of Sox1 expressing cells remain within the two stem/progenitor populations for up to 12 weeks. It is possible say that radial astrocytes that activate Sox1 can either commit to differentiate or remain as stem cells. However, the reduction in Sox1 expressing radial astrocytes over the 12-week suggests that some of these cells permanently exit the stem cell pool (Venere et al., 2012) and are likely to differentiate into astrocytes. Use of the Sox1-GFP transgenic mouse lines to study and define different stages of adult neurogenesis has several advantages. This technique has the advantage of allowing the identification neural precursors and the cytoplasmic localisation of GFP allows morphological analysis of labelled cells. Additionally, this transgenic mouse line, combined with immunostaining, can be used to quantify the size of particular subpopulations of cells. This particular transgenic system is also non-invasive, reducing the potential problem of confounding factors which can occur from multiple injections of labelling compounds.

1.6 Chemotherapy 5-Fluoruracil

5-Fluorouracil (5-FU), a fluorinated analogue of uracil was developed in 1957 as a potential antitumor drug. Since that time it has become one of the most useful antitumor agents and is widely used to treat breast and colorectal cancer (Heidelberger et al., 1957). In adjuvant chemotherapy for breast cancer it is used in combination with the other chemotherapy agents cyclophosphamide and methotrexate (CMF), doxorubicin and cyclophosphamide (FAC) or epirubicin and cyclophosphamide (FEC) (Guarneri et al., 2007).

1.6.1 Mechanism of action

5-Flurouracil is antimetabolite drug that works by irreversibly inhibiting the thymidylate synthase enzyme required for thymidine synthesis. 5-FU is able to cross the blood brain barrier (BBB) and reach high concentrations within the cerebrospinal fluid, but has a short half-life (Diasio and Harris, 1989). This drug passes into cells easily using the same transporter as uracil. Once 5-FU has entered a cell, it is transformed into cytotoxic metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). The most active metabolite is FdUMP which forms a covalent complex with thymidylate synthase preventing the binding of the enzyme with its normal substrate. The formation of the covalent complex requires folate metabolites that can be supplemented by administrating folinic acid in combination with 5-FU. Normally thymidylate synthase is involved in the synthesis of DNA base thymidine by converting deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP). The inhibition of thymidine synthesis has as a consequence, the inhibition of DNA synthesis during the cell cycle and an accumulation of dUMP. The metabolism of FdUMP produces metabolites able to bind directly to DNA and the incorporation of dUMP lead to the formation of DNA double strand breaks. Secondary 5-FU metabolites such as FUTP can be incorporated into RNA causing dysfunction of RNA processing. About 80% of administered 5-FU is catabolized by dihydropyrimidine dehydrogenase (DPD) to dihydrofluorouracil (DHFU). This primarily happens in the liver and in the gastrointestinal mucosa where the enzyme DPD is widely expressed (Longley et al., 2003) (See figure 1.4).

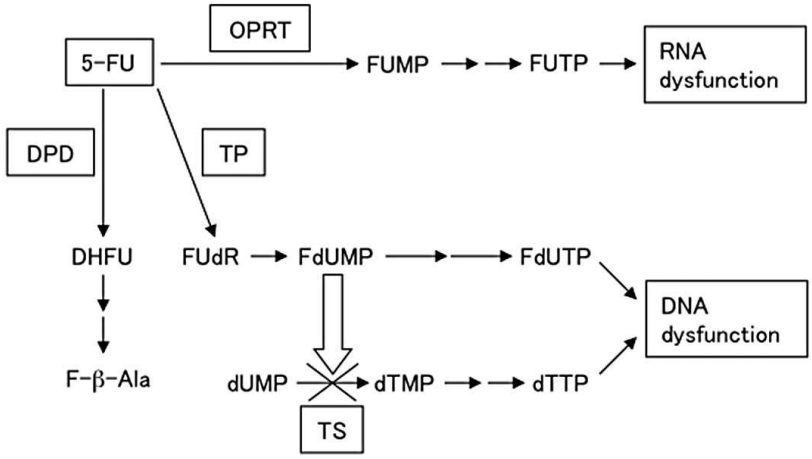


Figure 1.4 ***Activation pathways for 5-fluorouracil****. 5-FU, metabolic pathway of 5-fluorouracil; OPRT, ortate phosphoribosyl transferase; FUMP, 5-fluorouridine monophosphate; FUTP, 5-fluorouridine triphosphate; TP, thymidine phosphorylase; FUdR, 5-fluorodeoxyuridine; FdUMP, 5-fluorodeoxyuridine monophosphate; FdUTP, 5-fluorodeoxyuridine triphosphate; TS, thymidylate synthase; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; dTTP, deoxythymidine triphosphate; DPD, dihydropyrimidine dehydrogenase; DHFU, 5-fluoro-dihydrouracil; F-b-Ala, a-fluoro-b-alanine (Tamatani et al., 2012).*

1.6.2 Toxicity

5-FU affects rapidly proliferating cells in tissues such as the gastrointestinal mucosa and bone marrow. The pattern of toxicity associated with 5-FU varies considerably with the dose and the route of administration. If administered intravenously as a bolus, 5-FU can cause epithelial ulceration of the gastrointestinal tract, manifesting as mucositis, pharyngitis, dysphagia, esophagitis, gastritis and colitis (Macdonald, 1999). The diarrhoea may be aqueous or haemorrhagic and the combination with anorexia, nausea and vomiting, can lead to severe dehydration and low blood pressure of patients treated with 5-FU. Myelosuppression with granulomatous and thrombocytopenia may be present with this type of treatment, resulting in an increased susceptibility to infection and bleeding. Changes in the cardiovascular system such as acute chest pain, elevated cardiac enzymes and ischemic ECG changes have been temporally associated with the

administration of 5-FU as well as tachycardia and pulmonary oedema. Skin alterations are also possible such as modest hair loss, nail dystrophy, dermatitis, formation of vesicles, photosensitivity with increased pigmentation and atrophy skin (Macdonald, 1999).

5-FU can cause alterations in the CNS such as acute cerebellar ataxia (reported in about 2% of cases), and other symptoms such as mental confusion, difficulty in concentrating, decreased memory and mental deterioration (reported in up to 75% of cancer survivors (Grem et al., 1999, Ahles et al., 2002).

1.7 Potential protective strategies against the chemotherapy induced reduction in neurogenesis

1.7.1 Fluoxetine

The selective serotonin reuptake inhibitor (SSRI), fluoxetine, is primarily used as an antidepressant but has also been shown to increase neurogenesis (Malberg et al., 2000) and ameliorate impaired cognitive functions in both human (Boggio et al., 2005, Mowla et al., 2007) and animal subjects (Ivkovic et al., 2004). For this reason, it was chosen for one set of experiments presented in this thesis (Chapter 5) to investigate its ability to counteract the reduction in hippocampal cell proliferation caused by chronic 5-FU administration.

1.7.1.1 Mechanism of action

Selective serotonin reuptake inhibitors are the most frequently prescribed therapeutic agents in modern medicine. They are used to treat a wide range of psychiatric disorders

including obsessive compulsive disorder, depression, panic attacks and bulimia (Stahl, 1998). The main mechanism of action of SSRIs involves the regulation of 5-HT re-uptake (Wong et al., 1974). In normal conditions, when a neuron is activated, 5-HT is released from the presynaptic neuron and after diffusing across the synaptic cleft binds to specific 5-HT receptors on the post synaptic membrane. 5-HT is then rapidly removed from the synapse by reuptake transporters. SSRI antidepressants act by decreasing the 5-HT affinity of reuptake transporters and consequently causing 5-HT accumulation in the synaptic cleft (Stahl, 1998). Repeated long term treatment with SSRIs is able to stimulate BDNF gene expression in the hippocampus via cyclic adenosine monophosphate (cAMP), protein kinase A (PKA) and cAMP response element binding protein (CREB) pathways (Duman et al., 2001). Based on these findings, studies have tried to determine if the mechanism of action of antidepressants in treating depression involves increases in adult hippocampal neurogenesis as described previously in this chapter. It now appears that at least some behavioural aspects of antidepressants action require hippocampal neurogenesis (Santarelli et al., 2003, Perera et al., 2011, Yu et al., 2017).

1.7.1.2 Fluoxetine and cognitive functions

Due to the association between chronic fluoxetine treatment and increased neurogenesis and the important role of neurogenesis on cognitive functions such as learning and memory, some researchers have investigated if fluoxetine treatment is able to enhance cognition. In clinical studies, fluoxetine improves memory in patients with major depression (Gallassi et al., 2006), mild cognitive impairment (Mowla et al., 2007) and post-traumatic stress disorder (Davis et al., 2006). In addition to clinical studies, some experiments have been conducted using animal models which have shown that fluoxetine improved cognition when cognitive impairment was caused by stress (El

Hage et al., 2004) and stroke (Li et al., 2009). Furthermore, a previous study in our group demonstrated that fluoxetine can improve the memory impairment induced by chemotherapy administration in rats (Lyons et al., 2012) and after valproic acid treatment (Welbat et al., 2016).

1.7.1.3 Fluoxetine and inflammation

In recent years there has been increasing evidence for the role of the immune system in the pathogenesis of depression. According to this hypothesis, the therapeutic effects of antidepressants could be at least partly due to their ability to decrease the brain inflammation (Licinio and Wong, 1999). For this reason and because fluoxetine is known to have pro-neurogenic properties, in one experiment presented in this thesis (Chapter 4) we investigate the effect of this drug on microglia activation and inflammation.

Many studies have found possible anti-inflammatory actions of fluoxetine (Bianchi et al., 1994, Makkonen et al., 2011). It was for example found that fluoxetine reduced the number of macrophages, lymphocytes, neutrophils and eosinophils in a septic shock model (Roumestan et al., 2007). In a recent experiment in mice with LPS-induced sepsis, it has been shown that fluoxetine can attenuate affective behaviour in parallel with a decrease in microglia activation. Fluoxetine has also been shown to enhance the recovery in cell proliferation in the dentate gyrus, after sepsis (Anderson et al., 2016). Previous studies have reported that the anti-inflammatory action of fluoxetine could be related to the up-regulation of anti-inflammatory molecules such as interleukin 4 (Alboni et al., 2013) and to its ability to down regulate microglia mediated oxidative stress (Chung et al., 2010). In addition, it has been shown that microglial activation, iNOS, ROS, Nf-kB and the expression of pro-inflammatory factors (COX-2, TNF-α,

IL-1β andIL-6) decrease after fluoxetine administration (Tynan et al., 2012, Chung et al., 2010, Jin et al., 2009, Lim et al., 2009, Liu et al., 2011, Zhang et al., 2012a). Recently, it has been reported that chronic administration of fluoxetine in 6-hydroxydopamine-lesioned Parkinsonian rats restored central concentrations of inflammatory cytokines to basal levels (Sharifi et al., 2015). Fluoxetine has been shown to increase hippocampal neuronal survival following an ischemic injury by its anti-inflammatory effect on microglia (Dhami et al., 2013). The suppression of Nf-kB by fluoxetine was suggested as the mechanism involved in the anti-inflammatory effect of this antidepressant (Roumestan et al., 2007). Nf-kB is an important transcription factor involved in the production of pro-inflammatory molecules such as TNF-α, NO and IL-6. However, how exactly fluoxetine suppresses Nf-kB and produces other anti-inflammatory effects is still unknown (Lim et al., 2009).

1.7.1.4 Fluoxetine metabolism and side effects

Fluoxetine is one of the most frequently prescribed SSRIs and after oral administration is almost completely absorbed. It is metabolised in the liver by cytochrome P450 into the active metabolite, norfluoxetine (Vaswani et al., 2003). Norfluoxetine has a half-life of 7 to 15 days in humans (Vaswani et al., 2003) and 15 hours in rat (Caccia et al., 1990). At least 4 weeks of treatment are necessary to reach the steady state level of fluoxetine in both humans and rodents (Conley and Hutson 2007). The most important advantage of the SSRIs over older antidepressants is the absence of severe adverse effects from an over dose. Common side effects include gastrointestinal problems, such as nausea, anorexia and weight loss. It has also been reported that fluoxetine is effective in reducing body weight in obese patients (Epling, 2003). This was confirmed in a recent rodent experiment which produced weight loss after 2 weeks of fluoxetine treatment (Aggarwal et al., 2016). In some patients, SSRIs may induce or exacerbate

agitation and sleep disturbances. These side effects often attenuate with time or dose reduction. A variety of movement disorders have also been reported with SSRI treatment. These include akathesia, dystonia, dyskinesia and Parkinsonism (Vaswani et al., 2003).

1.7.1.5 Fluoxetine, cancer and cancer treatment

To evaluate the use of fluoxetine as a potential protective strategy against the cognitive side effects of chemotherapy in patients, it is important to understand any interactions between fluoxetine, cancer and cancer treatments. Antidepressants are widely prescribed for depression in both cancer and non-cancer patient populations but their impact on cancer patients has remained relatively unstudied. There are contradictory reports on the effect of fluoxetine on cancer. For example, it has been reported fluoxetine treatment increases the number of brain metastases in mice with induced breast cancer, by increasing blood brain barrier (BBB) permeability, inducing pro-inflammatory changes in the brain and glial activation (Shapovalov et al., 2014). Similarly, Brandes and colleagues, in 1992, reported that fluoxetine stimulated breast cancer growth in mice with transplanted tumours (Brandes et al., 1992). In contrast, Tutton and Barkla in 1982, reported an anticancer effect of FLX treatment in colon cancer (Tutton and Barkla, 1982) and this anti-proliferative effect of fluoxetine has been found in a number of in vivo and in vitro studies of many types of cancer, including leukaemia (Chamba et al., 2010), lymphoma (Cloonan et al., 2010, Cloonan and Williams, 2011, Frick et al., 2008, Frick et al., 2011), colon cancer (Kannen et al., 2015, Koh et al., 2011, Stopper et al., 2014), melanoma (Grygier et al., 2013, Kirkova et al., 2010), ovarian cancer (Lee et al., 2010) and glioblastoma (Liu et al., 2015). The mechanism behind the anti-tumour effects of fluoxetine are unclear but some researchers suggest the fact that the antidepressant treatment reduces dysplasia and pre-

neoplastic angiogenesis, and decreases epithelial and sub-epithelial cell proliferation (Kannen et al., 2012). Further it has been reported that by suppressing NF-κB nuclear activity, through increased expression of cytoplasmic NF-κB-inhibitor IκB-α and IκB-β proteins, fluoxetine is able to reduced *c-Myc* expression and then stromal proliferation (Stopper et al., 2014). As stated above other studies have shown that fluoxetine treatment arrested colon tumour cells at the G_0/G_1 phase of the cell cycle (Kannen et al., 2015). In addition, fluoxetine has been shown to decrease ROS generation, reducing the tissue oxidation in a mice melanoma model (Kirkova et al., 2010). Fluoxetine has also been shown to stimulate Ca^{2+} flux which reduced the expression of bcl-2 in B-cell lymphoma and mitochondrial membrane potential all of which induced DNA fragmentation and subsequent apoptosis in Burkitt's lymphoma cells (Serafeim et al., 2003). As the evidence of the effect of fluoxetine on cancer is contradictory more research is needed particularly as many cancer patients are on this drug.

Concerning the interactions between fluoxetine and chemotherapy treatment it has been reported that the antidepressant is an effective chemosensitizer (Peer et al., 2004). This action seems to be mediated by the inhibition of extrusion pumps such as P-glycoprotein and multidrug-resistance-associated-proteins responsible for chemotherapy resistance. These results suggest that fluoxetine may enhance the effect of chemotherapy treatment (Zhou et al., 2012). Chemotherapies such as tamoxifen, which target hormone dependant cancers, however may be inhibited by fluoxetine. Many SSRIs inhibit cytochrome P450 (CYP2D6) which can affect the metabolism of tamoxifen into its more potent metabolite endoxifen. Drugs, with a CYP2D6 inhibitory action can decrease the plasma concentrations of endoxifen. CYP2D6 inhibitors such as fluoxetine may increase the risk of relapse of breast cancer in patients being treated with tamoxifen (Desmarais and Looper, 2009). Due to these factors use of fluoxetine as

a treatment for chemotherapy induced cognitive impairment must take into account the type of cancer and treatment being given.

1.7.2 Indomethacin

The non-steroidal anti-inflammatory drug (NSAID), indomethacin has analgesic, anti-inflammatory and anti-pyretic properties and is used mostly for the treatment of rheumatic disorders (Waller, 1983). It has been indicated as effective in different experimental models of neuroinflammation (Monje et al., 2003). For this reason, it was chosen for the experiment presented in this thesis (Chapter 4) as a potential treatment to counteract the possible neuroinflammation caused by chronic 5-FU administration.

1.7.2.1 Mechanism of action

NSAIDs are the most widely used therapeutics for the treatment of pain and inflammation (Dannhardt and Kiefer, 2001). These anti-inflammatory drugs block the biosynthesis of prostaglandins (PGs) that are involved in the development of inflammation (See Figure 1.5).

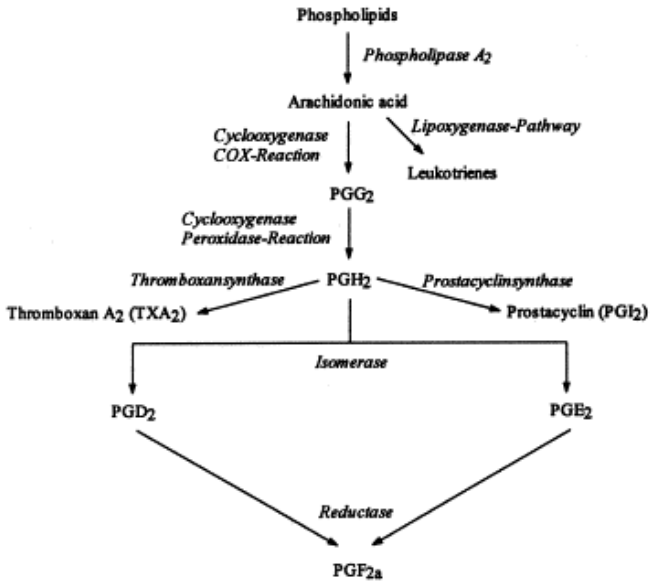


Figure 1.5. Biosynthesis of PGs*: the initial step of this process is the liberation of arachidonic acid (AA) from the phospholipids of the cell membrane catalysed by phospholipase A_2. The following step is the transformation of AA by cyclooxygenase (COX). The first product of this reaction is the unstable PGG_2 which is immediately converted into the more stable PGH_2 by the same enzyme in a peroxidase reaction. The final products of the AA metabolism are PGs, thromboxane and prostacyclin (Dannhardt and Kiefer, 2001).*

PGs mediate a number of characteristic features of the body's response to injury or inflammation. Moreover, PGs have cytoprotective properties in the gastrointestinal (GI) tract and control of renal functions in the kidney. PGE_2 is the most important PG which mediates the typical symptoms of inflammation, pain and fever. Like aspirin, all other NSAIDs such as ibuprofen, ketoprofen and naproxen act by blocking cyclooxygenase. Therefore, administration of NSAIDs, leads to a decrease in the prostaglandins required for the inflammatory and physiological functions previously mentioned (Dannhardt and Kiefer, 2001).

In particular, indomethacin, is known to inhibit two isoforms of COX, COX-1 and COX-2, with greater selectivity for COX-1. COX-1 is a constitutively expressed enzyme that is involved in gastric mucosal protection, platelet and kidney function. It catalyses the conversion of arachidonic acid to prostaglandin (PG) G2 and PGG2 to PGH2. COX-1 is involved in the synthesis pathways of PGE2, PGD2, PDF2a, PGI2

(also called prostacyclin) and thromboxane A2 (TXA2). COX-2 is constitutively expressed but is induced and unregulated by inflammatory stimuli, such as cytokines. Both COX-1 and COX-2 are expressed in the CNS; COX-1 is expressed in microglia and some neuronal populations (Hoozemans et al., 2001). COX-2 is expressed by neurons and microglia in many brain regions, both in humans and in rodents (Hoozemans et al., 2001, Yasojima et al., 1999).

1.7.2.2 Indomethacin and cognitive functions

It has been shown that prostaglandins do have some negative effects on learning and memory, particularly working memory (Matsumoto et al., 2004) and seem to impair contextual fear-conditioning (Hein et al., 2007). There is also evidence that COX-2-overexpression in aged animals can lead to behavioural deficits and neuronal loss (Andreasson et al., 2001). Previous epidemiological data revealed that the use of NSAIDs prevents cognitive decline in older adults and can be useful to prevent the Alzheimer's disease (AD) (Hayden et al., 2007). In a number of animal models of AD, NSAIDs have been applied with beneficial effects. For example, it has been shown that a long treatment with different kinds of NSAIDs such as flurbiprofen, ibubrofen and naproxen is able to ameliorate cognitive deficits in AD transgenic mice (Kukar et al., 2007, Lim et al., 2001, Kotilinek et al., 2008). Unfortunately the role of NSAIDs in other kinds of cognitive impairment models has not been elucidated yet.

1.7.2.3 Indomethacin and neurogenesis

At the moment there exist only a small number of studies on the potential protective effect of indomethacin against the impairment of neurogenesis induced by inflammation. It has been reported that indomethacin was able to regulate microglia activation and hippocampal neurogenesis in a mouse model of intracerebral

haemorrhage (Yang et al., 2015). Further results have come from a study where the level of hippocampal cell proliferation was restored by indomethacin treatment, which reduced systemic inflammation found in a mouse model of diabetes. A result which, may prevent the long-term cognitive deficits found in this model (Ho et al., 2015). It has also been demonstrated that indomethacin, when given after stroke, is able to increases the number of surviving new born cells in the hippocampus of rats (Hoehn et al., 2005). Chronic inflammation within the brain, produced by cranial irradiation, may contribute to the deficiency in neurogenesis and its related cognitive decline experienced by animals and patients receiving this treatment. It has been shown that treatment with indomethacin is able to restore neurogenesis after cranial irradiation in rats (Monje et al., 2003). However, in a mouse model of aging it has been found that indomethacin does not have a positive effect on hippocampal neurogenesis, but it is able to decrease the hippocampal expression ox COX-1 and COX-2 together with a reduced expression of the microglial marker Iba1 (Boehme et al., 2014).

1.7.2.4 Indomethacin metabolism and side-effects

Following oral administration, the absorption of indomethacin is rapid and complete. Generally, peak plasma concentrations are achieved within 1 to 2 hours. The half-life is reached after one hour in both humans and rodents (Yesair et al., 1970). Indomethacin has a bioavailability of about 98 %, plasma protein binding (albumin) is 90 %, and it is metabolized within the liver (Helleberg, 1981).

Side-effects of this class of anti-inflammatory drugs are related to their mechanism of action. Chronic NSAID users suffer from GI tract irritation or, in the worst cases, from the development of GI ulcers and bleeding (Allison et al., 1992). Long-term treatments with NSAIDs may also lead to renal disorders and hypertensive problems. These are

due to the reduced production of PGs, such as PGI_2, PGE_2 and PDG_2, which are involved in the regulation of renal blood flow and the rate of glomeruleric filtration both of which are reduced. This reduction, especially in subjects with a limited renal function, can lead to retention of water and hypertension (Clive and Stoff, 1984). Moreover, the inhibition of cyclooxygenase in thrombocytes results in decreased production of thromboxane A_2. This action prolongs bleeding time and leads to the inhibition of platelet aggregation (Dannhardt and Kiefer, 2001). CNS problems are observed in about 5% of patients using NSAIDs, Most commonly reported problems are dizziness, headaches, drowsiness, mood alteration and confusion. The CNS side effects differs between NSAIDs used, with a high prevalence found with indomethacin (Seideman and von Arbin, 1991).

1.7.2.5 Indomethacin, cancer and cancer treatment

Recently more attention has been given to the use of anti-inflammatory drugs as an adjunct in the treatment of cancer. Non-steroidal anti-inflammatory drugs (NSAIDs), have antineoplastic properties that can be attributed to their effect on inflammation (Grivennikov et al., 2010). The first evidence came from a study which reported that treatment with indomethacin, led to the regression of colorectal polyps in patients with familial adenomatous polyposis (Waddell and Gerner, 1980). Later preclinical studies demonstrated that indomethacin is efficacious against colon (Lundholm et al., 1994), lung (Cai et al., 2011) and breast cancer (Ackerstaff et al., 2007). However, the gastrointestinal toxicity produced by a chronic indomethacin administration suggests the use of safer NSAIDs with less side effects (Henry et al., 1996). Despite these limitations, several anti-inflammatory drugs, such as aspirin, have been found to reduce tumour incidence when used as a preventive drug, and to decrease the incidence progression and mortality of colon and breast cancer (Gupta and Dubois, 2001).

These results have led to the recommendation that anti-inflammatory drugs should be used in the prevention and treatment of cancer. In most cases therapy with anti-inflammatory drugs is likely to be effective only in combination with other chemotherapeutic drugs. For example, it has been demonstrated that administration of indomethacin alone, or in combination with oxaliplatin, may significantly inhibit the growth of lung cancer tumours transplanted into nude mice (Sun and Chen, 2016), suggesting that the combination NSAIDs with chemotherapeutic drugs may improve the antitumor effects. A study demonstrating this combined administration of indomethacin and the chemotherapy drugs adriamycin and cisplatin, decreased tumour growth in mice (Hattori et al., 2001). Indomethacin also increases the cytotoxic activity of doxorubicin against a doxorubicin-resistant SCLC cell line (de Groot et al., 2005). Due to this promising evidence, more investigation is necessary to understand the role of indomethacin in the protection and treatment of cancer.

1.7.3 Physical exercise

In recent years, physical activity has been considered an effective low cost non-pharmacological strategy to prevent and slow the cognitive decline associated with aging or diseases (Geda et al., 2010, van Uffelen et al., 2008, Lautenschlager et al., 2008). In support of this, it has been shown that 1 to 12 months of exercise in healthy adults caused a significant increase in memory, attention, processing speed and executive function (Smith et al., 2010). One possible means to reduce chemotherapy induced cognitive impairment, would be the application of physical activity programs. This approach has been shown to have positive effects on cognitive functions in both patients and animal models of this condition, in fact providing access to running wheels in animal models has a positive effect on neuronal development, in particular the brain regions involved in learning and memory including the hippocampus. (Vaynman et al.,

2004, Vaynman et al., 2006). In addition physical activity seems to have a protective role when cognitive functions are impaired by aging (Voss et al., 2013a), neurodegenerative disease (Heyn et al., 2004), traumatic brain injuries (Archer et al., 2012) and chemotherapy treatment (Winocur et al., 2013, Fardell et al., 2012a).

1.7.3.1 Neural mechanisms underlying the beneficial effect of physical activity on cognition

The CNS goes through molecular, cellular and functional changes when it is exposed to external cognitive, social and physical stimuli. Voluntary physical activity has been shown to have positive effects on cognition in physiological and pathological conditions in both human and animals (Voss et al., 2013b). In humans, non-invasive brain imaging techniques have been used to uncover the mechanisms involved in the beneficial effect of physical exercise on brain functions (Hotting and Roder, 2013). It has been found that aerobic exercise is able to increase blood flow and vascularization, which in turn can improve oxygen and nutrients supply. For example, magnetic resonance angiography in physically active older people demonstrated a higher number of small vessels in the brain as compared to less active older adults (Bullitt et al., 2009). Other studies using magnetic resonance imaging (MRI) to investigate structural changes after physical activity in humans, reported an increase in grey matter in frontal regions (Colcombe et al., 2006) and in the hippocampus (Erickson et al., 2011). Functional MRI studies reported an enhancement in neural efficiency during executive and memory tasks (Colcombe et al., 2004, Holzschneider et al., 2012).

As previously mentioned, it has been hypothesized that the positive effects of physical activity on cognition may be mediated in part by an increase in hippocampal neurogenesis (van Praag et al., 1999b). Van Praag et al, in 1999, have shown an

increase in cell proliferation and differentiation which led to a net increase in neurogenesis in mice housed with a free access to a running wheel for 2 weeks (van Praag et al., 1999b). The onset of the effect of physical exercise on neurogenesis is fast. The change in cell generation reaches a peak after three days but after a month of running the pro-proliferative effect returns to the baseline (Kronenberg et al., 2006). The increase in cell proliferation and survival is the effect most often observed after exercise treatment (Brown et al., 2003, van Praag et al., 1999a). This effect can be produced at all ages, in young, adult and aged rodents (Marlatt et al., 2012a, van Praag et al., 2005). The effect of exercise on neurogenesis is sustained throughout life in rodents. Indeed, in continuously exercised mice, from young to middle age, the age-related decrease of neurogenesis is less than in sedentary mice (Kronenberg et al., 2006) while in mice that start to do physical activity in middle or old age, the number of new born cells is elevated (Wu et al., 2008, van Praag et al., 2005). The enhancements of angiogenesis and neurogenesis by exercise seem to be closely related. Van der Borght and colleagues in 2009 have shown that in mice housed with a running wheel for 1, 3 or 10 days, the voluntary physical exercise produces a rapid increase in blood vessel density and in the generation of new cells in the dentate gyrus of the hippocampus. After running cessation in these animals, the levels of both blood vessels density and cell proliferation returned to those seen in sedentary animals within a day. Taken together these data demonstrate that the hippocampus shows a rapid adaptation to the stimuli of running. However, the number of cells expressing neural proliferation and differentiation markers, such as Ki67 and DCX, constantly increases with a protracted running wheel access over a period of 10 days (Van der Borght et al., 2009). This suggests that a prolonged period of running is necessary for exercise-induced neurogenesis. Also, in recent studies it has been demonstrated that the proliferative

effect of running is transitory and that running mainly enhances neurogenesis by increasing the survival of cells that have already been born (Snyder et al., 2009, Clark et al., 2010, Fuss et al., 2010). The overall mechanism of how physical activity increases hippocampal neurogenesis is still unknown. However, it has been shown that neurotrophic factors such as brain derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor (VEGF) are the principal mediators of hippocampal neurogenesis and increase during physical exercise (Cotman et al., 2007, Cotman and Berchtold, 2002, Trejo et al., 2008). The serum levels of both IGF and VEGF are also increased after voluntary physical activity, they cross the blood brain barrier to enter the brain where they can stimulate angiogenesis and the neurogenesis (Carro et al., 2000, Lopez-Lopez et al., 2004, Fabel et al., 2003). The impact of these growth factors in stimulating of neurogenesis and angiogenesis has been demonstrated by using blocking antibodies which can prevent their effects. In fact, blocking IGF and VEGF signals, by the blockade of peripheral growth factor passage into the brain, prevents the proliferation of neuronal progenitor cells induced by physical exercise in the dentate gyrus of the hippocampus (Trejo et al., 2001, Fabel et al., 2003). Similarly, the knockout of the BDNF receptor TrkB in hippocampal neuronal progenitor cells seemed to block the exercise induced increase in neurogenesis (Li et al, 2008). Moreover, the intracerebral infusion of BDNF enhances neurogenesis in the dentate gyrus of the hippocampus and simulates the effects of exercise in learning (Scharfman et al., 2005, Griffin et al., 2009). The expression of other neurotrophins, such as fibroblast growth factor 2 (FGF-2) and nerve growth factor (NGF) is increased in the hippocampus of exercising rodents and seems to be involved in the enhancement of the hippocampal neurogenesis (Gomez-Pinilla et al., 1997, Neeper et al., 1995).

Exercise induced neurogenesis is linked to enhanced hippocampal synaptic plasticity, in particular changes in long-term potentiation (LTP), which is widely considered to be one of the major cellular mechanisms that underlies learning and memory (Cooke and Bliss, 2006). It has been demonstrated that the LTP amplitude is enhanced in the dentate gyrus of hippocampal slice taken from running mice (van Praag et al, 1999). The positive effects of physical activity extend also to neuronal cell morphology. In particular, exercise influences the properties of dendritic spines, which are protrusions of dendrites on which are found excitatory synapses. In recent studies it has been shown that running enhances the dendritic spine density in dentate gyrus, CA1 and entorhinal cortex layer III and also accelerates the maturations of dendritic spines in new born neurons of the hippocampus (Stranahan et al., 2007, Redila and Christie, 2006).

1.7.3.2 Physical exercise, cancer and cancer treatment interactions

Contemporary medicine mostly focuses on the treatment of cancer rather than the prevention of cancer. It has been shown that physical exercise reduces the risk of breast, colon, pancreatic, prostate, endometrial, ovarian and lung cancer (Brown et al., 2012). Despite the large volume of epidemiologic literature on physical activity and cancer prevention, the molecular mechanisms behind these beneficial effects are not well understood. It has been hypothesized that the pathways relating to sex hormones, metabolic hormones, inflammation, immune function, oxidative stress and DNA repair are involved in the cancer prevention mediated by physical exercise (Rundle, 2005).

Despite the importance of physical activity in the primary prevention of cancer, physical exercise can provide health-benefits *after* diagnosis of cancer. Exercise seems to have a potent rehabilitative effect for cancer survivors, in fact there are numerous documented health-benefits among cancer patients, including improved disease-free

survival, muscular strength, aerobic capacity, quality of life and decreasing fatigue (Courneya, 2003, McNeely and Courneya, 2010). There is evidence indicating that physical activity, such as brisk walking, is effective in decreasing the symptoms and side effects of treatment (Schmitz et al., 2010). A 1989 a randomized trial of 45 women undergoing adjuvant chemotherapy for breast cancer demonstrated that 10 weeks of interval-based, aerobic exercise not only improved functional capacity, but also decreased chemotherapy-induced nausea. This work demonstrated that aerobic exercise was safe and beneficial for patients undergoing chemotherapy (MacVicar et al., 1989, Winningham and MacVicar, 1988).

During cancer treatment, cancer survivors may experience a variety of physiologic and psychological symptoms and side effects. Some of the most common side effects experienced during treatment include fatigue, pain, cardiovascular and pulmonary complications, endocrine changes, musculoskeletal fatigue and weakness, immune alterations, gastrointestinal upset and skin changes (Schmitz et al., 2010). It has been shown that aerobic fitness improved significantly cardiorespiratory function in cancer survivors during treatment over the exercise intervention period (Speck et al., 2010). Muscle fatigue and muscle weakness are also common side effects of cancer treatment but among 121 prostate cancer survivors undergoing radiation therapy, chest and leg strength improves over 24-weeks of exercise training (Segal et al., 2009). Furthermore, exercise interventions decrease the psychological side effects of cancer treatment, such as fatigue (Cramp and Byron-Daniel, 2012) and depression (Tomlinson et al., 2014). It has also been indicated that physical exercise is able to ameliorate chemotherapy induced cognitive impairment in breast cancer patients (Gokal et al., 2015). A reduction in cognitive disorganisation was also reported in breast cancer patients after a programme of yoga (Culos-Reed et al., 2006). There is evidence that

physical exercise improves the domains of cognition affected by chemotherapy; namely working memory and executive processing (Seigers and Fardell, 2011).

Considering the positive influence of exercise on the body and CNS, cancer patients suffering from cognitive impairments can benefit of regular physical activity and this may be an effective treatment for chemotherapy induced cognitive impairment.

1.8 Aims and objectives

The main aim of these studies was to investigate the effect of acute and chronic 5-fluorouracil treatment on adult hippocampal neurogenesis. Using the Sox1-GFP transgenic model it was possible to investigate which stage of neurogenesis and which types of neural stem cells are affected by chemotherapy treatment.

The second main objective was to evaluate the effectiveness of different potential protective strategies against chemotherapy induced cognitive impairment, including fluoxetine, indomethacin and physical exercise.

Therefore, the objectives of these studies were:

1. To determine which subpopulations of neural stem cells are most affected by acute and chronic 5-FU treatment in Sox1-GFP transgenic mice, using immunohistochemistry for different cellular markers
2. To determine the potential protective effect of the anti-inflammatory drug, indomethacin on adult hippocampal neurogenesis and microglia activation, in Sox1-GFP transgenic mice administered chronically with 5-FU.
3. To determine the potential protective effect of the antidepressant, fluoxetine and the anti-inflammatory drug, indomethacin, on hippocampal proliferation, neuroinflammation and DNA damage, in rats administered chronically with 5-FU and allowed to recover for 1 week.
4. To determine the effect of physical activity on adult hippocampal neurogenesis, in mice receiving an acute treatment with 5-FU.

Chapter 2 – EFFECT OF 5-FU ON HIPPOCAMPAL NEUROGENESIS IN SOX1-GFP TRANSGENIC MICE

2.1 Introduction

The following chapter will describe the effects of acute and chronic treatment with the chemotherapy agent 5-FU. The impact of 5-FU treatment will be analysed in term of the expression of GFAP in radial glia like neural stem cells, the proliferation marker Ki67 and the differentiation marker DCX in Sox1-GFP transgenic mice using fluorescence immunohistochemistry. This study investigated the immediate effect of acute (24hr) and chronic treatments (2 weeks) with 5-FU and for this reason 24hr were considered adequate to evaluate the effect of treatment.

A single dose of chemotherapy is not normally used for treatment of cancer in patients but this kind of acute treatment together with the evaluation and measure of neural stem cell subpopulations immediately after the chemotherapy administration, allowed us to identify the initial effects of chemotherapy on these cell populations. In the literature, the wide range of chemotherapy drugs and doses administered together with the different rodent strains used, has made it difficult identify an appropriate chemotherapy regimen for use in the present experiment. For example 75 mg/Kg of 5-FU has been administrated to both rats and mice (Fardell et al., 2012a, Foley et al., 2008) and produced similar cognitive impairments. However, recent studies have shown, that in order to see a decrease in cell proliferation in the hippocampus, it is necessary administer a higher dose of 5-FU in mice compared to rats (Krynetskiy et al., 2013, Walker et al., 2011, Elbeltagy et al., 2012). This likely due to the faster rate of metabolism in mice compared with rats so that mice require an increased dose to see an effect. A number of previous experiments by our group and others have investigated the effect of acute and chronic treatment with 5-FU in rodents (Elbeltagy et al., 2010, Han et al., 2008, Mustafa et al., 2008, Lyons et al., 2011a). These studies have demonstrated

that 5-FU treatment causes a reduction in cell proliferation in the SGZ of the hippocampus (Elbeltagy et al., 2012).

However, it is still unclear which type of neural stem progenitor is affected by 5-FU treatment. To investigate this aspect of the response of the hippocampal neurogenic niche to chemotherapy, we used a transgenic mouse line that allows the visualization of Sox1 positive cells as they express GFP as a reporter gene under the control of the Sox1 promoter (Aubert et al., 2003). This strain of GFP knock-in mice permits the visualization of Sox1 positive cells by fluorescence microscopy both in fixed tissue and in live cells. It also allows the identification and purification of Sox1-expressing cells by fluorescent activated cell sorting (FACS). Sox1 is expressed widely throughout the developing nervous system except the ventral midbrain and in the region that contains the precursors for dopaminergic neurons. GFP expression almost completely mimics that of endogenous Sox1 in the forebrain of Sox1–GFP knock-in mice (Barraud et al., 2005). Sox1 is localized in the germinal regions of the adult brain (hippocampus and lateral walls of the lateral ventricles) and is also expressed by a putative stem cell pool in the postnatal cerebellum (Aubert et al., 2003, Pevny and Rao, 2003, Barraud et al., 2005, Sottile et al., 2006, Kan et al., 2007). Sox transcription factors are central regulators of neurodevelopment (Pevny and Placzek, 2005, Favaro et al., 2009). Members of the SoxB1 subfamily, comprising of Sox1, Sox2 and Sox3, show distinct and overlapping expression patterns during embryogenesis (Pevny and Placzek, 2005). Sox1 is the earliest known specific marker of the neuroectoderm lineage, being activated during gastrulation. Sox1 in the adult brain plays an initial role in the maintenance of division within the progenitor pool, but, upon continued expression, caused neuronal differentiation (Kan et al., 2007). Its expression maintains stem/progenitor cells in an undifferentiated and proliferating state by inhibiting Hes1

that leads to a reduced Notch signalling, which is necessary for the progression of differentiation (Kan et al., 2004). At this point Sox1 is down-regulated and cells exit from the cell cycle (Kan et al., 2004). Gain-of-function studies in vivo have indicated that constitutive expression of Sox1 is sufficient to modestly expand the progenitor pool at a rate consistent with one extra round of the cell cycle (Kan et al., 2007). Interestingly, a previous *in vitro* study also found that constitutive overexpression of *Sox1*, in neural progenitors, appeared to cause one extra cell division before differentiation (Kan et al., 2004).

An important characteristic of adult NSCs is their ability to maintain a reversible quiescent state. This enables many stem cells in neurogenic regions, such as the SGZ of the hippocampus to be non-proliferative for most of the time but to have the ability to be activated and to start contributing cells to the process of neurogenesis. Because of this behaviour, NSCs can be divided into two subpopulations: quiescent NSCs (qNSCs) which remain in the G_0 phase of the cell cycle and active NSCs which are self-renewing, multipotent cells. Activated NSCs are derived from qNSC and give rise to transit amplifying progenitors (Wang et al., 2011). The expression of cell cycle inhibitors and the down regulation of proteins involved in cell cycle progression, such as p57, p27 and p21, in qNSCs allows the maintenance of the quiescent state (Kippin et al., 2005, Furutachi et al., 2013). The expression of Sox1 promotes the activation of NSCs and the progression of the cell cycle in active NSCs. Sox1 is therefore expressed in only a subset of stem/early progenitor cells and lineage tracing shows that these cells are responsible for the production of the majority of neuroblasts (Venere et al., 2012). These studies, however, did not fully characterize the cell types positive for Sox1 within the adult hippocampus. In this study, we describe the location, morphology and cell marker expression of GFP expressing cells in the Sox1-GFP transgenic mouse line.

NSCs populations were identified based on the presence GFAP immunoreactivity and by analysing the orientation of the main process extending from the cell body (radial or horizontal). This transgenic model has enabled the identification of the following stem cell populations: quiescent radial glia like neural stem cells (qRGLs) which have a radial morphology, are GFAP positive but Sox1 negative (Fig 2.1a); a population of activated NSCs which are still GFAP positive and also express Sox1 (Fig. 2.1b), a population of NSCs that express Sox1 but not GFAP (Fig. 2.1c) and finally a population of non-radial cells which continue to express Sox1 (Fig. 2.1d).

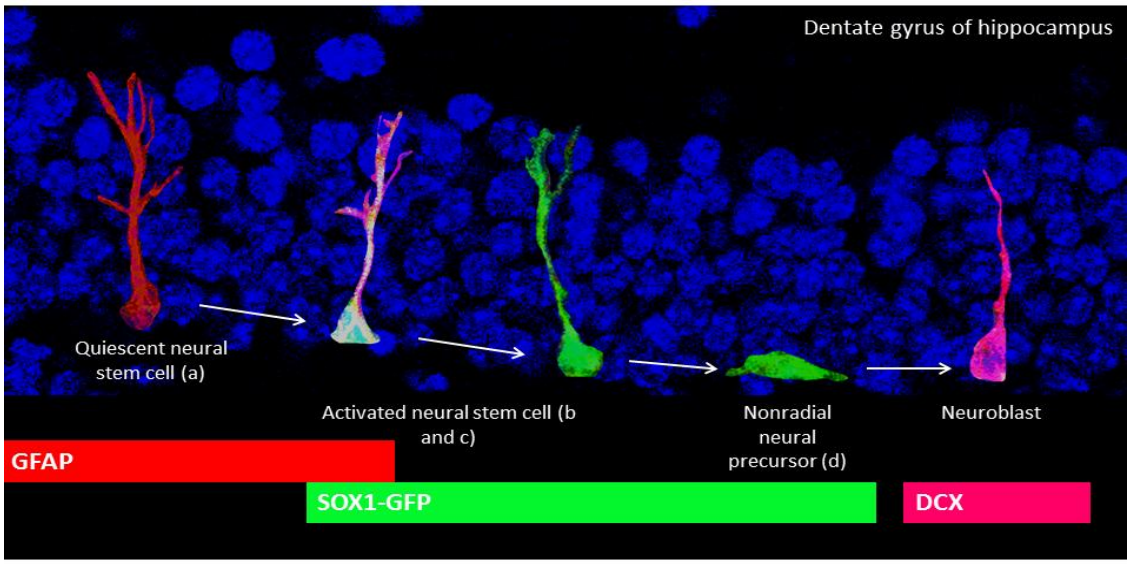


Figure 2.1. ***Model of neural stem cells (NCS) in the adult hippocampus of SOX1-GFP transgenic mice.***

Quiescent neural stem cells (qRGL) have a radial glia like morphology, characterized by a long process that extends through the granule cell layer and arborizes in the molecular layer (Kriegstein and Alvarez-Buylla, 2009). qRGLs in the SGZ are reported to be the primary precursors of new neurons in the dentate gyrus (Seri et al., 2001). They have the electrophysiological characteristics of astrocytes and express GFAP (glial fibrillary acidic protein), vimentin and brain lipid-binding protein (BLBP),

markers typically shown by astrocytes (Filippov et al., 2003). qRGL have a low rate of proliferation, in fact only 1-2% of cells in the SGZ are in a state of cell division at any one time. Recently, a subpopulation of neural stem cells with a radial morphology has been described in the SGZ (Lugert et al., 2010). These cells are able to generate neurons, astrocytes and radial astrocytes (Suh et al., 2007) and are capable of switching between quiescence and an activated state in which they divide. The coexistence in the SGZ of quiescent and activated neural stem cells has been further investigated using a downstream target of the canonical Notch signalling pathway, the transcription factor Hes5, to label type-1 cells of the SGZ. Two subpopulations of neural stem cells were identified: the first is in a quiescent state and the second expresses the proliferating cell nuclear antigen (PCNA) marker and is believed to be in an activated state (Lugert et al., 2010). Both populations of neural stem cells are capable of self-renewal. Different mechanisms control the balance between quiescence and entrance into the cell cycle; the transcription factor Sox1 is believed to be a component involved in the activation of neural stem cells (Venere et al., 2012). Once the cells cease to be quiescent, activate neural stem cells can generate other qRGLs and non-radial precursors or astrocytes. Non-radial precursors lack any radial process and elongate their extensions parallel with respect to the SGZ/granule cell boundary. These precursors express Sox2 and Sox1 but not GFAP, they are more proliferative than radial neural precursors but they are not always in the cell cycle. This population of cells has been proposed as the major source of new neurons (Bonaguidi et al., 2016). As a result of activation, activated neural stem cells divide several times asymmetrically or symmetrically before terminally differentiating into astrocytes (Encinas et al., 2011). If these cells divide with a mitotic cleavage plane which is horizontal (parallel to the SGZ) they generate a daughter cell located close to the activated neural stem cell. These cells are nestin positive and are

called type-2 cells or intermediate progenitor cells (Kempermann et al., 2004); if activated neural stem cells divide symmetrically they produce two neural stem cells that can return to quiescence. Type-2 cells have a round cell body and extend their short processes horizontally with respect to the SGZ and have a high rate of proliferation (Filippov et al., 2003). It is possible to divide this group of cells into two subpopulations: Type-2a or early amplifying progenitor cells which still express several markers of radial glia (Steiner et al., 2006) and Type-2b or late amplifying progenitors cells which although having the same morphology, lack the expression of radial glia markers (Wang et al., 2011). Type-2 cells undergo on average 2.5 symmetrical divisions to generate further Type-2 cells before exiting from the cell cycle and starting to differentiate into type-3 neuroblasts (Kempermann et al., 2004).

Use of the previously mentioned battery of markers together with the Sox1-GFP transgenic tool enabled the assessment of the impact of chemotherapy on different neural stem cells subpopulations of the hippocampal neurogenic niche. We initially hypothesized that activated neural stem cells with a high rate of proliferation could be the major cell population affected by chemotherapy treatment.

2.2 Materials and methods

2.2.1 Animals and treatment

Two studies were performed. The acute treatment with 5-FU will be called the "Acute Study" while chronic 5-FU treatment will be called the "Chronic Study". In each study, eight, 9 weeks-old male heterozygous Sox1-GFP$^{+/-}$ mice (Aubert et al., 2003), carrying a knocked in GFP reporter (BSU Transgenic Unit, University of Nottingham) were housed 4 to a cage under standard conditions of temperature (22 ± 1° C; 12/12h light/dark cycle) with free access to food and water and randomly assigned to controls

(CON, n=4) or the 5-FU treatment group (n=4). Animals were allowed to habituate for one week prior to treatment. 5-FU treated animals were i.p injected with 5-FU (75 mg/Kg dissolved in 0.9% sterile saline), twice a day (Medac, GmbH, Hamburg, Germany). This was done only for one day in the Acute Study and every second day for 2 weeks (4 days of injections in total) in the Chronic Study. Control mice were injected with the same volume of 0.9% sterile saline. The volume of injections were calculated for individual animals based on body weight. This dosing regimen was based on our previous pilot studies and in accordance relevant human doses as converted to mouse doses (Krynetskiy et al., 2013). The dosage of 5-FU selected for this experiment was well tolerated and did not influence activity levels. Throughout the experiment, mice were examined daily and weighed every 2 days. Principles of laboratory animal care were in accordance to UK Home Office Guidance regulations and with local ethical committee approval. Procedures were carried out under a Home Office project license No 30/3163 and personal license No I55DE633D.

2.2.2 Brain tissue preparation

24h after the last injection, in both studies, animals were euthanized with an overdose of pentobarbital sodium (Euthasol) and intracardially perfused with phosphate-buffered saline (PBS, pH 7.4) for 3 min followed by 4% paraformaldehyde (PFA) (Sigma-Aldrich, UK) in 0.1M PBS solution, for a further 5 min. Brains were rapidly removed and post-fixed overnight in 4% PFA at 4°C. The following day brains were cryoprotected in 30% sucrose at 4°C and then transferred in PBS at 4°C till sectioning. Brains were divided into two halves and coronally sectioned on a sledge microtome by being placed on a cooling plate with the rostral end facing up before sectioning at 60 µm thickness (Fig.2.2). Free-floating section were then placed into 24 well plates containing cryoprotective solution and were stored at -22°C.

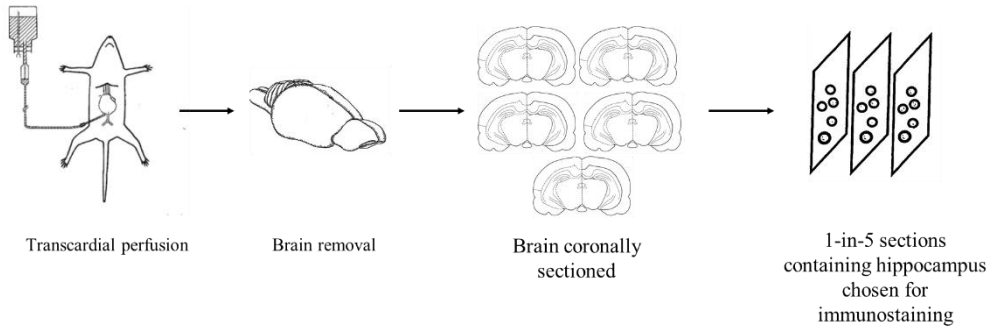


Figure 2.2 ***Drawing of brain tissue preparation***

2.2.3 Immunohistochemistry

The subgranular zone of the dentate gyrus was anatomically defined as the interface of the granular cell layer with the hilus (Duman, 2004). Sections were washed in phosphate-buffered saline (PBS) three times (5 min each) and then incubated in blocking solutions: 15% normal goat serum, 0.3% Triton X-100 in PBS (Ki-67 immunostaining), 5% bovine serum albumin (BSA), 2% goat serum and 0.3% Triton X-100 in PBS (GFAP immunostaining), 1.5% BSA, 0.3% Triton X-100 in PBS (DCX immunostaining) for 1h at room temperature. Primary antibodies were diluted in blocking solution and used as follows in an overnight incubation at 4°C: Ki67 (rabbit polyclonal, 1:1000, Vector Laboratories, Orton Southgate, UK), GFAP (1:500, rabbit polyclonal, Dako, Carpinteria, CA, USA) and DCX (1:100, rabbit polyclonal, Santa Cruz Biotechnology). All secondary antibodies were Alexa Fluor (1:500, Invitrogen, Gran Island, NY, USA), labelled with 568 and 594 fluorochromes, dissolved in 0.1% Triton X-100 in PBS and incubated for 1h at room temperature in the dark. After washing with PBS, sections were mounted on slides coated with gelatine and counterstained with DAPI nuclear stain (1:1000, Invitrogen, Gran Island, NY, USA).

2.2.4 Cell counting and imaging

Ki-67, GFAP and doublecortin (DCX) positive cells were stained in separate sections and observed by fluorescence or confocal microscopy. Only sections containing hippocampus were included in the sample slides and sampled in a systematic manner using 1: 5 series. Around 100 were obtained from the microtome sliced brain. For each animal, a total of 10 sections were used to stain for each marker. Staining with the nuclear marker DAPI were used to measure the area of the granule cell layer using ImageJ Software. Positive cells that were within the granular cell layer or within a two-cell diameter distance of this layer on the hilar side were counted along the entire length of the dentate gyrus. Sections were stained with GFAP and confocal z-stacks were taken of the dentate gyrus from one hemisphere randomly chosen using 40X objective. Cells positive for GFAP and Sox1-GFP were counted first in every single channel and then the co-localization was detected in the merged images. Co-localization was checked in the serial scan scans including the 3D projection of the z-stack.

2.2.5 Statistical analysis

Statistics were calculated using GraphPad Prism 6.0 software and significance was regarded as $p< 0.05$. Unpaired Student t-test (two-tailed) was used to compare controls and 5-FU treated groups. Repeated measures of 2-way ANOVA with Bonferroni's post hoc test were used to analyse the body weight of the animals.

2.3 Results

2.3.1 Acute study

2.3.1.1 5-FU acute treatment did not affect body weight

In this experiment animals were killed 24hr after the last injection. A two-way repeated measure ANOVA analysis on body weight data revealed a non-significant effect of treatment and time x treatment interaction (respectively $F_{1,5}$ = 1.49 and $F_{2,10}$ = 1.963). However, the statistical analysis highlighted a significant effect of time ($F_{2,10}$= 168.8, *p* <0.001) due to the measurement of body weight at different time points after the first 5-FU injection or vehicle (Fig.2.3). Mice were observed daily to determine other physical characteristics of the animals receiving chemotherapy; none showed hair loss or had apparent changes in overall activity.

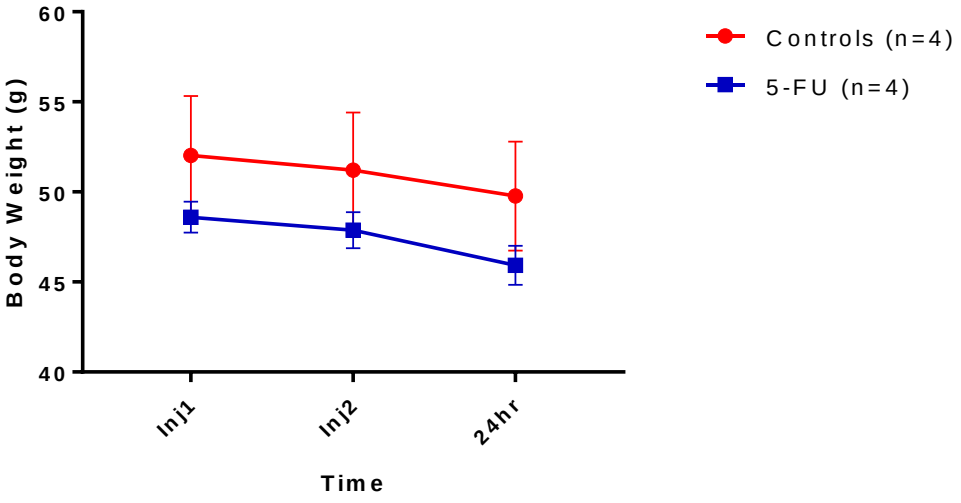
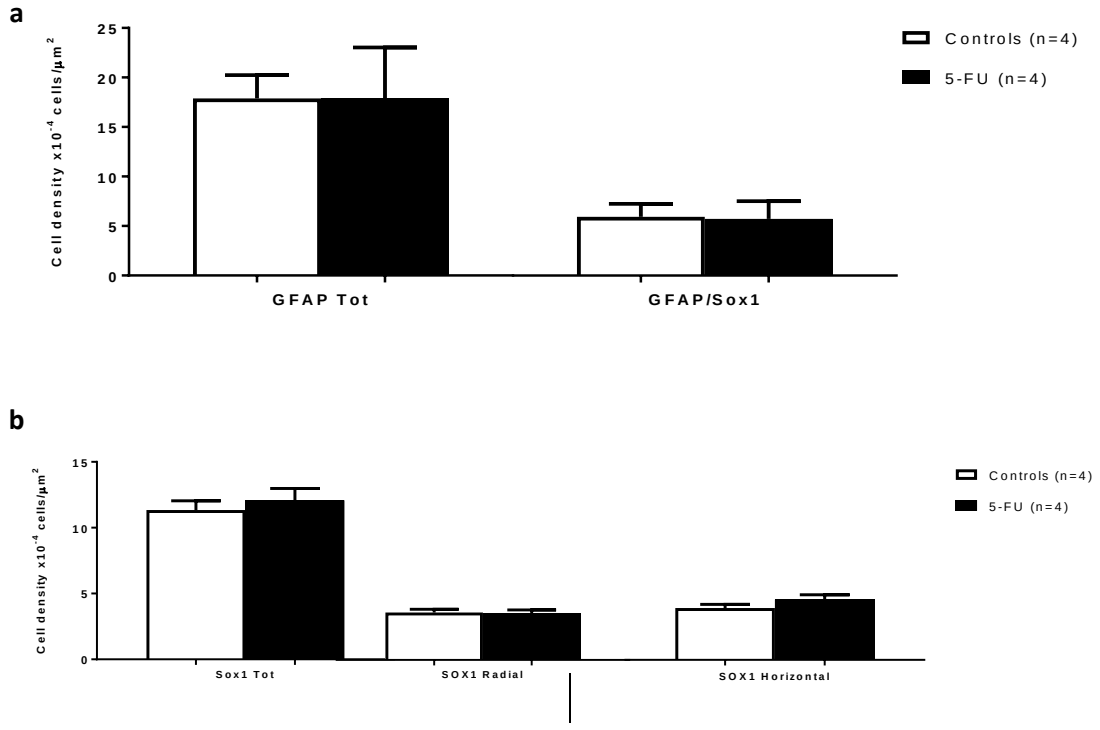


Figure 2.3 Body weight of mice (mean ± SEM, n=4). *Body weights were measured before the first and the second injection and 24hr after the last injection. Repeated measure two-way ANOVA showed a significant effect of time on body weight ($F_{2,10}$= 168.8, p <0.001).*

2.3.1.2 Effect of acute 5-FU treatment on neural stem cells markers

Overlaying the green fluorescent protein image with the red immunostained GFAP image, it was possible to measure the cell density of different subpopulations of neural stem cells in the SGZ of the hippocampus 24h after the last injection of 5-FU (Fig. 2.4 c). Unpaired t-test (two tails) showed that qRGL GFAP+ cells and activated neural stem cells that express both GFAP and Sox1 were not affected by acute chemotherapy treatment when compared to control animals (respectively $F_{14,11}$= 4.752 and $F_{14,11}$= 1.86, $p > 0.05$, Figure 2.4 a). The density of Sox1 expressing cells was also measured in the hippocampus and Sox1 positive cells that extend radial or horizontal process were counted separately. Data analysis with an unpaired t-test showed that the entire population of Sox1+ cells ($F_{14,11}$= 2.096, $p > 0.05$) and both subpopulations of activated neural stem cells with radial process and activated neural stem cells with horizontal process were not affected by acute 5-FU treatment when compared with controls (respectively $F_{14,11}$= 1.568 and $F_{14,11}$= 2.418, $p > 0.05$; Figure 2.4 b).



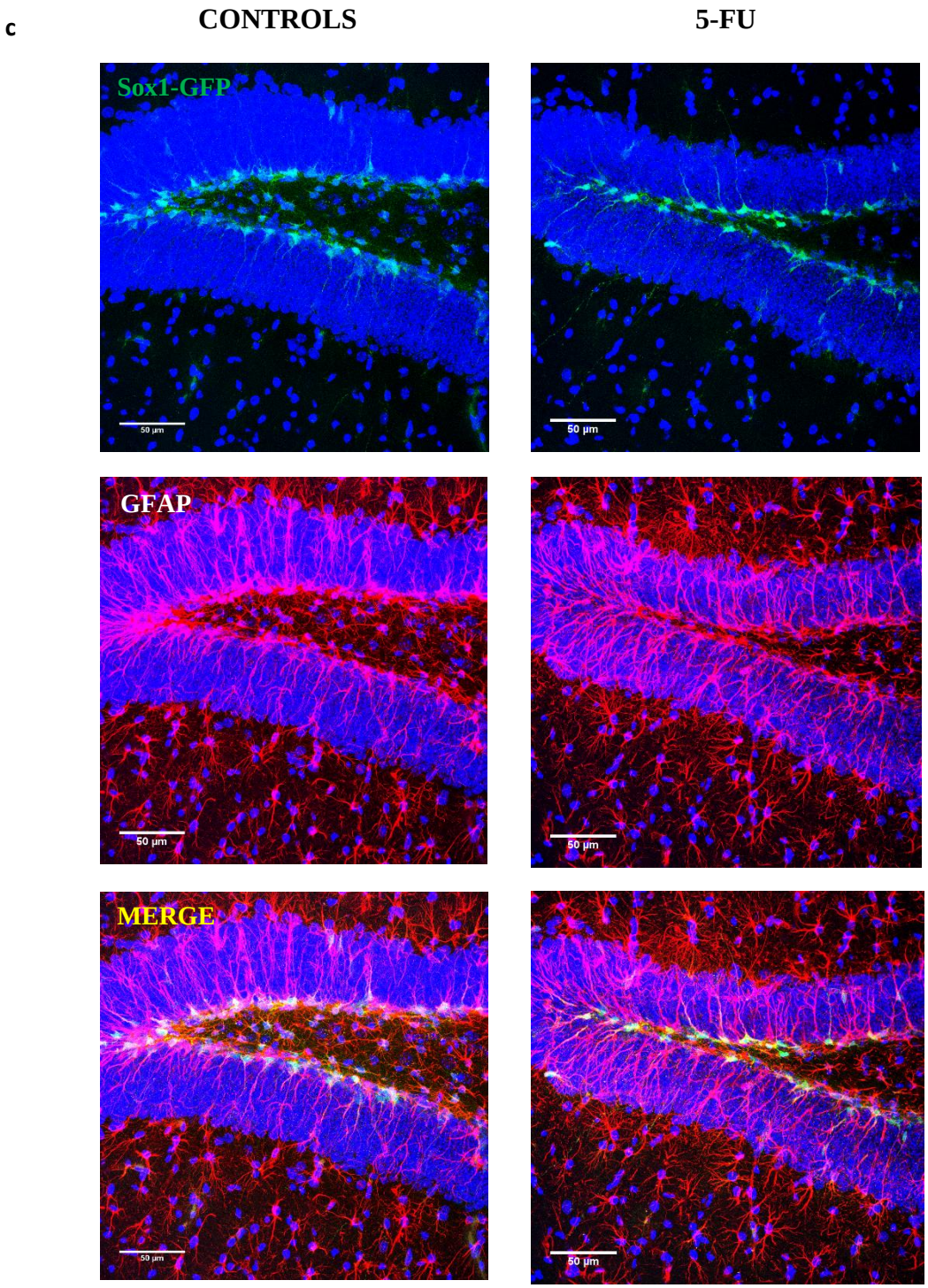


Figure 2.4. ***(a) GFAP and GFAP/Sox1 positive cells density in the SGZ (mean ± SEM, n=4).*** *Data analysis of GFAP+ and GFAP/Sox1+ cells shows no effect of acute 5-FU treatment on cell density.* ***(b) Sox1 in total and separated into cells with radial or horizontal process in the SGZ (mean ± SEM, n=4).*** *Data analysis of the cell density of Sox1 expressing cells and Sox1 positive cells with radial and horizontal process showed no significant effect of acute 5-FU treatment.* ***(c) Photographs of Sox1-GFP$^+$ positive cells*** *(green) located in the SGZ of control and 5-FU treated animals, showing co-labelling with GFAP (red); nuclei are highlighted by DAPI. Scale bar: 50 µm.*

2.3.1.3 Effect of acute 5-FU treatment on proliferation and differentiation

To determine the effect of acute treatment with 5-FU on proliferating cells in the SGZ, we analysed the expression of Ki67. Ki67+ cells were found in the SGZ of the hippocampus, a region where it was possible also to detect Sox1-GFP+ cells. Overlaying the green fluorescent protein image with red immunostained Ki67 image it was possible to quantify the number of dividing neural stem cells expressing Sox1 (Fig 2.5b). The remaining Ki67+ in the SGZ are assumed to be glia. Data analysis indicated that the total number of proliferating cells was not affected by acute 5-FU treatment ($F_{15,15}$= 2.381, $p > 0.05$). However, proliferating cells that express Sox1 are significantly reduced in animals treated with 5-FU ($F_{15,15}$= 3.079, $p < 0.05$; Fig. 2.5a). It was also observed that 98% of Ki67/Sox1+ cells have a non-radial morphology which is consistent with the high rate of proliferation previously described for this subpopulation of neural stem cells (Venere et al., 2012). To evaluate the number of neuroblasts produced in controls and animals under treatment with 5-FU were used the marker for differentiating cells doublecortin (DCX) (Fig. 2.5d). Unpaired t-tests showed that mice treated with 5-FU have a significant decrease in the number of DCX expressing cells when compared with control animals ($F_{9,11}$= 5.492, $p < 0.05$; Fig. 2.5c). Moreover, it was observed that DCX expression cells did not co-localize with Sox1-GFP expression, a result which is consistent with the previous description of these cells (Venere et al., 2012).

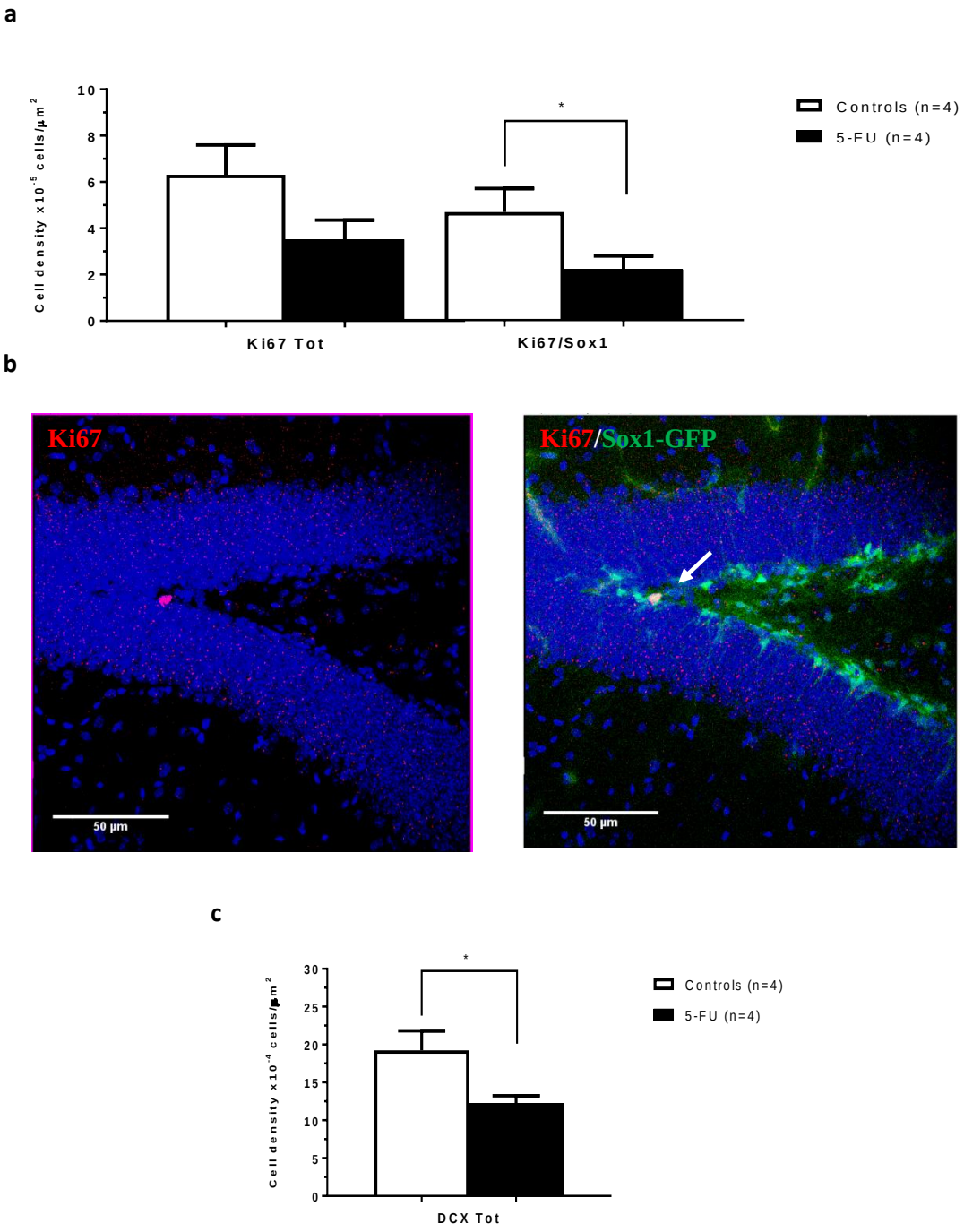


Figure 2.5. ***(a) Ki67+ and Ki67/Sox1+ cell density in the SGZ (mean ± SEM, n=4).*** *Quantification of Ki67/Sox1+ showed a significant decrease in animals treated with 5-FU when compared with control animals. The total number of proliferating cells (Ki67+), however, was not affected by acute chemotherapy treatment.* ***(b)*** *Photographs showing positive immunostaining for proliferating cell marker Ki67 (red), Sox1-GFP expressing cells (green) and photos merged. Arrow indicates a Sox1 positive cell in proliferation. Scale bar: 50 µm.* ***(c) DCX+ cell density in the SGZ (mean ± SEM, n=4).*** *Data analysis of DCX+ cells showed a significant reduction in animals receiving an acute treatment with 5-FU when compared with controls.*

2.3.2 Chronic study

2.3.2.1 5-FU chronic treatment affect body weight

In this experiment animals were killed 24h after the last injection of 5-FU. As expected animals in all groups gained or maintained their body weight over the period of the study. A two-way repeated measure ANOVA on body weight data revealed a significant effect of time and treatment × time interaction ($F_{9,45}$ = 5.898, $F_{9,45}$ = 13.14, respectively, p< 0.0001; Fig. 2.6). Mice were observed daily to determine any loss of conditions in animals receiving chemotherapy. No animals showed hair loss or had apparent changes in overall activity. However, one animal died following chemotherapy treatment.

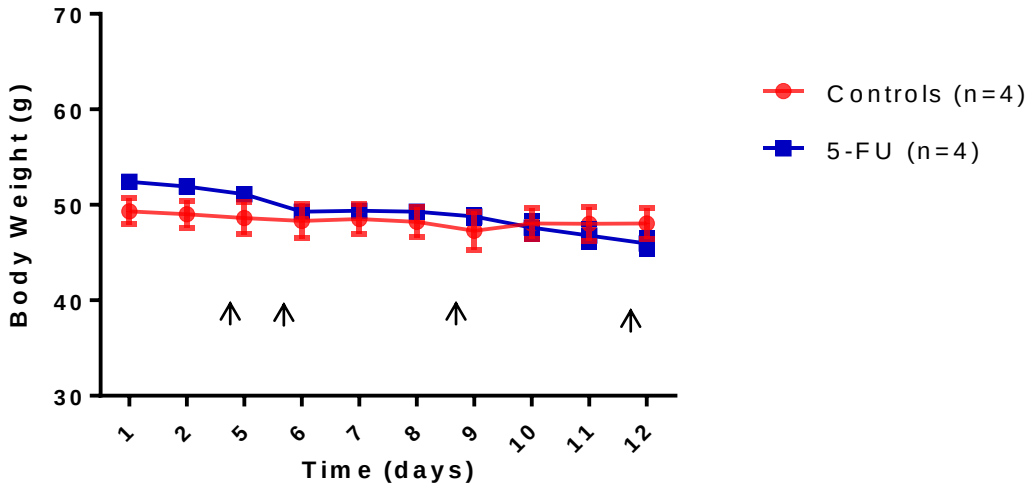
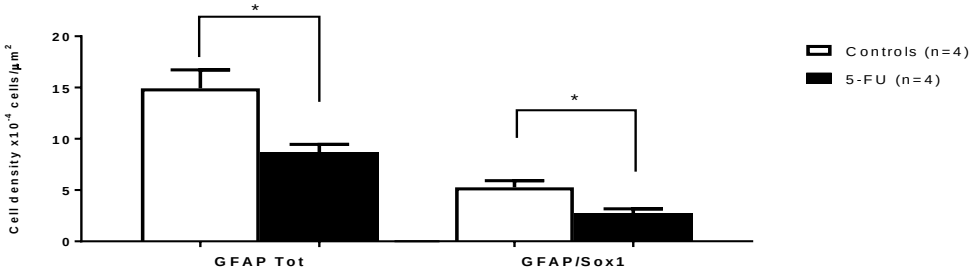


Figure 2.6. ***Body weight of Sox1-GFP transgenic mice (mean ± SEM, n=4)*** *from the day before the beginning of 5-FU treatment till the end of the experiment. A two-way repeated measure ANOVA on body weight data revealed a significant effect of time and treatment × time interaction ($F_{9,45}$ = 5.898, $F_{9,45}$ = 13.14, respectively, p< 0.0001). Arrows at day 2, 5, 8 and 11 indicate days of saline or 5-FU injection.*

2.3.2.2 Effect of chronic 5-FU treatment on neural stem cells markers.

Quantification of the cell density of different subpopulations of neural stem cells in the SGZ of the hippocampus was done 24h after the last injection of 5-FU (Fig. 2.7c). Unpaired t-test (two tails) showed that qRGL GFAP+ cells and activated neural stem cells that express both GFAP and Sox1 are both affected by chronic 5-FU treatment when compared to control animals (respectively $F_{8,9}$= 4.702 and $F_{8,9}$= 2.086, $p < 0.05$; Fig. 2.7a). Cells density of Sox1 expressing cells as well, was quantified 24h after the last 5-FU injection, in the SGZ of the hippocampus, discriminating between Sox1 positive cells that extend radial or horizontal process. Data analysis with unpaired t-test showed that the entire population of Sox1+ cells and the subpopulation of activated neural stem cells with radial process decreased significantly in 5-FU treated mice when compared with control animals (respectively $F_{13,15}$= 1.119 and $F_{12,10}$= 4.110 $p < 0.05$; Fig. 2.7b). However, in this case activated neural stem cells with horizontal process are not affect by acute 5-FU treatment when compared with controls ($F_{15,17}$= 2.806 $p > 0.05$; Fig. 2.7b).

a


Cell density x10^{-4} cells/µm^2
20
15
10
5
0
*
*
GFAP Tot
GFAP/Sox1
Controls (n=4)
5-FU (n=4)

b


Cell density x10^{-4} cells/µm^2
20
15
10
5
0
*
*
Sox1 Tot
Sox1 Radial
Sox1 Horizontal
Controls (n=4)
5-FU (n=4)

c

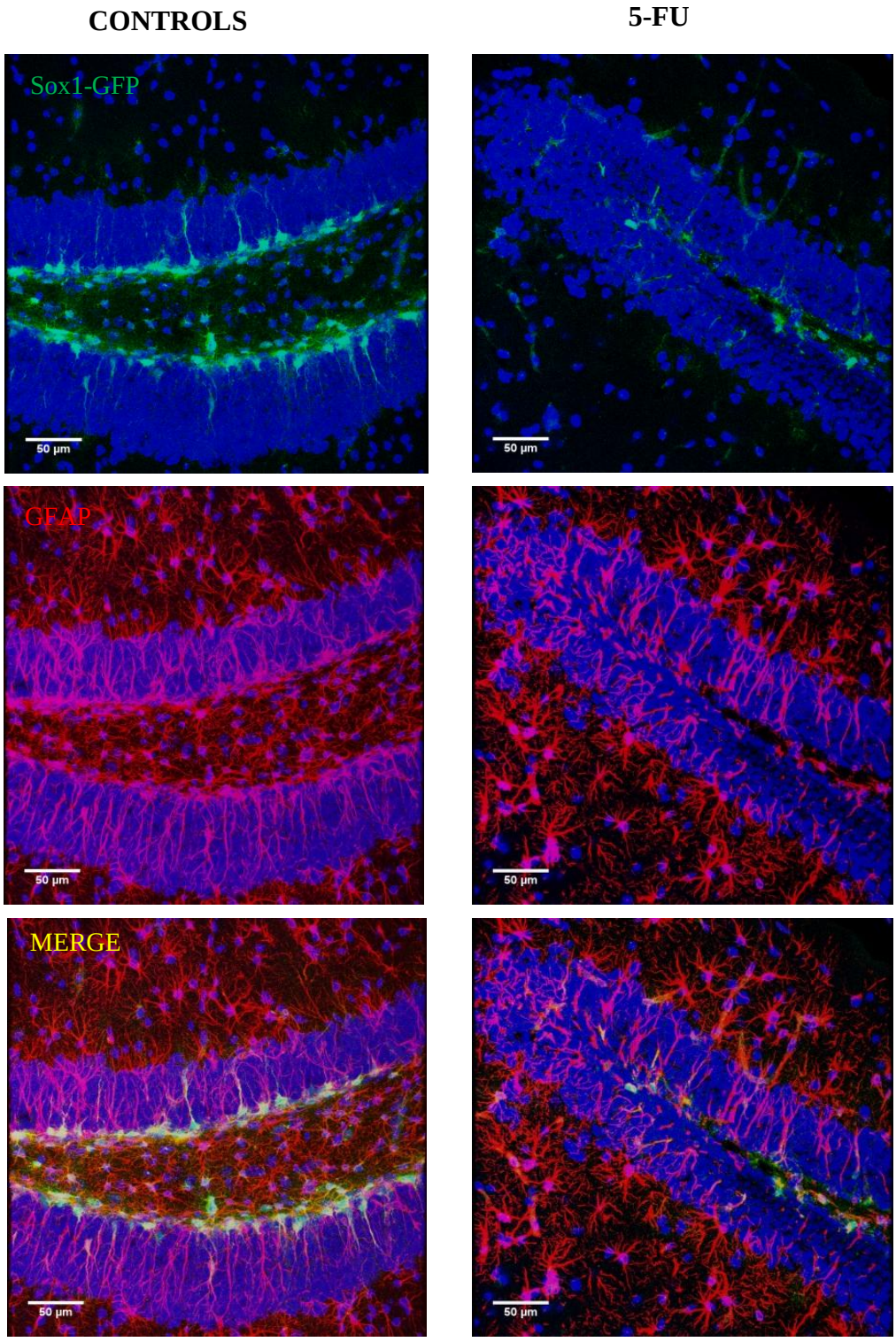
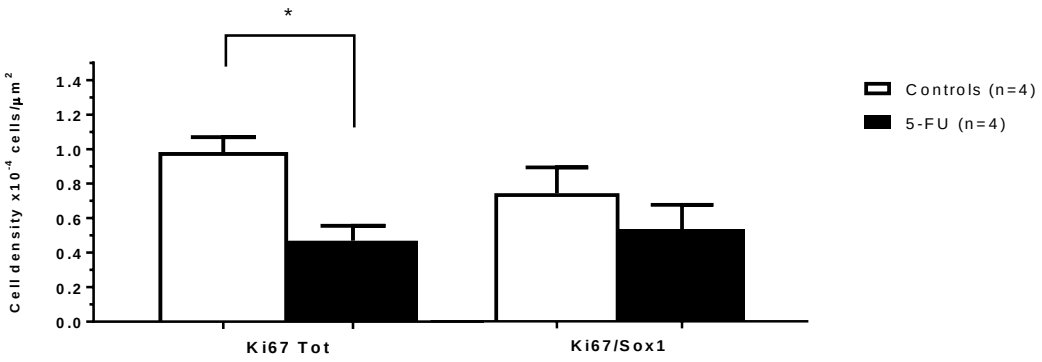


Figure 2.7 (a). ***GFAP and GFAP/Sox1 positive cells density in the SGZ (mean ± SEM, n=4).*** *Data analysis of GFAP+ and GFAP/Sox1+ cell density shows a significant effect of chronic 5-FU treatment.* ***(b) Sox1 in total, with radial and with horizontal process positive cells density in the SGZ (mean ± SEM, n=4).*** *Data analysis of cell density of Sox1 expressing cells and Sox1 positive cells with radial processes showed a significant effect of chronic 5-FU treatment. Sox1 positive cells with horizontal processes were not significantly affected by 5-FU treatment when compared with controls.* ***(c)*** *Sox1-GFP^{+} positive cells (green) located in the SGZ of control and 5-FU treated animals, showing co-labelling with GFAP (red); nuclei are highlighted by DAPI. Scale bar: 50 µm.*

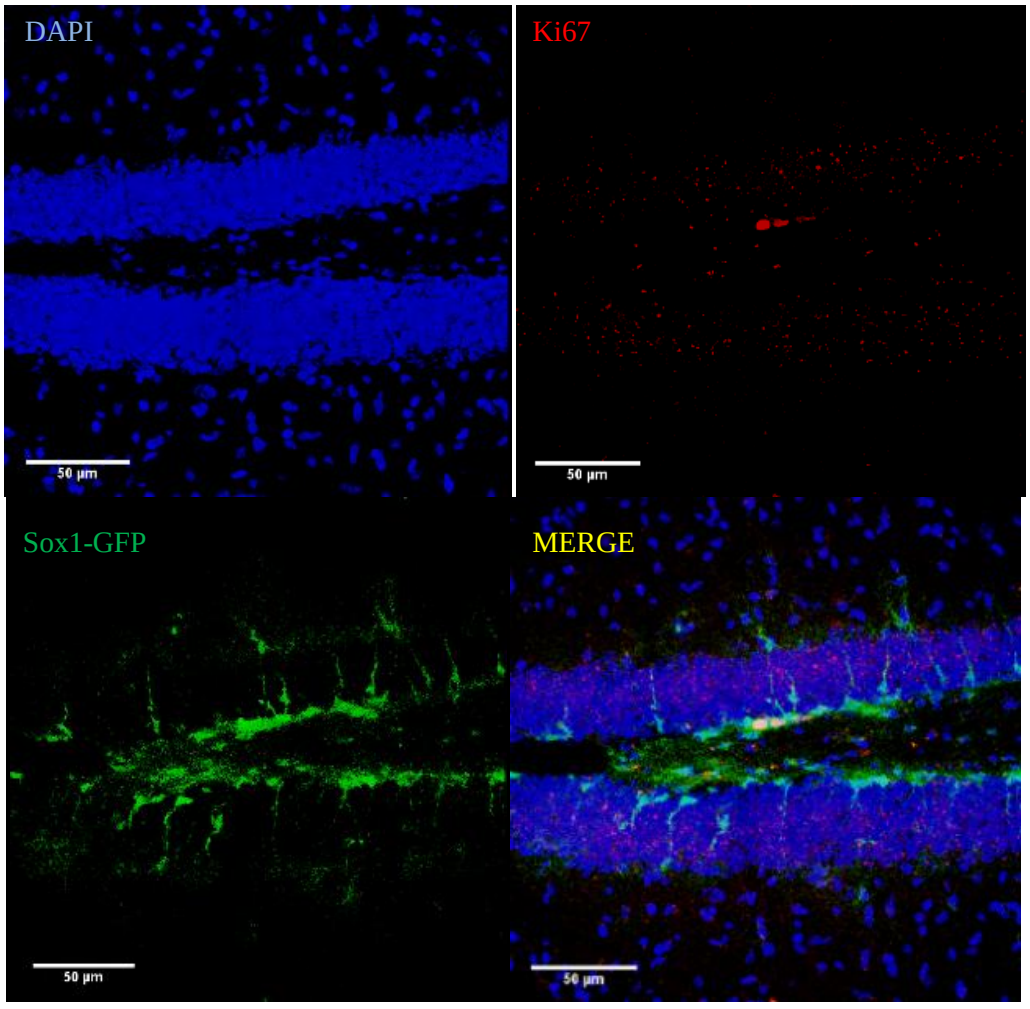
2.3.2.3 Effect of chronic 5-FU treatment on proliferation and differentiation

To evaluate the effect of the chronic treatment with 5-FU on proliferating and differentiating cells in the SGZ, we analysed the expression of Ki67 and DCX. Ki67+ and DCX positive cells were found in the SGZ of the hippocampus. Overlaying the green fluorescent protein image with the same red immunostained Ki67 image, it was possible to quantify dividing neural stem cells expressing Sox1 (Fig. 2.8 b). The remaining part of Ki67+ cells within the SGZ are assumed to be glia. Data analysis showed that the total number of proliferating cells decrease in animals treated with 5-FU when compared with controls ($F_{15,13}$= 1.172, $p < 0.05$, Fig. 2.8a). However, proliferating cells that express Sox1 were not significantly reduced in animals treated with 5-FU ($F_{15,13}$= 1.280, $p > 0.05$; Fig. 2.8a). Unpaired t-test showed that differentiating cells, DCX positive (Fig. 2.8d) of mice treated with 5-FU were not significantly affected by the treatment when compared with control animals ($F_{9,14}$= 2.386 $p > 0.05$; Figure 2.8c).

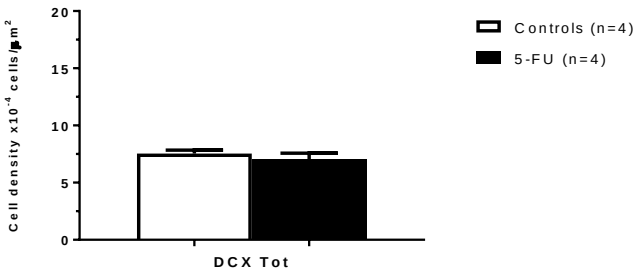
a


*
Cell density x10^{-4} cells/µm^2
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ki67 Tot
Ki67/Sox1
Controls (n=4)
5-FU (n=4)

b


DAPI
Ki67
Sox1-GFP
MERGE
50 µm
50 µm
50 µm
50 µm

c



d

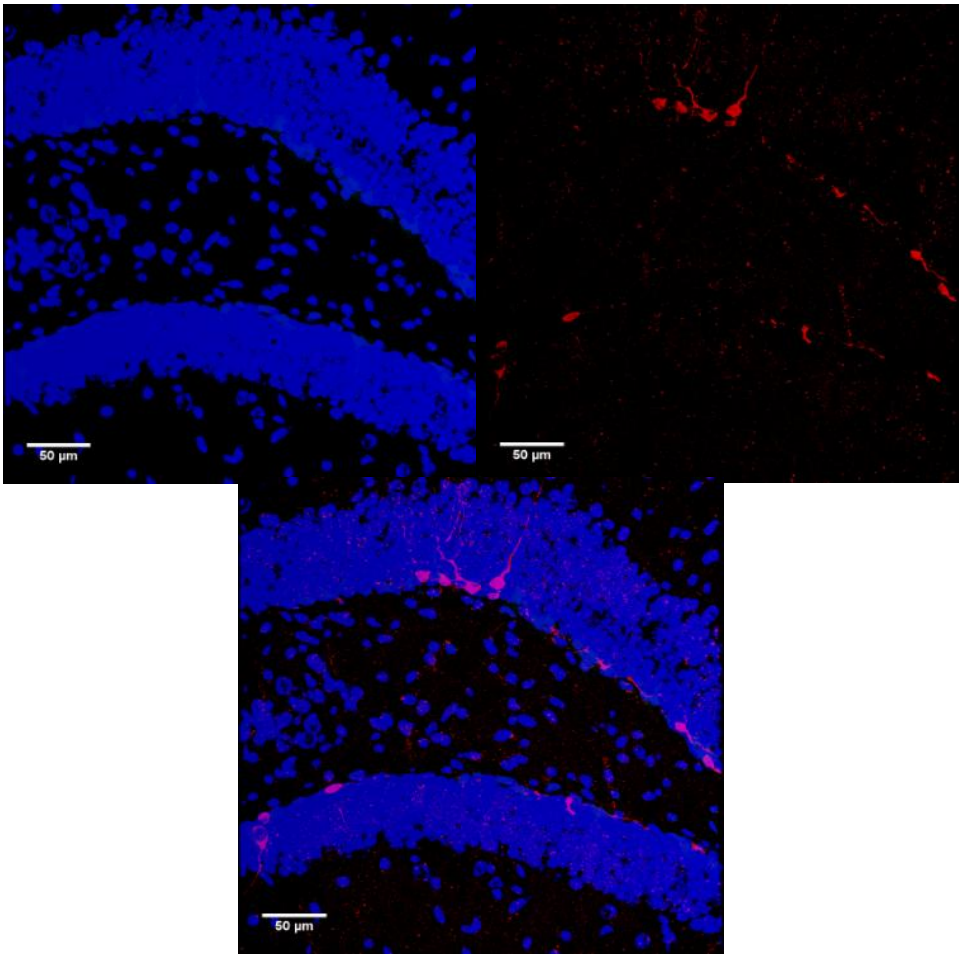


Figure 2.8. (a) ***Ki67+ and Ki67/Sox1+ cell density in the SGZ (mean ± SEM, n=4).*** *Quantification of Ki67+ showed a significant decrease in animals treated with 5-FU when compared with control animals. Cell density of proliferating cells Ki67+ also expressing Sox1 was not significantly affected by chronic treatment with 5-FU.* ***(b)*** *Photos showing positive immunostaining nuclear stain DAPI (blue), proliferating cell marker Ki67 (red), Sox1-GFP expressing cells (green) and photos merged. Scale bar: 50 µm.* ***(c) DCX+ cell density in the SGZ (mean ± SEM, n=4).*** *Data analysis of DCX+ cells showed a no significant reduction in animals receiving chronic treatment with 5-FU when compared with controls.* ***(d)*** *Photos showing nuclear stain DAPI (blue), positive immunostaining for neuroblasts marker DCX (red), and photos merged. Scale bar: 50 µm.*

A summary of the main results described in this chapter has been reported in the table below.

	GFAP+	GFAP+ Sox1+	GFAP-/Sox1+ with vertical processes	GFAP-/Sox1+ with horizontal processes	Ki67+	Ki67+/Sox1+	DCX
Acute treatment	=	=	=	=	=	↓	↓
Chronic treatment	↓	↓	↓	=	↓	=	=

Table 2.1. *Summary of effect of acute and chronic treatment with 5-FU on different markers used in this chapter*

2.4 Discussion

The aim of this study was to evaluate the immediate acute and chronic effects of 5-FU treatment on different populations of neural stem cells in the SGZ of the hippocampus. The effect of chemotherapy treatment on the proliferation and differentiation of neural stem cells was also evaluated. Animals were subjected to either two i.p injection of 75mg/kg 5-FU in one day, or 4 days of injections over a period of 10 days.

Chemotherapy treatment is aggressive and efficient at killing diving cells, including the proliferating cells of the gastrointestinal tract (Huang et al., 2002). As a consequence of 5-FU treatment, intestinal damage could lead to a loss of body weight (Song et al., 2013). As illustrated above (see Figure 2.3) acute treatment with 5-FU did not affect body weight, suggesting that two i.p injection of 5-FU, six hours apart, in a single day of administration are not sufficient to cause body weight loss. On the contrary the same chemotherapy dosing regimen applied in chronic treated animals, significantly affected their body weight. This is a well-known side effect of chronic 5-FU treatment which has been previously reported in other animal studies (Cao et al., 2014, Elbeltagy et al., 2012).

Clinical studies have highlighted an association between treatment with chemotherapeutic agents and long term cognitive dysfunctions in cancer patients (Vardy et al., 2007). These dysfunctions have been associated with a decrease in the number of proliferating cells in the SGZ which are needed for hippocampal neurogenesis. However, the populations of neural stem cells affected by chemotherapy treatment have not previously been identified. In the adult rodent dentate gyrus it is possible to identify using immunofluorescence techniques, different populations of neural progenitors including quiescent radial neural stem cells, activated radial and non-radial NSCs that give rise to intermediate progenitors which successively produce mitotic neuroblasts to become immature neurons and then mature granule neurons of the dentate gyrus (Crowther and Song, 2014). In this work, we identified different subpopulation of NSCs using the transgenic Sox1-GFP mice line. NSCs populations were identified based on the presence GFAP immunoreactivity by analysing process orientation (radial or horizontal). As described in the introduction of this chapter, we identified the following stem cell populations: quiescent radial glia like neural stem cells (qRGLs) which have a radial morphology, GFAP positive but Sox1 negative; a population of activated NSCs which are still GFAP positive and express Sox1, a population of NSCs that express Sox1 but not GFAP and finally a population of non-radial cells which continue to express Sox1 (see Figure 2.1). Investigating the impact of acute administration of 5-FU, it was possible show that chemotherapy does not significantly change the size of the subpopulation of NSCs in the SGZ of 5-FU treated Sox1-GFP mice. To determine the effect of 5-FU on proliferating cells, we have analysed the total expression of Ki67 and the expression of Ki67 in Sox1 expressing cells. A fraction of Sox1 positive cells co-labelled with Ki67 and showed a non-radial morphology. The cell density of proliferating cells present in the SGZ of Sox1-GFP

mice treated with 5-FU decreased significantly. It was possible see the same significant reduction of proliferating cells that express Sox1, namely, non-radial neural stem cells, in mice receiving 5-FU. To explain the apparent absence of an acute effect of 5-FU on NSCs not in a proliferative state, we hypothesize that this dose of chemotherapy was too low and the time of administration is too short to see a change in the behaviour of NSCs of the hippocampus. As mentioned previously, regimes of 5-FU dosing in the literature for mice show a wide range of 5-FU dosages with different reports of morbidity. In the Raghavendran et al study, for example, a single intra-peritoneal injection of 5-FU (200 mg/Kg) caused a significant body weight loss 5, 7 and 10 days after the injection (Raghavendran et al., 2012). In another study an intra-peritoneal single dose of 5-FU (160mg/Kg) reduced the blood cell count and a second injection at 3 or 7 days killed the mice (de Haan et al., 1996). In our previous studies (data not published) a single intra-peritoneal injection of 5-FU (50 mg/Kg) in male Sox1-GFP mice did not affect body weight or cell proliferation. Taking these results together, it is possible that in this case, the dose of 5-FU was too low to cause either body weight loss or a decreased number of NSCs, as shown in other previous studies, but was sufficient to induce a reduction in cell proliferation as expected. The evaluation of the impact of chemotherapy on DCX expressing cells showed that acute chemotherapy treatment caused a decrease in the number of cells expressing this marker. This result is supported by previous studies that have found a reduction of neuroblasts after chemotherapy treatment (Winocur et al., 2015, Winocur et al., 2014).

In the chronic study we showed that chronic 5-FU treatment modified the cellular composition of the germinal niche of Sox1-GFP transgenic mice. Firstly, we analysed the fraction of quiescent radial neural stem cells which were GFAP positive and the number of activated radial neural stem cells which were GFAP and Sox1-GFP positive.

Both populations showed a decrease in treated mice (see Table 2.1). However, quantification of the number of non-radial neural stem cells, showed that this population was unaffected by chemotherapy treatment. In agreement with what has been previously found, the total amount of proliferation decreased after treatment with 5-FU. However, in this case proliferating cells that express Sox1 seem to be unaffected by chemotherapy. According with that said above, it is evident that radial stem cells are the subpopulation of cells most affected by chemotherapy treatment. The decrease in the number of this kind of cell could result in a depletion of an essential early component of the neural stem cell pool within the SGZ of the dentate gyrus. This may explain the long term decline in hippocampal neurogenesis found in animal models and the long term cognitive effects found in animal models and patients after chemotherapy treatment. The cellular mechanisms underlying the depletion of the stem cell pool remain to be elucidated. Previous studies have never detected symmetrical division of quiescent neural stem cells, and throughout the course of time they may disappear from the subgranular zone of the dentate gyrus (Encinas et al., 2011). We suppose that chronic treatment with 5-FU accelerates this process of stem cells "disposal" increasing the rate of radial stem cells death due to an unrepairable level of DNA damage . We also hypothesize that this stem niche deforestation could be caused by the terminal differentiation of radial NSCs into astrocytes. As, astrocytes play a key role in maintaining cerebral homeostasis by reinforcing the blood-brain barrier, participating in neural signal transduction and modulating the blood flow in the brain (Allen and Barres, 2009). Under pathological conditions, astrogliosis is considered to be a defensive mechanism that limits the extent of tissue damage (Burda and Sofroniew, 2014). It has been shown that astrocytes are involved in restricting inflammation within the brain. During injury, reactive astrocytes form a scar tissue that demarcates and surrounds the

damaged tissue. Within the demarcated area, there is a robust inflammatory response that is cleanly separated from the immediately adjacent viable neural tissue by the scar, and inflammation resolves rapidly after injury (Sofroniew, 2005). Furthermore, a study has demonstrated that astrocytes protect melanoma cells from cell death induced by cytotoxic agents conferring resistance to chemotherapy treatment (Lin et al., 2010). Taken together, these and other findings allow us to hypothesise that radial NSCs of mice treated with chemotherapy undergo a terminal differentiation into astrocytes. These astrocytes could then form scar tissue around clusters of activated non-radial neural stem cells protecting them by giving resistance to the effect of 5-FU (See Figure 2.9). However, the mechanism involved in the depletion of the stem cells pool is still unclear and further work is needed to clarify this. This work needs to include an evaluation of astrocyte differentiation from quiescent neural stem cells, and an evaluation of the amount of apoptosis in the SGZ. Moreover, the lack of effect of chemotherapy on activated and proliferative populations of non-radial neural stems cells remains to be explained. Further investigations are necessary to elucidate the possible mechanisms that preserves this group of cells.

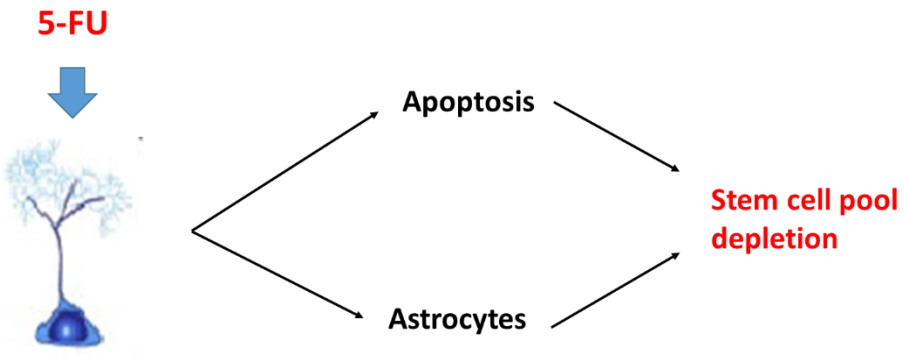


Figure 2.9. *Diagram to illustrate the possible consequence of chronic chemotherapy treatment on quiescent/activated neural stem cells with radial processes.*

As expected the total amount of proliferation decreased in animals injected with 5-FU and in accordance with what we hypothesized above, proliferating Sox1 expressing

cells were not affected by chemotherapy treatment. This result reinforces our hypothesis with regards to the possible protective activity of astrocytes on clusters of proliferating cells.

We also evaluated the amount of neural differentiation using the neuroblast marker doublecortin in controls and mice under treatment. In contrast with previous work in rats (Winocur et al., 2015, Mustafa et al., 2008), the number of DCX positive cells was not significantly affected by 5-FU chronic treatment. Different 5-FU doses and time of treatment make comparison with other studies difficult. See Table 2.1 for a summary of main results reported in this chapter.

In conclusion this study has shown that radial stem cells are particularly sensitive to chronic treatment with 5-FU. This treatment caused a reduction in cell proliferation in the SGZ, which particularly affected radial stem cells. In contrast non-radial stem cells were not affected by chemotherapy treatment. Using the Sox1-GFP transgenic model, it has been possible provide information on stem cell sub populations within the SGZ indicating the usefulness of this model in future work.

Chapter 3 - EFFECT OF 5-FU ON HIPPOCAMPAL NEUROGENESIS IN SOX1-GFP TRANSGENIC MICE: POTENTIAL PROTECTIVE EFFECT OF INDOMETHACIN.

3.1 Introduction

The following chapter describes the effect of chronic treatment with 5-FU on Sox1-GFP mice and evaluates the potential protective effect of the anti-inflammatory drug indomethacin. The parameters measured were, the number of GFAP expressing radial glia like neural stem cells, the number of dividing cells as shown by the proliferation marker Ki67 and the number of differentiating neural cells expressing DCX. This experiment also measured the extent of neuroinflammation by evaluating the number of microglia and their activation together with the number of cells expressing the inflammatory marker Cox-2.

A wide range of chemotherapy doses and a number of different rodent strains have been used in previous studies on the impact of chemotherapy. These have produced conflicting accounts of the effects, morbidity and mortality produced by different protocols which has made it difficult to identify a chemotherapy dose suitable for this kind of experiment. Most studies have found that in order to see a decrease in cell proliferation in the hippocampus, it is necessary give a higher dose of 5-FU to mice as compared to rats (Krynetskiy et al., 2013, Walker et al., 2011, Elbeltagy et al., 2012). This is explained by the faster metabolism and breakdown of the drug in mice as compared to rats. As described in Chapter 2 of this thesis a dose of 75mg/Kg injected twice a day in mice could result in some animals exceeding the licenced weight loss and animal death during chronic treatment. From our results certain strains of mice like Sox1-GFP transgenic mice, are more sensitive to the drug than wild type mice. For this reason the 5-FU dose chosen for this experiment was 75mg/Kg once a day, in accordance with previous studies of our group. Previous experiments in our and other labs have investigated the effect of chronic treatment with 5-FU in rodents (Elbeltagy et al., 2010, Han et al., 2008, Mustafa et al., 2008, Lyons et al., 2011a). These studies have

found that this dose caused a reduction in cell proliferation within the SGZ of the hippocampus (Elbeltagy et al., 2012).

As described in Chapter 2, the transgenic Sox1-GFP mouse model allows investigation of the effect of chemotherapy on the neural stem cell populations of the hippocampal neurogenic niche. This particular transgenic line allows the visualization of Sox1 positive cells using GFP as a reporter gene (Aubert et al., 2003). The GFP knock-in mice permit visualization of Sox1 expressing neural stem cells by fluorescence microscopy. Sox1 expressing cells are localized in the germinal regions of the adult brain (hippocampus and lateral walls of the lateral ventricles) and is also expressed by a putative stem cell pool in the postnatal cerebellum (Aubert et al., 2003, Pevny and Rao, 2003, Barraud et al., 2005, Sottile et al., 2006, Kan et al., 2007). The expression of Sox1 promotes the activation of NSCs and maintains proliferation in active NSCs. Sox1 is expressed in only a subset of stem/early progenitor cells and lineage tracing shows that these are the cells responsible for the production of the majority of neuroblasts (Venere et al., 2012). This transgenic tool allows us to describe the location, number and morphology of GFP expressing cells. NSCs populations were identified based on the presence GFAP immunoreactivity and by analysing the orientation of cell processes (radial or horizontal). The stem cell populations identified in this study are: a) quiescent radial glia like neural stem cells (qRGLs) which have a radial morphology, are GFAP positive but Sox1 negative; b) a population of activated NSCs which are still GFAP positive and express Sox1; c) a population of NSCs that express Sox1 but not GFAP and finally d) a population of non-radial cells which continue to express Sox1 (See Figure 2.1, Chapter 2).

Few experiments have looked at the neuroinflammatory effect of chemotherapy in the brain and particularly in the hippocampal neurogenic niche. A recent study has shown that treatment with a combination of cyclophosphamide, methotrexate and 5-FU caused an increase in the levels of the pro-inflammatory cytokines IL-β and TNF-α in the corpus callosum of rats when analysed 4 weeks after the end of chemotherapy treatment. This change in cytokine levels was associated with a reduced performance in memory tasks (Briones and Woods, 2014). Previous studies have shown that 5-FU treatment can induce inflammation in peripheral tissues (Lopes et al., 2009) but it now seem likely that this drug can also induce inflammation in the brain, either by direct action on neural tissue or indirectly via peripherally produced cytokines which can passage through the blood brain barrier (BBB) (Lopes et al., 2009, Elbeltagy et al., 2012). It has also been observed that the production in peripheral tissues of the inducible prostaglandin producing enzyme cyclooxygenase-2 (COX-2) is enhanced by systemic 5-FU treatment (Lopes et al., 2009). Neuroinflammation has been recently considered as one of the mechanism responsible for CICI (Seigers and Fardell, 2011, Ahles and Saykin, 2007) and for this reason some researchers are focused on the study of microglia and their activation. Christie et al, in 2012 have described an increased number of activated microglia in the hippocampus of rats 4 weeks after cyclophosphamide treatment (Christie et al., 2012), It has been hypothesized that CICI could be the result of a direct effect by chemotherapy on neurogenesis but may also be caused by a persistent inflammatory condition that can indirectly reduce neurogenesis.

The interaction between inflammation and neural stem cell behaviour has recently emerged as an important research area. It has been demonstrated that inflammation is involved in majority of human neurodegenerative diseases might exert specific deleterious effects on stem cells and in the microenvironment of neurogenic niches (Das

and Basu, 2008). The exact influence of inflammation on NSCs has been controversial, because both negative and beneficial effects have been described suggesting that the interactions between the immune system and NSCs are context dependent (Crutcher et al., 2006). However, that inflammatory processes in the neurogenic regions of the brain, greatly alters the microenvironment of neural stem cells and influences their fate has been well established. All stages of adult neurogenesis, namely, proliferation, differentiation, and migration of NSCs, are modulated differentially by the inflammatory response. It appears that an uncontrolled immune response impairs progenitor survival and proliferation and blocks repair processes and is associated with cognitive impairment (Ekdahl et al., 2003, Monje et al., 2003). One reason behind impaired neurogenesis in inflammatory conditions has been suggested to be a dysfunction in the progression of the cell cycle of NSCs and a reduction in the migratory behaviour of neuroblasts (Pluchino et al., 2008).

A number of studies have investigated pharmacological approaches to improve the quality of life and cognition of patients treated with chemotherapeutic agents. Treatment with non-steroidal anti-inflammatory drugs such as indomethacin, have been suggested to ameliorate Alzheimer`s disease and reduce the increased inflammation in the brain found during ageing (Cote et al., 2012, Waldstein et al., 2010). In a recent study it was shown that the co-administration of a COX-2 inhibitor with the chemotherapy agents cyclophosphamide, methotrexate and 5-FU, normalized the levels of inflammatory cytokines and ameliorated the deficits seen during memory tasks after chemotherapy treatment (Briones and Woods, 2014). This study however did not look at the effect of chemotherapy or anti-inflammatory drugs on hippocampal neurogenesis. Treatment of rodents with the NSAID indomethacin has also resulted in protection against the cognitive deficits induced by cranial irradiation. Histological analysis of the

brains of animals treated in this way showed, a decrease in the number of activated microglia and infiltrating monocytes found in the dentate gyrus of the hippocampus. Indomethacin treatment during and after radiation exposure, also partially restored the number of proliferating cells in the hippocampus (Monje et al., 2003). In our study indomethacin was administered in the drinking water at a dose of 2mg/Kg/day. This route of administration was used to reduce the stress induced by daily i.p injections (Sharp et al., 2002). This dose of indomethacin is based on a previous study which reported that a chronic treatment with this drug reduced tumour volume by 49% in a mouse model of cancer (Quidville et al., 2004). As previously mentioned the anti-cancer effects of anti-inflammatory drugs would make them a particularly suitable adjuvant treatment for patients receiving chemotherapy.

Use of the previously mentioned battery of markers together with the Sox1-GFP transgenic tool enabled the assessment of neural stem cells subpopulations of the hippocampal neurogenic niche and their behaviour after administration of the anti-inflammatory drug indomethacin and the chemotherapy drug 5-FU either alone or in combination.

To evaluate neuroinflammation, we used antibodies to detect microglia (Iba1) and expression of COX2 in the cells of the dentate gyrus of Sox1-GFP mice. The ionized calcium binding adaptor molecule 1 (Iba1) is a microglia/macrophage-specific calcium-binding protein (Ohsawa et al., 2004). Microglia can be in resting, pro-inflammatory or anti-inflammatory states. The stage of activation can be distinguished by changes in microglial morphology. Resting microglia show a stellar ramified morphology with many long fine processes and a small cell body, a morphology which is thought to enable them to sample a wide region of the surrounding tissue. Activated microglia, in contrast have an elongated cell body with few thick short processes. Assessing the size

of the cell body and the area covered by and the number of processes extending from the microglial cell has previously been used as indicators of microglia activation (Gebara et al., 2015). Indomethacin is a non-selective inhibitor of cyclooxygenease (COX), an enzyme that is required for the conversion of arachidonic acid to prostaglandins (Kohman and Rhodes, 2013). Cox2 is an inducible enzyme normally produced by macrophages and activated microglia at the sites of inflammation (Das and Basu, 2008). The density of hippocampal cells expressing COX-2 has been evaluated to determine the level of inflammation in this brain region.

This study aims to evaluate and compare the potential protective effects of indomethacin against the effect of chronic 5-FU treatment on neural stem cell subpopulations and on different phases of neurogenesis including proliferation and differentiation. The effect of these drugs has been also evaluated on neuroinflammation and microglia activation. The results of this experiment will enable a better understanding of the mechanisms involved in chemotherapy neurotoxicity and the action of drugs which may be neuroprotective and maintain neurogenesis.

3.2 Materials and methods

3.2.1 Animals and treatment

Fifteen 9 weeks-old male heterozygous Sox1-GFP$^{+/-}$ mice (Aubert et al., 2003), carrying a knocked in GFP reporter (BSU Transgenic Unit, University of Nottingham) were housed under standard conditions of temperature (22 ± 1° C; 12/12h light/dark cycle) with free access to food and water and randomly assigned to controls (n=4), 5-FU treatment (n=5), indomethacin treatment (n=3) and indomethacin + 5-FU treatment (n=3). Animals were allowed to habituate for one week prior to treatment. 5-FU treated animals were i.p injected with 5-FU (75 mg/Kg solved in 0.9% sterile saline, once a

day; Medac, GmbH, Hamburg, Germany) every third day for 10 days (4 days of injections in total). Control mice were injected with the same volume of 0.9% sterile saline. The volume of injections were calculated for individual animals based on body weight. This dosing regimen was based on our previous pilot studies and is comparable to human doses (Krynetskiy et al., 2013). Indomethacin (Sigma Aldrich, St. Louise, Usa) was administered in drinking water at a dose of 2 mg/Kg/day, based upon water consumption which was calculated every other day. An indomethacin stock solution was made by dissolving 50 mg of indomethacin in 50 ml of ethanol. Three times per week throughout the experiment indomethacin was added to the water bottles of animals receiving this drug. In controls and other groups not treated with indomethacin, a volume of ethanol equal to those receiving indomethacin was added to the drinking water. Indomethacin was administered to the animals for the duration of the experiment, starting one week prior to the first 5-FU injection. The total treatment time for animals was 17 days (Figure 1). Throughout the experiment, mice were examined daily and weighed every 2 days. Principles of laboratory animal care were in accordance to UK Home Office Guidance regulations and with local ethical committee approval. Procedures were carried out under a Home Office project license No 30/3163 and personal license No I55DE633D.

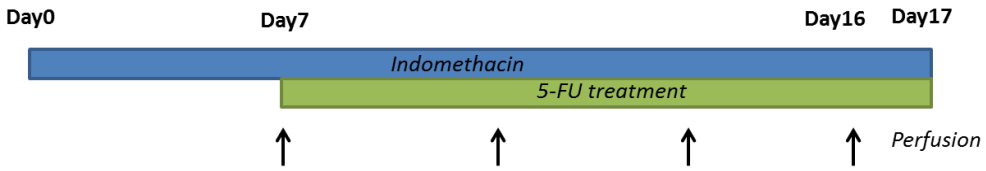


Figure 3.1. *Indomethacin (blue) treatment was given in drinking water throughout the experiment. 5-FU (green) was given after 7 days of indomethacin treatment, for 10 days every third day. Arrows indicates days of 5-FU injections. 24h after the last injection animals were put down.*

3.2.2 Brain tissue preparation

24h after the last injection, animals were euthanized with an overdose of pentobarbital sodium (Euthasol). Brains were processed as described in chapter 2.

3.2.3 Immunohistochemistry

Sections were washed in phosphate-buffered saline (PBS) three times (5 min each) and then incubated in blocking solutions: 15% normal goat serum and 0.3% Triton X-100 in PBS (Ki-67 immunostaining), 3% bovine serum albumin (BSA), 2% goat serum and 0.3% Triton X-100 in PBS (GFAP and Iba1 immunostaining), 1.5% BSA and 0.3% Triton X-100 in PBS (DCX immunostaining), 1% BSA and 0.3% Triton X-100 in PBS (Cox2 immunostaining) for 1h at room temperature. Primary antibodies were diluted in blocking solution and used as follows in an overnight incubation at 4°C: Ki67 (rabbit, polyclonal 1:1000, Vector Laboratories, Orton Southgate, UK), GFAP (1:500, rabbit, polyclonal, Dako, Carpinteria, CA, USA), DCX (1:100, rabbit, polyclonal, Santa Cruz Biotechnology), Cox2 (1:500, rabbit polyclonal, Abcam, UK) and Iba1 (1:2000, rabbit, polyclonal, Wako). All secondary antibodies were Alexa Fluor (1:500, Invitrogen, Gran Island, NY, USA), labelled with 568, 488 and 594 fluorochromes, diluted in 0.1% Triton X-100 in PBS and incubated for 1hr at room temperature in the dark. After washing with PBS, sections were mounted on slides coated with gelatine counterstained with DAPI nuclear stain (1:1000, Invitrogen, Gran Island, NY, USA).

3.2.4 Image analysis and cell counting

Images were acquired using a Nikon EFD-3 Fluorescence microscope, at 20x and 40x magnification and photographed with an Optiphot-2, Spot insight QE camera. For each animal, a 1-in-5 series of sections was stained with the nuclear marker DAPI and use to

measure the area of the granule cell layer. The granule cell area was measured using ImageJ Software. The total number of immunolabeled cells were counted when the positive immune labelling co-localised with DAPI stained cell nuclei and were within the granular cell layer or within a three cell diameter distance from the hilar side of SGZ. Positive cells were counted along the entire length of the dentate gyrus and the total number in each section was related to the granular cell layer sectional area to estimate the density of immunoreactive cells. At least five coronal sections were stained with GFAP and confocal z-stacks were taken of the dentate gyrus from one hemisphere randomly chosen using 40X objective. Cells positive for GFAP and Sox1-GFP were counted first in every single channel and then the co-localization was detected in the merged images. Co-localization was checked in the serial scan scans including the 3D projection of the z-stack. The total number microglia was investigated using immunostaining against the microglia specific marker Iba-1. As described in the chapter 2, the morphology of microglia was measured to evaluate the state of activation of these cells. The parameters measured were soma area, the territory occupied by projections from the cell body and the number of projections. All parameters have been measured with ImageJ software.

3.2.5 Statistical analysis

All data are expressed as group means ± SEM. Statistical analysis was performed using Prism v6.0 (GraphPad, San Diego, USA) using analysis of variance (Two Way ANOVA) with treatment in drinking water and drug injected as factors. When significant interaction effects were found, Bonferroni post-hoc analysis was performed to elucidate the difference between groups within conditions. For all statistical tests, the threshold of significance was set at $p \leq 0.05$.

3.3 Results

3.3.1 5-FU treatment did not affect body weight

In this experiment animals were killed 24hr after the last injection of 5-FU. As expected animals in all groups gained or maintained body weight over the period of the study. A two-way repeated measure ANOVA on body weights data revealed a significant effect of time, treatment and treatment × time interaction ($F_{6,66}$ = 28.04, $F_{3,11}$ = 22.94, respectively, p< 0.0001 and $F_{18.66}$ = 1.871, p< 0.05; Figure 2). The difference between subjects was also significant ($F_{25,66}$ = 25.96, , p< 0.0001). Animals treated with 5-FU showed a significant increase in body weight across with time when compared with controls (p< 0.0001), suggesting no significant effect of 5-FU treatment on body weight. This effect could be due to the higher body weight of mice allocated to the 5-FU group compared to animals in the control group at the beginning of the experiment. Mice were observed daily to determine any loss of conditions in animals receiving chemotherapy. No animals showed hair loss or had apparent changes in overall activity. None died following chemotherapy treatment.

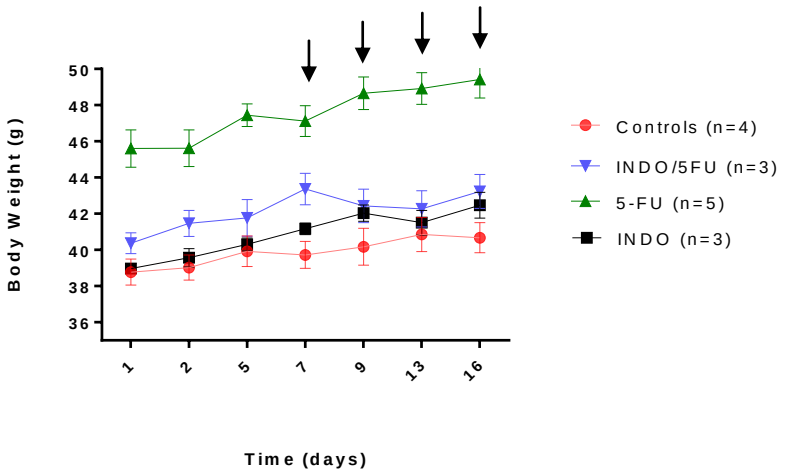


Figure 3.2. ***Body weight of mice (mean ± SEM) from the first day of indomethacin administration and during the 5-FU treatment.*** *Indomethacin was given from day 1 till day 17. Significant differences were determined by repeated measure ANOVA. Arrows at day 7, 9, 13 and 16 indicate days of saline or 5-FU injection.*

3.3.2 5-FU treatment and water intake

As indicated in the graph (Figure 3.3), a reduction in water intake for those animals receiving 5-FU was found. In contrast, it was observed that treatment with indomethacin and 5-FU in combination increased the fluid intake.

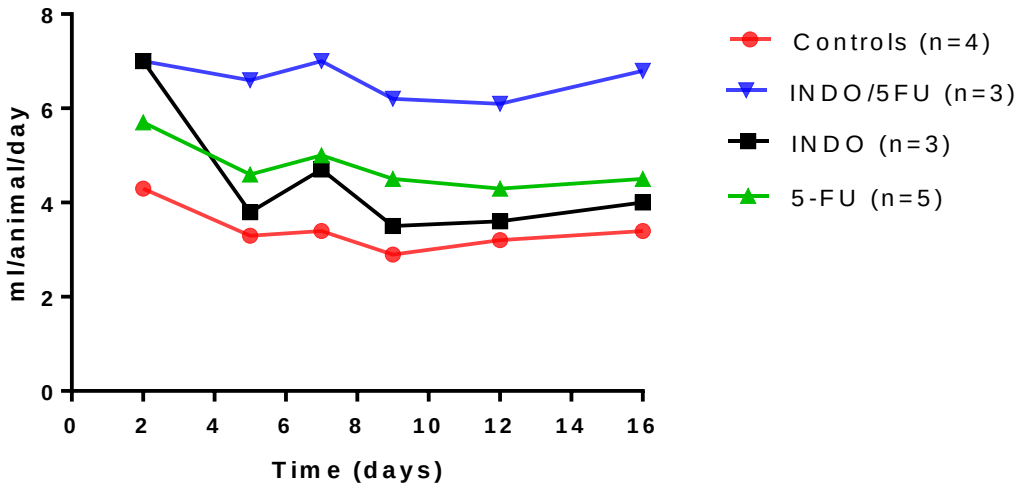
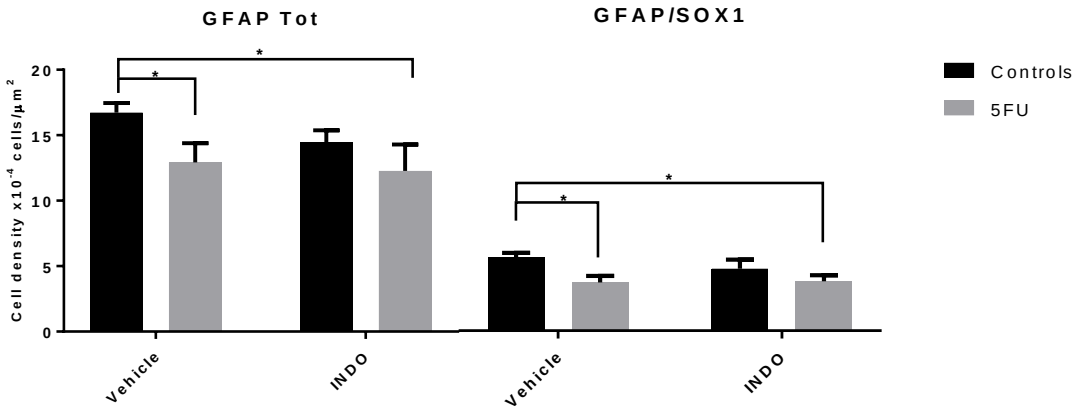


Figure 3.3 F*luid intake from the first day of indomethacin administration and during the 5-FU treatment of each group.* *Indomethacin was administrated from day 1 till day 16. Administration of 5-FU seems to reduce the fluid intake across the time, while the administration of indomethacin in combination with 5-FU apparently increased the water intake.*

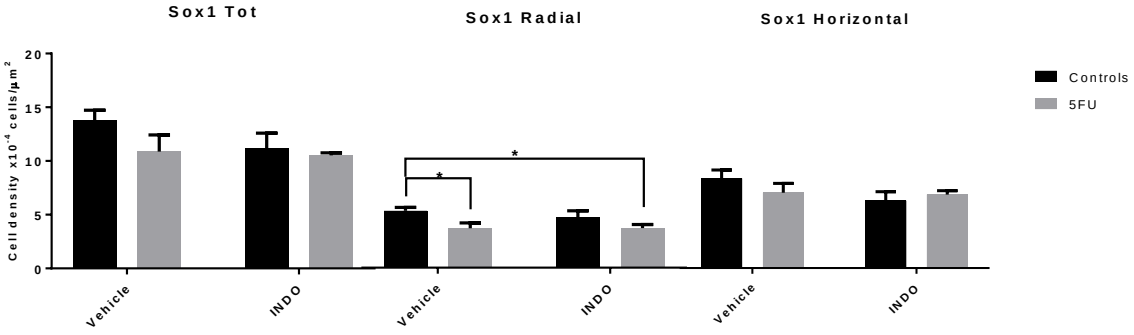
3.3.3 Effect of indomethacin and chronic 5-FU treatment on neural stem cells.

Quantification of cell density of different subpopulations of neural stem cells in the SGZ of the hippocampus was done 24h after the last injection of 5-FU. Two way ANOVA analysis showed that qRGL GFAP+ and GFAP/Sox1+ cells are affected by treatment with 5-FU (respectively, $F_{1,10} = 11.84$ and $F_{1,10} = 13.23$, $p < 0.05$). A post hoc Bonferroni test revealed a decrease in qRGLs and activated NSCs density after chronic treatment with 5-FU alone or in combination with indomethacin when compared with controls ($p < 0.05$; Figure 3.4a). The density of Sox1 expressing cells in the SGZ was also quantified. The analysis discriminated between Sox1 positive cells that extend radial or horizontal processes. Data analysis with a Two way ANOVA showed that the entire population of Sox1+ cells and the subpopulation of activated neural stem cells with horizontal processes was not significantly affected by treatment with 5-FU (respectively, $F_{1,10} = 3.685$ and $F_{1,10} = 0.22$, $p > 0.05$; Figure 3.4b,c). On the contrary, the subpopulation of activated neural stem cells with radial process resulted significantly affected by treatment with 5-FU ($F_{1,10} = 12.99$, $p < 0.05$). A Bonferroni post hoc test revealed that activated neural stem cells with radial process decreased significantly after treatment with 5-FU and with 5-FU/indomethacin when compared with control animals ($p < 0.05$; Figure 3.4 b,c).

a


GFAP Tot
GFAP/SOX1
Cell density x10-4 cells/µm2
20
15
10
5
0
*
*
*
*
Vehicle
INDO
Vehicle
INDO
Controls
5FU

b


Sox1 Tot
Sox1 Radial
Sox1 Horizontal
Cell density x10-4 cells/µm2
20
15
10
5
0
*
*
Vehicle
INDO
Vehicle
INDO
Vehicle
INDO
Controls
5FU

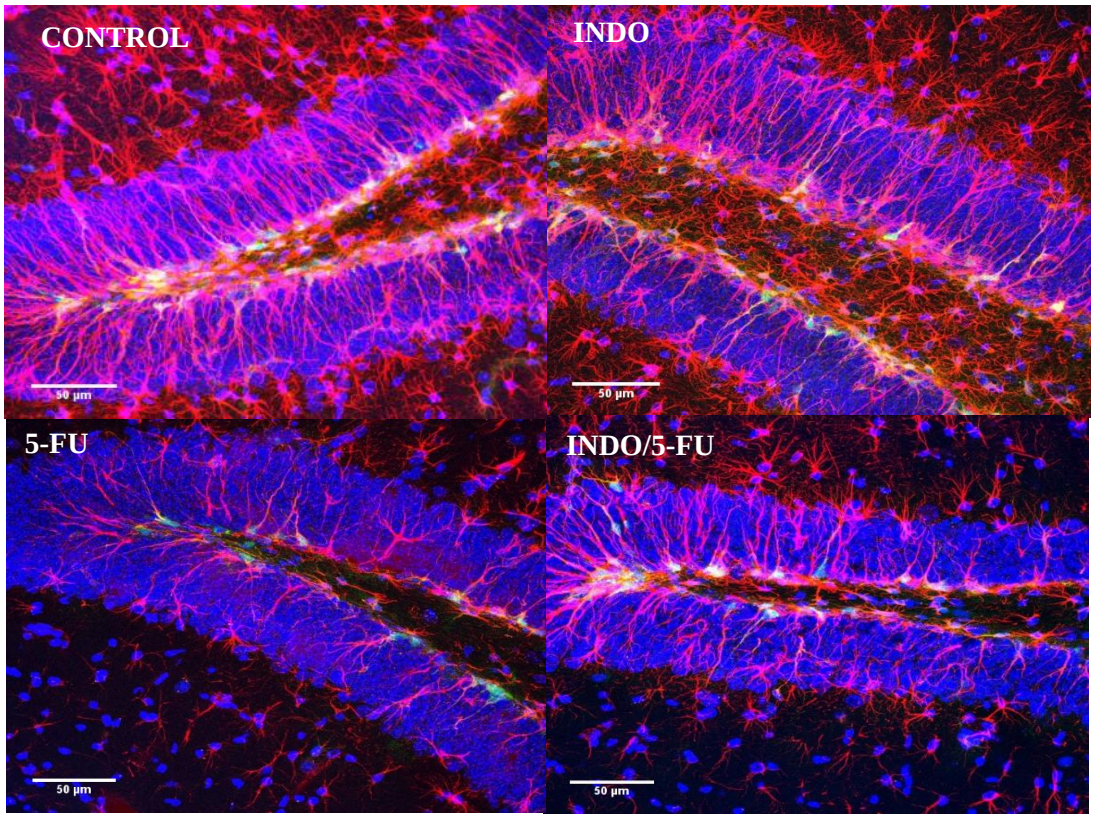


Figure 3.4 (a). ***GFAP and GFAP/Sox1 positive cells density in the SGZ of Sox1-GFP mice (mean ± SEM).*** *Data analysis of GFAP+ and GFAP/Sox1+ cell density shows a significant effect of 5-FU treatment alone or in combination. Activated neural stem cells that express both GFAP and Sox1 decreased in animals receiving 5-FU alone or in combination with indomethacin when compared with controls.* ***(b) Cell density of Sox1 expressing cells (Tot) and Sox1 cells with radial or horizontal processes in the SGZ of Sox1-GFP mice (mean ± SEM).*** *Data analysis of cell density of Sox1 expressing cells showed that Sox1 positive cells with radial processes were significantly affected by 5-FU alone or in combination with indomethacin. The total number of Sox1 positive cells and Sox1 positive cell with horizontal processes were not significantly affected by treatments.* ***(c)*** *Photos showing Sox1-GFP^{+} positive cells (green) located in the SGZ of control, 5-FU, indomethacin and indomethacin/5-FU treated animals, showing co-labelling with GFAP (red); nuclei are highlighted by DAPI. Scale bar: 50 µm.*

3.3.4 Effect of indomethacin and chronic 5-FU treatment on proliferation and differentiation

To evaluate the effect of chronic treatment with 5-FU and indomethacin on proliferating cells in the SGZ, we analysed the expression of Ki67. Ki67+ cells were found in the SGZ of the hippocampus but rarely elsewhere. It was possible to quantify dividing neural stem cells expressing Sox1, by overlaying the green fluorescent protein image with the red immunostained Ki67 image. Data analysis showed that the total number of proliferating cells and proliferating cells that express Sox1 (Ki67+/Sox1+) was significantly affected by treatment (respectively, $F_{1,9} = 8.571$ and $F_{1,9} = 6.504$, $p < 0.05$). A multiple comparison test showed decreased cells density of both populations of cells in animals treated with 5-FU when compared with controls ($p < 0.05$; Figure 3.5a). However, as shown in the graph (Figure 3.5a) animals co-treated with 5-FU and indomethacin showed an increased number of cells in both subpopulations of cells when compared with 5-FU group ($p < 0.05$; Figure 3.5a). This suggested that the effect of 5-FU on proliferating cells was abolished by co-administration with indomethacin. Cell differentiation in the SGZ was evaluated using the marker for differentiating cells doublecourtin (DCX) which marks neuroblasts. DCX positive cells were found in the SGZ of the hippocampus and Two way ANOVA analysis showed that neuroblast cell density is not significantly affected by treatments ($F_{1,10} = 2.347$, $p > 0.05$; Figure 3.5b).

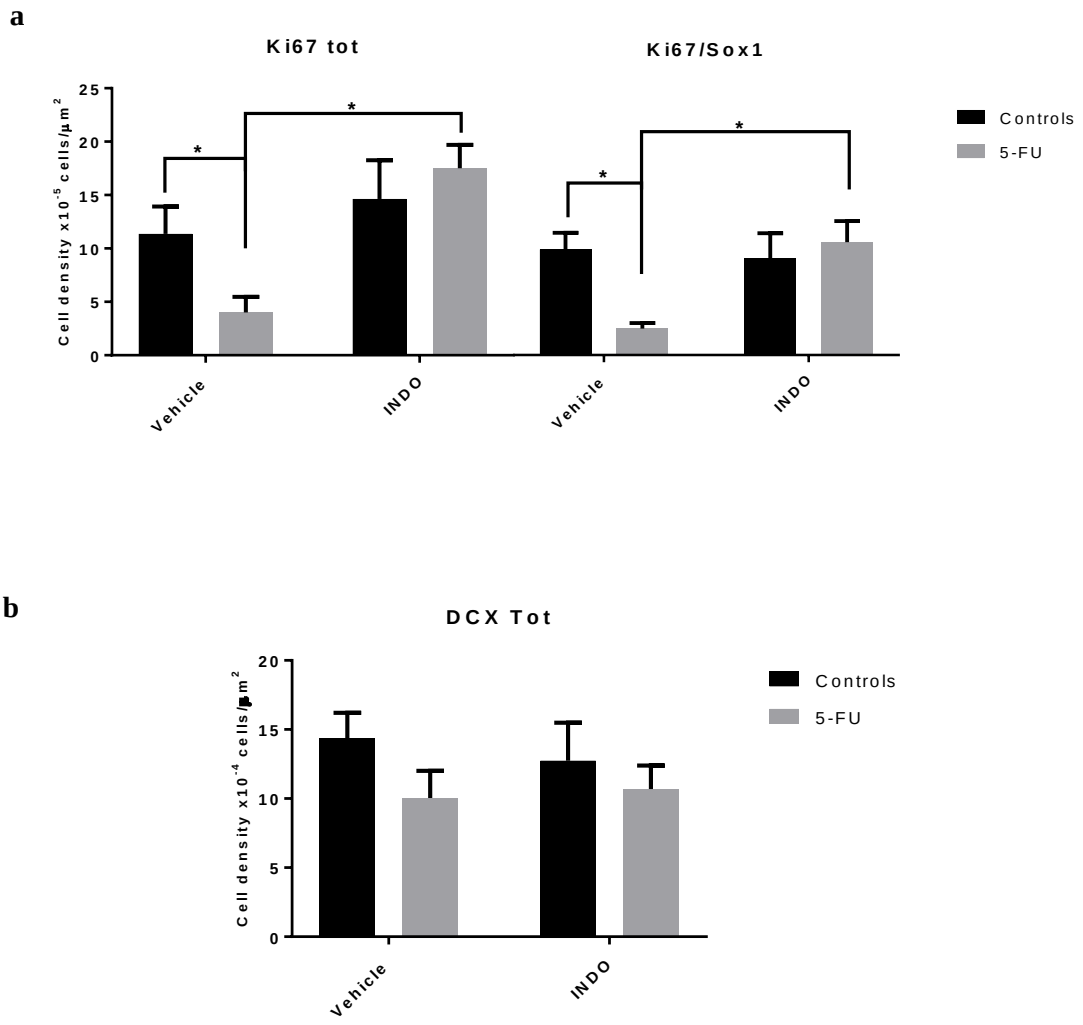


Figure 3.5. ***(a) Ki67+ and Ki67/Sox1+ cell density in the SGZ of Sox1-GFP mice (mean ± SEM).*** *Quantification of Ki67+ and Sox1+ proliferating cells showed a significant decrease in animals treated with 5-FU when compared with control animals. There is a significant increased number of cells in both subpopulation in animals co-treated with 5-FU and indomethacin when compared with 5-FU group ($p < 0.05$) suggesting that the effect of 5-FU on proliferating cells was abolished by co-administration with indomethacin.* ***(b) DCX+ cell density in the SGZ of Sox1-GFP mice (mean ± SEM).*** *Data analysis of DCX+ cells showed a non-significant reduction in animal receiving 5-FU alone or in combination with indomethacin.*

A summary of the effect of 5-FU and indomethacin treatment described in this chapter has been reported in the table below.

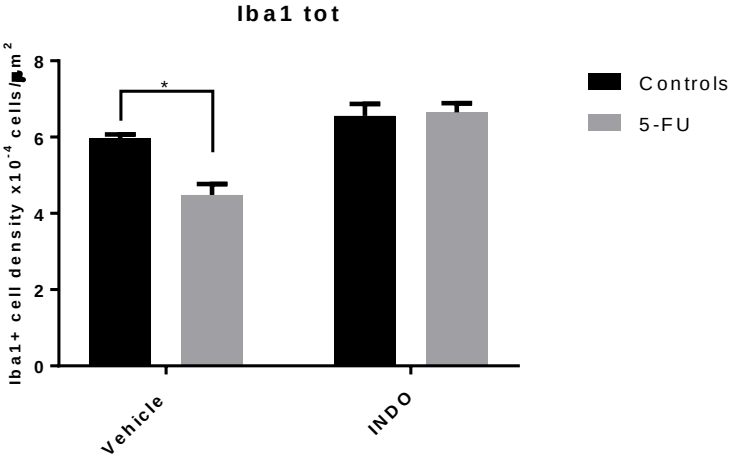
	Quiescent radial glia like neural stem cells GFAP+	Activated neural stem cells (radial morphology) GFAP+ Sox1+	Activated neural stem cells (radial morphology) Sox1+	Non-radial neural stem cells Sox1+	Proliferating Non-radial neural stem cells in proliferation Sox1+ Ki67+	Neuroblasts DCX+
5-FU	↓	↓	↓	=	↓	=
Indomethacin + 5-FU	↓	↓	↓	=	Protection	=

Table 3.1. ***Summary of chronic 5-FU and indomethacin treatment effect on different subpopulation of neural stem cells.***

3.3.5 Effect of 5-FU and indomethacin on microglia activation

The total number of microglia in the SGZ of the hippocampus was quantified 24h after the last 5-FU injection by immunostaining for the microglia specific marker Iba-1. Two way ANOVA data analysis showed a significant effect of treatment with 5-FU and Indomethacin on Iba-1 cell density (respectively, $F_{1,10} = 8.042$ and $F_{1,10} = 32.05$, $p < 0.05$). A Bonferroni post hoc test showed that 5-FU chronic treatment significantly affected the cell density of Iba-1 expressing microglia in the SGZ when compared with the control group ($p<0.05$; Figure 3.6a, b). However, animals co-treated with 5-FU and indomethacin showed an equal number of microglia when compared with control group ($p > 0.05$; Figure 3.6a, b) suggesting a potential protective effect of indomethacin treatment against the effect of 5-FU on microglia.

a



b

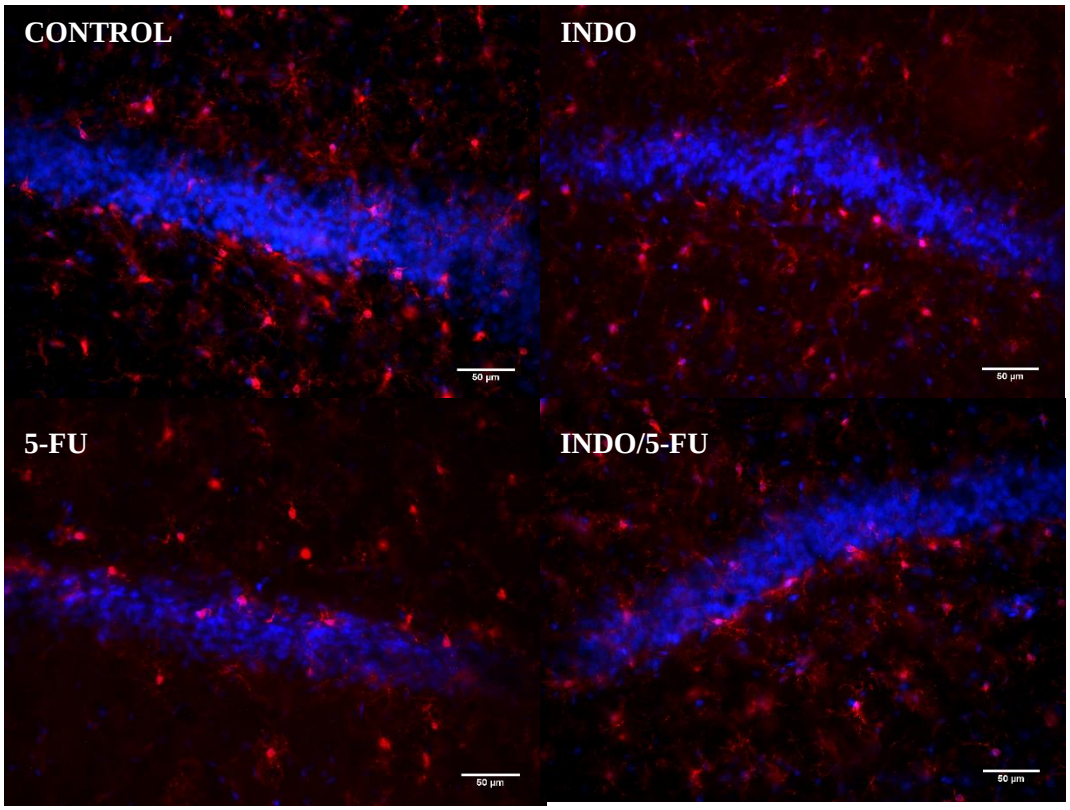


Figure 3.6. ***(a) Iba-1 positive cells density (mean ± SEM) in the SGZ of Sox1-GFP mice.*** *Mice injected with 5-FU had a significantly lower number of Iba-1 positive cells ($p<0.05$) in the subgranular zone. Other groups did not significantly differ from controls ($p>0.05$).* ***(b)*** *Photos showing Iba1 positive cells (red) in the SGZ of controls, indomethacin, 5-FU and indomethacin/5-FU treated animals. Scale bar: 50 µm.*

As reported in the Chapter 4, microglia seems to be particular sensitive to 5-FU treatment. To evaluate the level of microglia activation we analysed three morphological aspects of microglia: cell body size, territory projection area and number of process. Resting microglia are characterized by a round, small cell body and a high number of long processes while activated microglia show a big, elongated cell body with few short projections. 5-FU treatment does not change significantly the size of microglia cell bodies ($F_{1,9} = 2.16$, $p > 0.05$; Figure 3.7a). As expected, the indomethacin treatment significantly affected the territory area contacted by microglia ($F_{1,9} = 33.46$, $p < 0.05$; Figure 3.7b) A multiple comparison test with a Bonferroni post hoc test showed that 5-FU treatment significantly decreased the area contacted by microglia processes when compared with the indomethacin/5-FU group ($p<0.05$; Figure 3.7b). Furthermore, the territory covered by microglia processes increased in animals co-administered with indomethacin and 5-FU when compared with controls ($p < 0.05$) suggesting an increased proportion of microglia in a resting state (Figure 3.7b). However, the number of branches was not affected by any treatment ($F_{1,9} = 0.002$ and $F_{1,9} = 2.605$, $p < 0.05$; Figure 3.7c).

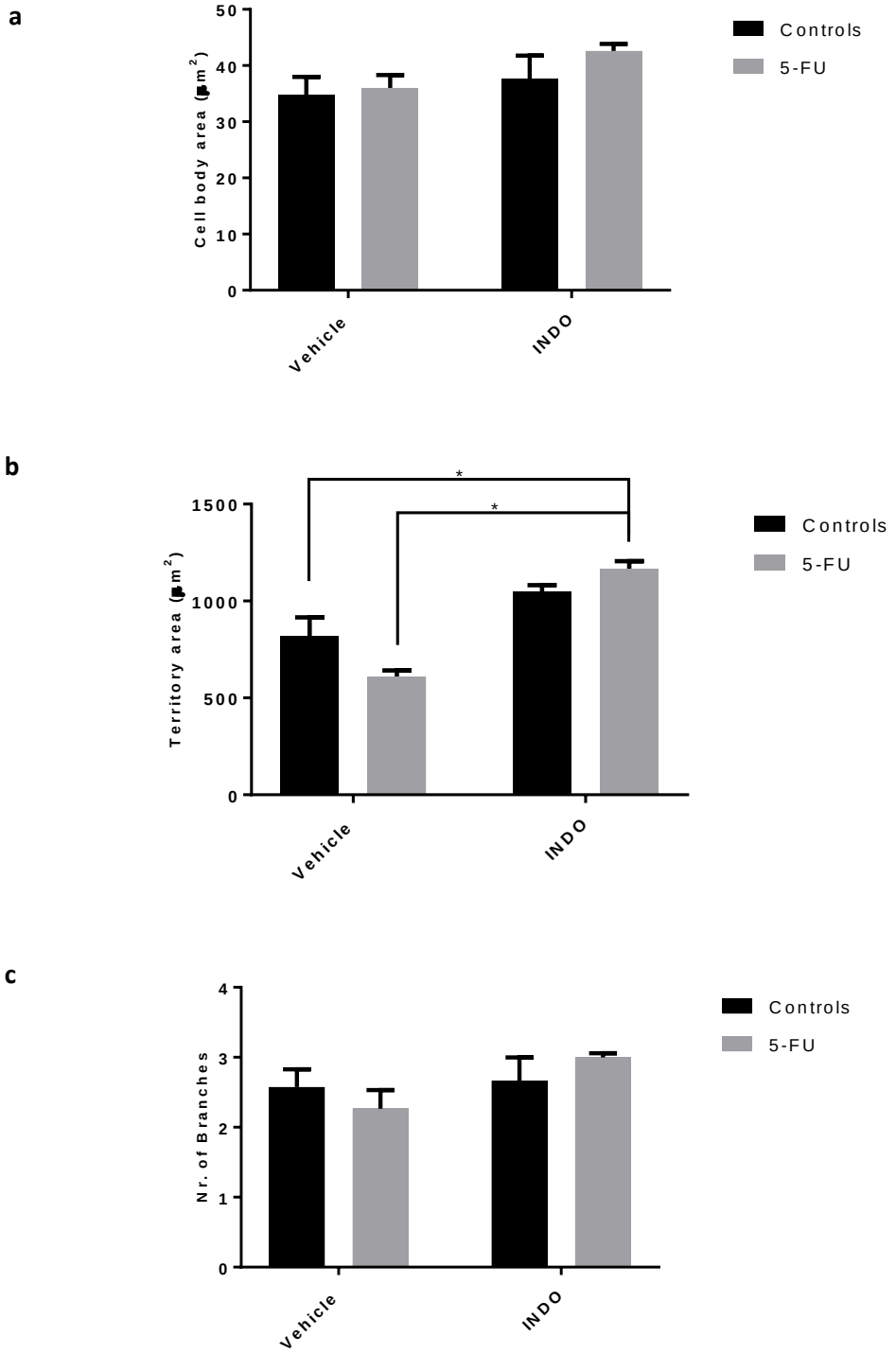


Figure 3.7. C***ell body area (a), territory area (b) and number of branches (c) of microglia in the SGZ of Sox1-GFP mice (mean ± SEM).*** *Microglia within the SGZ of mice injected with 5-FU showed no significant changes in cell body size but a decreased territory area when compared with indomethacin/5-FU group (p>0.05). The number of branches was not affected by any treatment.*

3.3.6 Effect of 5-FU and indomethacin treatment on COX-2 expressing cells

The total number of Cox-2 positive cells in the SGZ of the hippocampus was quantified 24h after the last 5-FU injection by immunostaining. A Two way ANOVA indicated a significant overall effect of 5-FU treatments ($F_{1,10}$ = 21.48, p < 0.05; Figure 3.8). Multiple comparison data analysis showed that co-administration of 5-FU and indomethacin significantly increased the cell density of Cox-2 positive cells in the SGZ when compared with controls and indomethacin group (Bonferroni multiple comparisons test, p<0.05; Figure 3.8).

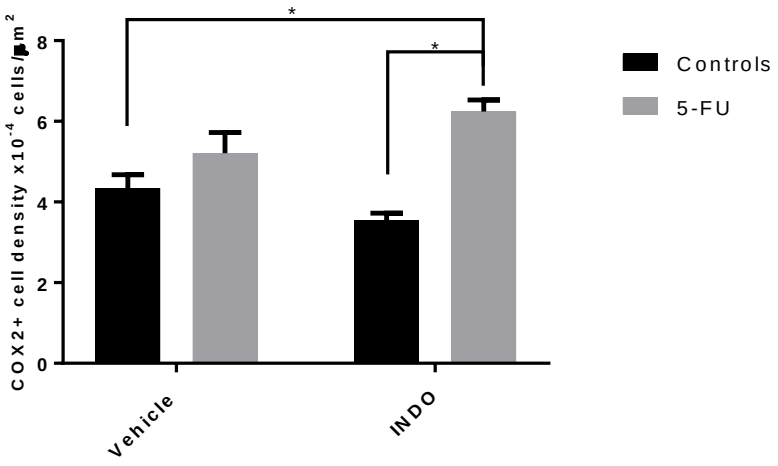


Figure 3.8. ***Cox-2 positive cells density (mean ± SEM) in the SGZ of Sox1-GFP mice.*** *Mice injected with 5-FU and receiving indomethacin in their drinking water had a significantly higher number of Cox-2 positive cells (p<0.05) when compared with indomethacin and control group.*

3.4 Discussion

The present study investigated the impact of chronic 5-FU treatment and the potential protective effects of the drug indomethacin, on different subpopulations of neural stem cells in the SGZ of the hippocampus. In addition, the effect of chemotherapy and anti-inflammatory drugs on proliferation, differentiation and neuroinflammation were quantified. Animals were dosed with 2mg/kg/day of indomethacin in their drinking water and i.p injected with 75mg/kg of 5-FU every second day over a period of 10 days.

The negative impact on body weight is a well-known side effect of chemotherapy treatment and has been found in previous animal studies (Cao et al., 2014, Elbeltagy et al., 2012). Chemotherapy drugs are effective killers of healthy proliferating cells, including cells of the gastrointestinal tract (Huang et al., 2002). Intestinal damage may lead to poor absorption of nutrients and cause body weight reduction (Song et al., 2013). However, in this experiment treatment with 5-FU alone did not cause a body weight decrease across the time of the experiment. This effect may have been due to the higher body weights of mice at the start of the experiment allocated to the 5-FU group compared with animals of the control group. If this is the case it may indicate that larger animals have a resistance to chemotherapy. In support of this hypothesis, it has been reported that a higher production of insulin and insulin-like growth factor which is related to high body weight could lead to the development of resistance to chemotherapy and radiotherapy in tumour cells (Turner et al., 1997).

Together with body weight, water intake may be affected by chemotherapy treatment. As indicated in figure 3.3, mice receiving 5-FU treatment showed a reduction in fluid intake. In contrast, animals co-treated with indomethacin and 5-FU showed an increase in water intake. The decreased production of prostaglandins induced by the

cyclooxygenase inhibitor indomethacin could change the physiological role of this protein in kidney function, inducing an increased water intake (Kim, 2008).

As described earlier previous studies, have demonstrated an association between treatment with chemotherapeutic agents and long term cognitive dysfunctions in cancer patients (Vardy et al., 2007). Similar cognitive declines have been associated with a decrease in the number of proliferating cells in the SGZ needed for hippocampal neurogenesis in animal models of the impact of chemotherapy. Using the same model described in Chapter 2, in this experiment we evaluated the impact of a chronic treatment with chemotherapy on different subpopulation of neural stem cells together with markers of neuroinflammation in Sox1-GFP transgenic mice. Additionally, we investigated the potential of the anti-inflammatory drug indomethacin to protect against the effect of chemotherapy.

As described in Chapter 2, NSCs populations were differentiated by the presence or absence of GFAP immunoreactivity and by analysing their process orientation. We described and measured the following stem cell population: quiescent radial glia like neural stem cells (qRGLs) which have a radial morphology are GFAP positive but Sox1 negative; a population of activated NSCs which are still GFAP positive and express Sox1; a population of NSCs that express Sox1 but not GFAP and finally a population of non-radial cells which continue to express Sox1.

In accord with our previous study of Sox1-GFP mice, chronic 5-FU treatment modified the cellular composition of the germinal niche in this animal model. The cell density of quiescent radial neural stem cells which were GFAP positive and of activated radial neural stem cells which were GFAP and Sox1 positive in the SGZ was reduced by 5-FU treatment alone or in combination with indomethacin. In agreement, with the previous

study the proportion of non-radial neural stem cells was not affected by chemotherapy treatment or co-administration with indomethacin. In terms of these chemotherapy induced changes, co-treatment with indomethacin was not protective.

In agreement with what has been previously said, it is evident that radial stem cells are the subpopulation of cells most affected by chemotherapy treatment. The decrease in the number of these cells indicates a depletion of this component of the neural stem cell pool within the SGZ of dentate gyrus. It is possible that these cells cannot be replaced and this may explain the long term cognitive effects found in animal models and patients after chemotherapy treatment. Previous investigators have never detected symmetric division of quiescent neural stem cells, and throughout the course of time they may disappear from the SGZ (Encinas et al., 2011). We propose that chronic treatment with 5-FU can accelerates this mechanism of stem cells "disposal" by increasing the rate of radial stem cells death. We also hypothesize that the depletion of the stem cell pool could be caused by the terminal differentiation of radial NSCs into astrocytes. Under pathological conditions, astrogliosis is considered to be a protective mechanism that limits the extent of tissue damage (Burda and Sofroniew, 2014). It has been shown that astrocytes are involved in the restriction inflammation within the brain. During injury, reactive astrocytes form a scar tissue that demarcates and surrounds damaged tissue enabling inflammation to rapidly resolves after injury (Sofroniew, 2005). Furthermore, a study has demonstrated that astrocytes protect melanoma cells from cell death induced by cytotoxic agents conferring resistance to chemotherapy treatment (Lin et al., 2010). Taken together, these and other findings allow us to hypothesise that the radial NSCs of mice treated with chemotherapy undergo a terminal differentiation into astrocytes that could form a scar tissue around clusters of activated

non-radial neural stem cells protecting them and giving them resistance to the effects of 5-FU.

Consistent with our previous studies in rats and mice, the evaluation of the density of doublecortin positive neuroblasts in the SGZ of Sox1-GFP mice did not reveal any significant effect of 5-FU when administered alone or in combination with indomethacin. Chemotherapy is believed to act mostly on proliferating cells, rather than cells that are not dividing. This would include differentiating neuroblasts which are migrating into the granule cell layer of the dentate gyrus. It was also hypothesized that changes to the neurogenic niche during chemotherapy treatment could push neural committed cells to differentiate. However, this mechanism has not been shown and further investigation may help to elucidate this process.

Measuring the cell density of Ki67 expressing cells, showed a reduction in the total number of proliferating cells in the SGZ of treated mice. However, in contradiction to the previous chronic study on this animal model, proliferating cells that express Sox1 also decreased after the chemotherapy injections. This difference between studies could be due to the different dose of chemotherapy administered to mice in the experiment described in the Chapter 2. Interestingly, the co-administration of 5-FU and indomethacin completely prevented the reduction in cell proliferation produced by 5-FU on its own. See Table 3.1 for a summary of results presented in this chapter. This suggests that the effect of chemotherapy on cell division in the SGZ may be indirect. Chemotherapy treatment may be inducing a systemic inflammatory response, can modify the neurogenic niche. This inflammatory reaction which is associated with an increase in microglia activation and release of inflammatory cytokines, could create an environment that may impair neural stem cell proliferation. The maintenance of cell proliferation within the SGZ when indomethacin was co-administered with 5-FU may

be mediated by a reduction in cytokine expression and microglia activation. Although we did not measure the levels of inflammatory cytokines in this study, our hypothesis is consistent with our results showing a reduction in the morphological markers associated with microglial activation in the SGZ of mice co-administered with 5-FU and indomethacin. Our results showed an increase in the territory area covered by microglia processes in animals after indomethacin and chemotherapy treatment when compared with mice treated with 5-FU alone. In contrast treatment with 5-FU on its own produced a decrease in the territory covered by processes. These morphological modifications suggest an increase in microglia activation in animals treated only with 5-FU and a decreased microglia activation when indomethacin is co-administered with the chemotherapy.

However by measuring the microglial cell density in the SGZ of Sox1-GFP mice, it was possible to show a significant reduction in the number of these cells in animals treated with 5-FU. As previously mentioned, investigations by our group using a microglial cell line, have shown that this cell type is particularly sensitive to 5-FU treatment when compared to endothelial cells, astrocytes and fibroblasts (Al-Bayti personal communication). We hypothesize that chronic treatment with 5-FU initially induces the proliferation of microglia but continued administration of 5-FU kills a significant fraction of these cells. Co-treatment with indomethacin ameliorates these effects by reducing microglial activation and maintaining their numbers. This action, supports, our hypothesis that this anti-inflammatory drug has a protective role, in the SGZ, during chemotherapy treatment. However, further investigations are required to elucidate the mechanism involved in this protective effect.

In contradiction with the previous study in rats, levels of COX-2 did not increase in mice injected with 5-FU and unexpectedly the expression of COX-2 increased in

animals co-administered with indomethacin and 5-FU. These observations are in contradiction with other studies where a reduction in inflammation in animal models of inflammation-associate damage have been described (Bastos et al., 2008, Monje et al., 2003). These results suggest that in our experiment indomethacin is not able to counteract the increased COX-2 expression induced by inflammation. We hypothesize that the faster drug metabolism in mice than in rats can affect the action of the anti-inflammatory drug and limit its protective effect.

In conclusion it is possible to suggest that chemotherapy treatment affected the neural stem cell pool in the SGZ of Sox1-GFP mice and that indomethacin is not able to ameliorate this condition. However, indomethacin was effective in protecting proliferating cells and microglia from the effect of 5-FU treatment. A slightly increased level of microglial activation was found but this was not associated with increased COX-2 expression in 5-FU treated mice. In this case, co-treatment with indomethacin is able to counteract the effect of 5-FU on microglia activation and seems to induce an increase in COX2 production. Taking these observations together, it is possible say that indomethacin is partially protective against the effects of chronic chemotherapy treatment.

Chapter 4 – THE POTENTIAL PROTECTIVE EFFECT OF FLUOXETINE AND INDOMETHACIN AGAINST THE SIDE EFFECTS OF CHRONIC 5-FU TREATMENT

4.1 Introduction

The following chapter will describe the effect of chronic treatment, with the chemotherapy agent 5-FU, on the expression of markers for proliferation, inflammation and DNA damage in the SGZ of the hippocampus of Lister Hooded rats. In addition, the potential protective effect of indomethacin and fluoxetine administration prior, during and after chemotherapy treatment has been assessed. The markers used to assess the impact of these drugs are as follows: for cell proliferation, Ki67; for microglia, Iba-1 together with a morphological study of these cells; for inflammation levels, the expression of the enzyme Cox-2; for DNA-damage, γ-H2AX and for neuroblasts, expression of doublecortin (DCX). Staining was carried out one week after the last injection of 5-FU. Use of this battery of markers makes it possible to assess the impact of a number ofaspects of chemotherapy treatment.

Lister Hooded rats were used in this experiment to enable comparison with previous studies within this lab. Male rats were used to exclude any confounding factors related to the oestrus cycle, as in female rats, changes in hormonal levels can influence neurogenesis and neuroinflammation, (Villa et al., 2016, Duarte-Guterman et al., 2015).

A number of previous experiments have looked at the effects of a chronic dosing regimen of 5-FU on cognition and neurogenesis in rats (El-Beltagy et al., 2009, Lyons et al., 2012, Mustafa et al., 2008). 5-FU is routinely used to treat a number of different cancers, often in combination with other chemotherapy drugs such as cyclophosphamide and methotrexate (Kreukels et al., 2008). Cognitive dysfunctions and a decrease in neurogenesis has been reported in previous studies from our group. These studies demonstrated that 5-FU treatment causes a reduction in cell proliferation and survival in the SGZ of the hippocampus (Elbeltagy et al., 2012, Lyons et al., 2012)

and a decrease in the performance of tasks requiring spatial memory (El-Beltagy et al., 2009, Lyons et al., 2012). Studies by other groups have used a wide range of doses and the number of injections of 5-FU and different strains of rats. This has made it difficult to choose the appropriate dosing protocol. In addition some studies have combined 5-FU with other chemotherapy drugs such as methotrexate or/and cyclophosphamide which has led to significant morbidity in the animals (Seigers et al., 2009). In the present study, the dose of 5-FU chosen was that which reduced the colon tumour load in rats (Au et al., 1983) and is within the equivalent human dose range (Reagan-Shaw et al., 2008). As such it is a therapeutically relevant dose which does not cause significant morbidity.

Few past experiments have looked at the neuroinflammatory effects of chemotherapy. This is despite the strong likelihood that these toxic drugs may produce a range of inflammatory reactions. One recent study has shown that treatment with a combination of cyclophosphamide, methotrexate and 5-FU caused an increase in the levels of the pro-inflammatory cytokines IL-β and TNF-α in the corpus callosum of rats when analysed 4 weeks after the end of chemotherapy treatment. This change in cytokine levels was associated with a reduced performance in memory tasks (Briones and Woods, 2014). Previous studies have shown that 5-FU treatment can induces inflammation in peripheral tissues (Lopes et al., 2009) but it now seems likely that this drug can induce inflammation in the brain either by direct action on neural tissue or indirectly via peripherally produced cytokines which can passage through the blood brain barrier (BBB)(Lopes et al., 2009, Elbeltagy et al., 2012). This suggestion is supported by observations that the production in peripheral tissues of the inducible prostaglandin producing enzyme cyclooxygenase-2 (COX-2) is enhanced by systemic 5-FU treatment (Lopes et al., 2009). For this reason, in the present study, it was decided

to evaluate the effect of chronic 5-FU treatment on the number of cells expressing COX-2 in the SGZ of the dentate gyrus.

Neuroinflammation has recently been considered as one of the mechanisms responsible for CICI (Seigers and Fardell, 2011, Ahles and Saykin, 2007), a hypothesis which has stimulated research into this topic. Christie et al, 2012 described an increased number of activated microglia in the hippocampus of rats 4 weeks after cyclophosphamide treatment (Christie et al., 2012). This suggests a persistent inflammatory condition which could itself cause CICI (Seigers and Fardell, 2011) and/or also result in a depletion in the number of stem cells. The interaction between these effects could exacerbate the decline in cognition experienced by many patients under chemotherapy treatment.

The cognitive enhancement reported after treatment with the selective serotonin reuptake inhibitor fluoxetine, by healthy individuals as well as patients affected by brain injuries or cognitive impairment, is now widely accepted (Levkovitz et al., 2002, Mowla et al., 2007). Previous work in our lab has shown that when fluoxetine is administered before and during chronic treatment with either methotrexate or 5-FU, it both prevents the reduction in cell proliferation in the SGZ of the hippocampus and ameliorates the performance rats during memory tasks. As such it prevents the reduction in hippocampal neurogenesis and the behavioural changes normally found after chemotherapy treatment (Lyons et al., 2011a, Lyons et al., 2012, Elbeltagy et al., 2010). As in previous studies, fluoxetine was administered in the drinking water at a dose of 10 mg/Kg/day. This dose has been established in previous studies, by our research group, to improve the performance in behavioural tasks and prevent the reduction in cell proliferation within the SGZ (Elbeltagy et al., 2012, Lyons et al., 2012). Investigations of the pharmacokinetics of FLX in previous studies have shown

that the 10mg/Kg dose produces a high bioavailability of the drug rats with a volume of distribution comparable with humans (Chambers, personal communication)(Caccia et al., 1990). Animals were group housed to avoid the social stress caused by isolation housing that could negatively impact neurogenesis (Lu et al., 2003). Fluoxetine was administered in drinking water to avoid the stress induced by repeated i.p injection over a long period of time. In a previous study it was shown that daily i.p injections for more than 3 weeks can produce enough chronic stress and negatively affect neurogenesis as well as inducing peritoneal irritation (Sharp et al., 2002).

As well as fluoxetine, the non-steroidal anti-inflammatory drug (NSAID) indomethacin was also given. This has been suggested to ameliorate cognitive decline of Alzheimer`s disease and reduce the increased inflammation found with ageing (Waldstein et al., 2010, Cote et al., 2012). A single animal study has reported that the co-administration of a COX-2 inhibitor, NS-393, in combination with cyclophosphamide, methotrexate and 5-FU, normalized the levels of inflammatory cytokines and reduced the deficits seen during memory tasks after chemotherapy (Briones and Woods, 2014). Treatment with the NSAID indomethacin resulted in protection against the cognitive deficits induced by cranial irradiation, and in addition, decreased the number of activated microglia and infiltrating monocytes in the dentate gyrus of the hippocampus. Indomethacin treatment during and after radiation exposure, also partially restored the number of proliferating cells in the hippocampus (Monje et al., 2003). These results suggest that chemotherapy or irradiation induced inflammation may be part of the mechanisms behind the cognitive declines found after these treatments. Indomethacin has previously been administered in drinking water at a dose of 1mg/Kg/day, a dosing route which avoids the stress of daily i.p injections (Sharp et al., 2002). This dose of indomethacin has also been reported to increase the survival time of tumour- bearings

rats (Sandstrom et al., 1990), a feature which would make it a particularly suitable treatment in cancer.

The assessment of neurogenesis was conducted by evaluating the expression of the proliferation marker Ki67 and the neural differentiation marker doublecortin. Ki67, which is commonly used to monitor neurogenesis, is an endogenous nuclear protein which is only found during cell proliferation and is detectable during all phases of the cell cycle (Scholzen and Gerdes, 2000). Doublecortin is a microtubule-associated protein expressed initially by precursor cells with a neural commitment and which continues to be expressed in immature neurons (von Bohlen und Halbach, 2011, Couillard-Despres et al., 2005).

To determine whether 5-FU induced neuroinflammation, we used antibodies to detect microglia (Iba1) and the expression of COX2 in the granular cells of the dentate gyrus. The ionized calcium binding adaptor molecule 1 (Iba1) is a microglia/macrophage-specific calcium-binding protein (Ohsawa et al., 2004). Microglia are the resident macrophages of the brain implicated in developmental, homeostatic, and disease processes (Bennett et al., 2016). Microglia can be in resting, pro-inflammatory or anti-inflammatory states. To some extent these can be distinguished by changes in microglial morphology. Resting microglia have a stellar, ramified morphology with many long fine processes and a small cell body, a morphology which is thought to enable them to sample a wide region of the surrounding tissue. Activated microglia, in contrast, have an elongated cell body with few thick short processes. In this state they are thought to be producing inflammatory cytokines (Kohman and Rhodes, 2013). Assessing the size of the cell body and the area covered by and the number of processes extending from the microglial cell has been used as indicators of microglia activation (Gebara et al., 2015). As indomethacin is a non-selective inhibitor of cyclooxygenease

(COX), an enzyme that is required for the conversion of arachidonic acid to prostaglandins (Kohman and Rhodes, 2013). Cox2 is normally produced by macrophages and activated microglia at the sites of inflammation (Das and Basu, 2008). The density of hippocampal cells expressing COX-2 has been evaluated to determine the level of inflammation in this brain region.

One of the main mechanisms of action of 5-FU is to cause DNA-damage which prevents cell replication (Parker and Cheng, 1990). To assess the level of DNA damage after chemotherapy treatment, the density of hippocampal cells expressing γ-H2AX was measured. H2AX is a histone protein whose phosphorylation into γ-H2AX occurs after DNA damage. This marker is used to assess the rate of formation of double strands DNA breaks and is a widely used marker to evaluate the grade of DNA damage resulting from chemotherapy or irradiation treatments (Kuo and Yang, 2008, Garcia-Villa et al., 2012, Bourton et al., 2011).

This study aims to evaluate and compare the potential protective effects of fluoxetine and indomethacin against the effect of a chronic 5-FU treatment on the cell proliferation required for neurogenesis, neuroinflammation and DNA damage. The results of this experiment will enable a better understanding of the mechanisms involved in chemotherapy neurotoxicity and the action of drugs which may be neuroprotective and maintain neurogenesis.

4.2 Materials and methods

4.2.1 Animals and treatment

3 months-old male Lister hooded rats (weighing 175-230 g, Charles River, UK, total n=24) were randomly allocated into 6 groups, with 4 rats in each group. The groups, consisted of: controls (n=4), fluoxetine (n=4), indomethacin (n=4), 5-FU (n=4), 5-FU and fluoxetine (n=4) and 5-FU and indomethacin (n=4).

Groups	**Drug added in drinking water**	**Injection**
Controls	Tap water + ethanol	Saline 0.9%
Fluoxetine	Tap water + FLX 10mg/Kg/day + ethanol	Saline 0.9%
Indomethacin	Tap water + Indomethacin 1mg/Kg/day solved in ethanol	Saline 0.9%
5-FU	Tap water + ethanol	35mg/Kg 5-FU
Fluoxetine/5-FU	Tap water + FLX 10mg/Kg/day + ethanol	35mg/Kg 5-FU
Indomethacin/5-FU	Tap water + Indomethacin 1mg/Kg/day solved in ethanol	35mg/Kg 5-FU

Rats in the 5-FU groups were administered 5-FU (35mg/Kg, Medac GmbH, Hamburg, Germany), in six i.p injections each injection given every three days. Rats in groups not treated with 5-FU were given i.p an identical volume of 0.9% sterile saline (DEMO SA,

Pharmaceutic Industry, Athens, Greece). The volume of each injection was calculated for individual animals based on body weight.

Fluoxetine (RTI International, Durham, USA) and indomethacin (Sigma Aldrich, St. Louise, Usa) were administered in drinking water at a dose of 10mg/Kg/day and 1 mg/Kg/day, respectively, based upon water consumption which was calculated every other day. An indomethacin stock solution was made by dissolving 50 mg of indomethacin in 50 ml of ethanol. Three times per week throughout the experiment fluoxetine and indomethacin were added to the water bottles of the animals. In controls and other groups not treated with indomethacin, an equal volume of ethanol was added to the drinking water. The total treatment time for the animals was 44 days (Figure 1). Fluoxetine and indomethacin-treated drinking water was administered to animals in these groups for the duration of the experiment, starting 21 days prior to the first 5-FU injection. All groups were then allowed to have one week of recovery after the last 5-FU injection. Fluoxetine and indomethacin treatments were given to all relevant groups prior, during, and after 5-FU treatment. All rats were maintained under standard rat housing guidelines with a 12 hours light/dark cycle and with free access to food and water. Laboratory animal care and procedures are in accordance to UK Home Office Guidance regulations and with local ethical committee approval. Procedures were carried out under a Home Office project licence No 31/3163 and personal licence No I55DE633D.

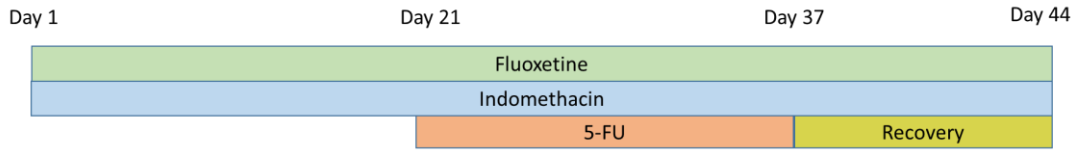


Table 4.1 ***The timeline of animal procedures***. *Indomethacin (blue) and fluoxetine (green) treatment were given in drinking water throughout the experiment. 5-FU (orange) was given after 21 days of fluoxetine or indomethacin treatment, for 16 days in a series of 6 injections every third day. Animals were allowed to recover for one week after the last injection before being put down on day 44.*

4.2.2 Brain Tissue Preparation

All animals were killed one week after the last 5-FU injection by dislocation of the upper cervical spinal column after concussion. Brains were immediately removed and cut sagittally along the mid line into two halves. One half of each brain was submerged in optimal cutting temperature (OCT) freezing medium (VWR International Ltd, Lutterworth, UK) and snap frozen in liquid nitrogen-cooled isopentane. They were stored at -80°C and then coronally sectioned at 20 μm thickness the following day using a Leica CM 100 cryostat (Leica Microsystems, UK). The sections were thaw mounted onto 3-aminopropylmethoxysaline-coated slides and stored at -20°C until used for immunohistochemistry. The second half of each brain was postfixed overnight in 4% paraformaldehyde (PFA) at 4°C. The following day these half brains were cryoprotected in 30% sucrose solution. They were stored at 4°C and then coronally sectioned at 60 μm thickness using a microtome. Free floating section were stored at -22°C in cryoprotectant solution.

4.2.3 Immunohistochemistry

Sections to be analysed were selected using a systemic sampling method in which brain 1 in 5 sections were chosen from each brain. At least 8 coronal sections from each brain

were selected for staining. The subgranular zone (SGZ) of the dentate gyrus was anatomically defined as the interface of granular cell layer with the hilus (Duman, 2004).

Ki-6, COX2 and γ-H2AX immunostaining

Mounted brain sections, produced from cryostat sectioning, were fixed with 2% PFA for 10 minutes at room temperature before being washed with phosphate-buffered-saline (PBS) 3 times for 5 minutes. Sections were then incubated in blocking solution (1% bovine serum albumin (BSA), 0.3% Triton-X, glycin (25mg/ml), and PBS) for 1 hour, followed by an overnight incubation in primary antibodies diluted in blocking solution as follows: Ki67 (Rabbit polyclonal, 1:300, Novus Biologicals, Littleton, USA), COX-2 (Rabbit polyclonal, 1:500, Abcam, UK), γ-H2AX (Mouse monoclonal, 1:1000, Merck Millipore). The sections were then washed with PBS 3 times (5 minutes each), before being incubated in the dark with goat anti-rabbit secondary antibody (conjugated either with AlexaFlour 488 or 568, Molecular Probes Inc., Eugene, USA) diluted 1:500 for Ki67 and 1:1000 for COX-2 in PBS and goat anti-mouse (conjugated with AlexaFluor 488) diluted 1:300 for γ-H2AX. After 3 washes (5 minutes each) of PBS, the sections were then incubated for 45 seconds with DAPI nuclear stain (Sigma-Aldrich, St. Louis, USA) diluted 1:1000 in PBS, before they were briefly washed with distilled water and cover slipped.

Iba-1 and DCX Immunostaining

Post fixed free floating sections were washed in phosphate buffered saline (PBS) three times (5 min each) and then incubated in blocking solutions: 3% Bovine Serum Albumin (BSA), 2% goat serum and 0.3% Triton-X 100 in PBS (Iba-1 Immunostaining) and 1.5% BSA and 0.3% Triton-X 100 in PBS (DCX

Immunostaining) for 1h at room temperature. Primary antibodies were diluted in blocking solution and used as follows in an overnight incubation at 4°C: Iba-1 (1:2000, rabbit, Wako) and DCX (1:100, rabbit, Santa Cruz Biotechnology). After another 3 washes in PBS secondary antibodies diluted 1:500 have been added and incubated in the dark at RT for 1h. Secondary antibodies were Alexa Fluor (Invitrogen, Gran Island, NY, USA), labelled with 568 or 594 fluorophores. After washing with PBS, sections were mounted on slides coated with gelatine and counterstained with DAPI nuclear stain (1:1000, Sigma-Aldrich, St. Louis, USA).

4.2.4 Image Analysis

All images were acquired using a Nikon EFD-3 Fluorescence microscope, at 20x and 40x magnification and photographed with an Optiphot-2, Spot insight QE camera. For each animal, a 1-in-5 series of sections was stained with the nuclear marker DAPI and use to measure the area of the granule cell layer. The granule cell area was measured using ImageJ Software. The total number of immunolabeled cells were counted when the positive immune labelling co-localised with DAPI stained cell nuclei and were within the granular cell layer or within a three- dell diameter distance from the hilar side of SGZ. Positive cells were counted along the entire length of the dentate gyrus and the total number in each section was related to granular cell layer sectional area to estimate the density of immunoreactive cells.

4.2.5 Morphological study of microglia

The total number microglia was investigated using immunostaining against the microglia specific marker Iba-1. The morphology of microglia was measured to evaluate the state of activation of these cells. The parameters measured were soma area,

the territory occupied by projections from the cell body and the number of projections. All parameters were measured with ImageJ software.

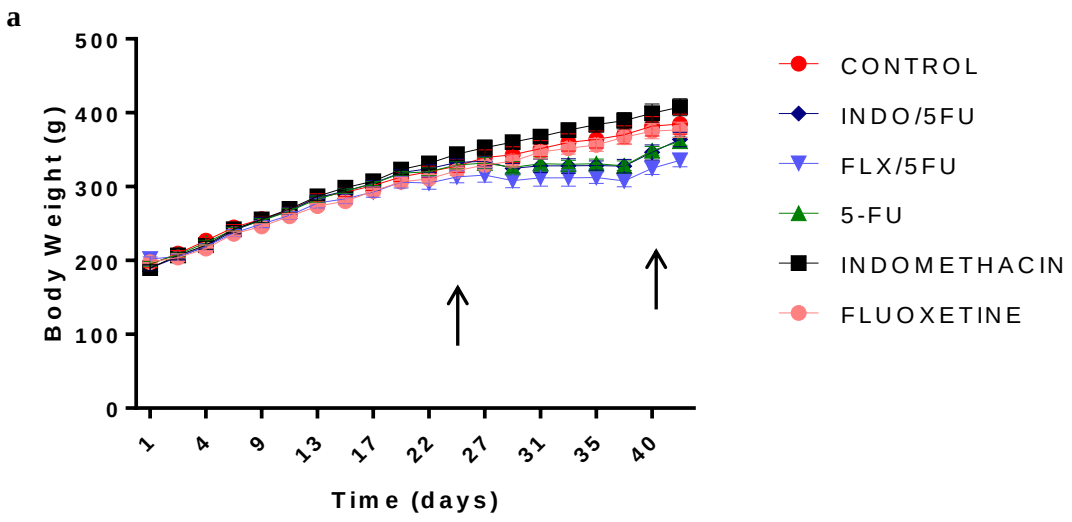
4.2.6 Statistical analysis

All data is expressed as group means ± SEM. Statistical analysis was performed using Prism v6.0 (GraphPad, San Diego, USA) using analysis of variance (Two Way ANOVA) with treatment in drinking water and drug injected as factors. When significant interaction effects were found, Bonferroni post-hoc analysis was performed to elucidate the difference between groups within conditions. For all statistical tests, the threshold of significance was set at $p \leq 0.05$.

4.3 Results

4.3.1 Body weight is reduced by 5-FU treatment

In this experiment animals were killed one week after the end of 5-FU treatment. As expected animals in all groups gained weight over the period of the study. A two- way repeated measure ANOVA analysis on body weight data revealed a significant effect of time and time x treatment interaction ($F_{19,342}$ = 1126, $F_{95,342}$ = 9053 respectively, p<0.0001 for both) (Fig. 4.1a). Rats treated with 5-FU and fluoxetine or indomethacin in combination with 5-FU gained significantly less body weight compared with saline treated controls and this change persisted till the end of the treatment with 5-FU ($F_{5,18}$ = 5188, p < 0.05). One week of recovery allowed body weight to return to control levels for 5-FU and indomethacin/5-FU treated groups but not for the fluoxetine/5-FU group (Bonferroni multiple comparison test, p< 0.0001) (Fig. 4.1b). Rats were observed daily to determine any loss of conditions in animals receiving chemotherapy and or fluoxetine and indomethacin. No animals showed hair loss or had apparent changes in overall activity. No animals died following chemotherapy treatment.



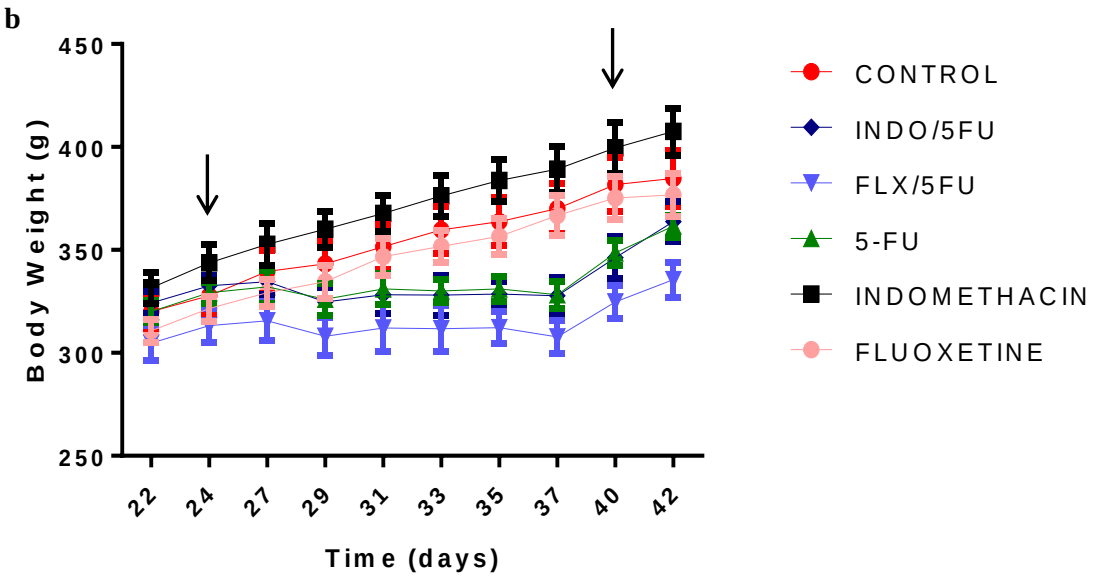


Figure 4.1 ***Body weight of rats (mean ± SEM, n=4) from the first day of fluoxetine and indomethacin administration (a) and during the 5-FU treatment and recovery period (in detail, b)***. *Arrows at day 22 and 37 indicate the beginning and the end of the 5-FU treatment. Fluoxetine and indomethacin were given from day 1 till day 44. Rats treated with 5-FU or in combination with indomethacin and fluoxetine gained significantly less weight compared to controls. After one week of recovery the body weight of 5-FU and indomethacin/5-FU treated groups returned to control levels while the fluoxetine/5-FU group had a lower body weight by the end of the experiment when compared with the control group. Significant differences were determined by repeated measure ANOVA*

4.3.2 Effect of drugs in drinking water and 5-FU treatment on water intake.

Across the treatment period it was observed that the daily water consumption per rat decreased in animals receiving treatment with FLX and 5-FU alone or in combination while the treatment with indomethacin alone or in combination with 5-FU seems does not induce changes on daily water intake (Fig. 4.2).

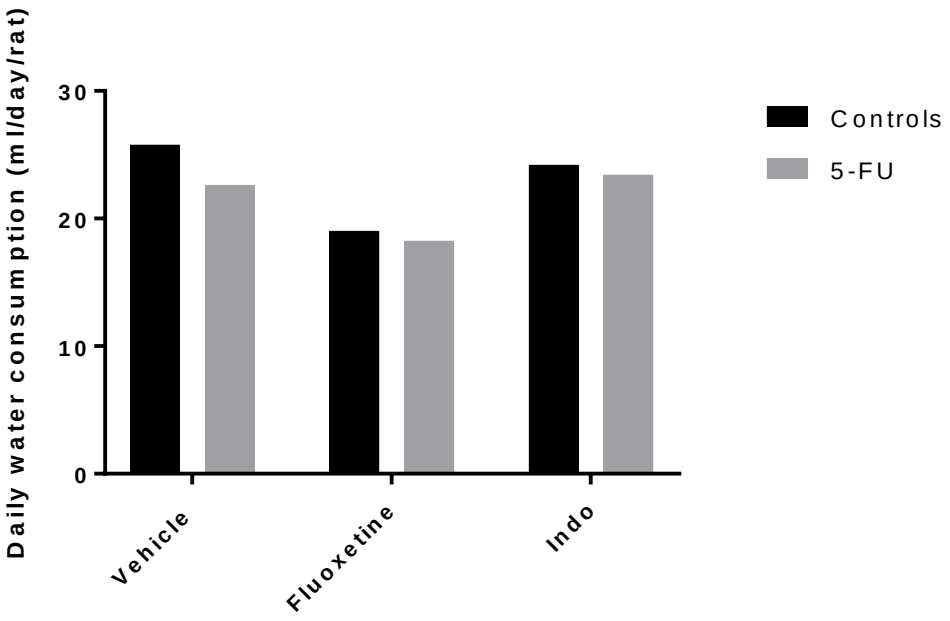
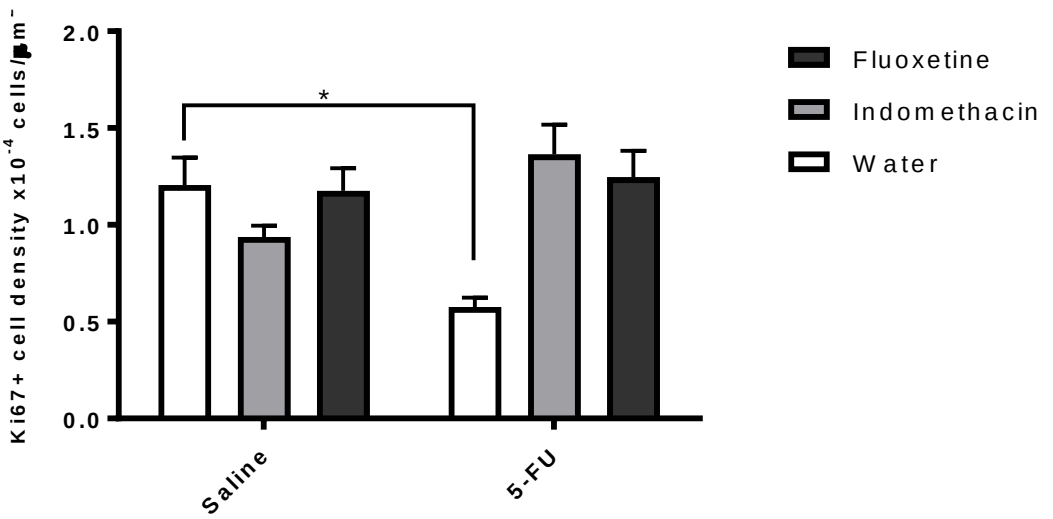


Figure 4.2 ***Daily water consumption per rat***. *Fluoxetine and indomethacin were administrated from day 1 till day 44. Administration of FLX and 5-FU alone or in combination seems to reduce the daily fluid intake.*

4.3.3 The 5-FU induced cell proliferation reduction is reversed by fluoxetine and indomethacin

Cell proliferation in the SGZ of the dentate gyrus was quantified one week after the last 5-FU injection by immune staining for Ki67 (Fig. 4.3b). Two way ANOVA indicated a significant interaction between drug treatments ($F_{2,18}$ =19.55, p<0.001) and a significant effect of drugs in the drinking water ($F_{2,18}$ = 7.862, p<0.05). Rats receiving only 5-FU had a significantly lower number of Ki67 positive cells when compared with controls (p<0.05; Fig. 4.3a). A multiple comparison test with a Bonferroni post-hoc test showed that the number of Ki67 positive cells in rats co-treated with 5-FU and fluoxetine or indomethacin did not differ significantly from the controls. From this it is reasonable to suppose that the effect of 5-FU on proliferation can be counteract by fluoxetine and indomethacin if they are administered before, during and after chemotherapy treatment.

a



b

CONTROLS **5-FU**

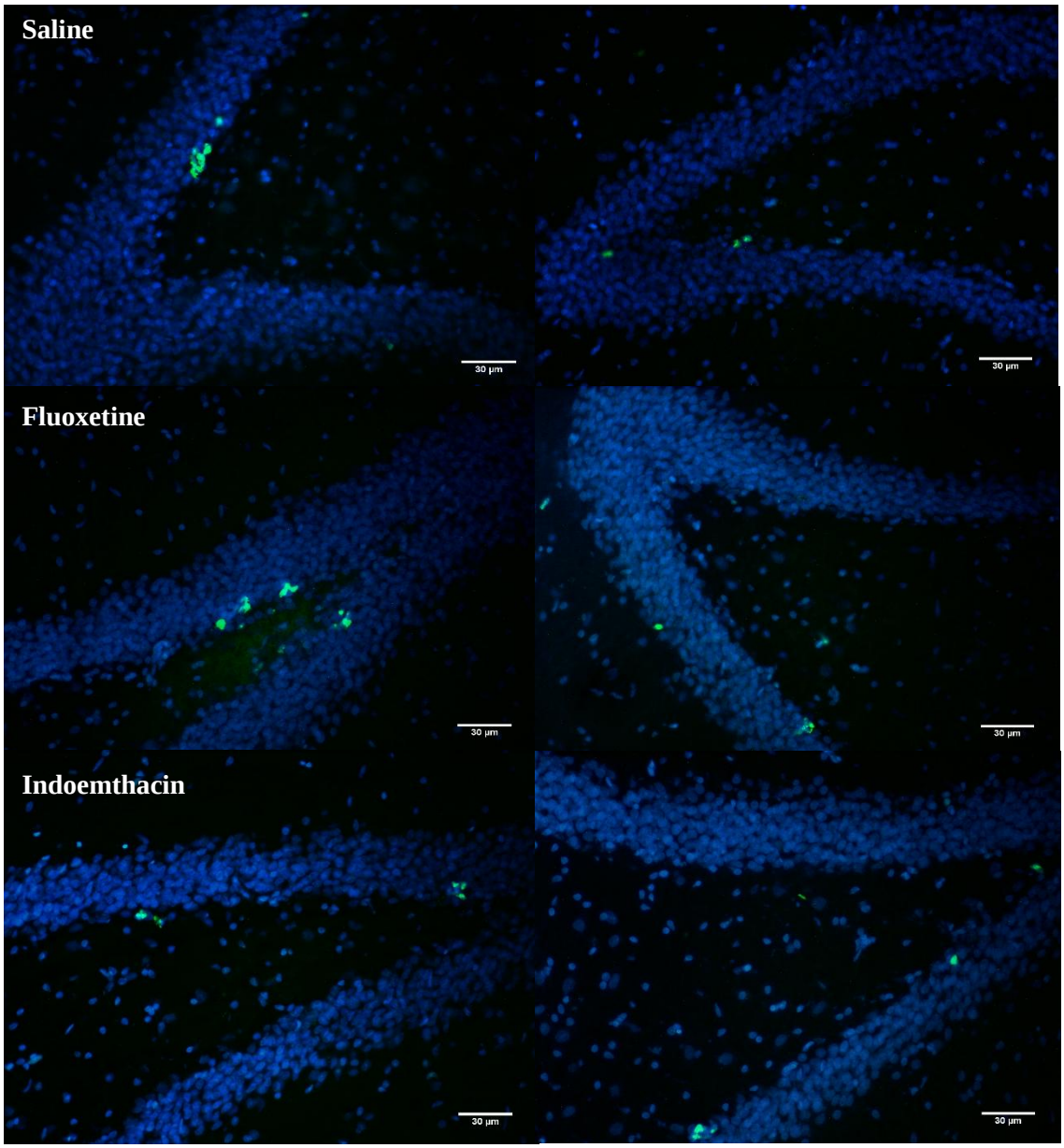


Figure 4.3 ***(a) Ki67 positive cells density (mean ± SEM, n=4) in the SGZ.*** *Rats injected with 5-FU had a significantly lower number of Ki67 positive cells ($p<0.05$) in the subgranular zone while the other groups did not significantly differ from controls.* ***(b)*** *Photos showing Ki67 positive cells (green) located in the SGZ of control, 5-FU, fluoxetine, fluoxetine/5-FU, indomethacin and indomethacin/5-FU treated animals. Nuclei are highlighted by DAPI. Scale bar: 30 µm.*

4.3.4 5-FU treatment did not affect DCX expressing cells

Cell differentiation in the SGZ of the dentate gyrus was quantified one week after the last 5-FU injection by staining for doublecortin (DCX) which marks differentiating neuroblasts. A Two way ANOVA indicated an overall significant effect of the drugs ($F_{2,18}$ = 5.898, p<0.05). However, multiple comparison data analysis showed that chronic 5-FU treatment did not significantly affect the density of neuroblasts in the SGZ of the hippocampus. The co-administration of fluoxetine or indomethacin had no effect on DCX positive cells density (Figure 4.4).

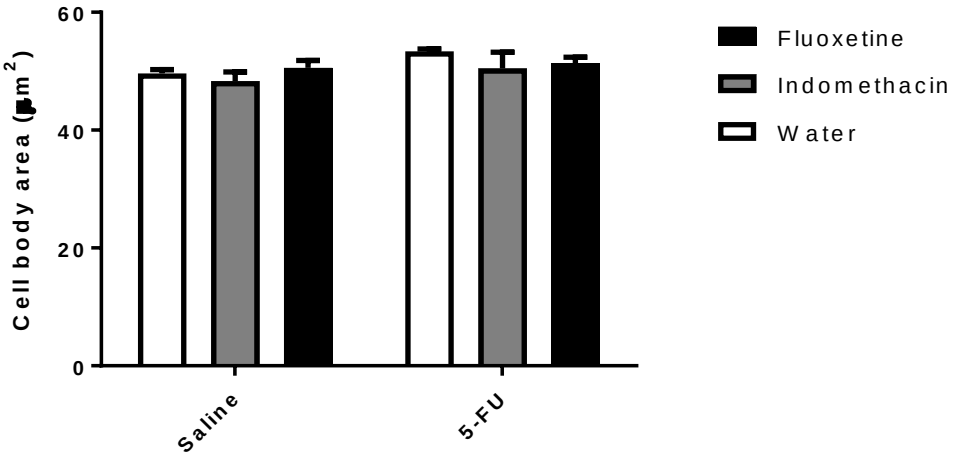


Figure 4.4. ***Doublecortin positive cells density (mean ± SEM, n=4) in the SGZ.*** *Data analysis of DCX positive cells density shows no significant effect of chronic 5-FU treatment. Nor did co-administration of fluoxetine or indomethacin affect DCX cell density.*

4.3.5 The 5-FU induced decrease in microglia was prevented by fluoxetine and indomethacin

The total number of microglia in the SGZ of the hippocampus was quantified one week after the last 5-FU injection by immunostaining for the microglia specific marker Iba-1 (Fig. 4.5b). 5-FU treatment and drugs given in drinking water significantly affected the total number of Iba-1 expressing microglia in the SGZ (respectively $F_{1,14}$ = 31.37, and $F_{2,14}$ = 8.812, p<0.05). A Bonferroni multiple comparison test showed that the total number of Iba1 positive cells significantly decrease in 5-FU treated rats when compared with the control group (p<0.05). However, co-administration with either fluoxetine or indomethacin ameliorated this effect. As shown in Figure 4.5a, the administration of fluoxetine alone increased the total number of Iba-1 positive cells when compared with controls (Bonferroni multiple comparisons test, p<0.05; Fig 4.5a).

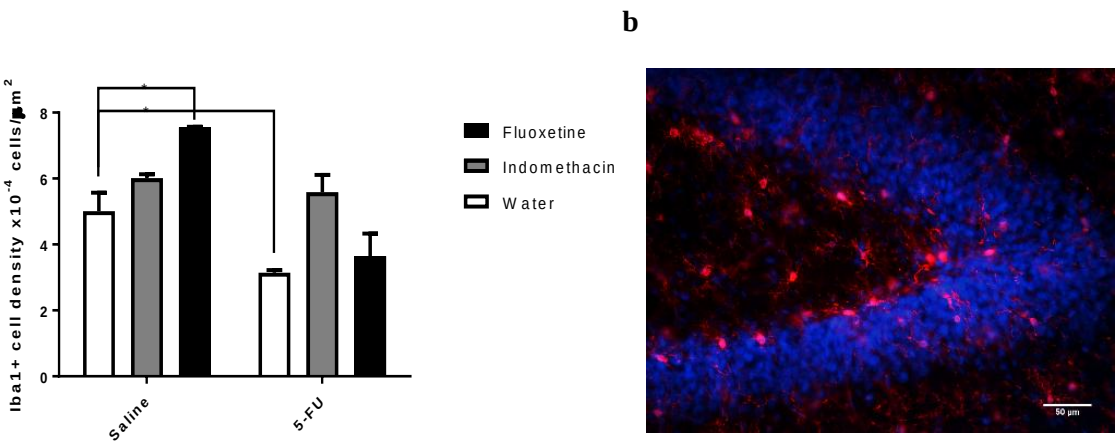


Figure 4.5. ***(a). Iba-1 positive cells density (mean ± SEM, n=4) in the SGZ.*** *Rats injected with 5-FU had a significantly lower number of Iba-1 positive cells (p<0.05) in the SGZ. Rats administered with fluoxetine in drinking water had a significant higher number of Iba-1 positive cells while the other groups did not significantly differ from controls (p>0.005). (b) Photos showing Iba1 positive cells (red) located in the SGZ. Nuclei are highlighted by DAPI. Scale bar: 50 µm.*

4.3.6 5-FU induced microglia activation is prevented by fluoxetine and indomethacin

In the present experiment we found a decrease in the total number of microglia in the SGZ of the 5-FU treated group (See Paragraph 4.3.5) when compared with the control group. Neuro-inflammation leads to the activation of microglia as shown by changes in their morphology. To evaluate the level of microglia activation we analysed three morphological aspects of microglia: cell body size, territory projection area and number of process (Figure 4.6) 5-FU alone or when co-administrated with either fluoxetine or indomethacin does not significantly change the size of the microglia cell body (respectively, $F_{1,14} = 3.771$ and $F_{2,14} = 1.192$, $p > 0.05$; see Fig. 4.6a). However, 5-FU treatment on its own and drugs added in drinking water significantly affected the territory over which the processes projected (respectively, $F_{1,14} = 11.2$ and $F_{2,14} = 4,79$; $p<0.05$; see Fig. 4.6b) and the number of processes (respectively, $F_{1,14} = 15.48$ and $F_{2,14} = 5.004$; $p<0.05$; see Fig. 4.6c). A Bonferroni multiple comparison test confirmed that the territory area and number of microglia process significantly decreased in 5-FU treated rats when compared with controls ($p<0.05$). This suggests that there is an increase in activation of the remaining microglia after chemotherapy treatment and that fluoxetine and indomethacin are able to counteract this effect.

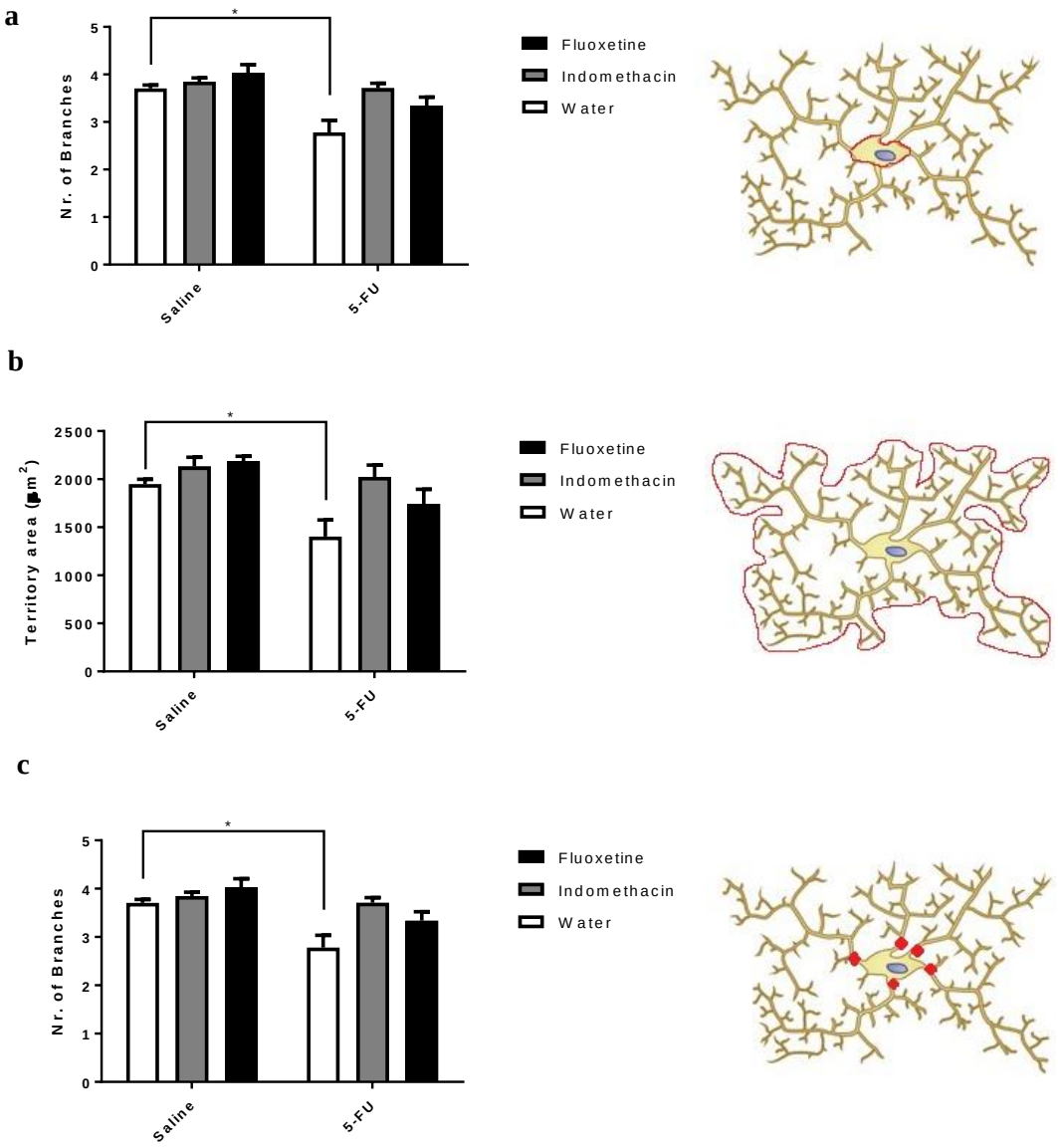


Figure 4.6. ***Graph and drawing showing the cell body area (a), territory area (b) and number of branches (c) of microglia ((mean ± SEM, n=4).*** *Microglia of the SGZ of rats injected with 5-FU showed no significant changes in cell body size but a decreased territory area and number of branches (p>0.05). The other groups did not significantly differ from controls.*

4.3.7 5-FU increased cell density of COX-2 expressing cells

The total number of Cox-2 positive cells in the SGZ of the hippocampus was quantified one week after the last 5-FU injection by immunostaining (Fig. 4.7b). Two way ANOVA indicated a significant interaction between drug treatments ($F_{2,18}$ = 7.311, p<0.05) and a significant effect of the drug injected ($F_{1,18}$ = 23.37, p<0.001). A two way ANOVA data analysis showed that chronic 5-FU treatment significantly increased the total number of Cox-2 positive cells in the SGZ when compared with the control group (Bonferroni multiple comparisons test, p<0.001; Fig. 4.7a). The co-administration of indomethacin reduced this effect. As shown in Figure 8a the co-administration of 5-FU and fluoxetine increased the total number of Cox-2 positive cells (Bonferroni multiple comparisons test, p<0.05), suggesting that fluoxetine treatment is not able to counteract the inflammatory effect of 5-FU.

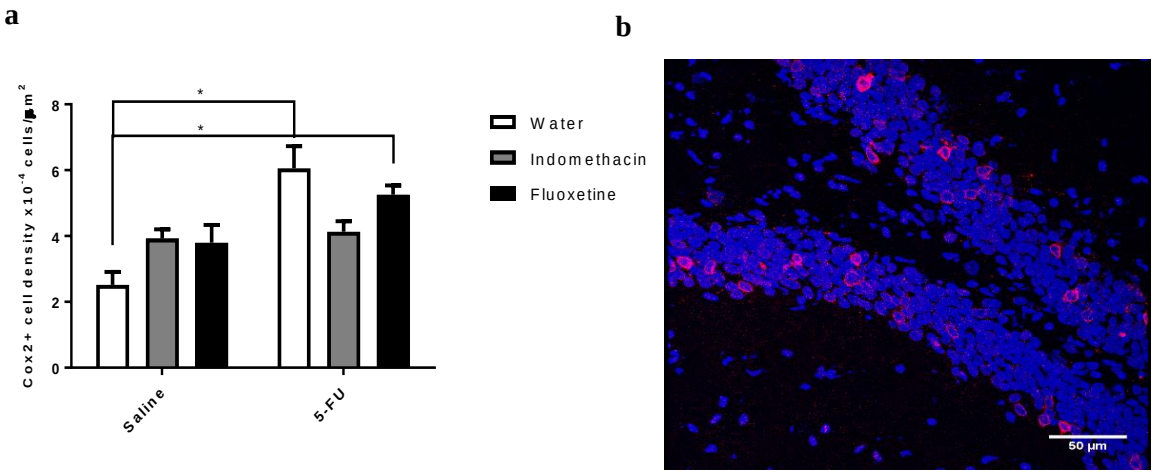


Figure 4.7. ***(a) Cox-2 positive cells density (mean ± SEM, n=4) in the SGZ.*** *Rats injected with 5-FU had a significantly higher number of Cox-2 positive cells (p<0.001) in the SGZ as did rats co-administered with fluoxetine and 5-FU (p<0.05). The co-administration of indomethacin and 5-FU counteracted the inflammatory effect of 5-FU.* ***(b) Confocal picture of Cox-2 (red) immunostaining.*** *Nuclei are highlighted by DAPI. Bar indicates 50 µm.*

4.3.8 5-FU induced DNA damage

To evaluate the level of DNA damage the total number of γ-H2AX positive cells was counted in the SGZ of the hippocampus by immunostaining (Fig. 4.8b). Two way ANOVA indicated no significant interaction between drug treatments but a significant effect of the drug injected ($F_{1,18}$ = 31.18, p<0.001) and administered in drinking water ($F_{2,18}$ =7.649, p< 0.05). Multiple comparison data analysis showed that chronic 5-FU treatment significantly increased the total number of γ-H2AX positive cells in the SGZ when compared with the control group (Bonferroni multiple comparisons test, p<0.001, Figure 4.8a). The co-administration of fluoxetine counteracted the effect of 5-FU on DNA damage. Data in figure 9a showed that co-administration of 5-FU and indomethacin increased the total number of γ-H2AX positive cells (Bonferroni multiple comparison test, p<0.05), suggesting that indomethacin treatment is not able to prevent the increases in DNA damage produced by 5-FU.

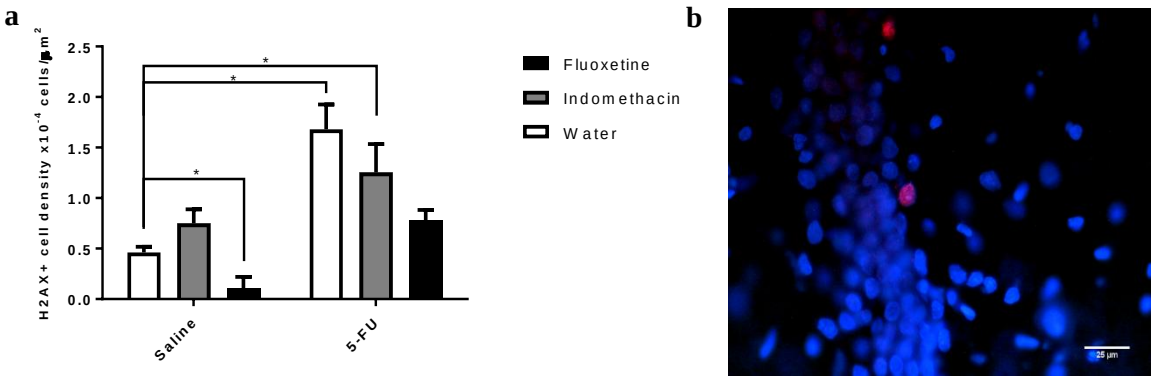


Figure 4.8. ***(a) γ-H2AX positive cells density (mean ± SEM, n=4) in the SGZ.*** *Rats injected with 5-FU had a significantly higher number of γ-H2AX positive cells (p<0.001) in the SGZ as well as rats co-administered with indomethacin and 5-FU (p<0.05). The co-administration of fluoxetine and 5-FU reduced the DNA damage induced by 5-FU treatment.* ***(b) Fluorescent microscope picture of γ-H2AX immunostaining.*** *Nuclei are highlighted by DAPI. Bar indicates 25 µm.*

A summary of main results of this Chapter are resumed in the Table 4.2

	FLX	INDO	5-FU	FLX/5-FU	INDO/5-FU
Ki67	=	=	↓	=	=
DCX	=	=	=	=	=
Iba1	=	=	↓	=	=
Microglia activation	=	=	↑	=	=
COX-2	=	=	↑	↑	=
Γ-H2AX	↓	=	↑	=	↑

Table 4.2 **Resume of main effects of 5-FU treatment and potential protective interventions.**

4.4 Discussion

This study investigated the effect of chronic 5-FU treatment on aspects of neurogenesis, neuroinflammation and DNA damage one week after the end of the treatment. The potential protective effect of fluoxetine and indomethacin was also investigated. Animals were dosed with 10 mg/Kg/day of fluoxetine and 1mg/kg/day of indomethacin starting 21 day before the chemotherapy treatment until one week after the last injection of 5-FU. Animals were i.p injected with 35mg/kg of 5-FU every second day for two weeks (See Table 4.1).

Chemotherapy drugs are efficient at killing dividing cells, including the healthy proliferating cells of the gastrointestinal tract (Huang et al., 2002). Intestinal damage consequent to 5-FU treatment could cause pour absorption of nutrients and could lead to a loss of body weight (Song et al., 2013). As illustrated above (see Figure 4.1), animals treated with 5-FU alone or in combination with fluoxetine or indomethacin showed a decrease in body weight during the chemotherapy administration period. This is a well-known side effect of 5-FU treatment which has been reported previously in animal models (Elbeltagy et al., 2012, Cao et al., 2014). Animals treated with 5-FU alone or in combination with indomethacin showed an increase in body weight after the end of 5-FU treatment, returning to the same weight as control animals. The body weight of animals treated with 5-FU in combination with fluoxetine remained significantly below that of controls, one week from the last injection of 5-FU. As previously reported the administration of fluoxetine can lead to a decrease in food intake beginning with the first dose which can lead to a decrease in body weight after chronic treatment in rats (Yen and Fuller, 1992). Additionally this loss of body weight may be caused by a reduction in water intake. Animals treated with FLX and 5-FU

alone or in combination showed a decreased daily water intake. The reduction in water intake found in the present study was likely to be due to the non-palatable flavour of FLX added to drinking water and is consistent with previous studies in our lab (Lyons et al., 2012).

The side effects of chronic exposure to chemotherapy in patients have attracted increasing attention and a number of publications over the past few decades. Chemotherapy treatment can have long lasting side effects on the cognitive functions of patients and a number of neurobiological, neuroinflammatory and neurovascular mechanisms seem to be related to this impairment. As shown by previous studies by our group, chronic 5-FU treatment causes impairment of spatial and working memory (Elbeltagy et al., 2010, Mustafa et al., 2008) and this deficit is associated with a decrease in the number of proliferating cells required for neurogenesis in the SGZ of the hippocampus. The integration, into the dentate gyrus, of new neurons derived from the proliferation and differentiation of neural stem cells in the SGZ is required for the formation of spatial memory (Toni and Sultan, 2011). It has been shown that a reduction in hippocampal neurogenesis is likely to be one of the causes of the long lasting cognitive impairment reported in patients and animal models after chemotherapy (Dupret et al., 2008, Wigmore, 2013). The interaction between this and other long term side effects of chemotherapy are still unknown but inflammation and DNA damage are likely to significantly contribute to the overall cognitive decline (Soussain et al., 2009). Inflammation significantly reduces hippocampal neurogenesis and is correlated with cognitive dysfunctions (Bastos et al., 2008, Monje et al., 2003, Hein and O'Banion, 2012).

It is now widely accepted that neurogenesis can be modified by pharmacological treatments such as antidepressants and environmental interventions such as physical

exercise (Ma et al., 2016, Zhou et al., 2016). The present investigation was carried out to compare the protective effects of the antidepressant fluoxetine and the anti-inflammatory indomethacin against the effects of 5-FU on neurogenesis, neuroinflammation and DNA damage.

The results presented here show that chronic treatment with 5-FU reduces the number of proliferating cells within the SGZ of the dentate gyrus when compared with other groups. One week of recovery after the last injection of chemotherapy is not enough time to allow the number of proliferating cells to return to control levels. This result has been found in previous studies using different chemotherapy drugs including methotrexate and cyclophosphamide, where the effects of chemotherapy on proliferation, can persist for at least six weeks (Elbeltagy et al., 2010, Lyons et al., 2012, Lyons et al., 2011a, Christie et al., 2012). This reduction in cell proliferation appears to be the main impact of chemotherapy treatment on hippocampal neurogenesis. In the present experiments, the failure of dividing cell numbers to return to control levels a week after the end of treatment could be related to persistent DNA damage which was still present when the animals were put down. DNA damage can lead to cell death or to the induction of a temporary quiescence which stops cells entering division until the DNA is repaired. One result of the DNA damage response, is the inhibition of the cell cycle and several observations suggest that cell cycle rates are not fully reversed following repair (Bailey et al., 2004). In this case the failure to recover to control levels of proliferation could be caused by these two factors. The evaluation of DCX+ neuroblasts density in the SGZ did not shown any significant effect of 5-FU when administered alone or in combination with fluoxetine or indomethacin. This result differs from some previous studies which have found a reduction of DCX+ cells after chemotherapy treatment (Mustafa et al., 2008). Different

5-FU doses and the time of recovery in previous studies make comparison with other studies difficult.

As reported in previous studies by our group and confirmed in the present experiment, co-administration of fluoxetine prevents the decrease in the number of proliferating cells found after 5-FU treatment (Lyons et al., 2012). The administration of fluoxetine induces an increased production of Brain Derived Neurotrophic Factor (BDNF) which could lead to an increase in neurogenesis as reported previously (Encinas et al., 2006). Enhancement of BDNF synthesis might not be the only mechanism involved in fluoxetine neuroprotection, in fact it has been shown that treatment with different types of anti-depressive drugs such as imipramine, desipramine and fluoxetine inhibit the expression of p21, a change which increases neurogenesis in the SGZ of the hippocampus (Pechnick et al., 2011). The protein p21 is a cyclin dependent kinase inhibitor expressed in quiescent cells. Its function is maintain the cell in a quiescent non dividing state by inhibiting the cell cycle and allowing DNA repair or differentiation. The down-regulation of this enzyme allows stem cells to enter in the cell cycle (Pechnick et al., 2011). It is likely that these two mechanisms, increased BDNF synthesis and induction of quiescence, act independently but simultaneously to produce the neurogenic effect of fluoxetine.

In the present study it has been shown that the administration of indomethacin as well as fluoxetine acts to prevent the reduction in the number of Ki67+ cells in the SGZ after chemotherapy treatment (See Figure 4.3). Chemotherapy treatment can lead to a systemic inflammatory reaction with consequent microglia activation and release of inflammatory cytokines. This could create an environment in the neurogenic niche of the SGZ which does not support neural stem cells proliferation (Wigmore, 2013). Recently it has been shown that an increased production of IL-1β, IL-6 and TNF-α and

microglia activation are associated with the inflammation related decrease in neurogenesis (Kohman and Rhodes, 2013). In line with this hypothesis we observed a significant increase in the level of microglia activation and an increased number of cells expressing COX-2 in rats administered with 5-FU when compared with controls. Surprisingly rats treated with 5-FU had significantly lower total numbers of microglia than controls. However, our in vitro studies, using a microglial cell line have shown that this cell type is very sensitive to chemotherapy compared to endothelial, astrocytic or fibroblast cell types (Al-Bayti, personal communication).

The maintenance of proliferation in the SGZ when indomethacin is co-administered with 5-FU could be mediated by a reduction in cytokines levels, microglia activation and COX2 expression. Although, we did not measure the levels of inflammatory cytokines in this study, this hypothesis is consistent with our evidence of a reduction in the morphological markers associated with microglia activation and the number of cells expressing COX-2 in the SGZ of rats administered with indomethacin and 5-FU. These observations confirm other studies where a reduction in inflammation has a positive effect on neurogenesis in animal models of inflammation-associate damage (Bastos et al., 2008, Monje et al., 2003).

Co-treatment of 5-FU with fluoxetine was also able to reduce the level of microglial activation induced by chemotherapy treatment. In recent years there has been an increasing amount of evidence for the potential anti-inflammatory role of SSRIs. It has been shown that fluoxetine can reduce the number of inflammatory cells and the production of cytokines such as TNF-α in an animal model of septic shock and allergic asthma (Roumestan et al., 2007). A recent review has reported that treatment with fluoxetine reduces microglia activation and the production of iNOS, ROS, Nf-kB and pro-inflammatory cytokines (COX-2, TNF-α, IL-6, IL-1β) in different rodent models

(Caiaffo et al., 2016). Although we did not detect any reduction in the number of cells expressing COX-2 in the SGZ of the hippocampus of animals co-administered with fluoxetine and 5-FU this may be due to the dosing regime, in fact as previously reported the anti-inflammatory effect of fluoxetine in the brain is dose and cell type dependent (Caiaffo et al., 2016). Previous studies have reported that the anti-inflammatory action of fluoxetine could be related to the up-regulation of anti-inflammatory molecules such as interleukin 4 (Alboni et al., 2013) and to its ability to down regulate microglia mediated oxidative stress (Chung et al., 2010). Fluoxetine has been shown to increase hippocampal neuronal survival following an ischemic injury by its anti-inflammatory effect on microglia (Dhami et al., 2013). The suppression of Nf-kB by fluoxetine was suggested as the mechanism involved in the anti-inflammatory effect of this antidepressant (Roumestan et al., 2007). Nf-kB is an important transcription factor involved in the production of pro-inflammatory molecules such as TNF-α, NO and IL-6 however it is not known in which way fluoxetine suppress Nf-kB production (Tynan et al., 2012, Lim et al., 2009).

The evaluation of the total number of microglia found a significant reduction in the number of these cells in the SGZ of rats treated with 5-FU. As previously mentioned, investigation by our group, of a microglial cell line show it to be particularly sensitive to 5-FU in vitro (data not shown). We hypothesize that chronic treatment with 5-FU initially induces the proliferation of microglia but that subsequent 5-FU treatment kills a significant portion of these proliferating cells. Indomethacin administration together with 5-FU had a positive effect on microglia density supporting the protective role of anti-inflammatory drugs during chemotherapy treatment. However, in this case the co-administration of fluoxetine and 5-FU was not protective. Rats treated only with

fluoxetine showed a significant increase in microglia density in the SGZ, however the reason for this unexpected result is unknown and should be further investigated.

In the complex mechanisms involved in the side effects of chemotherapy, inflammation and DNA damage appear to be correlated. DNA damage can be produced directly by the effect of 5-FU on the process of DNA synthesis, induction of DNA strand breaks or by mitochondria damage leading to the production of reactive oxygen species (ROS) and consequent oxidative DNA damage (Wigmore et al., 2010). An accumulation of DNA damage stimulates the activity of γ-H2AX (Cornelissen et al., 2011). Our experiment confirmed the increased level of DNA damage in rats treated only with 5-FU. The evaluation of the protective role of fluoxetine and indomethacin allows us to say that fluoxetine but not indomethacin is able to reduce the DNA damage induced by 5-FU. This action of fluoxetine has been described in a past study where fluoxetine has been found to restore the antioxidant system into the brain (Bilici et al., 2001). It has been shown one of the mechanism involved in the indomethacin induced gastrointestinal toxicity is an increase in DNA damage and oxidative stress (Boyacioglu et al., 2016). Due to this observation we can hypothesize that indomethacin is not protective in this case and it is likely to produce DNA damage itself.

In conclusion it is possible to suggest that chemotherapy treatment reduces cell proliferation in the SGZ and microglial number while increasing neuro-inflammation and DNA damage. These effects can be counteracted by the co-administration of fluoxetine or indomethacin. Indomethacin appears to be more effective than FLX in reducing inflammatory markers including COX-2 expressing cells and microglia activation while FLX seems to be more able to prevent 5-FU induced DNA damage. For a summary of main results presented in this Chapter see Table 4.2.

Chapter 5 - EFFECT OF ACUTE 5-FLUOROURACIL ON HIPPOCAMPAL NEUROGENESIS AFTER VOLUNTARY RUNNING

5.1 Introduction

This study looked at the effect of physical exercise prior a single dose of 5-FU and LCV on the expression of neurogenesis markers in the hippocampus of C57BL/6J mice. To evaluate the effect of a single dose of 5-FU on neurogenesis and neural differentiation, I used an endogenous marker of cell proliferation KI67, that labels proliferating cells (Lagace et al., 2010, Kee et al., 2002) and the immature neuron marker doublecortin (DCX) (Couillard-Despres et al., 2005).

C57BL/6J mice are frequently used in physical exercise experiments because they are more active in both light and dark cycle compared with other strains (Kaye and Kusy, 1995). The use of male mice eliminated any possible confounding factor related to the oestrus cycle of female mice, where fluctuation of hormonal levels could affect neurogenesis and levels of physical activity (Duarte-Guterman et al., 2015, Lightfoot et al., 2010).

Although a single dose of chemotherapy is not normally used for treatment of cancer in patients, this acute treatment together with the evaluation of neurogenic markers immediately after the chemotherapy administration allows us to identify the initial cell targets of chemotherapy. The wide range of chemotherapy drugs and dosing regimens together with number of rodents strain previously used for these types of experiment make it difficult to identify a suitable chemotherapy dosing protocol. For example, the same dose of 75 mg/Kg of 5-FU has been administrated in both rats and mice (Fardell et al., 2012a, Foley et al., 2008) which showed similar cognitive impairments induced by chemotherapy despite the different species used. Other studies have evaluated the behavioural effects of chemotherapy but they have not measured neurogenesis (Fardell et al., 2012b, Sharpe et al., 2012, Merzoug et al., 2011, Fardell et al., 2010, Liedke et

al., 2009, Foley et al., 2008, Reiriz et al., 2006). Some studies have shown a long term effect on cognitive functions and neurogenesis (Fardell et al., 2010, Seigers et al., 2008b, Sharpe et al., 2012) while others have described only a transitory decline in cell proliferation (Yang et al., 2011b, Morris et al., 1995). However, recent studies showed that in order to see a decrease in cell proliferation in the hippocampus it is necessary administrate a higher dose of 5-FU in mice compared to rats (Krynetskiy et al., 2013, Walker et al., 2011, Elbeltagy et al., 2012) probably due to the faster metabolism in mice compared with rats.

A number of studies have investigated non-pharmacological approaches to improve the quality of life and cognition of patients treated with chemotherapeutic agents. It is well known that physical activity gives a great number of benefits to the brain. In human and animal studies, physical activity improves cognitive functions (Rogers et al., 1990, van Praag et al., 1999b), prevents age-associated memory impairment (Laurin et al., 2001, van Praag et al., 2005), delays the onset of and reduces the risk of neurodegenerative diseases such as Alzheimer's disease (Friedland et al., 2001, Adlard et al., 2005), enhances healing after brain injuries (Grealy et al., 1999, Gobbo and O'Mara, 2005), has preventive and therapeutic effects on depression (Nabkasorn et al., 2006, Singh et al., 2005) and reduces chemotherapy-induced cognitive impairment (Galantino et al., 2012, Winocur et al., 2013). In animal models also, physical activity has been associated with the amelioration of declining cognitive functions, in addition an enriched environment with the inclusion of free access to exercise equipment, such as a running wheel, seems to have a positive effect on neuronal systems involved in memory and cognition like the hippocampus. It is also well known that the physical exercise, such as voluntary running, increases the proliferation and survival of cells in the dentate gyrus of the hippocampus during the neurogenic process (van Praag et al.,

1999a) and it has been speculated that this mechanism is involved in the positive effect of running on cognition. The mechanisms involved in the protective action of exercise against chemotherapy-induced cognitive impairment have not been elucidated but, in recent years, a great number of studies in rodent models have enlarged our knowledge of the molecular mechanisms associated with the prevention of cognitive deficits with physical exercise, including the increase of neurogenesis (Marlatt et al., 2012b), angiogenesis (Berggren et al., 2014), levels of neurotrophic factors (Berchtold et al., 2005), synaptic plasticity and dendritic spine density (Eadie et al., 2005).

Analysis of neurogenesis was carried out by evaluating the expression of the proliferation marker Ki67 and the differentiation marker doublecortin (DCX). Ki67 is an endogenous nuclear marker which is possible detect during all active phases of the cell cycle (Scholzen and Gerdes, 2000). Doublecortin is a marker widely used to study neurogenesis. Doublecortin is expressed neuro-precursor cells with a neural commitment, neuroblasts and immature neurons (von Bohlen und Halbach, 2011).

This study aims to evaluate the potential protective effect of physical exercise against the effect of a single injection of 5-FU+LCV on neurogenesis.

5.2 Material and methods

5.2.1 Animals and housing conditions

Sixteen, 9 weeks-old, male, C57BL/6J mice (Charles River, UK, total n =16) were housed individually under controlled standard environmental conditions of temperature (22 ± 1 °C) and light (12/12 h light/dark cycle) with free access to food and water. Animals were randomly assigned to 4 groups of 4 animals each: non-runners (NR), non-runners treated with 5-FU (75 mg/Kg) and leucovorin (75 mg/Kg) NR-5FU), runners (R) and runners treated with 5-FU (75 mg/Kg) and leucovorin (75 mg/Kg) (R). Two weeks before drug administration and for the duration of the experiment, each mouse in the running group was provided with a cage equipped with a running wheel of approximately 4.5 cm in diameter. Duration, speed and distance were measured with a meter attached to the cage. Mice in non-running groups were housed in identical cages equipped with a fixed running wheel. The mice were checked daily; duration, speed and distance of running were collected every day while the body weight was recorded every two days. All procedures were in accordance to UK Home Office Guidance regulations and with local ethical committee approval.

5.2.2 Drug administration

After two weeks of running, mice in NR-5FU and R-5FU groups received an intraperitoneal injection of 5-FU (75mg/Kg, Medac, Germany) + leucovorin (75mg/Kg, Hospira, UK). Mice in NR and R groups were injected with the same volumes of 0.9 % saline (DEMO SA, Pharmaceutic Industry, Athens, Greece). The volume of injections were calculated for individual animals based on body weight. The dosage selected for this experiment was well tolerated and did not influence the body weight and the activity levels.

5.2.3 Brain tissue processing

On the last day of the experiment, 24h after injection, animals were euthanized with an overdose of anaesthetic and perfused with 4% paraformaldehyde (PFA). Brains were rapidly removed and stored in 4% PFA overnight at 4°C. The following day brains were stored in 30% sucrose solution at 4°C until sectioning. Brains were coronally sectioned at 60 microns on a sledge microtome. Free floating sections were stored in cryoprotectant solution filled 48 wells (Thermo Fisher Scientific, Denmark) at -20°C until used for immunohistochemistry.

5.2.4 Immunohistochemistry

Using a systematic random sampling procedure (Mayhew and Burton, 1988), 8 sections from each brain were selected for staining. The subgranular zone (SGZ) of the dentate gyrus was anatomically defined as the interface of the granular cell layer with the hilus (Duman, 2004). Individual Ki67-positive proliferating cells and doublecortin (DCX) positive cells were stained in separate sections and observed under fluorescent microscopy after counter staining with the nuclear marker DAPI. Ki67 and DCX positive cells that were within the granular cell layer or within a two-cell diameter distance of the layer in the hilar side were counted along the entire length of the dentate gyrus (Palmer et al., 2000). All counting was performed blind to the treatment.

Ki67 Immunostaining

The sections were washed in phosphate-buffered-saline (PBS) three times (5 min each) and then incubated with 15% normal goat serum and 0.3% Triton X-100 in PBS (blocking solution) for 1h. They were then incubated overnight at 4°C with the primary polyclonal rabbit anti-mouse anti-Ki67 IgG (1:1000 Vector Laboratories, Orton

Southgate, UK) in blocking solution. After 3 washing of 5 min with PBS and 0.1% Triton X100, sections were incubated with a goat anti-rabbit secondary antibody AlexaFluor 568 (1:500) in the dark and at room temperature for 1h. After three washes in PBS, sections were mounted on slides coated with gelatine and counterstained with DAPI nuclear stain (1:1000). Ki67 positive cells were counted in a one-in-five series of sections (240 µm apart) at 40X throughout the rostro-caudal extent of the granule cell layer of the dentate gyrus of hippocampus.

Doublecortin (DCX) immunostaining

As above, sections were rinsed in PBS three times (5 min each) and then incubated with a blocking solution (3% bovine serum albumin and 0.3% Triton X100 in PBS) for 1h at room temperature. Afterwards, they were incubated overnight a 4 °C with the primary antibody against DCX (Santa Cruz, C-18, goat anti-doublecortin 1:100 in blocking solution). After the overnight incubation the sections were washed three times with a buffer solution prepared with 1% bovine serum albumin and 0.1% Triton X100 in PBS and then incubated in the dark for 1h at room temperature with the secondary antibody donkey anti-goat AlexaFluor 594 (1:500). Sections were then washed with PBS (3 times, 5 min each) and mounted on slides coated with gelatine and counterstained with DAPI nuclear stain (1:1000). Doublecortin positive cells were counted in a one-in-six series of sections (240 µm apart) at 40X throughout the rostro-caudal extent of the granule cell layer of the dentate gyrus of hippocampus.

5.2.5 Statistical analysis

All statistical parameters were calculated using GraphPadPrism 6.0 software and significance was regarded as $p<0.05$. Repeated measures two-way ANOVA was used to determine difference in animal weight between treatment groups. One-way ANOVA

was used to analyse data of the proliferating and differentiating cell count (Ki67 and DCX positive cells) in the SGZ between treatment groups. The models for these ANOVAs contained two between-subject factors, chemotherapy (5-FU + LCV) or saline and running (runners or non-runners); and the interaction between these two factors. Interactions between the two factors were included in the model.

5.3 Results

5.3.1 Effect of running and acute 5-FU treatment on body weight

The body weight of each mouse was monitored every two days throughout the study. The weights of the mice at the onset (24.5 ± 0.08 gr, no runners; 24.6 ± 0.06 gr, runners) and after 2 weeks of running (24.25 ±0.25, no runners; 24.5 ± 0.01 runners) did not differ between groups at any time point during the experiment (Repeated measure Two way ANOVA, $F_{5,60}$ = 1.192, p >0.05). The running × weight-change interaction was not statistically significant (Repeated measure Two Way ANOVA, $F_{5,70}$ = 0.23, p >0.05) as well as the chemotherapy × weight change $F_{1,12}$ = 1.174, p >0.05) (Fig 5.1a). Animals treated with chemotherapy in the running group exhibited a decrease in body weight the day following treatment (Fig.5.1b). However, this change in body weight was not significant.

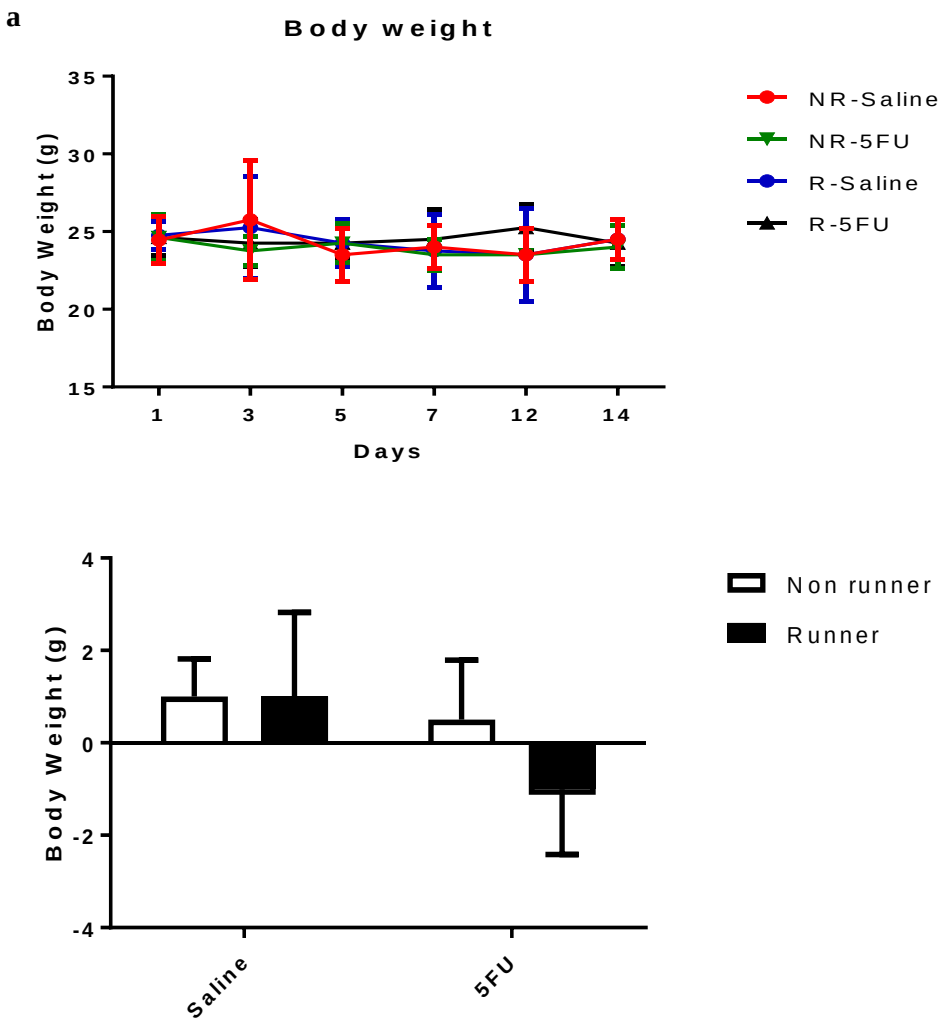


Figure 5.1. ***(a) Daily body weight in g (mean± SEM) over 14 days of running***. *The body weights do not differ between groups at any time point during the running period (p>0.05). The interaction between running and chemotherapy treatment with body weight changes is not significant.* ***(b)*** *Difference in body weight (± SEM) between the day before and 24h after the 5-FU injection (b). An acute single 5FU injection did not affect the body weight in runners treated with 5-FU when compared with runners injected with saline (post hoc test, p >0.05).*

5.3.2 5-FU treatment did not affect running distance

Over the 14 day period, mice in the RUN group spent 3.07 hrs/day (± 1.03) running and rotated the wheel an equivalent of 7.710 Km/day (± 2.5). They gradually increased their running activity until it reached a maximum on Day 9 with an average running distance of about 12.5 km/ day (Fig 5.2). The daily increase in running distance were significant across the time of the experiment (Repeated measure Two Way ANOVA, $F_{(8,48)}$=7.63,

p<0.05). This data is in accordance with previous studies that have shown a similar pattern of running in C57BL/6J male mice at the same age as the animals involved in our study (3.53 hrs/day (± 1.06) and 6.8 km/day (± 2.6) (Lightfoot et al., 2010). The acute chemotherapy treatment received on Day 13 significantly affected the running distance during the last day of the experiment (Repeated measure Two Way ANOVA, $F_{(1.6)}$=27.06, p<0.05), however the significance is due to difference on pattern of running between animals of group R-Saline (1,2,3,4) and animals of R-5FU (5,6,7,8) observed from the beginning of the experiment as shown in Fig 5.2

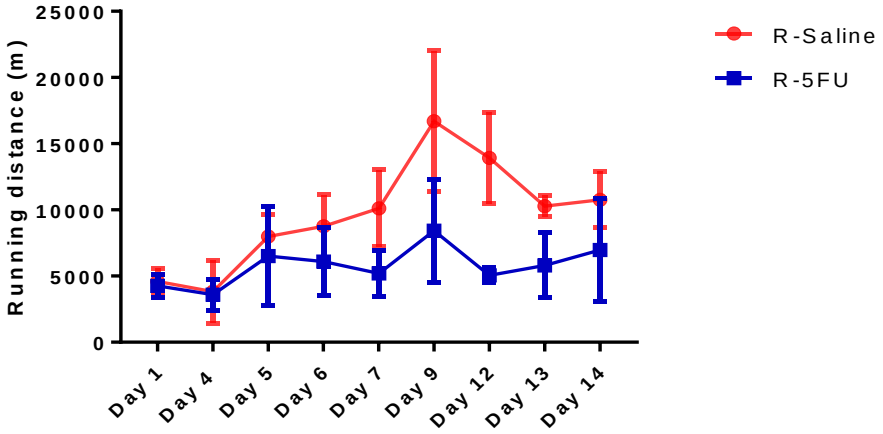


Figure 5.2. ***Daily running distance for each group (mean± SEM)***. *The running distance increases significantly daily until it reaches a peak on day 9 (p < 0.001). The injection of 5-FU on day 12 resulted in a significant decrease in running distance (p <0.05), but this variation it is likely to be related to the different pattern of running observed in the two different groups from the beginning of the experiment.*

5.3.3 Running but not chemotherapy affects cell proliferation in the SGZ

To determine the effects of running and chemotherapy on cells in the proliferative phase of hippocampal neurogenesis, we analysed the expression of Ki67, a nuclear protein that is expressed by proliferating cells in the all phase of the cell cycle (Scholzen and Gerdes, 2000). Two way ANOVA analysis showed that the overall effect of running on Ki67+ cell number was significant (Two way ANOVA, $F_{1,12}$=11.62, $p<0.05$). A Bonferroni post hoc test confirmed that Ki67 positive cells in the SGZ of mice in both the R-S and R-5FU groups displayed an increase when compared with their respective controls ($p < 0.05$; see Fig. 5.3). This data indicates that the basal proliferation was highly stimulated by running. However, the effect of the acute treatment with 5-FU resulted non-significant ($F_{1,12}$= 0.4311, $p>0.05$). A post hoc analysis as well did not show a significantly reduced number of Ki67 positive cells in the 5-FU treated groups compared to saline treated mice (NR-S vs NR-5FU, $p >0.05$; R-S vs R-5FU, $p > 0.05$). This demonstrates that an acute injection of 5-FU+LCV (75mg/Kg) in this case did not reduce the number of proliferating cells in the SGZ.

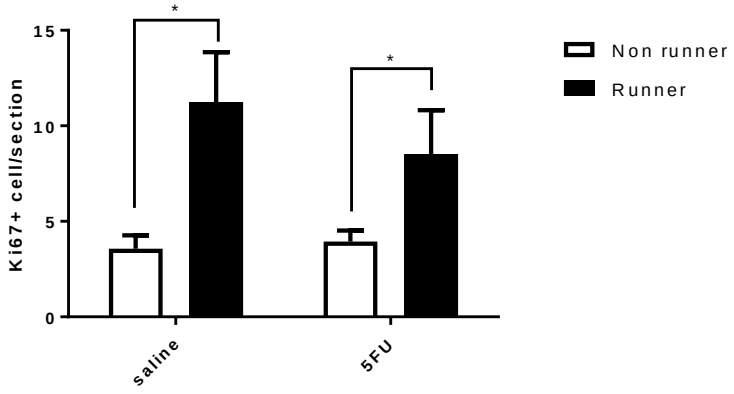


Figure 5.3. ***Ki67 positive cells counts per section (mean± SEM) in the SGZ of the dentate gyrus.*** *Quantification of Ki67+ cells showed a significant increase in the total number of Ki67+ cells in R-S and R-5FU mice when compared with their respective controls (p<0.05). Comparing the control group of non-runners injected with saline with non-runners injected with 5-FU there was no significant difference between these two groups (p>0.05).*

5.3.4 Running but not chemotherapy affects differentiation in the SGZ

We determined the number of neuroblasts produced under our housing conditions and chemotherapy treatment by quantifying the number of DCX positive cells. DCX positive cells were found in the SGZ of the dentate gyrus of the hippocampus and immunohistochemistry showed that the overall effect of running on the expression of DCX were highly statistically significant (Two way ANOVA, $F_{1,12}$=10.89, $p < 0.05$). A Bonferroni post hoc analysis showed that mice in both the R-S and R-5FU groups the number of DCX+ cells increased when compared with their respective controls ($p < 0.001$ Fig. 5.4). However, analysis did not show a significant reduction in the number of DCX positive cells in 5-FU treated groups ($F_{1,12}$= 0.5328, $p >0.05$). Post hoc analysis confirmed a no significant reduction in the number of DCX positive cells in the 5-FU treated groups compared to saline treated mice (NR-S vs NR-5FU, $p >0.05$; R-S vs R-5FU, $p > 0.05$). This suggests that an acute injection of 5-FU+LCV (75mg/Kg) in this case did not affect the number of differentiating cells in the SGZ.

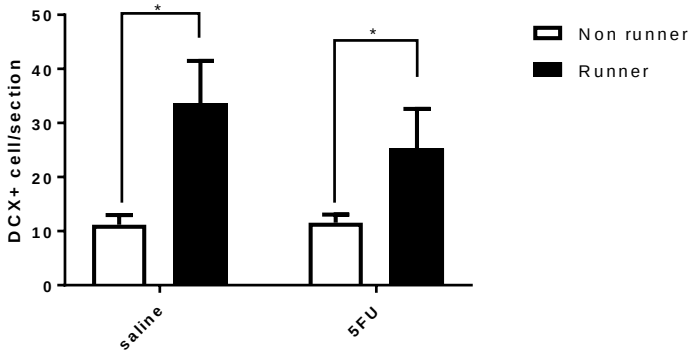


Figure 5.4. ***DCX positive cells counts per section (mean ± SEM) in the SGZ of the dentate gyrus.*** *Quantification of neuroblasts showed a significant increase in the total number of DCX+ cells in R-S and R-5FU mice when compared with their respective controls ($p<0.05$). Comparing the control group of non-runners injected with saline with non-runners injected with 5-FU showed that there was no significant difference between these two groups ($p>0.05$), suggesting that in this case a single 5-FU injection is not able to reduce the number differentiating cells in the SGZ of the hippocampus of treated mice.*

5.4 Discussion

The main purpose of the present study was to investigate if physical activity could be a potential strategy to prevent and reduce the negative consequence of acute chemotherapy treatment on neurogenesis. It has been suggested that the cognitive impairment following chemotherapy is mediated, at least in part, by a chemotherapy-induced decrease in hippocampal neurogenesis and there is evidence that physical activity can lead to increased neurogenesis in healthy animals (Olson et al., 2006, van Praag et al., 2005). The present study confirmed that physical exercise can stimulate the genesis of new hippocampal cells. The results showed that mice in both chemotherapy and control groups generally benefited from the free access to running wheel. Ki67 and DCX+ cell counts increased in both the R-S and R-5FU groups compared with their respective controls. These results highlight that voluntary exercise on a running wheel enhanced the neurogenesis and influenced two important aspects of new neuron maturation, including cell proliferation and differentiation in the DG. We also investigated if there was an association between the distance run and the number of new cells produced. The mechanism underlying the effect of running on neurogenesis is still unknown and other studies are necessary to explain this mechanism. Neurotrophins such as BDNF, VEGF and IGF-1 have been suggested to mediate the neurogenic effect of physical activity (Farmer et al., 2004, Marlatt et al., 2012b, Wu et al., 2008, Trejo et al., 2001). Running also induces the expression of immediate early genes (c-Fos, Zif 268, Arc) in new and pre-existing dentate granule cells (Clark et al., 2011), suggesting enhanced synaptic plasticity by physical exercise. Neurotransmitters systems, such as glutamatergic (Farmer et al., 2004, Vasuta et al., 2007), opioidergic (Colt et al., 1981, Sforzo et al., 1986), endocannabinoid (Hill et al., 2010), monoaminergic and

serotoninergic (Chaouloff, 1989) have also been take in consideration as possible factors involved in exercise- induced neurogenesis.

However, there was no difference in the number of proliferating or DCX expressing cells in mice treated with saline or 5-FU and leucovorin in contrast to our studies in rats (see previous chapters). To explain this apparent absence of an effect of 5-FU, we have hypothesized the involvement of a number of different factors. Firstly too low a dose of chemotherapy may have been given to produce neurogenic effects and secondly there may have been a potential protective effect with the addition of the folic acid (leucovorin) which was given with 5-FU. Regimes of 5-FU dosing in the literature for mice show a wide range of 5-FU dosages with different reports of morbidity; in the Raghavendran et al, 2012 study for example an intra-peritoneal single injection of 5-FU (200 mg/Kg) caused a significant body weight loss 5, 7 and 10 days after the injection (Raghavendran et al., 2012). Also in another study an intra-peritoneal single dose of 5-FU (160mg/Kg) reduced the blood cell count and a second injection at 3 or 7 days killed the mice (de Haan et al., 1996). In our previous studies (data not published) a single intra-peritoneal injection of 5-FU (50 mg/Kg) in male Sox1-GFP mice did not affect body weight or cell proliferation. Taking together these results, it is possible that in this case the dosage of 5-FU was too low to cause either body weight loss or a decrease in proliferation as shown in other previous studies. Another factor possibly involved is the potential protective role of folic acid against the deleterious effect of chemotherapy on brain. A maintained intake of folic acid is required for normal development of the nervous system, playing important roles regulating neurogenesis and programmed cell death (Zhang et al., 2012b). For example, maternal diets deficient in folate result in decreased neurogenesis and increased apoptosis in the forebrain of fetal mice (Craciunescu et al., 2004). It also has been shown that neuroprogenitor cells

cultured from fetal or neonatal rat brains respond to folic acid with increased proliferation (Ji et al., 2007). Furthermore folic acid is important for neuroprogenitor cell proliferation also in adults; indeed folic acid deficiency inhibits proliferation of neuroprogenitor cells in the hippocampus of adult mice (Kruman et al., 2005). However, the role of folic acid in regulation of neurogenesis is unknown. In the present study folic acid was administrated with 5-FU as this is done in some human chemotherapeutic regimes where it has been reported to enhance the anti-cancer activity of the 5-FU (Longley et al., 2003). If folic acid has a positive neurogenic effect on the dentate gyrus of the hippocampus, it could have a protective effect during treatment with chemotherapy. We hypothesise that in our study the decrease of proliferation given by 5-FU may have been balanced by folic acid-increased neurogenesis, resulting in the lack of change in neurogenesis showed in NR-5FU and R-S when compared with controls treated with saline.

In summary, the present study showed that physical activity can stimulate hippocampal neurogenesis but the design of the experiment does not enable us to explain the potential protective effect of physical activity against the chemotherapy-suppressed neurogenesis and consequently against chemotherapy induced cognitive impairment. This kind of non-pharmacological approach to prevent and reduce the side effect of chemotherapy may be useful in this regards. The present results suggest further investigations of this strategy.

Chapter 6 – GENERAL DISCUSSION

Progress in cancer treatments and regimes has led to a higher survival rate in a considerable number of cancer types. This growing number of cancer survivors, however, has led to an increased number of patients who are living with the chronic side effects of chemotherapeutic agents. Cognitive dysfunction is one of the commonest adverse effects among cancer survivors. Although cancer patients are subject to many factors that may impact on their ability to think, chemotherapy induced cognitive impairment (CICI) appears to be a major cause of post treatment cognitive decline. Chemotherapy itself, even when given systemically, affects many brain regions and cell types but a chemotherapy induced impairment of hippocampal neurogenesis has been proposed as an important mediator of CICI (Dietrich et al., 2008, Wigmore, 2013). Research is now looking at different approaches to reduce and prevent the reduction in hippocampal neurogenesis and the consequent decline in cognition.

The studies presented in this thesis first addressed the difference between the impact of acute and chronic 5-fluoruracil treatment on adult hippocampal neurogenesis. Using the Sox1-GFP transgenic mouse model it was possible investigate which stage of neurogenesis and the specific types of neural stem cells that were affected by chemotherapy treatment. The second main objective was to evaluate the effect of different potential protective strategies against chemotherapy induced cognitive impairment; these were chronic fluoxetine and indomethacin treatment and physical exercise. Fluoxetine and exercise are known to increase hippocampal neurogenesis while the anti-inflammatory indomethacin was chosen to determine if a reduction in chemotherapy induced inflammation would protect hippocampal neurogenesis.

6.1 Principal findings

The first study presented in this thesis focussed on the effects of acute and chronic treatment with 5-FU on different subpopulations of neural stem cells in the dentate gyrus of hippocampus. This experiment was performed using Sox1-GFP transgenic mice. Fluorescence microscopy and immunostaining enabled the evaluation of the location and morphology of different subpopulations of neural stem cells within the dentate gyrus of animals with and without different regimes of chemotherapy treatment. This study showed that acute chemotherapy treatment, consisting of a single injection of 5-FU, was not able to produce changes in the cell number of different populations of neural stem cells. However, it was sufficient to cause a reduction in the amount of cell proliferation in the SGZ. Chronic 5-FU treatment, consisting of 8 injections of chemotherapy modified the cellular composition of the germinal niche of Sox1-GFP transgenic mice. These results found that quiescent and activated radial stem cells are the subpopulation of cells most affected by chemotherapy treatment. We hypothesize that radial NSCs of mice treated with chemotherapy, undergo a terminal differentiation into astrocytes that could form “scar tissue” around clusters of activated non-radial neural stem cells protecting them by giving resistance to the effects of 5-FU. After neural stem cells finish their round of cell division, they can undergo a series of changes in their gene expression which is then translated into changes in biomarker expression and morphology. This model of neural stem cell pool deforestation could explain the long lasting chemotherapy related decline of hippocampal neurogenesis. The low number of NSCs remaining after chemotherapy treatment is not sufficient to maintain an adequate level of hippocampal neurogenesis leading to long term cognitive dysfunctions related to this impairment on neurogenesis. This hypothesis may explain

the long term cognitive effects showed in animal models and patients after chemotherapy treatment.

As expected the total amount of proliferation decreased in animals injected with 5-FU and as hypothesized above, proliferating Sox1 expressing cells, namely non-radial neural stem cells, were not affected by the chemotherapy treatment. This result supports our hypothesis regarding the possible protective activity of astrocytes on clusters of proliferating cells. Taking the results from chronic and acute treatments together is possible postulate that an entire cycle of chemotherapy, consisting of multiple injections, is necessary to induce changes in the composition of the NSCs niche of the hippocampus. The evaluation of the impact of chemotherapy in DCX expressing cells showed that only acute chemotherapy treatment caused a decrease in the number of this type of cell. A recent study showed that a single dose of chemotherapy, in this case methotrexate, induced an initial reduction of DCX+ cells which recovered after 3 days (Yang et al., 2011a). Our result is supported by previous studies that have found a reduction in neuroblasts after chemotherapy treatment (Winocur et al., 2015, Winocur et al., 2014). The lack of chronic effect of 5-FU on neuroblasts was unexpected, as past studies has found a reduction of DCX expressing cells after chemotherapy treatment (Winocur et al., 2015, Mustafa et al., 2008). The effect of chemotherapy on neuroblasts is difficult to elucidate due to the different experimental factors involved, such as different dosing regimens, time and strains of animals.

In the second study (Chapter 3), using Sox1-GFP, mice the impact of chronic 5-FU treatment and the potential protective action of indomethacin, on different subpopulations of neural stem cells in the SGZ were investigated. These results found that the number of quiescent radial neural stem cells which were GFAP positive and activated radial neural stem cells which were GFAP and Sox1 positive decreased after

treatment with 5-FU alone or in combination with indomethacin. In agreement, with the previous study, non-radial neural stem cells were not affected by chemotherapy treatment or co-administration with indomethacin. In this case, co-treatment with indomethacin was not protective, suggesting an indirect involvement of neuroinflammation on the depletion of the stem cell pool. Consistent, with our previous studies in rats and mice, the quantification of the number of doublecortin positive neuroblasts in the SGZ of Sox1-GFP mice did not reveal any significant effect of 5-FU when administered alone or in combination with indomethacin. In contrast to these results, the quantification of proliferation, showed a different result from the previous chronic study in Sox1-GFP mice. In this case, proliferating cells that express Sox1 decreased after chemotherapy injections. The co-administration of 5-FU and indomethacin ameliorates this effect of 5-FU on proliferation. The possible inflammatory reaction produced by 5-FU with consequent microglia activation and the release of inflammatory cytokines could create an environment that would impair neural stem cells proliferation. Our hypothesis is consistent with our evidence of a reduction in morphological markers associated with microglial activation in the SGZ of mice co-administered with 5-FU and indomethacin. Morphological modifications evaluated in this study, suggest an increased microglia activation in animals treated only with 5-FU and a decreased microglia activation induced by the anti-inflammatory drug, in animals co-treated with 5-FU and indomethacin. These observations are confirmed by other studies where a reduction in inflammation and a positive effect on neurogenesis in animal models of inflammation-associate damage have been described (Bastos et al., 2008, Monje et al., 2003). The SGZ of Sox1-GFP mice treated with 5-FU showed a significant reduction of microglia in animals treated with 5-FU. As previously mentioned, in vitro investigations by our group using a microglial cell line have shown

that this cell type is particularly sensitive to 5-FU treatment. (Al-Bayti, unpublished observations). Co-treatment with indomethacin reduces the effect of chemotherapy on microglia density, supporting a protective role for this anti-inflammatory drug in maintaining microglia cell numbers. It remains to be elucidated which mechanism is involved in this process and why indomethacin is protective just against the effect of chemotherapy on proliferating cells.

In the rat study (Chapter 4) the effect of chronic 5-FU treatment on aspects of neurogenesis, neuroinflammation and DNA damage one week after the end of the treatment together with the potential protective effect of fluoxetine and indomethacin was investigated. As reported in past studies of this group (Elbeltagy et al., 2012, Lyons et al., 2012) and confirmed in these experiments, chronic treatment with 5-FU has a negative effect on the number of proliferating cells within SGZ when compared with controls and one week of recovery after the last injection of chemotherapy is not enough time to allow the number of proliferating cells to return to control levels. As reported in previous studies by our group (Lyons et al., 2012) and confirmed in the present experiment, co-administration of fluoxetine or indomethacin prevented the decrease in the number of proliferating cells found after 5-FU treatment. Additionally, co-treatment of 5-FU with fluoxetine and indomethacin was also able to reduce the level of microglial activation induced by chemotherapy treatment inducing a reduction in morphological markers of microglia activation. As previously described in this thesis, the number of microglia was significantly decreased in the SGZ of rats treated with 5-FU, a result similar to that found in mice. Indomethacin and fluoxetine co-administration together with 5-FU had a positive effect on microglia density supporting the protective role of these two drugs during chemotherapy treatment. Neuroinflammation is not the only mechanism involved, in fact it has been

demonstrated that 5-FU treatment can cause DNA damage which stimulated the activity of γ-H2AX (Cornelissen et al., 2011). Our experiment confirmed the increased level of DNA damage in rats treated only with 5-FU. The evaluation of the protective role of fluoxetine and indomethacin allows us to say that fluoxetine but not indomethacin is able to reduce the DNA damage induced by 5-FU. Comparing the two potential protective drugs it is possible say that both are effective in the protection of proliferating cells but, Indomethacin appears to be more effective than FLX in reducing inflammatory markers including COX-2 expressing cells and microglia activation while FLX seems to be more able to prevent 5-FU induced DNA damage (See Table 6.1).

	5-FU	FLX/5-FU	INDO/5-FU
Proliferation	↓	Protection	Protection
Differentiation	=	=	=
Microglia	↓	Protection	Protection
Microglia activation	↑	Protection	Protection
COX-2 expression	↑	↑	Protection
DNA damage	↑	Protection	↑

Table 6.1. ***Summary of 5-FU and fluoxetine and indomethacin effects on proliferation, differentiation, microglia, microglia activation, COX-2 expression and DNA damage.***

The experiment that followed (Chapter 5) investigated the ability of physical activity to prevent and reduce the negative consequence of acute chemotherapy treatment on neurogenesis. As previously reported in the literature, we demonstrated that physical exercise can stimulate the genesis of new hippocampal cells (van Praag et al., 1999b). The results showed that mice in both chemotherapy and control groups had an increase in Ki67 and DCX+ cell counts when compared with their respective controls. However,

there was no difference in the number of proliferating or DCX expressing cells in mice treated with saline or 5-FU and leucovorin, in both the sedentary and running groups. Taking together these results, it is possible that in this case the dosage of 5-FU was too low to cause a decrease in proliferation. Another factor which may be involved, is the potential protective role of folic acid against the deleterious effect of chemotherapy on brain. A good intake of folic acid is required for normal development of the nervous system, as it plays important roles in regulating neurogenesis and programmed cell death (Zhang et al., 2012b). In the present study folic acid was administrated with 5-FU as this is done in some human chemotherapeutic regimes where it has been reported to enhance the anti-cancer activity of the 5-FU. If folic acid has a positive neurogenic effect on the dentate gyrus of the hippocampus, it could have a protective role during treatment with chemotherapy.

6.2 Stages of neurogenesis affected by chemotherapy treatment

There is a high number of clinical (Ahles et al., 2010, Koppelmans et al., 2012a, van Dam et al., 1998) and preclinical studies (Lyons et al., 2011a, Seigers et al., 2013) demonstrating the cognitive dysfunctions related to chemotherapy treatment. A number of previous experiments by our group and others have investigated the effect of acute and chronic treatment with 5-FU in rodents (Elbeltagy et al., 2010, Han et al., 2008, Mustafa et al., 2008, Lyons et al., 2011a) and have demonstrated that 5-FU treatment causes a reduction in cell proliferation in the SGZ of the hippocampus. a result which has suggested that a decline in hippocampal neurogenesis is one of the main mechanisms involved in CICI (Elbeltagy et al., 2012). However, it is still unclear which subpopulation of neural stem cells is affected by chemotherapy treatment. To investigate this aspect of the response of the hippocampal neurogenic niche to

chemotherapy, in Chapter 2 and 3 we used a transgenic mouse line that allows the visualization of Sox1 positive cells as they express GFP as a reporter gene under the control of the Sox1 promoter (Aubert et al., 2003). This strain of GFP knock-in mice permits the visualization of Sox1 positive cells by fluorescence microscopy both in fixed tissues and in live cells. Using immunohistochemistry, to detect cellular markers of specific neurogenesis stages, we identified different subpopulations of NSCs in the SGZ. This particular transgenic model enabled us to measure which subpopulation of NSCs changed after chemotherapy treatment and if co-administration with indomethacin was able to protect these cells from chemotherapy. Different NSCs populations were identified by the presence GFAP immunoreactivity and by analysing the orientation of the main process extending from the cell body (radial or horizontal). We identified the following NSCs populations: quiescent radial glia like neural stem cells (qRGLs); a population of activated NSCs; a population of NSCs that express Sox1 but not GFAP and finally a population of non-radial cells (See Figure 6.1)

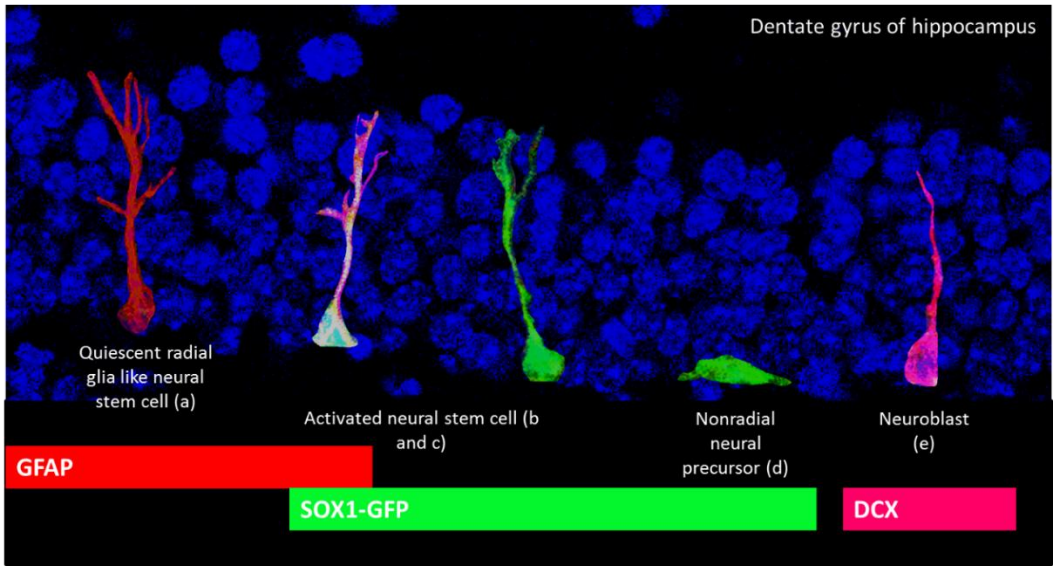


Figure 6.1. ***Subpopulations of neural stem cells under physiological conditions.*** *(a) Quiescent radial glia like neural stem cells: present a soma in the SGZ, extend a single apical process before arborizing in the molecular layer of the dentate gyrus. These cells are GFAP positive and do not co-localize with markers of proliferation. (b) Activated neural stem cells showing a*

radial morphology. The immunoreactivity for both GFAP and expression of Sox1 made it possible to define these cells as activated. (c) Sox1 positive activated neural stem cells with a radial morphology lacking the expression of GFAP. Few of these cells co-localize with markers of proliferation. (d) Sox1 positive activated neural stem cell with non-radial morphology. Their soma is localized in the SGZ and they extend processes horizontally to the SGZ. (e) Neuroblasts doublecortin (DCX) positive.

Acute treatment of Sox1-GFP mice with 5-FU caused a decrease in the number of proliferating non-radial neural precursors and in neuroblasts. It is possible that in this case neural stem cells entered a quiescent phase in which they stopped proliferating so as not to enter the cell cycle while they had DNA damage (See Table 6.2). Furthermore, it has been shown that an increased level of quiescence is related to a decrease survival and differentiation of neurons (Kandasamy et al., 2010), confirming our results on neuroblasts. As a part the cellular response to DNA damage, there is an increased production of p53 that mediates cell cycle arrest via the expression of p21. Recently it has been shown that p21 regulates the proliferation-quiescence switch by causing quiescence to maintain genomic stability if DNA damage occurs (Barr et al., 2017).

	Quiescent radial glia like neural stem cells GFAP+	Activated neural stem cells (radial morphology) GFAP+ Sox1+	Activated neural stem cells (radial morphology) Sox1+	Non-radial neural stem cells Sox1+	Proliferating Non-radial neural stem cells in proliferation Sox1+ Ki67+	Neuroblasts DCX+
Acute treatment	=	=	=	=	↓	↓

Table 6.2. ***Summary of acute 5-FU treatment effect on different subpopulation of neural stem cells***

Chapter 2 demonstrated that higher and more frequent doses of chemotherapy had a different effect on the neurogenic niche than single doses. Mice subjected to chronic treatment with 5-FU, showed a decreased number of both quiescent radial glia like neural stem cells and activated neural stem cells with a radial morphology. In contrast

to acute treatment, non-radial precursors whether proliferating or not, together with neuroblasts are not affected by chemotherapy treatment (See figure 3). The decrease in the number of quiescent and activated neural stem cells with radial morphology could be related to a depletion of a part of the neural stem cell pool within the SGZ which may explain the long term cognitive effects found in animal models and patients after chemotherapy treatment. The cellular mechanisms underlying the depletion of the stem cell pool remain to be elucidated but we have hypothesized that, as previously reported, quiescent neural stem cells are not able to undergo symmetric divisions, and throughout the course of time they may disappear from the subgranular zone of the dentate gyrus (Encinas et al., 2011). We suppose that chronic treatment with 5-FU accelerates this process of stem cell "disposal" increasing the rate of radial stem cell death due to an unrepairable level of DNA damage. Furthermore, quiescent cells can become senescent and are no longer able to replicate in an asymmetric way and replenish the stem cell pool required for neurogenesis. However, this hypothesis is not enough to explain why non-radial neural precursors, that have a high rate of proliferation, are not affected by chemotherapy treatment. To explain this effect we hypothesized that the loss of stem cells from the stem niche could be caused by the terminal differentiation of radial NSCs into astrocytes. It has been demonstrated that astrocytes are involved in defensive mechanisms that limit the extent of tissue damage under pathological conditions (Burda and Sofroniew, 2014). During injury, reactive astrocytes form a scar tissue that surrounds the damaged tissue which is separated from the immediately adjacent viable neural tissue, allowing repair and producing anti-inflammatory cytokines that help resolve inflammation rapidly after injury (Sofroniew, 2005). Considering this evidence, we hypothesise that the radial NSCs of mice treated with chemotherapy undergo a terminal differentiation into astrocytes that could form a scar tissue around clusters of

activated non-radial neural stem cells protecting them by giving resistance to the effect of 5-FU (see Table 6.3). This is similar to what has been previously reported by Schneider et all who found that irradiated neural stem cells become senescent, lose their stem cell features and become committed to an astrocytic fate (Schneider et al., 2013).

	Quiescent radial glia like neural stem cells GFAP+	Activated neural stem cells (radial morphology) GFAP+ Sox1+	Activated neural stem cells (radial morphology) Sox1+	Non-radial neural stem cells Sox1+	Proliferating Non-radial neural stem cells in proliferation Sox1+ Ki67+	Neuroblasts DCX+
Chronic treatment	↓	↓	↓	=	=	=

Table 6.3. ***Summary of chronic 5-FU treatment effect on different subpopulation of neural stem cells.***

The co-administration of indomethacin and 5-FU did not ameliorate this effect of chronic 5-FU treatment on different subpopulations of neural stem cells, suggesting that neuroinflammation is not responsible for neural stem cells depletion but seems to be one of the causes of the decreased proliferation induced by 5-FU treatment. In fact in Chapter 3 of this thesis we observed a protective effect of indomethacin when co-administrated with 5-FU only on proliferating cells (See Table 6.4). Indomethacin should induce a decrease in pro-inflammatory cytokines levels and microglia activaction that can be detrimental for neurogenesis (Yirmiya and Goshen, 2011). In this way, indomethacin, could act as a protective drug against the effect of 5-FU on proliferation. Taking these considerations together, it is possible to say that the depletion of the stem cell pool induced by 5-FU is the result of DNA damage while the impaired proliferation might be caused by an increase in cytokine production together with pro-inflammatory microglia activation.

	Quiescent radial glia like neural stem cells GFAP+	Activated neural stem cells (radial morphology) GFAP+ Sox1+	Activated neural stem cells (radial morphology) Sox1+	Non-radial neural stem cells Sox1+	Proliferating Non-radial neural stem cells in proliferation Sox1+ Ki67+	Neuroblasts DCX+
5-FU	↓	↓	↓	=	↓	=
Indomethacin + 5-FU	↓	↓	↓	=	Protection	=

Table 6.4. ***Summary of chronic 5-FU and indomethacin treatment effect on different subpopulation of neural stem cells.***

6.3 Protective effect of fluoxetine

The potential protective effect of fluoxetine in a rat model of chemotherapy treatment was investigated in the Chapter 4 of this thesis. Our results indicate a protective role of fluoxetine on proliferation, neuroinflammation and DNA damage (See Figure 1). It has previously been shown that fluoxetine is able to protect proliferating cells in the hippocampus from the effect of chemotherapy treatment (Lyons et al., 2012). This drug is also effective in the protection of microglia, a type of glial cell which in vitro studies show to be extremely sensitive to 5-FU treatment. Furthermore, in our model, fluoxetine was able to prevent microglial activation, as shown by the reduction of the morphological markers used to identify activated microglia. This antidepressant, was surprisingly able to reduce the number of cells expressing the DNA damage marker γ-H2AX after 5-FU treatment. In previous studies antidepressants have been shown to enhance hippocampal neurogenesis. For example, 14 or more days of treatment with

fluoxetine, increased the proliferation of neural progenitor cells and the survival of immature neurons in rats (Marcussen et al., 2008). Previous studies by our group, using similar animal models of chemotherapy treatment, have also reported a protective role for this antidepressant against the reduction in cell proliferation induced by chemotherapy treatment (Lyons et al., 2012, Lyons et al., 2011b). The mechanism by which this drug is able to maintain the levels of proliferating cells is still not understood but it is known that administration of fluoxetine increases levels of Brain Derived Neurotrophic Factor (BDNF) which could lead to an increase in neurogenesis (Encinas et al., 2006). Enhancement of BDNF synthesis might not be the only mechanism involved in fluoxetine neuroprotection, it has been shown that a number of anti-depressive drugs including imipramine and desipramine as well as fluoxetine inhibit the expression of p21. This effect will increase neurogenesis by maintaining cell proliferation (Pechnick et al., 2011). The function of p21 is to maintain the cell in a quiescent non dividing state by inhibiting the cell cycle and if necessary allowing DNA repair or differentiation. The down-regulation of this enzyme allows stem cells to enter in the cell cycle (Pechnick et al., 2011). It is likely that these two mechanisms, increased BDNF synthesis and induction of quiescence, act independently but simultaneously to produce the neurogenic effect of fluoxetine. As shown in the chapter 4, fluoxetine, is effective in the reduction of neuroinflammation related to chemotherapy treatment. Previous studies have reported that the anti-inflammatory action of fluoxetine could be related to the up-regulation of anti-inflammatory molecules such as interleukin 4 (Alboni et al., 2013) and to its effect in the down regulation of microglia mediated oxidative stress (Chung et al., 2010). The suppression of Nf-kB, an important transcription factor involved in the production of pro-inflammatory molecules such as TNF-α, NO and IL-6, induced by fluoxetine has been

suggested as being involved in the anti-inflammatory effect of this antidepressant (Roumestan et al., 2007). The underlying mechanisms behind the anti-inflammatory effects of fluoxetine are however still not fully explained and should be further investigated. Examination of the protective role of fluoxetine allows us to say that fluoxetine is also able to reduce the DNA damage induced by 5-FU. This action of fluoxetine has been described in a past study where fluoxetine has been found to restore the antioxidant system into the brain (Bilici et al., 2001). Oxidative stress alters the normal cell metabolism and can lead to cell death (Caiaffo et al., 2016). Analysing mice treated with fluoxetine, Novío et al. (2011) found a partial amelioration of oxidative stress caused by stress. This improvement was associated with the capacity of fluoxetine to restore endogenous components of the antioxidant defence system (SOD and catalase) as well as non-enzymatic antioxidant components, such as glutathione (Novio et al., 2011).

Despite all the protective effects of fluoxetine against the negative effects of chemotherapy, fluoxetine, has its own side effects which may be detrimental to cancer patients. One common side effect is gastrointestinal problem, such as nausea, anorexia and weight loss and in fact fluoxetine is effective in reducing body weight in obese patients (Epling, 2003). This was demonstrated in a recent rodent experiment which reported a weight loss after 2 weeks of fluoxetine treatment (Aggarwal et al., 2016). As weight loss can be a problem in cancer patients, chronic fluoxetine use may exacerbate this issue. More seriously, fluoxetine treatment has been reported to increase blood brain barrier permeability and to increase the number of brain metastases in a mouse model of breast cancer. It has also been reported that fluoxetine treatment for 5 days led to a 40% increase of the numbers of mammary fibrosarcomas in mice, which was followed by a 95% enhancement in breast cancer incidence after 15 weeks of fluoxetine

treatment (Brandes et al., 1992). In contrast, Tutton and Barkla reported an anticancer effect of FLX treatment in colon cancer (Tutton and Barkla, 1982) and this an anti-proliferative effect of fluoxetine has been found in a number of in vivo and in vitro studies of many types of cancer, including leukaemia (Chamba et al., 2010), lymphoma (Cloonan et al., 2010, Cloonan and Williams, 2011, Frick et al., 2008, Frick et al., 2011), colon cancer (Kannen et al., 2015, Koh et al., 2011, Stopper et al., 2014), melanoma (Grygier et al., 2013, Kirkova et al., 2010), ovarian cancer (Lee et al., 2010) and glioblastoma (Liu et al., 2015). The mechanism behind the anti-tumour effects of fluoxetine are unclear but some researchers suggest that the antidepressant treatment reduces dysplasia and pre-neoplastic angiogenesis and, decreases epithelial and sub-epithelial cell proliferation (Kannen et al., 2012). Further it has been reported that by suppressing NF-κB nuclear activity, through increased expression of cytoplasmic NF-κB-inhibitor IκB-α and IκB-β proteins, fluoxetine is able to reduce *c-Myc* expression and then stromal proliferation (Stopper et al., 2014). As stated above other studies have shown that fluoxetine treatment arrested colon tumour cells at the G_0/G_1 phase of the cell cycle (Kannen et al., 2015). Research data regarding the activity of fluoxetine treatment in cancer patients is still very limited and more investigations are necessary to determine if fluoxetine is safe enough to be use as a protective strategy to prevent CICI.

6.4 Protective effect of indomethacin

The potential protective effect of the anti-inflammatory drug indomethacin in this animal model of chemotherapy treatment, was investigated in Chapter 3 and 4 of this thesis. In Chapter 3 we used the Sox1-GFP transgenic mouse line to evaluate which subtype of NSCs is most affected by chemotherapy treatment and if indomethacin treatment was able to prevent these effects on NSCs. In Chapter 4, we evaluated the

protective effect of indomethacin in a rat model of chemotherapy treatment, against the reduction of proliferating cells numbers and the increase in microglia activation, neuroinflammation and DNA damage. In agreement with what has been previously said, it was evident that radial stem cells are the subpopulation of NSCs most affected by chemotherapy treatment. The decrease in the number of these cells would lead to a reduction in a key population of neural stem cells within the SGZ. Treatment with indomethacin was not able to protect these cells against the effects of chemotherapy. A high level of DNA damage has been proposed as consequence of 5-FU treatment and the lack of any effect of indomethacin on DNA damage (Chapter 4) could explain why NSCs remained affected by 5-FU in animals co-treated with indomethacin. However, indomethacin, in both experiments, was protective against the reduced proliferation in the SGZ of animals treated with 5-FU. The maintenance of proliferation in the SGZ when indomethacin is co-administered with 5-FU could be mediated by a reduction in cytokine levels and microglia activation. Although, we did not measure the levels of inflammatory cytokines in this study, this hypothesis is consistent with our evidence of a reduction in the morphological markers associated with microglia activation in the SGZ of rats and Sox1-GFP mice administered with indomethacin and 5-FU. Our results are confirmed by other animal models of neuroinflammation, where indomethacin is able to regulate microglia activation and hippocampal neurogenesis in a mouse model intracerebral haemorrhage (Yang et al., 2015). Further evidence has come from a study of chronic inflammation in the brain produced by cranial irradiation which may contribute to neural stem cell dysfunction. Irradiation decreased the proportion of proliferative cells adopting a neuronal fate but treatment with indomethacin was able to partially restore the portion of proliferating cells committed to become neurons in this rat model (Monje et al., 2003). In our experiments (Chapter 3 and 4), measuring the

microglial cell density within the SGZ of animals treated with 5-FU showed a significant reduction in microglia in animals treated with 5-FU. Co-treatment with indomethacin ameliorated the reduction in microglial numbers, supporting the idea that this drug has a protective role within the hippocampus during chemotherapy. However, further investigations are required to elucidate the mechanism involved in this protective effect. Analysing the data of COX-2 expression, we obtained contradictory results in mice and rats. In mice, Cox-2 expression showed no increase in animals treated with 5-FU alone. This was unexpected and contrasted with the results from rats. In rats treatment with 5-FU, increased COX-2 expression and when animals were co-administered with indomethacin, this reduced the numbers of COX-2 in the cells of SGZ. Due to the different experimental designs and dosage regimens of 5-FU and indomethacin it is difficult explain the differences in these results. Recently there has been interest in the use of anti-inflammatory drugs in treating and preventing cancer. It has been shown that non-steroidal anti-inflammatory drugs (NSAIDs), widely used in the treatment of inflammatory diseases, have antineoplastic properties that can be attributed to their effect on inflammation (Grivennikov et al., 2010). Preclinical studies have demonstrated that indomethacin is effective against colon (Lundholm et al., 1994), lung (Cai et al., 2011) and breast cancers (Ackerstaff et al., 2007). However, the gastrointestinal toxicity produced by a chronic indomethacin administration may indicate the use of safer NSAIDs with fewer side effects (Henry et al., 1996). Despite these limitations, several anti-inflammatory drugs, including aspirin, have been found to decrease cancer progression and mortality, particularly in colon cancer (Gupta and Dubois, 2001). Anti-inflammatory drugs are now, widely approved as adjuvant treatments for cancer. It has been shown that administration of indomethacin alone, or in combination with oxaliplatin, can significantly inhibit the growth of lung cancer in

nude mouse transplanted tumours (Sun and Chen, 2016). Due to this promising evidence, more investigations are necessary in this field to understand the role of indomethacin as a protective and cancer treatment and would be useful to investigate other classes of anti-inflammatory agents with less side effects than NSAIDs.

6.5 Protective effect of physical exercise

The potential protective effect of physical exercise in a mouse model of chemotherapy treatment was investigated in Chapter 5. Our results showed that physical exercise can stimulate the genesis of new hippocampal cells and that mice in both chemotherapy and control groups had an increase in Ki67 and DCX+ cell counts when compared with their respective controls. These results demonstrate that voluntary exercise on a running wheel enhanced neurogenesis and influenced two important aspects of new neuron maturation, cell proliferation and differentiation. A number of studies have investigated non-pharmacological approaches to improve the quality of life and cognition of patients treated with chemotherapeutic agents. It is well known that physical activity provides significant benefits to the brain. Voluntary physical activity has been shown to have positive effects on cognition in both physiological and pathological conditions in humans and animals (Voss et al., 2013b). Physical exercise increases the proliferation and survival of cells in the SGZ required for neurogenesis (van Praag et al., 1999a). It is thought that this mechanism is involved in the positive effect of running on cognition. In recent years, numerous studies in rodents have enlarged our knowledge of the molecular mechanisms associated with the prevention of cognitive deficits with physical exercise, including an increase in neurogenesis (Marlatt et al., 2012b), angiogenesis (Berggren et al., 2014), levels of neurotrophic factors (Berchtold et al., 2005), synaptic plasticity and dendritic spine density (Eadie et al., 2005).

Use of physical exercise as a non-pharmacological approach to prevent and/or ameliorate CICI may be a useful approach to prevent cancer itself. In fact it has been shown that physical exercise reduces the risk of breast, colon, pancreatic, prostate, endometrial, ovarian and lung cancer (Brown et al., 2012). Exercise seems to have a potent rehabilitative effect for cancer survivors, in fact there are numerous documented health-benefits among cancer patients, including improved disease-free survival, muscular strength, aerobic capacity, quality of life and decreased fatigue (Courneya, 2003, McNeely and Courneya, 2010). Considering the positive influence of exercise on the body and the CNS, cancer patients suffering from cognitive impairments would benefit from regular physical activity.

6.6 Conclusions and future directions

Results from the PhD project presented in this thesis have shown that the chemotherapy agent 5-FU affects the neural stem cell pool and the number of proliferating cells in the hippocampus. Chronic administration with FLX can prevent the decrease in the number of proliferating cells, microglia activation and DNA damage while treatment with Indomethacin protects only from the effect of 5-FU on proliferating cells and microglia activation. Physical exercise prior to acute treatment with 5-FU enhanced the overall amount of neurogenesis but, possibly, due to a low dose of chemotherapy it was not possible see a significant effect of chemotherapy.

This research area has the potential to improve the quality of life of significant numbers of cancer patients. For this reason further investigations are necessary to evaluate which cellular and molecular targets can be modified by potential protective treatments. The Sox1-GFP transgenic model was particularly useful in identifying these targets, but further experiments are necessary to elucidate the molecular pathways involved in the

passage from quiescence to activation and vice versa of neural stem cells together mechanisms involved in their fate modification under pathological conditions. This transgenic model together with immunostaining made it possible to evaluate the cellular modification of hippocampal neurogenic niche under chemotherapy treatment. Further studies would usefully measure levels of cytokines in the brain and plasma, to explain how systemic inflammation and neuroinflammation affects neurogenesis in subjects under chemotherapy treatment. To extend the results presented in this thesis, it would be useful to run behavioural tests to measure cognition in animals under different drug regimes. It is hoped that these studies will enable a useful strategy to be implemented to prevent chemobrain.

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