Effect of 5-Fluorouracil chemotherapy and the potential protective effect of the SSRI antidepressant Fluoxetine on memory and neurogenesis in the adult hippocampus

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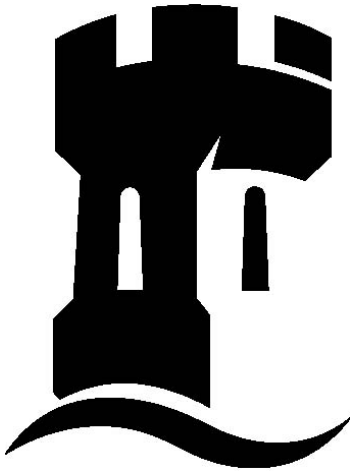

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Publications:

PAPERS

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BOOK CHAPTER

Peter M. Wigmore, Sarah Mustafa, Maha El-Beltagy, Laura Lyons, Jariya Umka and Geoff Bennett (2010) Effects of 5-FU. In 'Chemo-fog': Cancer Chemotherapy-Related Cognitive Impairment. Eds Raffa R.B. & Tallarida R. J. Landes Bioscience

ABSTRACTS

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El-Beltagy M. Mustafa S. Umka J. Lyons L. Salman A. Bennett G. Wigmore P.M. (2010) Effect of 5Fluoruracil on survival and proliferation of the hippocampal rat brain dividing cells.
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Abbreviations

5-FU	5 -Fluorouracil
ALDH	Aldehyde dehydrogenases
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
BLEB	Brain lipid binding protein
BrdU	Bromodeoxyuridine
cAMP	Cyclic adenosine monophosphate
CH2THF	5, 10-methylene tetrahydrofolate (folate co-factor)
CMF	Cyclophosphamide, methotrexate, and 5-Fluorouracil
CNS	Central nervous system
COX2	Cyclooxygenase 2
CREB	cAMP response element-binding
CSF	Cerebrospinal fluid
CYP	Cyclophosphamide
DCX	Doublecortin
DNA	Deoxyribonucleic acid
dTMP	2'-deoxythymidine-5 '-monophosphate
dUMP	2'-deoxyuridine-5 '-monposphate
FdUMP	5-fluoro-2'deoxyuridine-5 'monophsophate
FdUTP	5-fluorodeoxyuridine-5 '-triphosphate
FITC	Fluorescein isothiocyanate
FUTP	5-fluorouridine 5'-triphosphate
GCL	Granular cell layer

GFAP	Glial fibrillary acidic protein
i. p.	Intra-peritoneal
i.v.	Intra-venous
Ki67	Ki-67
LCV	Leucovorin
MAM	Methylazoxyrnethanol acetate
MTX	Methotrexate
MW	Molecular weight
NOD	Novel object discrimination
NSE	Neuron-specific enolase
OCT	Optimal cutting temperature
OD	Optical density
OLR	Object location recognition
PBS	Phosphate buffer solution
PFA	Paraformaldehyde
PI	Preference Index
RNA	Ribonucleic acid
SGZ	Sub-granular zone
SSRI	Selective serotonin reuptake inhibitors
SVZ	Sub-ventricular zone
TRITIC	Tetramethylrhodamine isothiocyanate
TS	Thymidyalte synthetase
VEGF	Vascular endothelial growth factor

Abstract

Cancer patients, treated with systemic adjuvant chemotherapy, have described experiencing persistent deteriorations in cognition. The nature of these effects is unclear, and although a wide range of theories have been advanced, there is currently no treatment.

This thesis uses an animal model to investigate the effects of a commonly prescribed chemotherapeutic agent, 5-fluorouracil (5-FU). The cognitive effects of 5-FU were examined using two behavioural tests, the object location recognition test (OLR) and the conditioned emotional response test (CER) both of which require input from the hippocampus, a brain region associated with memory. Memory consolidation by the hippocampus requires the continual production of new neurons (adult neurogenesis) from progenitor cells in the sub granular zone (SGZ) of the dentate gyrus. As an anti mitotic agent, 5-FU could be reducing the cell proliferation required for neurogenesis and this could be a cause of the cognitive deterioration. This hypothesis was tested by quantifying the numbers of proliferating cells ($Ki67^{+}$) in the SGZ in sections together with the levels of doublecortin (DCX), a neurofilament expressed in developing neurons and brain- derived neurotrophic factor (BDNF), a factor required for new neuron survival and synaptic plasticity, by Western blotting.

After developing the methodology (chapter 2); adult male Lister Hooded rats were given five i.v injections of 5-FU (25mg/kg) over a two week period and their behaviour and cellular aspects of the hippocampus compared with saline injected controls (chapter 3). 5-FU treated animals showed significant impairments in their performance of both the OLR and CER behavioural tests. Animals were sacrificed after the behavioural tests were performed and analysis showed they had significantly reduced numbers of dividing cells in the SGZ and non significant reductions in the levels of BDNF and DCX within the hippocampus. These results demonstrate that 5-FU treatment can produce cognitive impairments in this animal model which are similar in nature to those described by patients after chemotherapy. These behavioural changes are correlated with a reduction in the cell proliferation required for hippocampal neurogenesis providing support for the hypothesis that chemotherapy drugs are affecting this aspect of hippocampal function.

In order to develop a treatment for the cognitive effects of chemotherapy the antidepressant fluoxetine was co-administered with 5-FU (chapter 4). This approach was based on recent

evidence that fluoxetine can increase neurogenesis and protect neurons after damage. As with the experiment described above, performance in the CER test was impaired by five injections of 5-FU (25 mg/kg) as compared with saline treated controls. Similarly, animals treated with six injections of 5-FU (20mg/kg) were unable to discriminate between objects in novel and familiar locations in the OLR task. However co-administration of fluoxetine in drinking water (10mg/kg/day) for three weeks, starting a week before 5-FU treatment, prevented the impaired performance of this task found in the 5-FU only group. 5-FU chemotherapy caused a significant reduction in the number of proliferating cells in the SGZ compared to controls but this reduction was eliminated in the group co administered with fluoxetine. Fluoxetine on its own had no effect on proliferating cell number or behaviour. Moreover hippocampal BDNF or DCX protein levels in the co-treated group (5-FU+fluoxetine) were significantly increased compared to the 5-FU only treated group. These findings suggest that while 5-FU can negatively affect cell proliferation and hippocampal dependent memory, these deficits can be reversed by co- administration of fluoxetine.

To understand the long term effects of chemotherapy, the cellular effects of 5-FU treatment were quantified one day, 2 and 6 weeks after the end of two weeks of 5-FU (20mg/kg) treatment (chapter 5). The results showed that 2 weeks of 5-FU treatment did not significantly reduce cell proliferation in the SGZ when quantified one day after the end of treatment. However proliferating cell numbers were significantly reduced compared to controls two and six weeks after the end of treatment. This suggests that 5-FU has a delayed effect on cell proliferation with its maximum effect two weeks after the end of treatment. Cell survival was quantified by BrdU labelling cells immediately prior to 5-FU treatment, and quantifying the numbers of BrdU positive cells at the different time points. $BrdU^+$ cell numbers were significantly reduced at the end of treatment and continued to decline at 2 weeks but stabilised by 6 weeks. These results demonstrate that 5-FU has prolonged effects on neurogenesis after the end of chemotherapy treatment. The effects of 5-FU on cognition and neurogenesis are discussed and correlated with chemotherapy treated patient reports of continued cognitive impairment for months or years after completion of chemotherapy treatment.

CHAPTER 1

General Introduction

1.1 Chemobrain

Epidemiological studies have shown that one in every three people will develop cancer at some time in their life (Cancer Research UK, 2007). In most cancers, the treatment is surgical removal of the tumour followed by adjuvant chemotherapy or irradiation. The survival rate of cancer patients has increased due to the significant improvements in the treatment of such patients. However chemotherapy has a wide range of unwanted side effects which include mental symptoms such as confusion, memory deficits and difficulties in concentration. These finding are collectively named “Chemo-brain” or “chemo-fog” by patients and physicians (Bender et al., 2005; Wefel et al., 2004). Not only patients who have brain tumours suffer from these deteriorations in cognitive functions, but also patients with tumours in other locations such as breast cancer (Ahles and Saykin, 2002; Castellon et al., 2005). However, these cognitive deficits often last even after the treatment period ends and sometimes for up to 10 years after treatment (Ahles et al., 2002) in addition, these deteriorations have been found to be chemotherapeutic dose-dependent (van Dam, Schagen et al. 1998). In patients, major confounding variables such as depression or anxiety caused by the disease or its treatment might be responsible for the behavioural changes experienced and this has led some authors to ascribe the cognitive decline to these conditions rather than the chemotherapy itself (Jenkins, Shilling et al. 2006; Shilling, Jenkins et al. 2006).

This line of reasoning has lead to the idea that the incidence of these cognitive problems in women with breast cancer is unrelated to their treatment with chemotherapy and due primarily to psychological stress and changes in their quality of life (Shilling and Jenkins 2007). This highlights the requirement of better controlled studies and the use of animal models.

1.2 Chemobrain in clinical studies

Since chemobrain was identified, several studies have tried to determine the cognitive effects of the chemotherapy on patients. An early study (Peterson and Popkin 1980) reported that the neuropsychological deficits resulting from chemotherapy could be due to the specific chemotherapeutic agent used, the cancer itself or other generalised toxic effects on CNS. Later authors (Wieneke and Dienst 1995) have found that 75% of patients, 6 months after standard dose, (5-FU, doxorubicin, cyclophosphamide) or CMF chemotherapy (cyclophosphamide, methotrexate, 5-FU) suffered from deteriorations in attention, concentration, verbal, visual and visuospatial memories. A similar study (van Dam, Schagen et al. 1998) which compared the effect of a standard dose (SD) versus a high dose (HD) of systemic chemotherapy, 2 years after the end of treatment, showed that 17% of patients under the SD chemotherapy had cognitive impairments compared to 32% after HD chemotherapy. These impairments primarily affected their attention, visual memory and motor function. Similarly (Schagen, van Dam et al. 1999) found that the possibility of developing these cognitive impairments in breast cancer patients 1.9 years after a SD of CMF chemotherapy was 12%.

This led further authors to continue their investigations in this field. In 2000, (Brezden, Phillips et al. 2000) found that around 50% of breast cancer patients

having SD chemotherapy developed similar cognitive complications found in previous studies. A more recent study (Ahles and Saykin 2002) has also supported the concept of chemobrain and has shown that these cognitive impairments could last up to 10 years after the end of treatment. A recent study, (Nelson CJ 2007) has tried to list the possible mechanisms that could contribute to this disruption. Although this still remains largely unknown, the suggested mechanisms by which these deteriorations occur could be due to vascular injury and oxidative damage, inflammation, direct damage to neurons, or chemotherapy-induced anaemia. It is important to note that patients from these studies did not have any tumour metastases and did not have any other medical complications that might alter their cognitive function (Schagen, Muller et al. 2002; Rugo and Ahles 2003). Clinical, studies of patients after chemotherapy are continuing to try to understand the mechanism by which chemotherapy produces these cognitive deficits (Wefel, Lenzi et al. 2004; Wagner, Knaevelsrud et al. 2006; Stewart, Collins et al. 2008). Some studies have been limited by small sample sizes but four meta-analyses of the literature have concluded that working, visual and verbal memory appear to be consistently affected in patients who have completed a course of chemotherapy (Anderson-Hanley, Sherman et al. 2003; Falleti, Sanfilippo et al. 2005; Jansen, Miaskowski et al. 2005; Stewart, Bielajew et al. 2006). Imaging studies have also shown structural (brain region volume and white matter pathology) and functional (blood flow) changes in the brains of patients after chemotherapy with alterations in hippocampus, striatum and cingulate gyrus (Yoshikawa, Matsuoka et al. 2005; Bradbury 2006; Inagaki, Yoshikawa et al. 2007; Silverman, Dy et al. 2007). Similarly an EEG study has

shown differences in patients after receiving chemotherapy (Kreukels, Hamburger et al. 2008).

The duration after treatment during which deficits are manifested is of significant importance to patients. Some studies have found that deficits can last for several years (Schagen, Muller et al. 2002; Wefel, Lenzi et al. 2004) but a more recent longitudinal study, where patient's cognition was tested prior to chemotherapy, as well as at two points after treatment concluded that deficits are present one month after treatment but have disappeared by one year (Collins, Mackenzie et al. 2009). As indicated different studies have found differing effects and some authors have criticised the types of psychometric tests used, the subjects chosen as controls, the small sample sizes and confounding variables associated with depression and disease progression (Shilling, Jenkins et al. 2006; Pedersen, Rossen et al. 2009). Some of clinical studies on chemobrain are listed on (table 1.1).

(Wieneke and Dienst 1995)	6 months	Published norms	SD chemo: FAC/CMF	SD Chemo: 75%	Attention/concentration Verbal memory Visual memory Visuospatial ability Processing speed	Not applicable
(van Dam, Schagen et al. 1998)	2 years	BC patients received local therapy	SD chemo: FEC HD chemo: FEC+CTC	Controls: 9% SD: 17% HD: 32%	Attention/ concentration Processing speed Visual memory Motor function	HD and SD groups: Concentration Memory Thinking
(Schagen, van Dam et al. 1999)	1.9 years	BC patients received local therapy	SD chemo: CMF	Controls: 12% SD: 28%	Attention/concentration Mental flexibility Processing speed Memory Motor function Verbal functions	Concentration Memory
Studies (2000-2010)						
(Brezden, Phillips et al. 2000)	*During Chemo *2years	Healthy females	*SD chemo: CMF/CEF *SD chemo: CMF/CEF	Controls: 11% *48% *50%	*Memory& Language *Language& Visual-motor cognition	Not applicable
(Ahles and Saykin 2002)	10 years	Cancer patients received local therapy	SD chemo (combinations)	Controls: 14% SD chemo: 39%	Verbal memory Psychomotor function	Working memory
(Tchen, Juffs et al. 2003)	1-2 years	Healthy female controls	*CMF *CEF/AC(Adriamycin &cyclophosphamide)	symptoms not related to cognitive function	Attention& concentration Language Visuo-spatial	HSCS (test for detecting subtle Cognitive impairment).
(Castellon, Ganz et al. 2004)		Healthy female controls BC patients received local therapy	SD chemo (combinations)	Not applicable	Visual memory Visuospatial memory Verbal Learning	No correlation between this and neuropsychological assessments
(Hermelink, Henschel et al. 2008)	Before chemo 5 month after chemo 1 year after	Test norms	Epirupcin, Paclitaxel/cyclo CMF+Tamoxifen/ anastrozole or letrozole	Intelligence assessment and induced menopause cognitive problems	Verbal memory Verbal short-term memory Attention Verbal working memory Information processing	At the three time point there was fixed positive effect of menopause on cognitive parameters
(Schilder, Eggens et al. 2009)	2 years	Healthy females	AC doxorubicin/cyclophosphamide chemotherapy, randomized to tamoxifen or exemestane	28% AC/tamoxifen 29%AC/exmestane 6% controls	Verbal memory Mental flexibility Verbal Fluency Information processing speed Motor speed	memory concentration thinking language
(Collins, Mackenzie et al. 2009)	1 year	Hormonal treated post-monopausals	SD adjuvant chemotherapy	Standererdized Regression Based (SRP) approach	Processing speed Verbal memory	Not applicable

Table 1.1 Summary of cross sectional clinical studies reporting incidence of cognitive deficits, changes in neuropsychological domains and patient-reported cognitive measures caused by high-dose (HD) or standard-dose (SD) chemotherapy. BC: breast cancer; chemo: chemotherapy; FAC: 5-FU, doxorubicin, cyclophosphamide; CMF: cyclophosphamide, methotrexate, 5-FU; FEC: 5-FU, epirubicin, cyclophosphamide; CTC: cyclophosphamide, thiopeta, carboplatin. Adapted from (Rugo and Ahles 2003)

1.3 Animal models of chemobrain:

Clinical studies have suffered from a variety of methodological problems making the development of an animal model to test for the cognitive effects of chemotherapy a useful strategy. These problems have included the inability to randomly assign patients to experimental groups (Schagen, Muller et al. 2002). Also, in clinical studies, it is very difficult to specify which chemotherapeutic agent may have caused the cognitive effects because patients receive combinations of different drugs in different doses and routes. Applying behavioural tests to the animals is a useful, fast and efficient means to asses cognitive changes which has been extensively used and validated (Tannock, Ahles et al. 2004). Previous studies which have modelled the toxicity of chemotherapy in animals have provided important information about the neurobiological changes caused by chemotherapy (listed in table 1.2). Several of these studies investigated the effect of a single chemotherapy agent (Lee, Longo et al. 2006; Reiriz, Reolon et al. 2006; Seigers, Schagen et al. 2008) while others observed the effects of a combination of two drugs (Winocur, Vardy et al. 2006; Macleod, DeLeo et al. 2007).

Behavioural paradigms that model spatial learning as well as recognition and spatial memory in animals were commonly used in these animal models to test the effects of chemotherapy on cognition. For example, rats injected with vincristine in the dorsal hippocampus showed impaired spatial learning in the Morris water maze which was associated with a wide spread lesion in the dorsal hippocampus (Eijkenboom and Van Der Staay 1999). In another study, rats treated with methotrexate for 6 days showed impairments in their conditioned avoidance and histologically, their hippocampi showed decreased noradrenaline,

dopamine and serotonin (Madhyastha, Somayaji et al. 2002). Furthermore, mice treated with a mixture of 5-fluorouracil and methotrexate, showed not only an impairment in their performance in the Morris water maze but also a deficit in the delayed non-matching to sample test (Winocur, Vardy et al. 2006). In contrast, one study, (Lee, Longo et al. 2006), found that the performance of female mice treated with cyclophosphamide or 5-fluorouracil improved in both the water and T- mazes. However no explanation of this has been found. Using another behavioural test, (Macleod, DeLeo et al. 2007) have shown that doxorubicin and cyclophosphamide impaired a contextual conditioned emotional response (CER) whereas the cued CER remained unaffected in ovarictomized female rats treated with both drugs for three weeks. Recently, it has been shown that methotrexate impaired spatial memory as tested in the Morris water maze and recognition memory in the novel object discrimination tasks after rats were treated for 3 weeks (Seigers, Schagen et al. 2008). This study also gave strong evidence that methotrexate caused a dose-dependent decrease in the number of proliferating cells in the dentate gyrus within the hippocampus.

Using different behavioural tests, (Gandal, Ehrlichman et al. 2008) found that treatment with methotrexate and 5-FU decreased the ability of mice gate incoming auditory stimuli indicating that chemotherapy disrupted this function. These deficits were also accompanied by increased reaction to fear conditioning and reduced animals' response to novel objects. The generalised neurotoxicity of 5-fluorouracil has previously been demonstrated by showing that the drug caused a dose-dependent depression in neuronal activity as measured from hippocampal field potentials and a wide spread cellular necrosis in the brain (Berg-Johnsen, Heier et al. 1987; Okeda, Shibutani et al. 1990).

Recently, we have confirmed that 5-FU treatment impaired spatial memory in a rat model (Mustafa, Walker et al. 2008; ElBeltagy, Mustafa et al. 2010) and the studies done in this thesis are a continuation of these results.

Study	Drug	Dose	Period of treatment	Findings
(Phillips, Thaler et al. 1989)	MTX	250mg/kg 1000mg/kg 2500mg/kg	Intravenous cannula, infusion over 24hrs	Lethargy, hyperirrita seizures, reduces glu metabolism reversed high Leucovorin
(Shors, Townsend et al. 2002)	MAM	7mg/kg	Intraperitoneal daily for 14 days	*Water maze-no affe *CER/context altered *trace fear condition affected * Plus maze - no affe * Reduced BrdU labe
(Bruel-Jungerman, Laroche et al. 2005)	MAM	5mg/kg	Sub acute daily for 14 days BrdU injection	*MAM reduces NOR neurogenesis. *Enrichment improv NOR and neurogenes *MAM+enrichement reduces NOR and neurogenesis
(Winocur, Vardy et al. 2006)	*5FU *MTX	*75mg/kg *37.5mg/kg	3 weekly intraperitoneal injections	Variety of water maz affects spatial but no memory
(Lee, Longo et al. 2006)	*Cyclphosphamide *5FU	*100mg/kg *150mg/kg	Intraperitoneal 4 injections over 18 wks	*8-10 weeks recover *Water maze and T maze showed Improvement. * LTP down acutely but up wks.
(Dietrich, Han et al. 2006)	*BCNU *Cisplatin *Cytarabine	*10mg/kg *5mg/kg *250mg/kg	Intraperitoneal 3 injections over 5 days	*TUNEL increased * decreased in DG and
(Macleod, DeLeo et al. 2007)	*Doxorubicin *Cyclophosphamide	*4mg/kg *40mg/kg	Iv 3 injections weekly	*CER/audio no effec * CER context signif effect
(Seigers, Schagen et al. 2008)	MTX	37.5-300mg/kg	Iv single inject+ leucovorin	*Water maze and NO significant effects * Dose dependent reduction Ki67.

(Mustafa, Walker et al. 2008)	5FU	25mg/kg	Iv 5 injections over 12 days	*OLR sig effect, *Reduced BDNF and DCX *No sig effect on Ki6
(Konat, Kraszpulski et al. 2008)	*Doxorubicin *Cyclphosphamide	*2.5mg/kg *25mg/kg	4 intraperitoneal injections weekly	Significant effect on Passive avoidance, restored by antioxida
(Han, Yang et al. 2008)	5FU	40mg/kg	3 intraperitoneal injections every second day	*Maximum decline E at 14 days. *increase TUNEL *Delayed myelination.
(Foley, Raffa et al. 2008)	*MTX *5FU Single or combined	*3-32mg/kg *3-75mg/kg	Single intraperitoneal injection	*Reduced operant conditioning to tone 1 day after treatment *Enhanced effects w combined drugs.
(Bessa, Ferreira et al. 2009)	MAM	7mg/kg	Sub acute daily 2 weeks	Reduces BrdU, Ki67 60%. MAM did not b behavioural effects o fluoxetine in rats.
(Li, Cai et al. 2009)	AZT	100mg/kg	Intraperitoneally 28 days	Blocked the survival of Fluoxetine on hippocampal neuroge
(Ko, Jang et al. 2009)	MAM	1-5mg/kg 3mg/kg for behavioural tests	Sub acute daily 14 days	*Dose dependent dec in BrdU *No affect on CER
(ElBeltagy, Mustafa et al. 2010)	5Fluorouracil	20mg/kg	Intravenous Two weeks (6 injections)	Impaired OLR ,CEF and decreased positive cell counts

Table 1.2. Summary of the main studies that have examined the effect of chemotherapy on cognitive function and neurotoxicity in the CNS of adult rodents. Abbreviations: MTX, methotrexate; MAM, methylazoxymethanol acetate; BCNU, carmustine; AZT, 3
0-azido-deoxythymidine; 5FU, 5-fluorouracil; OLR, object location recognition; NOR, novel object recognition; CER, conditioned emotional response; LTP, long-term potentiation.

1.4 Chemotherapy:

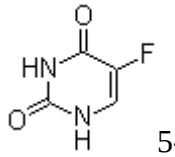
Systemic chemotherapy normally involves administration of a cocktail of agents making it difficult to ascribe cognitive effects to a specific drug. As previously described, a number of patient studies have tested the suggestion that systemic chemotherapy produces prolonged cognitive deficits independent of disease or other factors associated with treatment (Anderson-Hanley, Sherman et al. 2003; Castellon, Silverman et al. 2005; Falleti, Sanfilippo et al. 2005; Stewart, Bielajew et al. 2006). Most of these studies have examined breast cancer survivors and the majority of investigations have shown that patients who received systemic chemotherapy experienced cognitive problems which were attributable to their chemotherapy treatment (van Dam, Schagen et al. 1998; Schagen, van Dam et al. 1999; Brezden, Phillips et al. 2000; Ahles and Saykin 2002; Wefel, Lenzi et al. 2004; Ahles 2005; Ahles, Saykin et al. 2005; Bender, Sereika et al. 2005; Castellon, Silverman et al. 2005). Most of these studies have observed the association of "chemobrain" with the use of CMF chemotherapy which involves the combined administration of cyclophosphimide, methotrexate and 5-FU. More specifically, 5-FU has been particularly associated with patient descriptions of the cognitive side effects of chemotherapy as it has been shown that systemic treatment with this drug significantly increased the occurrence of cognitive problems when compared with treatment by local chemotherapy or local irradiation (Peterson and Popkin 1980; Hussain, Wozniak et al. 1993). Recent animal investigations have also found that 5-FU can reduce hippocampal neurogenesis and produce hippocampal specific behavioural deficits (van Dam, Schagen et al. 1998; Ahles, Saykin et al. 2002; Schagen, Muller et al. 2002; Winocur, Vardy et al. 2006; Mustafa, Walker et al. 2008). Studies in this thesis

are a continuation of these investigations into the effects of 5-FU on memory and the possible correlation between the cognitive deteriorations and a reduction of hippocampal neurogenesis.

1.5 5-FLUOROURACIL:

This section describes the commonly used chemotherapeutic drug which is used in the treatment of many cancers, 5-fluorouracil (5-FU).

1.5.1 5-Fluorouacil mechanism of action:



5-flurouracil was developed in 1957 as a toxic analogue of the RNA base uracil and it is widely used in cancer chemotherapy. It has previously been shown that the route of entry of 5-FU to the brain is mainly from the blood into the CSF (Bourke, West et al. 1973). It is a low molecular weight agent (see diagram at the start of this section) which enters the brain by simple diffusion through the blood brain barrier enabling it to have a direct effect on neural tissue. Clinically, it has been found that 5-FU reaches a higher concentration in cerebrospinal fluid than other chemotherapy agents (Grochow LB 1998). 5-FU is applied clinically in the treatment of a wide range of cancers including breast, ovarian, colorectal and gastro intestinal tract. It is occasionally used as a single agent but is usually combined with other chemotherapeutics especially cyclophosphamide and methotrexate in a triad known as CMF (Schagen, Muller et al. 2002). 5-FU is an anti-metabolite chemotherapeutic drug; however its major action is not on RNA, but in inhibiting the enzyme thymidilate synthetase (TS). This enzyme produces the DNA base thymidine which is required for DNA

synthesis during S-phase of the cell cycle (Chu, Callender et al. 2003). 5-FU is a pro-drug that has to be metabolized to produce the active metabolite 5-fluoro-2'deoxyuridine-5'monophosphate (FdUMP) that binds to and inhibits TS (Pinedo and Peters 1988).

Other cytotoxic metabolites produced from 5-FU metabolism are 5-fluorouridine 5`triphosphate (FUTP) and 5-fluorouridine-5`triphosphate (FdUTP) which can bind to DNA and also inhibit RNA synthesis. The 5-FU mechanisms of action are reviewed as follows.

DNA synthesis target mechanism (Figure 1.1)

When (FdUMP) binds to TS, the latter is inhibited and as a result the conversion of 2`- deoxyuridine-5`-monophosphate (dUMP) into 2`deoxythymidine-5`-monophosphate (dTMP) is disrupted. This step is an essential step in *de novo* synthesis of the DNA nucleotide base thymidine. The endogenous folate 5,10-methylene tetrahydropholate (CH2THF) potentiates the action of TS by facilitating the binding of dUMP to TS (Parker and Cheng 1990). Calcium folate (leucovorin) is a formyltetrahydrofolic acid derivative. Clinically, leucovorin is combined with 5-FU in cancer chemotherapy as it augments the binding of 5-FU metabolites to TS and so increases the 5-FU- induced inhibition of thymidylate synthase (Murakami, Fujii et al. 1998). Studies presented in this thesis used co-administration of leucovorin to potentiate the cytotoxic and the neurotoxic effect of 5-FU.

DNA and RNA target mechanisms:

FUTP, the active metabolite of 5-FU incorporates into all types of RNA affecting both transcription and translation processes required for protein synthesis. Also the other main active metabolite (FdUTP) is incorporated into DNA further disrupting its synthesis and function (Pinedo and Peters 1988; Parker and Cheng 1990; Chu, Callender et al. 2003).

The cytotoxic effect of 5-FU is not restricted to tumour cells but also targets rapidly proliferating cells at any place where growth or regeneration is taking place. The presence of proliferating cells in the brain is a relatively recent but now well established neurological phenomenon (Kempermann 2006). This fact led us to investigate the possibility that 5-FU could affect the number of the proliferating cells in the brain and whether this contributes as a causative mechanism to the drug's neurotoxicity.

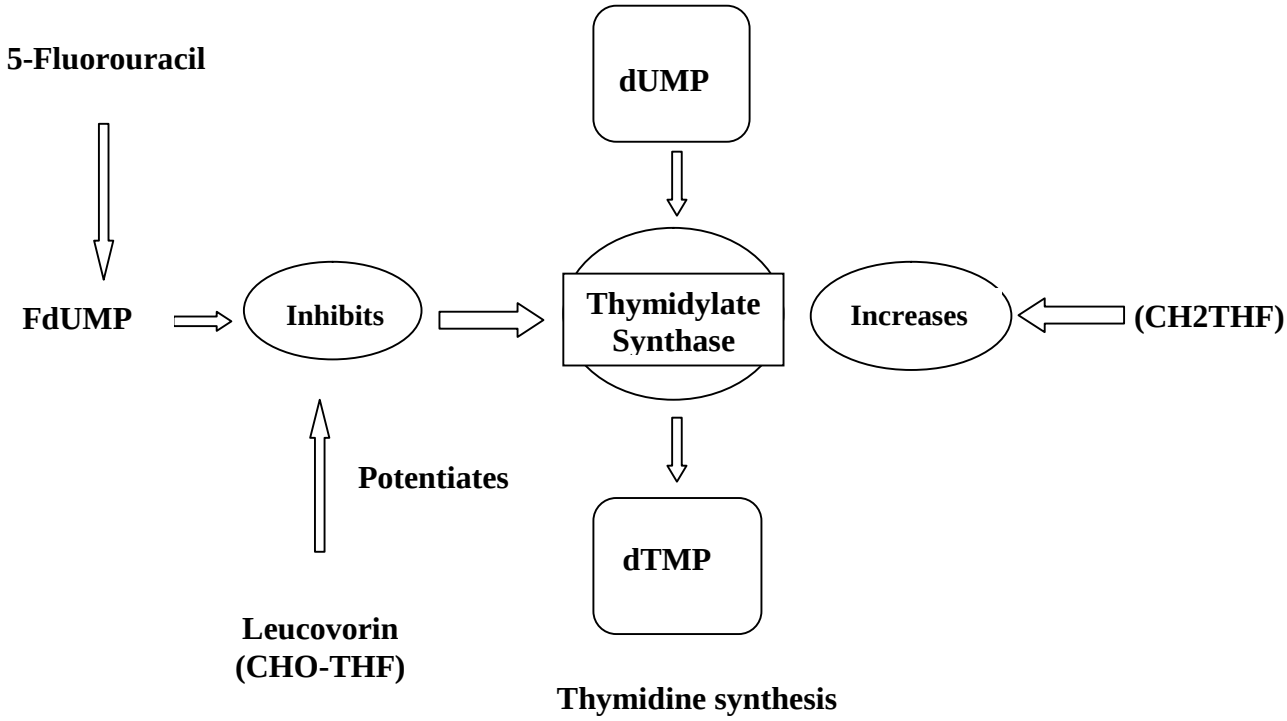


Figure1.1 the DNA-mediated mechanism of action of 5-fluorouracil modified and re-drawn from (Pinedo and Peters 1988). 2'-deoxyuridine-5'-monophosphate (dUMP) is converted into 2'-deoxy-thymidine-5'-monophosphate (dTMP) during the process of thymidine synthesis. This step requires the enzyme thymidylate synthase and is enhanced by the endogenous binding of the (CH2THF) to thymidylate synthase. In order to be active, 5-fluorouracil has to be converted to 5-fluoro-2'deoxyuridin-5'monophospate (FdUMP) which inhibits the enzyme thymidylate synthase, a process is potentiated by co-treatment with leucovorin.

1.5.2 5-Fluorouracil neurotoxicity:

Reviews on the side-effects of 5-FU have described relatively rare neurotoxic effects which have been ascribed to several mechanisms. For example, some reviewers have shown that high doses of 5-FU are associated with the occurrence of cerebellar syndrome and or organic brain syndrome which are reversible after stopping treatment (Lynch, Droszcz et al. 1981; Moore, Fowler et al. 1990; Atkins, Muss et al. 1991; Yeh and Cheng 1994). Cerebellar syndrome is characterized by ataxia and other deficits in motor coordination whereas organic brain syndrome mainly affects cognition and results in confusion and disorientation (Lynch, Droszcz et al. 1981; Moore, Fowler et al. 1990; Yeh and Cheng 1994). Another theory of 5-FU neurotoxicity relates to the ability of 5-FU to affect the vascular system of the brain (Weh, Bittner et al. 1993). There are also reports which believe that 5-FU neurotoxicity is due to a deficiency in the enzyme dihydropyrimidine dehydrogenase (DPD) (Tuchman, Stoeckeler et al. 1985; Takimoto, Lu et al. 1996). This enzyme is responsible for the metabolic degradation of 5-FU in the liver which accounts for 80% of the drug's metabolism (Pinedo and Peters 1988). In addition, there is also a possibility of neuronal death due to thiamine deficiency which takes part in inhibition of the Krebs cycle causing these neurotoxic effects (Lynch, Droszcz et al. 1981; Moore, Fowler et al. 1990).

1.6. Adult Neurogenesis:

The results of this thesis suggest that 5-FU chemotherapy reduces hippocampal neurogenesis. The following section reviews hippocampal neurogenesis.

1.6.1 History and definition of adult neurogenesis:

The adult mammalian central nervous system had been classified as a non-regenerative tissue for many decades. This was changed completely with the discovery of adult neurogenesis 40 years ago. The term neurogenesis means the continuous production of new, functioning, neurons which previously was believed to occur only during embryogenesis. As with most of other tissues in the body (e.g. the epidermis of the skin), neuronal tissue has tissue-specific stem cells which have the ability to generate cells with tissue specific functional capacity. This was demonstrated first by Altman and Das in 1965 when they discovered that proliferating cells appeared to form new neurons in the adult hippocampus of rats. Before this time it had been assumed that new neurons could not be formed once embryogenesis had finished (Altman and Das 1965; Kempermann 2006). Two areas of the brain have the ability to generate new neurons, the sub-ventricular zone (SVZ) of the lateral walls of the lateral ventricles and the sub granular zone (SGZ) of the dentate gyrus of the hippocampus, reviewed in (Gould and Gross 2002; Kempermann 2006). It has been proposed that the level of postnatal neurogenesis relates inversely to the complexity of the brain as ranked from lower to higher vertebrates including birds (Goldman SA 1983), rodent (Altman 1962) Primates (Gould, Reeves et al. 1999) and humans (Eriksson, Perfilieva et al. 1998).

Concerning neurogenesis in the SVZ, it appears to depend on the presence of astrocytic-like cells which have the stem cell-like properties of being able to self renew and to produce neuroblasts which begin their journey towards the olfactory bulb through the rostral migratory stream (Fig 1.2). During their migration, they form chains of neuroblasts which are independent of the radial glia normally required for guidance (Alvarez-Buylla, Garcia-Verdugo et al. 2001). After reaching the olfactory bulb, they mature into two types of interneurons, granule cells and periglomerular cells (Lois and Alvarez-Buylla 1994; Cameron and McKay 2001). During their two week maturation, the granule cells extend dendrites into the external plexiform layer of the olfactory bulb (Lledo, Alonso et al. 2006). The periglomerular cells mature over nearly 4 weeks (van Praag, Schinder et al. 2002).

The other neurogenic area in the brain is the SGZ of the dentate gyrus of the hippocampus. Nearly 9000 dentate gyrus progenitor cells are generated daily in the rat within the SGZ again from astrocytic like stem cells (Cameron and McKay 2001). The following section will discuss hippocampal neurogenesis in details.

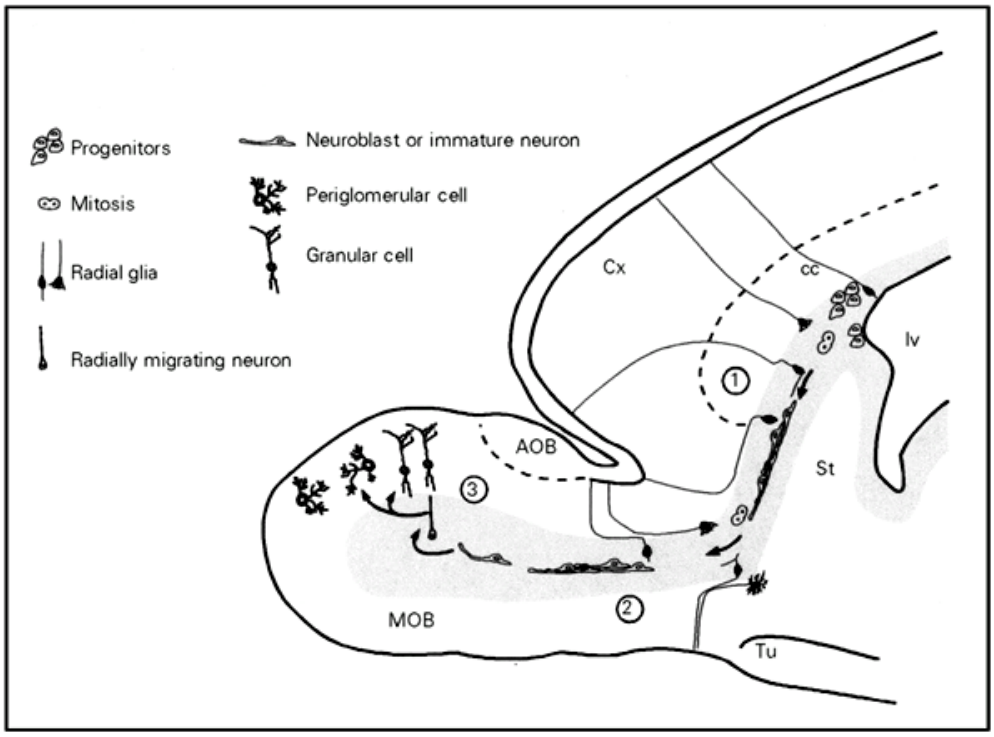


Figure 1.2. Summary of subventricular zone/rostral migratory stream (SVZ/RMS) migration towards the olfactory bulb (OB) in the anterior forebrain in the early postnatal animal modified from www.scielo.br/.../bjmbr/v35n12/html/4739i01.htm. Migration in the RMS is divided into three overlapping phases that correspond roughly to different portions of the pathway (numbers in circles). 1) Initially cells migrate but are still able to divide. That happens in regions of the SVZ close to the lateral ventricles where mitosis is more frequent. 2) Cells leave the cell cycle and continue migration towards the OB. 3) upon reaching the OB; cells penetrate the OB parenchyma and differentiate into granular and periglomerular cells. AOB, accessory olfactory bulb; Cx, cerebral cortex; cc, corpus callosum; lv, lateral ventricle; MOB, main olfactory bulb; St, striatum; Tu, olfactory tubercle.

1.6.2 Anatomy of Human Hippocampal Neurogenesis

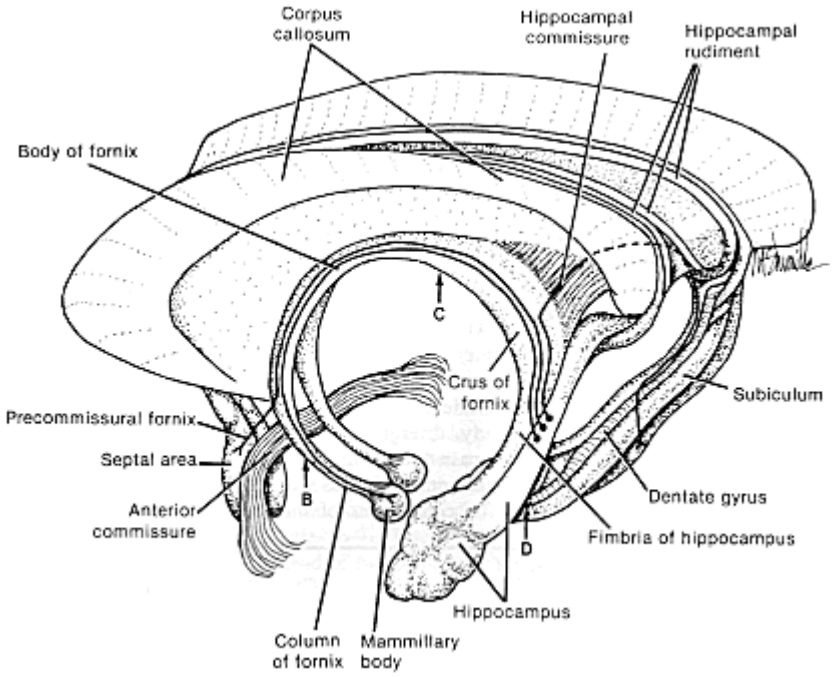


Figure 1.3. The Human Brain, Nolte (1993). Fig. 16-10A. p. 402.

The brain is divided into 2 hemispheres containing four lobes (parietal, frontal, occipital and temporal). The temporal lobe is the region largely responsible for the cognitive function of the brain such as memory and spatial learning. The hippocampus is a horse-shoe structure which lies within the temporal lobe (Fig 1.3). It is particularly involved in the consolidation of new memories, emotions and spatial orientation. The hippocampus is a component of the embryologicaly oldest part of the brain. It contains the dentate gyrus which is a slender gyrus running along the hippocampal formation. In humans, the hippocampal formation develops in a dorsal direction and then migrates ventromedially toward the temporal lobe.

The hippocampus starts forming at around 15-16 weeks of gestation and is fully formed by 18-20 weeks of gestation (Duan 2002). The hippocampus is a part of

the limbic system and consists of three main subfields: the dentate gyrus, area CA1 and area CA3 (area CA2 is very small). The dentate gyrus is composed of 3 layers. The external molecular layer which receives afferent fibres and contains the dendrites of the granule cells, the middle granule cell layer in the dentate gyrus and the pyramidal layer towards the hippocampus which contains the efferent fibres (Ming and Song 2005). The main afferents to the hippocampus originate from the entorhinal cortex. Axons projecting from the entorhinal cortex input into the dentate gyrus and area CA3 forming the perforant pathway. Axons project from the dentate gyrus to area CA3 forming the mossy fibre input to the pyramidal cells of area CA3 which in turn project to the pyramidal cells of area CA1 to form the Schaffer-collateral pathway. Efferent fibres then connect CA1 area to the subiculum (Fig 1.4).

From the above, it is clear that the dentate gurus acts as a gateway to the hippocampus (Kempermann 2002). Hippocampal neurogenesis is indicated by the presence of proliferating cells in the subgranular zone (SGZ) which lies adjacent to the granule cell layer (GCL) on the hilar side of dentate gyrus (Abrous, Koehl et al. 2005). To become functioning neurons, the cells generated in the SGZ, have to migrate from the SGZ into the GCL of the dentate gyrus where they are integrated in the dentate gyrus network (Abrous, Koehl et al. 2005). The cells produced by proliferation in the SGZ can differentiate into neurons, glia or endothelial cells but studies have shown that 70% of cells become granule cell neurons (Duman, Nakagawa et al. 2001). Therefore, the number of proliferating cells in the SGZ adjacent to the dentate gyrus has a strong relationship to the number of new neurons produced in adult hippocampal neurogenesis. The types of these cells are discussed in the following section.

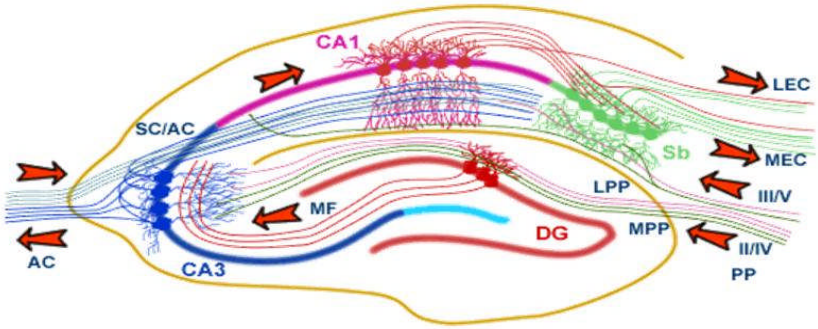


Figure 1.4. The hippocampal connections adapted from http://www.bristol.ac.uk/synaptic/pathways/. The input to the hippocampus comes from the entorhinal cortex (EC). Input signals travel from (EC) to the dentate gyrus (DG) and area CA3 through the perforant path (PP). In turn, the (PP) is divided into lateral and medial branches. Mossy fibres (MF) from the dentate gyrus send signals to area CA3. Fibres from CA3 are connected to area CA1 through the Schaffer collateral pathway (SC) and in addition, connect to the area CA1 cells in the contra lateral hippocampus through the associational commissural pathway (AC). Area CA1 sends its output to the subiculum (Sb) which in turn gives output signals to the EC, completing the circuit.

1.6.3 Types of Neurogenic Cells (Fig 1.5)

Developing cells found in the adult SGZ are divided into four cell types. These cells differ in their morphology, proliferative activity, migratory behaviour, and expression of key marker antigens (Fig 1.5 and Fig 1.6). Type 1 cells or the radial glia-like precursor cells, are morphologically similar to radial glia and have astrocytic and stem cell properties (Seri, Garcia-Verdugo et al. 2001). In the developing hippocampus, radial glia-like cells are necessary for the normal formation of the dentate gyrus (Kempermann 2006). These cells express glial fibrillary acetic acid (GFAP), nestin, Sox2 and brain lipid binding protein (BLBP) which can be identified immunohistochemically (Seri, Garcia-Verdugo et al. 2001; Kempermann 2006). The second and the third types of neurogenic precursor cell are type 2 and type 3 cells which are also called transient amplifying progenitors. Immunohistochemically, they are negative for expression of GFAP but are highly proliferative cells. Some of the type 2 cells express the immature neuronal marker DCX while some do not (Kronenberg, Reuter et al. 2003). Type 3 cells are DCX-positive, but nestin-negative and occasionally express polysialylated neural cell adhesion molecule (PSA-NCAM). This type of cell is involved in migration into the granule cell layer (Kempermann 2006). The final cell type is the maturing granule cell neurone which extends dendrites into the molecular layer and its axon to make contact with area CA3. It is clear that there are several markers which can be used to detect different stages of neurogenesis and the next section will describe the methods used in the detection of neurogenesis, particularly those used in this thesis.

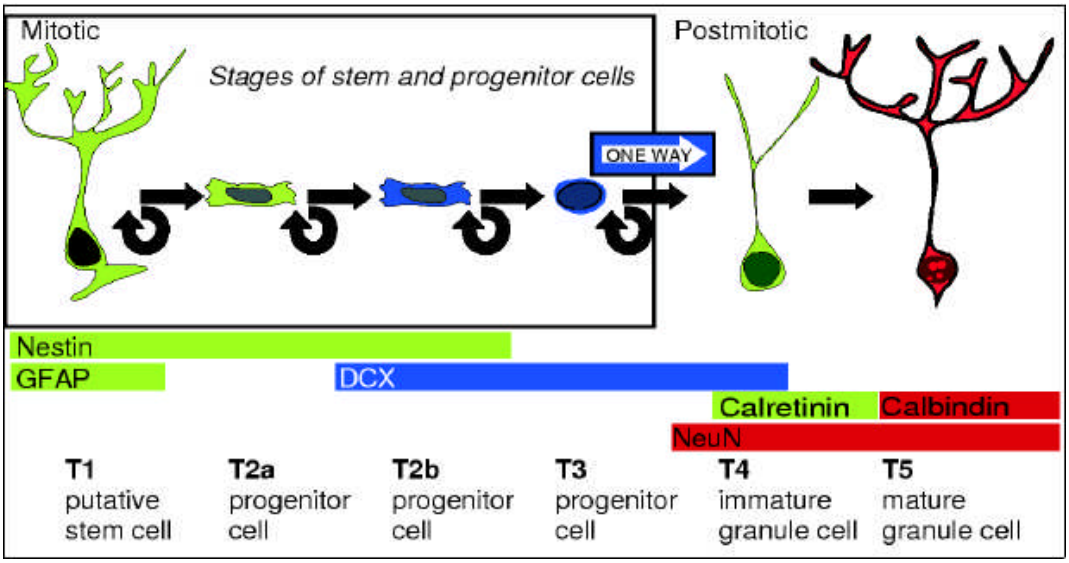


Fig 1.5. Cell types in the neurogenesis process that are distinguished by different markers adapted from (Kempermann, Jessberger et al. 2004). Six stages of neuronal development in the adult hippocampus are identified according to the cell morphology, proliferative ability, and expression of markers such as nestin, glial fibrillary acidic protein (GFAP), doublecortin (DCX), calretinin, calbindin and NeuN. Development originates from the putative stem cell (T1 cell; stage 1) that has radial glia and astrocytic properties and is identical to the astrocyte-like B-cell, Neuronal development then progresses over three stages of putative transiently amplifying progenitor cells (T2a, T2b and T3 cells; stages 2–4), which are determined to the neuronal lineage. This transient early postmitotic period is characterized by calretinin expression (stage 5).

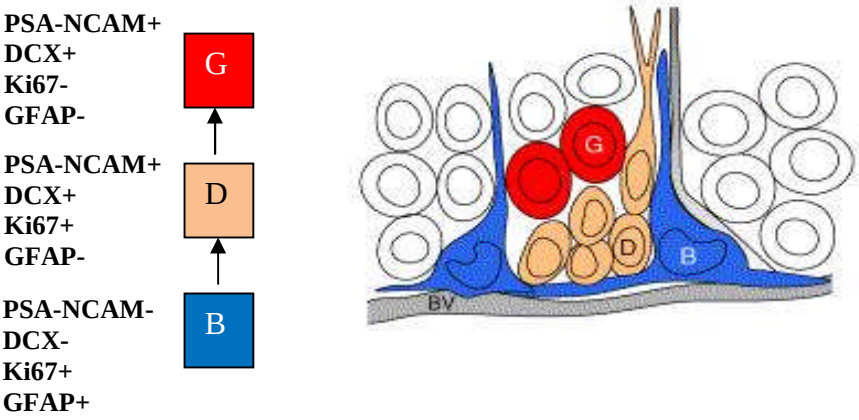


Figure 1.6. A diagram showing the three main cell types (left) undergoing neurogenesis and their anatomical locations (right) in the sub-granular zone (SGZ) of the adult rodent dentate gyrus, modified from (Doetsch 2003). B cells are the astrocytic cells (GFAP+) that are supportive to the stem cells. Asymmetric division of B cells gives rise to the transient amplifying progenitor cells or the D cells which express markers for immature neurons (DCX+ and PSA-NCAM) and will not express (GFAP+) after adopting a neuronal phenotype. Both B and D cells are positive for (Ki67+), the proliferative marker. G or the granule neuronal cells arise from D cells. B cells are usually located next to the endothelium of the blood vessels (BV). D cells arise from B cells and migrate into the granular cell layer giving rise to G cells. Abbreviations: PSA-NCAM, polysialyl neural cell adhesion; DCX, doublecortin; GFAP, glial fibrillary acidic protein.

1.6.4 Methods of Detecting Neurogenesis

1.6.4.1. Tritiated Thymidine and Bromodeoxyuridine

In most publications, demonstration of adult neurogenesis is based on the "birth-marking" of cells with bromodeoxyuridine (BrdU), a thymidine analogue and an exogenous marker which permanently labels cells which are in S-phase of the cell cycle at the same time of injection of BrdU and not when the tissue specimen was obtained. Labelling cells in this way is an effective way of visualizing and tracking cells and can be used to determine their survival rates, migration and phenotypic fate (Cameron and McKay 2001; Kee, Sivalingam et al. 2002). The first widely used substance that allowed permanent labelling of dividing cells was tritiated thymidine which was first used in 1956 (Friedkin and Wood 1956). All studies of adult neurogenesis from 1962 to 1993 were based on thymidine autography. The main disadvantage of this marker is that it is not easily combined with other cell type-specific markers that allow the phenotype of the labelled cells to be determined (Kempermann 2006). In 1993, Cameron and Gould used thymidine autoradiography together with immunohistochemistry against neuron-specific enolase (NSE) (Cameron, Woolley et al. 1993). This was followed by Frank Corotto who first applied the BrdU method of birth marking cells to adult neurogenesis (Corotto, Henegar et al. 1993). But the first big study that made full use of the BrdU method was that of George Kuhn (Kuhn, Dickinson-Anson et al. 1996) who demonstrated that the fluorescent visualization of BrdU can be combined with two or more other markers by using confocal microscopy. Protocols using the BrdU labelling method to study neurogenesis involve the injection of the thymidine analogue either shortly before termination of the study (2 to 6 hours) to observe cells proliferating at the time of death or (2

to 3 weeks) before termination to observe survival and final phenotype (Eisch 2002).

As mentioned previously, BrdU tracking methods have revealed that one month after labelling, 70% of precursor cells adopt a neuronal phenotype while of the remaining 30%; most become glial and a small number develop endothelial phenotypes, respectively (Palmer, Willhoite et al. 2000). However, one concern with the BrdU method is that BrdU may not only label dividing cells but can also pick up cell death, by labelling DNA fragmentation or DNA repair (Cooper-Kuhn and Kuhn 2002; Rakic 2002). Moreover, in animal studies of neurogenesis in which learning is being measured, BrdU administered by intraperitoneal injections could stress these animals adding a confounding variable to these studies (Kee, Sivalingam et al. 2002). In addition, BrdU can cause toxicity and modify the blood-brain barrier (Gould and Gross 2002). However, for most studies of neurogenesis that involve marking and tracking dividing cells, BrdU remains the most frequently used method (Kee, Sivalingam et al. 2002) and because of this, the BrdU labelling protocol in has been used in this thesis (Chapter 5) to detect possible 5-FU chemotherapy-induced changes in the survival of newly dividing cells in the rat dentate gyrus.

1.6.4.2. Labelling of Cell cycle-related antigens

Assessment of cell proliferation in tissues can now make use of the expression of cell cycle-related antigens detected immunohistochemically. In conditions where only proliferation at the time of death needs to be assessed, these cell cycle markers can be used instead of BrdU. These markers can be combined with detection of BrdU injected some time earlier and additionally combined with

detection of proteins such as nestin (neural stem cell marker), GFAP (astrocytes marker), doublecortin (DCX, expressed in differentiating/migrating and immature neurons (Abrous, Koehl et al. 2005).

These markers have been used widely to study different stages of neurogenesis including proliferation and differentiation. Ki67 is the name of the original antibody clone that identifies a cell-cycle associated protein (mki67) encoded on mouse chromosome 7 (Kempermann 2006). It appears to be essential for cell cycle progression (Starborg, Gell et al. 1996; Endl and Gerdes 2000). Expression of the Ki67 antigen identifies cells in late G1, S, G2, and M phase of the cell cycle (Scholzen and Gerdes 2000). MKi67 is the broadest known cell cycle-associated antigen. When BrdU is injected some hours before death and the tissue stained for both BrdU and Ki67 most authors report a higher number of Ki67 positive cells as this marker is present at all stages of the cell cycle while BrdU only labels cells in S phase (Kee, Sivalingam et al. 2002).

In our lab, we have found that the number of proliferating cells adjacent to the dentate gyrus of the hippocampus could be affected by the chemotherapy 5-FU by using Ki67 as a marker for those cells (Mustafa, Walker et al. 2008). Following this work, the studies in this thesis have also used detection of the Ki67 protein to determine changes in precursor proliferation.

1.6.5 Regulation of Hippocampal Neurogenesis

In the normal rodent dentate gyrus, the cell cycle takes approximately 25 hours and proliferating neural precursor cells yield 9000 new neurons per day (Cameron and McKay 2001). This is not a constant number and can increase or decrease according to intrinsic and extrinsic factors. First of all, there is a natural variation in the degree of adult hippocampal neurogenesis across different rodent strains (Kempermann, Kuhn et al. 1997; Kempermann and Gage 2002). For example, in rats, a comparison between two strains showed a significant difference in adult hippocampal neurogenesis (Perfilieva, Risedal et al. 2001). Many other parameters such as total granule cell number and hippocampal weight show a similar variability (Wimer and Wimer 1989; Peirce, Chesler et al. 2003). It has been shown that where a large difference (25 fold) in the rate of neurogenesis exists between two strains of mice, this is mainly due to the influence of their genetic background (Kempermann and Gage 2002). It is very important also to note that the regulation of the balance between cell production and cell death also plays a major role in the net regulation of neurogenesis, reviewed extensively in (Kempermann 2006). The following sections review the endogenous and external factors which regulate proliferation and differentiation/survival in hippocampal neurogenesis.

1.6.5.1. Intrinsic Factors

Hippocampal neurogenesis is subject to changes by extrinsic and intrinsic factors (Lledo, Alonso et al. 2006). The intrinsic modulators of hippocampal neurogenesis affect both the proliferation and or differentiation of cells via changes in internal conditions such as hormones, growth factors or neurotransmitters (Lledo, Alonso et al. 2006). These factors are summarized in the following table.

Intrinsic Factors	Proliferation	Differentiation	Study
Hormones			
Oestrogen	Increase	No effect	(TanapatP 1999)
Corticosteroids	Decrease	Decrease	(CameronHA 1994; Rodriguez, Montaron et al. 1998)
Neurotransmitters			
Glutamate	Decrease	Decrease	(CameronHA 1995) (Nacher, Alonso-Llosa et al. 2003)
5-HT	Increase	Increase	(Banasr, Hery et al. 2004)
Noradrenaline	Increase	No effect	(Kulkarni, Jha et al. 2002)
Growth Factors			
BDNF	Increase	Increase	(Lee, Duan et al. 2002)
VEGF	Increase	Increase	(Jin, Zhu et al. 2002; Cao, Jiao et al. 2004)
EGF	No effect	Decrease	(Kuhn, Winkler et al. 1997)
HB-EGF	Increase	Not applicable	(Jin, Xie et al. 2003)
IGF-1	Increase	Increase	(Lichtenwalner, Forbes et al. 2001)
CNTF	Increase	Increase	(Emsley and Hagg 2003)

Table 1.3 Studies identifying hormone, neurotransmitter, and growth factor regulation of proliferation and differentiation during neurogenesis in the dentate gyrus of the adult rodent brain, modified from (Abrous, Koehl et al. 2005). Abbreviations; EGF, epidermal growth factor; HB-EGF, heparin-binding epidermal growth factor; IGF-1, insulin like growth factor 1; CNTF, ciliary neurotrophic factor.

1.6.5.2. Extrinsic Factors

Extrinsic factors which affect neurogenesis are agents outside of the animal or person which have been shown to alter cell proliferation and or differentiation during the formation of new neurons in the dentate gyrus. These include antidepressants, anti-psychotics, cannabinoids, opiates, alcohol and mood stabilizers (Abrous, Koehl et al. 2005). Chemotherapy, the subject of the present study, has also been shown to affect neurogenesis (Mustafa, Walker et al. 2008). The interaction between depression, hippocampal neurogenesis and the mode of action of antidepressants has been extensively studied (Duman, Nakagawa et al. 2001). These investigations have provided important results on the effects of antidepressants, particularly fluoxetine, on memory and hippocampal neurogenesis and as described later in this thesis, provide a means to overcome the effects of chemotherapy. The extrinsic factors affecting neurogenesis are summarized in the following table.

Factors/Conditions	Proliferation	Differentiation	Reference
Learning (Morris water maze)	Increase	Increase	(Gould, Beylin et al. 1999; Dobrossy, Drapeau et al. 2003)
Environmental enrichment	No effect	Increase	(Nilsson, Perfilieva et al. 1999)
Exercise	Increase	Increase	(Uda, Ishido et al. 2006)
Dietary restriction	No effect	Increase	(Lee, Duan et al. 2000)
Seizures	Increase	Increase	(ParentJM 1997)
Irradiation	Decrease	Decrease	(Monje, Mizumatsu et al. 2002)
Chronic antidepressants	Increase	Increase	(Mayberg, Brannan et al. 2000)
Chronic stress	Decrease	No effect	(Pham, Nacher et al. 2003; Heine, Zareno et al. 2005)
Ageing	Decrease	Decrease	(Heine, Maslam et al. 2004)
Cerebral ischemia	Increase	Increase	(LiuJ 1998)
Depression model (bulbectomy)	Decrease	Decrease	(Keilhoff, Becker et al. 2006)

Table 1.4 showing studies which have examined the effect of extrinsic factors and pathological conditions on proliferation and or differentiation in hippocampal neurogenesis in the adult rodent brain, modified from (Parent 2003).

1.6.6. Neurogenesis and memory

1.6.6.1. Types of memory (Fig 1.7)

The effect of chemotherapy on cognition appears to involve changes in memory. It is important to differentiate types of memory. In outline, memory is divided into three types, which are sensory memory, short-term memory and long-term memory.

(I) Sensory memory

The sensory memories represent memories related to sensory stimuli. Each sensation has its own type of memory for example, echoic, iconic and haptic memories for aural, visual and touch stimuli respectively. If of interest, information will be transferred from sensory memory into short term memory (Neath, Gordon et al. 2005).

(II) Short-term memory

Short-term memory also called working memory is the ability to recall needed information temporarily. Short term memory stays for a very short time (around 200 ms). It involves a collection of structures and processes in the frontal cortex. The information is processed and subsequently can be transferred to long term memory by transformation of memories into a stable form. If this fails to occur, the information will be lost. Recalling of information can lead to an increase in the short term memory capacity. If this process is interrupted, the retention of information in short –term memory will be disturbed. In order for this not to happen, the person tries to finish the act in the short term memory very rapidly. Working memory is essential for the intelligence of humans (Smith, Jonides et al. 1996).

(III) Long-term memory

Long-term memory has a larger capacity than short-term memory and is designed to store information for a longer time. In order not to forget the information in the working memory, it tends to travel to the long term memory very rapidly. There are two types of long-term memory which are the episodic and semantic memories. Episodic memories represent our daily events and the experience gained from one's life. Semantic memory, on the other hand, is the type of memory related to the person behaviour and skills. In order to work effectively, information stored as semantic memories should be in a direct contact with episodic memory, in another words; the person should learn the things or adapt his behaviour according to his previously gained experience. There are three functions of long term memory: storage, deletion and retrieval. Short-term memory is converted to long-term memory through rehearsal (**www.cc.gatech.edu/classes/cs6751_97_winter/Topics/human-cap/memory)**. This occurs by repeated exposure to the stimulus of interest. Furthermore, the time during which we gain this information is vital in this process. Deletion occurs by decay or interference of a memory. It is also worth mentioning that emotional factors are involved in remembering and forgetting things. Retrieval, on the other hand requires recall or recognition of information (Smith, Jonides et al. 1996; Neath, Gordon et al. 2005). These different types of memory involve different processing mechanisms by inducing changes in both physiological (synaptic processing) e.g. long term potentiation and depression in the hippocampus (LTP and LTD respectively) (Bliss and Collingridge 1993) and longer term changes in synaptic proteins (e.g. synaptogenesis) which is related to the spatial learning function of the hippocampus (Ramirez-Amaya, Balderas et al.

2001; Shors 2004). Furthermore, the types of memory involve different brain structures and regional interactions. The following section will discuss the function of hippocampal neurogenesis in memory processing

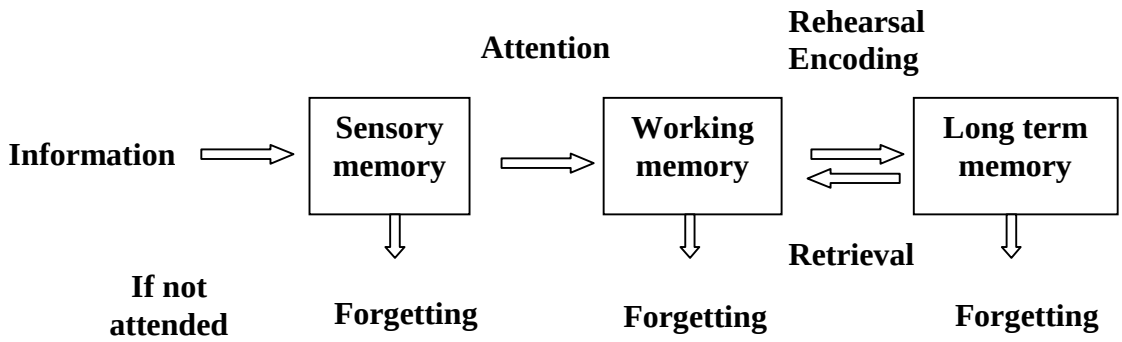


Figure 1.7 an outline of the types of human memory adapted from (www.cc.gatech.edu/classes/cs6751_97_winter/Topics/human-cap/memory). Incoming information is passed first to the sensory memory and is then stored in short -term (working memory) by attention. Short term memory is converted into long term memory by rehearsal. Information is then encoded in long -term memory. Retrieval of information from the long-term memory occurs through recall and recognition processes. If the process is interrupted at any stage, forgetting occurs (down arrows).

1.6.6.2. Role of Hippocampal neurogenesis in memory and learning

Procedural learning, a type of instinctive learning requires the acquisition of motor skills and does not depend on the hippocampus. However, one type of learning that requires the hippocampus is the learning of declarative memories (Kempermann 2006). Learning in the hippocampus is believed to use the process of long-term potentiation or depression by which conductance at particular synapses within the hippocampus is made either stronger or weaker (Duan 2002). This is believed to be responsible not only for the consolidation of memories by the hippocampus but also the recall of stored information (Kempermann 2006). Another type of learning which is believed to be largely a function of the hippocampus is spatial learning. In patients with Alzheimer's disease, the hippocampus is affected from the onset of the disease and spatial memory impairment and disorientation are early symptoms (Kempermann 2006). One of the most widely used tests of spatial memory, especially in rodents, is the Morris water maze (Morris 1984).

A number of studies have demonstrated that learning increases hippocampal neurogenesis. For example, in the study of Gould (Gould, Beylin et al. 1999), adult rats which were trained for 1 week to acquire the location of the platform in the Morris water maze or trained for one week in the conditioned eye blink response (a measurement of associative learning). The number of proliferating cells in the SGZ as demonstrated by BrdU labelling, increased significantly. In contrast the training of animals on a non-hippocampal dependent task did not alter cell proliferation indicating the effect of certain types of learning on hippocampal neurogenesis (Gould, Beylin et al. 1999).

While learning increases neurogenesis, blocking of neurogenesis impairs memory. For example, in their study, (Snyder, Hong et al. 2005), prevented neurogenesis by irradiation of the hippocampus which caused rats to become significantly impaired in acquiring long term spatial memory in the Morris water maze. Irradiation also impaired performance in the contextual conditioned fear response task which is hippocampal dependent (Winocur, Wojtowicz et al. 2006). Similarly administration of the proliferative cell toxin methylazoxymethanol acetate (MAM) impaired performance in two hippocampal dependent tasks, the object recognition memory test (Bruel-Jungerman, Laroche et al. 2005) and trace memories for a conditioned emotional response in a trace paradigm (Shors, Townsend et al. 2002). These animal models have demonstrated that hippocampal neurogenesis is playing an important role in mediating memory and learning. In humans, the hippocampus is believed to be involved in verbal, spatial and recognition memories (Reed and Squire 1997; Carrozzo, Koch et al. 2005; Grunwald and Kurthen 2006). It is very important to note that chemotherapy negatively affects verbal, visual and working memory in cancer patients (van Dam, Schagen et al. 1998; Ahles and Saykin 2002; Castellon, Ganz et al. 2004). Thus there is a strong relationship between hippocampal function and the types of memory which are affected by chemotherapy. This evidence directed the present investigation of the effect of chemotherapy on the types of memory mediated by the hippocampus and in turn the relationship between the cognitive impairments produced by chemotherapy and hippocampal neurogenesis.

1.7. Behavioural testing

In order to establish a suitable animal model of chemobrain, it was necessary to choose a proper test to assess hippocampal memory function. Animal behavioural testing has been extensively used in memory and neurogenesis studies as it provides a good parameter to detect intact hippocampal function. In humans, the hippocampus is clearly important in mediating certain types of memories. This was evidenced by (Scoville and Milner 2000) who reported severe declarative memory loss in an epileptic patient after bilateral hippocampal removal in order to alleviate his symptoms. From this, there is a strong hypothesis that the cognitive deficits seen after or during chemotherapy treatment are caused by targeting a specific anatomical locus in the brain which is the hippocampus. Moreover, the recognition that neurogenesis and continuous neuronal regeneration are occurring in the human hippocampus (Eriksson, Perfilieva et al. 1998), made it fundamental to test our animal models for their memory function after their treatment with chemotherapy and the possible correlation between this and the chemotherapy-induced reduction in hippocampal neurogenesis. For this reason we chose two different behavioural parameters to assess two different types of hippocampal memories. These were the object location recognition test (OLR) to test for animals' spatial working memory and the conditioned emotional response (CER) to test for the animals contextual fear conditioning. The following sections review these tests.

1.7.1. Object Location Recognition Test

Spatial working memory is a part of hippocampal function (Ennaceur and Meliani 1992; Dix and Aggleton 1999; Ennaceur, Michalikova et al. 2005). Human studies have revealed that hippocampal lesions reduce patient ability to spatially remember objects (Nunn, Graydon et al. 1999). Animal studies that tend to test spatial working memory have extensively used the Morris water maze and they have shown that this task is specific for assessing an intact hippocampal spatial working memory function. Moreover, it was found that hippocampal damage impaired rat performance in the water maze task (Morris, Garrud et al. 1982; Sutherland, Whishaw et al. 1982). However, this test has the disadvantage of a lengthy training protocol which may induce stress in rats (Ennaceur and Delacour 1988; Ennaceur and Meliani 1992). Another test which has been shown to accurately assess the spatial orientation memory of rats without primary reinforcement (e.g. electric shock) or long pertaining period is the object location recognition (OLR) task. For this reason, we chose the OLR as a test for spatial working memory of our rats. In this test, two identical objects are presented for rats to explore in the familiarization trial for three minutes. After 5 minutes inter- trial interval, one object is moved to a new location (choice or test trial) and rats are allowed to explore both objects for 3 minutes again (Ennaceur, Neave et al. 1997; Dix and Aggleton 1999). This test has been previously used in our lab to test rats' spatial working memory and showed that rats treated with 5-fluorouracil had a chemotherapy-induced memory impairment compared to controls (Mustafa, Walker et al. 2008). Rats usually tend to explore the new location of an object more that the old one (Mumby, Gaskin et al. 2002; Dere,

Huston et al. 2007) which highlights the ability of rats to remember the general architecture of an abject in a certain spatial location.

Rats' exploration of novelty is displayed usually by approaching, inspecting, sniffing and manipulating the object with their paws (Dere, Huston et al. 2007). It has been shown that hippocampal lesioning impairs performance in the OLR task (Mumby, Gaskin et al. 2002; Dere, Huston et al. 2007). Furthermore, (Lee, Hunsaker et al. 2005) reported that dentate gyrus lesions reduce rats exploration for new locations of objects. This adds further evidence to the theory that hippocampal neurogenesis (which is occurring mainly in the dentate gyrus) is linked to memory and that the chemotherapy induced memory impairments could be related to the reduction of hippocampal neurogenesis.

1.7.2. Conditioned Emotional Response Test

Conditioned emotional response is an emotional response that is acquired by conditioning. The mechanism by which this conditioning happens is the occurrence of fear which is a protective behavioural response for both human and animals against dangerous or unpleasant stimuli. A well known example of conditioned response is the "Pavlovian" example in which the dog salivates on hearing the bell ringing as food is always offered after the sound of the bell. Conditioning or extinction means the reduction in the response to an unpleasant stimulus with time (Barad 2005). Extinction requires a certain type of learning which is different from ordinary learning theories. In other words, it is a type of inhibitory learning (in which the response to a stimulus is reduced). On the other hand, excitatory learning occurs where there is an increased response to a stimulus (Barad 2005). As it is learning dependent, extinction requires functional long term potentiation in the brain (LTP). The molecular mechanism essential for this process to occur depends on the NMDA type of receptors (N-methyl-D-aspartate) which are activated by calcium influx (Falls, Miserendino et al. 1992; Lin, Yeh et al. 2003).

It is crucial to differentiate between contextual conditioning which is occurring due to pairing of a stimulus (e.g. foot shock) to the context (background stimuli present in the test chamber) and the cue-specific conditioning which occurs due to pairing of a tone to a foot shock (Fendt and Fanselow 1999; Fanselow 2000). There is increasing evidence that contextual conditioning is a part of hippocampal functions whereas the cue-specific conditioning requires both an intact amygdala and hippocampus (Phillips and LeDoux 1992; Fanselow 2000).

Moreover, it was reported that amygdaloidal lesions interfere with both contextual fear and the cue-specific conditioning whereas hippocampal lesions interfere only with contextual fear conditioning sparing the cue-specific conditioning (Kim and Fanselow 1992; Phillips and LeDoux 1992; Fanselow 2000). Fear associated tachycardia, analgesia, freezing, startle vocalization and increased levels of several hormones especially corticosteroids are detected in animals in fear conditioning states (Fendt and Fanselow 1999). Freezing which is defined as absence of all movement of the animal except that for respiration along with hunched posture and piloerection, has been used as an index to measure the conditioned fear of the animal during the CER test (Fendt and Fanselow 1999; Fanselow 2000).

The above review has evidenced that the CER test is recognized as a behavioural model for testing hippocampal dependent memory and learning. For this reason we tended to use the CER to assess another aspect of hippocampal function. The CER test which was performed in this thesis to assess an intact hippocampal function was adapted from (Resstel, Joca et al. 2006) in which animals were habituated in the first day followed by application of 10 foot shocks (0.4 Ma each). Moreover, there is evidence that X ray-induced disruption of hippocampal neurogenesis impaired animals contextual conditioning to fear (Saxe, Battaglia et al. 2006). Because our project aimed to investigate the effect of 5-FU chemotherapy on hippocampal memory and neurogenesis, it was necessary to assess the effect of the drug on animal performance in the CER test as well as its effect on hippocampal neurogenesis and the possible correlation between both in our developed animal model of chemotherapy.

1.8. Antidepressants

Antidepressants were first introduced at a similar time to antibiotics, antihypertensives, and a range of other drugs. The following section reviews the different types of antidepressant and their more recent relationship with neurogenesis.

1.8.1. Types and mechanisms of action of Antidepressants

1.8.1.1. Tricyclic antidepressants (TCA):

The tricyclic antidepressants are frequently used in the treatment of depression. They were designed and developed to block the reuptake pumps for both serotonin and noradrenaline (and to a lesser extent, dopamine). In addition, TCAs block the muscarinic cholinergic receptors, H1-histamine receptors, and alpha-1 adenoreceptors. Some TCAs also block 5-HT2 receptors, which may contribute to the therapeutic action of these agents. Antidepressants bind to an allosteric site close to the neurotransmitter uptake site on nerve terminal and block its synaptic reuptake thereby increasing neurotransmitter concentration in the synaptic cleft (Carlsson 1984; Range 1996; Gareri, Falconi et al. 2000; Range 2007).

1.8.1.2. Monoamine oxidase inhibitors (MAOIs)

Drugs classed as MAOIs were amongst the first clinically used antidepressants. However, the side effects of MAOIs and the discovery of other more efficient categories of antidepressants decrease their clinical applicability. The main action of MAOIs is the irreversible inhibition of the enzyme monoamine oxidase. MAO exists in two subtypes, A and B. The MAO- A form acts mainly on the neurotransmitter most closely linked to depression (serotonin and noradrenaline).

Moreover, it acts on the amine which controls blood pressure (noradrenaline). Thus, MAO- A inhibition is associated with antidepressant action and the hypertensive side effects of MAOIs (Carlsson 1984; Range 1996; Range 2007).

1.8.1.3. Selective serotonin reuptake inhibitors (SSRIs), the drug of choice

Selective serotonin reuptake inhibitors (SSRIs) are widely used for the treatment of moderate to severe depression. Their effectiveness has been assessed clinically and shows a similar response rate to tricyclic antidepressants. SSRIs are powerful antidepressants that are used to treat many psychological disorders such as anxiety, aggression, obsessive compulsive disorders and post traumatic stress disorder. These psychiatric conditions are all associated with serotonin dysfunction which reflects the primary role of serotonin in the regulation of these disorders (Wong, Bymaster et al. 1995; Mancini and Ameringen 1996). The major differences between various SSRIs are in their abilities to inhibit the reuptake of serotonin. The serotonin transporter is a molecular complex that has an enzyme binding site (energy producing Na+/ -K ATPase) and other binding sites (e.g. serotonin, sodium ion, SSRI) (Frazer 2001). When sodium binds to its location on the molecule, it increases the transporter affinity for serotonin, permitting serotonin binding with its transporter (Stahl 1999). In contrast, when an SSRI binds to its site on the molecule, this decreases the affinity for the serotonin transporter, resulting in an inhibition of serotonin binding to the transporter. Several drugs within the SSRI class have been introduced recently, including fluoxetine. Clinically, fluoxetine is currently the most prescribed antidepressant. This may be because it has a higher selectivity to 5-HT re- uptake compared to noradrenalin re- uptake and because it has fewer side effects

compared with TCAs. As with other antidepressants it usually requires 2-4 weeks of treatment before a therapeutic effect is produced (Range 1996; Range 2007). Fluoxetine has been shown to improve cognition in a variety of situations involving memory impairment (Cassano, Puca et al. 2002; Horsfield, Rosse et al. 2002; Blaney, Berg et al. 2004; Mowla, Mosavinasab et al. 2007).

The actions of fluoxetine on the hippocampus include an increase in neurogenesis, a phenomenon known to be associated with improved memory (Levkovitz, Caftori et al. 2002; Vermetten, Vythilingam et al. 2003; Chen, Pandey et al. 2006; Hitoshi, Maruta et al. 2007; Mowla, Mosavinasab et al. 2007; Marcussen, Flagstad et al. 2008; Monleon, Vinader-Caerols et al. 2008). One of the studies presented in this thesis, shows that co-administration of fluoxetine with 5-FU chemotherapy helped to prevent the cognitive deficits and decreased neurogenesis found after chemotherapy in a rat model (ElBeltagy, Mustafa et al. 2010).

1.8.1.4. Others

A newly discovered class of antidepressants known as, serotonin antagonists reuptake inhibitors (SARIs). They are powerful antagonists of serotonin receptors with a weaker blocking of 5-HT reuptake (Stahl 1998; Gareri, Falconi et al. 2000). Noradrenergic and specific serotonergic antidepressant (NASSA) is another category of antidepressants which act by antagonising alpha-2 adenoreceptors and increase of 5-HT receptors and cause postsynaptic blocking of 5-HT2 and 5-HT3 receptors. This mechanism provides an effective way to control depression with fewer side effects than those produced by SSRIs (Stimmel, Dopheide et al. 1997). However, Electroconvulsive therapy (ECT) is

the most reliable treatment in cases of psychotic and treatment-resistant depressions as it has the advantage of a faster action than drug therapy However, The major side effects of ECT are those related to cognition e.g., confusion and memory impairments (Range 1996; Range 2007).

1.8.2. Antidepressants and neurogenesis

It has been suggested that the formation of new memories is a function of the hippocampus (McClelland, McNaughton et al. 1995). Recent theories have suggested that the reduction in hippocampal neurogenesis produced by stress could lead to a disruption of adaptive behaviour and thus potentiate depression. Evidence for this has come from observations that stress- exposed animals have a much lower rate of neurogenesis in the hippocampus (Gould, Tanapat et al. 1998; Tanapat, Galea et al. 1998; Gould and Tanapat 1999). Cell proliferation in the SGZ is affected by both chronic and acute stress (GouldE 1997). In addition, stress has been reported to produce atrophy of the pyramidal cells of the dentate gyrus and CA3 as well as decreasing the formation of granule cells in the dentate gyrus which results in a decrease in hippocampal volume, a feature also observed in chronic depression (Sapolsky 1996; Sheline 1996; Duman, Malberg et al. 1999). These stress-induced changes as well as the use of antidepressants to treat depression are very important modulators of the course of depression. These findings have been shown in both preclinical studies where atrophy and death of stress-exposed neurons in the hippocampus has occurred (Sheline, Wang et al. 1996; Shah, Ebmeier et al. 1998; Bremner, Narayan et al. 2000) and also in post-mortem studies where atrophy and cell death occurred in the hippocampus and the prefrontal cortex (Kumar, Schweizer et al. 1997; Kumar, Jin et al. 1998;

Narayan, Bremner et al. 1999). As mentioned above, several studies have shown that antidepressants increase neurogenesis (Levkovitz, Caftori et al. 2002; Vermetten, Vythilingam et al. 2003; Chen, Pandey et al. 2006; Hitoshi, Maruta et al. 2007; Mowla, Mosavinasab et al. 2007; Marcussen, Flagstad et al. 2008; Monleon, Vinader-Caerols et al. 2008).

In their study, (Manev, Uz et al. 2001) showed that S100ß, a neurotrophic regulatory factor, was increased by the selective serotonin reuptake inhibitor, fluoxetine and its immunoreactivety was reduced with the reduction in serotonin synthesis. It is clear that treatment with antidepressants increases neurogenesis. Furthermore, it is hypothesized that one mechanism by which antidepressants act via up regulation of neurogenesis. In addition, the time required by antidepressant treatments to produce a behavioural effect (i.e. chronic not short-term course of treatment), may be due to the time required for the increased numbers of new neurons to become integrated into the dentate gyrus. It is also important to note that recovery from depressive behaviour extensively depends on different mechanisms of treatment (Duman, Heninger et al. 1997).

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1.9. HYPOTHESIS

1.9.1. Chemotherapy induces cognitive deficits

Our hypothesis is that 5-FU easily crosses the blood brain barrier due to its low molecular weight, producing cognitive impairment symptoms such as confusion, memory deteriorations and attention and concentration disruption. These symptoms which have been claimed by patients undergoing 5-FU chemotherapy treatment have been investigated in a rat model by testing their behavioural response in two hippocampal specific behavioural tasks, the object location recognition and the conditioned emotional response which showed memory impairments in 5-FU chemotherapy-treated rats.

1.9.2. Chemotherapy reduces neurogenesis in the hippocampus

According to the literature reviewed earlier in this chapter, our hypothesis is that cytotoxic drugs, particularly those with high blood-brain barrier permeability which includes 5-FU, are targeting the proliferation and survival of dividing cells in the SGZ of the dentate gyrus. This was tested by quantifying the numbers of proliferating cells in the SGZ in control and 5-FU treated animals. Dividing cells were identified by immunohistochemical staining for the endogenous proliferative marker Ki67 as described in (Mustafa, Walker et al. 2008), on one half of each brain obtained from animals after behavioural testing. Cell survival was tested (Chapter 5), by injecting BrdU immediately prior to drug administration and quantifying BrdU positive cell numbers, by immunohistochemistry, at different time points after drug treatment. Cell proliferation, at the time of death, was also quantified in these experiments, by

staining for Ki67, to determine if drug treatment has a prolonged effect on cell proliferation in the SGZ.

Contralateral half brains were processed for Western blotting to quantify DCX and BDNF proteins (Chapters 3 and 4). DCX is transiently expressed in new born neurons and levels in the hippocampus and frontal cortex give a measure of the rate of neurogenesis. Changes in BDNF protein levels in the hippocampus were also quantified to determine the effect of 5-FU treatment on neurotrophic factor levels. These are believed to be important in cell survival and maturation during neurogenesis. Measures of Ki67, BrdU, DCX and BDNF aimed to determine the proliferation, differentiation and survival aspects of neurogenesis in the present animal model of chemobrain.

1.9.3. A reduction in hippocampal neurogenesis will correlate with and may be the cause of the cognitive impairment found after chemotherapy treatment.

Our hypothesis is that the disruption of hippocampal neurogenesis, as a result of chemotherapy treatment, will cause deficits in memory processes mediated by it. In the animal model used in this thesis, rats were tested using two behavioural tasks that require hippocampal-mediated memory. These were the object location recognition (OLR) and the conditioned emotional response (CER) tests. The OLR test depends on the inherent ability of rats to explore the new location of the object in the presence of familiar stimuli (Mustafa, Walker et al. 2008). The CER test is testing the contextual fear conditioning of the rats which was shown to be a hippocampal specific task (Resstel, Joca et al. 2006).The advantages of these tests is that they do not require long training and learning protocols as in Morris water maze. Adult male rats were used and behavioural testing was carried out

immediately after the period of animal treatments with the cytotoxic drug 5-FU. The details of these tests are described in (Chapter 2, Materials and Methods).

1.9.4. Treatment with antidepressants will prevent the deficits in both neurogenesis and cognition found after chemotherapy.

In addition to determining the cognitive and cellular changes brought about by chemotherapy, one study in this thesis also examined the effect of chronic treatment with the antidepressant fluoxetine co administered with 5-FU (Chapter 4). This was done to test the ability of chronic treatment with antidepressants to improve the disrupted picture of neurogenesis and memory induced by chemotherapy.

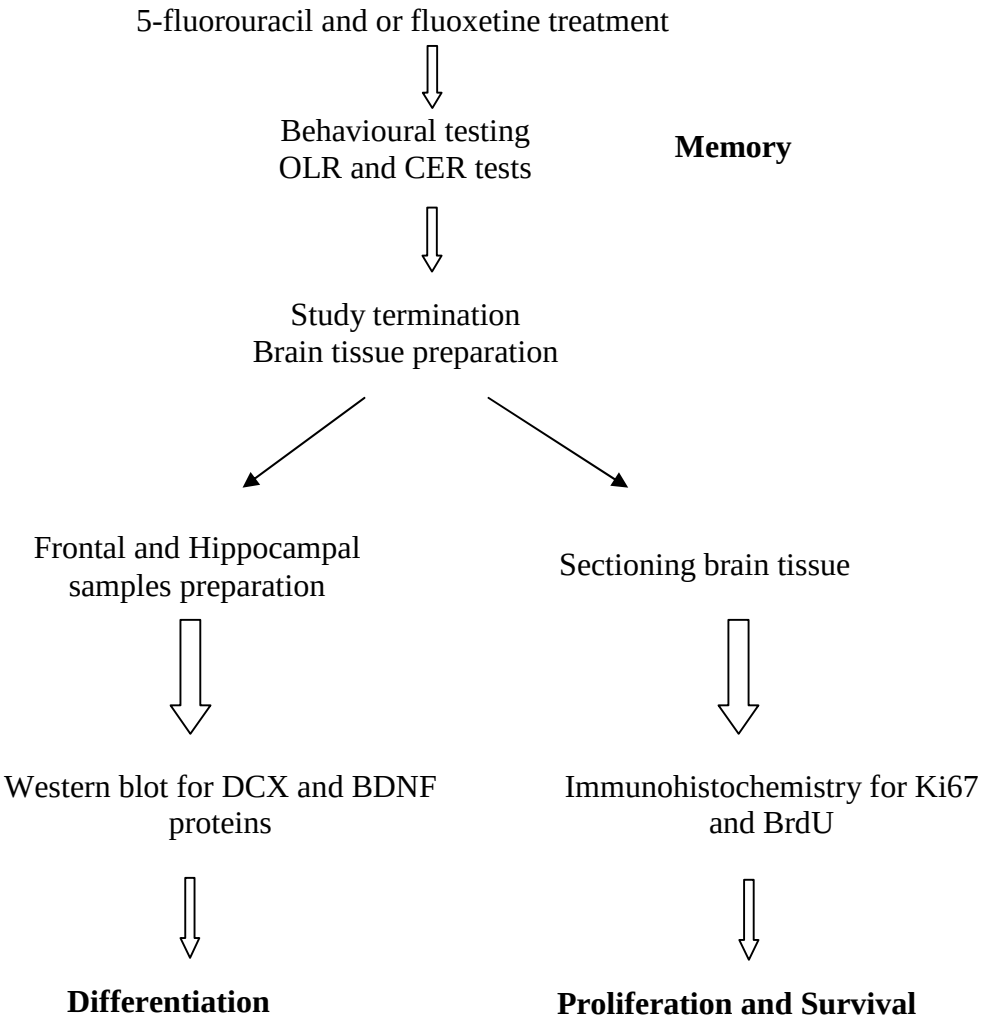


Figure 1.8. Outline of the methods used to test the effect of 5-fluorouracil and or fluoxetine treatment on hippocampal specific memory and neurogenesis in a rat model in this thesis. Hippocampal recognition memory was tested using the (OLR) test. The contextual conditioning of rats was measured by carrying out the (CER) test. At the end of the study, one cerebral hemisphere of brain tissue was processed for Western immunoblotting to quantify brain derived neurotrophic factor (BDNF) and doublecortin (DCX) protein levels in the hippocampus and frontal cortex. The other cerebral hemisphere was processed for immunohistochemical staining for the proliferative markers (Ki67 and BrdU).

1.10 AIMS AND OBJECTIVES

The objectives of these studies were as follows:

1- To study the behavioural effects of 5-FU chemotherapy in two behavioural paradigms (OLR and CER) both of which require hippocampal memory.

2- To study the effect of 5-FU chemotherapy on hippocampal neurogenesis by measuring cellular proliferation and differentiation in the dentate gyrus of the hippocampus. At the same time to quantify the effect of 5-FU on the neurotrophic factor BDNF and the neuronal differentiation marker doublecortin (DCX).

3- To study the long term effects of 5-FU treatment on the survival of new born cells in the dentate gyrus and the rate of cell proliferation at different time points after the end of drug treatment.

4 - To study the effect of the anti-depressant fluoxetine, on both memory and neurogenesis when given during 5-FU treatment.

CHAPTER 2

Preliminary Studies to evaluate:

1- 5-Flurouracil toxicity (pilot study)

2- The parameters for behavioural testing

2.1.1. INTRODUCTION

As 5-FU is a toxic drug, it was necessary to establish a suitable dosage and regime of administration which produced limited morbidity and after which the animals would be capable of performing the proposed behavioural tests. 5-FU is known to cause weight loss and, if this is too pronounced acutely or caused a cumulative weight reduction of more than 20%, Home Office licensing regulations would require termination of the experiment. From the literature a wide range of doses from 20 – 150 mg/kg have been administered to rodents with a variety of acute and chronic dosing regimens (Migone et al. 2006; Lee et al 2006; Mustafa et al. 2008; Foley et al 2008; Han et al. 2008). Studies using a chronic dosing regimen to reduce tumour load have commonly used 25-30mg/kg which is believed to be within the human dose range used in treatment of breast and other cancers (Au et al 1983; Watson et al 1998; Reagan et al. 2008). However previous studies in this laboratory, using 25mg/kg in a series of injections over two weeks, had caused some morbidity, with one animal out of ten having to be put down (Mustafa et al. 2008).

As the dose of 25mg/kg has been used previously in chronic studies it was decided to try to use this dose again in combination with the same concentration of leucovorin while paying particular attention to daily body weight changes.

At the end of chemotherapy administration it is important that animals are capable of performing the proposed behavioural tests. General activity behaviour can be assessed by videotaping locomotor activity and using the Etho Vision tracking software (Noldus Information Technology UK) to measure movement. Comparison of activity of control and treated animals enables the measurement of any drug effect on activity which might alter normal exploratory behaviour.

A wide variety of objects and box sizes have been used in different studies of object recognition and object location behavioural studies. Discussions with investigators familiar with these types of test indicated that these parameters could have a significant effect on the ability of animals to perform the tests (K. Fone personal communication). It was decided to try 2 different objects in the novel object location test (OLR) to find the most appropriate ones to use.

The CER behavioural test requires animals to remember the context of an unpleasant stimulus (electrical shock) over 24 hours. Memory is assessed by measuring the freezing behaviour when the animal is returned to the original context (CER box). It is important to establish that untreated animals, which have not experienced unpleasant stimuli, do not freeze on being returned to the CER box and that animals which have been shocked are able to remember the experience after 24 hours and show a behavioural change.

The studies reported in this section establish a suitable drug dosage which should enable animals to perform the behavioural tests and also carry out the controls necessary to establish the validity of the CER test.

Experiment1: (5-FU toxicity): Effect of 5-Fluorouracil on Hippocampal Recognition Memory, and Fear Conditioning of Rats

2.1.2. MATERIALS AND METHODS

2.1.2.1. Animals

All animals in this and future studies were Lister Hooded male rats obtained from Charles River Laboratories. Animals were delivered at between 2-3 months and 270-300gms, housed in the Bio-Medical school Service Unit (BMSU) and allowed to acclimatize for 5 days prior to any procedures. All procedures were conducted in accordance with Home Office guidelines and local ethical approval and carried out under a Home Office personal licence (40/8761) and project licence (40/3283). For this experiment animals were randomly divided into control (saline-injected, n=5) and treated (5-FU-injected, n=5) groups and housed 5 per cage under standard conditions (12hr light / dark cycle and free access to water and food).

2.1.2.2. 5-FU Chemotherapy Treatment

All rats received 5 intra-tail vein (i.v.) bolus injections, under general anaesthesia (hypnorm), over the course of 10 days (every other day). Animals were warmed in a heated box and the tails thoroughly cleaned with surgical scrub prior to injection. Treated animals received 5-FU (25mg/kg) and leucovorin (25mg/kg) both from (Mayne Pharma, UK) in the same injection (Au, Walker et al. 1983). The control group received 0.9% sterile saline injections in the same volume as the treated group. Weights were monitored daily during treatment.

2.1.2.3. Behavioural Testing

Behavioural testing to all animals was carried out before and after drug injection. The locomotor test was done during the habituation for the OLR test. Animals were videoed during one hour of habituation and their distance and velocity analysed using the Ethovision software.

Rats were tested for their ability to perform the two behavioural tests (OLR and CER) both before and after treatment, to make sure that rats were able to perform these tasks and to determine the effect of drug treatment.

Day 1-5	**Day 6 – 10**	**Day 11 -20**	**Day 21 - 25**	**Day 26**
Weighing and habituation	OLR then CER behavioural tests	5-FU+LCV or saline intravenous injections	OLR then CER behavioural tests	Killing of animals

Table 2.1.1. A protocol table of the study. Rats were habituated for 5 days in BMSU. Over 10 days, rats were injected by sterile saline or 5-FU+LCV every other day (5 injections). Rats were tested for their performance in the OLR and CER tests before and after treatment respectively. Rats were killed on the following day of the CER test.

2.1.2.3.1. The Object Location Recognition Test

The OLR test was modified from the method of Dix and Aggleton, 1999. Equipment consisted of two large opaque plastic boxes (66cm length, 41cm width and 40 cm in height) set up in a well aired room at a temperature of 22^0C with maximal lighting. These boxes (Arenas) were labelled as Arena1 and Arena 2 and were kept with the same label until the end of this test. Both boxes were in the same room but an opaque divider prevented animals seeing the other arena. Animal activity was remotely monitored by a ceiling mounted video camera which relayed images to an adjacent room. This test was done over two days; on the 1st day animals were put into an arena for one hour to habituate before returning to their home cages. Arenas were cleaned and swabbed with Alcosan, water and 20% ethanol between all trials.

The second day of the test involved the familiarization trial (sample trial), the inter trial-interval (ITI) and the choice trial. For the sample trial, two identical objects (either weighted striped plastic bottles or toy penguins) were placed in each arena at specific locations. The rats were introduced into their arenas and left for 3 minutes. Animal behaviour was displayed and recorded in the adjacent room.

After 3 minutes, the rats were removed from the arenas and returned back into their home cages for the inter-trial interval time (ITI) which lasted for 5 minutes. For the choice trial, the objects were placed back into the arenas, but, one object in was placed in a different location. Rats were left to explore the objects for another 3 minutes (Fig 2.1). Exploration of the object was defined as when the animal sniffed, licked, chewed or directed its nose at a distance $\leq$ 1cm from the

object (King, Sleight et al. 2004). Exploration time was obtained from the recorded video tapes.

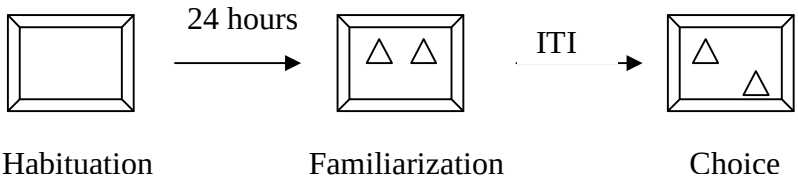


Figure 2.1.2 Schematic representation of the object location recognition (OLR) task. Animals habituate for 1 hr on day one and on day two they complete the familiarisation, inter-trial interval and choice trials.

2.1.2.3.2. The Conditioned Emotional Response Test

The CER test was carried out in a protocol modified from Resstel et.al, 2006. On the first day (the shock day), each animal was put individually into the CER box, which is a metal box (25 cm height, 24 cm width and 25 cm length) with a metal grid floor connected to a shock generator to give a 0.4ma electric shock. Shocks were monitored using an oscilloscope. 10 shocks, one every minute lasting for one second, were given to the rat in over a period of ten minutes (total 10 shocks). The box was cleaned between trials with 20% alcohol to avoid olfactory cues.

On the following day, each animal was returned to the same box in the same conditions for ten minutes without shocking. The duration of freezing behaviour as defined as the absence of all movement with the exception of respiration, was measured from the videos using a stop watch.

Figure 2.1.3. A picture modified from pnf.ruhosting.nl/Skinner.htm **showing the conditioned emotional response apparatus (the modified Skinner box) used in the CER test.**

2.1.2.4. Statistical analysis

All statistical parameters were calculated using GraphPad Prism 4.0 software. Weight data were analysed using repeated measures two-way ANOVA followed by the Bonferroni post-test. Locomotor activity parameters (distance and velocity) were analysed by using one way ANOVA. For behavioural testing before and after treatment, the Student's t-test was used. Within treatment group comparisons (e.g. comparison of drug-treated group exploration of object RT versus object LT) were analysed using a paired t-test expressed as Mean±S.E.M. Between groups comparisons (e.g. drug-treated group versus vehicle-treated group) were analysed using the unpaired version of the t-test. For analysis of results from the CER test an unpaired t- test was used to compare between controls and treated animals.

For all tests, a significance level less than 0.05 was used ($P<0.05$).

2.1.3. RESULTS

2.1.3.1. Weight changes during treatment.

The body weight of rats was monitored daily throughout the experiment. Rats receiving 5-FU chemotherapy exhibited a significant decrease in the amount of weight gained during the treatment period compared to their controls (P**=0.005) and appeared to transiently lose weight on most days following injections (Fig.2.1.4).

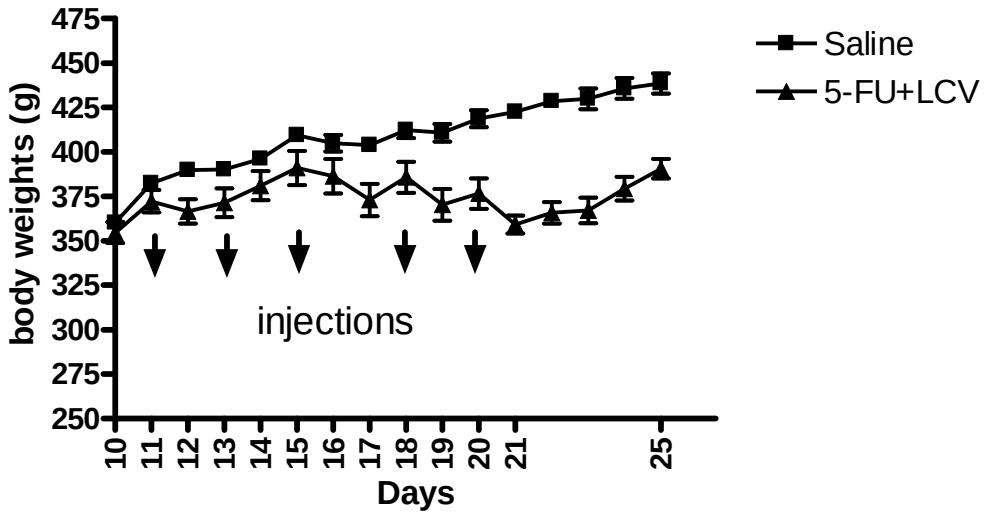


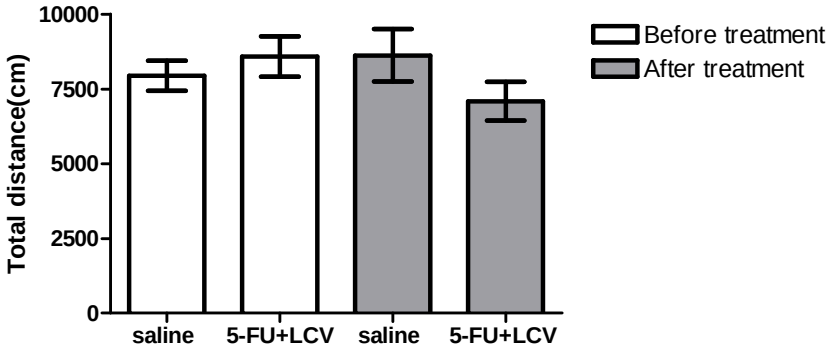
Figure 2.1.4. Body weights (Mean±S.E.M) of saline and 5-FU+LCV treated groups (n=5 each) recorded daily during the study. Injections were administered as indicated on the graph by arrows.

5-FU+LCV injections significantly decreased weight gain compared to saline (P=0.005) over the time period (P***<0.0001). The interaction between time and treatment factors was also significant (p***<0.0001); repeated measures two-way ANOVA with Bonferroni post-hoc test).**

2.1.3.2. Locomotor activity (distance and velocity) measurement.

Locomotor activity was measured in saline and drug treated groups during the habituation period prior to the OLR test both before and after treatment. There was no significant difference between groups in the mean velocity (cm/sec) or in the total distance (cm) moved by the animals (P=0.3) (one-way ANOVA), Fig 2.1.5. A and B. This indicates that drug treatment (5-FU+LCV), did not alter these aspects of locomotion and that any differences in the behavioural tests were not due to the treated animals being unable to perform these tests.

A



B

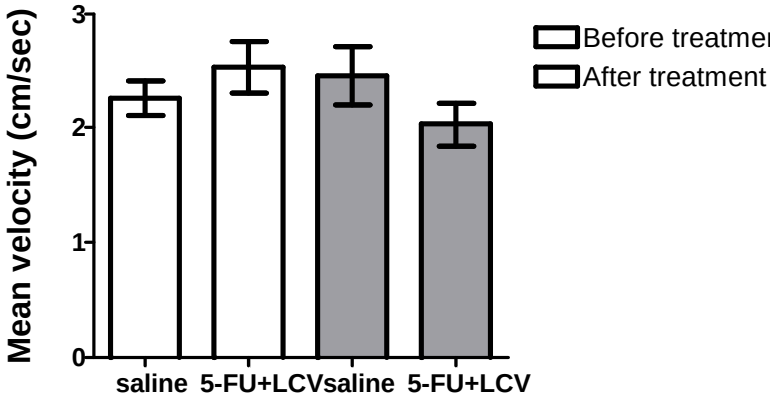
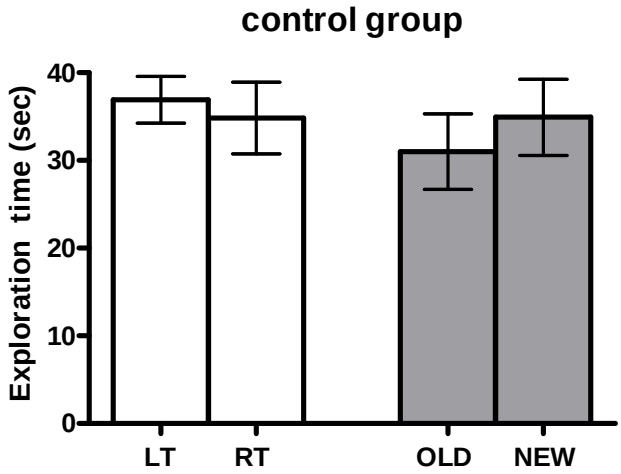


Figure 2.1.5. (A) The total distance and (B), the mean velocity of animals (Mean±S.E.M) in each group (n=5) during the habituation period (1 hour) of the OLR test before and after treatment. There was no significant difference between any groups in both parameters (P=0.3; One-way ANOVA).

2.1.3.3. Object Location Recognition before Treatment

The OLR test was performed for all animals before treatment to test whether all animals were able to perform the task before starting the treatment. In this experiment striped water bottles and penguin toys were used. As shown in Fig 2.1.6. Both controls and the group to be treated with 5-FU+LCV explored both objects in the sample trial equally ($P>0.05$). After changing the one location of one object in the choice trial, neither controls nor the group to be treated with 5-FU+LCV exhibited any significant difference in exploratory time between objects in the novel or familiar location ($P=0.6$ and 0.2 respectively).

A



B

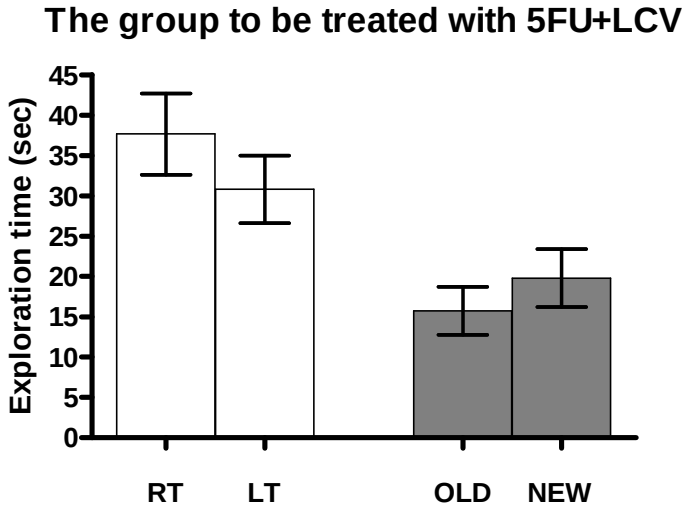


Fig 2.1.6.A and B Exploration time (Mean±S.E.M) of rats performing OLR task before treatment in the two groups (n=5 each). In the sample trial (white shaded), objects were placed in either RT (right) or LT (left) locations. In the choice trial (dark shaded). Replicas of the same object used in the sample trial were placed in either locations (OLD or NEW) (designed the old location (OLD) or a novel location (NEW). There was no significant difference in the exploration time spent by animals in either sample or choice trials (P<0.05; paired Student's t-test).

2.1.3.4. The CER results before treatment

The (CER) test was performed for all animals before treatment to ensure that all animals were able to perform the task before starting the treatment. As shown in Fig 2.1.7. There was no statistically significant difference in the amount of freezing between control and the group to be treated with 5-FU+LCV (P=0.7).

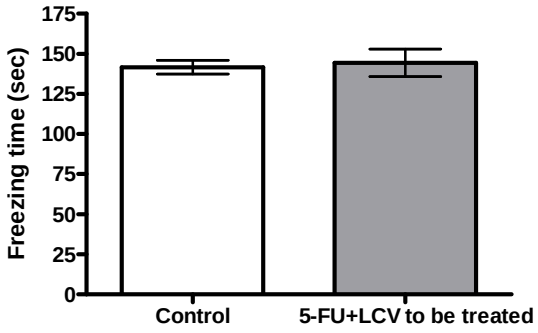
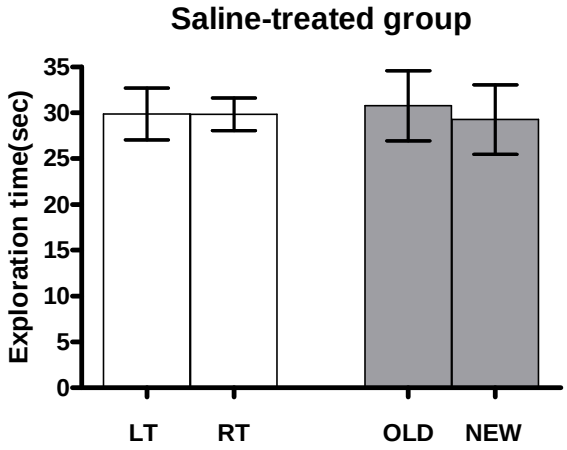


Fig 2.1.7. The freezing times (Mean±S.E.M) exhibited by the rats in each group before treatment (n=5). There was no statistically significant difference in the measured freezing time between the control and the group will be treated with 5-FU+LCV (P=0.7; paired Student's t-test).

2.1.3.5. Object Location Recognition after drug treatment.

Both saline and 5-FU-treated rats displayed similar amounts of exploration time for both objects and locations in the sample trial indicating that rats did not have a preference for either of the objects or their locations in the box. In the choice trial, after the 5 minute inter-trial interval, there was no difference between the exploration time of the familiar (Old) and novel (New) locations in both saline and 5-FU+LCV treated groups (P=0.5 and 0.7 respectively) as shown by a paired Student's t-test Fig 2.1.8 [A and B]). Indicating that even the saline treated animals were not able to differentiate between familiar and novel locations in this task.

A



B

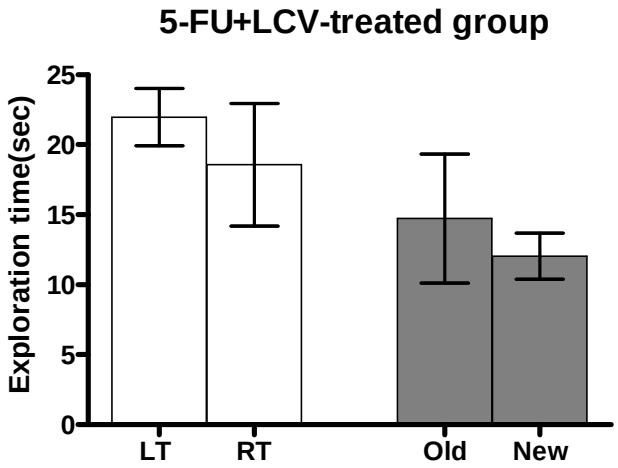


Fig 2.1.8. A and B, Exploration time (Mean±S.E.M) of rats in OLR task after treatment (n=5 in each group). In the sample trial (white shaded), objects were placed in either RT (right) or LT (left) locations. In the choice trial (dark shaded). Replicas of the same object used in the sample trial were placed in either locations (OLD or NEW) designed the old location (OLD) or a novel location (NEW). There was no significant difference in the exploration time spent by all animals in either sample or choice trials ($p<0.05$; paired Student's t-test).

2.1.3.6. The CER test results after treatment

For the CER test, freezing behaviour was recorded 24 hours after conditioning when animals were returned to the test box. Vehicle-treated animals spent an average of 132 seconds in this behaviour. In contrast 5-FU-treated animals spent an average of only 94 seconds exhibiting this type of behaviour, which was not significantly less than controls (P=0.07; unpaired Student's t-test; Fig 2.1.9).

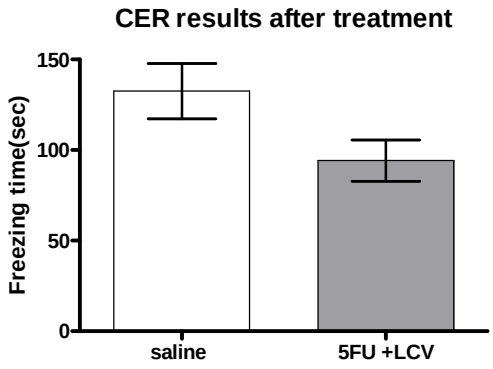


Fig 2.1.9. Freezing time (Mean±S.E.M) exhibited by the rats in each group after treatment (n=5). There 5-FU+LCV measured freezing time, although less, but was not significantly different from the measured freezing time in the saline treated rats (p=0.07; unpaired Student's t-test).

Experiment 2: Optimization of the parameters for behavioural testing

2.2.1 INTRODUCTION

As shown in the first experiment, control rats were not able to perform the object location recognition task. In the conditioned emotional response test, there was a tendency towards a decrease in the measured freezing time of the 5-FU+LCV treated animals compared to the saline- treated animals. These results could be attributed to the small sample number (5 animals per group) which made it difficult to reach a statistically different conclusion. Another factor which could affect the accuracy of the result obtained from the object location recognition task was the unintended variation of the objects used during both sample and choice trials (a toy penguin and a stripped water bottle). It has been noticed from the replayed videotapes which recorded the behaviour of the animals during the object location recognition task, that the rats were so much interested in exploring the toy penguins much more than exploring the stripped water bottles. For these reasons, it was necessary to design a new experiment (without treatment) with bigger sample number which avoids these confounding variables to test whether Lister Hooded rats are able to perform the OLR and the CER behavioural tasks or not before establishing these two tests in future studies. To optimize the object location recognition test, decorated water bottles were used either in the familiarization or the choice trials and the test was run as usual. For the conditioned emotional response test, half of the rats was shocked and the other were not and their freezing behaviour was recorded.

2.2.2 MATERIALS AND METHODS

2.2.2.1. Animals and Housing

For this project 20 adult Lister Hooded rats were obtained from BMSU, University of Nottingham. Rats weighed approximately between 220-255g when they arrived. Animals spent one week acclimatizing the new environment in BMSU .They were housed in four cages 5 animals each and weighted daily. This assignment was done randomly. The rats were housed in appropriate conditions according to BMSU standard procedures. Their diets consisted of water and dry rat chow which were readily available at all times for both cages.

2.2.2.2. Behavioural Testing (modified object location recognition and conditioned emotional response tests):

Object location recognition testing was done for all animals as described in (Experiment 1, Chapter 2) with the previously described considerations (using the decorated water bottle in all test trials). Animals then were divided randomly into two groups (10 rats each) for the CER test. The first 10 rats were put individually in the CER box for 10 minutes without interference and the freezing behaviour was recorded for each animal separately. The second ten rats were tested for their conditioned emotional response as described in (Experiment 1, Chapter 2).
On the last day of the CER test, animals were killed. Their termination involved stunning, followed by dislocation of the cervical spine in the neck using a blunt instrument. Animal bodies were disposed of in the -80ºc freezer of the BMSU.

Day 1 to day 5	Days 8 and 9	Days 10 and 11	Day 12
Habituation in BMSU	Object location recognition test for all animals	Conditioned emotional response test for half of the rats	Putting down of all animals

Table 2.2.1. A protocol table of the study is presented above. On arrival, rats were left to habituate the new condition of the BMSU for one week then on days 8 and 9 rats were tested for their performance in the OLR and on days 10 and 11, half of the rats were tested for their response in the CER tests. At the end of the third week all rats were killed.

2.2.2.3. Statistical Analysis

All statistical parameters were calculated using GraphPad Prism 4.0 software. . For the OLR behavioural, paired Student's t-test was used expressed as Mean±S.E.M. For CER analysis, Student's unpaired t- test was used to compare between shocked and non-shocked animals freezing time.

For all tests, a significance level less than 0.05 was chosen ($P<0.05$).

2.2.3. RESULTS

2.2.3.1. Object Location Recognition

Animals displayed similar amounts of exploration time for both objects in the sample trial (exploring objects in RT and LT) locations indicating that rats did not have a preference for either of the objects or their locations in the box. After the 5 minute inter-trial interval in the choice trial, animals spent significantly more time exploring the object in the novel location compared to that in the familiar location (P***<0.0001; Fig 2.2.2).

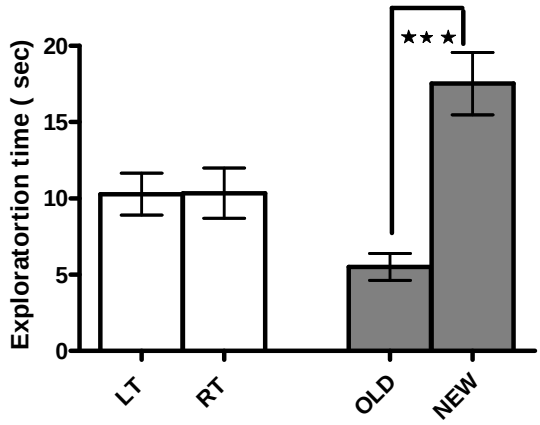


Figure 2.2.2. Exploration time (Mean±S.E.M) of rats performing the object location recognition (OLR) task. In the sample trials (white shaded), (LT and RT) locations, all rats (n=20) spent the same amount of time exploring replicas of an object in both locations (RT and LT)) (p=0.9). In the choice trials (dark shaded), replicas of the same object used in the sample trial were placed in either locations (OLD or NEW). All rats explored the NEW location significantly more than the OLD location (p*<0.0001). All comparisons were calculated using paired Student's t-test.**

2.2.3.2. Conditioned Emotional Response Test

For the shocked animals, freezing behaviour was recorded 24 hours after conditioning when animals were returned to the test box (during 10 minutes). The freezing behaviour of the non-shocked animals was recorded during the 10 minutes of their testing. Shocked animals spent an average of 154 seconds in this behaviour. In contrast the non-shocked animals spent an average of only 10 seconds exhibiting this type of behaviour, which was significantly much less than in shocked animals (P***=0.0001; unpaired Student's t-test; Fig 2.2.3). This confirms that this is a good test for memory and those animals were able to perform this task.

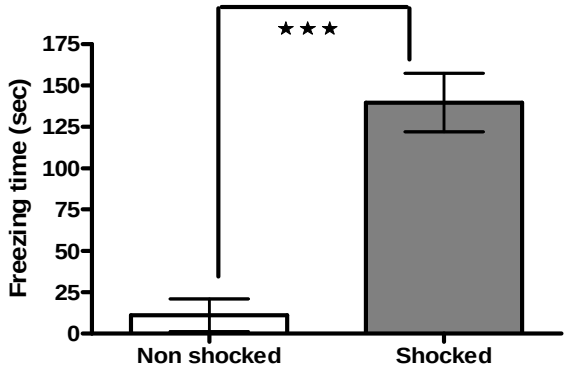


Figure 2.2.3. The freezing times (Mean±S.E.M) exhibited by the rats in each group (n=10). The shocked animals freezing time was significantly greater than the non-shocked animals freezing time (P*=0.0001). The analysis was done by using unpaired Student's t-test.**

2.2.4. DISCUSSION

The aim of this pilot study was to establish suitable parameter for an animal model to study effects of the anticancer drug 5-FU on the behaviour of Lister Hooded rats. In particular it was necessary to ascertain that the drug dose (25mg/kg) was suitable and that two behavioural tests chosen could be used to test cognition under these conditions.

2.2.4.1. 5-FU significantly reduced weight gain

Previous studies have shown that 5-FU exerts cytotoxic effects on the proliferating cells of the intestinal wall reducing nutrient absorption (Houghton, Houghton et al. 1979; Huang, Kemp et al. 2002). In the present experiments, 5-FU+LCV treated rats (n=5) showed a significant reduction in their body weights compared to the saline treated animals (Fig 2.1.4). The oscillating pattern in the weight of 5-FU treated animals indicates the possible transient effect on feeding from which the animals recovered on the following day. Although weight gain was transiently affected by the injection protocol, overall morbidity was low and the dose used appeared to be suitable for use in further studies. This conclusion was reinforced by the locomotor data (distance and velocity) which showed that drug- treated animals showed no difference from saline treated animals (Fig 2.1.5. [A&B]).

2.2.4.2. OLR test.

In Experiment 1, we found that none of the groups of animals could discriminate between objects in novel and familiar locations in the choice trial, either before or after drug treatment (Fig 2.1.6 [A&B] and 2.1.8 [A&B] respectively). A

possible cause was the low number of animals per group (n=5) compared to other studies that have used a minimum of 6 animals per group. The other factor which might explain these results was the type of object used during these trials as it was noticed that animals seemed more interested in exploring the toy penguins rather than the water bottles.

On the basis of this, objects were replaced by decorated water bottles which when combined with an increased sample size, showed that the Lister Hooded rats (Experiment, 2) were capable of performing this test and showed significantly increased preference for the object in the novel location.

2.2.4.3. CER test.

In Experiment1, when drug treated animals were compared with a saline-treated group, a non -significant difference in freezing time was found between the two groups (Fig 2.1.9). The difference in freezing behaviour was seen particularly in the first three minutes of the test but declined after this (data not shown). Although this result was not significant, it is likely that a larger sample size (10 rather than 5) wouldshow a significant result and this was confirmed in subsequent experiments. In Experiment 2, CER test where unshocked animals were compared with those who received shocks during their first period in the CER box, the shocked animals showed significantly more freezing time than the non- shocked group (Fig2.2.3) Indicating that memory of the context in which the shocks were experienced is maintained for 24 hours and showing that this is an appropriate test of contextual memory (Frankland, Josselyn et al. 2004).

2.2.5. CONCLUSION

These preliminary experiments have demonstrated that the dose of 5-FU+LCV of 25mg/kg is suitable for future studies and does not cause excessive morbidity. This was confirmed by the locomotor data which showed that the activity of drug-treated animals was not different to controls and that they should be able to perform the behavioural tests after treatment. It was found that n=5 was too small a sample size and that appropriate objects must be used for these animals to be able to perform the OLR test. The CER test appears to be a useful test of memory over 24 hours. It clearly differentiates between animals experiencing an adverse stimulus in a particular context from those that have not experienced this stimulus, but have been in the same context. Although not reaching significance in these experiments, larger sample sizes should be able show differences between drug and saline- treated animals. Further experiments detailed in the rest of this thesis make use of these preliminary results which give greater confidence in the results obtained.

CHAPTER 3

Effect of 5-FU on memory and neurogenesis in the adult male rat hippocampus

3.1 INTRODUCTION

In the preliminary study (Chapter 2); adult L.H male rats were given 5 tail vein injections of either saline or 5-FU at a dose of 25 mg/kg in combination with leucovorin (25 mg/kg) in the same syringe. This did not cause significant morbidity and these preliminary findings form the basis for the present study in which a larger number of rats were treated with the same 5-FU+LCV protocol. This study looks at the effect of 5-FU chemotherapy on three aspects of neurobiology. First, the effect of drug treatment on cognitive behaviour as measured in the OLR and CER tests; second on the number of proliferating cells in SGZ of the dentate gyrus as a measure of this aspect of hippocampal neurogenesis and third on the levels of doublecortin (DCX), a marker of newly formed neurons and brain derived neurotrophic factor (BDNF) were measured by Western blotting, in the hippocampus and frontal cortex. DCX is a microtubule-associated protein which is transiently expressed in developing neurons in which it is required for their migration (Friocourt, Koulakoff et al. 2003; Couillard-Despres, Winner et al. 2005; Plumpe, Ehninger et al. 2006). In the adult brain this protein is only expressed in neurogenic areas such as the dentate gyrus of the hippocampus and the rostral migratory stream within the frontal lobe (Nacher, Crespo et al. 2001; Kempermann 2006; Gage, Kempermann et al. 2008). DCX is expressed by dentate gyrus granule cell precursors for between three days and several weeks during their final rounds of cell division and as they migrate into

the dentate gyrus and start making connections (Brown, Couillard-Despres et al. 2003; Plumpe, Ehninger et al. 2006).

Due to its restricted expression pattern, the quantification of DCX - positive cells has been suggested as a measure of adult hippocampal neurogenesis (Couillard-Despres, Winner et al. 2005). In the present study, DCX protein was quantified by Western blotting to see if 5-FU chemotherapy affected the levels of this protein. Protein levels were measured in both hippocampus and frontal cortex to see if the effects of chemotherapy were region specific.

BDNF is a key component in both the formation of memories and hippocampal neurogenesis. This secreted neurotrophic factor is produced by both neurons and endothelial cells and produces its effects by binding to the TrkB receptor (tyrosine receptor kinase B) on target cells (Leventhal, Rafii et al. 1999). During the process of learning, BDNF expression is up-regulated and plays a key role in both early and late phase long-term potentiation (LTP) (Lu, Christian et al. 2008; Waterhouse and Xu 2009).

BDNF levels in the hippocampus increase with antidepressant treatment and exercise, both of which cause an increase in hippocampal neurogenesis (Sairanen, Lucas et al. 2005). Conversely BDNF levels and neurogenesis decrease with lack of exercise and stress (Yasuhara, Hara et al. 2007). The exact role of BDNF in hippocampal neurogenesis is still not clear but both mature dentate gyrus granule cells and neural progenitors in the SGZ express both BDNF and the TrkB receptor (Li, Luikart et al. 2008). Expression of TrkB, however, increases as the neural progenitors in the SGZ mature (Donovan, Yamaguchi et al. 2008). If the BDNF / TrkB signalling pathway is blocked, both progenitor cell proliferation (Lee, Duan et al. 2002; Li, Luikart et al. 2008) and the survival of newly born

granule cells is reduced (Lee, Duan et al. 2002; Sairanen, Lucas et al. 2005) while infusion of BDNF into the hippocampus increases neurogenesis (Scharfman, Goodman et al. 2005). Interestingly, genetic ablation of either TrkB or BDNF reduces the neurogenic and behavioural response to antidepressants (Sairanen, Lucas et al. 2005; Li, Luikart et al. 2008).

The first step of neurogenesis is the division of neural stem and progenitor cells. In this chapter, this was quantified by counting the number of Ki-67 positive cells in the SGZ of the dentate gyrus (Kee, Sivalingam et al. 2002).

By combining investigations of these parameters it was hoped to determine if 5-FU chemotherapy affects cognitive behaviour and to provide a cellular mechanism for this effect.

3.2 MATERIALS AND METHODS

3.2.1. Animals

20 male Lister Hooded rats, 2-3months age and their weights between 200-250 grams, were bought from Charles River (UK) and left to habituate to the new environment of the Biomedical Services Unit (BMSU), University of Nottingham for one week. Rats were housed in groups of 5 under standard laboratory conditions with free access to food and water all the time and weighted daily. Rats were randomly assigned to vehicle (n=10) or 5-FU+LCV chemotherapy (n=10) groups.

3.2.2. 5-FU chemotherapy treatment

Over the course of 2 weeks (every other day excluding weekends) rats received 5 i.v. injections into the tail vein of 5-FU (Mayne Pharma, PLC) and leucovorin (LCV, Teva, UK LTD) at a dose of 25 mg/kg in the same syringe while under (0.2 ml intraperitoneal) hypnorm general anaesthesia. The vehicle group received 0.9% sterile saline at an equivalent injection volume equivalent to drug treated animals. Two animals from the chemotherapy- treated group died in this experiment, one failed to recover from the anaesthetic and the other died after 2 days for unknown reasons. Animals were weighed daily during the treatment period.

Days 1-7	Days8&9	Days10&11	Days 12-20	Days 21& 22	Days 23 & 24	Day 26
Habituation in the BMSU	OLR test	CER test	5-FU+LCV or saline, every other day	OLR test	CER test	Sacrifice

Table 3.1. A protocol table of the study. Rats were tested in the OLR and the CER tests respectively prior to treatment to ensure that they were able to perform the tasks. Rats then received 5 intravenous injections of 5-FU+LCV (25mg/kg) as shown in the table. Animals then were tested for their performance in the OLR then the CER tasks. On day 26 of the experiment, all animals were sacrificed.

3.2.3. Behavioural Testing

The OLR and the CER tests were carried out and analysed as described in (Chapter 2, section 2.1.2.3.1 and 2.1.2.3.2). Animals in the object location recognition test that did not explore in behavioural testing sample or choice trials (i.e. combined exploratory time of 0 seconds for both object/objects locations) were excluded.

3.2.5. Brain Tissue Preparation

Two days after performing the CER test, (Fig 3.1) rats were killed by rapid stunning and decapitation. Whole brains were dissected from the skull and divided into two along the longitudinal fissure. One half was used for histology and the other for Western blot analysis.

3.2.6. Preparation of half brain for histology.

Half brains were cryoprotected in 30% sucrose for 2 hours before being frozen in isopentane cooled in liquid nitrogen. Tissue was stored at -80^{0}C before being warmed to -20^{0}C in a cryostat (Microm), embedded in OCT compound (VWR International Ltd.) and serially sectioned at 20μm. Sections were thaw mounted onto APES coated slides (aminopropyltriethoxysilane) and air dried before being stored at -20^{0}C. The hippocampus was identified from Toluidine blue- stained sections using information from a rat brain atlas (Paxinos, Watson et al. 1985).

3.2.7. Immunohistochemistry for Ki67

Sections for immunostaining were selected from the whole length of the hippocampus using a systematic random sampling procedure (Mayhew and Burton 1988). Slides were defrosted at RT and sections ringed with a PAP pen (Vector Laboratories). All washes were carried out in PBS and all procedures were at RT.

Sections were washed 3 times with phosphate buffered saline to remove OCT compound, fixed in 0.5% paraformaldehyde (PFA) for 3 minutes and washed again 3 times.

Primary antibody (anti Ki-67 Rabbit Polyclonal Vector Laboratories) diluted 1: 250 in PBS was applied for one hour.

Sections were washed and secondary antibody (Alexa 488; goat anti- rabbit, Molecular Probes) diluted 1: 250 applied for one hour.

Sections were washed, counter- stained with the red nuclear dye, propidium iodide (Molecular Probes; 1:3000 in PBS), mounted in glycerol and viewed under fluorescence illumination on a Nikon Optiphot-2 microscope. Images were

captured using a Spot Insight QE camera and Spot Advanced software (Image Solutions Ltd.). All counting was done blind to whether the material was from control or drug- treated animals.

3.2.8. Western Immunoblotting

3.2.8.1. Sample preparation and protein quantification using Lowry assay

Hippocampal and frontal cortex tissue samples were homogenized at 4°C to produce a 100mg/ml solution in lysis buffer. An equal volume of x2 solubilisation buffer was added to the homogenized sample to solubilise the protein. The Lowry assay was used to determine the protein concentration within the tissue samples before protein separation and immunoblotting. 20µl of the homogenized and solubilised samples was diluted (1:10) in distilled water to a total volume of 200 µl. A standard curve of bovine serum albumin (BSA) dilutions (in distilled water, total volume of 200 µl) (which protein concentrations of the tissue samples will be compared with) was prepared within the concentration range of 0 to 0.4 mg/ml. 1 ml of Lowry AB solution was added to all diluted tissue and BSA samples and left to incubate at room temperature for 10 minutes. Folin reagent (Sigma) was diluted 1:1 using distilled water and added at 100 µl per tissue and BSA samples. Tissue and BSA samples were then transferred onto a 96 well plate at 200 µl per well and allowed 45 minutes at room temperature for the Folin reagent to react with Lowry AB solution until development of the characteristic blue colour of samples. The colour density depends on the protein concentration present in the sample. The 96 well plate was loaded onto a Dynex MRX Model 96 well plate reader (MTX Lab Systems Inc., USA) which was used to colorimetrically detect the protein concentration

within the samples. Revelation software (MTX Lab Systems Inc., USA) was used to analyze the colorimetric reading and provided the protein concentration value for each sample. Details of solutions used in sample preparation and protein quantification are described in Appendix I.

3.2.8.2. Protein separation

The solubilised samples were warmed on a heating block at 95°C for 5 minutes, vortexed (5 min), centrifuged (1300 rpm, 1 min) and loaded onto a 16% SDS-polyacrylamide gel. Samples were run in parallel to 15µl of a molecular weight marker (PageRuler plus Prestained protein ladder manufactured by Fermentas UAB, Lithuania). Proteins were separated for 45 minutes (200 V at room temperature) with SDS-gels immersed in electrophoresis buffer. The protein separation was followed by transfer of proteins from the gel onto a nitrocellulose membrane for 90 minutes (200V at 4°C) in transfer buffer. Ponceau solution (Sigma, UK) was used to confirm successful transfer of protein to the nitrocellulose membrane. Details of solutions used in this step are described in Appendix I.

3.2.8.3. Protein transfer and band detection

Nitrocellulose membranes were blocked with a 5% milk solution (dried milk powder dissolved in Tris-buffered saline-Tween 20 (TBST) while shaking (1 hour at room temperature). All primary and secondary antibody dilutions were prepared in 5% milk solution. Membranes were incubated for 60 minutes on a constant shaker at room temperature with the following primary antibodies: polyclonal rabbit doublecortin (DCX) (1:1000; Cell Signalling Technology,

USA) and polyclonal rabbit BDNF N-20 (BDNF) (1:500; Santa Cruz, USA). Membranes were washed 3 times for 5 minutes each using TBST. Afterwards, all membranes were incubated with different secondary antibodies according to the method used for detecting protein bands. For visualization using the Odyssey system, membranes were incubated with fluorescent ®-conjugated secondary antibody, polyclonal goat anti-rabbit IgG (LICOR Bioscience, UK; 1:10000 dilution of 1 mg/ml stock in 10 ml of antibody dilution buffer with gentle shaking for 1 hour at room temperature).

Membranes were washed again in the same way following primary antibody incubation and rinsed with distilled water to prepare them for protein detection. Membranes were drained of excess fluid and allowed to dry just before scanning. Membranes were scanned using the infra-red Odyssey imaging system (LICOR Bioscience, UK).

For protein bands detection with the ordinary ECL (enhanced chemiluminescence) detection method, membranes were incubated with polyclonal goat anti-rabbit horse radish peroxidase (HRP)-conjugated secondary antibody (1:2000; DAKO Cytomation, Denmark) on a shaker for one hour at room temperature. Membranes were then washed in a similar way as after primary antibodies and rinsed with distilled water in preparation for band detection. Solutions from the chemiluminescence's kit (Amershham, GE Healthcare, UK) were applied onto the membranes for one minute then dried with filter paper. Membranes then were sealed in developing cassettes with photographic film (Kodak) in a dark room. After 10 minutes, the film was developed, rinsed with distilled water, fixed and left to dry.

3.2.8.4. The control protein

Glyceraldehyde 3- phosphate dehydrogenase (GAPDH; 37 kiloDaltons), a constitutively expressed housekeeping gene, was used as a loading control against which values of BDNF (15 kiloDaltons) and DCX (47 kiloDaltons) protein bands were normalized. GAPDH was immunoblotted on BDNF/DCX-containing membranes by incubating them with mouse GAPDH primary antibody (Abcam, UK). For the Odyssey method, secondary antibody was then incubated (IRD 700 ®-conjugated secondary antibody polyclonal goat anti-mouse IgG (LICOR Bioscience, UK; 1:10000) and the same protocol as described above was continued. For the ECL detection method, secondary antibody was polyclonal rabbit anti-mouse HRP-conjugated secondary antibody (1:2000; DAKO Cytomation, Denmark) then the same protocol was continued.

3.2.8.5. Quantification and data analysis

18 hippocampal or frontal cortex tissue samples from both vehicle and 5-FU+LCV chemotherapy group (10 controls and 8 5-FU) were run on one gel and the assay was repeated 3 times. Quantitative measurement of protein level was performed using (LI-COR Bioscience, USA) software specialized for the Odyssey infrared viewing images by measuring the infrared wave length of the protein band against the black background of the scanned area (700 nm wave length florescence is shown in red channel and 800 nm fluorescence wave length is shown in green channel). For ECL detection method, quantitative measurement of protein level was performed using the Quantity One 1-D analysis software (BioRad Laboratories, USA) by measuring the optical density (OD) of the protein band against the light coloured background of the photographic film. The values

for BDNF and DCX for each sample were divided by their corresponding GAPDH value which was run on the same gel and the product was then multiplied by 100. The percentage values of each experimental group were averaged from the 3 trials and presented as the mean expression of BDNF and DCX proteins as a percentage of GAPDH expression.

3.2.9. Statistical Analysis

All statistical analyses were performed using Prism Version 4.0 (GraphPad Software Inc., USA). Weight data were analysed using repeated measures of two-ways ANOVA with Bonferroni post-hoc test. Within-group analysis of OLR task was done using the paired Student's t-test to compare the mean exploratory times of objects in the sample and choice trials. Unpaired Student's t-test was used for between-group analysis comparing the mean exploratory time values of vehicle and 5-FU treated rats. OLR data were converted to percentages to compare for the preference index (PI) between groups was analyzed by using unpaired Student's t-test. Data for CER test, proliferating cell counts and Western immunoblotting were analyzed by unpaired Student's t-test. P-values less than 0.05 were considered significant ($P<0.05$).

3.3 RESULTS

3.3.1. Weight

The body weight of control and drug treated rats was monitored daily throughout the study. Rats receiving 5-FU+LCV chemotherapy exhibited a significant decrease in weight gain during the treatment period compared to their controls (P**=0.006), (repeated measures Two-ways ANOVA with Bonferroni post-hoc test, Fig.3.2), although they rapidly increased in weight after the last injection. Animals on 5-FU+LCV treatment showed a 24 hr drop in weight after each injection which recovered during the following day.

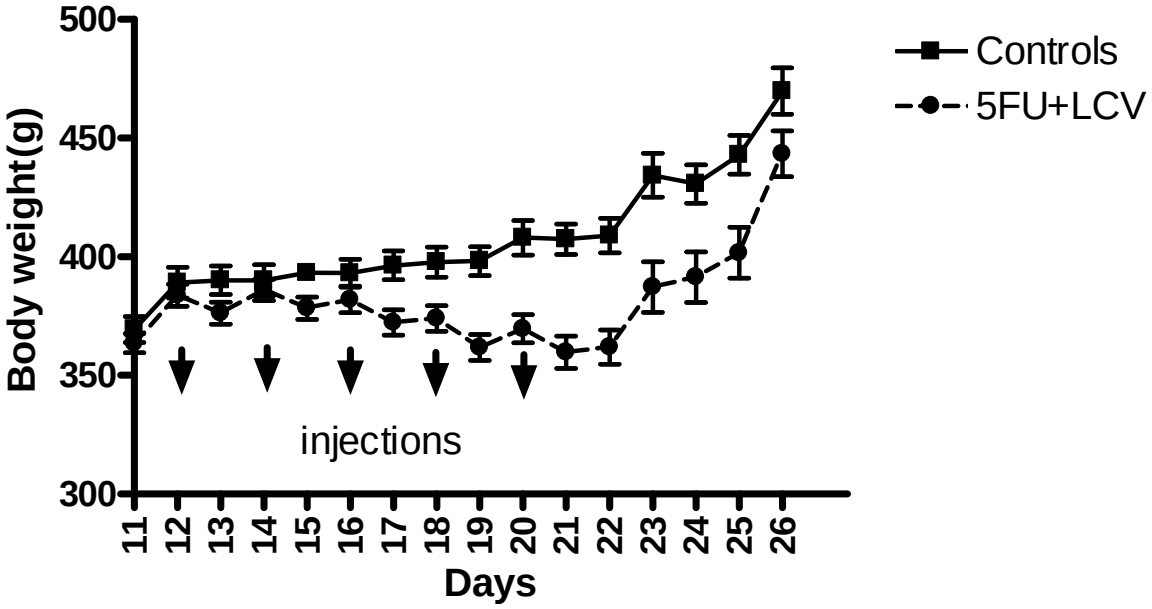
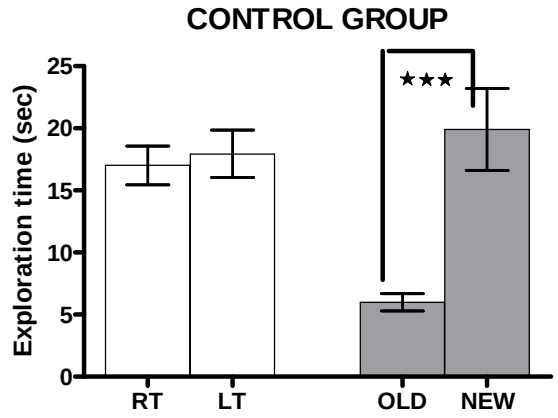


Figure 3.2. Body weight (Mean±S.E.M) of saline and 5-FU+LCV-treated groups on each day (n=10 and 8 respectively). The injections are labelled on the graph by arrows. There was a significant decrease in the amount of weight gained by rats treated with 5-FU+LCV when compared to their controls (p=0.006) over the time (p**= 0.001). The interaction between the time and the treatment factors was significant (p***<0.0001; repeated measures two-way ANOVA with Bonferroni post-hoc test.**

3.3.2. Object location recognition test before treatment

The OLR test was carried out on all animals before treatment to ensure that they were capable of performing the task. As shown in Fig 3.3. both groups explored the objects in the two locations equally in the sample trial (P>0.05). After changing one location of one object in the choice trials both groups spent significantly more time exploring the object in the (NEW) location compared to the object in the (OLD) location (P***=0.0001 and ***0.0006 respectively).

A



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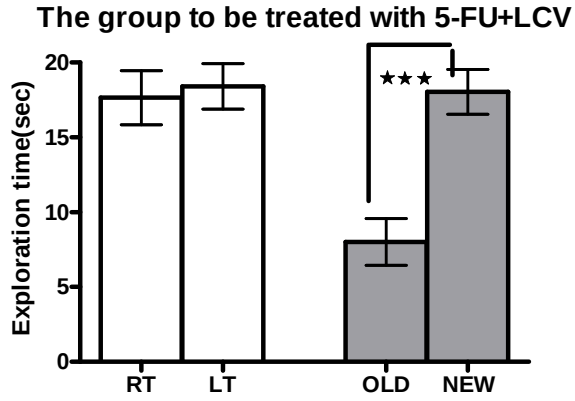


Fig 3.3.A and B Exploration time (Mean±S.E.M) of rats performing the OLR task before treatment (n=10 each). In the sample trial (un shaded), (RT and LT locations), both controls and the group to be treated with 5-FU+LCV spent the same amount of time exploring both locations of the objects (RT&LT) (P>0.05). In the choice trial (dark shaded), replicas of the same object used in the sample trial were placed in either locations (OLD or NEW) designed the old location (OLD) or a novel location (NEW). Vehicle treated rats or the group to be treated with 5-FU+LCV spent a significantly more time exploring the object in the (NEW) location compared to the (OLD) location (P*=0.0001) and (P***=0.0006) respectively. All comparisons were calculated using paired Student's t-test**.

3.3.3. CER test before treatment

The (CER) test was performed before treatment to ensure that all animals were able to perform this task before starting the experiment. As shown in Fig 3.4. There was no statistically significant difference in the amount of freezing between control and the group to be treated with 5-FU+LCV (P>0.05).

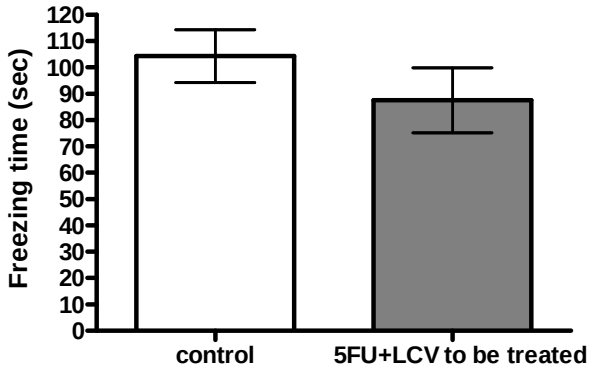
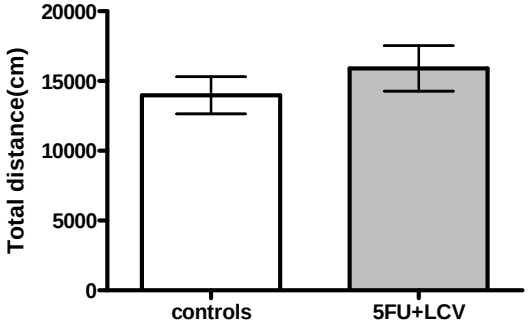


Fig 3.4. Freezing times (Mean±S.E.M) exhibited by the rats in each group before treatment (n=10). There was no statistically significant difference in the freezing times between the control and the group to be treated with 5-FU+LCV (p>0.05; paired Student's t-test).

3.3.4. Locomotor activity (total distance and Mean velocity) after treatment

Locomotor activity was measured during the habituation period prior to the OLR test after treatment. There was no significant difference in the mean velocity (cm/sec) or in the total distance (cm) performed by the animals (P=0.3) unpaired Student's t-test, Fig 2.1.5. A and B) which means that 5-FU+LCV did not alter animals' movement during the habituation phase of the OLR test suggesting that the changes measured during the test trials were due to true changes in behaviour of the animals not due to restriction of their movements.

A



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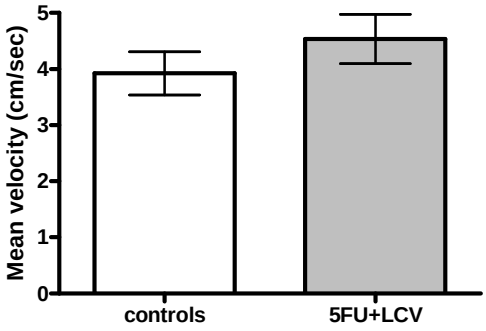
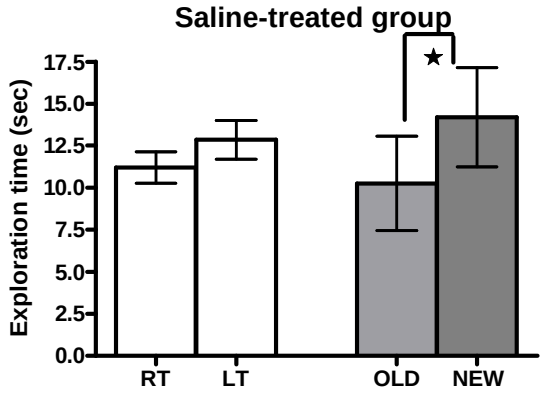


Figure 3.5. (A) The total distance and the mean velocity (B) (Mean±S.E.M) performed by saline and 5-FU+LCV-treated groups (n=10 and 8 respectively) during the habituation period (1 hour) of the OLR test after treatment. There was no difference between groups in the mean velocity or the total distance after treatment during the habituation period of the object location recognition test (p=0.3 in both; unpaired Student's t-test).

3.3.5. The object location recognition test after treatment

After 5-FU+LCV treatment, animals' spatial working memory was again tested using the OLR test. Both saline and 5-FU+LCV- treated rats displayed similar amounts of exploration time for both objects in the sample trial (exploring objects in RT and LT locations indicating that the rats had no preference for either of the objects or their locations in the box. In the choice trial, after the 5 minutes inter-trial interval, saline- treated animals proceeded to explore the object in the new location significantly more than the object which remained unmoved (P*=0.014, paired Student's t-test), Fig.3.6. However, 5-FU+LCV-treated rats showed no significant difference in the mean exploration time between objects in the new and old locations (P>0.05, Fig 3.6). These results indicate that animals in the control group retained the ability to discriminate between objects that had remained unmoved and those in a novel location but that the 5-FU+LCV chemotherapy treatment had caused a deficit in the spatial recognition memory of these rats. Object exploratory times in the choice trial were converted into a preference index (PI) to allow for the between-experimental-group comparison. The preference index of the 5-FU+LCV treated groups was significantly lower than the preference index of the saline treated rats (as shown in Fig. 3.7 ; P**=0.001) confirming the memory deficits caused by 5-FU+LCV chemotherapy in those animals.

A



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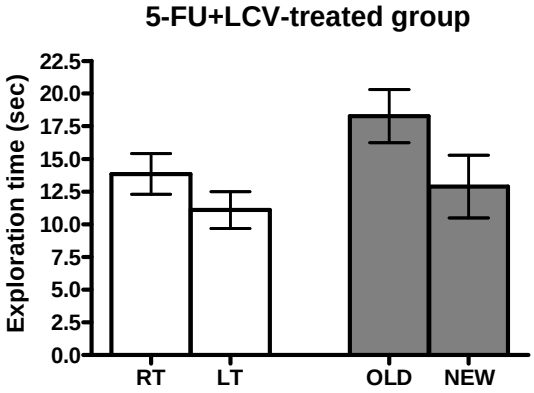


Fig 3.6. A and B, Exploration time (Mean±S.E.M) of saline and 5-FU+LCV treated groups in the (OLR) task after treatment (n=10 and 8 respectively). In the sample trial (white bars), LT and RT locations, both saline and 5-FU+LCV-treated rats spent the same amount of time exploring replicas of an object in both locations (RT and LT) (P>0.05). In the choice trial (dark shaded), replicas of the same object used in the sample trial were placed in either locations (OLD or NEW). Saline-treated rats spent a significantly greater time exploring the object in the new location (NEW) compared to the old location (OLD) (P*=0.014).

There was no significant difference in the time spent exploring the object in the new location compared to the old location by rats treated with 25 mg/kg of 5-FU+LCV (P>0.05; paired Student's t-test).

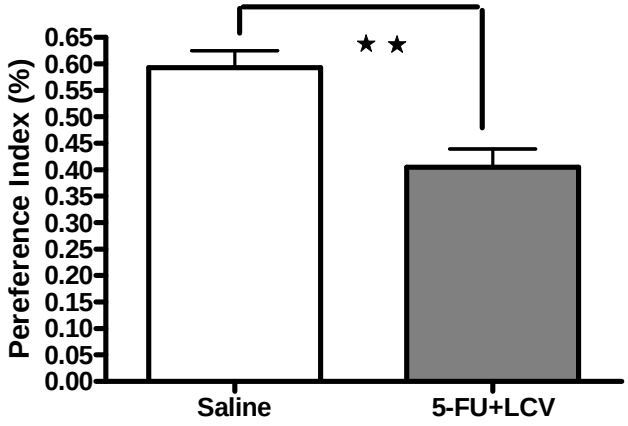


Fig 3.7. The preference index (PI) (Mean±S.E.M) of saline and 5-FU+LCV chemotherapy-treated rats performing the object location recognition task (n=10 and 8 respectively). The 5-FU+LCV (25 mg/kg)-treated group had a significantly lower PI than the saline treated (p=0.001; unpaired Student's t-test).**

3.3.6. The CER test after treatment

For the CER test, freezing behaviour was recorded 24 hours after conditioning when the animals were returned to the test box. Vehicle-treated animals spent an average of 148 seconds in this behaviour. In contrast 5-FU+LCV treated animals spent an average of only 70 seconds exhibiting this type of behaviour, which was significantly less than controls; Fig 3.8, (P*=0.01; unpaired Student's t-test). These results indicate that the 5-FU-treated animals had poorer recognition of the context of the unpleasant stimuli compared to control animals.

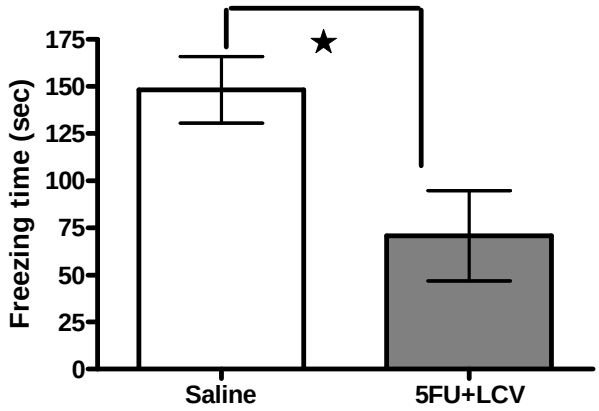


Fig 3.8. Freezing time (Mean±S.E.M) exhibited by the rats in saline and 5-FU+LCV-treated groups after treatment (n=10 and 8 respectively.

The 5-FU+LCV freezing time was significantly less than the freezing time for the saline-treated rats (P*=0.01; unpaired Student's t-test).

3.3.7. Proliferating cell counts

Five brains from each group were analysed and 12 sections, distributed along the length of the hippocampus, were stained from each brain. Ki67-positive cells were easily identified in the SGZ adjacent to the dentate gyrus. Figure 3.9 (A; B; C) displays representative images of Ki67-positive cells in sections counter-stained with the nuclear dye PI. Only cells which had both a red PI stained nucleus and nuclear Ki67 staining were counted. 5-FU+LCV-treated rats had a significantly lower number of positive Ki67 proliferating cells within the SGZ compared to the saline-treated group (P*=0.03, unpaired Student's t-test, Fig.3.10), indicating that the number of proliferating cells in the SGZ is reduced after 5-FU+LCV chemotherapy.

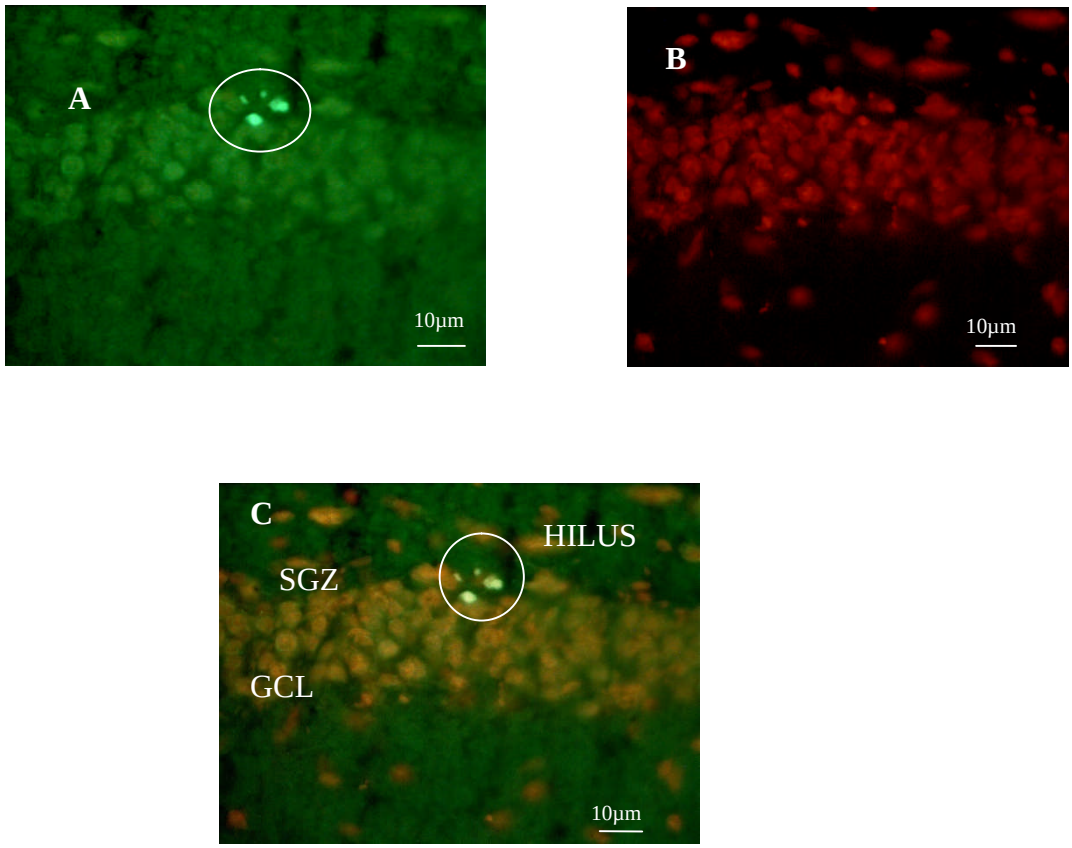


Figure 3.9. Representative images of rat dentate gyrus by fluorescent microscopy (A) Ki67- positive cells (circled) on the edge of the dentate gyrus. (B) Propidium Iodide (PI) nuclear staining showing all nuclei. (C) Merged image of A and B. Regions of the DG are highlighted: granule cell layer (GCL); sub-granular zone (SGZ) and the hilus (HILUS). All images were taken at x40 magnification.

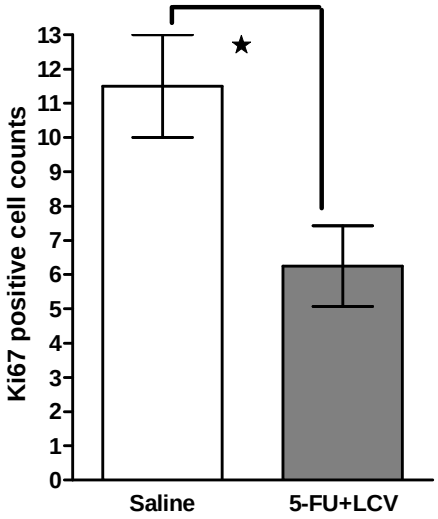


Figure 3.10. Ki67-positive cell counts in the dentate gyrus (Mean±S.E.M) of the saline and 5-FU+LCV-treated rats (n=5 each). There was a significant decrease in the mean numbers of Ki67-positive cells in the 5-FU+LCV-treated rats compared to the saline-treated rats (p*=0.03; unpaired Student's t-test).

3.3.8. Western blot results

Levels of DCX and BDNF proteins were measured in the hippocampus and frontal cortex to determine whether the 5-FU+LCV-induced memory deficits could be attributed to changes in a measure of neurogenesis (DCX) or in the levels of a neurotrophic factor (BDNF) known to be involved in memory and neurogenesis.

3.38.1 Levels of DCX and BDNF proteins in the hippocampus.

5-FU+LCV treatment produced a trend (non-significant) towards a reduction in DCX levels in the hippocampus (P=0.1, unpaired Student's t-test, Fig 3.11.A). The same treatment also produced a non-significant decrease in the level of BDNF protein as compared to the saline-treated animals (P*=0.09; unpaired Student's t-test, Fig 3.11.B).

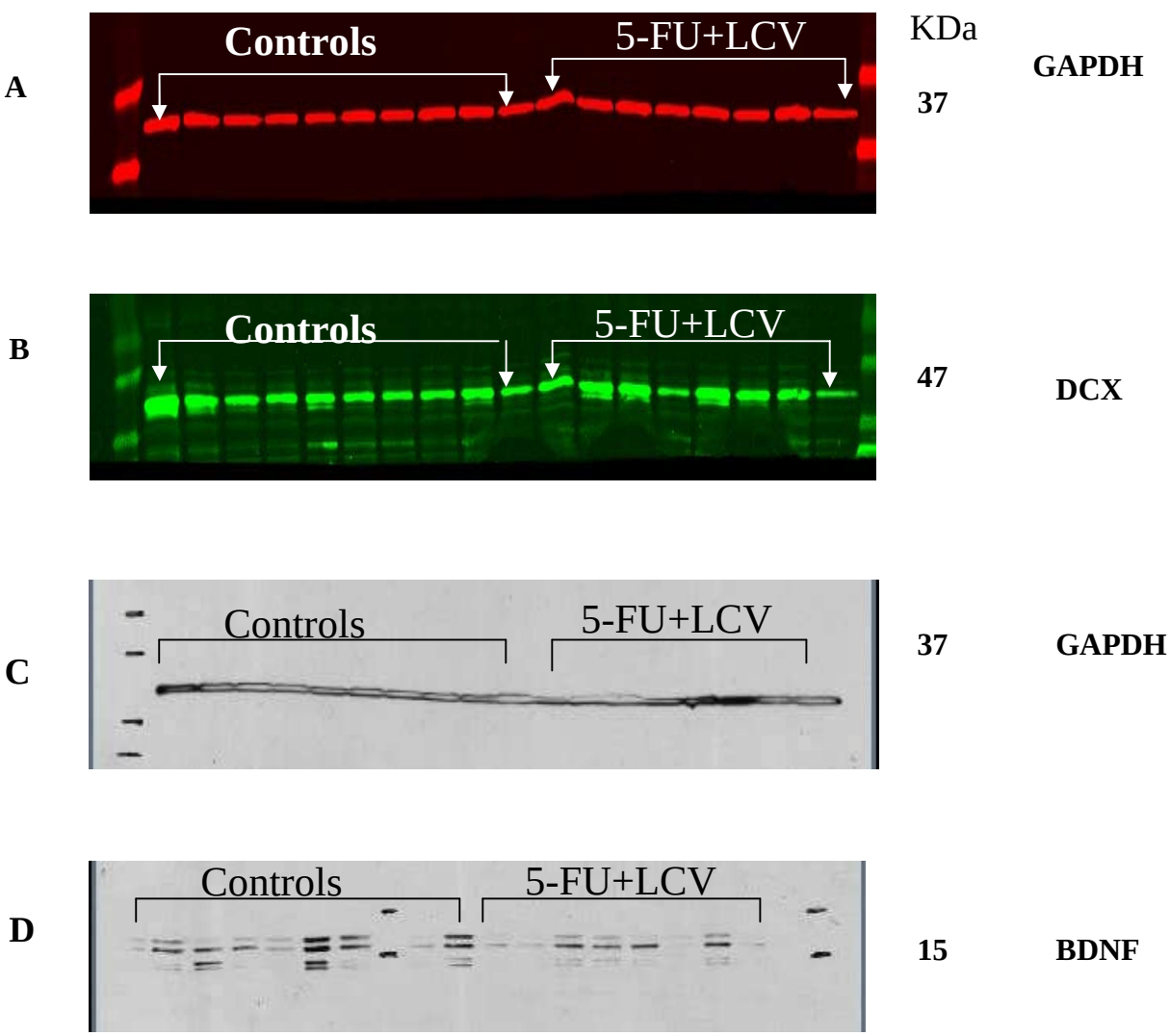
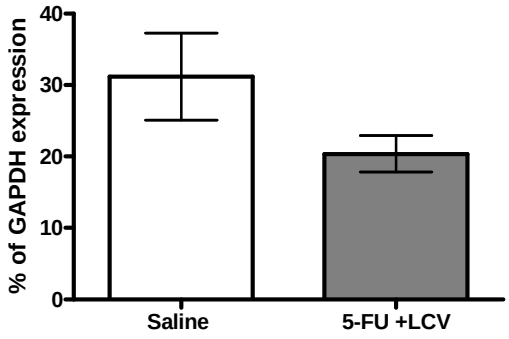


Figure 3.10. A, B, C and D. Representative Western immunoblots of rat hippocampal tissue. (B) Protein bands at the molecular weight corresponding to doublecortin (DCX) protein are indicated at 47 kiloDaltons (kDa) using the Odyssey system. On the same blot, corresponding levels of GAPDH protein from same samples using Odyssey system are shown (A). (D) Representative Western immunoblot of hippocampal BDNF protein bands are indicated at 15 KiloDalton (KDa) using the ELC developing system. On the same blot, corresponding levels of GAPDH protein from same samples using ELC method were shown (C). GAPDH was used as a loading control to ensure that equal amounts of proteins were loaded in each well (n=8-10).

(A) Hippocampal DCX protein



(B) Hippocampal BDNF protein

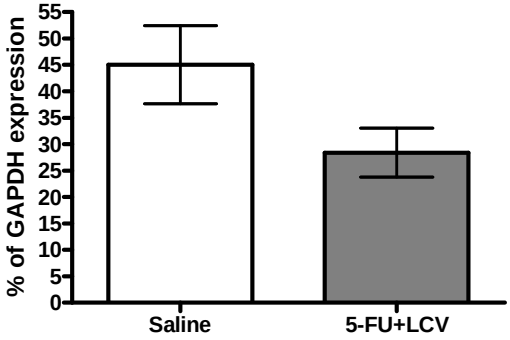
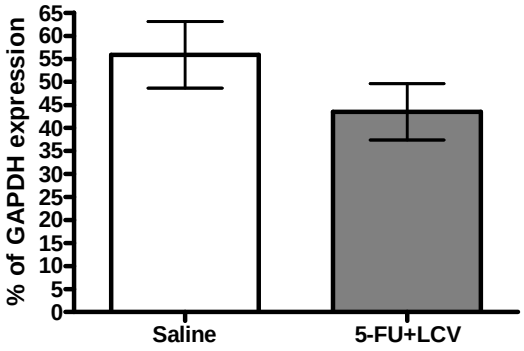


Figure 3.11. A and B. Graphs represent levels of DCX and BDNF respectively in the hippocampus (Mean±S.E.M; n= 10-12). There was no significant difference in the levels of DCX protein in the hippocampus between the saline-treated and the 5-FU+LCV-treated groups (P=0.1; unpaired Student's t-test). BDNF protein levels in the 5-FU+LCV treated group was not significantly decreased compared to the saline-treated group (P=0.09; unpaired Student's t-test). Data are the mean intensity/OD of either DCX or BDNF protein bands displayed as a percentage of the corresponding value of the GAPDH loading protein.

3.3.8.2. Levels of DCX and BDNF proteins in the frontal cortex

In the frontal cortex, DCX levels were not significantly decreased by 5-FU+ICV chemotherapy when compared to saline-treated animals (P=0.2, unpaired Student's t-test, Fig 3.12 (A). However, frontal cortex tissue levels of BDNF were significantly decreased due to 5-FU+LCV chemotherapy compared to the saline treated rats (P**=0.005, unpaired Student's t-test, Fig 3.12. (B)

(A) DCX levels in the frontal cortex



(B) BDNF levels in the frontal cortex

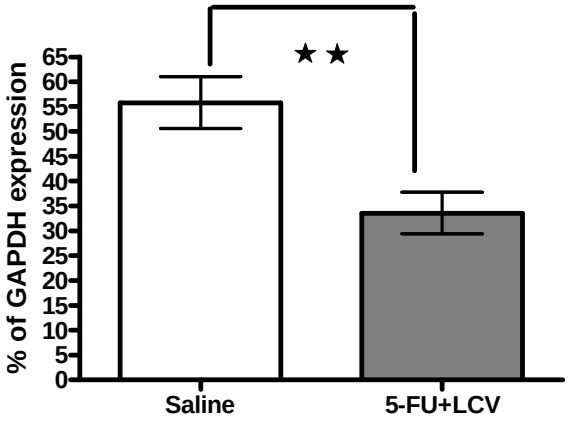


Figure 3.12 (A) Graph represents levels of the DCX protein in the frontal cortex (Mean ±S.E.M; n= 10-15). Although decreased, there was no significant difference in the levels of DCX protein between the saline treated and the 5-FU+LCV treated groups (P=0.2; unpaired Student's t-test).

(B) Graph represents BDNF levels in the frontal cortex which were decreased significantly by 5-FU+LCV treatment (P=0.005; unpaired Student's t-test) compared to the saline-treated rats. Data are the mean intensities/OD of DCX and BDNF protein bands displayed as a percentage of the corresponding wave length of the GAPDH loading protein.**

3.4. DISCUSSION

The reported incidence of cognitive problems associated with chemotherapy is very high (Moleski 2000; Wefel, Lenzi et al. 2004; Shilling, Jenkins et al. 2005). These cognitive deficits, as described by patients, mostly affect their memory, attention and information processing abilities. The verbal, spatial working and the recognition aspects of memory, which are mediated by the hippocampus, are considered the most vulnerable parameters in chemobrain sufferers (Reed and Squire 1997; Carrozzo, Koch et al. 2005; Grunwald and Kurthen 2006). The present study aimed to use an animal model to investigate the effect of systemically administered 5-FU chemotherapy on hippocampal memory tasks and to correlate these with changes in DCX and BDNF protein levels in the hippocampus and with different measures of hippocampal neurogenesis. Treatment involved two weeks of 5-FU+LV injections (5 injections; 25mg/kg) into the tail vein followed by behavioural testing which started one day after the final injection.

3.4.1. 5-FU chemotherapy decreased weight gain but did not affect locomotor activity.

There was a significant decrease in the amount of weight gained by rats treated with 5-FU+LCV during the treatment period when compared to the saline treated rats (Fig.3.2). This is a common observed effect of systemically administered 5-FU (Lee, Kim et al. 2006) and is attributed to the cytotoxic effect of the 5-FU on the proliferating cells of the gastrointestinal tract lining which reduces its ability to absorb nutrients (Huang, Kemp et al. 2002). Animals rapidly gained weight after the end of the treatment.

The measurement of the mean velocity and the total distance travelled by the 5-FU+LCV-treated rats (locomotor activity parameters, Fig 3.5.A and B) over the course of 1 hour prior to behavioural testing, did not show any difference from the same parameters measured in the saline-treated group. This indicates that the deficits which occurred in the performance of the behavioural tests in the 5-FU+LCV-treated rats were not attributed to a reduction in their locomotor activity compared to the saline treated rats. This is important as behavioural changes produced by the anti-mitotic drug methylazoxymethanol acetate (MAM) have been suggested to be due to reduced movement (Dupret, Montaron et al. 2005).

3.4.2. 5-FU altered the spatial memory of the rats

Hippocampal memory function was initially measured by testing the rats using the OLR behavioural test. The OLR test is a non-stressing spatial recognition memory testing for rats (Mustafa, Walker et al. 2008) which uses the hippocampus and requires an intact dentate gyrus of the rat to be able to perform this task (Lee, Hunsaker et al. 2005). Both control and drug-treated groups could successfully perform this test prior to the start of treatment. 5-FU+LCV treatment however significantly impaired the ability of animals to remember the locations of objects as shown by their failure to spend more time on the object in the novel location (Fig.3.6). This impairment in spatial memory was further analysed by conversion of the exploratory activity data into preference indices which also showed a significant difference between treated and control groups (Fig.3.7).

Apart from an earlier paper from our research group, the OLR test has not been used to examine the effects of chemotherapy on cognition (Mustafa, Walker et al. 2008). Several groups have used a related test, the object recognition test (ORT), which tests the ability of rats to recognise that the appearance of an object has changed without any change in its location (Ennaceur and Delacour 1988). Treatment of rats with the chemotherapeutic drug methotrexate (Seigers, Schagen et al. 2008) or the anti proliferative and cytotoxic drug MAM (Bruel-Jungerman, Laroche et al. 2005), produced a deficit in the ORT task. Both tests (ORT and OLR) require an intact hippocampus but the OLR has been shown to specifically require the dentate gyrus (Lee, Hunsaker et al. 2005). The OLR results presented here are in line with earlier work from our group which showed that a lower dose of 5-FU+LCV (20mg/kg) caused significant deficits in the performance of this task (Mustafa, Walker et al. 2008) and suggest that a general effect of anti-proliferative drugs may be to produce deficits in this type of memory task. The specificity in the requirement of the OLR test for an intact dentate gyrus strongly suggests that this part of the hippocampus is at least one of the brain regions adversely affected by 5-FU.

3.4.3. 5-FU impaired the contextual fear conditioning of the rats

The CER test as carried out here, measures recall of the context of an unpleasant stimulus after a 24 hour interval. Memory is quantified by the amount of freezing behaviour exhibited by the animal on being returned to the same arena (context) as the delivery of the unpleasant stimulus. Our results showed that treatment with 5-FU+LCV significantly reduced the amount of time animals spent in a freezing posture when returned to the arena compared to saline treated animals, indicating a failure in the recall of the context of this experience. (Fig.3.8). Performance of the CER behavioural test, where the stimulus is paired to a context, is impaired by hippocampal lesions (Matus-Amat, Higgins et al. 2004) but is also thought to involve input and modulation from other brain structures, notably the amygdala and anterior cingulate gyrus, which are involved in the emotional and nociceptive aspects of the test (Huff and Rudy 2004; McGaugh 2004; Malin and McGaugh 2006). If the unpleasant stimulus is paired with a tone (cued fear conditioning), hippocampal lesions have much less effect on performance indicating that the cue specific response is largely mediated only by the amygdala (Kim and Fanselow 1992; LeDoux 2000). Clinically it has been shown that sustained contextual anxiety is associated with an increase in cerebral blood flow in the right hippocampus (Hasler, Fromm et al. 2007) and the CER rodent test as performed here is a good measure of human hippocampal-dependent declarative memory (Maren, Aharonov et al. 1997; Rudy, Huff et al. 2004). From the above, it is clear that the CER test, although not as specific to the dentate gyrus as the OLR test, is still testing hippocampal function.

The CER test has been used more frequently than the OLR test in examining the cognitive effects of chemotherapy or other mitotic inhibiting drugs; MAM (Ko,

Jang et al. 2009); MTX and 5-FU (Gandal et al 2008); cyclophosphimide and doxorubicin (Macleod et al. 2007); cyclophosphimide (Reiriz et al 2006). Although doses and administration vary significantly between studies, only two of these investigations found that drug treatment caused a deficit in the performance of the CER test (Macleod et al. 2007; Reiriz et al. 2006).

The present investigation is the first to demonstrate that 5-FU+LCV treatment can impair performance in this test and together with the OLR test, supports the idea that drug treatment is affecting the hippocampus. The CER result from this chapter has recently been published (ElBeltagy, Mustafa et al. 2010).

3.4.4. 5-FU decreased proliferating cell counts in the hippocampus

One of the hypotheses of this investigation was that chemotherapy might be reducing the levels of cell proliferation in the SGZ of the dentate gyrus and in this way affect the formation of hippocampal dependent memories (Madsen, Kristjansen et al. 2003) . Because changes in human neurogenesis are not detectable clinically, it was important to use an animal models to test this. In the present study, 5-FU chemotherapy significantly reduced the number of proliferating (Ki67 positive) cells in the dentate gyrus of the hippocampus of adult rat brains compared to the saline treated rat brains (Fig.3.10). This result provides significant support for this hypothesis and a mechanism which may be contributing to the cognitive deficits experienced by patients. Proliferating cells in the SGZ can be either dividing stem cells or transit amplifying cells (Fig.3.9.A and C). Neural stem cells are slow dividing cells and most dividing cells in the SGZ are rapidly dividing transit amplifying cells which are often found in small clusters as shown in (Fig 3.9 A and C) (Kempermann, 2006). It is likely that

most of the reduction in dividing cells seen is due to a reduction in proliferating transit amplifying cells.

An earlier study from our group (Mustafa et al. 2008) found a non-significant reduction in Ki67 positive cell numbers in the SGZ after a lower dose of 5-FU+LCV (20mg/kg). Together with the present results this shows that the reduction in cell proliferation is dose-dependent.

Several other investigators have looked at the effects of antimitotic drugs on cell proliferation in the SGZ. These have included the cytotoxic drug MAM (Ko, Jang et al. 2009); which produced significant reductions in cell proliferation. Chemotherapeutic drugs, e.g MTX (Seigers et al. 2008); BCNU, cisplatin and cytarabine (Dietrich et al. 2006); thioTEPA (Migone and Weber 2006) and 5-FU (Han et al. 2008) have also been investigated in this way. All of these drugs caused a reduction in cell proliferation but only (Seigers et al. 2008) have previously correlated this change with changes in cognitive behaviour. As well as showing a dose-dependent effect of MTX on cell proliferation. These authors found that high doses of this drug impaired the performance of rats in the Morris water maze test.

A post-mortem study which was done by (Monje, Vogel et al. 2007) has provided evidence that patients undergoing chemotherapy treatment for tumours in their CNS show reduced hippocampal neurogenesis, particularly a dramatic reduction in the immature neurons suggesting that neurogenesis is severely affected. We have shown that treated rats with 5-FU+LCV chemotherapy displayed significantly reduced numbers of proliferating cells in the dentate gyrus of the hippocampus by using the proliferative marker Ki67 (ElBeltagy, Mustafa et al. 2010) . Furthermore, it has been reported that adult neurogenesis is the most

likely target of 5-FU chemotherapy (Mustafa, Walker et al. 2008). These findings are in line with the current results obtained from this study which strengthen the idea of the possible association between the memory impairments and the 5-FU induced reduction in hippocampal neurogenesis.

3.4.5. 5-FU chemotherapy reduced BDNF levels in frontal cortex not in the hippocampus

In the present study, although there was a tendency towards reduction of the BDNF levels in the hippocampus of the 5-FU+LCV treated rats compared to the saline treated ones, this reduction was not significant (Fig.3.11.B). The effect of 5-FU chemotherapy on BDNF levels seems to be regionally specific as the frontal cortex BDNF levels were significantly reduced (Fig.3.12.B). Previously, it was reported that BDNF levels in the frontal cortex of dopamine transporter knockout mice were reduced as brain plasticity (which depends on the brain dopaminergic system) also requires BDNF (Fumagalli, Racagni et al. 2003). Moreover, it was found that there is a strong relationship between the deteriorated memory and reduced levels of frontal cortex BDNF in mice tested for their performance in the water radial-arm maze (Bimonte-Nelson, Hunter et al. 2003). It is possibly that 5-FU chemotherapy targets neurons within the dentate gyrus because BDNF mRNA expression is prevalent in the granular cell layer of the dentate gyrus (Wetmore, Ernfors et al. 1990; Conner, Lauterborn et al. 1997). However, it was reported that there might be different roles of endogenous BDNF splice variants in protecting neuroblastoma cells from death induced by chemotherapy (Baj and Tongiorgi 2009) which might give a reason why BDNF level was reduced in frontal cortex but not in the hippocampus of 5-FU treated rats. Furthermore, it

was found that BDNF neurotrophic activation of TrkB protects neuroblastomas cell from chemotherapy-induced cell death via the phosphatidylinositol 3'-kinase pathway (Jaboin, Kim et al. 2002) and because there are many other suggested pathways by which BDNF might be involved in brain response to chemotherapy, it is possibly that the effect of 5-FU chemotherapy on BDNF protein level is regionally specific.

Another issue was raised in this study, which was the use of the CER as a test for contextual conditioning of rats as there is strong relationship between stress and elevated corticosteroid levels in the brain (de Kloet, Oitzl et al. 1999). The pairing of foot shocks with the context is stressful for animals which leads to elevated levels of corticosteroids (Davis 1997; Korte 2001). It has been proposed that BDNF levels did not change in rat hippocampus with elevated corticosteroid levels (Schaaf, De Kloet et al. 2000). Moreover, it has been found that eosinophils BDNF levels were not dramatically reduced by dexamethasone treatment in asthmatic patients (Noga, Hanf et al. 2005). From these findings, we decided not to test rats for the CER test in future experiments especially that it has been reported that stress could affect the level of hippocampal neurogenesis, our main target in future experiments (Lee, Kim et al. 2006).

3.4.6. 5-FU chemotherapy did not change DCX levels in the hippocampus or frontal cortex.

In the present study, 5-FU+LCV+LCV chemotherapy didn’t change the DCX levels in the hippocampus (Fig.3.11.A) or in frontal cortex (Fig.3.12.A). DCX is detected early in embryonic life and further expressed at high levels in most cells of the developing brain. Although expressed during neuronal development, DCX

is no longer expressed after neuronal maturation is completed. However, in the adult mammalian nervous system, its expression does still continue in restricted areas such as the dentate gyrus of the hippocampus and the lateral wall of the olfactory bulb axis which illustrates the continuous neurogenesis in the adult brain in these areas (Brown, Couillard-Despres et al. 2003; Couillard-Despres, Winner et al. 2005). DCX expression in neurons is transient and present throughout stages of final mitosis of neurogenic precursors and their differentiation while Ki67 is an endogenously expressed proliferative marker expressed in all stages of the cell cycle except G0 phase (Kempermann 2006).

Therefore, the lack of DCX changes in the present study may suggest that 5-FU chemotherapy targets late stages of proliferation especially since there was a gap between the last 5-FU+LCV treatment and termination of the study (the period of behavioural testing). This possibility was consistent with the studies of Chapter 4 and 5 which showed that 5-FU neurotoxicity requires at least 2 weeks to occur (ElBeltagy, Mustafa et al. 2010). As discussed above, the unchanged levels of BDNF protein between controls and 5-FU+LCV treated animals could be attributed to the stress to which animals were exposed during the CER test. The same reason could be addressed for the unaffected DCX levels in either hippocampus or frontal cortex. There is evidence that chronic mild stress or the stress caused by transient ischemia in the brain elevate numbers of DCX immunoreactive cells in the dentate gyrus of the hippocampus of rats which highlights that stress could modulate DCX in the hippocampus even in the opposite way to chemotherapy (Nakatomi, Kuriu et al. 2002; Tanaka, Yamashiro et al. 2004).

3.5. CONCLUSION

The results from this study indicated that 5-FU+LCV chemotherapy produces deficits in memory tasks dependent on the hippocampus of adult rats as measured by the OLR and CER tests. These deteriorations were strongly related to the reduction in the amount of neurogenesis of the 5-FU-treated rats compared to the saline-treated ones. However, this study failed to resolve the effect of chemotherapy on the neurotrophic factor, BDNF or the immature neuronal marker, DCX in regulating neurogenesis. The next chapter shows the effects of the antidepressants, namely fluoxetine, on memory and neurogenesis of the adult male hippocampus which will help in establishing the possible protective role of antidepressants against chemotherapeutic-induced deficits in a rat model.

CHAPTER 4

Fluoxetine reverses the memory deficits caused by the chemotherapy agent 5-Fluorouracil

4.1 INTRODUCTION

Systemic adjuvant chemotherapy is used in the treatment of many cancers including breast cancer. For some time breast cancer patients have reported that chemotherapy can have negative effects on cognition, a phenomenon which has led to the use of the terms “chemobrain” or “chemofog” (Anderson-Hanley, Sherman et al. 2003; Myers 2009). This together with the realisation that many chemotherapy agents can cross the blood brain barrier has led to cross sectional and longitudinal studies of patient cognition during and after chemotherapy has been carried out. The majority of these studies have shown mild to moderate cognitive deficits as reviewed in (Collins, Mackenzie et al. 2009). In particular four meta-analyses of the literature have concluded that working, visual and verbal memory appear to be consistently affected in patients who have completed a course of chemotherapy (Anderson-Hanley, Sherman et al. 2003; Falleti, Sanfilippo et al. 2005; Jansen, Miaskowski et al. 2005; Stewart, Bielajew et al. 2006).

A treatment for “chemobrain” would provide significant benefits to the large number of cancer survivors who have had chemotherapy. Development of such a treatment would be helped, if it was understood what the underlying causes were and why the condition persists after the end of chemotherapy. This question has attracted numerous suggestions reviewed in the following literatures (Barton and

Loprinzi 2002; Saykin, Ahles et al. 2003; Verstappen, Heimans et al. 2003; Jansen, Miaskowski et al. 2005; Ahles and Saykin 2007; Taillibert, Voillery et al. 2007; Dietrich, Monje et al. 2008; Wefel, Witgert et al. 2008; Soussain, Ricard et al. 2009; Chamberlain 2010).

No agreement has however been reached on the cause of chemobrain leading Saykin et al. to state "the greatest gap in our knowledge regarding chemotherapy-related cognitive changes is a lack of understanding of the mechanism or mechanisms that account for the observed changes" (Saykin, Ahles et al. 2003). Numerous suggestions that have been put forward from both human and animal studies and these have included the following:

Inhibition of hippocampal neurogenesis: (Dietrich, Han et al. 2006; Han, Yang et al. 2008; Mustafa, Walker et al. 2008; Seigers, Schagen et al. 2009); **neuroinflammation:** (Hook, Kimmel et al. 1992; Chen, Hinton et al. 1994; Cleeland, Bennett et al. 2003; Tangpong, Cole et al. 2007; Han, Yang et al. 2008; Seigers, Timmermans et al. 2010); **damage to the brain micro vasculature:** (Merkle, Moore et al. 2000; Dietrich, Marienhagen et al. 2004; Rzeski, Pruskil et al. 2004; Moore, Merkle et al. 2006); **anaemia:** (Cunningham 2003); **damage to white matter tracks (leukencephalopathy) including demylination and death of oligodendrocytes and their precursors:** (Akiba, Okeda et al. 1996; Brown, Stemmer et al. 1998; Choi, Lee et al. 2001; Moore, Somers et al. 2002; Cho, Choi et al. 2004; Yamashita, Yada et al. 2004; Dietrich, Han et al. 2006; Inagaki, Yoshikawa et al. 2007; Abraham, Haut et al. 2008; Baehring and Fulbright 2008; Han, Yang et al. 2008); **induced menopause or hormonal changes:** (Bender, Paraska et al. 2001; Shilling, Jenkins et al. 2003; Tchen, Juffs et al. 2003; Fan, Houede-Tchen et al. 2005; Bender, Sereika et al. 2007; Hermelink, Henschel et

al. 2008); **metabolic changes including glucose metabolism and oxidative stress:** (Silverman, Dy et al. 2007; Joshi, Aluise et al. 2010).

Reflecting the uncertainty in the cause of chemotherapy induced cognitive deteriorations, a wide range of possible treatments have been suggested, reviewed in (Gehring, Sitskoorn et al. 2008). These have included **cognitive behavioural or psychological therapies** (Cimprich and Ronis 2003; Biegler, Chaoul et al. 2009; Poppelreuter, Weis et al. 2009), **acupuncture** (Johnston, Yang et al. 2007), pharmaceutical interventions including **erythropoietin** (O'Shaughnessy 2003; Chang, Couture et al. 2004; Fan, Park et al. 2009), pychostimulants such as **methylphenidate and mondafinil** (Rozans, Dreisbach et al. 2002; Mar Fan, Clemons et al. 2008; Kohli, Fisher et al. 2009); **glutamate receptor antagonists** (Rzeski, Pruskil et al. 2004), and **stem cell treatment** (Noble and Dietrich 2002). Results from these interventions have been variable, where patient trials have been carried out, some are open label and may suffer from placebo or practice effects and none appears to have been taken up in general clinical practice.

In this thesis, the hypothesis is that the cognitive deficits produced by chemotherapy are, at least in part, caused by a reduction in hippocampal neurogenesis. With this in mind, an intervention which would increase hippocampal neurogenesis and cognition was sought. There is good evidence that antidepressants can fulfil these criteria especially the selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine (Malberg, Eisch et al. 2000; Herrera-Guzman, Gudayol-Ferre et al. 2009). Fluoxetine is one of the most commonly prescribed antidepressants which inhibit serotonin reuptake from the synaptic cleft (Wong, Bymaster et al. 1995). Moreover, a number of human studies have found that treatment with fluoxetine, can improve cognition and

memory in patients suffering from mild to moderate cognitive impairment (Cassano, Puca et al. 2002; Horsfield, Rosse et al. 2002; Levkovitz, Caftori et al. 2002; Mowla, Mosavinasab et al. 2007). The improvement in memory appears to be independent of depressive symptoms and the original causes of cognitive decline. One study of human post-mortem material has also shown that antidepressants including, fluoxetine, increase hippocampal cell proliferation in the SGZ of the dentate gyrus (Boldrini, Underwood et al. 2009). Similarly, animal studies have shown that chronic but not acute treatment with fluoxetine improves cognition and increases neurogenesis in the SGZ (Kodama, Fujioka et al. 2004; Chen, Pandey et al. 2006; Marcussen, Flagstad et al. 2008; Monleon, Vinader-Caerols et al. 2008).

The present chapter investigates whether treatment with fluoxetine can prevent the cognitive and cellular changes produced by 5-FU treatment. As shown in Chapter 3, in the present study we further demonstrate that 5-FU causes impairments in spatial memory as demonstrated by the OLR behavioural test, and reduces the number of the proliferating cells in the SGZ. Co-administration of fluoxetine, however reduced the impact of these cognitive deficits and prevented the decrease in cell proliferation in the SGZ. As BDNF is implicated in the neuroprotective and neurogenesis-promoting effects of antidepressants (Duman, Nakagawa et al. 2001), BDNF levels in the hippocampus were measured to test the effect of chronic 5-FU+LCV chemotherapy and fluoxetine on this growth factor. Also it has been reported that antidepressant treatment increases expression of phosphorylated cyclic-AMP response element binding protein (pCREB) and PSA-NCAM in the hippocampus both of which proteins are expressed in newborn neurons (Sairanen, O'Leary et al. 2007). In the present

study, the effect of fluoxetine and 5-FU+LCV chemotherapy on hippocampal levels of DCX, another protein expressed by newborn neurons, was also determined. Both chemotherapy and antidepressants may be altering hippocampal function and an understanding of this may provide a mechanism for improving cognition during cancer treatment. Results from this chapter have recently been published (ElBeltagy, Mustafa et al. 2010).

4.2 MATERIALS AND METHODS

4.2.1. Animals, drug administration.

Adult male Lister Hooded rats (Charles River UK) of 150-170gms at the start of experiments were maintained in the BMSU and weighed daily. Animals were allowed to habituate for 2 weeks before treatment and housed in groups of 4 under standard conditions of 12-h light – 12-h dark cycle (From 8.30am to 8.30 pm) with free access to food and water. Behavioural testing of all animals was performed one day after the end of drug treatment between (8.30am and 2pm). Doses given of 5-FU are the standard doses used to investigate the effects of 5-FU and are within the range which reduces tumour load in rats (Au et al 83; Watson et al 98) and are equivalent to the human dose range (Reagan, Nihal et al. 2008).

45 rats were randomly assigned to 4 groups (saline (n=12), 5-FU+LCV (n=12), fluoxetine (n=11) and combined 5-FU and fluoxetine treated (n=10). 5-FU+LCV-treated animals received 6 intravenous (i.v) bolus injections of 5-FU 20mg/kg/day (MAYNE Pharma PLC) together with leucovorin, 20mg/kg/day (TEVA UK LTD) i.v. under isoflurane anaesthesia every other day over two weeks. Saline-treated control animals (n=12) or fluoxetine treated (n=11) received an identical sequence of normal saline (0.9%) injections. Animals receiving fluoxetine (PINEWOOD Healthcare Colonel, Ireland) were given the drug in the drinking water with a dose of 10mg/kg/day over 3 weeks (started one week before 5-FU+LCV injection) being calculated from the amount of daily water intake (Yau, Hibberd et al. 2002). The concentration of fluoxetine given in drinking water was calculated to give the animals 25mg/5mls of water consumed.

Water intake decreases with fluoxetine treatment but dosages were recalculated every other day depending on water consumption and animal weights.

Drinking water containing fluoxetine was freely available to the animals. The solution of fluoxetine was changed every other day.

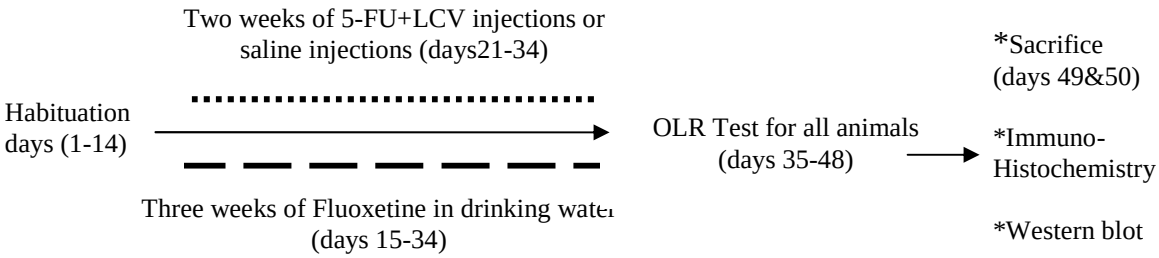


Table 4.1. A protocol table of the study is presented above. A chronic period of treatment lasted 21 days during which rats received injections of 5–FU+LCV chemotherapy 20mg/kg (short dotted bar, 2 weeks) and antidepressant fluoxetine 10mg/kg/day (long dotted line, three weeks) in drinking water. After drug treatment rats were tested for their performance in the OLR test. One day after the OLR test, animals were killed.

4.2.2. Behavioural testing, object location recognition task

This was modified from a previous protocol (Dix and Aggleton 1999) and was recorded by video camcorder as done previously in our laboratory (Mustafa, Walker et al. 2008). The locomotor activity (the distance and velocity of rats) was measured throughout the habituation period of the test. Exploratory activity of the object was measured as described earlier (Chapter 2, section 2.1.2.3.1). Behavioural data analysis was done as previously described (Chapter 2, section 2.1.2.6).

4.2.3. Brain tissue preparation

On the day following the OLR test, rats were killed by rapid stunning followed by decapitation. 29 Brains from all groups were randomly chosen from animals undergoing behavioural testing. These brains were prepared for immunohistochemistry as previously described (Chapter 3, section 3.2.6).

A systemic random sampling technique (Mayhew and Burton 1988) was used to choose every 21st section throughout the length of the dentate gyrus (overall 10 sections).

4.2.4. Ki67 Immunohistochemistry

Immunohistochemistry for Ki67 protein, along with sterology and microscopy for Ki67 were carried out as described previously (Chapter 3 section 3.2.7).

4.2.5. Western Immunoblotting of BDNF and DCX proteins

Hippocampus and frontal cortex sample preparation and determination of sample protein concentration using Lowry assay were carried out as detailed previously (section 3.2.7.1., Chapter 3). In the present study, a new system was used for Western blotting to optimize the protein band signal strengths and to shorten the time needed to obtain the final results.

This new system is the SNAP i.d, protein detection system and blotting membranes (Millipore, UK) which unlike conventional Western blotting, where diffusion is the primary means of reagent transport, the SNAP i.d. system applies a vacuum to actively drive reagents through the nitrocellulose membrane. After protein separation was done as previously described (section 3.2.7.2., Chapter 3), nitrocellulose membranes were placed directly in the SNAP i.d. champers and blocked in 0.5% Fish Gelatine Buffer (NBS, Biologicals, Ltd, UK) for 20 seconds. All primary and secondary antibody dilutions were made in the same blocking solution. Primary antibodies (polyclonal rabbit doublecortin (DCX) (1:1000; Cell Signalling Technology, USA) and polyclonal rabbit BDNF N-20 (BDNF) (1:500; Santa Cruz, USA). were then poured over the nitrocellulose membranes into two different chambers for each antibody for 10 minutes. Membranes then washed with Tris-buffered saline-Tween 20 [TBST] (Appendix I) 3 times 20 seconds each. After this, membranes were incubated with secondary antibodies (fluorescently -conjugated secondary antibody, polyclonal goat anti-rabbit IgG [LICOR Bioscience, UK; 1:10000 dilution of 1 mg/ml stock in 5 ml of antibody dilution buffer (fish gelatine buffer) at room temperature] in the same chambers for 10 minutes while keeping the chambers in an aluminium foil wrapped box to protect the nitrocellulose membranes from

light which could interfere with the infra-red detection method used to visualise the protein bands. Secondary antibodies were removed by washing using Tris-buffered saline-Tween 20 [TBST] 3 times 20 seconds each. The control protein was used as described previously (section 3.2.7.4, Chapter 3) and was incubated with each nitrocellulose membrane separately in each chamber of the SNAP i.d. system to avoid cross reaction between antibodies. Finally, membranes were drained of excess fluid and allowed to dry just before scanning. Membranes were scanned using an appropriate filter on the infra- red Odyssey imaging system (LICOR Bioscience, UK). Quantification and data analysis were carried out similar to that in chapter 3 section 2.7.5 except that 12 hippocampal (3 from each group) or 8 frontal cortex tissue samples (2 from each group) were run on one gel and the experiment was repeated 3 times.

4.2.6. Statistical Analysis

All statistical parameters were calculated using Graph pad Prism 4.0 software, USA. Paired student t- test (two-tailed) and repeated- measures of ANOVA were used to analyse data of the OLR task. Weight and water intake data were analysed using repeated measures Two-way ANOVA.

One- way ANOVA with Bonferroni post-hoc test was used to analyse data of the proliferating cell count, the preference index, Western immunoblotting data, the mean velocity and the total distance moved by the animals. A probability level of $P<0.05$ was considered statistically significant

4.3 RESULTS

4.3.1. Body weight of rats

Animals were weighed daily. Animals on 5-FU+LCV showed a weight loss during drug administration and, although they showed improved weight gain after the end of drug treatment (data not shown), they were still significantly below control weights (P**=0.007, repeated measures of Two-way ANOVA, Fig.4.2). Animals on fluoxetine or fluoxetine and 5-FU+LCV were significantly lighter than controls during drug administration but recovered to control weights by the end of the experiment. Animals on 5-FU+LCV showed a 24 hr drop in weight after each injection which recovered in the following day. Animals treated with fluoxetine also put on less weight than controls.

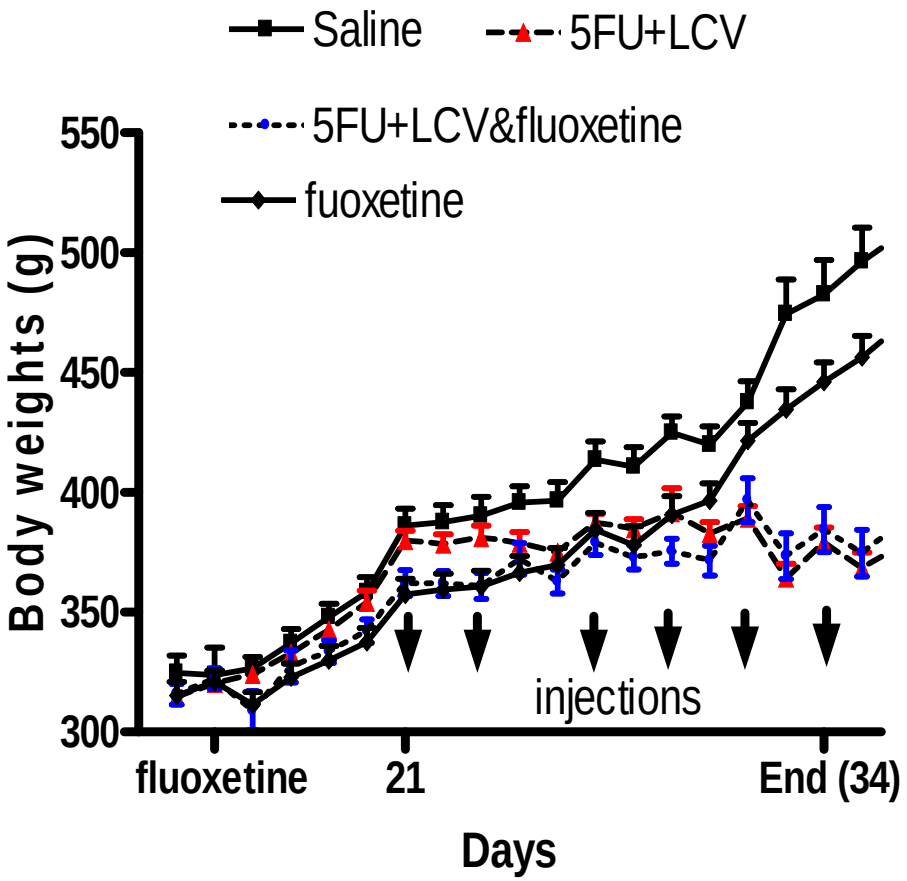


Figure 4.2. Body weight for each experimental group (Mean±S.E.M; n=10-12) were measured daily throughout the course of treatment (fluoxetine and 5-FU+LCV). There was a significant difference across all groups due to treatment (P=0.007) over the period of time (p***<0.0001). Also the interaction between time and treatment was highly significant (p***<0.0001; repeated measures Two-way ANOVA with Bonferroni post-hoc test). There was significant reduction in the mean body weight of fluoxetine, 5-FU+LCV or fluoxetine&5-FU+LCV-treated groups compared to the saline-treated group during the treatment period. On the graph, injections (arrows) are indicated and on the X axis, the start of fluoxetine and the end of drug treatment are indicated.**

4.3.2. Water intake

Water bottles of each cage were weighed daily during the period of fluoxetine treatment (3 weeks) and the amount of water intake was calculated and averaged for each experimental group. Fluoxetine was changed in the water bottle every other day with recalculation of the dose to keep it unchanged over the chronic course of treatment (10mg/kg/day). Fluoxetine alone or combined with 5-FU+LCV significantly decreased the amount of water intake by the animals compared to the saline treated animals on the following day after fluoxetine administration ($P^{**}<0.001$, repeated measures Two-way ANOVA, Bonferroni post test, Fig. 4.3). There was no significant difference in the amount of water intake between saline and 5-FU+LCV-treated groups ($P>0.05$) during the 5-FU+LCV injection period. Again there was no significant difference between the 5-FU+LCV combined with fluoxetine and the 5-FU treated groups in the amount of water intake during the drug treatment period ($P>0.05$).

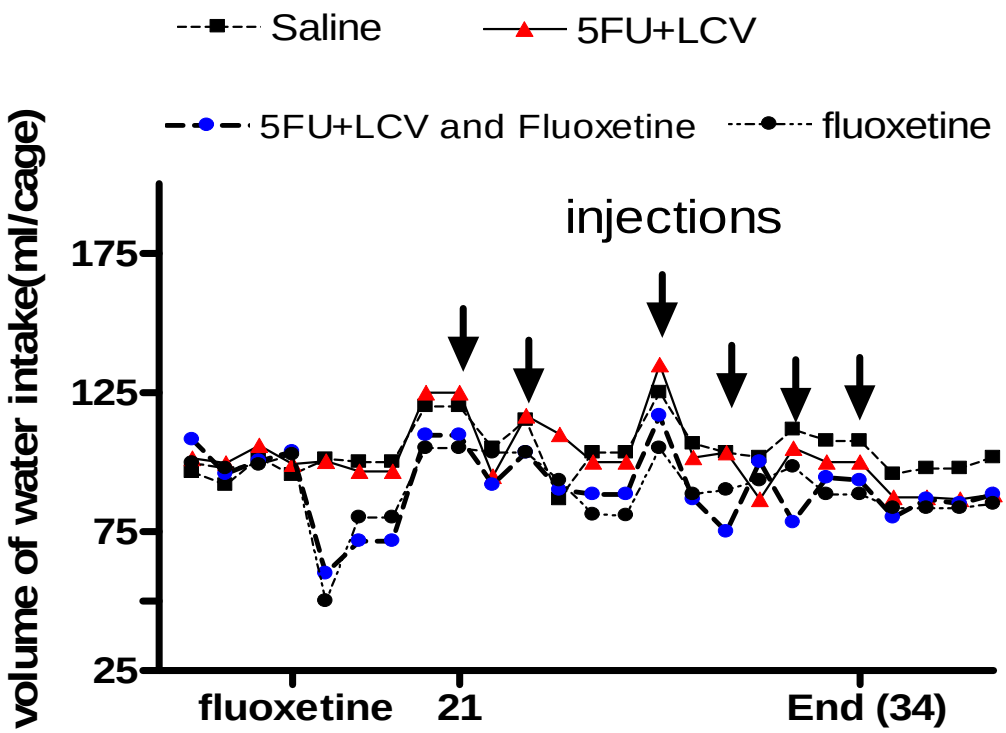
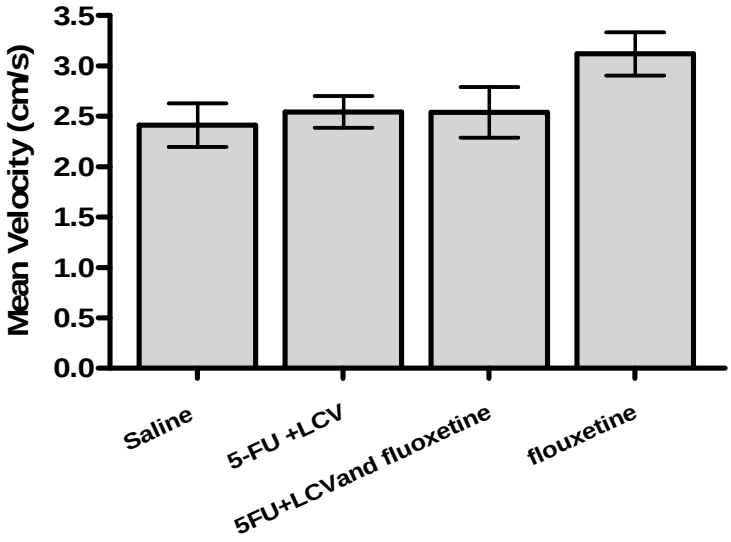


Figure 4.3. Volume of water intake for each experimental group (Mean while S.E.M was removed for clarity; n=10-12) were observed daily throughout the course of drug treatment (fluoxetine and 5-FU+LCV). There was a significant difference across all groups over the chronic period of time (P*<0.0001, repeated measures Two-way ANOVA with Bonferroni post-hoc test). T-here was a significant reduction in the mean volume of water intake in both fluoxetine and fluoxetine and 5-FU+LCV treated groups compared to the saline treated group (P**<0.001) on the following day of fluoxetine treatment. There was no significant difference in the mean volume of water intake between saline and 5-FU+LCV treated groups (P>0.05) during the treatment period. Also there was no significant difference between fluoxetine only and 5-FU+LCV and fluoxetine treated groups (P>0.05) during the treatment period. The interaction was not significant. On the X axis, the start of fluoxetine and the end of drug treatment are labelled. On the graph, injections (arrows) are indicated.**

4.3.3. Velocity and Distance (Locomotor activity)

Locomotor activity was measured during the habituation period prior to the OLR test. There was no significant difference in the mean velocity (cm/sec) or in the total distance (cm) performed by the animals (P=0.1 and 0.6 respectively, one-way ANOVA, Fig 4.4 A and B) which means that fluoxetine on its own or combined with 5-FU+LCV did not alter animals movement during the habituation phase of the OLR test suggesting that the changes measured during the test trials are due to true changes in behaviour of the animals not due to restriction of their movements.

A



B

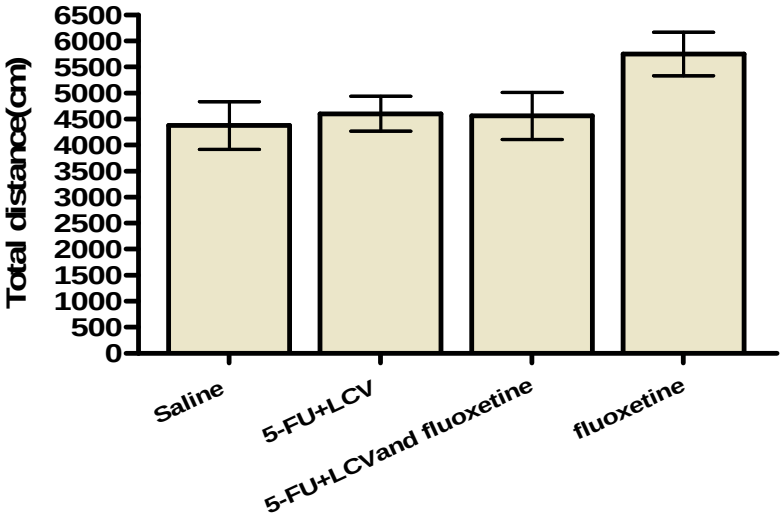


Figure 4.4. (A) Mean velocity and total distance (B) (Mean±S.E.M) moved by saline and 5-FU+LCV-treated groups (n=10-12) during the habituation period (1 hour) of the OLR test after treatment. There was no difference between all groups in the mean velocity or the total distance after treatment during the habituation period of the object location recognition test (P=0.1 and 0.6 respectively; one way ANOVA with Bonferroni post-hoc test.

4.3.4. Object Location Recognition

The OLR test measures interactions with objects either in familiar or novel locations within a test arena. During the familiarization trial, when animals explore two identical objects, both vehicle and drug treated groups showed no preference for either object or the total exploration time (data not shown). Following a 5 min inter-trial interval, one object is moved to a new location (choice trial) and object preference is recorded. Saline-injected controls and animals receiving fluoxetine in their drinking water both explored the novel object location significantly more than the familiar location (P=0.01 and 0.02 respectively) (Fig.4.5). However, animals treated with 5-FU+LCV by iv injection failed to show discrimination between objects in familiar and novel locations (P=0.06). Surprisingly 5-FU+LCV-treated animals spent more time examining the familiar object although this was not significantly different from the time spent on the novel object.

Animals which had received fluoxetine in their drinking water throughout the experiment as well as i.v injections of 5-FU+LCV spent more time exploring the novel object but this was not significantly different from the time spent exploring the familiar object. Further analysis using the PI was done to compare between the four groups, see (Fig 4.6).

Comparing the PI between the control and the 5-FU+LCV-treated group demonstrated a significant preference decrease after treatment with the chemotherapy drug (P*<0.01). Similarly fluoxetine on its own showed a significantly higher preference index to 5-FU+LCV treated animals (P*<0.05). Comparing the preference index (PI) of animals treated with 5-FU+LCV on its own and that of animals simultaneously given fluoxetine and 5-FU+LCV showed

that co- administration of fluoxetine significantly improved preference compared with animals on 5-FU+LCV alone ($P^{*}<0.05$). These findings indicate that animals receiving 5-FU+LCV show deficits in spatial memory which are improved by simultaneous administration of fluoxetine.

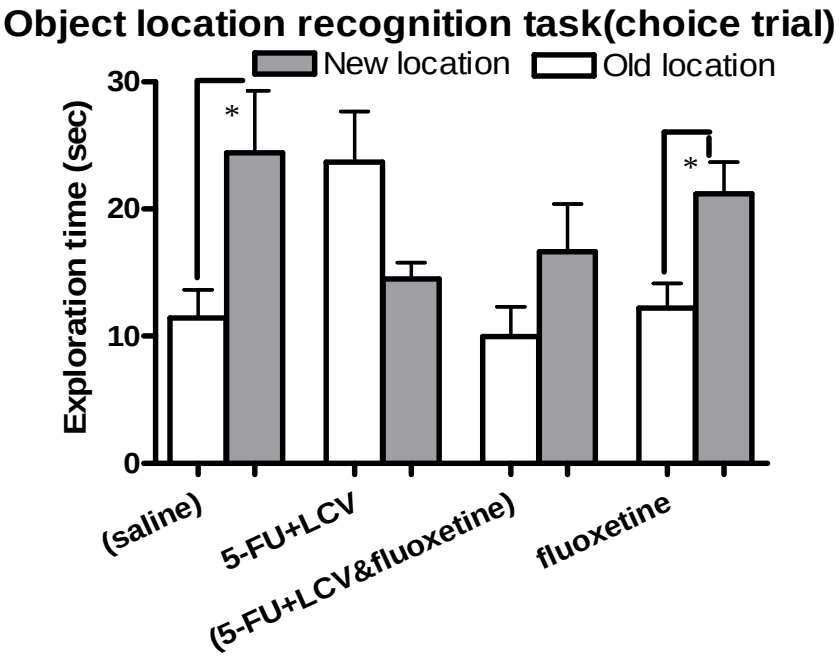


Figure 4.5. Exploration time (Mean±S.E.M) of groups (n=10-12) for replicates of an object viewed in the new and the old locations. The figure shows that the saline treated animals explored the new location (dark shaded) significantly more than the old location (un-shaded) (p*=0.01). Whereas the 5-FU+LCV treated animals failed to discriminate between the new and the old objects (P=0.06). There was a non-significant increase in the exploration time of the new object than the old one in the combined (5-FU+LCV and fluoxetine) group (p=0.1). Concerning fluoxetine-treated rats, their exploration of the new location was significantly greater than the old location (p*= 0.02). The analysis was done using paired T test (two tailed) to compare between the mean exploration time of the old and the new location in the choice trial after treatment within each group.

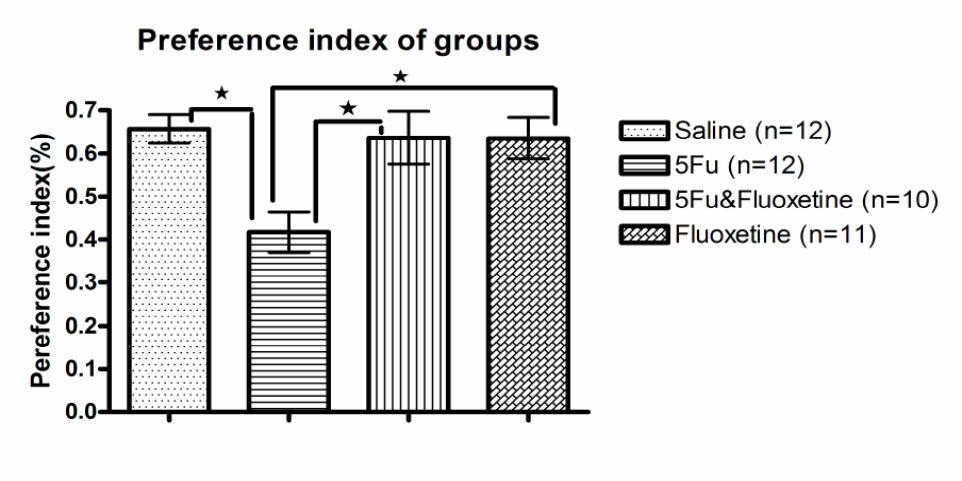
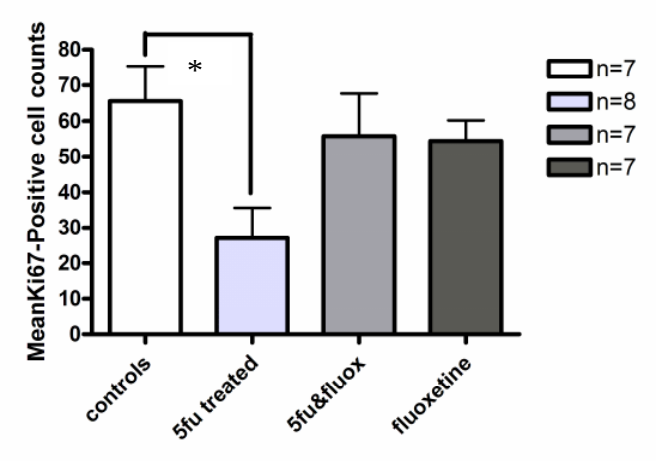


Figure 4.6. Exploration time was converted into mean preference index (PI) (Mean±S.E.M) for each experimental group in the OLR task. The preference index is defined as the calculated exploration time of the new location, represented as a percentage of the combined exploration time of both novel and familiar locations of an object. The preference index showed a significant decrease in the 5-FU+LCV treated rats in comparison to the other three groups (P=0.001; one way ANOVA with Bonferroni post-hoc test.

4.3.5. Proliferating Cell Counts

As shown in Fig 4.7. A there was a significant reduction in the total number of Ki67-positive cells of the 5-FU+LCV-treated group compared to saline-treated rats ($P^*<0.05$). This difference was abolished by co-treatment with fluoxetine ($P>0.05$) while fluoxetine alone did not have any effect on the total number of Ki67-positive cells compared to controls ($P>0.05$). Fig 4.7. B shows a representative image of Ki67-positive proliferating cells within the dentate gyrus displayed with PI nuclear counterstaining. Although 5-FU+LCV affected behaviour and reduced cell proliferation across the groups, there was no direct correlation between proliferating cell number and performance in the behavioural test for individual animals (data not shown).

A



B

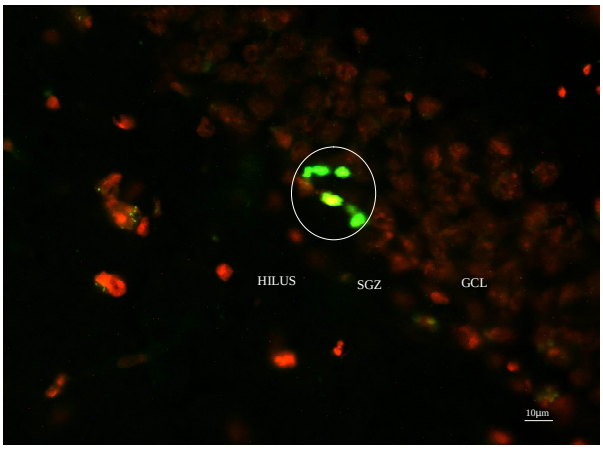


Figure 4.7. Ki67 Proliferating cell counts (Mean±S.E.M) in the sub-granular zone (SGZ) of the dentate gyrus (7-8). (A)There was a significant reduction of the number of Ki67 positive cells of the 5-FU+LCV-treated group compared to controls ($p^* < 0.05$) which was abolished after co-treatment (fluoxetine and 5-FU) ($p > 0.05$). There was no difference in the proliferating cell count between controls and fluoxetine only-treated group ($p > 0.05$; one way ANOVA with Bonferroni post-hoc test. (B) Representative image of rat DG by fluorescent microscopy. Proliferating cells in cluster (circled) within the dentate gyrus (DG) are immunostained for Ki67 (green) and counterstained with propidium iodine nuclear strain (red). Regions of the DG are highlighted: granular cell layer (GCL) ; subgranular zone (SGZ) and the hilus (HILUS).

4.3.6. Hippocampal BDNF and DCX Levels

The effect of 5-FU+LCV chemotherapy and fluoxetine on levels of BDNF neurotrophic factor and DCX neurogenic protein was measured in the hippocampus and the frontal cortex. BDNF levels in the hippocampus differed significantly between the 5-FU+LCV and the combined 5-FU+LCV&fluoxetine-treated groups (P*=0.02, one way ANOVA. Fig.4.10 [A]). There was no difference in the level of hippocampal BDNF protein between the 5-FU+LCV and the saline-treated groups (P>0.05). Moreover there was no significant difference in the same protein levels between the saline and fluoxetine only-treated groups (P>0.05, one way ANOVA. Fig.4.10 [A]). Hippocampal DCX levels showed a marginally non-significant difference between groups (P=0.053, one way ANOVA. Fig.4.10 [B]). Fontal cortex BDNF protein level showed a marked significant difference between groups (P**=0.008, one way ANOVA. Fig.4.11 [A]). There was a significant increase in the level of frontal cortex BDNF protein in the fluoxetine-treated group compared to the saline-treated (P*<0.05) while there was no change in the level of frontal cortex BDNF between the 5-FU+LCV and the combined 5-FU+LCV & fluoxetine-treated groups (P>0.05, one way ANOVA. Fig.4.11 [A]). Also co-treatment with fluoxetine did not alter the same protein level in the same tissue (P>0.05). On the other hand, levels of frontal cortex DCX differed significantly between groups (P=0.019, one way ANOVA. Fig.4.11 [B]). Bonferroni post-hoc test revealed a significant difference in the protein level between the 5-FU+LCV and the combined 5-FU+LCV&fluoxetine treated groups (P*<0.05). Also the level of frontal cortex DCX protein in the fluoxetine only-treated group was significantly higher than

the same protein levels in the 5-FU+LCV treated group (P*<0.05, one way ANOVA. Fig.4.11 [B]).

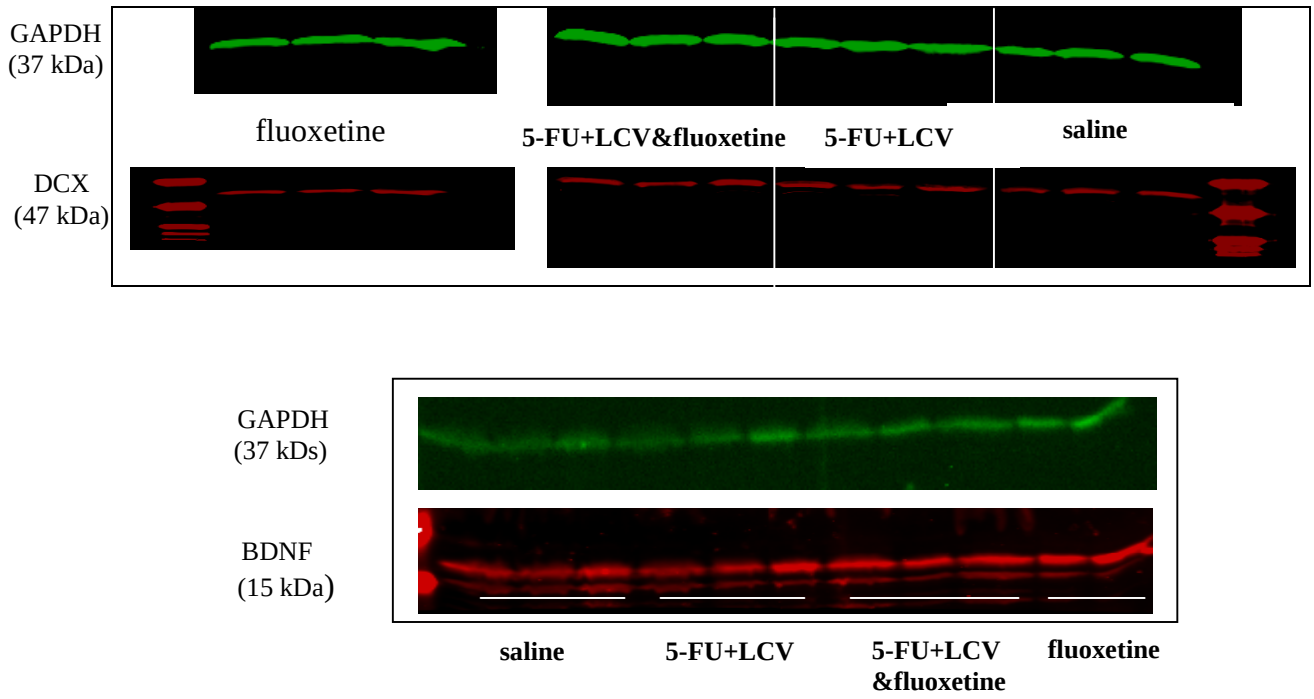
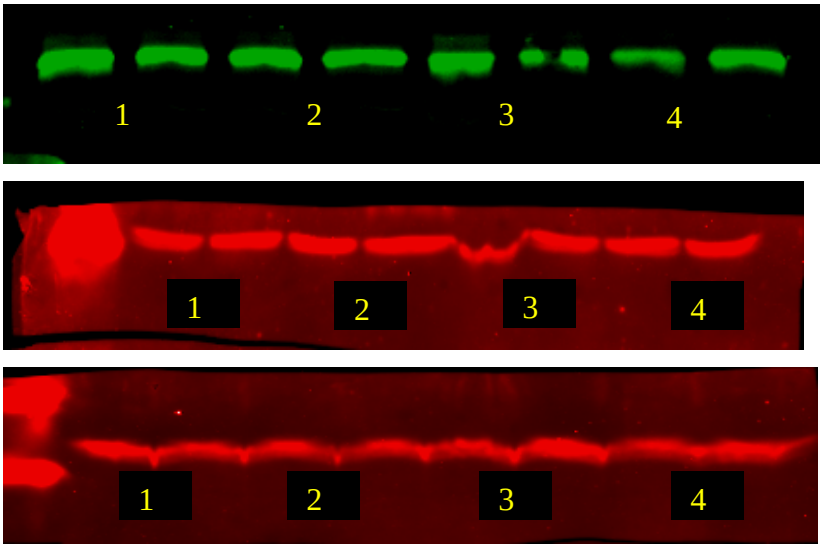


Figure 4.8. Representative Western immunoblots of hippocampal tissue are displayed. In the top box, protein bands at the molecular weight corresponding to (DCX) protein are indicated at 47 kiloDaltons (kDa). On the same immunoblots, corresponding GAPDH protein bands detected in the same samples from which DCX was detected at 37 kDa. The bottom box displays protein bands at the molecular weight corresponding to (BDNF) protein as indicated at 15 kDa. Corresponding GAPDH protein bands were observed on the same immunoblots as BDNF bands. GAPDH was used as a loading control to ensure that equal amounts of protein were loaded into each well. From each experimental group 3 tissue samples were immunoblotted for both BDNF and DCX except for fluoxetine samples in the lower box whereas 2 samples only were loaded for BDNF protein

4.3.7. Frontal Cortex BDNF and DCX Levels



GAPDH
(37 kDa)

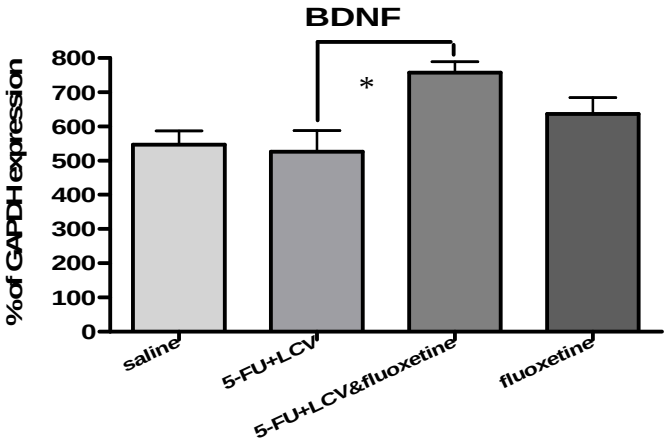
DCX
(47 kDa)

BDNF
(15 kDa)

Figure 4.9. Representative Western immunoblots of frontal cortex tissue are displayed. The middle blot displays protein bands at the molecular weight corresponding to (DCX) protein are indicated at 47 kiloDaltons (kDa). The bottom blot displays protein bands at the molecular weight corresponding to (BDNF) protein as indicated at 15 kDa. On the same immunoblots, corresponding GAPDH protein bands detected in the same samples from which DCX and BDNF were detected are observed at 37 kDa. (top blot). GAPDH was used as a loading control to ensure that equal amounts of protein were loaded into each well. From each experimental group 2 tissue samples were immunoblotted for BDNF and DCX where 1 labels (saline treated samples), 2 labels (5-FU+LCV treated samples), 3 labels (5-FU+LCV&fluoxetine treated samples) and 4 labels (fluoxetine treated samples).

HIPPOCAMPUS

A



B

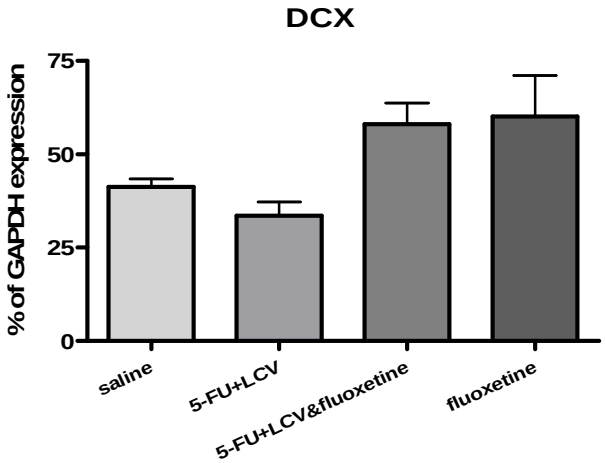
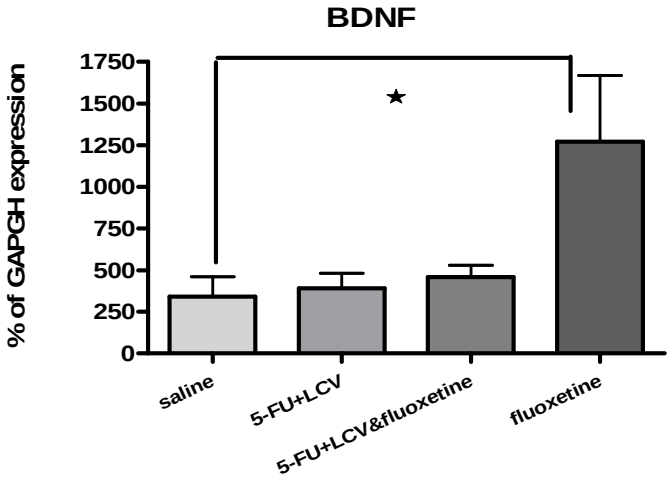


Figure 4.10. Graphic representation of levels of brain-derived neurotrophic factor (BDNF) and doublecortin (DCX) proteins in the hippocampus (Mean±S.E.M; n= 7-9). Graphs represent the mean amplitude of the wave lengths emitted from the protein bands relative to that of GAPDH protein that was blotted on the same gel. (A) BDNF levels in the hippocampus differed significantly between groups (p*=0.02, one way ANOVA with Bonferroni post-hoc test). There was a significant difference only between the5-FU+LCV and the combined 5-FU+LCV&fluoxetine-treated groups (P*<0.05). (B) DCX levels in the hippocampus were marginally different between groups (p=0.053, one way ANOVA with Bonferroni post-hoc test). There was no significant difference between group pairs (p>0.05).

FRONTAL CORTEX

A



B

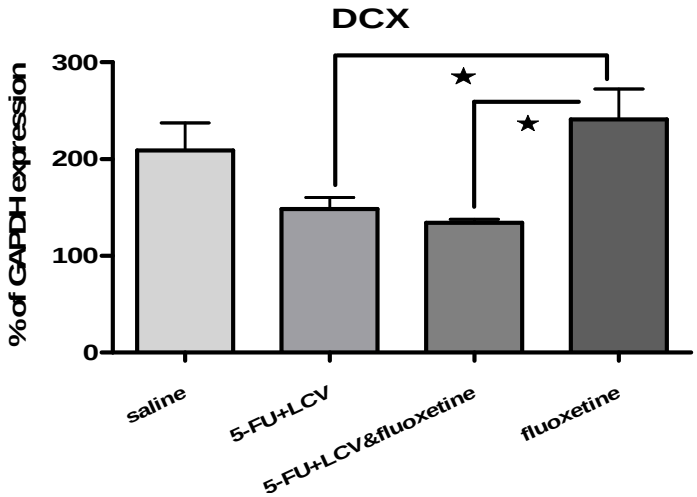


Figure 4.11. Graphic representation of levels of brain-derived neurotrophic factor (BDNF) and doublecortin (DCX) proteins in frontal cortex (Mean±S.E.M; n= 6-9). Graphs represent the mean amplitude of the wave lengths emitted from the protein bands relative to that of GAPDH protein that was blotted on the same gel. (A) BDNF levels in the frontal cortex differed significantly between groups (p*=0.019, one way ANOVA with Bonferroni post-hoc test). There was a significant difference only between the saline and the fluoxetine treated groups. (B) DCX levels in the frontal cortex differed significantly between groups (p=0.008, one way ANOVA with Bonferroni post test). There was a significant difference between the 5-FU+LCV and the 5-FU+LCV&fluoxetine-treated groups (P*<0.05). Also there was a significant difference in the level of DCX protein between the 5-FU+LCV and fluoxetine- treated groups (p*<0.05).**

4.4 DISCUSSION

Reports by patients undertaking chemotherapy have indicated that systemic chemotherapy can produce a wide range of cognitive symptoms including difficulties in concentration, attention and memory which have become collectively known as “chemobrain” (Wefel, Lenzi et al. 2004). These effects can have a significant impact on patient quality of life and in their ability to return to work (Ahles, Saykin et al. 2005; Scherwath, Mehnert et al. 2006). Psychometric testing of patients during and after systemic chemotherapy has confirmed patient reports and indicates that chemotherapy can produce a range of mild to moderate cognitive deficits which are dose dependent and can last for several years after the cessation of treatment (van Dam, Schagen et al. 1998; Anderson-Hanley, Sherman et al. 2003; Castellon, Silverman et al. 2005; Falleti, Sanfilippo et al. 2005). The symptoms described by patients after chemotherapy treatment almost always include deficits in declarative memory. These involve difficulties in the recall of both semantic (factual) and episodic (temporal) aspects of memory for which the hippocampal formation is known to be required (Eichenbaum, Yonelinas et al. 2007). Clinically, it has been reported that antidepressants can improve memory function in patients suffering from a variety of conditions (Horsfield, Rosse et al. 2002; Levkovitz, Caftori et al. 2002; Vermetten, Vythilingam et al. 2003). Similarly, in animal models, SSRI antidepressants have been reported to improve cognition after stroke or stress (Ramanathan, Kumar et al. 2003; Song, Che et al. 2006; Li, Cai et al. 2009) but not in normal animals (Stewart and Reid 2000; Yau, Hibberd et al. 2002). In the present study, chronic treatment with the SSRI fluoxetine was integrated with 5-FU+LCV chemotherapy to determine whether fluoxetine could alter the cognitive

and neuropathological changes induced by chemotherapy. Fluoxetine treatment improved the memory deficits caused by 5-FU chemotherapy in a spatial working memory paradigm. The proliferation aspect of neurogenesis was quantified and it revealed that fluoxetine treatment reversed the reduction in neurogenesis caused by the chemotherapy 5-FU+LCV. Conversely 5-FU+LCV chemotherapy did not change the level of BDNF protein in the hippocampus or frontal cortex compared to the control group. However, co-treatment of fluoxetine with 5-FU+LCV significantly altered the BDNF levels in the hippocampus from that of the 5-FU+LCV treated group whereas there was a marginally significant difference in the level of DCX protein in the hippocampus between all groups. Evaluating frontal cortex BDNF, showed a marked significant difference between the saline and the fluoxetine only-treated groups however there was a significant difference in the DCX protein level in the same tissue between the 5-FU+LCV chemotherapy and the combined 5-FU+LCV&fluoxetine treated groups. Also a significant difference was found in frontal cortex DCX level between the 5-FU+LCV chemotherapy and fluoxetine only-treated groups.

4.4.1. Fluoxetine and 5-FU reduced weight gain

Fluoxetine and 5-FU+LCV displayed a significant reduction in weight gain over the chronic treatment period (Fig 4.2). The effect of 5-FU+LCV chemotherapy on weight gain is a consistent one (Chapter 2 and 3) and it is largely associated with reduced ability of gastrointestinal tract to absorb nutrients due to cytotoxic damage to the proliferating cells lining the intestinal wall (Huang, Kemp et al. 2002). Fluoxetine also significantly reduced the body weight gain compared to those saline-treated as found in previous studies (Mancini and Halpern 2006). It

is possible that absorption of fluoxetine could have been affected by 5-FU+LCV treatment. Rats that had received both 5-FU+LCV chemotherapy and fluoxetine gained the least weight out of all four groups indicating that combining both drug treatments resulted in a compound effect on weight gain of the animals.

4.4.2. Fluoxetine and fluoxetine with 5-FU reduced water intake

Water bottles of animals were measured on a daily basis (Fig.4.3). The amount of daily water intake was calculated. There was a significant decrease in amount of water intake in (fluoxetine) and combined (fluoxetine&5-FU+LCV)-treated groups compared to the saline-treated animals following fluoxetine administration (Fig 4.3). It has been shown that fluoxetine reduces the amount of water intake by Sprage-Dawley rats (Lee, Lee et al. 2000; Rezvani, Overstreet et al. 2000). It seems that the reduction IN the amount of water intake in the 5-FU+LCV&fluoxetine-treated group was due to the effect of fluoxetine not 5-FU+LCV chemotherapy as there was no significant difference in the water intake between 5-FU+LCV-only treated animals and the saline-treated ones.

4.4.3. Fluoxetine improved the memory and neurogenic deficits caused by 5-FU chemotherapy

We have used the OLR test of hippocampal function as a means of assessing the cognitive effects of a therapeutically relevant dose of the commonly used chemotherapy agent 5-FU. This task has been shown to require an intact hippocampal dentate gyrus (Mumby, Gaskin et al. 2002). Confirming our previous results (Mustafa, Walker et al. 2008), these results show that 5-FU+LCV treatment caused deficits in this task. Control animals were readily able

to discriminate between the novel and familiar locations of objects and spent significantly more time on the objects in the novel location. In contrast 5-FU+LCV treated animals showed no significant difference in the time spent on the two objects and in fact spent more time on the object in the familiar location. The total distance moved (cm) and the mean velocity (cm/sec) performed by animals during the test were not different between all groups after treatment (Fig.4.4 [A, B]).

Animals receiving both i.v injections of 5-FU+LCV and fluoxetine in their drinking water, although showing an improvement in their discrimination between familiar and novel object locations, still did not significantly discriminate between these positions (Fig. 4.5). However calculation of the preference index, a measure of percentage of the combined exploration time of both novel and familiar locations of an object (Bruel-Jungerman, Laroche et al. 2005) showed a significant improvement in selecting the object in the novel location over the 5-FU+LCV treated animals (Fig. 4.6).

Regarding the effect of 5-FU chemotherapy on memory, one possible cause could be disruption of adult hippocampal neurogenesis. The production of new neurons in the SGZ for incorporation into the dentate gyrus, is a well-characterized phenomenon in all mammals including humans (Eriksson, Perfilieva et al. 1998; Imayoshi, Sakamoto et al. 2009). Reductions in cell proliferation in the SGZ by pharmacological, environmental or genetic means are associated with deficits in memory (Bruel-Jungerman, Laroche et al. 2005; Zhao, Deng et al. 2008; Jessberger, Clark et al. 2009). In the present study, we showed that 5-FU+LCV chemotherapy significantly decreased the number of proliferating cells in the SGZ of the dentate gyrus using immunohistochemistry

for the proliferative marker Ki67. Animals were put down four weeks after the start of drug treatment. Other studies have suggested that this period is long enough for changes in neurogenesis to affect behaviour (Abrous, Koehl et al. 2005; Lee, Kim et al. 2006).

In line with previous investigations on changes in neurogenesis this was associated with a decline in the performance of memory specific tasks. Understanding the effects of chemotherapy on patients is complicated by other aspects of the disease and treatment and cancer patients can exhibit cognitive impairments prior to treatment (Wefel, Lenzi et al. 2004). The present study shows that 5-FU can produce cognitive decline in the OLR behavioural test and that this is associated with a decrease in proliferation in the neurogenic region of the hippocampus. These results are in line with animal studies of other chemotherapy agents which have shown cognitive declines after treatment (Winocur, Vardy et al. 2006; Foley, Raffa et al. 2008; Gandal, Ehrlichman et al. 2008; Han, Yang et al. 2008; Konat, Kraszpulski et al. 2008; Mustafa, Walker et al. 2008; Seigers, Schagen et al. 2008). Our previous investigation showed that 5-FU+LCV produced a non-significant reduction in cell proliferation in the SGZ (Mustafa, Walker et al. 2008). The present investigation increased the number of injections which was sufficient to significantly reduce cell proliferation. Further, because our study was based on the whole dentate gyrus, future studies will have to investigate functional specificity of different regions of the dentate gyrus in controlling neurogenesis, such as ventral vs. dorsal areas of the hippocampus (Moser, Moser et al. 1993). Most reports have found that antidepressants increase proliferation of cells in the SGZ (Kodama, Fujioka et al. 2004) but recently there have been reports which have failed to find this effect (Cowen,

Takase et al. 2008; Holick, Lee et al. 2008). Our results show that co-treatment of fluoxetine with 5-FU+LCV abolished the reduction in cell proliferation in the SGZ caused by 5-FU+LCV and improved performance in the OLR test. Fluoxetine on its own had no effect on proliferation or behaviour. It is unlikely that the effects of fluoxetine are limited to neurogenesis in the hippocampus as several studies have shown positive effects on other brain regions associated with cognition notably frontal, cingulate and temporal lobes (Mayberg, Brannan et al. 2000; Brody, Saxena et al. 2001; Smith, Reynolds et al. 2002; Saxena, Brody et al. 2003). The action of fluoxetine on these regions as well as the hippocampus may therefore underlie its effects on cognition.

4.4.4. Co-treatment (fluoxetine&5-FU+LCV) elevated hippocampal BDNF

Western immunoblotting showed that 5-FU+LCV chemotherapy did not significantly change BDNF protein levels in the hippocampus compared to saline treatment (Fig.4.10 [A]). This effect is similar to that reported in the previous experiment (Chapter 3) and strengthens the hypothesis that the deteriorated cognition of the animals treated with 5-FU+LCV chemotherapy was mainly due to 5-FU-induced reduction of hippocampal neurogenesis as shown in the results (Chapters 3 and 4). However, fluoxetine co-treatment with 5-FU+LCV elevated hippocampal BDNF level from that of 5-FU+LCV only treated group. Surprisingly, fluoxetine on its own did not produce the same effect. It has been reported that chronic fluoxetine treatment up-regulates cellular BDNF expression in rats (Molteni, Calabrese et al. 2006). These effects are also consistent with the reports that fluoxetine has been shown to increase BDNF levels in the hippocampus of female mice (Engesser-Cesar, Anderson et al. 2007).

It could be that 5-FU+LCV chemotherapy non-significantly reduced the hippocampal BDNF levels from that of saline treatment and that co-treatment of fluoxetine with 5-FU could reverse this condition. This experiment showed that there was a marked significant increase in frontal cortex BDNF level compared to saline treated BDNF level. In line with this finding, it was found that frontal cortex BDNF level in female mice chronically treated with fluoxetine was significantly more than the same protein level in the frontal cortex of the controls (Mannari, Origlia et al. 2008). These findings suggest that 5-FU- induced behavioural changes were mainly due to the reduction in dentate gyrus neurogenesis caused by the chemotherapy drug while the improvement in cognition noticed in rats after co-treatment with fluoxetine could be attributed to two reasons. One of these is an increase of hippocampal neurogenesis and or alternatively an up-regulation of hippocampal BDNF level which is mainly linked to the improvement in both differentiation and survival aspects noticed after chronic antidepressants treatment.

4.4.5. Fluoxetine elevated hippocampal and Frontal cortex DCX

The results in the present experiment showed a significant difference in the DCX protein level in the frontal cortex tissue between the 5-FU+LCV chemotherapy and the combined 5-FU+LCV&fluoxetine-treated groups. Also a significant difference was found in frontal cortex DCX level between the 5-FU+LCV chemotherapy and fluoxetine only-treated groups. Moreover, hippocampal level of DCX protein was up-regulated by fluoxetine compared to both 5-FU+LCV and co-treatment (5-FU+LCV&fluoxetine)-treated groups. Considering that DCX is a transiently expressed protein which is found during

early mitosis and continues until 30 days of maturation (Marcussen, Flagstad et al. 2008), fluoxetine could have affected the protein level in hippocampal or frontal cortex dividing cells at early or late stages of 5-FU treatment (2 weeks). The same cause could be attributed to the significant increase in hippocampal or frontal cortex protein level in the combined treatment group compared to the 5-FU+LCV chemotherapy treated group. In agreement with these findings, it was found that fluoxetine treatment for 28 days increased levels of hippocampal neurogenesis as well as hippocampal DCX level in Wister rats (Marcussen, Flagstad et al. 2008). Similar findings were also observed by (Wang, David et al. 2008). Furthermore, (Namestkova, Simonova et al. 2005) found that fluoxetine reversed the reduced neurogenesis after testing rats for their response in water maze task. This was indicated by the increased number of DCX protein marker positive cells. Also there is evidence that chronic fluoxetine treatment targets early dividing cells in the dentate gyrus of the hippocampus as measured by increased cellular immuno-reactivity for the DCX marker (Encinas, Vaahtokari et al. 2006). In addition, it was not unexpected that fluoxetine up-regulates DCX (as an early proliferation marker protein) in frontal cortex tissue as there is evidence that cerebral cortex neuronal cells are positive expressers for DCX protein in animals (Xiong, Luo et al. 2008) as well as in humans (Varea, Castillo-Gomez et al. 2007). Previously, it has been demonstrated that fluoxetine treatment increases cellular proliferation in both hippocampus and frontal cortex (Kodama, Fujioka et al. 2004) as well as increases in hippocampus and frontal cortex reactivity to plasticity marker proteins in rats such as polysialylated nerve cell adhesion molecule (PSA-NCAM), phosphorylated cyclic-AMP response

element binding protein (pCREB) and growth-associated protein 43 (GAP-43) (Sairanen, O'Leary et al. 2007). Added to this, the fluoxetine-induced improvement in cellular proliferation in the co-treatment group in the dentate gyrus of the hippocampus compared to 5-FU+LCV only-treated group as measured by increased cellular immunoreactivity for the proliferative marker Ki67, these finding collectively support the observed increase in DCX protein (as an immature proliferative marker) in both hippocampus and frontal cortex as a result of chronic fluoxetine treatment.

4.5. CONCLUSION

In conclusion, our results show that 5-FU treatment caused cognitive deficits which were associated with a reduction in cell proliferation in the SGZ of the dentate gyrus. These deficits were reduced when fluoxetine was administered throughout the 5-FU+LCV treatment period. However, it is unclear how and when 5-FU chemotherapy exerts its effect on neurogenesis. For this reason, a new experiment (Chapter 5) was carried out in which the long term effects of 5-FU on cell proliferation and survival were quantified by combining BrdU tracking of cells born at the start of drug treatment with Ki67 quantification of cell proliferation at different time points after drug treatment.

Chapter 5

Effect of 5-Fluoruracil on survival and proliferation of the hippocampal rat brain dividing cells

5.1. INTRODUCTION

A substantial proportion of patients undergoing chemotherapy, for a variety of cancers, have reported problems with their memory, attention, and cognition which affected their daily lives profoundly (Rugo and Ahles 2003; Bower 2008). One of the puzzling features of the cognitive decline experienced by patients after chemotherapy is that many patient studies have shown that this condition can persist for up to several years after the completion of drug treatment (Castellon, Silverman et al. 2005; Falleti, Sanfilippo et al. 2005; Fan, Houede-Tchen et al. 2005; Scherwath, Mehnert et al. 2006; Bower 2008; Correa and Ahles 2008). Although all sufferers seem to ultimately make a full recovery, cancer survivors resume their educational, social and familiar roles suffering from a long-term side effect of their cancer treatment, which can have resounding effects on their lives (Ahles and Saykin 2002). This is especially prevalent in young survivors who still contribute socially and vocationally in their society and, as such, these patients are of particular interest, in terms of the prevalence of neurological and neuropsychological side effects (Anderson-Hanley, Sherman et al. 2003). There are some similarities to the long term pattern of symptoms found after chemotherapy with those found after cranial irradiation where cognitive symptoms can also persist and, in fact, increase after treatment and full remission from the original cancer (Soussain, Ricard et al. 2009). These treatment

strategies, chemotherapy and irradiation, designed to target cancer cells, are commonly associated with harmful effects on multiple organ systems, including the central nervous system (CNS).

Cancer patients may experience a wide range of neurotoxic adverse symptoms, including vascular complications, seizures, mood disorders, and cognitive dysfunction (Dietrich, Monje et al. 2008; Minisini, Pauletto et al. 2008).

Recent studies have stressed the biological basis of chemotherapy and radiation-associated CNS toxicity. The most vulnerable cell populations susceptible to these treatments are self – renewing neural precursor cells (NPCs), which are the direct ancestors of all differentiated cell types of the CNS, but which are particularly important in the neurogenic regions (SVZ and SGZ) and oligodendrocytes (myelin forming cells) precursors which are required for the maintenance of white matter tracts. Additionally non-dividing mature oligodendrocytes also appear to be susceptible and demyelination has been reported with both irradiation and chemotherapy (Dietrich, Han et al. 2006; Soussain, Ricard et al. 2009). In contrast, mature astrocytes and neurons are significantly less vulnerable at comparable doses of drugs or irradiation. In vitro studies have similarly shown that application of chemotherapy agents is associated with increased cell death of oligodendrocytes and NPCs (Doetsch, Caille et al. 1999; Dietrich, Han et al. 2006; Han, Yang et al. 2008).

It has been shown that the initial reduction in cell proliferation and increased cell death following a single dose of chemotherapy is followed by a marked rebound in cell proliferation. However, repetitive drug exposure resulted in long–term suppression of cell division and prolonged cell death in the subventricular zone, the hippocampus, and the white matter tracts (Dietrich, Monje et al. 2008).

We and others have shown that 5-FU treatment of rats, causes a reduction in neurogenesis in the dentate gyrus of rat brain hippocampus (Han, Yang et al. 2008; ElBeltagy, Mustafa et al. 2010) and that this is associated with cognitive decline (Mustafa, Walker et al. 2008; ElBeltagy, Mustafa et al. 2010). However, it is still not clear how the decrease in neurogenesis occurs or what its duration is. One possibility is that a reduction in the numbers of stem or progenitor cells in the SGZ during chemotherapy causes a prolonged reduction in the number of proliferating cells in this region (Eisch and Mandyam 2007). Similarly increased death of dividing cells may also contribute to the overall reduction in the number of these cells in the SGZ of the dentate gyrus (Arguello, Harburg et al. 2008).

In this part of the investigation into the effects of 5-FU on hippocampal neurogenesis, it was decided to look at the longer term (up to six weeks) effects of treatment and in addition to look at the survival of cells dividing at the start of chemotherapy treatment. A reduction in hippocampal neurogenesis due to decreased proliferation or survival of NPCs is a possible causative mechanism by which 5-FU could induce long term and progressive cognitive deteriorations in cancer patients.

To investigate this, 2 successive intra peritoneal injections of the exogenous S phase proliferative marker bromodeoxyuridine (BrdU) were given over two days immediately prior to 5-FU+LCV treatment. This was designed to mark cells dividing (specifically those in S phase) at the start of 5-FU+LCV administration. Comparison with the numbers of BrdU positive cells in control animals, given saline injections, at different time intervals after 5-FU+LCV treatment will

quantify the survival of this population proliferating cells. Animals were killed 24 hours, 2 weeks or 6 weeks after treatment with 5-FU+LCV.

Sections of hippocampus were stained for BrdU and the endogenous cell cycle marker Ki67. Comparison of Ki67 positive cell number with controls could indicate changes in cell proliferation at the different time intervals after treatment. The results demonstrate that the longer term rate of cell division and the survival of neurogenic cells are both highly sensitive to the effect of 5-FU, and this provides a novel insight into long term effects of 5-FU on neurogenesis in the rat brain. The causes of the long term effects of chemotherapy or radiotherapy are currently unknown (Soussain, Ricard et al. 2009). One suggestion is that these treatments induce an inflammatory response in the brain which persists after treatment has finished. It is known that inflammation reduces hippocampal neurogenesis (Ekdahl, Claasen et al. 2003; Monje, Toda et al. 2003; Bastos, Moriya et al. 2008) and is associated with cognitive impairment (Hein and O'Banion 2009).

The evidence for inflammation having a role in the inhibition of hippocampal neurogenesis is particularly strong after cranial irradiation where not only is there a significant increase in activated microglia, but treatment with a non steroidal anti inflammatory drug, partially prevents the reduction in cell proliferation in the SGZ after treatment (Monje, Toda et al. 2003; Monje, Vogel et al. 2007). Chemotherapy is not associated with particularly high levels of inflammation but 5-FU treatment has been reported to induce inflammation in peripheral tissues in animal models of chemotherapy (Logan, Stringer et al. 2008; Lopes, Plapler et al. 2009). This suggests that it could have a similar direct effect on the brain or that proinflammatory cytokines produced peripherally, could cross the blood brain

barrier and induce inflammatory changes indirectly. However two groups have recently published reports which failed to find evidence for inflammation in the brain after either 5-FU (Han, Yang et al. 2008) or MTX (Seigers, Timmermans et al. 2010) chemotherapy treatment. Han et al. used a marker for activated glia while Seigers et al. measured cytokine levels in plasma and brain. Although Seigers et al. found no significant changes in inflammatory cytokine levels, they did report changes in microglial morphology.

It was decided to look at a different inflammatory marker to these studies to see if treatment affected other aspects of the inflammatory response. As 5-FU has been reported to increase expression of the inducible prostaglandin producing enzyme cyclooxygenase 2 (COX-2) in peripheral tissues (Lopes, Plapler et al. 2009), it was decided to look at the effect of chemotherapy on the number of cells expressing this marker in the brain. COX-2 synthesises prostaglandins from arachidonic acid and is constitutively expressed at low levels in microglia and neurons in the intact brain but expression is strongly increased after infection or damage (Bastos, Moriya et al. 2008; Ahmad, Zhang et al. 2009). Interestingly COX-2 expressing microglia appear to be closely associated with dividing cells in the neurogenic regions of the brain and inhibition of COX-2, with specific inhibitors, under non-inflammatory conditions, strongly reduces neurogenesis (Goncalves, Williams et al. 2010). These results suggest that prostaglandin synthesis by COX-2 is required for neurogenesis in non-inflammatory conditions but that increased COX-2 expression during inflammation is associated with decreased neurogenesis (Ekdahl, Kokaia et al. 2009). As part of the investigations into the longer term effects of 5-FU treatment, it was decided to quantify the number of COX-2 expressing cells in both the dentate gyrus itself

and the hilus of the dentate gyrus by immunohistochemistry at different time points after drug treatment.

5.2. MATERIALS AND METHODS

5.2.1. Animals and drug administration.

All experiments and animal care were in accordance with UK Animals (Scientific procedures) Act 1986. All experiments were on male Lister hooded (LH) rats (Charles River UK) of 150-170gms at the start of experiments and animals were weighed daily. Animals were allowed to habituate to the animal house for 2 weeks before treatment and housed in groups of 4 under standard conditions of 12-h light – 12-h dark cycle (light from 8.30am to 8.30 pm) with free access to food and water. Doses given of 5-FU are within the range which reduces tumour load in rats (Au, Walker et al. 1983; Watson, Michael et al. 1998), and are the standard doses used to investigate the effects of 5-FU which are within the human dose range (Reagan, Nihal et al. 2008).

48 L.H male rats were randomly divided equally into 2 main groups:

Group (A) Controls received 6 intravenous (i.v) injections of (0.9% normal) saline under isoflurane anaesthesia every other day over two weeks.

Group (B) received 6 intravenous (i.v) bolus injections of 5-FU 20mg/kg (MAYNE Pharma PLC) together with leucovorin, 20mg/kg (TEVA UK LTD. into the tail vein under isoflurane anaesthesia. All animals were injected intraperitoneally (i.p) with 2 successive BrdU (Sigma, St. Louis; in 0.9% saline) 150mg/kg each. one day before treatment started. On the last day of treatment, all animals were subdivided into 6 subgroups as follows:

Group A1 and B1: 1st controls and 5-FU+LCV respectively (8 animals each) were killed one day after treatment

Group A2 and B2: 2nd controls and 5-FU+LCV respectively (8 animals each) were killed two weeks after treatment

Group A3 and B3: 3rd controls and 5-FU respectively (8 animals each) were put down 6 weeks after treatment.

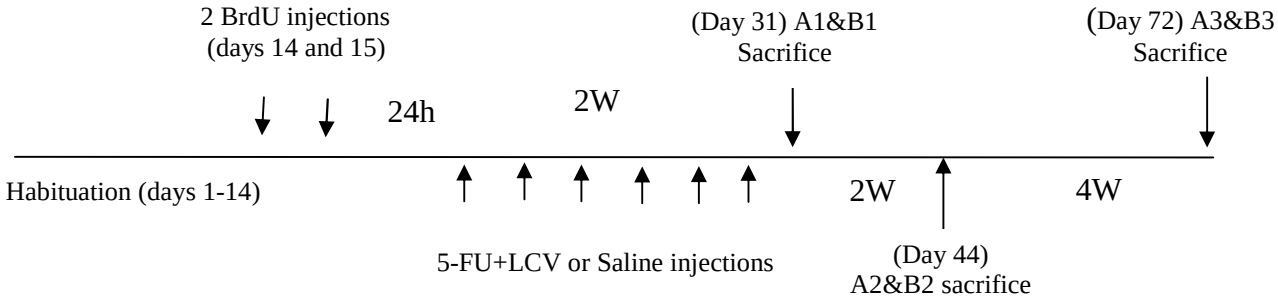


Figure 5.1, a protocol line of the study is presented above. Rats received 2 intraperitoneal injections of BrdU 24 hours before injecting them with sterile normal saline or 5-FU+LCV (6 intravenous injections over 2 weeks in a dose of 20 mg/kg). Groups of animals were killed one day, two weeks and 6 weeks after treatment as indicated on the figure.

5.2.2. Brain tissue preparation

Rats were killed by rapid stunning followed by decapitation. 40 brains were randomly chosen for immnohistochemistry. These brains were divided sagittally; one half was cryoprotected in a 30% sucrose solution for 3 hours at 4°C. Before embedding in OCT (VWR International Ltd, Lutterworth, UK), embedding medium for frozen tissue specimens and stored at -80°C. Frozen brains were serially sectioned (20-μm) in the coronal plane from Bregma point-2.12 to -6.08 mm(Paxinos and Watson 1997) to include the entire hippocampus using a Leica CM 100 cryostat (Leica Microsystems, Knowlhill, UK). Sections were mounted onto 3-aminopropyl-methoxysilane (APES) - coated slides and stored at -80 for immunohistochemistry which was done according to the manufacture's protocols. The other half of brains were frozen at -80ºc for Western blotting.

5.2.3. Immunohistochemistry

A systemic random sampling technique (Mayhew and Burton 1988) was used to choose every 21st section throughout the length of the dentate gyrus (overall 10 sections).

For Ki67 staining, sections were rinsed in PBS followed by fixing with 0.5% paraformaldehyde (PFA) for 3 minutes, before incubation with monoclonal mouse anti-Ki67 (1:100); Vector Laboratories, Orton Southgate, UK) for 1 h, followed by 1h incubation with Alexa 566 (Invitrogen/molecular Probes, UK) goat anti-mouse secondary antibody (1:300).

For BrdU staining, sections were washed three times in PBS and then fixed with 4% PFA for 3 minutes. Sections then were incubated in 2M HCL containing 0.3% Triton X100 for 20 min at room temperature (r.m.) then with 5M HCL for

10 minutes to open the DNA structure of the labelled cells. Immediately after acid washes, borate buffer (0.1M; pH 8.5) was added to neutralise the acid for 12 mins at r.t. Sections were then blocked in 0.1M PBS (Ph 7.4) + 1% TritonX100 + 5% normal goat serum (1h) prior to incubating with sheep anti-BrdU (Abcam,UK 1:100 in the blocking solution) for 1hr. Samples are then incubated with anti-sheep secondary (Vector labs. UK 1:300) for 1h at r.t. Washing in-between steps was done with 0.1M borate solution (3x5 mins).

For COX-2 staining, sections were rinsed in PBS and fixed in 2% PFA, washed in PBS and incubated with polyclonal anti-COX-2 AB(Abcam 15191-500) diluted 1:100 in PBS for 1hr. After washing, sections were incubated in secondary antibody, goat, anti-rabbit AB (Alexa fluro 546) diluted 1:300 in PBS for 1hr.

For Ki67, BrdU and COX-2 staining, diaminobenzidine (DAPI) nuclear stain (Vector shield, mounting medium in glycerol) was used as a counterstain and sections were viewed at 40x magnification on a Nikon EFD-3 fluorescence microscope. Ki67/BrdU-positive cells were counted within the SGZ, defined as within 3 cell diameters of the inner edge of the dentate gyrus (Mustafa, Walker et al. 2008). COX 2-positive cells were counted in both the blades and the hilus of the DG. Counts from all sections of one dentate gyrus were averaged to provide the number of positive cells per section (Mustafa, Walker et al. 2008; ElBeltagy, Mustafa et al. 2010).

5.2.4. Statistical analysis

All statistical parameters were calculated using Graph pad Prism 4.0 software, USA. Unpaired student *t*- tests (two-tailed) was used to compare the number of immunohistochemically positive cells between controls and 5-FU+LCV-treated groups for each time interval. One and two- way ANOVAs with Bonferroni post-hoc tests were used to analyse data of the proliferating and survival cell count. Repeated measures two way ANOVA with Bonferroni post-hoc tests were used to analyse the weights of the animals. A probability level of $P<0.05$ was considered statistically significant.

5.3. RESULTS

5.3.1. Effect of treatment on weight of animals:

As shown in Fig 5.2, animals on 5-FU+LCV showed a loss in body weight during the drug administration period as shown previously in studies in Chapters (2-4). This effect is a well known side effect of 5-FU attributed to effects on the gastrointestinal tract lining (Huang, Kemp et al. 2002) and although they showed improved weight gain after the end of drug treatment (data not shown); they were still significantly below control weights.

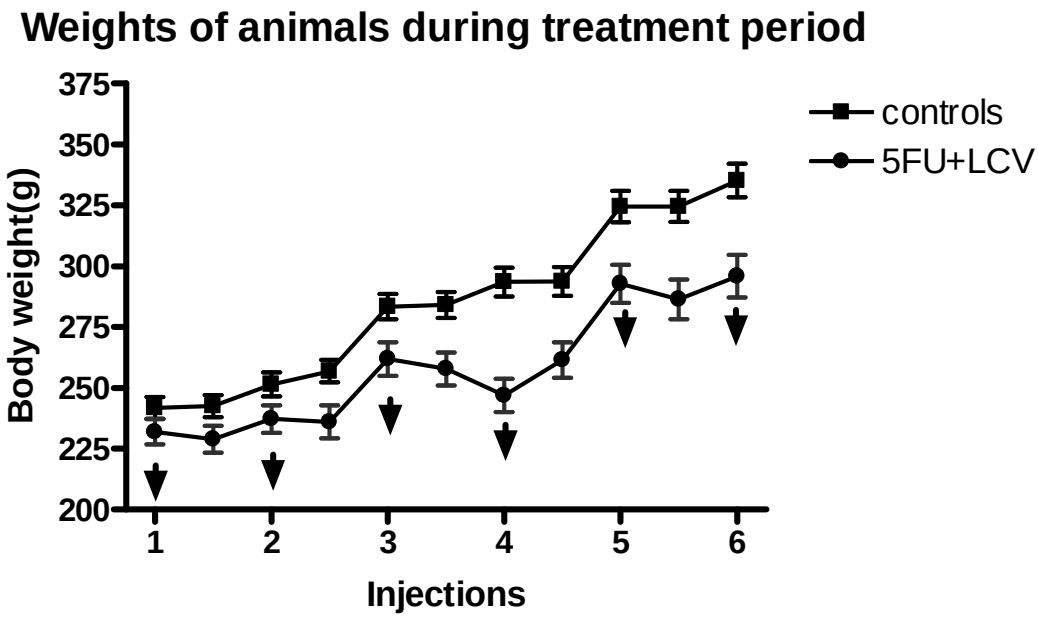


Figure 5.2, Body weight of rats during the treatment period of the experiment (Mean±S.E.M; n=24 each group). There was a significant difference in weights between the two groups throughout the whole time (p*<0.0001; repeated measures two- way ANOVA with Bonferroni post test). 5-FU+LCV treatment significantly reduced body weight gain throughout the treatment period (p**=0.003; repeated measures two- way ANOVA with Bonferroni post test). Also the interaction between both time and treatment was significant [p***<0.0001; repeated measures two- way ANOVA with Bonferroni post-hoc test). The injections are indicated on the graph by arrows and on X axis by numbers.**

5.3.2. Effect of 5-FU treatment on the rate of proliferation of the rat brain dividing cells:

Ki67 positive cells were found in the SGZ of the dentate gyrus (Fig 5.3). As shown in the figure, control animals showed no variation in the number of dividing cells between post-mortem time points. 5-FU+LCV treated animals showed no difference in the mean Ki67 positive cell counts in the SGZ of the dentate gyrus of the hippocampus from the controls one day post-treatment (P=0.3). However 2 weeks after treatment, there was a marked and significant reduction in these numbers (P**=0.008). This reduction still persisted even 6 weeks post treatment but at a lower significance level (P*=0.02). There was a tendency for a progressive reduction in the mean number of Ki67 positive cell counts in the 5-FU+LCV treated groups with time; however, this was not significant (P>0.05).

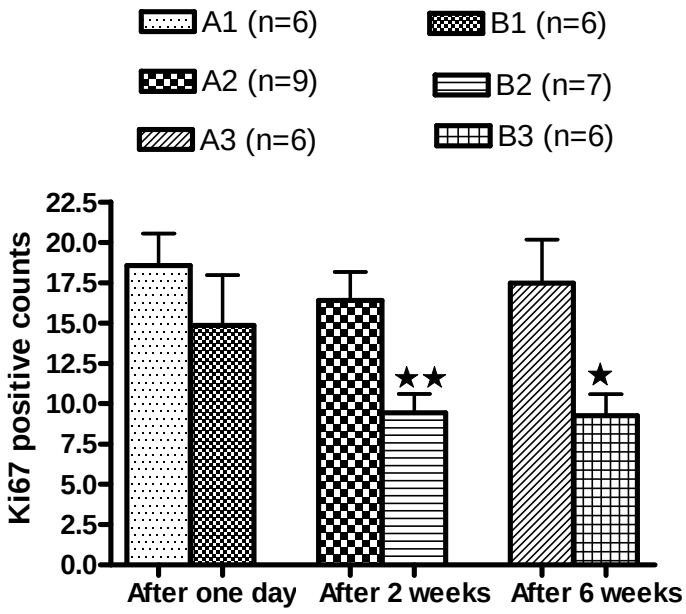
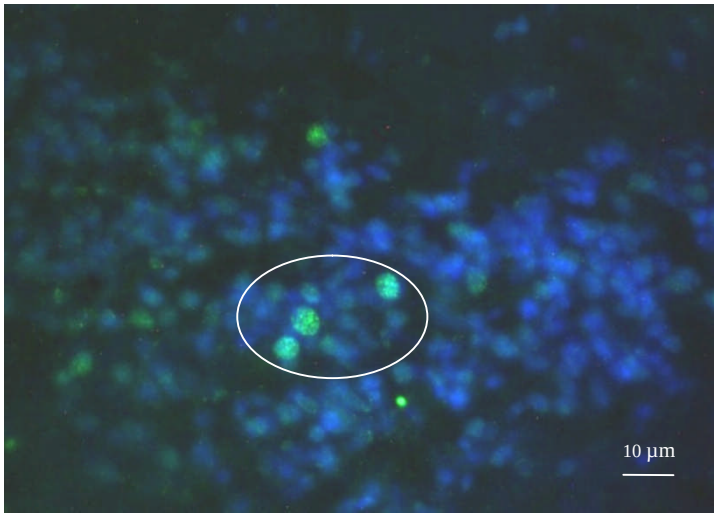


Figure 5.3, Ki67 positive cell count (Mean ± SEM; n=6-9). Control animals showed no significant difference in the numbers of dividing (Ki67-positive) cells over time. There was no difference in the mean Ki67 number between controls and 5-FU+LCV treated one day after treatment (P=0.3). In contrast there was a marked and significant difference in the number of Ki67 positive cells two weeks after treatment (p=0.008) and 6 weeks after the end of treatment (p*=0.02). One way ANOVA with the Bonferroni post-hoc test was used to compare between all groups and unpaired t-test (two-tailed) was used to compare the counts between controls and 5-FU+LCV-treated groups of each time point separately.**

5.3.3. Effect of 5-FU treatment on the survival of cells dividing at the start of treatment:

BrdU had been injected prior to the start of 5-FU+LCV treatment and BrdU positive cells were detected at all ages in the inner edge of the blades of the dentate gyrus. As shown in Fig 5.5, control animals showed no significant change in the number of BrdU positive cells with time although the numbers at 6 weeks showed a non-significant decline. 5-FU+LCV-treated animals, unlike the results, from Ki67 quantification, showed a significant reduction in the mean BrdU positive cell counts in the SGZ of the dentate gyrus of the hippocampus compared with controls one day after the end of treatment (P*=0.01) . Moreover, after 2 weeks from treatment, there was still a marked significant reduction in these numbers (P***=0.0003). This marked reduction persisted even 6 weeks after treatment (P***=0.0002).

A



B

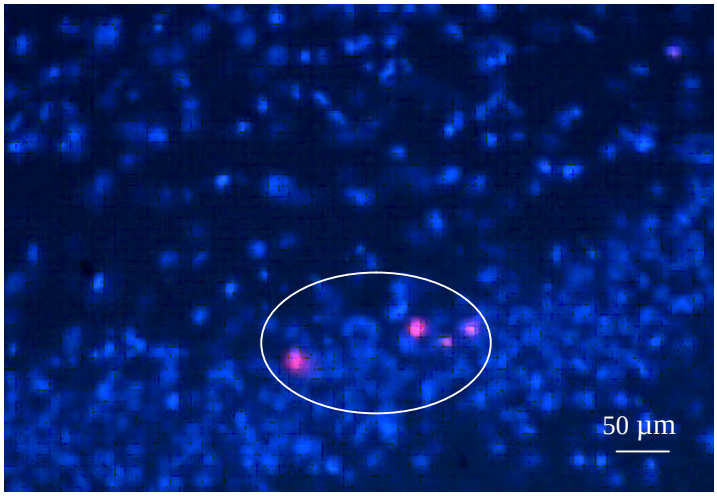


Figure 5.4, Representative images of rat dentate gyrus by fluorescence microscope. (A) Proliferating cells (circled) within the dentate gyrus are immunostained using BrdU (green) or Ki67 (red) (B). DAPI nuclear staining of the dentate gyrus is observed under the ultra-violet light filter of the fluorescence microscope in both A and B.

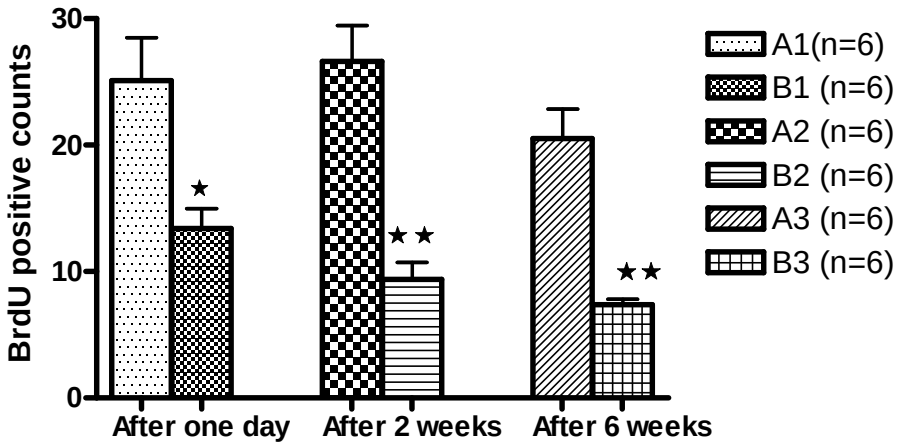


Figure 5.5, BrdU positive cell counts in all groups (Mean ± SEM; n=6-9). BrdU positive cells in control groups, show no significant variation during the period of investigation. There was asignificant reduction in the mean number of BrdU positive cells in 5-FU+LCV treated animals compared with the controls one day after treatment (p*=0.01), Also there was a marked and significant reduction in the number of BrdU positive cells two weeks after treatment between controls and 5-FU+LCV treated groups (p*=0.0003). After 6 weeks from treatment there was still a marked significant reduction between control and 5-FU+LCV treated groups in the mean numbers of BrdU positive cell counts (p***=0.0002). One way ANOVA with Bonferroni post-hoc test was used to compare between all groups and unpaired t-test (two-tailed) was use to compare the counts between controls and 5-FU+LCV- treated groups of each time point separately.**

5.3.4. Effect of 5-FU on the rate of both survival and proliferation of the rat dentate gyrus dividing cells (Fig 5.6 and 5.7):

Comparing the effect of 5-FU+LCV treatment on both the survival and proliferation of cells in the SGZ of the dentate gyrus between all groups, there was a highly significant difference in the mean numbers of both Ki67- and BrdU- positive cell counts between all controls and all 5-FU+LCV-treated groups (P***<0.0001). Also the effect of time was significant (P*=0.04) indicating that the numbers of positive cells were significantly lower in the animals killed two weeks after treatment compared with those killed after one day and the same numbers were significantly much lower in the animals killed 6 weeks after treatment from those killed 2 weeks after treatment. However, the effect of 5-FU+LCV treatment on survival (BrdU) of the dentate gyrus dividing cells was not found to be different from its effect on proliferation (Ki67) as indicated from the mean Ki67/BrdU positive cell counts (P>0.05). The analysis was done by using two-way ANOVA with Bonferroni post-hoc tests to compare between all groups.

Fig 5.6

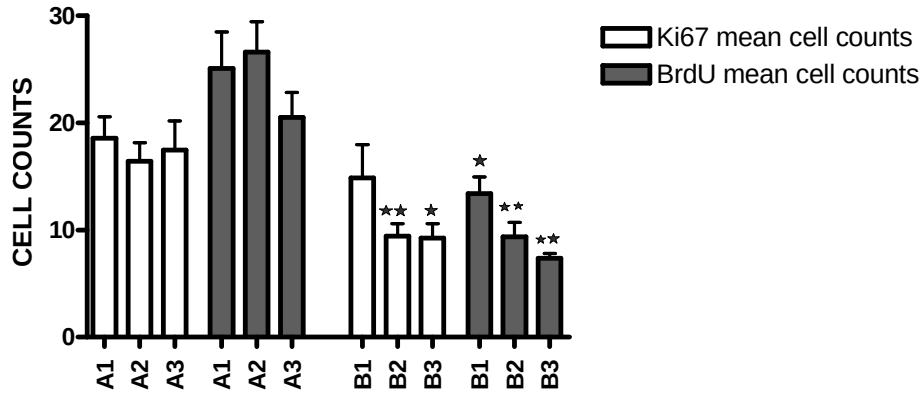


Fig 5.7

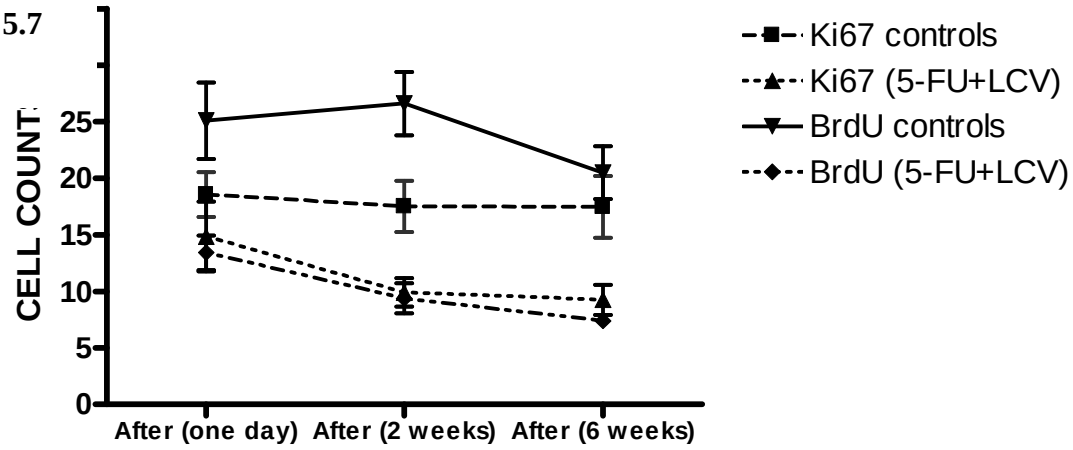


Figure (5.6) and (5.7) Ki67 and BrdU positive cell counts between all groups during the three different time intervals (Mean ± SEM; n=6-9). (A) There was a significant difference in the mean numbers of either Ki67 or BrdU positive cell counts between all controls and all 5-FU+LCV treated groups except for the Ki67 cell count between A1 and B1 which was not significant (P*<0.0001; One-way ANOVA with Bonferroni post-hoc test. (B)The effect of 5-FU+LCV treatment on survival (BrdU) of the dentate gyrus dividing cells was not different from its effect on proliferation (Ki67) as indicated from the mean Ki67/BrdU positive cell. The effect of time on either Ki67 or BrdU cell counts was significant (P*=0.04). The interaction**

between time and treatment was not significant; Two-way ANOVA with Bonferroni post-hoc test was used to compare between all groups.

5.3.5. Effect of 5-FU treatment on the number of COX2-labelled cells

COX-2 positive cells were found in both the blades of the dentate gyrus and the region of the hilus between the blades (Fig 5.8 and 5.9). The number of cells per section was counted, averaged from all sections and results are shown in Fig 5.10. One day after the end of treatment the number of COX-2 positive cells was significantly greater in the blades of the treated animals compared to controls. No difference was found at other ages or for counts of COX-2 positive cell numbers in the hilus.

A B C

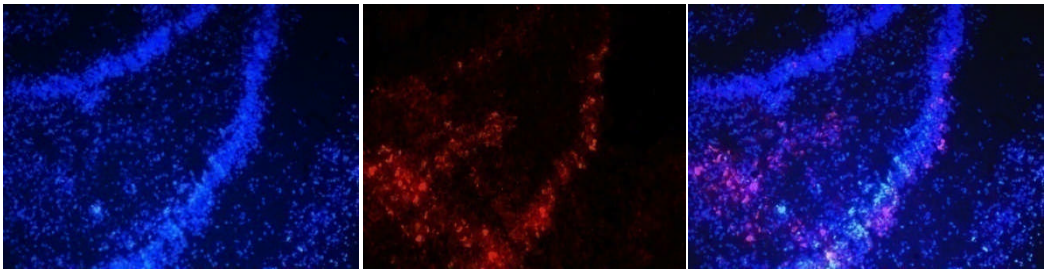


Fig 5.8, Representative images from the dentate gyrus of a control rat 24 hours after completion of 5-FU treatment. A. DAPI staining showing all nuclei in the dentate gyrus and hilus. B. COX-2 immunostaining. C. Merged image of A and B.

A B C

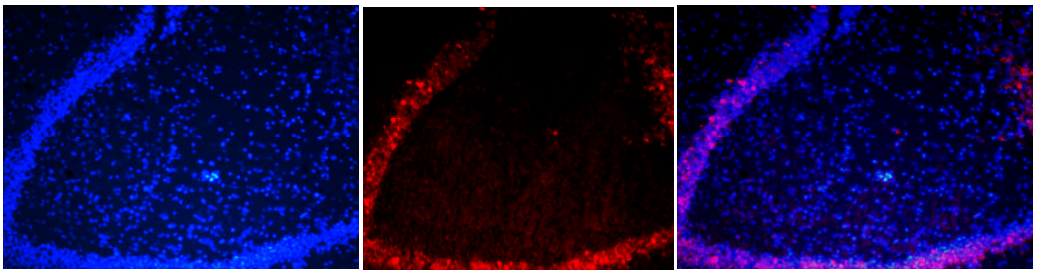
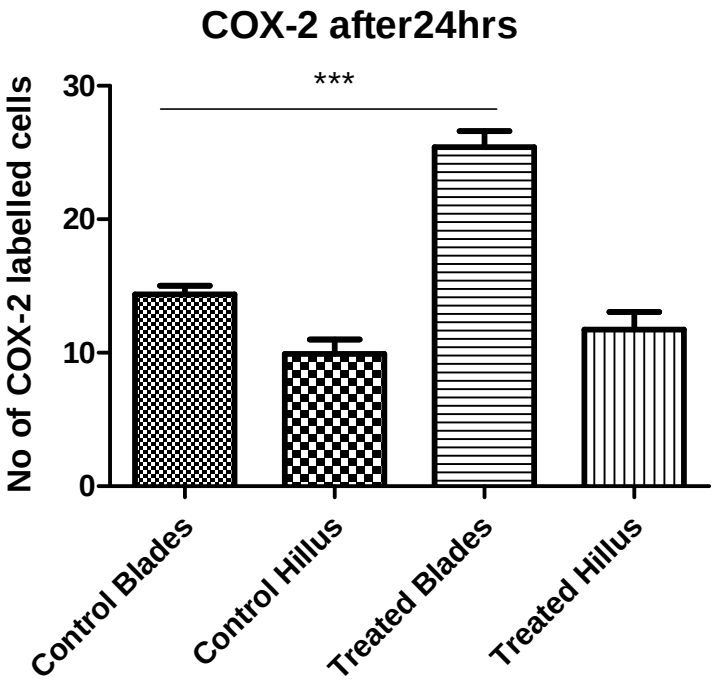
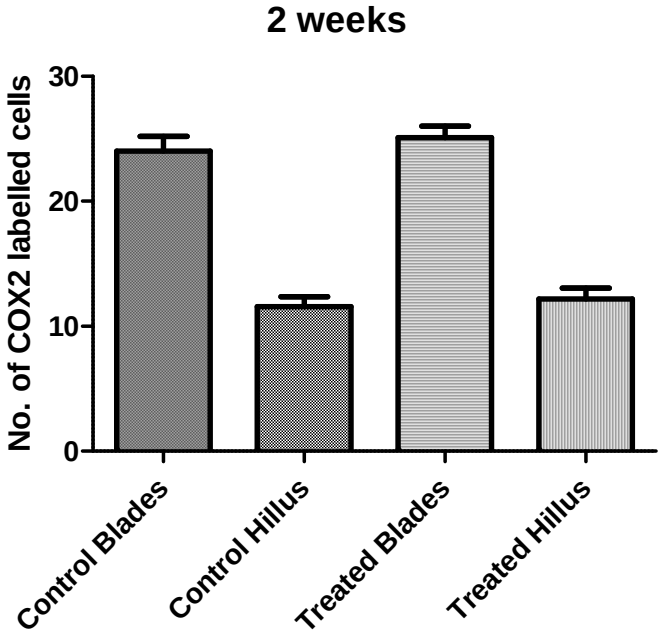


Fig 5.9, Representative images from the dentate gyrus of a 5-FU treated rat 24 hours after completion of 5-FU+LCV treatment. A. DAPI staining showing all nuclei in the dentate gyrus and hilus. B. Cox-2 immunostaining. C. Merged image of A and B.

A


COX-2 after24hrs
***
No of COX-2 labelled cells
30
20
10
0
Control Blades
Control Hillus
Treated Blades
Treated Hillus

B


2 weeks
No. of COX2 labelled cells
30
20
10
0
Control Blades
Control Hillus
Treated Blades
Treated Hillus

C

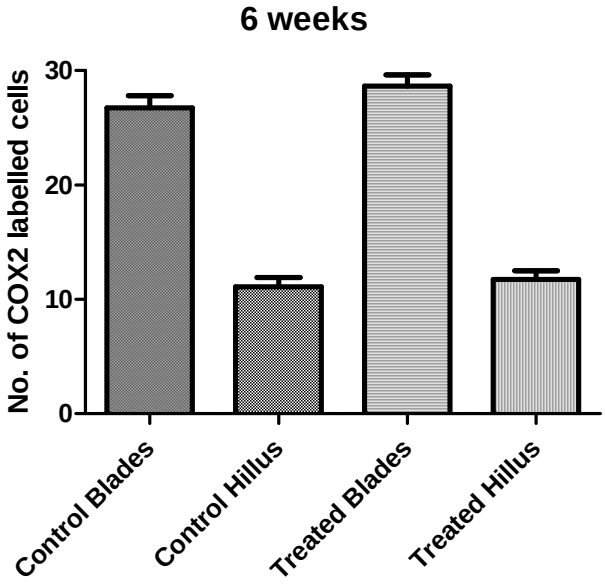


Fig 5.10 A, B and C. Numbers of Cox-2 positive cells per section in control and 5FU+LCV treated animals at different time points after the end of treatment (Mean ± SEM; n=6-9). At 24 hrs the number of COX-2 positive cells was significantly higher in the dentate gyrus blades of treated animals compared to the same number in the dentate gyrus blades of control animals (P*<0.0001; One-way ANOVA with Bonferroni post test). No difference was found at other ages or in the counts for cells in the hilum.**

5.4. DISCUSSION

It is now well established that new neurons are produced in discrete regions of the adult brain throughout life. It has been shown that although some neurons die in the dentate gyrus, the total number of granule cells increases during adulthood (Imayoshi, Sakamoto et al. 2009). Therefore, it has been suggested that neurogenesis is the cause of this increase in the number of neurons in the dentate gyrus of the hippocampus (Bayer, Yackel et al. 1982; Boss, Peterson et al. 1985; Dayer, Ford et al. 2003; Kempermann, Gast et al. 2003). The rate of adult neurogenesis can be up or down-regulated by many factors (Kempermann 2006). In our previous investigation we showed that, 5-fluorouracil, a well known chemotherapeutic agent caused a reduction in cell proliferation in the SGZ of the adult rat brain and that this was accompanied by cognitive deteriorations which manifested as an inability of the rats to perform hippocampal-specific behavioural tasks (Mustafa, Walker et al. 2008; ElBeltagy, Mustafa et al. 2010). Numerous neurogenesis studies have demonstrated that SGZ cells of the dentate gyrus in different stages of progenitor cell maturation are sensitive to physiological and pharmacological stimuli (Kronenberg, Reuter et al. 2003; Encinas, Vaahtokari et al. 2006; Mandyam, Crawford et al. 2008; Mandyam, Wee et al. 2008). Although the effects of stimuli such as running, antidepressants, and environmental enrichment on the maturation of progenitor cells have been well established (Ming and Song 2005; Plumpe, Ehninger et al. 2006; Wang, David et al. 2008), the effect of 5-FU on proliferation and or survival had not been assessed. In the present work, we aimed to examine the effect of 5-FU on the survival and proliferation of the rat brain dentate gyrus dividing cells at different time intervals

using the two markers, Ki67 and BrdU. Ki67-positive cell numbers indicated the rate of cell proliferation at different time points while BrdU-positive cell numbers indicate the survival of cells which were dividing at the start of 5-FU treatment. We found that 2 weeks of 5-FU+LCV treatment did not significantly reduce the number of SGZ proliferating cells one day after treatment had finished. However, proliferating cell number was significantly reduced, compared to controls two weeks after the end of treatment. This significant reduction was still present even 6 weeks after treatment. This result is surprising as it suggests that 2 weeks of 5-FU+LCV treatment does not have an immediate effect on the rate of cell proliferation in the SGZ but rather has a delayed effect such that cell proliferation is only significantly reduced two weeks after treatment. This result is in line with our previous investigation which showed that the number of Ki67-positive cells in the SGZ was significantly reduced 2 weeks after 5-FU+LCV treatment compared to controls (ElBeltagy, Mustafa et al. 2010). Other groups have reported similar findings after systemic application of thiotepa (Mignonea and Weberb 2006) methotrexate (Seigers, Schagen et al. 2008) and 5-FU (Han, Yang et al. 2008). Systemic application of these drugs was associated with a dose-dependent inhibition of hippocampal cell proliferation *in vivo*. In addition, methotrexate has been shown to impair cognitive performance in animal models (Winocur, Vardy et al. 2006; Seigers, Schagen et al. 2008). In breast cancer patients, CMF chemotherapy is associated with cognitive deteriorations (which likely reflect an inhibition of neurogenesis) that are mostly noticed from 1 month to several years after treatment (Wieneke and Dienst 1995; Schagen, van Dam et al. 1999; Kreukels, Schagen et al. 2005). The BrdU results showed that the survival of cells dividing immediately prior to 5-FU+LCV treatment was reduced

at the earliest time point studied (one day after 5-FU treatment). However, the degree of this effect was more obvious with time (2-6 weeks from treatment) as indicated from the BrdU-positive cell counts. These results show that 5-FU has the ability to increase the level of neurogenic cell death inside the hippocampus early during treatment and that the level of cell death increases with time even after cessation of treatment.

Taken together, the Ki67 and BrdU results indicate that 5-FU+LCV treatment decreases the survival of newly born cells both during treatment and for some time afterwards while its effects on cell proliferation appear to be delayed and, becoming significant several weeks after the end of treatment.

The delayed and continuing effects of chemotherapy treatment found in the present study may provide an explanation for the patient reports of prolonged cognitive impairment. The mechanism behind these effects remains unclear but it has previously been reported that 5-FU exposure induces G1/S phases arrest and apoptosis by a P53-dependent molecular pathway, in HPV 18 positive cells (Didelot , Mirjolet et al. 2003). Moreover, Dietrich et al. found that three intraperitoneal injections of the alkalylating agent in chemotherapy, carmustine (BCNU) significantly increased cell death for up to 10 days after treatment in the corpus callosum and the hippocampal dentate gyrus, and up to 6 weeks in the sub-ventricular zone. Similar effects were noticed after 3 intraperitoneal injections of cisplatin (Dietrich, Han et al. 2006; Seigers, Schagen et al. 2008).

In this chapter a marker for inflammation, COX-2, was quantified over the 6 week period after 5-FU+LCV treatment. Although there was a significant difference between COX-2 cell numbers in treated and control groups, one day after treatment (Fig 10 A), this is due to a reduction in the number of COX-2

positive cells in the control group at this age compared to other ages. There appears to be no change in COX-2 positive cell number across the three ages studied in the treated animals and there is no significant difference between controls and treated groups at 2 and 6 weeks. It is possible that the low numbers of COX-2 cells in the controls at one day are due to stress as a similar effect , in this case a reduction in cytokine levels, was found by Seigers et al. in controls immediately after treatment, but further work will be need to prove this (Seigers, Timmermans et al. 2010). Overall it does not seem that 5-FU+LCV treatment has increased COX-2 positive cell numbers compared to controls. This would suggest that prolonged inflammation is not the cause of the long term depression in cell proliferation and survival found in this chapter. As noted in the introduction, several other groups have also looked for inflammation within the brain after chemotherapy treatment and in agreement with the results found here, have failed to demonstrate that this is occurring.

Another possible mechanism by which survival of the dentate gyrus dividing cells could be affected is the possible alteration of the dentate gyrus BDNF levels. It has been demonstrated that incubation with BDNF increases the differentiation of progenitor cells into neurons but it does not directly influence the division of progenitor cells (Palmer, Takahashi et al. 1997). Moreover, increased apoptosis among hippocampal progenitor cells and excessive neuronal cell death were reported in BDNF knockout mice (Linnarsson, Willson et al. 2000), indicating that BDNF is essential for the survival of neurons especially those which continuously regenerated in the brain (Lee, Kim et al. 2006). Furthermore, one of our studies has shown that rat brains hippocampal BDNF levels were reduced by 2 weeks of 5-FU treatment which were not associated with an immediate

reduction in the number of SGZ proliferating cells (Mustafa, Walker et al. 2008). Indeed, both mechanisms (reduced hippocampal neurogenesis and hippocampal BDNF levels) could be included in 5-FU effect on the survival of dentate gyrus dividing cells and further investigations on the dentate gyrus BDNF levels should be done to establish this effect.

The continued loss of cells which had been marked with BrdU at the start of 5-FU+LCV treatment indicates that cells dividing at the start of treatment had a lower survival after treatment compared to controls. Treatment of dividing cells with 5-FU has a number of effects the most important of which is thought to be the inhibition of the enzyme thymidylate synthase however in addition, it has been shown that 5-FU causes the incorporation of the RNA base uracil into genomic DNA during S phase of cell division. Failure to repair this has been suggested as a cause of subsequent cell death if the cell attempts to re enter mitosis (Wyatt and Wilson 2009). This mechanism could explain the continued loss of cells marked at the start of drug treatment.

5.5. CONCLUSION

Overall, this chapter shows that 5-FU+LCV treatment causes prolonged effects on neurogenesis in the hippocampus. Further work on whether behaviour is also affected over this time scale will need to be done but, if shown, will indicate a possible mechanism for the long term effects of chemotherapy on cognition.

CHAPTER 6

GENERAL DISCUSSION

The studies presented in this thesis are a continuation of a project which aims to investigate the possible effect of chemotherapy on memory and neurogenesis by developing and applying an animal model. The process of adult hippocampal neurogenesis is now well established and studies using animal models have shown that it is involved in an increasing number of normal and pathological processes and that modulation of adult neurogenesis may open new doors to therapeutic treatments (DeCarolis and Eisch 2010). Cancer patients, especially young patients, often complain of cognitive problems, especially those related to memory, attention and concentration which produce adverse effects on their lives. This has led to many clinical investigations focusing on the underlying causative mechanisms for this cognitive deterioration. Clinical cancer studies have ascribed these cognitive deteriorations to the disease itself, the possible side effects caused by drug combinations or to the metastasis of the original cancer into the CNS (see references in the introduction to Chapter 4). However the most accepted theory is that the chemotherapy itself is the most likely cause of these cognitive symptoms. One of the drugs frequently associated with these CNS symptoms is 5-fluorouracil (5-FU), used throughout the present thesis. This drug has been consistently associated with descriptions of cognitive impairment by cancer patients, especially breast cancer survivors. 5-FU can be used either alone or in combination with other chemotherapeutic drugs especially cyclophosphamide and methotrexate in CMF combination therapy.

Previous studies by the Nottingham group have provided an indication that 5-FU chemotherapy affects spatial working memory and the formation of newborn neurons in the adult rat hippocampus (Mustafa, Walker et al. 2008). The present thesis extends these studies and further work is continuing to investigate the cytotoxic effects of this drug on cell proliferation, differentiation and survival during adult neurogenesis.

Quantification of hippocampal-specific memory was undertaken using the object location recognition test (OLR) which measures spatial working memory and the conditioned emotional response test (CER) which measures contextual fear conditioning to unpleasant stimuli. Both of these two memory tests have been shown to require input from the hippocampus (Mumby, Gaskin et al. 2002; Resstel, Joca et al. 2006; Mustafa, Walker et al. 2008). Adult neurogenesis is affected by a number of external or internal modulators. One of the most widely studied classes of pharmacological modulators of neurogenesis is the SSRI antidepressants such as fluoxetine. For this reason, we examined the effect of fluoxetine, which has been shown to improve both neurogenesis and memory in both clinical and animal studies. In particular it is important to know when and how both 5-FU and fluoxetine exert their effects on neurogenesis and memory which in turn will help to improve future animal models and aid in establishing the potential role of antidepressants as a cognitive enhancer in clinical chemotherapy studies. In order to do this, we followed cell survival using BrdU marking of dividing cells to determine how and when they are affected by chemotherapy.

6.1 SUMMARY OF RESULTS (table 6.1)

The preliminary experiments, testing the effect of 5-FU on memory using OLR and CER behavioural tests are detailed in the pilot studies (Chapter 2 Experiment1). The OLR test did not show a novel response even in the control group while CER test showed a subtle deficit in the 5-FU treated group. Because of the inability of control animals to perform the OLR task, it was necessary to design a separate experiment in which optimization of these two tasks was done before continuing to use them in the following studies in this animal model. This was achieved in chapter 2, Experiment 1 where control animals were able to successfully undertake both the OLR and the CER tasks taking into consideration the methodological problems discussed in chapter 2. Animals spent significantly more time exploring the new location of the object more than the old location. Furthermore, the shocked animals measured freezing time was significantly more than the freezing time of non-shocked animals. The choice and use of these cognitive tests are discussed in section 6.2 below. The results in chapter 2 led us to rely on these tests as measures of hippocampal specific function in latter studies. In chapter 3, a further experiment with larger numbers of animals was performed to investigate the effect of 5-FU on memory and neurogenesis within the adult male hippocampus. Moreover, immunohistochemistry for the marker (Ki67) was used successfully to measure changes in the number of dentate gyrus dividing cells between saline and 5-FUtreated groups.

The levels of hippocampal neurotrophic factor (BDNF) and the immature proliferative marker (DCX) were also compared between saline and 5-FU treated animals by doing Western blot analysis. The level of these two proteins was also

measured in the frontal cortex to provide information about their regional specificity. The results obtained in this study from the OLR confirmed that 5-FU impaired memory in both spatial and contextual fear conditioning. The number of Ki67 immunoreactive cells in the 5-FU treated group was significantly lower than the number in the control group. Although there was a trend towards reduction in hippocampal BDNF levels, this was not significant. Similarly there was no difference in hippocampal DCX levels between 5-FU and saline treated group. In contrast, frontal cortex BDNF levels were significantly reduced by 5-FU treatment compared to controls while frontal cortex DCX levels were unchanged comparing saline and 5-FU treated groups. In chapter 4, the effect of a major modulator of neurogenesis, the SSRI antidepressant fluoxetine, on memory and neurogenesis of the adult male hippocampus was examined using the methods outlined in the previous chapter. As shown previously the results obtained from the OLR test showed a reduction in the performance of this test in 5-FU treated animals but this effect was prevented by co-treatment with fluoxetine. Furthermore, the neurogenesis of 5-FU treated animals in the dentate gyrus was reduced compared to the controls as shown by quantifying the Ki67 immunoreactive cells. This significant reduction was abolished following co-treatment with fluoxetine suggesting an improvement in either cell proliferation or survival or both.

The hippocampal BDNF levels were significantly increased by the co-treatment (5-FU+LCV&fluoxetine) compared to 5-FU+LCV treatment alone while DCX levels in the same tissue also showed a marginally significant enhanced effect. On the other hand, the frontal cortex BDNF levels were increased by fluoxetine

treatment compared to controls. Moreover, fluoxetine significantly increased frontal cortex DCX levels compared to 5-FU+LCV and the co-treatment group.

The final study of this thesis was designed to gain insight into how and when 5-FU was exerting its effect on neurogenesis. In this study we used the method of birth dating and tracking of cells using BrdU which was administered by intraperitoneal injection prior to 5-FU+LCV treatment. This was followed by assessing the dividing cell survival, one, two weeks and six weeks after 5-FU+LCV treatment by quantifying immuno-positive BrdU cells as well as examining changes in proliferation by using immunohistochemistry for the proliferative cell marker (Ki67). Our results suggested that 5-FU was maximally affecting cellular proliferation two weeks after treatment and that this persisted for at least 6 weeks. In contrast the effect of 5-FU on the survival of the dividing cells in the dentate gyrus is early in onset (being significant one day after 5-FU treatment) and continued over the course of the experiment. A summary of these finding is presented in the following table (Table 6.1).

Table 1.6

6.2. Effect of Chemotherapy on memory of the adult hippocampus

Clinical studies of chemobrain reviewed in (Chapter 1) have provided strong evidence that chemotherapy induces cognitive problems mainly in memory, attention and concentration. These studies have linked these cognitive symptoms to many types of chemotherapeutic drugs rather than ascribing them to a single chemotherapy agent (Falleti, Sanfilippo et al. 2005; Bender 2006; Jenkins, Shilling et al. 2006; Ahles, Saykin et al. 2008; Castellon and Ganz 2009; Collins, Mackenzie et al. 2009; Schilder, Eggens et al. 2009). Application of animal models in chemobrain studies provides a better clarification of the mechanism of action of these chemotherapeutics and throws light on the possible anatomical locations affected by chemotherapy within the brain. A large number of these studies have investigated the effect of chemotherapy, either as a single agent or combined drugs (commonly used in therapy), on the hippocampus especially the dentate gyrus, because this is the gateway to memory in the brain (Dietrich, Han et al. 2006; Mignonea and Weberb 2006; Macleod, DeLeo et al. 2007; Mustafa, Walker et al. 2008; Seigers, Schagen et al. 2008). Because of the difficulties in interpretation the effects of combined drug action, in the present thesis, the effect of a single chemotherapy agent; 5-FU+LCV, on hippocampal memory has been investigated using two hippocampal specific cognitive tasks. These tests have been shown previously to require hippocampal input to spatial and contextual fear conditioning memories respectively. The object location recognition task has been used successfully previously in our lab (Mustafa, Walker et al. 2008). However, this thesis is the first report to use the conditioned emotional response test as a test for hippocampal memory after chemotherapy. The following section will discuss each of these two tasks.

6.2.1. Effect of chemotherapy on hippocampal spatial memory

The OLR test is a reliable test for the spatial working memory in rats as normal animals tend to explore the new location of an object more than the old location (Ennaceur and Delacour 1988). A wide range of animal models of chemobrain have used this task to test for the hippocampal spatial memory orientation (Bruel-Jungerman, Laroche et al. 2005; Mustafa, Walker et al. 2008). Another behavioural task which has been shown by a number of studies to be a good test for modelling this hippocampal specific memory is the Morris water maze (Shors, Townsend et al. 2002; Lee, Kim et al. 2006; Winocur, Vardy et al. 2006; Seigers, Schagen et al. 2008). Although, it is a specific task for measuring the spatial memory orientation of rats, the water maze test requires a long training duration for the animals which obviates the chronic testing with chemotherapy drugs. For this reason, it was decided to choose the object location recognition task to test for the effect of chemotherapy on hippocampal spatial working memory of rats in a protocol modified from (Mustafa, Walker et al. 2008). The preliminary results in the present studies (see table 6.1) have shown that even control rats were not able to perform this task (Chapter 2, experiment1). This was possibly due to the small sample number (n=5) used in this study. It was shown that the larger the sample size the more significance was gained in the preference index for the new location in the object location recognition task between animal groups (Ennaceur, Michalikova et al. 2008).

This observation was confirmed by the present studies where the OLR test was more successful modelling the hippocampal spatial memory and the results obtained from this task confirmed the effect of 5-FU-induced cognitive deficits

compared to control rats (Chapter 3 and 4. table 6.1) as reported previously (Mustafa, Walker et al. 2008).

Studies in the present thesis (see chapter 4) showed the effect of 5-FU included disruption of hippocampal spatial memory as measured using object location recognition task and analysed using preference index statistical analysis (ElBeltagy, Mustafa et al. 2010).

6.2.2 Effect of chemotherapy on contextual conditioning of rats

It has been shown that contextual conditioning to stimuli is a part of hippocampal function (Rudy, Barrientos et al. 2002; Huff and Rudy 2004). However, the hippocampus is not the only brain region responsible for the integration of this test since an intact amygdala and the anterior cingulated gyrus are required for performance in this task (McGaugh 2004; Ponnusamy, Poulos et al. 2007). For this reason it was of interest to look at the effect of chemotherapy on this task. In a protocol modified from (Resstel, Joca et al. 2006), we tested Lister hooded rats for their conditioning to electric shocks as unpleasant stimuli. Initially no difference was found in the measured freezing time between the saline and the 5-FU treated rats (Chapter 2, experiment 1). This again was likely due to the small sample size (n=5). In (Chapter 3) when the sample size was increased to 10 per group, significantly less freezing time was shown by the 5-FU treated group compared to the saline treated group (table 6.1). This result strengthens our hypothesis that 5-FU chemotherapy impairs memories which are not only hippocampal specific. Previously, it has been shown that chemotherapy impairs contextual fear conditioning, a task requiring an intact hippocampus, but did not

affect cued fear conditioning which is less hippocampal dependent (Macleod, DeLeo et al. 2007).

Moreover, it has been recently shown that 5-FU alone or combined with methotrexate impairs conditioning to a tone stimulus and this happens one day after treatment (Foley, Raffa et al. 2008). However, it has been argued by some authors that this is a stressful test as indicated from the measurement of elevated corticosteroid levels (Davis 1997; Korte 2001). There is good evidence that stress modulates neurogenesis by either producing an increase (Lee, Kim et al. 2006) or a decrease (Heine, Zareno et al. 2005), therefore, it was decided to avoid using such a stressful test (CER) for rat hippocampal memory testing in subsequent studies.

6.3. Effect of chemotherapy on adult hippocampal neurogenesis

Adult hippocampal neurogenesis is now a well characterized phenomenon (Imayoshi, Sakamoto et al. 2009). Hippocampal neurogenesis is subject to influence by both external and internal manipulations as described in general introduction (Chapter 1). The use of animal models has enabled measures of both neurogenesis and cognition to be carried out after chemotherapy. Many studies, using animal models of chemotherapy or other anti mitotic drugs have reported a reduction in all aspects of hippocampal neurogenesis (Bruel-Jungerman, Laroche et al. 2005; Dietrich, Han et al. 2006; Han, Yang et al. 2008; Seigers, Schagen et al. 2008; Ko, Jang et al. 2009). In order to study the effect of 5-FU chemotherapy on the cell division required for hippocampal neurogenesis, we measured the endogenous proliferation marker Ki67, which is only expressed in proliferating cells (Kee, Sivalingam et al. 2002).

In order to characterize the type of cell proliferation taking place in hippocampal neurogenesis, the immature neuronal marker DCX was used to measure neural cell changes. Furthermore, changes in hippocampal levels of the neurotrophic factor BDNF after 5-FU chemotherapy treatment was examined to determine possible trorhic factor changes associated with cell proliferation. In addition, one study of this thesis investigated the possible effect of 5-FU chemotherapy-induced changes on the survival of cells produced during neurogenesis by quantifying BrdU immuno-reactive cells (Chapter 5).

6.3.1. Effect of chemotherapy on cell proliferation aspect of neurogenesis

Previous animal studies on the effects of chemotherapy on cell proliferation have reported a negative effect by counting dentate gyrus Ki67 immuno-reactive cells (Seigers, Schagen et al. 2008; Bessa, Ferreira et al. 2009). In the present study (Chapter 5) the results obtained from Ki67-positive cell counts showed that 5-FU is exerting its effect on proliferation at least two weeks after the end of treatment and this effect persists for at least 6 weeks. The reasons for this are unclear, as the effect of 5-FU on the survival of cells dividing at the start of treatment is apparent at the end of treatment and continues throughout the experiment.

A possible mechanism by which 5-FU could exert prolonged affects on the proliferation of cells during neurogenesis is by altering the levels of a brain cyclooxygenase (COX-2), a prostaglandin-synthesizing enzyme up regulated in inflammatory processes (Goncalves, Williams et al. 2010; Menter, Schilsky et al. 2010). Previously, it has been shown that while basal levels of COX-2 are required for cell proliferation during neurogenesis in the dentate gyrus of the hippocampus (Sasaki, Kitagawa et al. 2003; Goncalves, Williams et al. 2010) up-

regulation of COX-2 during inflammation reduces the production and survival of new cells in adult neurogenesis (Bastos, Moriya et al. 2008). It was thought that 5-FU chemotherapy might induce inflammation and increase COX-2 expression which in turn could reduce hippocampal neurogenesis. However, as COX-2 levels do not appear to change after chemotherapy this seems unlikely.

6.3.2. Effect of chemotherapy on the survival aspect of proliferating cells

Neurogenic studies on animals highlighted the priority of the BrdU tracking method over positive Ki67 counting method for detecting changes in the survival of early dividing cells (Kee, Sivalingam et al. 2002; Kempermann 2006; Lee, Longo et al. 2006). Several animal studies have shown that chemotherapy-induced changes in the survival of proliferating cells using the BrdU protocol (Shors, Townsend et al. 2002; Mignonea and Weberb 2006; Zhou, Hu et al. 2007; Han, Yang et al. 2008; Bessa, Ferreira et al. 2009; Li, Cai et al. 2009). In this thesis, the BrdU protocol was used to test the effect of 5-FU chemotherapy on the survival of the dentate gyrus dividing cells (Chapter 5). The result demonstrated that there was a significant reduction of the survival of dividing cells one day after treatment. This reduction was more obvious 2 and 6 weeks after treatment which highlighted that the onset of the 5-FU induced action on the survived dividing cells was very early (one day) after treatment and this action remained and even increased with time (2 and 6 weeks after treatment). 5-FU targets dividing cells during S-phase of the cell cycle (Pinedo and Peters 1988). Moreover, BrdU is marking cells by incorporation into their DNA during the S-phase of the cell cycle (Kempermann 2006). For this reason, it was not surprising

that 5-FU reduced the survival of dividing cells in the SGZ by the end of treatment (one day after treatment) because it is assumed that 5-FU had killed cells during the chronic period of treatment (2 weeks). In a recent study (Han, Yang et al. 2008) it was found that 3 intraperitoneal injections of 5-FU every other day reduced the BrdU positive cell counts only after 14 days whereas increased TUNNEL expression, a marker for apoptotic cells, was found after 1 day. In addition, they reported that 5-FU chemotherapy produced destruction of the white matter tract of CNS. Moreover one drawback with the BrdU cell tracking method is that inclusion of apoptotic cells and fragmented DNA generated during the cell cycle (Kee, Sivalingam et al. 2002; Kempermann 2006). Considering that chemotherapy increases apoptosis in neuronal cell cultures and within the dentate gyrus *in vivo* (Rzeski, Pruskil et al. 2004) and neuronal precursors (Dietrich, Han et al. 2006) besides that 5-FU chemotherapy and its metabolites have been shown to alter the Krebs cycle resulting in increased apoptosis (Pinedo and Peters 1988; Yamashita, Yada et al. 2004) thus it is inaccurate to say that the 5-FU induced reduction in dentate gyrus dividing cell survival is the sole mechanism responsible for the reduction in the number of BrdU dentate gyrus positive cells. However, at present the BrdU method is the most reliable available method for detecting changes in the proliferating cell survival.

6.3.3 Effect of Chemotherapy on the differentiation aspect of neurogenesis

The effect of chemotherapy on the differentiation aspect of neurogenesis was measured in (Chapters 3 and 4) by using Western blot techniques to identify the immature proliferative marker (DCX) and the brain derived neurotrophic factor (BDNF) in both hippocampus and frontal cortex tissues. The results obtained suggested that 25mg/kg of 5-FU for two weeks did not alter DCX levels in either hippocampus or frontal cortex tissues compared to controls. In contrast, BDNF levels in the hippocampus were marginally reduced by this treatment regime compared to controls. In addition, frontal cortex BDNF level was significantly reduced by the same dose compared to controls. From the above it could be concluded that 5-FU intravenous injections in a dose of 25mg/kg reduced both hippocampal and frontal cortex BDNF levels while sparing DCX protein in same tissues.

Also using 6 intravenous injections of (20mg/kg) of 5-FU did not change either hippocampal or DCX levels between controls and 5-FU treated groups (Chapter 4, see table 6.1). These results suggest that levels of DCX protein are not affected by 5-FU chemotherapy while the neurotorphic factor BDNF could be affected. BDNF has been shown to potentiate differentiation and survival of dividing cells in the brain (Zigova, Pencea et al. 1998).

Observing the effect of 5-FU chemotherapy on the proliferation of cells in the dentate gyrus using the proliferative marker Ki67 in the same studies showed, a significant reduction in the mean numbers of Ki67 positive cells in the 5-FU compared to the saline treated group. This was correlated with the reduction in 5-FU treated animals memory compared with controls as measured by OLR and CER test in (Chapter 3) and OLR only in (Chapter 4, see table 6.1). This

indicates that proliferating cells in the dentate gyrus were indeed affected by 5-FU chemotherapy by mechanisms which spared the hippocampal DCX protein. Taking into consideration that DCX protein is transiently expressed as progenitors start to differentiate (Couillard-Despres, Winner et al. 2005; Kempermann 2006) , those cells which were targeted by 5-FU could be at a different stage of maturation to those expressing DCX or 5-FU could be affecting those cells after they had stopped expressing DCX. This problem could be solved by injecting BrdU at the start of experiment and detecting cells double labelled for BrdU and DCX by immunohistochemistry but this was outside the scope of the present experiments and will have to be considered in future studies. Previous results from our lab have highlighted the possibility that 5-FU chemotherapy could alter hippocampal BDNF levels without affecting proliferation. This also was accompanied by a reduction in hippocampal specific memory as measured by OLR task (Mustafa, Walker et al. 2008). These results suggest that there could be several different mechanisms affecting hippocampal cognition and memory namely a reduction of hippocampal neurogenesis or a reduction in hippocampal BDNF. Previously it has been reported that dentate gyrus neurogenesis could be affected by chronic mild stress without affecting proliferation. This study has also given evidence that the survival of dentate gyrus dividing cells could be changed without altering the hippocampal BDNF l**ev**els (Lee, Kim et al. 2006). Moreover, it was shown that the cognitive functions of the brain could be disrupted by alteration in hippocampal BDNF levels alone as BDNF is required for the process of long term potentiation in the hippocampus (Korte, Carroll et al. 1995; Lee, Duan et al. 2002). Furthermore, it should be noted that not only BDNF acts as a neurotrophic factor responsible

for aspects of neurogenesis but also other intrinsic factors influence neurogenesis, such as vascular endothelial growth factor (VEGF), which has been shown to have a positive effect on neuronal differentiation as well as improving the performance of animals undertaking the Morris water maze task (Cao, Jiao et al. 2004). From the above, it is clear that several factors could be participating in 5-FU chemotherapy-induced changes in neurogenesis in the hippocampus and future studies are needed to understand the mechanisms behind this.

6.4. Correlation between chemotherapy induced memory impairments and hippocampal neurogenesis

This thesis provides evidence from a rat model of chemotherapy that whenever memory impairment was present, proliferation of neural progenitors in the dentate gyrus of the hippocampus was reduced (Chapter 3 and 4). However, the differentiation aspect of hippocampal neurogenesis was not affected as indicated from the unchanged level of DCX protein between the saline and 5-FU-treated groups in the hippocampus (Chapter 3 and 4). Moreover, in one study, BDNF level in the hippocampus showed a marginal reduction in the 5-FU+LCV treated group compared to controls (Chapter 3). These findings suggest a positive correlation between cognitive impairment the reduction of proliferation of hippocampal neurogenesis. In a review of adult hippocampal neurogenesis, (Abrous, Koehl et al. 2005) it has been suggested that the differentiation and survival parts of rat hippocampal neurogenesis increase during the early stages of Morris water maze learning paradigm. However, in late stages of this task, there is a transient increase in cell death which is followed by increased proliferation. In addition, a more recent study (Dupret, Fabre et al. 2007) supported the idea

that both cellular proliferation and apoptosis are required for spatial learning and memory mediated by the hippocampus. Taking into consideration the later findings in this thesis (Chapter 5), it appears that proliferation is a target of chemotherapy at least two weeks from the end of treatment and lasts up to 6 weeks after treatment whereas survival of dividing cells is affected early (one day) after treatment and this effect lasts up to (2-6) weeks after treatment. One possibility is that the survival and proliferation of neural precursor cells in the dentate gyrus are controlled by two distinct mechanisms both of which could affect the process of hippocampal learning and memory independently. Using the present animal model of chemotherapy, it should be possible to observe the changes at different stages of hippocampal neurogenesis while hippocampal learning is talking place. This will give a better understanding of the correlation between hippocampal neurogenesis and the chemotherapy-induced cognitive symptoms.

6.5. Effect of chemotherapy and antidepressants on memory and neurogenesis of the hippocampus

The effect of antidepressants, namely the selective serotonin reuptake inhibitor fluoxetine, on memory and neurogenesis in a rat model of chemotherapy was tested in Chapter 4. Our results show that fluoxetine improved the memory deteriorations caused by 5-FU chemotherapy. This was determined using the OLR test analysed using the preference index (PI). Fluoxetine also increased the number of dividing cells in the SGZ which were reduced by 5-FU chemotherapy. Moreover, measuring hippocampal BDNF levels showed that there was a significant up-regulation in this neurotrophic factor in the co-treated group (fluoxetine+5-FU+LCV) compared to the 5-FU only-treated group, see table 6.1.

Although not significant, there was also a marginal increase of the hippocampal DCX level in the co-treated group compared to 5-FU+LCV only treated group. It was concluded that fluoxetine improved memory in a rat model of chemotherapy and this cognitive enhancement was mediated by an increase in neurogenesis and or the up-regulation of hippocampal BDNF levels. Previous studies on the effect of fluoxetine on neurogenesis and cognition in animal models have shown an improvement in these parameters (Kodama, Fujioka et al. 2004; Perera, Coplan et al. 2007; Valluzzi and Chan 2007; Li, Cai et al. 2009). In addition there is clinical evidence that fluoxetine increases hippocampal progenitor cells (Boldrini, Underwood et al. 2009). It has also been suggested that chronic treatment with fluoxetine can improve the cognition of Alzheimer's patients (Mowla, Mosavinasab et al. 2007). In the study of (Santarelli, Saxe et al. 2003), it was shown that hippocampal neurogenesis was important in the action of antidepressants including fluoxetine. The other mechanism through which antidepressants are suggested to exert their effect on hippocampal memory is the up-regulation of hippocampal BDNF levels which was a positive finding in the present thesis (Chapter 4, see table 6.1). Previous reports, (Sairanen, Lucas et al. 2005), have found that treatment with antidepressants (imipramine and fluoxetine) increased hippocampal BDNF levels as well as enhancing the proliferation of precursor cells in the dentate gyrus of mice and that BDNF promotes the survival of these cells. Added to this, it was found that rats treated with imipramine had increased expression of three proteins associated with neural plasticity, PSA-NCAM, pCREB, and GAP-43 in their hippocampi and frontal cortices (Sairanen, O'Leary et al. 2007). This also could account for the elevated frontal cortex BDNF level in the co-treated group compared to the group treated

with 5-FU chemotherapy in the present study. The use of antidepressants to treat chemotherapy impaired memory has not been fully assessed but the improvement in memory as a result of chronic antidepressant treatment seems to be a positive finding although, the mechanism of the effect is not fully understood.

According to the work of the present thesis and work of other colleagues in the group, alteration of neurogenesis is one of the likely mechanisms behind this but further work is needed to establish the exact mechanism through which antidepressants exert their behavioural improving effect in animal models of chemotherapy.

6.6 Future work

6.6.1. Future consideration in chemobrain modelling

6.6.1.1. The use of leucovorin (LCV)

The 5-FU chemotherapy method used in this thesis was accompanied by co treatment with LCV. The latter is a folate derivative that is used to potentiate the cytotoxic effect of 5-FU chemotherapy. LCV has a chemical structure which is similar to vitamin B9 and when metabolized it enhances the process of DNA synthesis. The effect of LCV on animal cognition however has not been assessed. However, in their study,(Phillips, Thaler et al. 1989) have provided evidence that leucovorin eliminated the methotrexate-induced neurotoxicity in a rat model of chemotherapy. In a more recent study, (Seigers, Schagen et al. 2008) also supported this finding. In addition, clinically, it has been found that leucovorin reverses toxic brain encephalopathy resulting from Methotrexate (Jaksic, Veljkovic et al. 2004). Observing its mechanism of action, methotrexate inhibits dihydrofolate reductase which results in depletion of the endogenous folate so that when combined with LCV, the later reverses the chemotherapeutic action while in the case of 5-FU chemotherapy LCV potentiates the drug-induced cytotoxicity by increasing the conjunction between the drug and the enzyme thymidylate synthetase which is essential for DNA synthesis (Herrmann, Reuter et al. 1988). Moreover, the promoting role of folinic acid in DNA synthesis has been previously established (Nixon 1979). It is clear that in future chemobrain models, the effect of leucovorin alone on memory and neurogenesis needs to be determined.

6.6.1.2. The use of single agent vs. combined chemotherapy

The animal model of chemotherapy developed in this thesis aimed to continue earlier investigations on the effect of single agent chemotherapy (5-FU), on memory and adult neurogenesis. Clinical reviews of chemobrain (table 1.1) have highlighted the observation that chemotherapy involving several agents has a greater effect on cognition than treatments composed of only a single agent (van Dam, Schagen et al. 1998; Ahles, Saykin et al. 2002; Schagen, Muller et al. 2002). Some animal models have supported this finding, for example Wincour et al (2006a) and Foley et al (2008). have reported greater impairments in performance in tasks including the Morris water maze and the delayed non matching sample tasks after combined 5-FU and methotrexate treatment compared to either drug on its own (Winocur, Vardy et al. 2006; Foley, Raffa et al. 2008). These results suggest that the combination of methotrexate and 5-FU produce a more potent deleterious effect on memory and learning in mice than either drug on its own. Future work should consider combined chemotherapy regimes, as two or more mechanisms of action of chemotherapeutic drugs could be more powerful than one mechanism. Future animal models on chemobrain therefore, should investigate the effect of combined chemotherapy on memory and neurogenesis in the adult rat hippocampus.

6.6.1.3. Chemobrain in female rats

Most clinical reviews dealing with chemobrain have investigated the cognitive symptoms found after breast cancer treatment. This has involved female cancer survivors who may respond differently to the male rats commonly used in animal tests of cognition. One feature of this is that female breast cancer patients are often treated with the oestrogen receptor antagonist tamoxifen or other hormonal therapies along with chemotherapy. Some reported a worsening effect of tamoxifen on cognition (Paganini-Hill and Clark 2000; Shilling, Jenkins et al. 2003; Jenkins, Shilling et al. 2004; Bender, Sereika et al. 2006; Palmer, Trotter et al. 2008) while other studies found no effect (Schagen, van Dam et al. 1999).

Most studies, including the present one, use male animals to avoid the possible confounding variables caused by the oestrous cycle however as it appears that the oestrous cycle can affect cognitive behaviour in rats and humans a study using female animals is possibly more relevant to the study of chemobrain in breast cancer (Sutcliffe, Marshall et al. 2007; Galea, Uban et al. 2008; Spencer, Waters et al. 2008; Maki and Sundermann 2009). Interestingly one of the few studies using female ovariectomised rats found that cyclophosphamide enhanced their performance in the Morris water maze task (Lee, Longo et al. 2006). Animal studies of the effects of hormonal treatment have found that while tamoxifen alone impaired hippocampal specific spatial memory as modelled by the water maze task (Chen, Wu et al. 2002) oestrogen enhances hippocampal neurogenesis in female rats (Tanapat, Hastings et al. 1999). This may open up further avenues of research into the combined effects of tamoxifen or oestrogen with chemotherapy in future animal studies of chemotherapy.

6.6.2. Future consideration in studying the cellular effects of chemotherapy

6.6.2.1. Regional specificity of chemotherapy

The effect of chemotherapy on hippocampal neurogenesis was investigated in this thesis by counting the number of positive Ki67cells throughout the whole length of the hippocampus to obtain an average number for each brain regardless of the region e.g. (ventral vs. dorsal portions of the hippocampus). However, as shown in Chapter 4, the effect of the antidepressant, fluoxetine, on different sub-regions of the hippocampus was quantified but no difference in the mean numbers of positive Ki67 cells between the ventral and the dorsal portions of the hippocampus was found in any of the animal groups (data not shown). This does not exclude the possibility that chemotherapy could affect different brain regions independently. Observing the results obtained from determining BDNF protein levels in both frontal cortex and the hippocampus, after saline or chemotherapy treatment (Chapter 3), we found that while the protein level was marginally decreased in the hippocampus there was a significant reduction in the same protein levels in the frontal cortex tissue due to 5-FU+LCV chemotherapy treatment. This adds further evidence suggesting a regional specificity of the effect of chemotherapy on different parts of the brain. Moreover, it has been found that induction of seizures affects proliferation and differentiation in ventral and dorsal portions of the hippocampus independently (Ferland, Gross et al. 2002). Future animal models should take this regional specificity into consideration as this may help in better understanding of the different roles of these sub regions in controlling memory mediated by the hippocampus.

6.6.2.2. The value of double-labelling

The current animal model depended mainly on single immunolabelling of dentate gyrus dividing cells to detect changes in hippocampal neurogenesis after 5-FU chemotherapy. Either the endogenous proliferative marker (Ki67) or the exogenous marker (BrdU) was used to investigate the chemotherapy induced changes in proliferation or survival of cells respectively during hippocampal neurogenesis. However, the exact fate regarding either neuronal or glial cells and which was affected by 5-FU chemotherapy, remains unclear. Previously it has been reported that cranial irradiation particularly affects dividing cells which would mature into neurons (Monje, Mizumatsu et al. 2002; Monje, Toda et al. 2003).

It would be interesting to look at cells double labelled for proliferation and differentiation markers to see the effect of chemotherapy on different cell types (Couillard-Despres, Winner et al. 2005). Co-labelling of markers for proliferation such as DCX, a marker of developing neurons; NeuN; a marker for mature neurons; or GFAP, a marker of astrocytes, along with the BrdU would help to clarify the picture of chemotherapy induced changes in hippocampal neurogenesis.

6.6.2.3. Apoptosis and neurogenesis

The work of Abrous and her group (Abrous, Koehl et al. 2005) has highlighted the importance of apoptosis as a process occurring in both neurogenesis and learning. As chemotherapy agents, including 5-FU, are designed to induce apoptosis and cell death, it will be important to observe how increased cell death (detected by TUNNEL staining) in the dentate gyrus as a result of chemotherapy interacts with or differs from the normally occurring cell death in untreated

animals. This will also shed light on the underlying mechanism by which chemotherapy produces the observed behavioural changes.

6.6.2.4. VEGF and differentiation

Vascular endothelial growth factor has also been claimed to increase neuronal differentiation and to improve the performance of rats in the Morris water maze (Cao, Jiao et al. 2004). Moreover, VEGF has been shown to promote proliferation in neural stem cells (Xiao, Kong et al. 2007). Studying the effect of chemotherapy on VEGF will be essential to determine its role in modulating the chemotherapy induced cognitive changes as well as hippocampal neurogenesis and the possible links between both.

6.6.2.5. Stress and neurogenesis

As indicated previously, the effect of stress can alter neurogenesis in either directions. Thus, future studies are needed to determine the effect of stress induced by chemotherapy by examining possible changes in the stress hormone, corticosterone.

6.6.3. Future considerations regarding the use of antidepressants in animal model s of chemotherapy

The study in Chapter 5 was designed to investigate the long term effect of 5-FU chemotherapy on dividing cells in the dentate gyrus and the survival of new cells by looking at different time points after treatment. This will not only help in understanding how and when 5-FU chemotherapy exerts its effect on hippocampal neurogenesis but also will provide information about the most effective time point for antidepressant therapy to counteract the effects of chemotherapy. Clinically, although antidepressants are sometimes given to cancer patients for depression, there is no systematic use nor are they given specifically to prevent the cognitive effects of chemotherapy. As noted previously, fluoxetine improves cognition in patients with traumatic brain injury (Horsfield, Rosse et al. 2002) and depression (Cassano, Puca et al. 2002; Levkovitz, Caftori et al. 2002) and this is backed up by animal studies showing that this drug improves their behavioural performance (Holick, Lee et al. 2008) and neurogenesis (Kodama, Fujioka et al. 2004; Marcussen, Flagstad et al. 2008). However the exact mechanism by which antidepressants improve cognition after chemotherapy is unknown and needs further study.

The results presented in Chapter 4 suggest that the 5-FU chemotherapy reduces hippocampal neurogenesis and that this is responsible for the cognitive changes. However, these behavioural changes could also be due to the reduced levels of the neurotrophic factor (BDNF) in the hippocampus and this will need further investigation. For example, chemotherapy induced changes in the levels of the transcription factor, cAMP response element binding protein (CREB) could subsequently affect BDNF levels (Nibuya, Nestler et al. 1996). Antidepressant

treatment for longer periods up-regulate the levels of plasticity associated proteins within the hippocampus (Sairanen, O'Leary et al. 2007) thus investigating the effect of chemotherapy on these proteins as well as BDNF will be essential in order to reach a better understanding about the mechanism by which antidepressants improve chemobrain symptoms.

6.7. Conclusion

The findings obtained from the present animal model of chemotherapy have demonstrated the possible association between 5-FU chemotherapy and memory impairments. Treatment was associated with a reduction in neural progenitor proliferation in the SGZ of the hippocampus which suggests that a reduction in neurogenesis may provide a possible mechanism by which 5-FU is exerting its behavioural effects. Interestingly the reduction in neurogenesis continues for at least 6 weeks after treatment providing an explanation for the long term effects of chemotherapy described by patients. However, it is possible that the impairment of hippocampal neurogenesis is not the sole mechanism by which 5-FU chemotherapy causes the symptoms of "chemobrain" as results presented here also suggest that other factors could be implicated such as alteration in the level of the neurotrophic factor BDNF or stress hormones. The project also demonstrated that the SSRI antidepressant, fluoxetine can prevent the memory and neurogenesis impairments caused by chemotherapy. Although, we provide evidence that an antidepressant improved neurogenesis in this animal model, the exact mechanism by which it acts on the brain needs more clarification. Indeed, investigating the effect of 5-FU chemotherapy on different aspects of hippocampal neurogenesis could answer the question of how and when the drug targets the brain and also could give a better understanding about how to incorporate antidepressants in the overall treatment of cancer with chemotherapy. Taking into consideration the information discussed in the section on future work, these studies will be of great value in establishing the causes and treatment of the effects of chemotherapy on memory and neurogenesis of adult brain hippocampus.

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Appendix I

WESTERN BLOTTING SOLUTIONS

(All materials are from Sigma, unless otherwise indicated)

Lysis Buffer: 20 mM Tris-hydroxymethylaminomethane (Tris), 1 mM ethylene iycol tetra-acetic acid (EGTA), 320 mM sucrose, 0.1% Triton Xl 00, 1mM NaF, 10 mM f3-glycerophosphate dissolved in 500 ml distilled water at pH 7.6.
1 tablet of ethylenediamine tetra-acetic acid (EDTA)-free protease inhibitor cocktail 'Roche Diagnostics, Germany) is added to 10 ml of lysis buffer solution just before .use.

x2 Solubilisation Buffer: 2.5 ml of 0.5 M Tris, 2 ml of glycerol, 2 ml of 10 % sodium dodecyl sulfate (SDS) solution, 2.5 ml of distilled water, 1ml of β-mercaptomethanol, and 40 µl of 2.5 % bromophenol blue.

Lowry Solution: 20 ml of Solution A (2g of NaOH, 1g of SDS and l0g of NaCO3 dissolved in 500 ml of distilled water) and prior to Lowry assay, solution A is mixed with Solution B (2% NaK tartrate and 1 % CuSO4).

Electrophoresis Buffer (x10): 30.3g of Tris, 144g of Glycine, and 10g of SDS dissolved in 1 litre of distilled water.

Transfer buffer: 30.3g Tris and 144g glycine dissolved in 8 litres of distilled water and 2 litres of methanol added (kept at 4°C).

Tris-buffered saline-Tween 20 (TBST): 25 mM Tris and 125 mM NaC1 dissolved in distilled water and adjusted to pH 7.6 (using concentrated hydrochloric acid). Tween 20 is then added to make a final concentration of 0.1 %.

One SDS gel is comprised of the following:

1- **4% stacker sodium dodecyl sulphate (SDS)-polyacrylamide gel:** 3.12 ml of 30% acrylamide (Protogel, Geneflow), 6 ml of 0.5 M Tris-HCL, 0.24 ml of 10% SDS, 0.12 ml of 10% ammonium-perisulfate (APS) and 0.024 ml of tetramethylenenediamine (TEMED) dissolved in 14.6ml of distilled water.

2- **Resolving sodium dodecyl sulphate (SDS)-polyacrylamide gel:** 30% acrylamide (protogel, Geneflow), 1.5 M Tris-HCL, 10% SDS and 10% APS dissolved in TEMED and distilled water. Quantities of these solutions are altered according to achieve the required percentages (e.g. 15%) SDS gel.

Appendix II

Throughout all studies treatment and care of animals was in accordance with Home Office guidelines on the use of animals in scientific research in the United Kingdom.

All procedures were carried out under the project licence 40-3283KF and personal licence 40/8761.

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