FLUOXETINE PREVENTS THE COGNITIVE AND CELLULAR EFFECTS OF CHEMOTHERAPY IN THE ADULT HIPPOCAMPUS

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Publications and Presentations

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Abbreviations

5-FU	5-Fluorouracil
5-HT	5-Hydroxytryptamine/ serotonin
ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
BrdU	5-Bromo-2-deoxyuridine
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CH_2THF	5,10-Methylene-tetrahydrofolate
CMF	Cyclophosphamide, methotrexate and 5-fluorouracil
CNS	Central nervous system
CP	Cyclophosphamide
CREB	cAMP response element binding protein
CNTF	Ciliary neurotrophic factor
DCX	Doublecortin
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNMTS	Delayed non-matching to sample
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate
EGF	Epidermal growth factor
FBS	Foetal bovine serum
FGF-2	Fibroblast growth factor-2
FH2	Dihydrofolate
FLX	Fluoxetine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
i.p.	Intraperitoneal
i.v.	Intravenous
LCV	Leucovorin/calcium folinate
LTP	Long-term potentiation
LTD	Long-term depression
MAM	Methylazoxymethanol acetate
MAP	Microtubule-associated protein
MRI	Magnetic resonance imaging
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTX	Methotrexate
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Noradrenaline
NeuN	Neuronal Nuclei
NLR	Novel location recognition
NMTS	Non-matching to sample
NOR	Novel object recognition
NSC	Neural stem cell
OCT	Optimal cutting temperature
PBS	Phosphate buffered saline
Pen/Strep	Penicillin and streptomycin
PFA	Paraformaldehyde
PI	Preference index/indices
PKA	Protein kinase A
rBDNF	Recombinant brain-derived neurotrophic factor
RNA	Ribonucleic acid
rpm	Rotations per minute
SDS-PAGE	Sodium dodecyl (lauryl) sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SFM	Serum free medium
SGZ	Subgranular zone
SSRI	Selective serotonin reuptake inhibitor
SVZ	Subventricular zone
TBST	Tris-buffered saline tween-20
THFA	Tetrahydrofolate
TS	Thymidine synthetase

Abstract

Rationale: CMF (cyclophosphamide: CP; methotrexate: MTX; 5-fluorouracil: 5-FU) is a chemotherapy combination associated with the cognitive impairments which many cancer patients experience after treatment. A reduction in hippocampal neurogenesis is a known means by which cytotoxic drugs alter cognition and is the mechanism investigated in the present study. There is currently no way of treating or preventing the cognitive deficits produced by chemotherapy and a simple pharmacological approach to achieving this could potentially have significant benefits for patients.

Objectives: The studies in the present thesis use an animal model to investigate the effects of the individual agents in the CMF combination on spatial working memory and the proliferation and survival of neural precursors involved in hippocampal neurogenesis. It was also investigated whether the cognitive impairment produced by chemotherapy could be reversed or prevented by the antidepressant fluoxetine.

Methods: In 4 separate experiments, adult male Lister-hooded rats were chronically administered with CP (30mg/kg, 4 or 7 i.v. doses), MTX (75mg/kg, 2 i.v. doses) or 5-FU (25mg/kg, 5 i.p. doses). Some rats were co-administered with fluoxetine (10mg/kg/day, in drinking water) for different time periods. Spatial memory was tested using the novel location recognition (NLR) task and the spontaneous alternation in the T-maze memory tasks. Proliferation and survival of hippocampal cells was quantified using immunohistochemistry and the levels of doublecortin (DCX) and brain-derived neurotrophic factor (BDNF) were quantified in the hippocampus and frontal cortex using Western blotting. Neural stem cells (NSC) were also isolated from the adult mouse hippocampus, to examine the direct effects of 5-FU, fluoxetine and its active metabolite, norfluoxetine (0.01 - 100μM) *in vitro*.

Results: Rats treated with 5-FU and MTX showed impairment in the NLR task but not the spontaneous alternation in the T-maze task. They also exhibited a reduction in cell proliferation (Ki67-positive cells) and survival (BrdU-positive cells) in the dentate gyrus, compared to saline treated controls, but no difference was seen in the levels of DCX of BDNF. The induced cognitive and cellular impairments were not seen when fluoxetine was co-administered with the chemotherapy. The impairments caused by 5-FU were counteracted when fluoxetine was co-administered before and during 5-FU treatment but not when it was only administered after treatment. CP did not impair performance in the NLR task or hippocampal cell proliferation; however it significantly reduced cell survival. 5-FU, fluoxetine and norfluoxetine all decreased cell viability *in vitro*.

Conclusions: These results demonstrate that MTX and 5-FU have more pronounced effects on spatial memory and hippocampal cell proliferation than CP in the CMF combination. Furthermore these impairments can be reversed by fluoxetine in a mechanism of prevention but not recovery. Although further work is required, it would be beneficial to establish an *in vitro* model of chemotherapy-induced cognitive impairment to justify this conclusion and to investigate the potential benefits of fluoxetine in cancer patients.

Chapter 1

General introduction

1.1 Chemotherapy-induced cognitive impairment

In the present thesis a rat model is created to study the effects of different chemotherapy agents on cognition, cell proliferation and cell survival in the adult hippocampus. The selective serotonin reuptake inhibitor (SSRI) antidepressant, fluoxetine, is investigated to determine its ability to prevent both the cellular and behavioural deficits found after chemotherapy treatment.

1.1.1 An introduction to chemobrain

It is estimated that more than one in three people will develop a form of cancer within their lifetime (Cancer Research UK, 2011). As treatment is continually improving, leading to a reduced risk of reoccurrence and a higher survival rate for patients, it is becoming increasingly important to research possible improvements for the quality of life of cancer survivors.

Many cancer patients have a tumour removed surgically followed by adjuvant chemotherapy. Although this treatment is often effective, chemotherapy is notorious for its many side effects. One area of side effects, reported by a substantial number of patients, is problems with cognition including working memory, concentration and general confusion (Matsuda et al. 2005). Chemotherapy-induced cognitive impairment is also colloquially called "chemobrain" or "chemofog" (Anderson-Hanley et al. 2003) and can occur from immediately after chemotherapy treatment to ten years after completion of treatment (Ahles and Saykin 2002). It has been described in patients who have received treatment for solid tumours including breast, lung, prostate and ovarian cancers (Argyriou et al. 2011), with the majority of clinical studies being carried out on survivors of breast cancer (Castellon et al. 2005). This cognitive deficit is usually subtle (Matsuda et al. 2005), but it has been reported to affect the ability of cancer survivors to return to work and resume a normal life (Boykoff et al. 2009). It has been difficult to evaluate the prevalence of chemotherapy-induced cognitive impairment and estimations suggest between 17% and 75% of patients who have received chemotherapy treatment suffer from some form of cognitive decline (van Dam et al. 1998; Wieneke and

Dienst 1995). One reason for this may be the controversy that initially surrounded chemotherapy-induced cognitive impairment. Clinical studies are confounded by a number of variables which could contribute to the cognitive deficit observed in patients. Such factors include behavioural aspects such as the stress and fatigue cancer patients are likely to experience during the course of treatment, as well as the effect of the cancer itself. This led to some dispute about the existence and prevalence of chemotherapy-induced cognitive impairment (Hermelink et al. 2007; Jenkins et al. 2006) and consequently the proposition to standardise research methods including the neuropsychological tests used (Shilling et al. 2006; Wefel et al. 2011). With the significantly increased number of clinical studies carried out recently (Table 1.1), which take into consideration these possible confounding factors, strong evidence in support of the existence of chemotherapy-induced cognitive impairment is now available.

1.1.2 Clinical studies of chemotherapy-induced cognitive impairment

One of the earliest studies of chemotherapy-induced cognitive impairment was carried out by Peterson and Popkin (1980), who estimated between 5% to 86% of cancer patients experience neurophsychological deficits due to chemotherapy treatment. This huge range was attributed to the different combinations of chemotherapeutic agents used, variations in the type of cancer, with the possibility of metastasis, and the effect of other drugs co-administered with the chemotherapy. During the subsequent 30 years a large number of studies have been carried out, summarised in Table 1.1, with many controlling a number of these variables. In the majority of these studies, the controls consisted of cancer patients who had received treatment other than chemotherapy such as surgery or radiation treatment. This was necessary to reduce the possibility of stress, anxiety and depression of the cancer patients confounding results (Ahles et al. 2002; Schagen et al. 2002b). Furthermore, the groups compared were appropriately matched in terms of demographics, such as their levels of education and age group, although these attributes were not found to affect the results (Ahles et al. 2002; Schagen et al. 2002b). In many studies groups were also often divided into pre- and post-

menopausal patients (Collins et al. 2009; Schilder et al. 2009) and those with and without adjuvant hormonal treatment (Bender et al. 2001; Schilder et al. 2009). Hormonal therapy is thought to be a significant confounder of chemotherapy-induced cognitive impairment as it may itself be able to induce cognitive decline (Collins et al. 2009). It is possible that some patients included within these studies had been concomitantly prescribed with antidepressants which could have affected their cognition and this was not taken into account (Table 1.1).

Wieneke and Dienst (1995) specifically investigated the effects of two chemotherapy combinations used to treat breast cancer: 5-Fluorouracil (5-FU), doxorubicin and cyclophosphamide (FAC) and CP: cyclophosphamide, MTX: methotrexate and 5-FU (CMF). They found that 75% of patients suffered deterioration in a range of cognitive tests 6 months after they received these drugs. An important study by van Dam et al. (1998) compared the effects of both standard dose and high dose chemotherapy, 2 years after treatment. They found that the standard dose impaired 17% of patients in comparison to 32% of patients who had been treated with the high dose, with respect to attention, visual memory and motor function. This dose-dependent effect was confirmed by Ahles et al. (2002), who studied long-term survivors of breast cancer. Patients who had received a larger number of chemotherapy cycles performed significantly lower on a range of neuropsychological tests.

More recent studies investigating the CMF chemotherapy combination were carried out by Hurria et al. (2006b) and Kreukels et al. (2008). Hurria and colleagues studied older women with breast cancer, finding 25% of them to be impaired in psychomotor speed, visuospatial abilities and visual memory, 6 months after completion of chemotherapy. Kreukals and his group compared breast cancer patients treated with CMF to healthy controls and found them to be impaired in a battery of cognitive tests 1 and 4 years after treatment.

Due to the nature of clinical studies, small sample size may limit the information obtained from the results. However, four meta-analyses of the literature have been compiled, and these have concluded that chemotherapy consistently affects working, visual and verbal memory

and processing speed in survivors of cancer (Anderson-Hanley et al. 2003; Falleti et al. 2005; Jansen et al. 2005b; Stewart et al. 2006). Although the mechanisms which contribute to this cognitive dysfunction still remain largely unknown, a list of potential mechanisms has been suggested by Jansen et al. (2005a). These include chemotherapy-induced anaemia or menopause, cytokine-induced inflammatory response and leukoencephalopathy. Leukoencephalopathy refers to structural alterations in cerebral white matter as was demonstrated by Inagaki (2007) using magnetic resonance imaging (MRI) in cancer survivors who received adjuvant chemotherapy treatment. Alterations in cerebral white matter has been demonstrated with MRI after treatment with a number of chemotherapy drugs and is accompanied by headaches, nausea and changes in mental status (Kastrup and Diener 2008; Onujiogu et al. 2008; Rajasekhar and George 2007; Soussain et al. 2009). However the majority of these symptoms resolve after time (Rajasekhar and George 2007; Soussain et al. 2009).

Although there is now strong evidence in the clinical literature for chemotherapy-induced cognitive impairments, clinical investigations are not without their limitations. Many variables including the dose, type and administration of chemotherapy, the cancer itself and patient individuality (e.g. age, menopausal status) have proved difficult to control, thereby contributing to a reduced sample size. It is also difficult to find appropriate controls to compare with chemotherapy treated patients. Furthermore, it is unethical to use invasive procedures to examine the underlying mechanisms of the impairment and methods to counteract it. For these reasons several animal models have been utilised to investigate different areas of chemotherapy-induced cognitive impairment, including the studies presented in this thesis.

Study	Time period post-chemo	Control group	Experimental groups	Cognitive impairment (%/description)	Neuropsychological domains	Patient reported measures
de Ruiter et al., 2011	10 years	BC patients treated without chemotherapy	HD chemo: FEC+CTC	Control: 5% HD chemo: 11%	Verbal function Memory Attention/concentration Processing speed Mental flexibility	Memory Attention Thinking Language
Tager et al., 2010	a) Prior to chemo b) 6 months c) 1 year	BC patients treated without chemotherapy	SD (various combinations)	SD chemo motor ability decreased over time	Verbal, visual and working memory Attention/concentration Motor Language Visuospatial ability	Perceived memory ability
Ahles et al., 2010	a) 1 month b) 6 months c) 18 months	BC patients treated without chemotherapy and healthy females	SD (various combinations)	SD chemo had a short-term impact on verbal ability	Verbal ability Verbal, visual and working memory Processing speed Sorting Distractibility Reaction time	Anxiety Fatigue Cognitive ability
Quesnel et al., 2009	a) Prior to chemo b) Immediately after c) 1 month	BC patients treated without chemotherapy	SD (various combinations)	SD chemo groups had impaired verbal fluency	Verbal and visual memory Executive function Attention/concentration Processing speed Verbal fluency	Cognitive failures Quality of life Adult intelligence
Collins et al., 2009	a) 1 month b) 1 year	BC patients received adjuvant hormonal therapy	SD (various combinations)	a) Control: 14% a) SD chemo: 34% b) Control: 11% b) SD chemo: 10%	Executive function Language Motor Processing speed Verbal, visual and working memory Visuospatial ability	Not applicable
Schilder et al., 2009	2 years	Healthy females	SD chemo: AC Randomised with tamoxifen or exemestane	Control: 6% SD chemo/ tamoxifen: 28% SD chemo/ exemestane: 28%	Verbal memory Mental flexibility Verbal fluency Processing speed Motor speed	Memory Concentration Thinking language
Kreukels et al., 2008	a) 1 year b) 4 years	a) Healthy females b) SD chemo patients who were cognitively unimpaired at 1 year	SD chemo: CMF	a) Control: 10% a) SD chemo: 33% b) SD chemo patients impaired at 1 year were still more cognitively impaired	a) Verbal function Memory Attention/concentration Processing speed Mental flexibility b) P3 latency P3 amplitude Reaction time Information processing	Not applicable
Schagen et al., 2008	3 years	TC patients received a) surgery b) surgery + radiotherapy	SD chemo: BEP	No difference between groups	Verbal function Memory Attention/concentration Processing speed Mental flexibility	Memory Attention Thinking Language
Bender et al., 2006	a) Immediately after b) 1 month c) 1 year	BC patients treated without chemotherapy	SD (various combinations) SD (various combinations) with tamoxifen	SD chemo: Impaired verbal working memory SD chemo/ tamoxifen: Impaired visual memory and verbal working memory	Visual memory Verbal working memory Cognitive/intellectual memory	Memory
Jenkins et al., 2006	a) Immediately after b) 18 months	Healthy females and BC patients treated without chemotherapy	SD (various combinations)	a)Healthy: 18% Control: 26% SD chemo: 20% b)Healthy: 11% Control: 14% SD chemo: 18%	Intelligence Verbal, visual and working memory Executive function Processing speed	Cognitive failures
Hurria et al., 2006	6 months	Published norms	SD chemo: CMF SD (various combinations)	SD chemo: 25%	Orientation Registration Attention Calculation Visuospatial ability Language Psychomotor speed	Psychological state
Shilling et al., 2005	18 months	Healthy female controls	SD chemo: CMF/FEC	Control: 19% SD chemo: 34%	Intelligence Verbal, visual and working memory Executive function Processing speed	Cognitive failure
Donovan et al., 2005	6 months	BC patients treated without chemotherapy	SD chemo: CMF SD (various combinations)	No difference between groups	Episodic memory Motor function Language Motor function	Cognitive complaints

Wefel et al., 2004	Before treatment	Published norms	Not applicable	SD chemo: 35%	Attention Memory Language Visuospatial ability Executive and motor function	Anxiety Depression
Castellon et al., 2004	2-5 years	Healthy female controls BC patients treated without chemotherapy	SD (various combinations)	Not quantified	Visual memory Visuospatial function Verbal learning	No correlation between self-reported measures and neuropsychological assessment scores
Tchen, et al., 2003	1-2 years	Healthy female controls	SD chemo: CMF/FEC/AC	Symptoms not related to cognitive function	Attention/ concentration Language Visuospatial ability	Subtle cognitive impairment
Schagen et al., 2002	4 years	BC patients treated without chemotherapy	SD chemo: CMF SD chemo: FEC HD chemo: FEC+CTC	Control: 11% SD CMF: 13% SD FEC: 9% HD FEC+CTC: 14%	Verbal function Memory Attention/concentration Processing speed Mental flexibility	Memory Attention Thinking Language
Ahles and Saykin, 2002	10 years	Cancer patients received local therapy	SD (various combinations)	Control: 14% SD chemo: 39%	Verbal memory Psychomotor function	Working memory
Schagen et al., 2001	1 week	BC patients treated without chemotherapy	SD chemo: FEC HD chemo: FEC+CTC	Control: 9% SD chemo: 17% HD chemo: 32%	Verbal function Memory Attention/concentration Processing speed Mental flexibility	Memory Attention Thinking Language
Brezden et al., 2000	a) During chemo b) 2 years	Healthy female controls	SD chemo: CEF/CMF	Controls:11% a) SD chemo:48% b) SD chemo: 50%	a) Memory and language b) Language and visual-motor cognition	Not applicable
Schagen et al., 1999	1.9 years	BC patients treated without chemotherapy	SD chemo: CMF	Controls: 12% SD chemo: 28%	Attention/concentration Mental flexibility Processing speed Memory Motor and verbal function	Concentration Memory
van Dam et al., 1998	2 years	BC patients treated without chemotherapy	SD chemo: FEC HD chemo: FEC+CTC	Controls: 9% SD chemo: 17% HD chemo: 32%	Attention/concentration Processing speed Visual memory Motor function	HD and SD groups: Concentration Memory Thinking
Wieneke and Dienst, 1995	6 months	Published norms	SD chemo: FAC/CMF	SD chemo: 75%	Attention/concentration Verbal and visual memory Visuospatial ability Processing speed	Not applicable
Kaasa et al., 1988	After treatment	Cancer patients treated without chemotherapy	SD chemo: cisplatin and etoposide	SD chemo impaired cognition	Trail Making Visual Retention Verbal Learning	Not applicable

Table 1.1 Summary of clinical studies investigating the incidence of cognitive deficits caused by high dose (HD) and standard dose (SD) chemotherapy (chemo) adapted from Rugo and Ahles (2003), listed in chronological order from most recent. It is possible that some patients in these studies were concomitantly prescribed with antidepressants, which may have affected their cognition, however this was not taken into account in these studies. Abbreviations: Cancers; BC: breast cancer, TC: Testicular cancer. Chemotherapy combinations; AC: adriamycin, cyclophosphamide, BEP: bleomycin, etoposide and cisplatin, CMF:

cyclophosphamide, methotrexate, 5-FU, CTC: cyclophosphamide, thioTEPA, carboplatin, FAC: 5-FU, doxorubicin, cyclophosphamide, FEC: 5-FU, epirubicin, cyclophosphamide

1.1.3 Animal models of chemotherapy-induced cognitive impairment

The recent use of *in vivo* animal models has provided a faster, more detailed method to research chemotherapy-induced cognitive impairment. In addition, they have enabled the investigation of the underlying mechanisms and neuropathological changes to discover the neurological basis for cognitive dysfunction (reviewed in Seigers and Fardell 2011). Existing models (summarised in Table 1.2) have used a number of cytostatic drugs, investigating the effects of both single agents and drugs used in combination. A range of cognitive tasks have been used to investigate different learning and memory paradigms, and in most studies animals are impaired in tasks associated with hippocampal spatial learning, such as the Morris water maze (MWM) and novel location recognition (NLR) task. In contrast it appears that animals were not as robustly impaired in fear conditioning tasks which are dependent on areas such as the amygdala and hippocampus, including the conditioned emotional response (CER), passive avoidance and delay conditioning tasks (Parkes and Westbrook 2011). This provides evidence that the hippocampus may be more involved in the mechanism underlying chemotherapy-induced cognitive impairment than other brain regions such as the amygdala. This is particularly relevant as working and visuospatial memory are frequently targeted in clinical studies of chemotherapy-induced cognitive impairment (Anderson-Hanley et al. 2003; Falleti et al. 2005; Jansen et al. 2005b; Stewart et al. 2006) (see Table 1.1) which are functions of the hippocampus (Baddeley et al. 2011; Loureiro et al. 2011; Sharma et al. 2010).

In a number of these studies, the neurobiological changes and possible anatomical loci for the cognitive impairments have also been investigated, and these have included the effect of chemotherapy on neurogenesis. Nearly all studies have found that administration of chemotherapy to rodents produces behavioural deficits. One study that is an exception to this is by Lee et al. (2006) in which an improvement in ability to perform the MWM was found after either 5-FU or CP treatment. The reasons for the difference in this result are unclear but have not been replicated and have received adverse comments in the subsequent literature (Reiriz 2006).

Study	Chemotherapy agent	Animals	Dose and administration	Time post chemotherapy	Cognitive assessment and outcome	Comments
Antimetabolites						
Winocur et al. (2011)	MTX+5-FU	Female BALB/C mice (3 months old)	MTX+5-FU: 50+75mg/kg all i.p. weekly for 4 weeks	1 to 5 weeks	Impaired in MWM, NMTS, and dNMTS tasks Unimpaired in cued memory	All MTX+5-FU impairments reversed by donepezil (3mg/kg/day)
Walker et al. (2011)	MTX 5-FU MTX+5-FU	Male Swiss-Webster mice (20-25g)	MTX: 3.2 or 32mg/kg 5-FU: 75mg/kg MTX+5-FU: 3.2/32+75mg/kg all i.p. weekly for 3 weeks	8 and 9 days	Impaired acquisition and retention response (autoshaping)	Effects of MTX and 5-FU were potentiated in combination Impaired acquisition and retention response (autoshaping) with chronic tamoxifen treatment
Yang et al. (2011)	MTX	Male C57BL/6 mice (8-9 weeks old)	40mg/kg, single i.p. injection	1 and 7 days	Impaired in NOR	Dose-dependent reduction in proliferation of hippocampal cells Increased depression–like behaviour
Lyons et al. (2011b)	MTX	Male Lister-hooded rats (150-200g)	75 mg/kg, 2 i.p. weekly injections followed by LCV rescue	1 month	Impaired in NLR	Reduced proliferation and survival of hippocampal cells MTX impairments reversed by fluoxetine (10mg/kg/day)
Fardell et al. (2010)	MTX	Male Wistar rats (303-460g)	250mg/kg, single i.p. injection followed by LCV rescue	11, 95 and 255 days	Impaired in NOR at all time points Impaired in MWM at 95 days	
ELBeltagy et al. (2010)	5-FU	Male Lister-hooded rats (150-170g)	20mg/kg, 6 i.p. injections every other day with LCV	1-3 days	Impaired in NLR and CFC	Reduced proliferation and survival of hippocampal cells All 5-FU impairments reversed by fluoxetine (10mg/kg/day)
Li et al. (2010b)	MTX	Acute: Male Long-Evans rats (12 weeks old) Chronic: Male and female Long-Evans rats (2 weeks old)	Acute: 250mg/kg, single i.p. injection Chronic: 1mg/kg, 10 i.p. injections. 2 weekly for 2 weeks then weekly for 6 weeks	3-7 days	Acute and chronic: Impaired in NLR Unimpaired in NOR	Acute and chronic: No impairment in activity or motor coordination Reduced folate levels in both CSF and serum and decreased ratio of CSF S-adenosylmethionine to S-adenosylhomocysteine
Li et al. (2010a)	MTX	Male Long-Evans rats (12 weeks old)	0.5mg/kg, intrathecal over 1min	3-7 days	Impaired in NOR and NLR	Reduced folate levels in both CSF and serum and increased CSF homocysteine No effect on locomotor, exploratory and anxiety-like behaviour
Seigers et al. (2009)	MTX	Male Wistar rats (3 months old)	250mg/kg, single i.v. injection followed by LCV rescue	MWM: 1 week CFC: 1 month	Impaired in MWM and CFC	Reduced proliferation of hippocampal cells at 7 days, but not 1 day Reduced white matter density at 1 day and 1 and 3 weeks No reduction in apoptosis of hippocampal cells
Foley et al. (2008)	MTX 5-FU MTX+5-FU	Male Swiss-Webster mice (20-35g)	MTX: 1-32mg/kg 5-FU: 3-75mg/kg MTX+5-FU: 3.2+75mg/kg All single i.p. injections	1 and 2 days	MTX and 5-FU (3-30mg/kg) did not reduce operant conditioning either day 5-FU (75mg/kg) reduced operant conditioning on day 2 but not 1 MTX+5-FU reduced operant conditioning on both days	Enhanced effects with combined drugs

Gandal et al. (2008)	MTX+5-FU	Male C57BL/6Hsd mice (7-8 weeks old)	MTX+5-FU: 19+37.5mg/kg 37.5+75mg/kg 4 i.p. weekly injections	6-7 weeks	Unimpaired in NOR and CFC	Impaired gating of auditory stimuli (electrophysiology)
Mustafa et al. (2008)	5-FU	Male Lister-hooded rats (200-250g)	25mg/kg, 5 i.v. injections over 12 days	5 days	Impaired in NLR	No reduction in proliferation of hippocampal cells Reduced level of BDNF and DCX in hippocampal tissue
Seigers et al. (2008)	MTX	Male Wistar rats (3 months old)	250mg/kg, single i.v. injection followed by LCV rescue	3-4 weeks	Impaired in MWM and NOR	Dose-dependent reduction proliferation of hippocampal cells
Li et al.(2008)	Ara-C	Male Sprague-Dawley rats (200-250g)	400mg/kg, 4 i.p. daily injections	7 days	Impairment in remote recall of MWM Unimpaired spatial learning and recent memory of MWM	Retraction of the neuronal apical dendrites in the anterior cingulate cortex but not the CA1
Lee et al. (2006)	5-FU	Female Fischer-344 rats (7 months old)	150mg/kg, i.p., every 4 weeks for 18 weeks	MWM: 7 or 29 weeks; Stone maze: 9 or 42 weeks	Improvement in MWM Unimpaired in Stone maze	
Winocur et al. (2006)	MTX+5-FU	Female BALB/C mice (2 months old)	MTX+5-FU: 37.5+75mg/kg, single i.p. injection	1 month	Impaired in MWM, NMTS and dNMTS Unimpaired in cue memory and discrimination learning	
Madhyastha et al. (2002)	MTX	Male Wistar rats (4 months old)	1.5 or 2mg/kg, cannula into lateral ventricle, 3 doses, every other day	7 days	Impaired in conditioned avoidance learning and memory	Reduced concentrations of neurotransmitters (noradrenaline, dopamine, and serotonin) and 5-hydroxyindoleacetic acid No effect on anxiety-like behaviour
Sieklucka-Dziuba et al. (1998)	MTX	Male and female Albino Swiss mice (20-25g)	10 mg/kg, single i.p. injection	24 h, 7 days and 14 days	Impaired passive avoidance at 14 days Unimpaired passive avoidance at 24 h and 7 days	Decreased GABA in brain tissue Increased susceptibility to seizures
Stock et al. (1995)	MTX	Male and female Sprague-Dawley rats (17 days old)	0.005mg/kg, single i.p. injection	10 weeks	Unimpaired in taste aversive conditioning	
Yanovski et al. (1989)	MTX	Male and female Lewis-inbred rats (16-17 days old)	0.005mg/kg, single i.p. injection	10 weeks	Impaired in CER and taste aversive conditioning	
Alkylating agents						
Lyons et al. (2011a)	CP	Male Lister-hooded rats (125-150g)	30 mg/kg, 7 i.v. injections every 3 days	5 days	Unimpaired in NLR	No effect on proliferation of hippocampal cells Reduced survival of new hippocampal cells
Mondie et al. (2010)	thioTEPA	Male C57BL/6J mice (5 weeks old)	10mg/kg, 3 i.p. daily injections	1-30 weeks	Impaired in NOR at 12 and 20 weeks Impaired in NLR at 20 weeks	Reduced proliferation of hippocampal cells at 1, 8 and 12 weeks No effect on depression behaviour
Yang et al. (2010)	CP	Male ICR mice (8-10 weeks old)	40mg/kg, single i.p. injection	12 h and 10 days	Impaired in NOR and passive avoidance at 12 h but not 10 days	Reduced proliferation of hippocampal cells No reduction in apoptosis of hippocampal cells
Konat et al. (2008)	CP+Doxo-rubicin	Female Sprague-Dawley rats (10 months old)	CP+Doxorubicin: 25+2.5mg/kg 4 i.p. weekly injections	7 days	Impaired in passive avoidance	Impairments restored by *N*-acetyl cyteine No effect on locomotor, exploratory and anxiety-like behaviour
Macleod et al. (2007)	CP+Doxo-rubicin	Female ovariectomised Sprague-Dawley rats (8 weeks old)	CP+Doxorubicin: 40+4mg/kg 3 i.v. weekly injections	7 days	Impaired in CFC Unimpaired in CER	

Reiriz et al. (2006)	CP	Male CF1 mice (70-90 days old)	8,40 or 200mg/kg, single i.p. injection	1 and 7 days	Impaired in inhibitory avoidance by 40 and 200mg/kg at 1 day Unimpaired in inhibitory avoidance at 7 days	
Lee et al. (2006)	CP	Female Fischer-344 rats (young: 7 and aged: 18 months old)	Young: 100mg/kg, i.p., every 4 weeks for 18 weeks Aged: 80mg/kg, i.p., for 16 weeks	MWM: 7 or 29 weeks; Stone maze: Young: 9 or 42 weeks Aged: 16 weeks	Improvement in MWM Unimpaired in Stone maze	Young: Enhanced neural synaptic function
Topoisomerase inhibitors						
Liedke et al. (2009)	Doxorubicin	Male Wistar rats (180-350g)	0.5, 2 or 8mg/kg, single i.p. injection	3 h, 1 day and 7 days	Impaired in inhibitory avoidance at 1 and 7 days with 8 and 2 mg/kg. Unimpaired in inhibitory avoidance at 3 h or 0.5mg/kg	No effect on locomotor and anxiety-like behaviour
Konat et al. (2008)	CP+Doxo-rubicin	Female Sprague-Dawley rats (10 months old)	CP+Doxorubicin: 25+2.5mg/kg 4 i.p. weekly injections	7 days	Impaired passive avoidance	Impairments restored by *N*-acetyl cyteine No effect on locomotor, exploratory and anxiety-like behaviour
Macleod et al. (2007)	CP+Doxo-rubicin	Female ovariectomised Sprague-Dawley rats (8 weeks old)	CP+Doxorubicin: 40+4mg/kg 3 i.v. weekly injections	7 days	Impaired in CFC Unimpaired in CER	
Sieklucka-Dziuba et al. (1998)	Doxorubicin	Male and female Albino Swiss mice (20-25g)	5 or 10 mg/kg, single i.p. injection	24 h, 7 days and 14 days	Unimpaired passive avoidance	Decreased GABA in brain tissue Increased susceptibility to seizures
Antimicrotuble agents						
Boyette-Davis and Fuchs (2009)	Paclitaxel	Male Long-Evans rats	1mg/kg, single i.p. injection	24 h	Unimpaired in five choice serial reaction time test	
Methylating agents (used as a model to reduce neurogenesis)						
Ko et al. (2009)	MAM	Male C57BL/6NCrljBgi mice (6-8 weeks old)	3mg/kg, 14 s.c. daily injections	2 days	Unimpaired in CER	Dose-dependent reduction proliferation of hippocampal cells
Bruel-Jungerman et al. (2005)	MAM	Male Sprague-Dawley rats (220-250 g)	5mg/kg, 14 s.c. daily injections	3 days	Impaired in NOR	Reduced hippocampal neurogenesis Environmental enrichment improves NOR and increases neurogenesis. These effects are prevented by MAM treatment during enrichment
Shors et al. (2002)	MAM	Male Sprague-Dawley rats (220-250 g)	7mg/kg, 14 i.p. daily injections	1-14 days	Impaired in CER, CFC and trace fear conditioning Unimpaired MWM and plus maze	Reduced hippocampal neurogenesis No effect on anxiety-like behaviour
Shors et al. (2001)	MAM	Male Sprague-Dawley rats (adult)	5mg/kg, 14 i.p. daily injections	5 days	Impaired in trace conditioning Unimpaired in delay conditioning	Reduced hippocampal neurogenesis No effect on anxiety-like behaviour

Table 1.2 Please see overleaf for legend

Table 1.2 Summary of rodent models investigating the effect of chemotherapy on cognition, listed in chronological order from most recent. Abbreviations: Chemotherapy agents; MTX: methotrexate, 5-FU: 5-fluorouracil, CP: cyclophosphamide, Ara-C: cytosine arabinoside, LCV: Leucovorin, MAM: methylazoxymethanol acetate. Cognitive tasks; MWM: Morris water maze, NOR: novel object recognition, NLR: novel location recognition, NMTS: non-matching to sample, dNMTS: delayed non-matching to sample, CER: conditioned emotional response, CFC: contextual fear conditioning. Other; CSF: cerebrospinal fluid, BDNF: brain derived neurotrophic factor, DCX: double cortin.

1.1.3.1 The role of antimetabolites in chemotherapy-induced cognitive impairment

Antimetabolites are metabolic substances which disrupt nucleic acid synthesis or nucleotide synthesis, leading to an arrest in the cell cycle (Perry and McKinney 2008). The most commonly investigated antimetabolites in rodent models of cognitive dysfunction are MTX and 5-FU (described in detail in section 1.2.3 and 1.2.4 of the present chapter), administered either individually or in combination (Table 1.2). These chemotherapy agents were selected for use in the present study. A large number of studies have shown that MTX can cause cognitive impairment in an array of cognitive tasks (Fardell et al. 2010; Li et al. 2010a; Madhyastha et al. 2002; Seigers et al. 2008; Seigers et al. 2009; Sieklucka-Dziuba et al. 1998; Yanovski et al. 1989), when animals were tested between 3 and 225 days after completion of chemotherapy treatment. Li et al. (2010b) found that MTX impaired performance in the spatial NLR task but not in the novel object recognition (NOR) task (for detailed descriptions of these tasks see section 1.8 of the present Chapter). Conversely, there is one study by Lee et al. (2006) where MTX treated rats showed an improvement compared to controls in the MWM and this group also observed that MTX had no effect on the Stone maze task. In addition, Foley et al. (2008), did not see an effect of MTX alone on operant conditioning, but found it to potentiate the effects of 5-FU. Another study showed that rats treated with MTX were unimpaired in appetitive or aversive conditioning (Stock et al. 1995) although here an extremely low dose was used (0.005mg/kg).

Previous studies have also found that 5-FU impaired cognition in rodents in NLR (ElBeltagy et al. 2010; Mustafa et al. 2008) and CER (ElBeltagy et al. 2010) tasks and operant conditioning (Foley et al. 2008), 1 to 5 days after administration. Conversely, one study observed that rats treated with 5-FU were unimpaired in the Stone maze but showed an improvement in the MWM (Lee et al. 2006).

When 5-FU and MTX were administered together, Winocur et al. (2006) found treated mice showed cognitive impairment in spatial MWM, non-matching to sample (NMTS) and delayed

non-matching to sample (dNMTS) tasks but were unimpaired in cue memory and discrimination learning. Furthermore, in a later study, the same group found that these behavioural deficits could be counteracted with co-administration of donepezil, a drug most commonly used to treat Alzheimer's disease (Winocur et al. 2011). Another study showed that MTX enhanced the reduction in operant conditioning caused by 5-FU (Foley et al. 2008). In contrast, Gandal et al. (2008) found this combination had no effect on performance in the NOR and contextual fear conditioning (CFC) tasks.

In many of these studies, neurobiological changes in brain tissue which are associated with the alterations in cognition after MTX and 5-FU administration have been investigated. Both MTX (Seigers et al. 2008; Seigers et al. 2009; Seigers et al. 2010b) and 5-FU (ElBeltagy et al. 2010) were reported to reduce proliferation of hippocampal cells in the dentate gyrus. However, this was not found for 5-FU in one study (Mustafa et al. 2008). MTX was also shown to decrease levels of folate in both cerebrospinal fluid (CSF) and blood serum (Li et al. 2010a; Li et al. 2010b) and reduce the concentrations of the neurotransmitters, noradrenaline, dopamine, and serotonin (5-HT) and the 5-HT metabolite 5-hydroxyindoleacetic acid in the brain (Madhyastha et al. 2002). Furthermore, Han et al. (2008a) observed that 5-FU caused delayed damage to progenitor cells and to myelinated tracts in the central nervous system.

One study found the antimetabolite cytosine arabinoside (Ara-C) impaired remote recall, but not spatial learning and recent memory in the MWM in rats. In addition, they showed that Ara-C treated rats had retraction of the apical dendrites in the neurons in the anterior cingulate gyrus but not in the pyramidal neurons in the hippocampal region CA1 (Li et al. 2008).

1.1.3.2 The role of alkylating agents in chemotherapy-induced cognitive impairment

Alkylating agents directly damage a cell's DNA by forming covalent bonds with guanine nucleotide bases. They can be monofunctional, reacting with only one DNA strand or bifunctional, forming alkyl cross-linkages between both DNA strands (Perry and McKinney 2008). CP (described in detail in section 1.2.2 of the present chapter) is the most investigated

alkylating agent in the literature of animal models of chemotherapy-induced cognitive impairment (Table 1.2) and was used in the experiments in this thesis. The range of cognitive tests and dosing regimens used has produced different outcomes. This drug has been shown to cause cognitive impairment in inhibitory avoidance tasks at 1 day post CP treatment (Reiriz 2006), in passive avoidance (Konat et al. 2008) and in the NOR (Yang et al. 2010) task. However, CP did not reduce performance in inhibitory avoidance tasks at 7 days post CP treatment (Reiriz 2006) or in the Stone maze (Lee et al. 2006) and it even caused an improvement in performance in the MWM (Lee et al. 2006). Studies have also shown that CP decreases cell proliferation in the hippocampus (Yang et al. 2010), although it did not affect apoptosis (Yang et al. 2010) and it enhanced neural synaptic function in young rats (Lee et al. 2006).

Another alkylating agent, thioTEPA was investigated by Mondie et al. (2010). Rats treated with thioTEPA were impaired in the NOR and NLR tasks and all had reduced hippocampal cell proliferation. MacLeod et al. (2007) observed that CP in combination with the topoisomerase inhibitor, doxorubicin, inhibited performance in the CFC but not the CER task and also reduced proliferation of hippocampal cells. Konat et al. (2008) found the same combination impaired passive avoidance, although interestingly, this was prevented by co-administration of the antioxidant, *N*-acetyl cyteine.

1.1.3.3 The role of topoisomerase inhibitors, antimicrotuble agents and methylating agents in chemotherapy-induced cognitive impairment

Although topoisomerase inhibitors, antimicrotuble agents and methylating agents were not used in the studies reported in the present thesis, their effects on cognition have been looked at in previous rodent models. Topoisomerase inhibitors inhibit the enzymes topoisomerase I and II, which catalyse the cleaving and rebinding of the DNA double helix, regulating DNA replication and RNA transcription. Inhibition of these enzymes causes cell necrosis, apoptosis or cell cycle arrest (Perry and McKinney 2008). Doxorubicin is a topoisomerase inhibitor

used in rodent models of cognitive impairment. Liedke et al. (2009) found it impaired inhibitory avoidance in rats at 1 and 7 days after administration but not 3 hours. However a different study found that it had no effect on passive avoidance in mice (Sieklucka-Dziuba et al. 1998). Its effects have also been investigated when it is administered in combination with CP and are discussed in the previous paragraph.

Antimicrotubule agents are compounds often derived from plants. They prevent cell division during M phase by interfering with microtubules which form the mitotic spindle necessary for separation of replicated DNA (Perry and McKinney 2008). Paclitaxel is such an agent but to date, only one animal study has investigated this drugs effect on cognitive function, showing that rats treated with paclitaxel were unimpaired in the five choice serial reaction time test (Boyette-Davis and Fuchs 2009).

Although the methylating agent methylazoxymethanol acetate (MAM) is not used as a chemotherapeutic agent, it is a cytostatic and has been used in rodent models of cognitive dysfunction to block neurogenesis. In all the models hippocampal cell proliferation was successfully reduced (Bruel-Jungerman et al. 2005; Ko et al. 2009; Shors et al. 2001; Shors et al. 2002). One study showed that MAM impaired NOR performance in rats, but this was improved and neurogenesis was increased by environmental enrichment (Bruel-Jungerman et al. 2005). Shors and his group demonstrated that rats treated with MAM were impaired in CER, CFC and trace fear conditioning but not MWM, plus maze and delay conditioning (Shors et al. 2001; 2002).

1.2 Drugs used in the present study implicated in chemotherapy-induced cognitive impairment

1.2.1 Cyclophosphamide, methotrexate and 5-fluoruracil (CMF)

Of all the drug combinations investigated in clinical studies of chemotherapy-induced cognitive impairment, it is difficult to determine which cytostatic agent, or indeed, which particular combinations of agents lead to cognitive dysfunction. Some studies have focused on single chemotherapy combination regimens (Kreukels et al. 2008; Schagen et al. 2008; Schagen et al. 1999), whilst others have obtained results from a number of combination regimens grouped together (Ahles et al. 2010; Bender et al. 2006; Quesnel et al. 2009; Tager et al. 2010). Most of the existing studies have investigated the effects of drugs used as adjuvant chemotherapy for treatment of breast cancer (Castellon et al. 2005) and have most frequently investigated the effects of the CP, MTX and 5-FU (CMF) combination (Brezden et al. 2000; Donovan et al. 2005; Hurria et al. 2006b; Kreukels et al. 2008; Schagen et al. 2002a; Schagen et al. 1999; Shilling et al. 2005; Tchen et al. 2003; Wieneke and Dienst 1995). The majority of these studies have found that CMF causes a deficit in one or more neuropsychological domain (Brezden et al. 2000; Hurria et al. 2006b; Kreukels et al. 2008; Schagen et al. 2002a; Schagen et al. 1999; Shilling et al. 2005; Wieneke and Dienst 1995). Furthermore, there is evidence for the involvement of these cytostatic drugs in cognitive dysfunction and reduced neurogenesis in animal studies (Table 1.2). In the work of this thesis, the effects of these three chemotherapy agents are investigated.

1.2.2 Cyclophosphamide

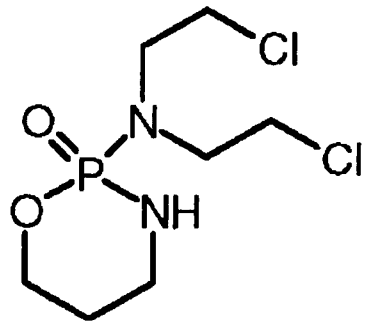

Figure 1.1 Chemical structure of CP (*N,N*-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine 2-oxide)

CP (Fig. 1.1) is a widely used chemotherapeutic drug developed in the late 1960s. It is used to treat a range of cancers including leukaemia, lymphomas, soft tissue and osteoginic sarcomas, paediatric malignancies and adult solid tumours; in particular breast and lung carcinoma (Allwood et al. 1997). It also acts as an immunosuppressant agent, by attacking leukocytes in the treatment of non-malignant diseases including rheumatoid arthritis, chronic interstitial pneumonia, multiple sclerosis as well as for organ transplant (Anderson et al. 1995). It is easily absorbed, given orally or by i.v. route and has a relatively low toxicity compared to other anti-cancer drugs, so is often given in a high dose to both adults and children (Anderson et al. 1995). It is able to cross the blood-brain barrier due to its low molecular weight (Perry and McKinney 2008). It has a half-life of 3 to 10 hours in humans and its primary active metabolite, phoshoramide mustard, has a half-life of 8 to 9 hours (Fischer et al. 2003).

CP is a very effective chemotherapeutic, but it can also cause some side-effects, predominantly due to its most toxic metabolite, acrolein. These include myelosuppression and sterility and if given during pregnancy, it can also cross the placenta and cause malformations in the foetus (Matalon et al. 2004). A frequent complication of this drug is due to its toxicity to bladder epithelial cells causing hemorrhagic cystitis, although this can be

prevented (Hu et al. 2008; Toren and Norman 2005; Wantuch et al. 2007). CP is carcinogenic itself as it has the ability to produce free oxygen radicals (Matalon et al. 2004).

1.2.2.1 Mechanism of action of CP

CP is an alkylating agent and belongs to the group of anti-cancer drugs, oxazaphosphorines. It is initially metabolised by the liver by cytochrome P450 into 4-hydroxycyclophosphamide and its tautomer, aldophosphamide. Aldophosphamide is further metabolised within peripheral tissue and tumour cells to phoshoramide mustard and acrolein (Matalon et al. 2004). Phoshoramide mustard is the major metabolite to kill neoplasmic cells by forming covalent linkages of alkyl groups, between and within DNA strands of mitotic cells. This disrupts the DNA and causes the cell to undergo apoptosis. As with all alkylating agents, this is independent of the stage of cell cycle (Matalon et al. 2004).

Aldophosphamide is oxidised by aldehyde dehydrogenase (ALDH), therefore CP can only be efficient in cells which contain low levels of ALDH. This also allows relatively low toxicity as bone marrow stem cells, liver cells and intestinal epithelial cells all contain a large amount of ALDH. ALDH3, a type of ALDH which can breakdown CP into non-toxic metabolites, is present in the brain (Bunting and Townsend 1996).

1.2.3 5-Fluorouracil

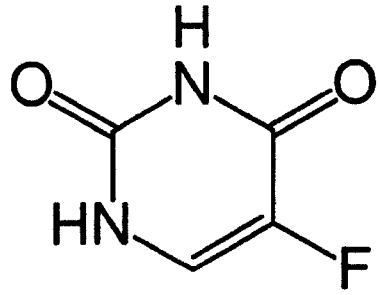

Figure 1.2 Chemical structure of 5-FU (5-fluoro-1*H*-pyrimidine-2,4-dione)

5-FU (Fig. 1.2) has been used for over 40 years to treat breast, colorectal, head and neck, bladder, gastrointestinal and ovarian cancer and hepatoma (Fischer et al. 2003). When used as adjuvant chemotherapy to treat breast cancer, it is systemically administered by the i.v. route along with other chemotherapy agents. The most common regimens involve the combinations CMF, 5-FU, epirubicin and CP (FEC) and 5-FU, doxorubin and CP (FAC) (Guarneri et al. 2007). It able to cross the blood-brain barrier by passive diffusion and reaches a concentration of 48% CSF: plasma ratio after being administered as a bolus dose (Patel et al. 1998). However, it has a short half-life of 10 to 25 minutes in serum, for both humans (Diasio and Harris 1989) and rats (Celio et al. 1983) and is broken down in the liver by dihydropyridimine dehydrogenase (Longley et al. 2003). Chronic administration of 5-FU is reported to cause neurological toxicity with cerebellar ataxia, myelosuppression and rarely encephalopathy (Perry and McKinney 2008), which is unsurprising given the high levels of 5-FU which are possible in the brain. Other toxicities of 5-FU include diarrhoea, hand foot syndrome and cardiac complications (Perry and McKinney 2008).

1.2.3.1 Mechanisms of action of 5-FU

5-FU is a fluorinated analogue of uracil (Fig. 1.3) which acts as an antimetabolite. It is intracellularly converted into its three main active metabolites; fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) (Fig. 1.3). These active metabolites are cytotoxic to the cell by disrupting thymidine synthetase (TS) and RNA synthesis (Longley et al. 2003).

TS inhibition is the major mechanism of action for 5-FU (Fig. 1.3). TS catalyses the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), which is necessary for the synthesis of the DNA pyrimidine base, thymidine. The conversion of dUMP to dTUMP is facilitated by the methyl donor, 5,10-methylenetetrahydrofolate (CH_2THF). The active metabolite FdUMP binds to TS, blocking the binding site of dUMP and inhibiting the synthesis of dTMP, during S-phase of the cell

cycle leading to cell death resulting in lethal DNA damage. Furthermore, inhibition of TS leads to the build up of dUMP. dUMP and 5-FU metabolite FdUTP can be misincorporated into DNA, causing the strands to break, leading to cell death (Longley et al. 2003) (Fig. 1.3). Despite its short half-life, 5-FU can lead to reductions in TS levels for several days (Longley et al. 2004).

The folate co-factor, CH_2THF is required for optimal binding of FdUMP to TS, enhancing FdUMP's effects. Leucovorin (LCV) is a synthetic co-factor of folate which can enhance the effects of the 5-FU metabolite by this mechanism. It is therefore often administered clinically with 5-FU to potentiate its cytotoxic effect (Herrmann et al. 1988) (Fig. 1.3). It is to be noted that LCV is also given after MTX chemotherapy treatment as a rescue therapy and not to potentiate its effects.

The third active metabolite of 5-FU, FUTP, is extensively misincorporated into RNA. This disrupts the normal processing of the RNA affecting cellular metabolism and viability (Longley et al. 2003).

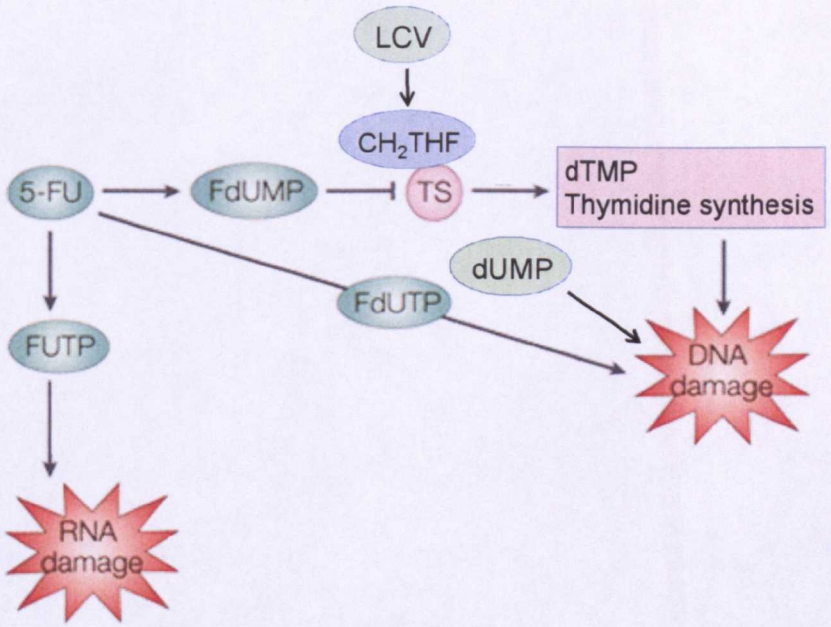

Figure 1.3 Mechanisms of action of the antimetabolite 5-FU, modified from Longley et al. (2003). Normally deoxyuridine monophosphate (dUMP) is converted to deoxythymidine monophosphate (dTMP) which is necessary for synthesis of the DNA pyrimidine base, thymidine. This is catalysed by thymidine synthetase (TS) and facilitated by the binding of 5,10-methylenetetrahydrofolate (CH_2THF) to TS. The major active metabolite of 5-FU, fluorodeoxyuridine monophosphate (FdUMP), competitively binds to TS (also facilitated by the binding of CH_2THF) inhibiting the production of dTMP, leading to DNA damage and cell death. This disruption also causes a build up of dUMP. Both dUMP and another active metabolite of 5-FU, fluorodeoxyuridine triphosphate (FdUTP) get incorporated into the DNA causing lethal damage. The third active metabolite of 5-FU, fluorouridine triphosphate (FUTP), is misincorporated into RNA affecting cellular metabolism and viability. Leucovorin (LCV) increases intracellular levels of CH_2THF, enhancing binding of FdUMP to TS, enhancing the effects of the 5-FU metabolite (Longley et al. 2003).

1.2.4 Methotrexate

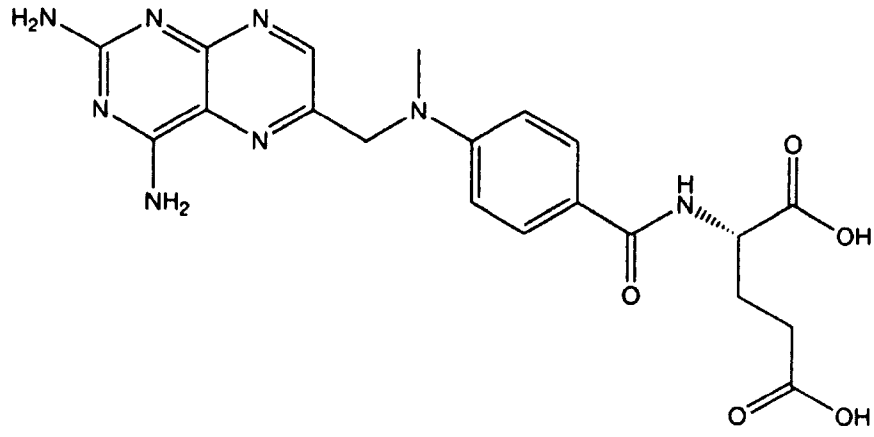

Figure 1.4 Chemical structure of MTX ((2*S*)-2-[(4- phenyl)formamido]pentanedioic acid)

MTX (Fig. 1.4) has been in use since the 1950s and is used to treat many cancers including breast, head and neck, bladder and colorectal and meningeal leukaemia (Fischer et al. 2003). It is also used to terminate pregnancies and as anti-inflammatory and/or immunosuppressive treatment for autoimmune diseases (Genestier et al. 2000). Methotrexate is well absorbed when administered orally, by the i.v. route or intrathecally (Perry and McKinney 2008). It has a half-life of approximately 3 hours (Fischer et al. 2003). It can be neurotoxic, inducing cerebellar ataxia. Other effects include mylosuppression and encephalopathy and renal and pulmonary toxicities (Fischer et al. 2003).

1.2.4.1 Mechanisms of action of MTX

Like 5-FU, MTX also affects the TS pathway (Fig. 1.5). TS catalyses the formation of dTMP which is facilitated by the binding of CH_2THF (Genestier et al. 2000). This converts CH_2THF to dihydrofolate (FH2) which is reduced by dihydrofolate reductase (DHFR) in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) to tetrahydrofolate (THFA). THFA is necessary for DNA synthesis and repair (Cheok and Evans 2006). MTX inhibits the enzyme DHFR, preventing synthesis of THFA. In addition, inhibition of DHFR prevents regeneration

of CH_2THF from FH2 which is required for the synthesis of the DNA base thymidine causing cell death during S-phase of the cell cycle (Genestier et al. 2000) (Fig. 1.5).

LCV antagonises MTX by increasing the intracellular level of CH_2THF and replenishing folate pools depleted by MTX. Therefore LCV is often administered clinically as a rescue therapy after patients have been treated with MTX (Genestier et al. 2000) (Fig.1.5). This is unlike the co-administration of LCV during 5-FU treatment, where it used to enhance the effects of the chemotherapy.

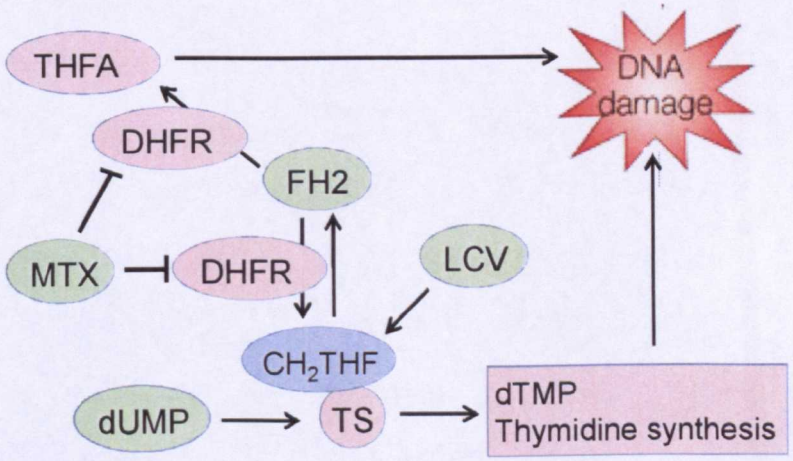

Figure 1.5 Mechanisms of action of the antimetabolite MTX. This diagram was created by use of information from Genestier et al. 2000. 5,10-methylenetetrahydrofolate (CH_2THF) is converted to dihydrofolate (FH2) when it binds to thymidine synthetase (TS) to catalyse the formation of deoxythymidine monophosphate (dTMP). FH2 is reduced by dihydrofolate reductase (DHFR) to tetrahydrofolate (THFA), which is necessary for DNA synthesis and repair. DHFR is inhibited by MTX, preventing synthesis of THFA. DHFR inhibition also prevents regeneration of CH_2THF from FH2 which is required for the synthesis of the DNA base thymidine causing cell death. Leucovorin (LCV) replenishes intracellular folate pools and increases levels of CH_2THF, antagonising the effects of MTX (Genestier et al. 2000).

1.3 Adult hippocampal neurogenesis

The experiments in this thesis investigate the effects of the chemotherapy agents in the CMF medication combination on memory. A possible underlying cause for the cognitive impairments observed is the cytotoxic effect of these chemotherapy drugs on hippocampal neurogenesis, which will therefore be discussed below.

1.3.1 Neural stem cells (NSC)

New neurons in the adult brain originate from dividing precursor cells, therefore neural stem cells (NSC) are the basis for adult hippocampal neurogenesis. Stem cells are undifferentiated cells which have the ability to continually divide by mitosis and differentiate into numerous cell types (Kosodo et al. 2004; Mongiat and Schinder 2011). Stem cells have different levels of diversity depending on from where they originate. A newly fertilised egg is totipotent and is able to generate all the cells of an organism (Schöler 2007). Stem cells from an embryo more than a few days old are classed as pluripotent. These cells are descended from totipotent cells and can give rise to almost all cell types. Multipotent cells can differentiate into a limited number of cell types but not all body tissue (Rao 2004). There are also oligopotent cells which have the ability to differentiate into a few cell types and unipotent cells, which are able to self-renew, but only into their own cell type (Schöler 2007).

The most important attribute to a stem cell is that it has the unlimited ability to self-renew whilst remaining undifferentiated (Rao 2004). However, some scientists classify them as also having the potency to generate a minimum of two different types of cell (McKay 1997; Weissman et al. 2001), which would indicate that unipotent cells would not be within the stem cell definition. Embryonic stem cells can be defined as true stem cells as they are pluripotent and are able to continuously self-renew. Adult stem cells, however, can only be multipotent, oligopotent or unipotent and their capacity for self-renewal is limited and are therefore better described as precursors. Although precursor cells do not have the ability to continuously self-renew, they have a large capacity for proliferation at a greater rate than stem cells. Therefore,

they can greatly increase a population of cells and are referred to as "transit amplifying cells" (for review see Kempermann 2006; Kriegstein and Alvarez-Buylla 2009).

During brain development, division of NSC can be symmetric, resulting in two identical daughter stem cells or asymmetric, producing one stem cell and one transit amplifying progenitor cell (Fig. 1.6). These progenitor cells can further divide symmetrically to produce two identical daughter cells, similar to the mother cell or divide asymmetrically to produce a similar daughter cell and one with different characteristics to the mother. In addition, these progenitor cells can symmetrically divide into two cells identical to each other but different from the mother (Kempermann 2006) (see Fig. 1.6). This is referred to as a neurogenic division and these cells have the ability to differentiate into neurons (Kosodo et al. 2004).

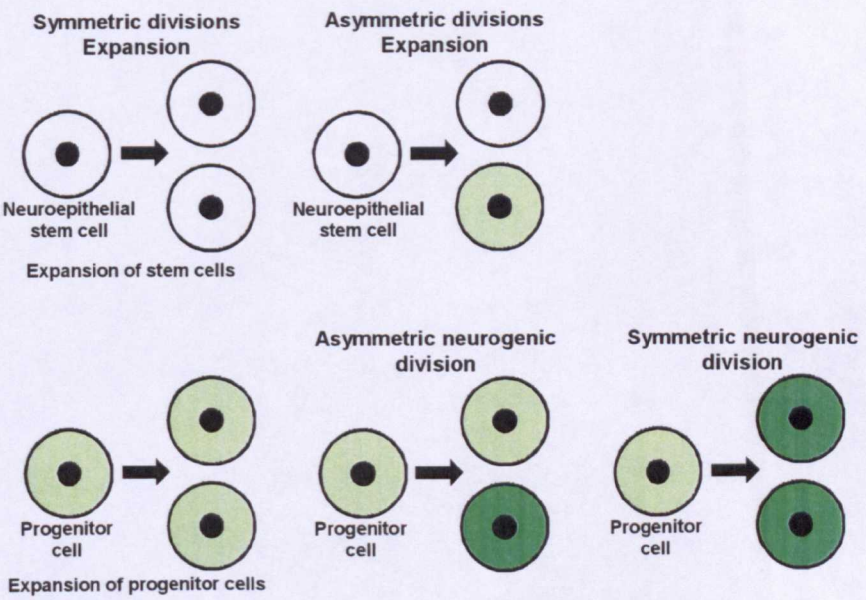

Figure 1.6 Diagram illustrating the symmetric and asymmetric divisions of stem cells and progenitors in brain development, modified from Kempermann (2006). Neuroepithelial stem cell division can be symmetric, resulting in two identical daughter cells similar to the mother or asymmetric resulting in a stem cell and a transit amplifying progenitor cell. These progenitor cells can than divide symmetrically to produce identical daughter cells similar to the mother or asymmetrically to produce one cell similar to the mother and one with different characteristics. Progenitor cells can also undergo neurogenic symmetric division generating two cells which differ from the mother which can differentiate into neurons.

1.3.2 Adult Neurogenesis

It was once thought that new neurons could not be created in the mammalian adult brain and once complex neural systems had been formed they could not be modified. Indeed, it was assumed that the central nervous system had no plasticity after embryogenesis (Altman 1962). However, in the sixties, Josef Altman discovered the generation of new neurons in the adult rat brain using radioactive labelled thymidine-incorporation (Altman 1962; Altman and Das 1965). This was termed adult neurogenesis, and although originally met with some scepticism, it is now accepted that new neurons are formed in the CNS in all mammals studied to date (Ehninger and Kempermann 2008). Adult neurogenesis not only refers to the proliferation of neural progenitors, but the migration, differentiation and functional integration of these new neurons (Knobloch and Jessberger 2011).

Generation of neurons in the adult CNS has been documented in numerous species from invertebrates, fish (Wullimann 2009; Zupanc 2006) and reptiles (Garcia-Verdugo et al. 1989) to birds (Goldman 1998) and mammals, including rodents (Altman and Das 1965), primates (Gould et al. 1999) and humans (Curtis et al. 2011; Eriksson et al. 1998). However, the occurrence of postnatal neurogenesis appears to be inversely related to brain complexity and is restricted to limited brain regions in higher vertebrates. For example, in fish, it is reported that almost all brain regions are prolific (Fernández et al. 2011; Zupanc 2006), whereas in mammals neurogenesis appears to be limited to specific areas (Gage 2000; Imayoshi et al. 2009). The predominant neurogenic regions in the mammalian adult brain are the dentate gyrus within the hippocampus and the subventricular (SVZ) zone of the lateral ventricles (Imayoshi et al. 2009). Neurons born in the mammalian SVZ migrate along the rostral migratory stream to the olfactory bulb, where they differentiate into granule cell and periglomerular cell interneurons (Zhao et al. 2008). Newly generated neurons in the mammalian hippocampus are born in the subgranular zone (SGZ) and migrate up the granular cell layer (GCL) in the dentate gyrus. Additionally, there is evidence that adult neurogenesis occurs in other brain regions such as the neocortex in primates (Gould et al. 1999) and the

rodent hypothalamus (Fowler et al. 2008), although relatively little is known about the functional relevance of these new neurons compared to those in the hippocampus.

1.3.3 Anatomy, circuitry and neurogenesis in the adult hippocampus

The hippocampus is a structure located in the medial temporal lobe of the mammalian brain, so called because of its resemblance to a seahorse. There are two hippocampi, one residing in each hemisphere of the brain. They consist of the cornu ammonis (CA) region comprising of CA1, CA2, CA3 and CA4 (or hilus) sub-regions and the dentate gyrus (Toni and Sultan 2011) (Fig. 1.7). The major form of neocortical input to the hippocampus is from the entorhinal cortex through the perforant path axon bundle (Fig. 1.8). These axons synapse onto granule cell neurons in the dentate gyrus which are the main constituent for the granule cell layer of the hippocampus. The granule cell axons, or mossy fibres, project to the pyramidal cells of the CA3 sub-region, then to the CA1, through the Schaffer collateral pathway. The signal is returned from the CA1 sub-region to the entorhinal cortex via the subiculum (Aimone et al. 2011; Toni and Sultan 2011) (Fig. 1.8). This circuitry constitutes the main flow of information through the hippocampus.

The dentate gyrus is the gateway to hippocampal circuitry. It consists of two blades, which occlude as it progresses caudally. The neurogenic region of the dentate gyrus is the SGZ which is between the granule cell layer (GCL) and the hilus (Fig. 1.7). The neural precursors in this region consist of two types of cell. Type 1 cells are radial glia-like stem cells, also known as B cells which have astrocytic properties with respect to their morphology and labelling (expressing glial fibrillary acidic protein, or GFAP) (Fukuda et al. 2003; Suh et al. 2009). Type 2 cells (or D cells) are non-radial transit amplifying progenitors which arise from asymmetric division of the stem cells. They are highly proliferative and differentiate into neurons after limited self-renewal. They can be identified by expression of the early neuronal marker, doublecortin (DCX) and do not express the glial marker GFAP (Fukuda et al. 2003). After 1 to 3 days the type 2 D cells start to differentiate and move up through the GCL as

immature neurons. They extend dendrites into the molecular layer at 1 to 3 weeks of age and develop an axon which contributes to the mossy fibre pathway, feeding back to the CA3 sub-region, eventually differentiating into mature granule cell neurons or G cells at about 4 weeks old (Suh et al. 2009; Toni and Sultan 2011) (Fig. 1.7).

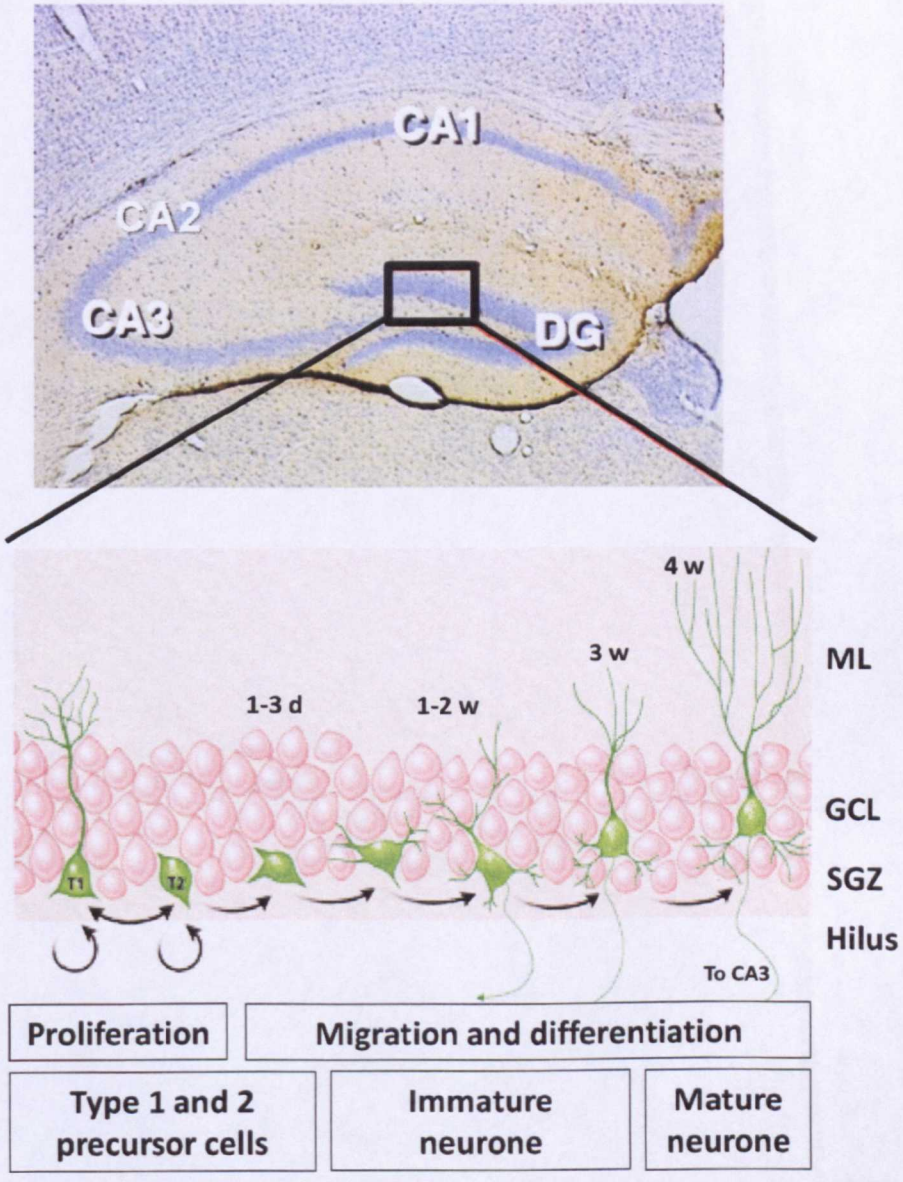

Figure 1.7 Photograph of the adult rat hippocampus highlighting the cornu ammonis (CA) sub-regions, CA1, CA2 and CA3 and the dentate gyrus (DG) modified from Abrous et al. (2005). Below is a diagram illustrating the proliferation, migration and differentiation of newly generated neurons modified from Suh et al. (2009). Type 1 (T1) radial glia-like stem cells and type 2 (T2) non radial transit amplifying progenitor cells proliferate in the subgranular zone (SGZ) of the DG. At 1 to 3 days, they start to migrate up through the granule cell layer (GCL). As they migrate as immature neurons, they differentiate and start to protrude axons up through the GCL to the molecular layer (ML) and CA3 sub-region at 1 to 2 weeks. By 4 weeks they are mature granule cell neurons.

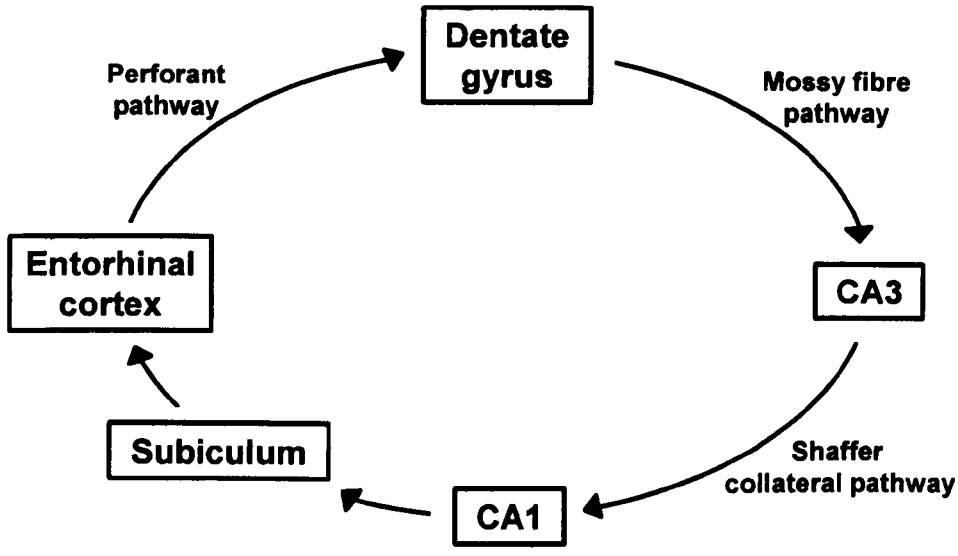

Figure 1.8 Diagram of hippocampal circuitry. The signal enters the hippocampus from the entorhinal cortex via the dentate gyrus, along the perforant pathway. It then travels along the mossy fibre pathway to the cornu ammonis 3 (CA3) sub-region then to the CA1 region along the Shaffer collateral pathway. From the CA1 the signal is returned to the entorhinal cortex via the subiculum (Toni and Sultan 2011).

1.3.4 Techniques for the detection of neurogenesis

1.3.4.1 Thymidine and bromodeoxyuridine

Adult neurogenesis was first detected by using tritiated thymidine (Altman 1962). In this method, radioactively labelled thymidine is systemically injected into the blood stream and is incorporated into the DNA of dividing cells during S-phase of the cell cycle. It can then be detected by autoradiography. However, this technique became impractical as it is very difficult to combine with other markers specific to cell type is now rarely used. Currently, the most commonly used method to investigate neurogenesis is immunohistochemistry labelling with 5-bromo-2-deoxyuridine (BrdU). Like tritiated thymidine, BrdU is a thymidine analogue which is systemically administered. It competes with endogenous thymidine to be incorporated during S-phase into the DNA of dividing cells. BrdU can be detected by antibodies and visualised by fluorescence. Therefore, a major benefit of this technique is that it can be combined with different markers when viewed on a confocal microscope (Kempermann 2006).

The dose of BrdU administered to rats has varied between studies. It has been reported that single doses of 20, 50 and 100mg/kg (body weight, i.p.) label 8, 40 and 65% of cells in S-phase in the dentate gyrus of the adult rat, respectively (Cameron and McKay 2001). For a full saturation of S-phase labelled cells, doses of up to 300mg/kg need to be administered (Cameron and McKay 2001). However very high doses of BrdU can be toxic to cells, although this can be overcome by administering multiple injections of a lower dose over days or hours (Taupin 2007). This method is also beneficial for long-term studies as BrdU labelled cells can still be detected 2 years after injection in rats (Kempermann 2006).

There are, however a few concerns using the BrdU technique. There has been suggestion that BrdU may detect cell repair and apoptosis (Cooper-Kuhn and Georg Kuhn 2002). In addition, it has been proposed that BrdU may disrupt the permeability of blood-brain barrier (Gould and

Gross 2002). Despite these potential experimental confounds, BrdU labelling remains the most commonly used method for detection of neurogenesis.

1.3.4.2 Endogenous cell markers

As well as BrdU detection, immunohistochemistry can also be utilised to investigate endogenous antigens within cells which are specific to cell type or stage of the cell cycle. These can be used when observing cell proliferation at the time of death and are summarised in Fig. 1.9. Markers of the early stages of neurogenesis include nestin (an intermediate filament protein specific to NSC) and GFAP (an intermediate filament protein which is only expressed in glial cells). DCX is a microtubule associated protein expressed in transit amplifying progenitors which are differentiating into immature neurons and Neuronal Nuclei (NeuN), a nuclear protein, is present in both immature and mature neurons (Zhao et al. 2008). Endogenous markers used independently or in conjunction with BrdU have enabled investigation of each stage of neurogenesis in the adult rat hippocampus (illustrated in Fig. 1.9).

Ki67 is a protein which is expressed in all stages of the cell cycle but not in resting cells (Scholzen and Gerdes 2000). This makes it an excellent marker for cell proliferation and one of the most widely used tools. However, unlike BrdU, it cannot be used to trace cells over long periods.

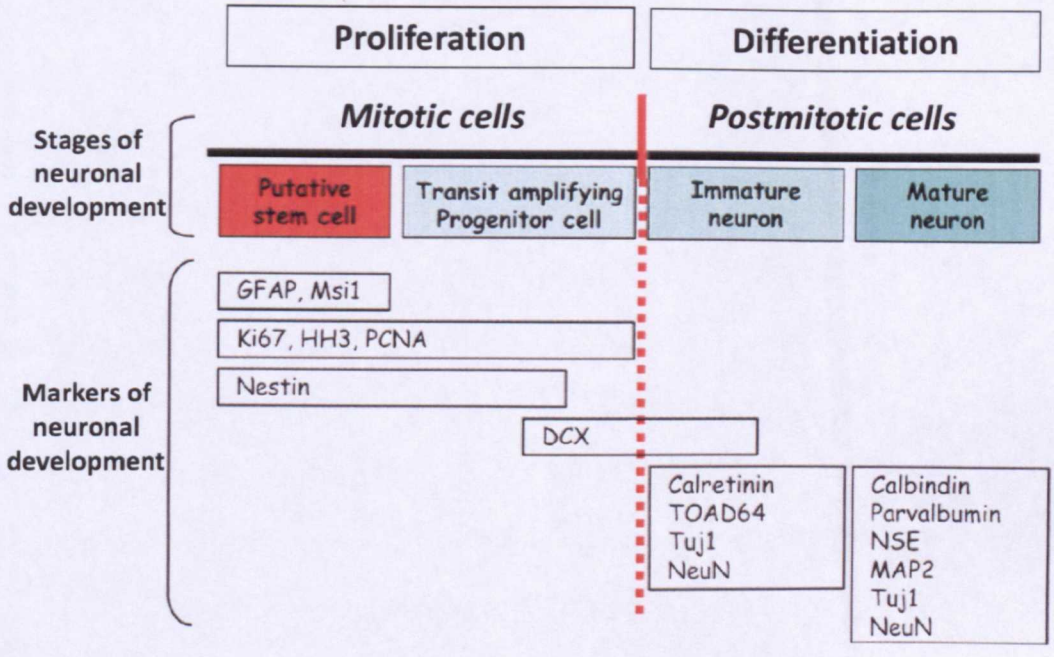

Figure 1.9 Diagram illustrating markers of different stages of granule cell development from stem cell proliferation to mature neuron, modified from Abrous et al. (2005). In studies in the present thesis Ki67 was utilised as it is expressed in all stages of the cell cycle, but not resting cells, however it is not specific to neurons. Doublecortin (DCX) was also selected for use in work in the present thesis as it is expressed in neural progenitor cells and immature neurones. Abbreviations; GFAP: glial fibrillary acidic protein, Msi1: musashi1, HH3: histone H3, PCNA: proliferating cell nuclear antigen, DCX: doublecortin, TOAD64: turned on after division-64 kDa, Tuj1: neuronal class III β-tubulin, NeuN: neuronal nuclei, NSE: neuron-specific enolase, MAP2: microtubule-associated protein 2.

1.3.4.3 Cell proliferation markers used in the present studies: BrdU, Ki67 and DCX

In the experiments discussed in this thesis, immunohistochemistry was used to detect BrdU and Ki67 in the rat hippocampus and DCX was quantified in the hippocampus and frontal cortex by Western blot analysis. DCX is ideal for investigating neurogenesis as it is a marker for young neurons and highly expressed in neurogenic regions in the adult rat brain and is expressed almost exclusively in neural progenitor cells and immature neurones (Nacher et al. 2001). Ki67 was used as a proliferative marker to quantify proliferating hippocampal cells at the time of death. As it is endogenous, it is non-invasive to the cell and it is present in all mammalian neuronal tissue (Scholzen and Gerdes 2000). With respect to adult neurogenesis, Ki67 is present in proliferating stem cells and transit amplifying progenitor cells but not in immature neurons (Abrous et al. 2005) (Fig. 1.9). For comparison, BrdU was selected as it allowed the tracing of cells dividing at a different time point than at the time of animal death. BrdU was administered at the beginning of the experiments to investigate the survival of the cells proliferating at that time. Initially, a single i.p. dose of 250mg/kg was used, but in the later experiments this was switched to 3 daily doses of 100mg/kg in order to label more dividing cells whilst avoiding toxicity. Neither Ki67 nor BrdU are specifically expressed in neurons and in the immunohistochemistry protocols of Ki67 and BrdU, the cells were not double labelled with a neuronal marker. However, it has been reported that 89% of proliferating cells in the rat hippocampus become neurons (Snyder et al. 2009).

1.3.4.4 NSC in vitro

To investigate many aspects of NSCs including their development potential, homeostasis and their direct response to pharmaceuticals, it is often necessary to grow them in cell cultures. NSCs are isolated and grown either as an adherent monolayer or in floating, aggregated neurospheres (Galli et al. 2003). They are cultured in serum free medium in the presence of growth factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF-2) to prevent them from differentiating and to encourage proliferation. To culture a monolayer, stem cells must be grown on surfaces with substrates such as laminin and fibronectin which allow

them to adhere to and often differentiate on the bottom of a flask or well (Palmer et al. 1999). The neurosphere method of culturing spherical clusters of cells was first described by Reynolds and Weiss (1992) and is now widely used as it rapidly produces large numbers of dividing cells and simplifies preparations of single cells (Gritti et al. 2008; Reynolds and Weiss 1992). However, it is to be noted that not all cells in floating neurospheres are NSCs. The spheres are originally generated from dividing NSCs and other dividing NSCs which collide and adhere together. These can undergo symmetric division or divide asymmetrically to generate a stem cell and progenitor. Therefore a neurosphere contains about 10 to 50% of cells with NSC-like properties combined with differentiating progenitors and even some differentiated neurons and glia, depending on the size of the neurosphere and its length of time in culture (Galli et al. 2003) (Fig. 1.10). Most of the proliferating cells are found on the outside of the neurosphere, whereas the differentiating cells are within (Gritti et al. 2008; Kempermann 2006). However, dissociation and re-plating of the neurosphere cell cultures causes the more differentiated cells to die and thus the undifferentiated NSC are forced to proliferate, generating secondary neurospheres (Fig. 1.10). This passage process can be repeated for over a year to produce large cultures of proliferating stem cells, or the growth factors can be removed allowing the neurospheres to differentiate into neurons, astrocytes and oligodendrocytes (Galli et al. 2003).

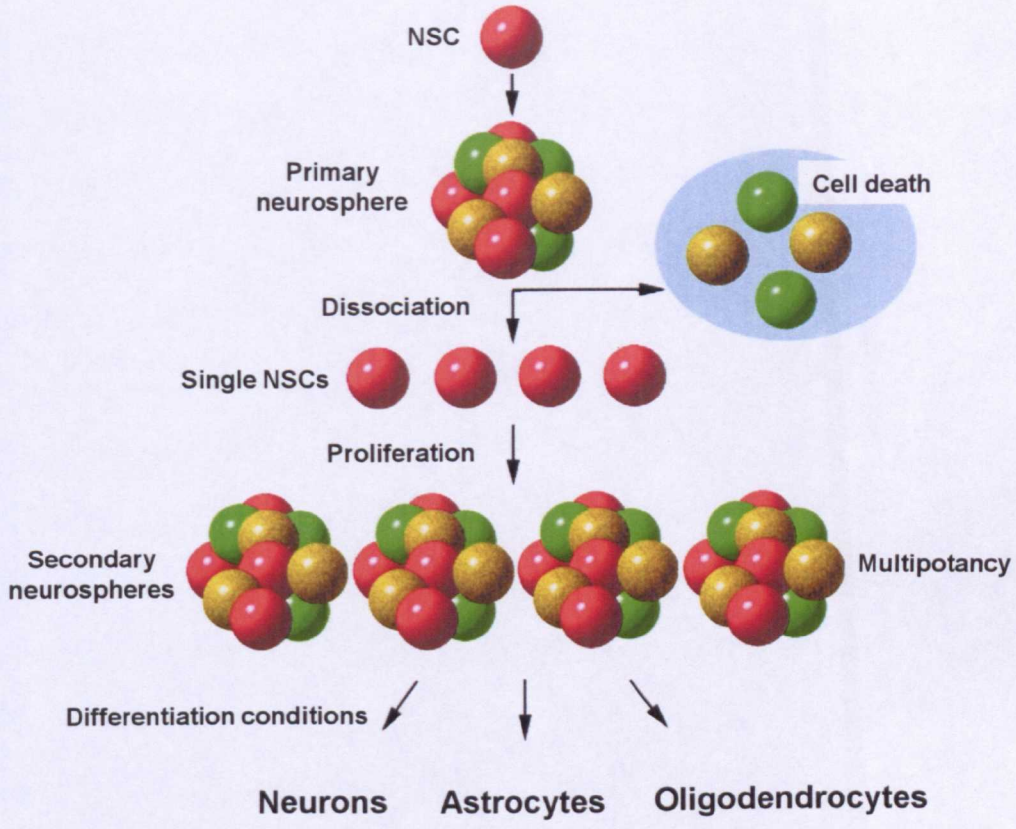

Figure 1.10 Diagram illustrating growth of neurospheres in culture adapted from Galli et al. (2003). Single neural stem cells (NSC) are grown in serum free medium in the presence of growth factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF-2) and proliferate to generate a spherical cluster of dividing cells. They also collide with other proliferating cells to produce larger neurospheres. These consist of cells with NSC-like properties, differentiating progenitors and differentiated neurons and glia. Neurospheres can be dissociated and re-plated under the same conditions, causing the more advanced differentiating cells to die, leaving the NSC to proliferate into secondary neurospheres. This process can be repeated to produce large numbers of proliferating cells. Alternatively the growth factors can be removed allowing the neurospheres to differentiate into neurons, astrocytes and oligodendrocytes (Galli et al. 2003).

1.4 The scale and regulation of neurogenesis

It has been estimated that in the dentate gyrus of the young adult rat, 9,000 new cells are generated per day with an approximate cell cycle time of 25 hours (Cameron and McKay 2001). Many of these differentiate into adult neurons, however a large proportion undergo cell death. It has been reported that nearly 50% of new newly generated neurons die in the first 4 weeks before reaching maturity (Dayer et al. 2003). Indeed, it appears that neuronal death is as important as neuronal birth in functional neurogenesis and only specific newborn neurons will be recruited into circuitry in the hippocampus (Dupret et al. 2007). It is to be noted that the scale of neurogenesis differs between species. Studies by Snyder et al. (2009) have shown that although neurogenesis occurs in the hippocampus of both rats and mice, there is a larger quantity of young neurons in rats. Furthermore, they are reported to mature faster, have better survival rates and are more likely to be integrated into hippocampal circuits (Snyder et al. 2009). This species difference needs to be taken into account when comparing models of neurogenesis. Furthermore, processes of neurogenesis can be regulated by a range of endogenous and external factors reviewed in the following sections.

1.4.1 External factors which regulate neurogenesis

Adult neurogenesis is a dynamic process which is responsive to a number of factors which are external from the organism. These refer to environmental manipulations such as physical activity, learning, irradiation and environmental enrichment which can alter the proliferation, differentiation or survival of newly generated neurons (key studies from rat models are summarised in Table 1.3). Environmental enrichment describes objects such as toys, platforms or a running wheel that laboratory animals are exposed to in contrast to standard laboratory conditions (Bruel-Jungerman et al. 2005) and is reported to have a positive effect on neurogenesis (Bruel-Jungerman et al. 2005; Ueda et al. 2005).

Stress is another factor which is reported to affect adult neurogenesis (reviewed in Dranovsky and Hen 2006; Samuels and Hen 2011). A study by Heine et al. (2005) demonstrated that a

multiple unpredictable stress paradigm caused cell proliferation in the rat hippocampus to be reduced by 32%. This was supported by another study by Rosenbrock et al. (2005) who showed that hippocampal cell proliferation in the dentate gyrus was reduced by chronic intermittent restraint of rats. Furthermore, progression of newborn neurons can be manipulated by pathogenesis including models of depression, ischemia and induced seizures, as well as physiological states such as ageing Table 1.3. Although these factors are not looked at directly in the experiments in this thesis, certain aspects have been considered, such as keeping the stress of the animals to a minimum so as not to confound results.

External factors which affect neurogenesis also include pharmacological and recreational drugs. Examples of these include antidepressants, alcohol, opiates and cannabinoids (Table 1.3). In addition certain cytostatic agents may reduce neurogenesis and are investigated within this thesis. The antidepressant, fluoxetine, is also investigated as a neurogenic enhancer and is discussed in detail in section 1.6 of the present chapter.

1.4.2 Endogenous factors which regulate neurogenesis

In addition to external factors, there are also a number of endogenous factors which influence the proliferation, differentiation and survival processes of neurogenesis and are summarised in Table 1.4. These include hormones such as corticosterone and oestrogen and neurotransmitters such as 5-hydroxytryptamine (5-HT), glutamate and noradrenaline. In addition, certain growth factors and neurotrophin factors have been shown to have positive and negative influences on neurogenesis including brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), ciliary neurotrophic factor (CNTF), EGF, FGF-2 and insulin-like growth factor one (IGF-1). Of particular interest in this study is BDNF which is discussed in the following section (1.4.3).

External factor	Proliferation	Differentiation	Survival	References
Regulation of systems, behaviour and pathogenesis				
Environmental enrichment and learning	Increase	Increase	Increase	Drapeau et al. (2007) Dupret et al. (2007) Ueda et al. (2005)
Physical activity	Increase	Increase	Increase	Kim et al. (2010) Uda et al. (2006)
Stress	Decrease	No effect	No effect	Heine et al. (2005) Rosenbrock et al. (2005)
Depression	Decrease	Decrease	Decrease	Jaako-Movit et al. (2006) Keilhoff et al. (2005)
Irradiation	Decrease		Decrease	Limoli et al. (2004) Madsen et al. (2003)
Age	Decrease	Decrease		Driscol et al. (2006) Heine et al. (2004)
Seizures	Increase	Increase	Increase	Scott and Burnham. (2006) Snyder et al. (2009)
Ischemia	Increase	Increase		Jin et al. (2001) Zhu et al. (2003)
Legal and illegal drugs				
Antidepressants	Increase	Increase	Increase	ElBeltagy et al. (2010) Malberg et al. (2000) Wang et al. (2008)
Alcohol	Decrease		Decrease	McClain et al. (2011) Morris et al. (2010)
Cannabinoids	Increase/no effect		No effect	Downer et al. (2007) Jiang et al. (2005) Kochman et al. (2006)
Opiates	Decrease			Eisch et al. (2000) Kahn et al. (2005)

Table 1.3 Summary of key studies which illustrate the effect of external factors which regulate neurogenesis in the adult rat hippocampus. Information for this table was partially sourced from Zhao (2008)

Endogenous factor	Proliferation	Differentiation	Survival	References
Hormones				
Oestrogen	Increase		No effect	Mazzucco et al. (2006) Tanapat et al. (1999)
Corticosterone	Decrease	Decrease		Ambrogini et al. (2002) Cameron and Gould (1994) Hellsten et al. (2002)
Neurotransmitters				
5-HT	Increase	Increase		Banasr et al. (2004) Brezun and Daszuta (1999) Jha et al. (2006)
Glutamate	Decrease	Decrease		Cameron et al. (1995) Gould et al. (1994)
Noradrenaline	Increase	No effect	No effect	Kulkarni et al. (2002)
Acetylcholine	No effect	Increase/no effect	Increase/no effect	Cooper-Kuhn et al. (2004) Kotani et al. (2008)
Growth or neurotrophin factors				
BDNF	Increase	Increase	Increase	Lee et al. (2002) Scharfman et al. (2005)
VEGF	Increase	Increase	Increase	Cao et al. (2004) Jin et al. (2002) Schanzer et al. (2004)
EGF	No effect	Decrease		Kuhn et al. (1997)
FGF-2			Increase	Rai et al. (2007)
IGF-1	Increase	Increase	Increase	Åberg et al. (2003) Lichtenwalner et al. (2001)
CNTF	Increase	Increase	Increase	Chohan et al. (2011) Müller et al. (2009)

Table 1.4 Summary of key studies which illustrate the effect of endogenous factors which regulate neurogenesis in the adult rat hippocampus. Information for this table was partially sourced from Balu and Luki (2009). Abbreviations; 5-HT: 5-hydroxytryptamine, BDNF: brain-derived neurotrophic factor, VEGF: vascular endothelial growth factor, EGF: epidermal growth factor, FGF-2: fibroblast growth factor-2, IGF-1: insulin-like growth factor one, CNTF: ciliary neurotrophic factor.

1.4.3 The role of BDNF in neurogenesis

Brain-derived neurotrophic factor (BDNF) is a polypeptide growth factor widely expressed in the hippocampus. Despite its name, it is also produced in peripheral tissues such as liver, muscle and adipose (Cassiman et al. 2001; Lommatzsch et al. 1999; Ukropec et al. 2008). In the dentate gyrus, it is produced by both mature granule cell neurons (Conner et al. 1997) and by surrounding glia and endothelial cells (Linnarsson et al. 2000). It is reported to nurture newly generated neurons by increasing their proliferation (Lee et al. 2002; Scharfman et al. 2005), differentiation (Lee et al. 2002) and survival (Sairanen et al. 2005) and preventing apoptosis (Linnarsson et al. 2000). Indeed, it is one of the most extensively studied neurotrophins and BDNF levels in the SGZ tend to correlate with cell proliferation (Kempermann 2006). Infusion of BDNF into the hippocampus of adult rats is shown to enhance proliferation of cells (Scharfman et al. 2005). Furthermore, in studies using heterozygous BDNF knockout mice, both proliferation and the amount of 4 week old neurons was reduced in the knockouts compared to the wild type (Lee et al. 2002; Rossi et al. 2006). Conversely, Sairanen et al. (2005) found BDNF knockout mice had increased numbers of proliferating cells, reporting that the only effect of BDNF was on neuronal survival.

Functionally, BDNF expression has been reported to be increased by antidepressants, whereas BDNF expression is reduced by chronic stress and depression (Duman and Monteggia 2006; Lee and Kim 2010; Sairanen et al. 2005). In rodents BDNF expression is also associated with the process of learning and memory. It is upregulated with enriched environment and enhanced learning and memory paragdims (Cheng et al. 2003; Lu et al. 2008; Rossi et al. 2006) and has also been shown to be involved in long-term potentiation (LTP) which is required for consolidation of short-term to long-term memories (Farmer et al. 2004; Korte et al. 1996; Lu et al. 2008) (see section 1.5.2 of the present chapter).

1.5 Adult hippocampal neurogenesis and memory

In the studies of this thesis the effects of chemotherapy on cognition are investigated, in particular the effects of hippocampal-dependent spatial working memory. In the present section, memory and the role of the hippocampus in memory processing are reviewed.

1.5.1 Types of memory

Learning is the acquisition of new information and memory is the retention of this information. The process of memory formation and retrieval occurs in three main stages: encoding, storage and retrieval. Encoding is the acquisition, processing and consolidation of information, storage is the formation of a permanent record of this information and retrieval is the recall of the information into consciousness or to execute an activity (reviewed in Gazzaniga et al. 2009).

Memory can be divided into three main types: sensory, short-term and long-term memory (Fig. 1.11). Sensory memory is perceived information which lasts only a few milliseconds to seconds which degrades rapidly unless given attention and transferred to short-term memory (Atkinson and Shiffrin 1971). Short-term memory has a limited capacity and is stored for seconds to minutes. If not encoded into long-term memory by rehearsal, the information is lost (Walker and Stickgold 2006) (Fig. 1.11). Working memory is a form of short-term memory originating from sensory information or retrieved from long-term memory. It is a limited-capacity storage of information to perform mental operations which process and utilise this held information (Gazzaniga et al. 2009).

In contrast to short-term memory, long-term memory is a stable form of information with a much larger capacity and is able to hold information indefinitely (up to a whole lifespan) (Mecklinger 2010). Long-term memory can be divided into two broad categories: declarative (explicit) and procedural (implicit). Declarative memory can be subdivided into semantic memory, which consists of facts and general knowledge and episodic memory, which consists of memories for autobiographical events. Both semantic and episodic memories are dependent

on the medial temporal lobe (a structure that includes the hippocampus) and diencephalon (Henke 2010). In contrast, procedural memory is the memory of learned skills of motor tasks (e.g. how to ride a bike) or cognitive skills (e.g. reading) which is independent from the medial temporal lobe (Henke 2010).

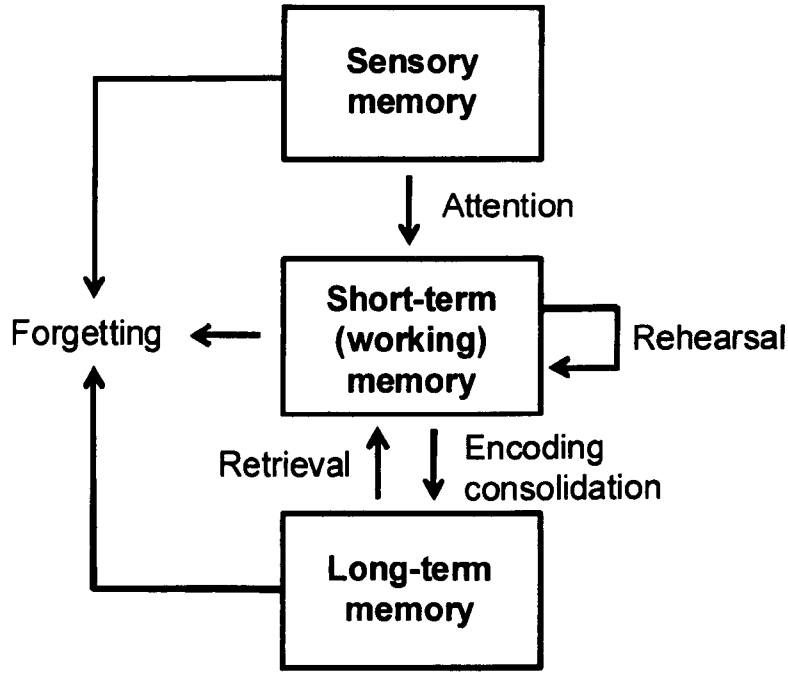

Figure 1.11 Types of memory. Perceived information is held in sensory memory for a few milliseconds to seconds and is lost unless given attention and is transferred to short-term memory. Short-term memory lasts from seconds to minutes and is consolidated to long-term memory if rehearsed, otherwise it will be lost. Working memory is a form of short-term memory necessary to perform mental operations. It requires information retrieved from long-term memory and from sensory memory. Long-term memory has a large capacity and can last indefinitely (Gazzaniga et al. 2009).

1.5.2 The role of the hippocampus in memory

Declarative memory, as discussed in the preceding section, requires the hippocampus whereas procedural memory is independent of the hippocampus (Henke 2010). Memory acquisition and forgetting in the hippocampus is reliant a process of LTP and long-term depression (LTD) (Lynch 2004). LTP is a process in which synapses are strengthened and is required for memory consolidation. In contrast, LTD is regarded as a complimentary mechanism to LTP and selectively weakens specific synapses (Bear et al. 2007).

The hippocampus is also believed to play a strong role in spatial learning and memory. This has been demonstrated by a number of studies involving hippocampal lesions. One study compared the ability of rats, with and without hippocampal lesions to perform spatial and non-spatial working memory tasks. Those with hippocampal lesions showed severe impairment in performance of the forced choice alternation task, a task of spatial working memory, but they were not impaired in the dNMTS non-spatial working memory task (Aggleton et al. 1986). Other studies have observed that rodents with hippocampal lesions were also impaired in the MWM, radial maze and spontaneous alternation in the T-maze spatial working memory tasks (Bannerman et al. 2004; Deacon and Rawlins 2005; Lalonde 2002; Morris et al. 1982; Olton and Papas 1979).

The NLR task is a spatial variant of the NOR task (discussed in detail in section 1.8 of the present chapter) and rodents with hippocampal lesions show a reduction in ability to perform the NLR task but not the NOR task (Dere et al. 2007; Mumby et al. 2002). These studies provide strong evidence of the involvement of the hippocampus in spatial memory. This is further supported by the existence of place cells in the hippocampus. These are neurons which selectively respond when a rat is in a specific location in its environment (O'Keefe 1979). Although at present, there is no evidence for place cells in the human hippocampus; studies of patients with hippocampal damage provide evidence that the hippocampus also plays a critical

role in spatial memory in humans (Abrahams et al. 1997; Astur et al. 2002; Goldstein et al. 1989; Maguire et al. 1996).

1.5.3 The involvement of neurogenesis in learning and memory

Adult neurogenesis in the hippocampus has given rise to questions about its functional relevance. There are now major implications for the involvement of neurogenesis in systems of stress, depression and learning and memory (reviewed in Aimone et al. 2011; Balu and Lucki 2009; Dranovsky and Hen 2006; Koehl and Abrous 2011). A number of rodent studies have shown that stress caused by factors such as isolation, footshock or physical restraint reduces neurogenesis in the SGZ (David et al. 2010; Mirescu and Gould 2006). However, a causal relationship between neurogenesis and behaviour was shown by Santerelli et al. (2003). This group observed that animals which had been irradiated to ablate hippocampal neurogenesis did not respond to antidepressant drugs in a test of anxiety (Santarelli et al. 2003). This landmark study demonstrated the importance of neurogenesis in behaviour. The relationship between antidepressants and neurogenesis is discussed in more detail in section 1.6.3 of the present chapter.

Possibly the most widely studied functional implication of neurogenesis is its involvement in learning and memory. Memory consolidation and working memory are both functions largely mediated by the hippocampus (Clark et al. 2007; Deng et al. 2010) (see section 1.5.2 of the present chapter). The functional relevance of synaptic plasticity to memory is well documented in the hippocampus, but the idea of plasticity on a cellular level is still relatively new. Numerous studies in rodents have observed correlations between hippocampal neurogenesis and cognition, extensively reviewed by Zhao et al. (2008) and Castilla-Ortega et al. (2011), with the majority finding that environmental factors which increase hippocampal cell proliferation also have a positive effect on hippocampal dependent learning. In addition, genetic knockout mice with decreased hippocampal proliferation were impaired in memory tasks (Castilla-Ortega et al. 2011; Zhao et al. 2008).

Ablation of neurogenesis, achieved by toxicity or irradiation, is relatively straightforward and has been used in numerous studies to investigate the role of neurogenesis in learning and memory. Irradiation of proliferating hippocampal cells in the rat has been shown to impair spatial memory in the MWM (Madsen et al. 2003; Snyder et al. 2005). A recent study by Drew et al. (2011) showed that irradiation of hippocampal neurogenesis in mice impaired their performance in the CFC task when a single-trial procedure, but not when multiple-trial procedures were used, indicating that adult hippocampal neurogenesis is only required for CFC in mice when they have received training. Conversely, another study demonstrated that hippocampal irradiation in mice impaired CFC but not CER which would suggest that neurogenesis is necessary for hippocampal-dependent learning. However, the same animals were not impaired in the MWM or Y-maze. One possible explanation of these contradictory findings is that hippocampal neurogenesis is required for some, but not all hippocampal functions (Saxe et al. 2006).

The antiproliferative drug MAM has also been exploited in reducing neurogenesis in rodent models of learning and memory and its effects are summarised in Table 1.2 and discussed in section 1.1.3 (animal models of chemobrain).

In a study by Dupret et al. (2007) MWM spatial learning was shown to depend on proliferation, survival and apoptosis of new hippocampal neurons, and blocking of apoptosis impaired memory and cell survival and proliferation induced by learning. The same group (Dupret et al. 2008) used a genetic mouse model to demonstrate that adult-born hippocampal neurons are necessary for complex, but not simple spatial memory functions. Furthermore, Kee et al. (2007) found that not only were newly generated neurons incorporated into hippocampal circuitry during MWM learning, but they were preferentially recruited over pre-existing granule cell neurons. These animal models all give strong evidence of a role for adult neurogenesis in the mediation of hippocampal spatial learning.

In humans there is a great deal less evidence of the functional relevance of adult neurogenesis. However, it is widely accepted that the hippocampus as a structure has a vital role in memory (see section 1.5.2 of the present chapter). The hippocampus is believed to play a role in spatial working (Carrozzo et al. 2005), verbal (Meyer et al. 2005) and recognition (Reed and Squire 1997) memory. Patients affected by chemotherapy-induced cognitive impairment experience problems primarily in working, visual and verbal memory as discussed in section 1.1 and presented in Table 1.1 of the present chapter. This correlation gives primary evidence that the hippocampus may be directly involved in the mechanisms behind chemotherapy-induced cognitive impairment. Furthermore, neurogenesis is known to occur in the hippocampus of humans (Eriksson et al. 1998) and thus could be a prospective target for cytotoxic drugs administered to treat cancer. Therefore, although the evidence linking chemotherapy, hippocampal neurogenesis and memory impairment is not direct, there is strong evidence for a mechanism connecting them together and this is the basis for the animal model presented in this thesis.

1.6 Fluoxetine

The selective serotonin reuptake inhibitor (SSRI) antidepressant, fluoxetine (Fig. 1.12), is reported to increase neurogenesis and enhance cognition in both human and animal subjects with a cognitive deficit (Monleon et al. 2007; Mostert et al. 2008). In the experiments in this thesis (Chapters 3 and 4) it was co-administered with MTX and 5-FU to counteract the chemotherapy induced reduction in neurogenesis and cognitive ability.

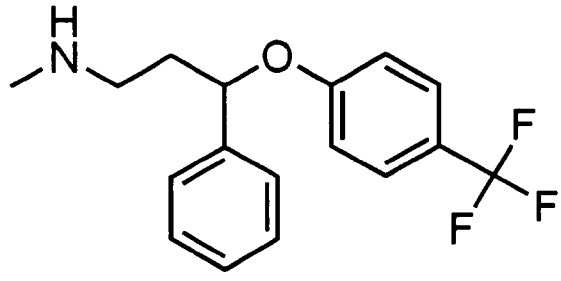

Figure 1.12 Chemical structure of the selective serotonin reuptake inhibitor (SSRI) antidepressant, fluoxetine.

1.6.1 Selective serotonin reuptake inhibitors

SSRIs are antidepressants used for the treatment of depression and anxiety disorders and they are the most widely used group of antidepressants (Preskorn et al. 2004). As well as depression they have also been used to treat psychological disorders including obsessive compulsive disorder, panic attacks and post traumatic stress disorder. These conditions are all related by involvement of serotonin (5-HT) and its receptors (Wong et al. 2005). Indeed the main mechanism of action though which SSRIs act is by regulation of 5-HT uptake. In normal circumstances, when a neuron is activated, 5-HT is released from the presynaptic neuron and after diffusing across the synaptic cleft binds to receptors on the post synaptic terminal. 5-HT is then rapidly removed from the synapse via reuptake transporters. SSRI antidepressants bind to these transporters decreasing their affinity for 5-HT, causing 5-HT to accumulate in the synapse (Stahl 1998). On binding to its receptors on the postsynaptic

neuron, 5-HT can stimulate BDNF gene expression via cyclic adenosine monophosphate (cAMP), protein kinase A (PKA) and cAMP response element binding protein (CREB) pathways (see Fig. 1.13) (Duman et al. 2001b). Fluoxetine (Fig. 1.12) is one of the most frequently prescribed SSRIs (Preskorn et al. 2004) and is metabolised in the liver by cytochrome P450 into its one active metabolite, norfluoxetine (Gordon and Hen 2004; Ni and Miledi 1997). Norfluoxetine has a half-life of 7 to 15 days in humans (Burke et al. 2000) and 15 hours in rat (Caccia et al. 1990) and antidepressant action takes 2 to 4 weeks to develop in both humans and rodents (Conley and Hutson 2007). Fluoxetine (and norfluoxetine) were used in some of the experiments presented in this thesis (Chapters 3, 4 and 5).

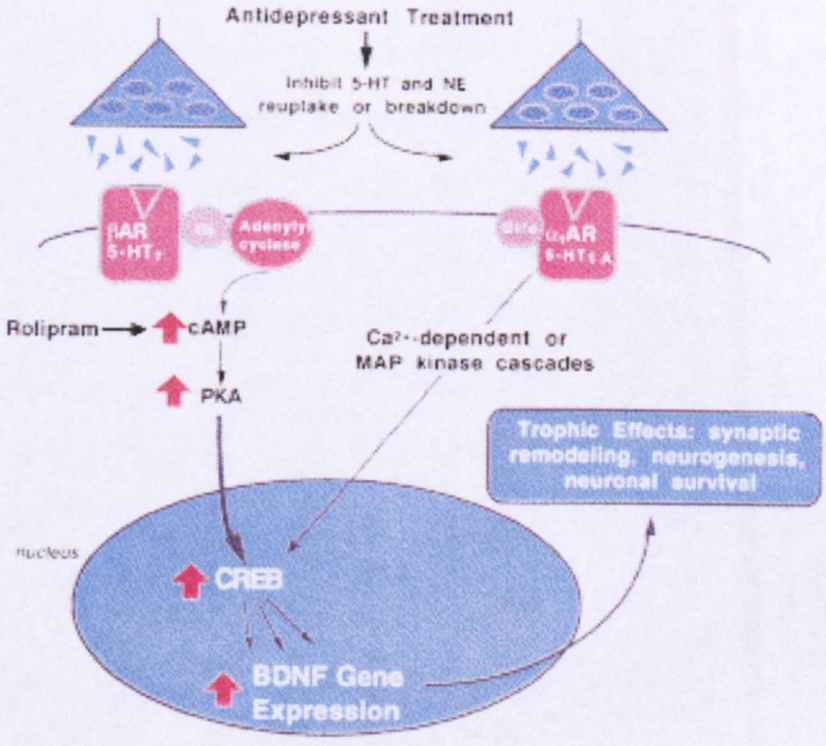

Figure 1.13 Diagram illustrating the upregulation of intracellular BDNF by antidepressant drugs (Duman et al. 2001). Antidepressants can inhibit the re-uptake of serotonin (5-HT) or noradrenaline (NE) into the presynaptic neuron. This increases the availability of these neurotransmitters to bind to mood regulating receptors on the post synaptic neuron (5-HT receptors shown in the diagram as 5-HT_7 and 5-HT_{5A} and adrenal receptors βAR and α_1 AR). This binding results in increase of the cyclic adenosine monophosphate (cAMP), protein kinase A (PKA) cascade and the Ca2+ dependent or microtubule-associated protein (MAP) kinase cascade. Both these cascades upregulate the expression of cAMP response element binding protein (CREB) and BDNF gene expression (Duman et al. 2001).

1.6.2 Fluoxetine and adult hippocampal neurogenesis

An additional mechanism by which fluoxetine could act is by up-regulating hippocampal neurogenesis (Castrén and Rantamäki 2010; David et al. 2010; Dranovsky and Hen 2006; Malberg 2004; Santarelli et al. 2003). The exact pathways by which this is achieved remains uncertain but it has been shown to increase the proliferation of rapidly dividing type 2 progenitors in the SGZ while leaving the slowly dividing NSC unaffected (Encinas et al. 2006). This effect may be via upregulation of BDNF which increases neurogenesis (see section 1.4.3 of the present chapter). BDNF availability is regulated by fluoxetine by the increased binding of 5-HT to receptors, which up-regulates intracellular MAP kinase or cAMP cascades. This increases CREB in the cell nucleus and leads to increased transcription of BDNF (Duman et al. 2001b; Merz et al. 2011) (see Fig. 1.13).

Evidence supporting that the action of fluoxetine is mediated by neurogenesis has come from a number of animal models. Santarelli et al. (2003) observed that blocking neurogenesis with irradiation also blocked the effect of fluoxetine in reducing anxiolytic behaviour. In contrast Holick et al. (2007) used a similar method to Santaralli et al., ablating neurogenesis with irradiation, and found that the action of fluoxetine on performance in the forced swim and novelty-induced hypophagia tests did not require neurogenesis. One group showed that chronic fluoxetine treatment increased the hippocampal expression of a number of genes associated with the promotion of neurogenesis including BDNF (Miller et al. 2007) and numerous studies have demonstrated increased neurogenesis in the rodent hippocampus after treatment with fluoxetine (Chen et al. 2006; Hitoshi et al. 2007; Levkovitz et al. 2002; Marcussen et al. 2008; Miller et al. 2007). Work *in vitro* has also shown that fluoxetine increases proliferation in NSC that are 10 days old but not when they are 1 day old (Manev et al. 2001). The correlation between animals exposed to stress and the reduction in the rate of hippocampal neurogenesis (Aimone et al. 2011; Balu and Lucki 2009; Gould and Tanapat 1999) also provides evidence to suggest that adult neurogenesis is involved in the action of fluoxetine.

1.6.3 Cognitive enhancement by fluoxetine

Due to the correlation between fluoxetine and neurogenesis and neurogenesis and memory, it is reasonable to question if fluoxetine is able to enhance cognition. Although fluoxetine is not thought to have a cognitive effect on healthy subjects (Monleon et al. 2007), some studies provide evidence that fluoxetine improves cognition when this has been impaired. In clinical studies, fluoxetine has been reported to improve deficits in memory in patients suffering from moderate to severe depression (Gallassi et al. 2006; Levkovitz et al. 2002; Vythilingam et al. 2004), mild cognitive impairment (Mowla et al. 2007), traumatic brain injury (Horsfield et al. 2002) and post traumatic stress disorder (Vermetten et al. 2003).

In addition several experiments have been carried out to investigate the effects of fluoxetine on memory in rodents, reviewed by Monleon et al. (2007). Collectively these yield a range of results which could be due to the type of memory tested in the tasks or be due to animals having no cognitive impairment before fluoxetine administration (Monleon et al. 2007). Positive effects of fluoxetine on cognition have also been reported in rodent models when memory impairment has been caused by stress (El Hage et al. 2004), hypoxia and stroke (Li et al. 2009; Strek et al. 1989), olfactory bulbectomy (Broekkamp et al. 1980; Garrigou et al. 1981), scopolamine and electroconvulsive shock (Nowakowska et al. 1996). Furthermore, a recent study in our laboratory found that fluoxetine can improve the memory impairment caused by 5-FU administration in rats (ElBeltagy et al. 2010).

Due to the ability of fluoxetine to enhance neurogenesis and counteract cognitive deficits, it was chosen for the experiments presented in this thesis to potentially counteract the deficits in hippocampal cell proliferation and memory caused by chemotherapy agents, 5-FU and MTX.

1.7 Animals chosen for this study

In order to investigate the cognitive and neurobiological effects of chemotherapy-induced cognitive impairment, it was necessary to select a suitable model. To date, most of the animal studies utilised rat or mouse models (Seigers and Fardell 2011), so a rodent model was considered appropriate for consistency and comparability with these studies. It was not considered necessary to use an animal model with a higher level of sentiency to investigate mild cognitive impairment. Moreover, a vast amount of literature is available on a range of established behavioural tests for these species. In this thesis, the effects of chemotherapy on adult neurogenesis are investigated. It has been shown that new-born adult hippocampal neurons are more numerous in rats than mice and they are more likely to be recruited into learning circuits (Snyder et al. 2009). Specifically, Lister-hooded rats were chosen as they have superior eyesight compared to albino, outbred rat strains and are highly inquisitive, which is beneficial in behavioural tests where they are intended to respond to environmental stimuli (Manahan-Vaughan and Schwegler 2011; Neophytou et al. 2000). Although clinical studies on chemotherapy-induced cognitive impairment are often in breast cancer patients, male rats were chosen in this study to avoid the effects of the oestrogen cycle on behaviour and neurogenesis (Galea et al. 2008). For these reasons, adult male, Lister-hooded rats were used in the *in vivo* experiments discussed in this thesis.

1.8 Behavioural tests used in this study

To establish a rodent model of chemobrain, animals' cognition was tested in each experiment. A variety of cognitive tests were used to investigate different types of memory, with particular focus on spatial working memory, which is a part of hippocampal function (Clark et al. 2007) (see section 1.5.2 of the present chapter). This approach was taken to make the study relevant to clinical studies of chemotherapy-induced cognitive impairment, in which memory tasks involving the hippocampus such as working and visuospatial memory are often affected (Baddeley et al. 2011; Loureiro et al. 2011; Sharma et al. 2010) (see Table 1.1). None of the tests used relied on positive or negative reinforcers so as not to confound results. Aversive stimuli, such as foot shocks, instil fear in and cause stress to an animal which would be undesirable for the model in this study. Firstly, because, although specific types of fear conditioning, such as contextual fear conditioning, involve the hippocampus, they are more strongly reliant on the amygdala (McHugh et al. 2004). Secondly, stress can reduce hippocampal neurogenesis (Zhao et al. 2008), a factor which will also be investigated in this study. Food or sweet liquid is often given as a positive reinforcer, which might skew results, as chemotherapy and fluoxetine have both been shown to suppress appetite and food intake (Garattini et al. 1989). Furthermore, starving an animal overnight may cause it stress which again could affect neurogenesis. Therefore, all the behavioural tests used in this thesis relied on the rats' natural preference for novel stimuli (Hughes 2007).

1.8.1 Novel object recognition (NOR) task

The NOR task used in the first experiment in this thesis was a modified version of the one developed by Ennaceur and Delacour (1988). It is a two trial object recognition task, and has previously been used in our laboratory to assess working memory. In the familiarisation trial the time the animals spend exploring two identical objects in an arena is recorded. Animals are removed from the arena and after an inter-trial interval they are returned for the choice trial, in which one of the objects is replaced with a new object and exploration time of each is again recorded. If a rat has unimpaired working memory, it will normally explore the novel

object for longer during the choice trial. However, the test is not hippocampal dependent (Dere et al. 2007) and it was possible that it may affect the rats' performance in the NLR task as the arena and objects used were the same. Therefore this task was not used in the second and subsequent experiments in this thesis.

1.8.2 Novel location recognition (NLR) task

The NLR task is a spatial variant of the NOR task developed by Dix and Aggleton (1999). It requires animals to remember the positions of two identical objects in the initial familiarisation trial, and therefore preferentially explore the object with the novel location in the choice trial after an inter-trial interval. Lesions in the hippocampus have been shown to impair performance in the NLR task (Dere et al. 2007; Mumby et al. 2002). More specifically, it has been reported that dentate gyrus lesions reduce the rats' ability in the task (Lee et al. 2005). The dentate gyrus is one place where adult neurogenesis continually occurs providing further evidence that adult neurogenesis is involved in memory formation. Hence this task was particularly appropriate for this study. Furthermore, the NLR task is short, only taking 2 days to complete with habituation. Therefore it was possible to examine neurogenesis in the hippocampus at a comparable time point to the behavioural tests.

1.8.3 Spontaneous alternation in the T-maze

The T-maze has been utilised in rodents to test a range of cognitive paradigms (Deacon and Rawlins 2006). In this thesis, spatial working memory was investigated using spontaneous alternation in the T-maze. This test relies on rodents' natural ability to alternate their choice destination arm of the T-maze in subsequent trials (Dember and Fowler 1958). Similar to the NLR task, animals are impaired in spontaneous alternation after hippocampal lesions (Deacon and Rawlins 2005; Lalonde 2002), making the task appropriate for this study. Again, the task is short, with each animal tested within 10 min. This task was used in the final two behavioural studies in this thesis.

1.9 Aims and objectives

The studies presented in this thesis were carried out to investigate the effects of agents in the CMF chemotherapy combination; CP, MTX and 5-FU, on memory and neurogenesis in the adult rat hippocampus. Co-administration of the SSRI antidepressant, fluoxetine, was investigated as a potential method to counteract any chemotherapy-induced cognitive and neurobiological changes. In addition, an *in vitro* model was used to study the direct effects of chemotherapy, fluoxetine and its active metabolite, norfluoxetine, on primary hippocampal NSC from the adult mouse. The principal objectives of the present studies were as follows:

1. To establish a rodent model of chemotherapy-induced cognitive impairment.

2. To investigate the effects of the individual chemotherapies from the CMF treatment combination on hippocampal dependent spatial memory utilising the NOR, NLR and spontaneous alternation in the T-maze behavioural tasks.

3. To determine if any changes in cognition correlate with the proliferation and survival of new cells in the adult hippocampus, using Ki67 and BrdU immunohistochemistry, thereby providing evidence that alterations in neurogenesis may be an underlying mechanism for these deficits.

4. To investigate if the cognitive and cellular deficits induced by chemotherapy can be prevented or reversed by the co-administration of fluoxetine and to determine the optimum time period to administer the drug.

5. To create an *in vitro* model to observe the direct effects of 5-FU, fluoxetine and norfluoxetine on the viability and proliferation of adult NSC from the mouse hippocampus.

Chapter 2

The effects of cyclophosphamide on spatial working memory and hippocampal cell proliferation in the rat

2.1 Introduction

In the present chapter two studies are discussed which were carried out to investigate the effects of the chemotherapy agent, cyclophosphamide (CP) on cognition and cellular changes in the hippocampus using a rat model. Firstly, a preliminary study was performed to assess the toxicity of CP and to optimise the novel object recognition (NOR) and novel location recognition (NLR) spatial memory tasks. The second study in this chapter is a continuation from the work completed in the preliminary experiment, investigating the cognitive effects of CP, using a modified version of the NLR task. Furthermore, in the second study cell proliferation and survival in the dentate gyrus of the hippocampus were examined and levels of DCX were quantified in the hippocampus and frontal cortex.

Patients who have received adjuvant chemotherapy as part of their treatment for cancer often report problems in cognition, encompassing memory impairment, a short concentration span and general confusion (Taillibert et al. 2007). These effects can last up to several years after completion of the treatment (Ahles and Saykin 2002; Matsuda et al. 2005). However combination therapies are often used in clinical treatment, so the actions of individual drugs are unclear. CP, MTX and 5-FU (CMF) are all chemotherapy drugs, commonly used in combination to treat breast cancer (Goldhirsch et al. 1998). This combination is reported to have an effect on cognition in human studies (Kreukels et al. 2008; Schagen et al. 2002a; Schagen et al. 1999). Previous work in our laboratory and elsewhere has shown that both MTX and 5-FU have a negative effect on memory and proliferation in the hippocampus (ElBeltagy et al. 2010; Mustafa et al. 2008; Seigers et al. 2008) and the experiments presented in the present chapter focus on the effects of CP.

CP is an alkylating agent, with its metabolites causing alkyl crosslinks within and between DNA strands of dividing cells, an action that causes them to apoptose (Matalon et al. 2004) (see section 1.2.2, Chapter1). CP is used to treat several types of cancer including breast and lung carcinoma (Allwood et al. 1997) and is able to cross the blood brain barrier (Janelsins et

al. 2010). CP is used clinically in chemotherapy combinations which have been reported to cause chemotherapy-induced cognitive impairment (Ahles and Saykin 2002; Brezden et al. 2000; de Ruiter et al. 2011; Hurria et al. 2006a; Kreukels et al. 2008; Schagen et al. 2002b; Schagen et al. 1999; Shilling et al. 2005; Wieneke and Dienst 1995). Neuropsychological domains affected include verbal, visuospatial and working memory, processing speed and attention/concentration (Table 1.1). However, the effects of CP alone on cognition have not been examined in patients. Rodent models have also been used to investigate the effects of chemotherapy on cognition (summarised in Table 1.2) with many researchers observing that chemotherapy causes animals to be impaired in a range of cognitive tasks, although other studies found chemotherapy had no effect and in a single study improvement was even seen in the MWM task (Lee et al. 2006; Seigers and Fardell 2010). The hippocampus is believed to play an important role in visuospatial and working memory in humans (Baddeley et al. 2011; Loureiro et al. 2011; Sharma et al. 2010). In many of the pre-clinical studies investigating chemotherapy-induced cognitive impairment the ability of rodents to perform tasks which are reliant on the hippocampus such as the NLR and MWM that are most greatly affected, compared with tasks such as fear conditioning tasks which are more dependent on the amygdala (Parkes and Westbrook 2011) (see Table 1.2, Chapter 1).

Furthermore, many of these studies also found that chemotherapy reduced proliferation of neural progenitors in the dentate gyrus of the hippocampus (ElBeltagy et al. 2010; Seigers et al. 2010a; Yang et al. 2010). This reduction in hippocampal neurogenesis has been considered as one possible cause of the cognitive impairment seen. Throughout life, neuronal progenitors in the SGZ of the dentate gyrus divide to produce new neurons, which get integrated into existing neural circuits (Lledo et al. 2006b) and are thought to have a functional role in learning and memory consolidation (Ehninger and Kempermann 2008; Zhao et al. 2008). Ablation of neurogenesis by means of irradiation (Wojtowicz 2006), hippocampal lesions (McGregor et al. 2004; Morris et al. 1982) or cytotoxic drugs (Bruel-Jungerman et al. 2005; Ko et al. 2009) has been shown to cause impairment in cognition. Previous work in our

laboratory also found that rats administered with 5-FU chemotherapy had reduced hippocampal levels of DCX, (Mustafa et al. 2008). DCX is a microtubule-associated protein which is expressed transiently in newly differentiated neurons (Zhao et al. 2008). Therefore its reduction by 5-FU strengthens the argument that chemotherapy is able to negatively affect neurogenesis.

In both studies presented in this chapter, CP was administered chronically in a rat model to mimic clinical administration. A dosage of 30mg/kg was chosen which is sufficient to cause weight loss but is well below the predicted median lethal dose of 200mg/kg (Branda et al. 2002) and below the amount administered which causes pain or cystitis (Wantuch et al. 2007). It is also within the range of doses (8-200mg/kg) which have been previously shown to have an effect in cognition in rodents, although the majority of these studies used a dose within the range of 10-40mg/kg (see Table 1.2, Chapter 1). Plasma concentrations of the active metabolite of CP, phosphoramide mustard, have been shown to peak at 23μM, 40 min after i.p. injection in rat, although a slightly higher dose of 50mg/kg was used in the study (Powers and Sladek 1983) and the brain/plasma concentration-integral ratio of phosphoramide mustard was found to be 0.18 (Genka et al. 1990). CP is reported to have an increased effect on proliferation and had less toxicity when adult rats were dosed at 14.00 h compared with 8.00 h (Pérez-López et al. 1984), therefore in the studies in this chapter all injections were administered between 14.00 and 16.00 h.

The NOR and NLR tasks described in section 1.8, Chapter 1, were used to test working memory of the rats after CP administration. However, in the preliminary study, the animals were unable to perform the tasks even before treatment, indicating that the tasks needed optimising in order for conclusions to be drawn. Consequently, in the second study presented in this chapter, the NLR task was amended and the NOR task was removed to eliminate the possibility of confounding the results by using both tasks in the same animals.

2.2 Preliminary study to assess the toxicity of CP and optimise the NOR and NLR tasks

2.2.1 Materials and methods

2.2.1.1 Animals and treatment

Male Lister-hooded rats (150-200g; Charles River, UK, total n=16) were administered CP (30 mg/kg, 4 i.v. doses each 3-4 days apart, into the tail vein, at a volume of 3ml/kg, dissolved in 0.9% sterile saline; Medac, Germany) or 0.9% sterile saline at an equivocal volume (both groups n=8). CP/saline was administered under gaseous isofluorane (4% to initiate anaesthesia then reduced to 2%). This method of administration was previously used in our laboratory (ElBeltagy et al. 2010; Mustafa et al. 2008). Both groups were administered BrdU (250mg/kg, i.p., at a volume of 4ml/kg; Sigma Aldrich, UK) immediately after their first CP/saline injection.

Rats were group housed (4 per cage) in cages (dimensions: 52 x 32 x 20 cm) with sawdust bedding and maintained with a 12 h light/dark cycle (7.00/19.00 h). Room temperature (21±2 °C) and humidity (60±5%) were kept constant. Food (2018 rodent diet, Harlan) and water was provided *ad libitum.* They were weighed every 1-3 days from arrival and allowed to habituate 1 week prior to behavioural testing. All procedures were in accordance to UK Home Office Guidance regulations and with local ethical committee approval under project license 40/2715 and personal license 40/8883.

2.2.1.2 Behavioural testing

2.2.1.2.1 Novel object recognition (NOR) task

The NOR task described (Fig. 2.1) is a two trial object recognition task adapted from Ennaceur and Delacour (1988). The apparatus consisted of an arena (a semi-transparent Perspex box; dimensions: 49 width x 66 length x 40 height cm) and the objects were weighted water bottles (15cm high, 7cm diameter) dark blue, with white tape horizontal stripes, or light pink with no stripes. Arenas and water bottles were cleaned with 20% ethanol prior to each experiment and

between trials to remove olfactory cues. Experiments were conducted at an illumination of 80 Lux between 9.00 and 14.00 h.

NOR testing was carried out 8 days before drug treatment and 3 days after the final CP/saline injection. Rats were habituated to the arena for 1 h, 24 h prior to testing. During the 3 min familiarisation trial rats were placed in the arena to explore two identical objects (objects A and B in results) in opposite corners. Rats were returned to their home cage for 15 min and then returned to the arena for the 3 min choice trial. In this trial, one of the objects was replaced with a novel object (which of the objects was novel was randomised). Exploration was defined as the rat directing its nose in the direction of the object less than 1 cm from the object, and actively exploring it (Dix and Aggleton 1999). Gnawing or climbing the object was not considered exploration. Exploration times of both objects and trials were recorded blind by 2 people, and averaged using a stopwatch from digitised recordings, so no observer was in the room during the trials. The exploration data of all animals was included in the analysis.

2.2.1.2.2 Novel location recognition (NLR) task

The NLR task (Fig. 2.2) is a spatial variant of the NOR task and was adapted from Dix and Aggleton (Dix and Aggleton 1999). The same apparatus and method was used as in the NOR task, with the variance being that in the choice trial, neither object was replaced (weighted, pink water bottles) but one was moved to a novel location in the arena. The starting location of the objects was randomised. It was carried out 4 days after the NOR tasks. The exploration data of all animals was included in the analysis.

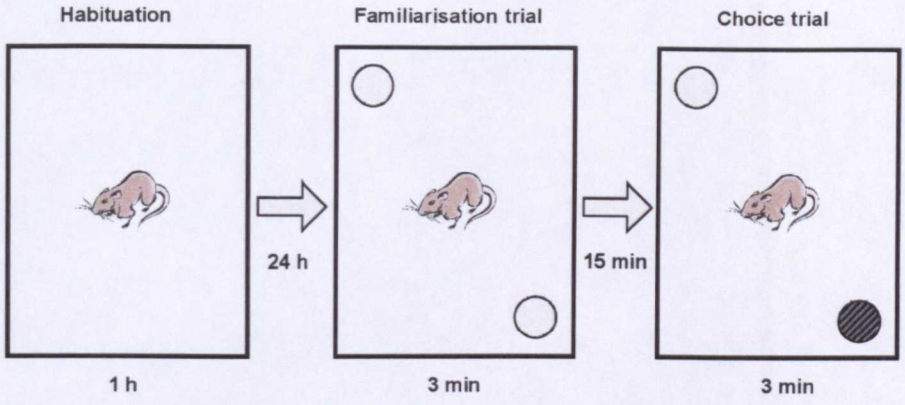

Figure 2.1 The novel object recognition protocol (NOR) was carried out over 2 days. Rats were habituated in the arena for 1 h. They were removed for 24 h and two identical objects were placed in 2 corners of the arena. Rats were replaced in the arena and allowed to explore the objects for 3 min (familiarisation trial) then removed again for 15 min. When returned again, one object had been replaced with a novel object and rats were again left to explore for 3 min (choice trial).

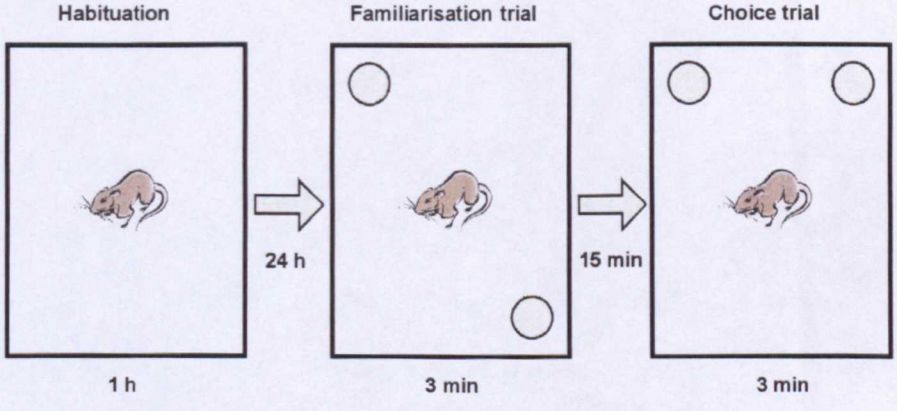

Figure 2.2 The novel location recognition protocol (NLR) was carried out over 2 days. Rats were habituated in the arena for 1 h. They were removed for 24 h and two identical objects were placed in 2 corners of the arena. Rats were replaced in the arena and allowed to explore the objects for 3 min (familiarisation trial) then removed again for 15 min. When returned again, one object had been moved to a different corner and rats were again left to explore for 3 min (choice trial).

2.2.1.3 Statistical analysis

Body weight was analysed using two-way repeated measures ANOVA. When ANOVA was significant Bonferonni post-hoc test was performed. Student's paired *t*-tests were used to compare exploration times of animals in the familiarisation and choice trials in both NOR and NLR tasks. Student's unpaired *t*-tests were used to compare total exploration time and average velocity of the animals. Preference indices (PI) were created for the NOR task by expressing time spent exploring the novel object in the as a percentage of the sum of exploration time of novel and familiar objects and from the NLR task by expressing time spent exploring the object in the novel location as a percentage of the sum of exploration time of novel and familiar locations in the choice trial, to create a single value to compare between groups. PI values were compared to 50% chance using a one-sample *t*-test and PI of both groups were compared using Student's unpaired *t*-test. Statistical analysis and graphs were created using GraphPad Prism 5 and significance was regarded as $p<0.05$.

2.2.2 Results

2.2.2.1 Cyclophosphamide does not reduce weight gain

CP had no effect on the amount of weight gained compared to vehicle treated controls ($F_{1,238}=4.16$, two-way repeated measures ANOVA, Fig. 2.3), although it was close to significant $p=0.079$. However, a significant effect of time and treatment × time interaction was confirmed ($F_{17,238}=515.33$, $F_{17,238}=10.39$ respectively, $p<0.0001$ for both). The animals remained in good health throughout the study never lost more than 10% of their maximum body weight.

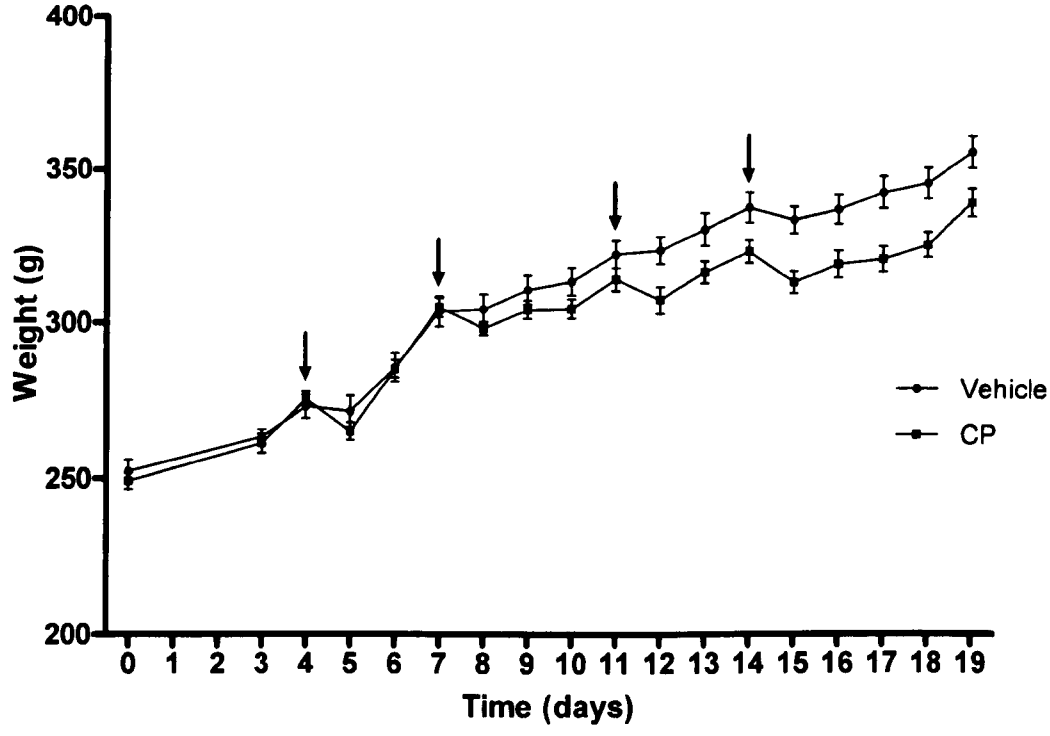

Figure 2.3 Body weights of rats (mean ± SEM, n=8) throughout the study. Arrows indicate CP/saline injections. CP had no effect on the amount of weight gained compared to vehicle treated controls ($F_{1,238}$=4.16, two-way repeated measures ANOVA). The effect of time and the treatment × time interaction was significant ($F_{17,238}$=515.33, $F_{17,238}$=10.39 respectively, $p<0.0001$ for both).

2.2.2.2 NOR task

No significant difference was found between the exploration times for either object for both groups in the familiarisation trials of the NOR task both before and after CP/saline treatment (Student's paired *t*-test, Fig. 2.4a and c). In the choice trial before treatment both groups spent more time exploring the novel object but this was not significant (Student's paired *t*-test, Fig. 2.4b). This was supported by PI analysis from the choice trial before treatment, as neither the PI of the CP nor the saline group significantly differed from 50% chance (one-sample *t*-test, Fig. 2.4e) nor did the PI differ between groups (Student's unpaired *t*-test). In the choice trial after treatment, the saline treated controls spent significantly more time exploring the novel object ($p<0.05$, Student's paired *t*-test, Fig. 2.4d) and the PI was significantly different from chance ($p<0.05$, one-sample *t*-test, Fig 2.4f) whilst CP treated animals showed no significant difference in exploration of either object (Student's paired *t*-test, Fig. 2.4d) nor was the PI significantly different from chance (one-sample *t*-test, Fig 2.4f). The PI of the saline and CP treated animals were not significantly different after treatment (Student's unpaired *t*-test).

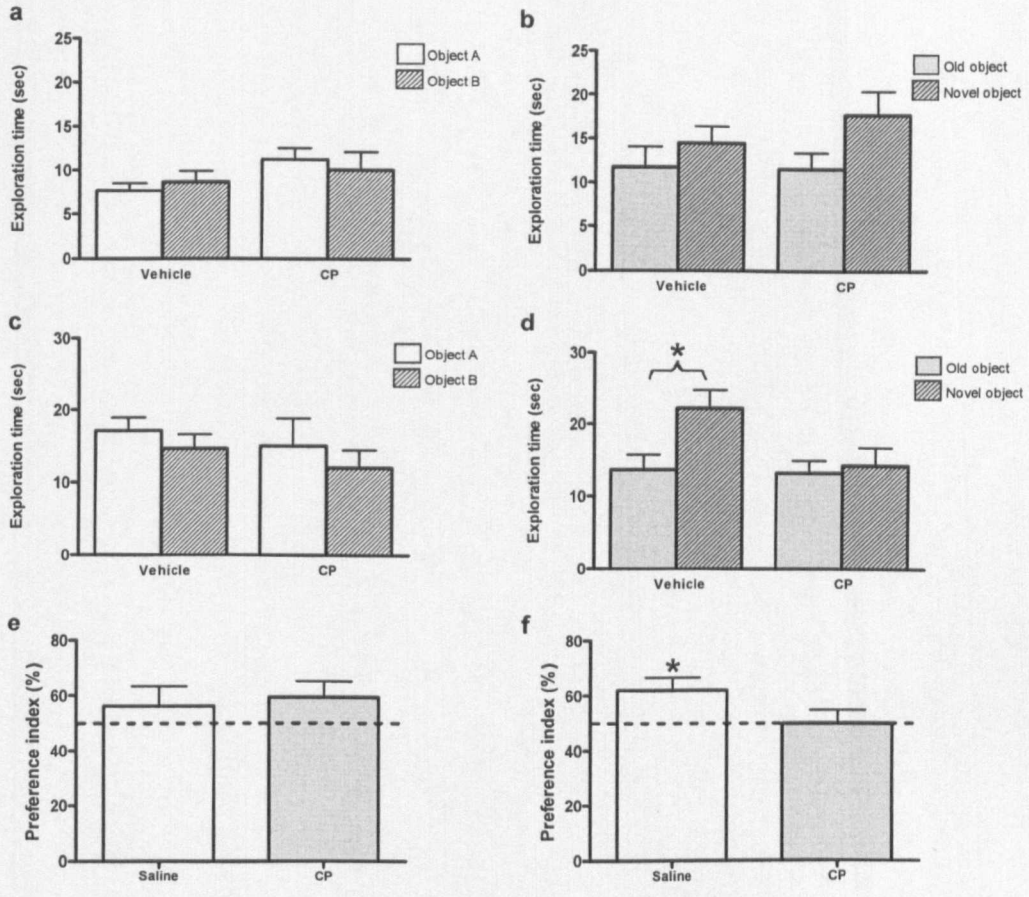

Figure 2.4 Exploration times (mean ± SEM, n=8) of the rats for each object for the novel object recognition (NOR) task from before (a and b) and after (c and d) CP/saline treatment. In the familiarisation trials (a and c) there was no significant difference in exploration time of either object for either group (Student's paired *t*-test). In the choice trial before treatment (b), neither groups spent significantly longer exploring the novel object (Student's paired *t*-test). In the choice trial, after completion of treatment (d), vehicle treated animals spent significantly longer exploring the novel object ($p<0.05$), but there was no significant difference in exploration time for the CP treated animals (Student's paired *t*-test). Preference indices (PI, mean ± SEM, n=8) were created by expressing time spent exploring the novel object as a percentage of the sum of exploration time of novel and familiar objects in the choice trial (Bruel-Jungerman et al. 2005). Before treatment (e), the PI of vehicle-treated animals and those to be CP-treated animals did not differ from 50% chance ($p<0.05$, one-sample *t*-test) or

between groups (Student's unpaired *t*-test). After treatment the PI of the CP and saline-treated groups did not differ from each other (Student's unpaired *t*-test, f). The PI of the saline-treated group differed from 50% chance after treatment ($p<0.05$, one-sample *t*-test) whereas the PI of the CP-treated group did not.

2.2.2.3 NLR task

There was no significant difference between the exploration times for either object for both groups in the familiarisation trials of the NLR task both before and after CP/saline treatment (Student's paired *t*-test, Fig. 2.5a and c). In the choice trial before treatment, the group to be treated with CP spent significantly longer exploring the object in the novel location ($p<0.01$, Student's paired *t*-test) whilst the group to be treated with saline showed no significant difference in exploration of either object (Student's paired *t*-test, Fig. 2.5b). The PI were significantly higher in animals to be treated with CP before treatment compared to the saline group ($p<0.01$, Student's unpaired *t*-test, Fig 2.5e). Before treatment the PI of the group to be treated with CP were significantly difference from 50% chance ($p<0.05$, one-sample *t*-test), whereas the vehicle-treated group were not. It is to be noted that at this stage the groups had not been administered with CP/saline. In the choice trial after treatment both groups spent more time exploring the novel object but this was not significant (Student's paired *t*-test, Fig. 2.5d). The PI of the saline and CP treated group did not differ from 50% chance (one-sample *t*-test, Fig 2.5f) or between groups after treatment (Student's unpaired *t*-test).

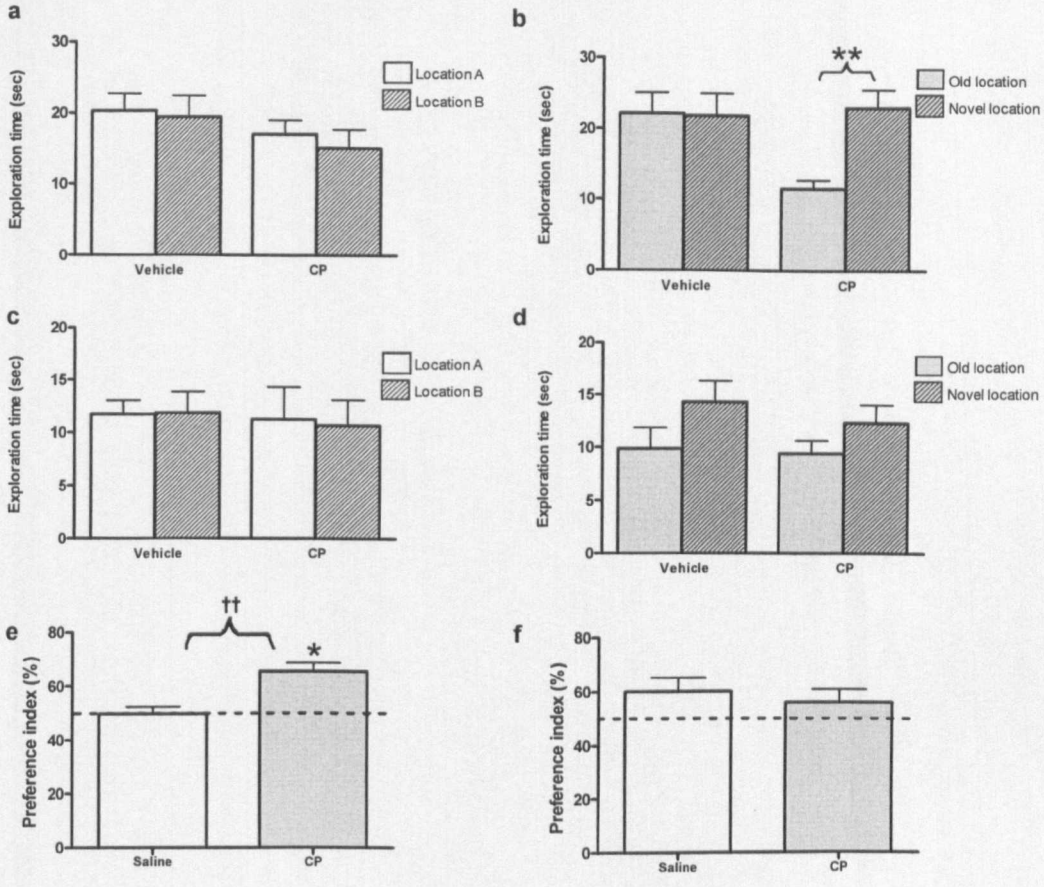

Figure 2.5 Exploration times (mean ± SEM, n=8) of the rats for each object for the novel location recognition (NLR) task from before (a and b) and after (c and d) CP/saline treatment. In the familiarisation trials (a and c) there was no significant difference in exploration time of either object for either group (Student's paired *t*-test). In the choice trial, before treatment (b), the vehicle treated group showed no significant difference in exploration time of either object (Student's paired *t*-test), whilst the CP treated group spent significantly longer exploring the object in the novel location ($p<0.01$, Student's paired *t*-test). In the choice trial, after completion of treatment (d), neither group spent significantly longer exploring the object in the novel location (Student's paired *t*-test). Preference indices (PI, mean ± SEM, n=8) were created by expressing time spent exploring the object in the novel location as a percentage of the sum of exploration time of novel and familiar locations in the choice trial (Bruel-Jungerman et al. 2005). The PI values were significantly higher in animals to be treated with CP before treatment (e) compared to the saline group ($^{\dagger\dagger}p<0.01$, Student's unpaired *t*-test).

Before treatment the PI of the group to be treated with CP were significantly difference from 50% chance ($p<0.05$, one-sample t-test), whereas the vehicle-treated group were not. After treatment (f), the PI of vehicle-treated animals and the CP-treated animals did not differ from 50% chance ($p<0.05$, one-sample t-test) or between groups (Student's unpaired t-test).

2.2.3 Discussion

In the present preliminary study, the NOR and NLR working memory tasks were used to investigate the effects of CP on cognition using a rat model. Behavioural experiments were carried out before and after CP treatment to ensure that the animals were able to perform the task. The usual expected outcome for these tasks would be that animals with no impairment in cognition would spend a similar amount of time exploring objects in the familiarisation trial, but spend significantly longer exploring the novel object or object in the novel location in the choice trial (Dix and Aggleton 1999; Ennaceur and Delacour 1988). From results of the NOR task in the present experiment, animals explored both objects for a similar amount of time in the familiarisation trials before and after treatment. In the choice trial before treatment (where no cognitive impairment should be seen) both groups appeared to explore the novel object for longer, although this was not significant. When the group numbers were combined (n=16) this difference became significant ($p<0.05$, Student's paired *t*-test, data not shown), suggesting significant variation between animals and indicating the task may be more reliable with larger n numbers (an n number of 8 was used in the present experiment). In the choice trial of the NOR task carried out after treatment, the saline treated animals spent significantly longer exploring the novel object. This would normally suggest that the CP treated animals display cognitive impairment; however, as neither of the groups was able to perform the task before treatment, this result remains inconclusive.

In the familiarisation trial of the NLR tasks before and after treatment, no significant difference was seen between exploration times for either object, which was expected. In the choice trial before treatment the group to be treated with CP was able to differentiate the objects in a novel and familiar location, although the group to be treated with saline could not. When the exploration times of the two groups were combined the overall difference in exploration time became significant ($p<0.05$, Student's paired *t*-test, data not shown), again indicating large variation between animals and suggesting that a larger number of animals were needed per group. Using data from the choice trial before treatment a power analysis was

performed to determine the number of animals that would be required to obtain significance of $p<0.05$. Data from the vehicle and control group combined suggested that a sample size of 33 would be required for an 80% chance for statistical significance. In the choice trial after treatment, both groups appeared to preferentially explore the object with the novel location, but this was not significant and a power analysis showed the probability of the effect of CP was only 30%, so no conclusions can be drawn from this study. Additionally, there may have been other complications with the tests. The tests were carried out 3 days apart and as the same objects were used for both tests, the animals may have remembered the object locations from the NOR task in the NLR task, which might have confounded the results. Based on this finding, in the rest of the experiments presented in this thesis, only one of these two tasks, the NLR task, was used.

CP is reported to cause weight loss when used in chemotherapy combinations clinically (Vettori et al. 2010) and in rat models (Lee et al. 2006). However, in the present study, rats treated with CP did not gain weight at a significantly lower rate than the control animals. This could indicate that the dose given was not clinically relevant and could be slightly increased, or be given for a longer time period in future studies.

In conclusion, although there was a slight indication that CP causes the ability of rats to be impaired in the NOR task, it is unjustifiable to draw these conclusions from this result as the animals were not able to perform the task before treatment. The experimental procedure used in the present study needs to be altered by increasing the number of rats in each group and only performing one behavioural test, so the possibility of the animals remembering previous objects and their locations is eliminated. These issues are addressed in the subsequent studies presented in this thesis.

2.3 *The effects of cyclophosphamide on hippocampal cell proliferation and spatial working memory in rat*

2.3.1 Introduction

In the preliminary study presented in this chapter, both the NOR and NLR tasks were used to test working memory of CP treated rats, however, the animals were not able to perform the tasks correctly even before treatment, indicating the task needs to be modified and higher numbers of rats need to be used. In the present study, a modified version of the NLR task was used to test spatial memory 6 days after the final CP injection. The NLR task was selected over the NOR task due to its dependence on the hippocampus (Dere et al. 2007). The number of CP injections was increased from 4 to 7 since no significant weight loss was observed in the preliminary study, although the dose of 30mg/kg remained the same. The modifications to the NLR task from that used in the previous study were as follows; the number of animals in each group was increased from 8 to 12; a visual cue (a square of black card) was placed on the wall of the room in which the test took place to aid rats with orientation; the habituation period 24 h before the familiarisation trial was decreased from 1 h to 30 min to reduce stress to the animal; a second habituation period of 3 min was added 1 min before the familiarisation trial. The NOR task was not utilised to avoid results being confounded by any recognition of the objects and their locations between tests.

Ki67 is a protein which is expressed in all stages of the cell cycle (Scholzen and Gerdes 2000) and was used to quantify cells which were proliferating in the dentate gyrus at the end of the experiment (see section 1.3.4.2, Chapter 1.). To investigate the effect of CP on the survival of newly generated hippocampal cells, BrdU was injected at the beginning of CP treatment to be incorporated into cells proliferating at that time. The surviving cells which expressed BrdU at the end of the experiment were quantified. Levels of DCX were quantified in the hippocampus to investigate changes in differentiation of newly born neurons in the hippocampus and also in the frontal cortex to indicate the regional specificity of changes induced by CP.

2.3.2 Materials and methods

2.3.2.1 Animals and treatment

Male Lister-hooded rats (125-150g; Charles River, UK, total n=24) were administered CP (30 mg/kg, 7 i.v. doses each 2 days apart, into the tail vein, at a volume of 1.5ml/kg, dissolved in 0.9% sterile saline; Medac, Germany) or 0.9% sterile saline at an equivocal volume (both groups n=12). Both groups were administered BrdU (250mg/kg, i.p., at a volume of 4ml/kg; neutralised to pH 7.0 with 0.1M NaOH, Sigma Aldrich, UK) immediately after their first CP/saline injection. All injections were given under gaseous isofluorane (4% to initiate anaesthesia then reduced to 2%) and administered between 14.00 and 16.00 h.

Rats were housed as described in section 2.2.1.1 of the present chapter. They were weighed daily from arrival and allowed to habituate 2 weeks prior to behavioural testing. All procedures were in accordance to UK Home Office Guidance regulations and with local ethical committee approval.

2.3.2.2 Behavioural testing

2.3.2.2.1 NLR task

The NLR task was carried out as described in section 2.2.1.2.2 of the present chapter, with a modified habituation procedure (Fig. 2.6). Animals were habituated for 30 min, 24 h prior to testing (during which EthoVision 4.1 was used to measure the mean velocity of the rats) and for a further 3 min, 5 min before the familiarisation trial (King et al. 2004). A black square of card was added on the wall of the room during trials to provide a prominent cue for spatial orientation. Exploration times of both objects and trials were recorded blind to the identity of the rat twice using a stopwatch and averaged. The exploration data of all animals was included in the analysis.

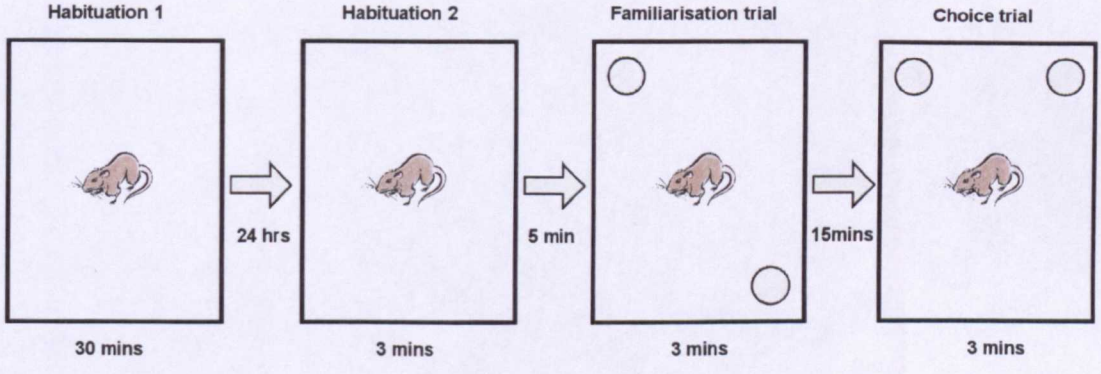

Figure 2.6 A modified version of the novel location recognition (NLR) task. Rats were habituated in the arena for 30 min and for a further 3 min 24 h later. They were then removed for 1 min whilst two identical objects were placed in 2 corners of the arena. Rats were replaced in the arena and allowed to explore the objects for 3 min (familiarisation trial) then removed again for 15 min. When returned again, one object had been moved to a different corner and rats were again left to explore for 3 min (choice trial).

2.3.2.3 Brain tissue preparation

Rats were killed by rapid concussion and cervical dislocation the day after behavioural testing. Brains were removed, cut sagittally and one half was cryopreserved in 30% sucrose solution for three hours at 4°C, then submerged in OCT-compound (VWR International Ltd, UK) and snap frozen in liquid nitrogen-cooled isopentane. These were stored at -80°C until being sectioned along the coronal plane using a Leica CM 100 cryostat (Leica Microsystems, UK) at 20μm thickness at -20°C. The sections were thaw mounted onto 3-aminopropylmethoxysaline (APES)-coated slides and stored at -20°C until used for immunohistochemistry. From the contralateral brain, the hippocampus and frontal cortex were dissected, placed in eppendorfs and immediately snap frozen in liquid nitrogen and stored at -80°C until used for Western blotting.

2.3.2.3.1 Immunohistochemistry

For both Ki67 and BrdU staining a systematic random sampling technique was used (Mayhew and Burton 1988). Every 20th section throughout the entire length of the dentate gyrus was selected, resulting in a total of 9-11 sections per brain. All immunohistochemistry incubations were carried out at room temperature in a light-proof humidity chamber.

2.3.2.3.2 Ki67

All dilutions and washes (each time performed thrice) were carried out with phosphate buffered saline (PBS). Sections were fixed using 0.5% paraformaldehyde (PFA) for 3 min, washed then incubated with monoclonal mouse anti-Ki67 primary antibody (1:100; Vector laboratories, UK) for 1 h, followed by a further wash and 1 h incubation with Alexa 555 donkey anti-mouse (1:300; Invitrogen, UK). Sections then had a final wash, were mounted with (diamidinophenylindole) DAPI (1.5μg/ml) nuclear marker (Vector laboratories, UK) and cover-slipped.

2.3.2.3.3 BrdU

All washes were performed three times with 0.1M sodium borate adjusted to pH 8.5 (borate buffer) unless otherwise stated. Sections were fixed in 4% PFA for 3 min, washed, then DNA was denatured by incubation in 2M hydrochloric acid (HCl) containing 0.3% tritonX100 (Sigma Aldrich, UK) for 20 min, immediately followed by 10 min incubation with 5M HCl. Sections were then washed and neutralised for 12 min in the borate buffer then blocked with 5% bovine serum albumin (BSA; Sigma Aldrich, UK) in PBS containing 0.15% triton X-100 for 30 min. After washing sections were incubated for 16-20 h with polyclonal sheep anti-BrdU primary antibody (1:100; Abcam, UK) in the blocking solution, followed by washing and incubation with Alexa 488 donkey anti-sheep secondary antibody (1:300; Invitrogen, UK) in PBS. Sections then had a final wash in PBS, were mounted with DAPI and cover-slipped.

All staining was viewed and quantified at ×40 on a Nikon EFD-3 fluorescence microscope. BrdU and Ki67 positive cells which co-localised with the DAPI nuclear staining within both blades of the dentate gyrus (or within a 3 cell diameters of the inner edge) were counted. By combining cell counts per section for the whole dentate gyrus and multiplying by 20, an estimate of total co-stained cell numbers was produced (Huang and Herbert 2006). All counting was performed blind.

2.3.2.4 Western blot analysis of DCX expression in hippocampus and frontal cortex

2.3.2.4.1 Sample preparation, Lowry assay and protein separation

Sample preparation was carried out on either ice or at 4°C. Hippocampus and frontal cortex samples (n=6) were homogenised in lysis buffer (see Appendix I) to produce a 100mg/ml solution. The samples were rotated on a spinning wheel for 20 min then centrifuged at 13,000g for 10 min. 20µl of the supernatant was taken to be used for the Lowry test. The remainder of the supernatant was added to an equal volume of 2X solubilisation buffer (see Appendix I) and stored at -20°C.

Protein concentrations within each sample were determined using a Lowry assay (Lowry et al. 1951). A standard curve of BSA dilutions (ranging from 0 to 0.5 mg/ml, in distilled water, total volume 200 µl) was created. The 20µl homogenised sample was diluted (1:10) in distilled water. 1ml of Lowry AB solution (see Appendix I) was added to the sample and all standards and incubated at room temperature for 10 min. 100 µl of Folin reagent (diluted 1:1 with distilled water) was added to each sample and incubated at room temperature for 45 min. Each standard and sample was loaded in triplicate into a 96 well plate and the optical density was read at 750nm wavelength using a Dynex MRX Model 96 Well Plate Reader (MTX Lab Systems Inc., USA). Protein concentration was detected colourimetrically and presented with Revelation software (MTX Lab Systems Inc., USA). All samples were then normalised with 2X solubilisation buffer diluted with lysis buffer (1:1)

The samples were denatured by heating to 95°C for five minutes, then vortexed and centrifuged for 1 min at 13,000g. 15µl of each were loaded onto a 10% SDS-polyacrylamide gel (see Appendix I) and run parallel to 1µl of a molecular weight marker (PageRuler plus Prestained protein ladder manufactured by Fermentus UAB, Lithuania). Protein separation occurred for 45 min (200V at room temperature) with gel immersed in electrophoresis buffer (see Appendix I). Subsequently the proteins from the gel were transferred onto a nitrocellulose membrane (Hybond, Amersham Biosciences, UK) for 60 min (100V at 4°C) in transfer buffer (see Appendix I). Successful transfer of protein was confirmed with Ponceau solution (Sigma-Aldrich, USA).

2.3.2.4.2 Immunodetection, quantification of protein and data analysis

The nitrocellulose membrane was blocked at room temperature, rocked for 1 h in 5% milk solution (dried milk powder in Tris-buffered saline tween-20 (TBST) solution, see Appendix I). All antibodies were diluted by TBST with 5% milk powder. The nitrocellulose was rocked overnight at 4°C with polyclonal rabbit anti-DCX (1:1000; Cell Signalling) and mouse anti-β-actin (1:1000; Cell Signalling) primary antibodies, washed several times with TBST to remove

primary antibodies. It was then rocked in IRDye 700CW goat anti-rabbit and IRDye 800CW goat anti-mouse (both 1:20000; LI-COR Biosciences) for 1 h and again washed in TBST. The membrane was scanned on an Odyssey scanner (LI-COR) at wavelengths of 700nm (red emission) and 800nm (green emission) and analysed using Odyssey software (Application version 3.0). β-actin (43 kDa) was used as a loading control that DCX (47 kDa) protein bands could be normalised against.

Tissue samples of the hippocampus and frontal cortex from both CP and vehicle treated groups were run on the same gel and repeated 3 times. Protein was quantified (and presented) using Odyssey software (Application version 3.0) by measuring the intensity of the protein band. Intensity levels of DCX expression was measured at a wavelength of 700nm and normalised against β-actin measured at 800nm. Mean DCX protein expression of the samples is presented.

2.3.2.5 Statistical analysis

Statistical analysis and graphs were created using GraphPad Prism 5 and significance was regarded as $p<0.05$. Body weight was analysed using two-way repeated measured ANOVA. When ANOVA was significant Bonferonni post-hoc test was performed. Student's paired *t*-tests were used to compare exploration times of animals in the familiarisation and choice trials. Preference indices (PI) were created by expressing time spent exploring the object in the novel location as a percentage of the sum of exploration time of novel and familiar locations in the choice trial, to create a single value to compare between groups (Bruel-Jungerman et al. 2005). PI were compared to 50% chance using a one-sample *t*-test. Student's unpaired *t*-tests were used to compare PI, total exploration time and average velocity of the animals and DCX expressed from Western blotting.

2.3.3 Results

2.3.3.1 Cyclophosphamide reduces weight gain

Both treatment and time had a significant effect on body weight ($F_{1,418}$=5.51, p<0.05 $F_{19,418}$=367.9, p<0.001 respectively, two-way repeated measures ANOVA, Fig. 2.7) and a significant effect of treatment × time interaction was also confirmed ($F_{19,418}$=30.23, p<0.001). Animals remained in good health throughout the study and never dropped more than 10% of their highest body weight.

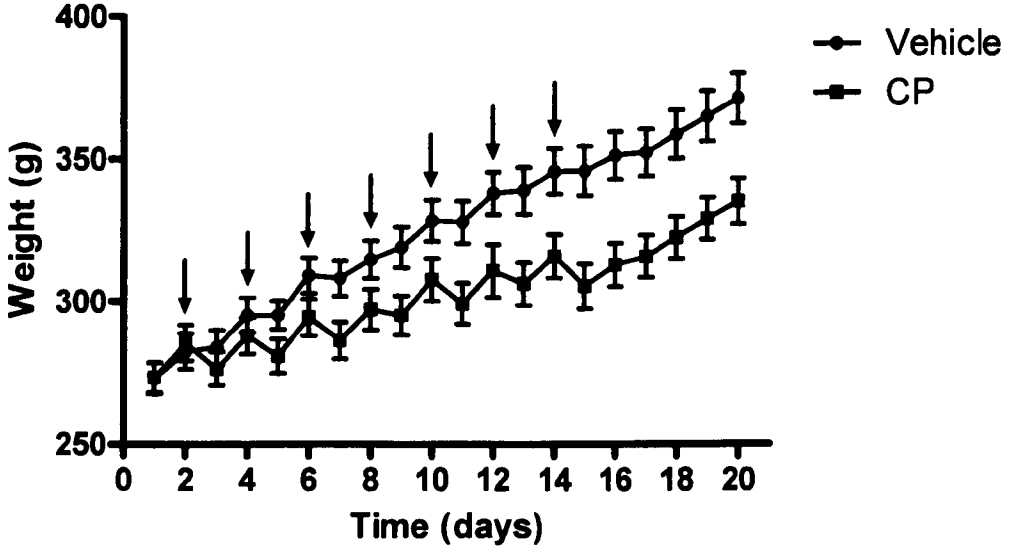

Figure 2.7 Body weights of rats (mean ± SEM, n=12) throughout the study. Arrows indicate CP/saline injections. Treatment and time significantly affected body weight ($F_{1,418}$=5.51, p<0.05 $F_{19,418}$=367.9, p<0.0001 respectively, two-way repeated measures ANOVA) and the treatment × time interaction was also significant ($F_{19,418}$=30.23, p<0.0001).

2.3.3.2 CP does not affect spatial working memory

In the familiarisation trials, both before and after animals were treated with CP or saline, neither the groups explored the objection in locations A or B for significantly longer, showing no preference for either location (Student's paired *t*-test, Fig. 2.8a and 2.9a). In the choice trial before treatment, both groups spent longer exploring the object in novel location ($p<0.001$, Student's paired *t*-test, Fig. 2.8b). In the choice trial after treatment both groups spent significantly longer exploring the novel object ($p<0.05$, Student's paired *t*-test, Fig. 2.9b). The exploration time data was converted into PI for further analysis (Fig. 2.8c and 2.9c). Both the vehicle and CP treated groups significantly differed from 50% chance in the choice trials ($p<0.001$), both before and after treatment, indicating neither group had impaired cognition. No significant difference was found between the PI of each group (Student's unpaired *t*-test) before and after treatment. No difference was found in total exploration time or mean velocity of the vehicle and CP treated animals both before and after treatment (Fig. 2.8d and 2.9d and Fig. 2.10).

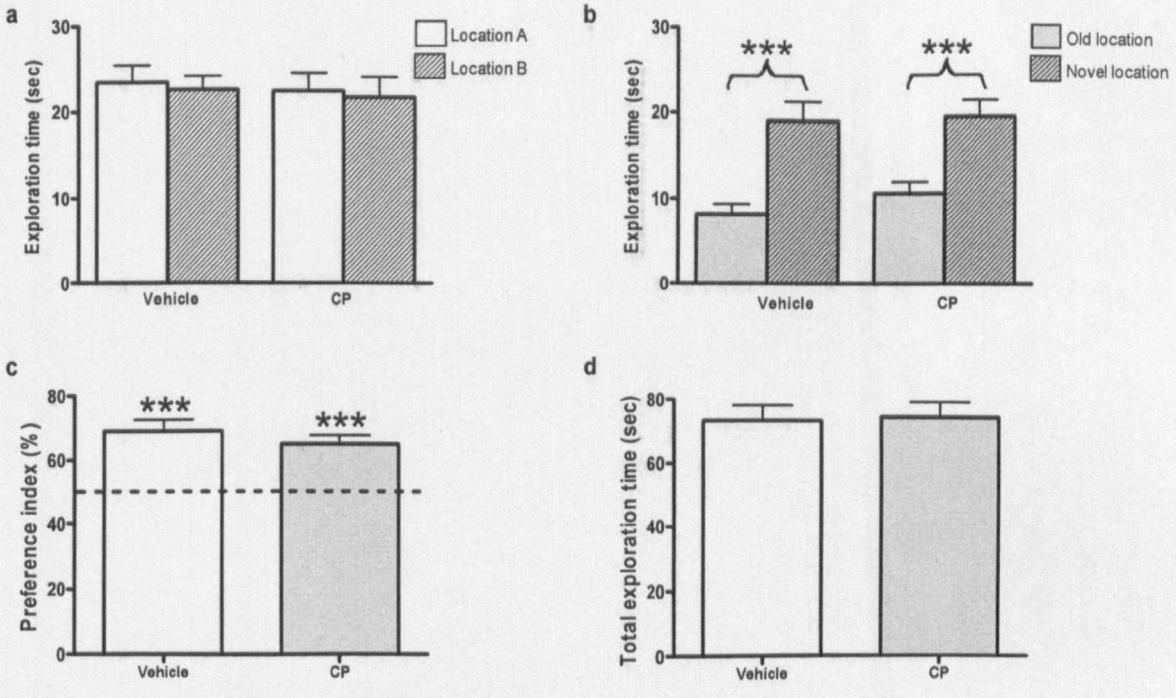

Figure 2.8 Exploration times (mean ± SEM, n=12) of the rats for each object in the familiarisation (a) and choice (b) trials in the novel location recognition (NLR) task, before CP/saline treatment. There was no significant difference in exploration time of either object for both groups in the familiarisation trial (Student's paired *t*-test). In the choice trial, both groups spent significantly longer exploring the object in the novel location ($p<0.001$, Student's paired *t*-test). Preference indices (PI, (c), mean ± SEM, n=12) were created by expressing time spent exploring the object in the novel location as a percentage of the sum of exploration time of novel and familiar locations in the choice trial (Bruel-Jungerman et al. 2005). The PI were not significantly different between groups (Student's unpaired *t*-test) but PI of both groups were significantly different from chance ($p<0.001$, Student's unpaired *t*-test). The total exploration time (mean ± SEM, n=12) for both trial combined (d) did not differ significantly between groups (Student's unpaired *t*-test).

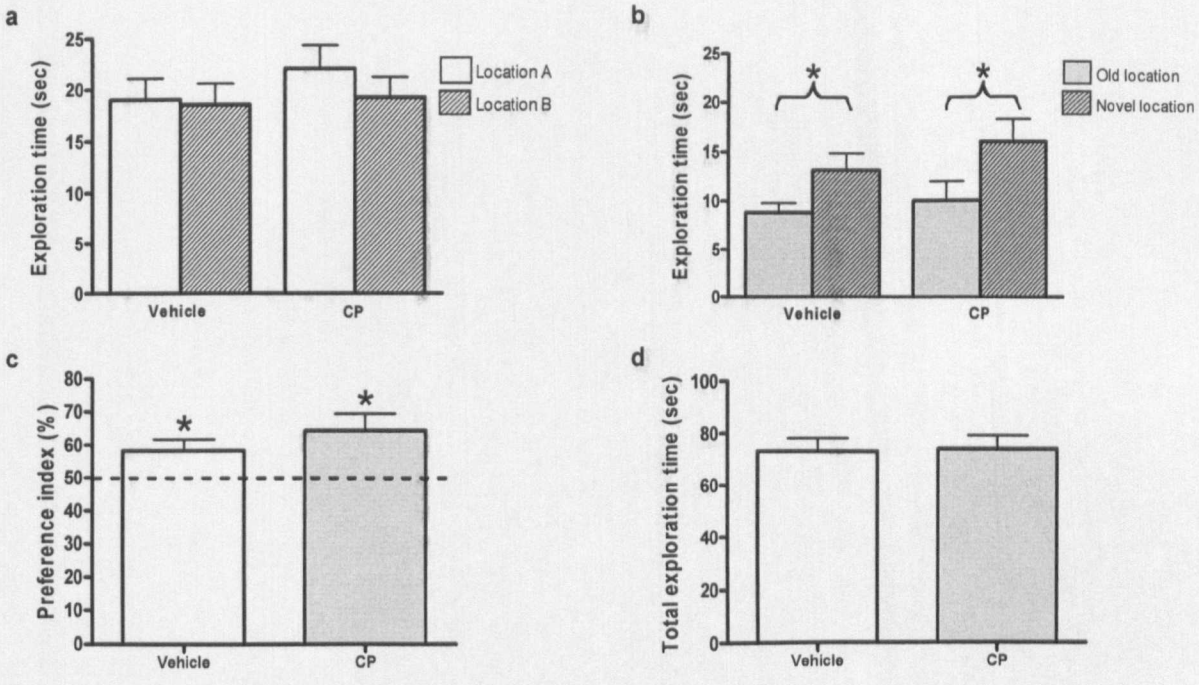

Figure 2.9 Exploration times (mean ± SEM, n=12) of the rats for each object in the familiarisation (a) and choice (b) trials in the novel location recognition (NLR) task, after treatment with CP/saline. There was no significant difference in exploration time of either object for both groups in the familiarisation trial (Student's paired *t*-test). In the choice trial, both groups spent significantly longer exploring the object in the novel location ($p<0.05$, Student's paired *t*-test). Preference indices (PI, (c), mean ± SEM, n=12) were created by expressing time spent exploring the object in the novel location as a percentage of the sum of exploration time of novel and familiar locations in the choice trial (Bruel-Jungerman et al. 2005). The PI were not significantly different between groups (Student's unpaired *t*-test) but PI of both groups were significantly different from chance ($p<0.05$, Student's unpaired *t*-test). The total exploration time (mean ± SEM, n=12) for both trials combined (d) did not differ significantly between groups (Student's unpaired *t*-test).

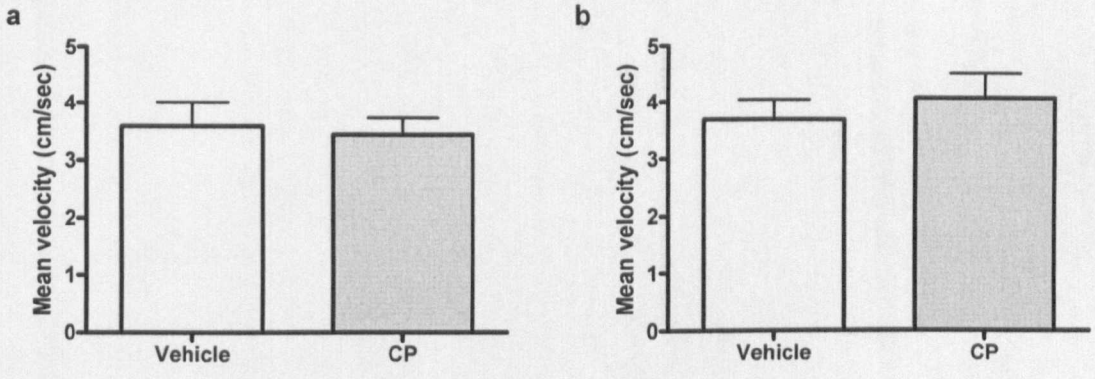

Figure 2.10 Velocity (mean ± SEM) of rats (n=12) was not significantly different between groups either before (a) or after (b) CP/saline treatment (Student's unpaired *t*-test).

2.3.3.3 CP does not reduce cell proliferation in the SGZ

Ki67 was used to investigate the effect of CP on the number of cells proliferating in the SGZ of the dentate gyrus (Fig. 2.11a-c). No significant difference was found for the amount of Ki67-positive cells between the groups (Student's unpaired *t*-test, Fig. 2.11d), suggesting that the CP regimen in the present study does not affect cells dividing in the dentate gyrus 7 days after it was administered.

2.3.3.4 CP reduces the survival of new-born hippocampal cells

BrdU was injected on the day of the first CP/saline injection to investigate the survival of the cells which were dividing at that time (Fig. 2.12a-c). Animals receiving CP had significantly fewer BrdU-positive cells in the dentate gyrus ($p<0.05$, Student's unpaired *t*-test, Fig. 2.12d), indicating that CP reduced the survival of the cells dividing in the SGZ of the dentate gyrus at the start of treatment.

2.3.3.5 Expression of DXC

Western blotting analysis was performed to determine the effects of CP on levels of DCX in the hippocampus and frontal cortex (Fig. 2.13). Whole brain was used as a positive control for DCX, but it is not shown on this immunoblot. Protein levels of DCX, a cytoskeletal protein transiently expressed in immature neurons, were normalised by comparison with the β-actin loading control. A student's unpaired *t*-test showed no significant difference in DXC expression between the CP treated group and saline treated controls in either the hippocampus (Fig. 2.13c) or the frontal cortex (Fig. 2.13d).

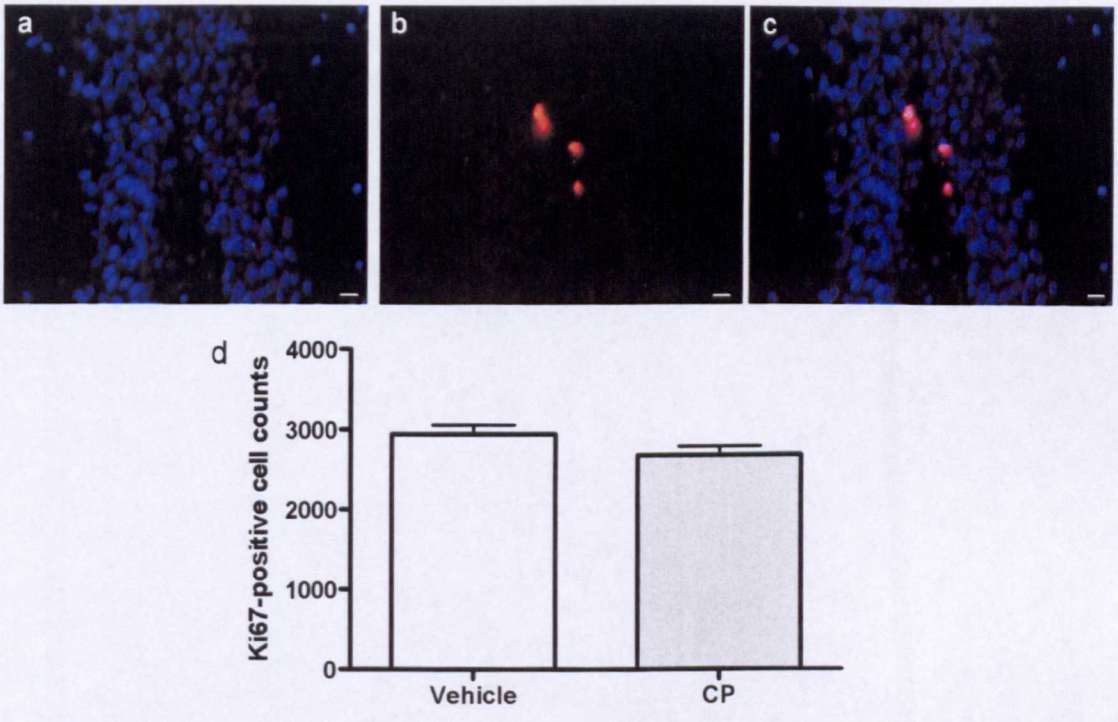

Figure 2.11 Representative photographs of the nuclei of cells in the SGZ of the dentate gyrus from the vehicle group (blue, a), Ki67-positive cells (red, b) and the photos merged (c). Bar scales indicate 20μm. There was no significant difference (Student's unpaired *t*-test) between groups of the total numbers of Ki67-positive cells (mean ± SEM, n=12, d).

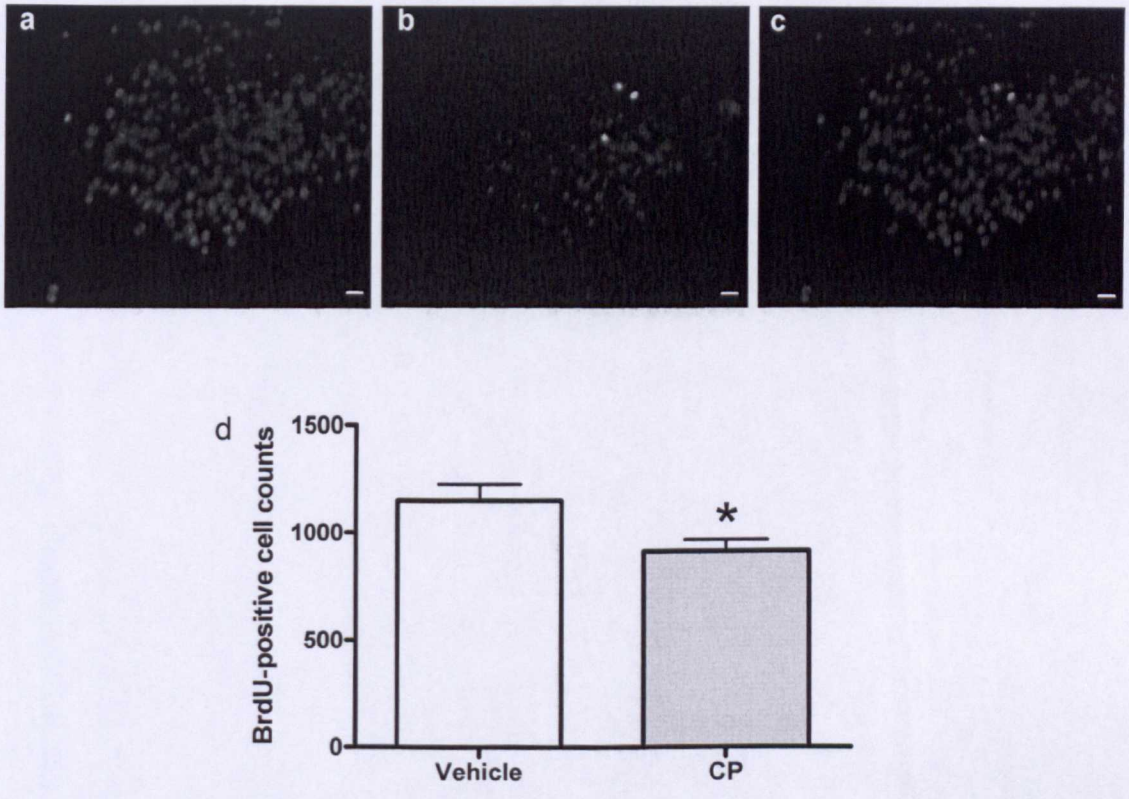

Figure 2.12 Representative photographs of the nuclei of cells in the dentate gyrus from the vehicle group (blue, a), BrdU-positive cells (green, b) and the photos merged (c). Bar scales indicate 20μm. Total numbers of BrdU-positive cells (mean ± SEM, n=12) in the dentate gyrus were estimated from cell counts (d). Rats receiving CP had significantly fewer BrdU-positive cells ($p<0.05$, Student's unpaired *t*-test) in the SGZ than the saline-treated control group.

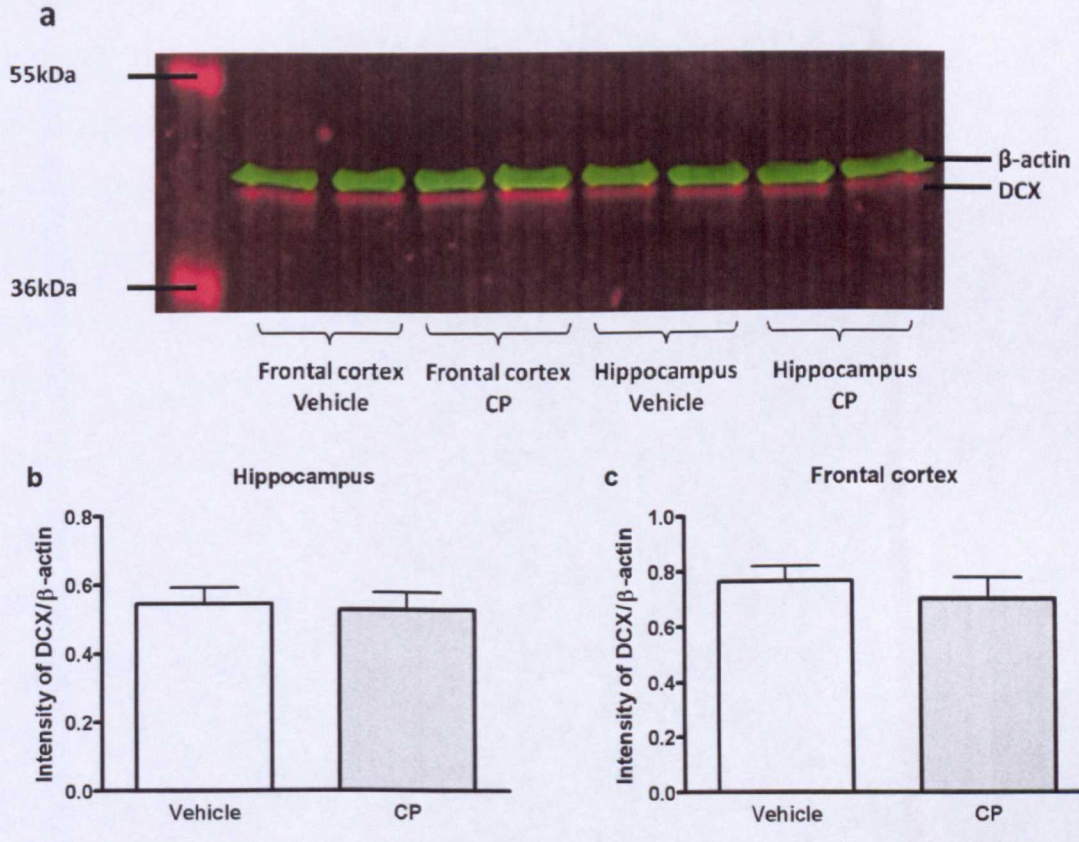

Figure 2.13 Example photomicrograph of immunblot bands from Western blotting (a) showing expression of DCX (red, at 47 kDa) and β-actin (green, at 43 kDa). Levels of DCX (mean ± SEM, n=6) in the hippocampus (b) and frontal cortex (c) of control and CP treated animals, normalised by β-actin loading control, were not significantly different (Student's unpaired *t*-test).

2.4 Discussion

In the present study a rat model was used to investigate the effects of CP on cognition and the survival and proliferation of newly generated hippocampal cells.

One memory task (the NLR task) was used in the present study, it was chosen over the NOR task to test spatial working memory, as it is hippocampal dependent (Dere et al. 2007; Mumby et al. 2002). Furthermore, it does not rely on any positive or negative reinforcers which may confound results. The results showed that before any treatment both groups of animals were able to distinguish an object in a novel location from that in a familiar, demonstrating that they were able to perform the task correctly. After treatment, animals which had received a series of CP injections still spent significantly longer exploring the object in the novel location, and did not significantly differ from the control group. This indicates that the dose and administration of CP used in the present study did not affect the rats' ability in this task. Other studies using rodent models within the literature have shown a mixture of results in regard to the effect of CP on cognition. Lee at al. (2006) found that 4, 100mg/kg doses of CP, 4 weeks apart, caused an improvement in Morris water maze performance in rat. However, other authors giving 4 weekly doses of 25mg/kg CP found an impairment in a passive avoidance test a week after treatment in female rats (Konat et al. 2008). Furthermore, Macleod et al. (2007) found a sub-chronic weekly dose of 40mg/kg of CP impaired context specific, but not cue specific conditioned emotional response in rat a week after the final injection. These differences may be explained by the different dosages and different behavioural tests used. It would be interesting to use further behavioural tests to investigate the effect of dosing regimen used in the present study on different cognitive domains in rat. Studies on mice have found an acute affect (within 24 hours) on memory but recovery within a period of days (2006; Yang et al. 2010). The results in the present study demonstrate that CP causes no deficit in the NLR task 6 days after the final CP injection. However this does not preclude the possibility that CP may have acute effects on memory which recover over a short time period. It is also possible

that CP may have longer term effects which have not been examined in the present study. Such time course issues are further explored in later studies using 5-FU in the present thesis.

New neurons in the dentate gyrus have been shown to be preferentially used in spatial learning tasks (Kee et al. 2007) and reductions in dentate gyrus neurogenesis impair the ability of animals to perform these tasks (Imayoshi et al. 2008). It is estimated that over 80% of dividing cells in the adult rat SGZ are destined to become dentate gyrus neurons (Snyder et al. 2009). In the present study, no significant difference was seen between control and CP treated groups in levels of DCX in the hippocampus and the frontal cortex. DCX is a protein expressed in immature neurons so is used as a marker for neurogenesis rather than proliferating cells (Zhao et al. 2008), suggesting that CP did not reduce the number of newly generated neurons in the hippocampus. Levels of DCX were also investigated in the frontal cortex to determine the effect of CP on other brain regions, and again no significant difference was found between CP and control groups. DCX in the frontal cortex most probably arises from newborn neurons migrating along the rostral migratory stream from the SVZ to the olfactory bulb (Zhao et al. 2008). Furthermore, the number of cells proliferating (Ki67-positive) in the SGZ of the dentate gyrus, a week after the final injection, was not affected by CP compared to the control group, although the number of BrdU-positive cells was significantly reduced. This reduction suggests that the survival of the cells which were dividing at the beginning of the experiment was lower in rats that received CP, indicating the drug is cytotoxic to newly generated hippocampal cells. This might be an explanation for the acute effects on behaviour found in some studies (Reiriz 2006; Yang et al. 2010). This conclusion is in line with recent studies showing that cell proliferation is reduced the day after CP administration (Janelsins et al. 2010) but gradually recovers over the following days (Yang et al. 2010), correlating with cognitive performance. It would be interesting to look at the effects of CP on cognition, cell proliferation and survival over a longer time period as another alkylating agent, thioTEPA, caused an initial reduction in hippocampal cell proliferation in mice, followed by a transient 3 week recovery. This in turn was followed by a long-term

deficiency in cell proliferation lasting for 3 months and these deficiencies were roughly correlated with spatial cognitive decline (Mondie et al. 2010).

Collectively, the results of different studies of CP on cognition and neurogenesis, still do not paint a clear picture. Drug delivery, with respect to route of administration, dosage and time course has differed between studies as have the behavioural tests used. Evidence from the previous studies suggests that CP may have an acute effect on cells proliferating in the SGZ of the adult dentate gyrus during which time animals may display cognitive deficits. However, it appears likely that the reduction in cell proliferation and spatial cognition is subtle and reversible. CP is broken down by aldehyde dehydrogenase 3 (ALDH3) present in the brain which converts CP into non-toxic metabolites (Bunting and Townsend 1996). The presence of this enzyme may make the effects of CP relatively short lasting compared with other chemotherapy agents.

CP is often administered with MTX and 5-FU, a combination known as CMF. This drug cocktail has been associated with cognitive impairment in patient studies (Kreukels et al. 2008; Schagen et al. 2002a) and several rodent models have been used to investigate the individual drugs. In the majority of studies, 5-FU has been shown to chronically impair memory and reduce proliferation in the dentate gyrus of the hippocampus for weeks after treatment has ended (ElBeltagy et al. 2010; Gandal et al. 2008; Winocur et al. 2006). Likewise, the negative effect of MTX on cognition and proliferation has also been shown to last for weeks (Foley et al. 2008; Gandal et al. 2008; Seigers et al. 2008). Indeed, a previous experiment within our laboratory demonstrated that 5-FU caused rats to be impaired in the NLR task and reduced the proliferation and survival of hippocampal cells (ElBeltagy et al. 2010). Furthermore, in experiments which are presented in Chapter 3 and Chapter 4 of this thesis, administration of both 5-FU and MTX cause cognitive impairment in an identical NLR task as described in the present study and significantly reduce hippocampal cell proliferation and survival. When comparing this to the effect of CP in the present study, it indicates that CP

is less toxic than the other drugs in the CMF combination. As no dose-response analysis was executed, it is possible that a higher dose of CP may have yielded different results. However, the present findings suggest that although CP may acutely impair spatial cognition and reduce the survival of newly generated hippocampal cells, this is reversible in a matter of days and it is likely to be the other chemotherapy drugs which cause long-term cognitive impairment. This work has been recently published (Lyons et al. 2011a).

Chapter 3

Fluoxetine reverses the memory impairment and reduction in proliferation and survival of hippocampal cells caused by methotrexate chemotherapy in the rat

3.1 Introduction

As discussed in the previous chapters of this thesis, many patients who have received adjuvant chemotherapy to treat cancer have experienced long-lasting cognitive deficits including the reduced ability to form new memories, lack of concentration and general confusion (Ahles et al. 2002; Matsuda et al. 2005; Taillibert et al. 2007). There is evidence to suggest that the effects of cytotoxic drugs on hippocampal neurogenesis is a potential mechanism for the cognitive impairments seen (ElBeltagy et al. 2010; Mustafa et al. 2008; Seigers et al. 2007; Yang et al. 2010). The effects of the chemotherapy agent, CP, on cognition and hippocampal changes were investigated in the previous chapter. In the present chapter the effects of MTX, another chemotherapy agent from the CMF combination (see section 1.2, Chapter 1), are examined to investigate whether this chemotherapeutic drug has more significant effects on spatial memory and cellular changes in the hippocampus. Furthermore, in this chapter, the reversal of cognitive and cellular deficits caused by MTX, by the co-administration of the SSRI antidepressant, fluoxetine is demonstrated.

Despite the increasing body of evidence for chemotherapy-induced cognitive impairment, the mechanisms causing this are still not understood and there have been few attempts to counteract it. Fluoxetine is an SSRI antidepressant reported to improve the memory deficits seen in patients with mild cognitive impairment (Mowla et al. 2007), depression (Gallassi et al. 2006; Levkovitz et al. 2002; Vythilingam et al. 2004), post traumatic stress disorder (Vermetten et al. 2003) and traumatic brain injury (Horsfield et al. 2002). These results are supported by rodent investigations showing that fluoxetine increases the levels of brain derived neurotrophic factor (BDNF) (Alme et al. 2007; Duman and Monteggia 2006), the rate of neurogenesis (Chen et al. 2006; Duman 2004; Marcussen et al. 2008) and the survival of new neurons (Duman et al. 1999; Hitoshi et al. 2007) in the hippocampus. All of these factors are thought to play a role in memory consolidation (Kitabatake et al. 2007; Lledo et al. 2006a; Zhao et al. 2008). While fluoxetine may not have any beneficial effects on healthy subjects

(Monleon et al. 2007), a recent study from our group in Nottingham found that it can improve cognition after 5-FU chemotherapy in rats (ElBeltagy et al. 2010).

The present study investigates whether fluoxetine alters the cognitive deficits induced by MTX chemotherapy in rats, and examines its effect on neurogenesis in the hippocampus. MTX is an antimetabolite, commonly used to treat several types of cancer including breast cancer (Rousseau et al. 2000) and is often used in adjuvant chemotherapy combinations which have been associated with impaired cognition (Falleti et al. 2005; Matsuda et al. 2005) (see section 1.1.3.1, Chapter 1). When MTX is used in high doses, LCV is often administered chronically after it to reduce toxicity. MTX is an inhibitor of tetrahydrofolate (THFA) reductase and THFA is necessary for DNA synthesis. LCV is a THFA which doesn't require THFA reductase, replenishing pools depleted by MTX (Genestier et al. 2000; Seigers et al. 2007) (see section 1.2.4.1, Chapter 1). The dose of MTX and protocol chosen in the present study matches that used clinically (Lobo and Balthasar 2002; Peters et al. 1993), and has been used by Seigers et al. who found that it significantly suppressed hippocampal cell proliferation in the adult rat (Seigers et al. 2007). Fluoxetine was delivered orally to mimic clinical administration and to prevent the stress of injection. The dose was chosen from previous work in our laboratory which showed that it could counteract the memory deficits caused by the chemotherapy agent 5-FU (ElBeltagy et al. 2010). Fluoxetine was given for 40 days before during and after MTX treatment as it takes at least 21 days in rat to have anxiolytic effects (Conley and Hutson 2007) and increase neurogenesis in the hippocampus (Kodama et al. 2004). Spatial memory was tested 6 days after the end of fluoxetine treatment, as it takes 3 days to wash out of the system (Caccia et al. 1990), using the NLR task (Dix and Aggleton 1999) and a further 6 days later using the spontaneous alternation in the T-maze task. The effects of MTX on both survival and proliferation of new-born hippocampal neurons was determined.

3.2 Materials and methods

3.2.1 Animals and treatment

Male Lister-hooded rats (150-200g; Charles River, UK, total n=47) were randomly allocated to vehicle (n=12), MTX/LCV (n=12), fluoxetine (n=12) or MTX/LCV + fluoxetine (n=11) groups. Rats were housed as described in section 2.2.1.1, Chapter 1 and allowed to habituate for 2 weeks prior to drug administration.

Rats in MTX/LCV groups were administered MTX (75mg/kg, two i.v. doses a week apart, at a volume of 0.5ml/kg; Mayne Pharma Plc, UK) and rats in non-MTX/LCV groups were given an identical volume of 0.9% sterile saline (i.v. , Fig. 3.1). LCV (CP Pharmaceuticals, UK), or saline for non-MTX/LCV groups, was administered i.p.18 h after each MTX injection at 6mg/kg and 26, 42 and 50 h after at 3mg/kg (at a volume of 1ml/kg). BrdU was administered to all groups immediately after their first injection (250mg/kg, i.p., at a volume of 5ml/kg; pH7; Sigma Aldrich, UK, Fig. 3.1).

Mean water consumption and animal weight were monitored throughout the experiment. Fluoxetine (Pinewood Healthcare, Ireland, Oral solution) was administered in drinking water at a dose of 10mg/kg/day, estimated from the water consumption within each cage and calculated on a daily basis. This was seen to be advantageous as it avoided the possible stress caused by isolation housing (Sharp et al. 2002). Fluoxetine-treated drinking water was administered to animals in these groups for 40 days, starting 9 days prior to first MTX injection (Fig. 3.1).

MTX or saline equivalent was administered by i.v. injection to the tail vein in 0.9% saline under isofluorane anaesthetic. Injection courses began three weeks after animal arrival. Throughout the experiment, rats were maintained with a 12 h light/dark cycle (7.00/19.00 h) with *ad libitum* food and water. Principles of laboratory animal care were in accordance to UK Home Office Guidance regulations and with local ethical committee approval.

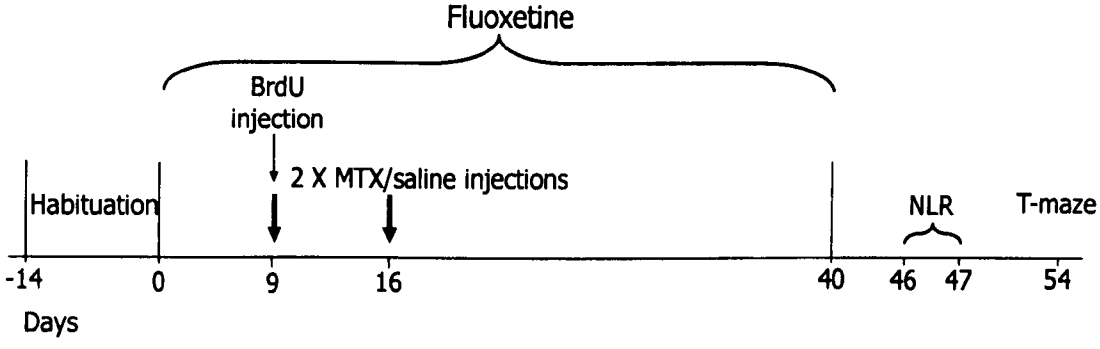

Figure 3.1 Time line showing protocol of drug administration and behavioural testing. Arrows represent single i.p. injections of BrdU (fine) and MTX/saline (thick). MTX/saline injections were followed by 4 leucovorin/saline i.p. injections 18, 26, 42 and 50 h after (not shown). The bracket represents the period of time for which fluoxetine was administered in the drinking water. The day after novel location recognition (NLR) behavioural testing, animals were killed and their brains were removed.

3.2.2 Behavioural testing

3.2.2.1 NLR task

The NLR test was carried out (as described in section 2.3.2.2.1., Chapter 2) 6 days after fluoxetine treatment ended. The task was only carried out after treatment with MTX and not before so the rats would have no prior experience of the objects and locations before the test was carried out to avoid any possibility of confounding the results. EthoVision 4.1 was used to measure the mean velocity of the rats during the 30 min habituation, 24 h prior to testing. Exploration times of both objects and trials were recorded blind twice and averaged using a stopwatch from digitised recordings, so no observer was in the room during the trials. The exploration data of all animals was included in the analysis.

3.2.2.2 Spontaneous alternation in the T-maze

The spontaneous alternation in the T-maze task was adapted from Deacon and Rawlings (2006) and carried out 6 days after completion of the NLR task. It relies on the nature of rats to preferentially choose the novel path in subsequent trials in the maze (see section 1.10, Chapter 1). Immediately before the start of the test, the rat was handled for 1 min. The rat was placed in the base of the T-maze (a black, opaque, Perspex box; dimensions: 111 total width x 49 arm length x 42 height x 13 arm width cm, Fig 3.2) facing the wall of the maze. It was left to run down the maze and turn into the left or right arm where a partition was put down, to trap the rat in the arm for 25 sec (Fig 3.2). The rat was handled in between trials for 30 sec, placed back in the base of the maze and allowed to run. It was again trapped in the arm of the maze it chose. This was repeated a total 7 times. If the rat chose the opposite arm from the previous run, it was scored as a correct trial (a total of 6 test trials). If the rat did not run for 1 min, the trial was restarted. The maze was cleaned with 20% ethanol between experiments to remove olfactory cues, but not between trials. The percentage of correct trials was recorded for each animal. The T-maze data of all animals was included in the analysis.

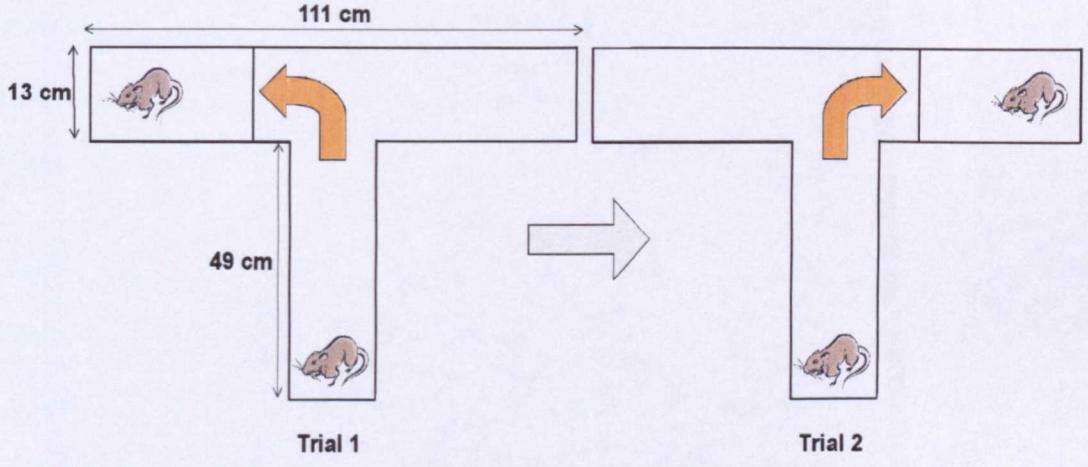

Figure 3.2 Spontaneous alternation in the T-maze task. Rats were placed in the base of the T-maze and allowed to run down one arm of the maze, where they were trapped by a partition for 25 sec. They were then removed for 30 sec, then placed back at the base of the T-maze and allowed to run down one arm of the maze again. They were then trapped for a further 25 seconds. This was repeated a total of 7 times (6 trials). A rat without impaired memory will normally alternate between arms each trial.

3.2.3 Brain tissue preparation

The day after behavioural testing was completed, rats were terminated by rapid concussion and confirmed by immediate cervical dislocation. Brains were prepared (as described in section 2.3.2.3, Chapter 2) and sections were mounted on to APES-coated slides and stored at -20°C until used for immunohistochemistry.

3.2.4 Ki67 and BrdU immunohistochemistry

Immunohistochemistry, microscopy and quantification of Ki67 and BrdU-positive cells were carried out as described previously (section 2.3.2.4, Chapter 2). All counting was performed blind.

3.2.5 Statistical analysis

Statistical analysis and graphs were created using GraphPad Prism 5 and significance was regarded as $p<0.05$. Student's paired *t*-tests were used to compare exploration times of animals in the familiarisation and choice trials. Preference indices (PI) were created by expressing time spent exploring the object in the novel location as a percentage of the sum of exploration time of novel and familiar locations in the choice trial, to create a single value to compare between groups. PI were compared to 50% chance using a one-sample *t*-test. One-way ANOVA was used to compare PI, total exploration time and average velocity of the animals, percentage correct trials in the T-maze and cells counts. Two-way repeated measures ANOVA was run to determine difference in animal weight between treatment groups. When ANOVA was significant Bonferonni post-hoc test was performed.

3.3 Results

3.3.1 MTX and fluoxetine reduce weight gain and fluid intake

Rats treated with MTX/LCV, fluoxetine or both in combination gained significantly less weight compared to vehicle treated controls and this change persisted to the end of the study ($F_{3,43}=4.16$, $p<0.001$, two-way repeated measures ANOVA, Fig. 3.3a and b). This was

attributed to disruption of intestinal absorption caused by MTX (Carneiro-Filho et al. 2004) and fluoxetine (Freeman et al. 2006). A significant effect of time and treatment × time interaction was also confirmed ($F_{56,2408}$=1793, $F_{168,2408}$=4.93 respectively, p<0.0001 for both).

Treatment and time significantly affected the amount of water drunk ($F_{3,384}$=5.82, p<0.05, $F_{48,384}$=29.34, p<0.001 respectively, two-way repeated measures ANOVA, Fig 3.4). However, by the last day of fluoxetine treatment no significant difference was seen (one-way ANOVA). The animals remained in good health throughout the study and never lost more than 10% of their maximum body weight.

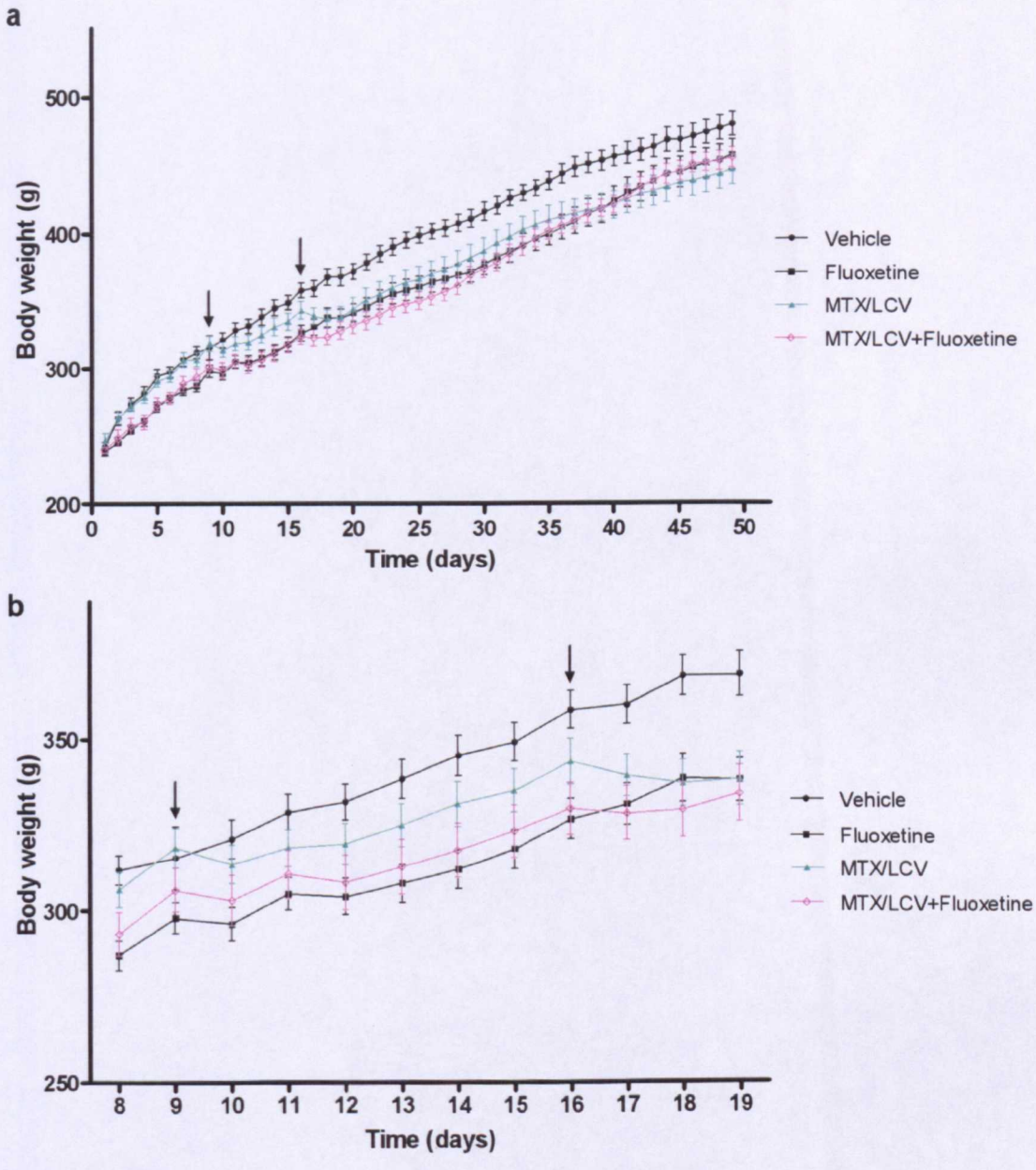

Figure 3.3 Body weights of rats (mean ± SEM, n=11-12) from the first day of fluoxetine administration (a) and during the MTX treatment period only (in detail, b). Arrows at day 9 and 16 indicate MTX/saline injections (BrdU was also administered on day 9). Fluoxetine was given in drinking water from day 1 to day 40. Rats treated with MTX/LCV, fluoxetine or both in combination gained significantly less weight compared to vehicle treated controls ($F_{3,43}$=4.16, p<0.001, two-way repeated measures ANOVA). The effect of time and treatment × time interaction was significant ($F_{56,2408}$=1793, $F_{168,2408}$=4.93 respectively, p<0.0001 for both).

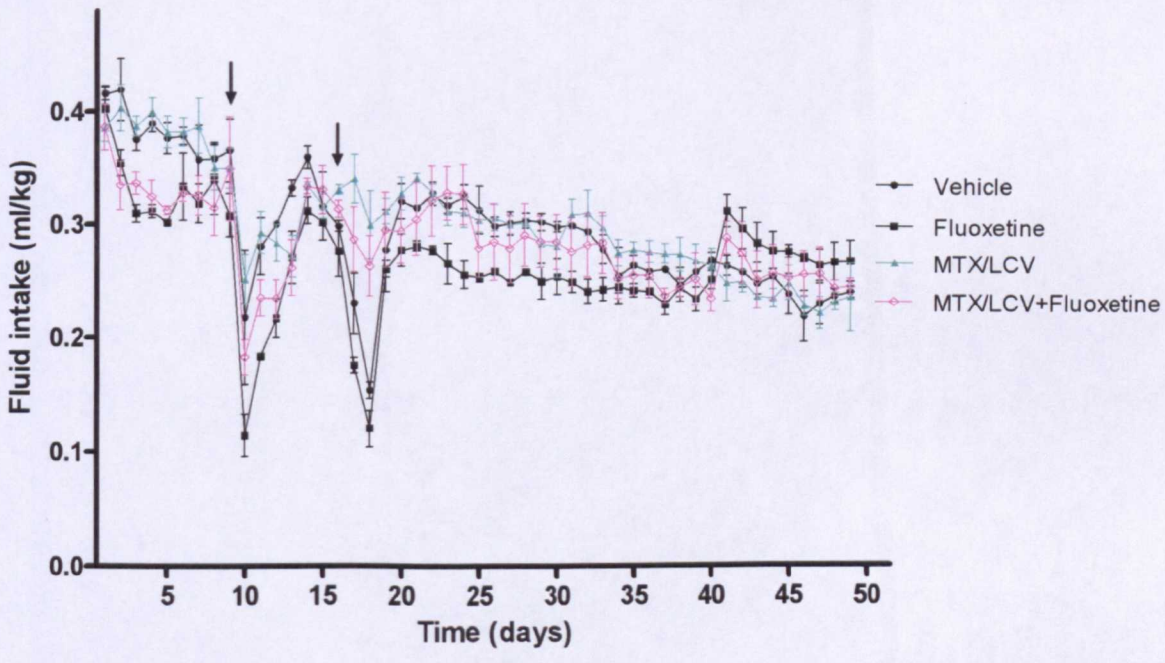

Figure 3.4 Fluid intake of rats (mean ± SEM, n=11-12) from the first day of fluoxetine administration represented as ml/kg. Treatment and time significantly affected the amount of water drunk ($F_{3,384}$=5.82, p<0.05, $F_{48,384}$=29.34, p<0.001 respectively, two-way repeated measures ANOVA).

3.3.2 Fluoxetine prevents the spatial working memory deficits in the NLR task caused by MTX

The NLR test was used to observe the effects of MTX/LCV and fluoxetine on spatial working memory. None of the treatment groups showed a significant difference in the exploration time of the two objects in the familiarisation trial (Fig. 3.5), indicating no preference for the location of either object. In the choice trial, Student's paired *t*-tests revealed that the vehicle and fluoxetine-treated groups explored the object in a novel location to a significantly greater extent ($p<0.01$, Fig. 3.6), suggesting unaffected spatial working memory (Dix and Aggleton 1999). In contrast, the MTX/LCV group showed no preference for either the novel or familiar object, showing no significant difference between exploration time for each object. This result indicates that MTX treatment caused a deficit in spatial working memory. The rats which were administered both MTX/LCV and fluoxetine showed a significant preference for the object in the novel location ($p<0.05$) in the choice trial, indicating that this group behaved similarly to control or fluoxetine-treated rats and did not show the spatial memory deficit exhibited by the MTX group. Further analysis was carried out on exploration times within the choice trial by conversion of raw data into a PI (Bruel-Jungerman et al. 2005) (Fig. 3.7). PI were created by expressing time spent exploring the object in the novel location as a percentage of the sum of exploration time of novel and familiar locations. The present results showed that the mean PI of the vehicle, fluoxetine and MTX/LCV + fluoxetine groups were significantly different from 50% chance (one-sample *t*-test), while the PI of the MTX group was not. Using the PI for comparison between groups using ANOVA however, showed no significant difference between treatment groups ($F_{3,43}=1.85$). Unexpectedly one-way ANOVA revealed that the total exploration time in the familiarisation trial (Fig. 3.8a) was significantly higher for the fluoxetine group compared to each other group ($F_{3,44}=12.11$, $p<0.05$), suggesting that fluoxetine may cause the animals to be more inquisitive. The extra time spent exploring and learning the object locations in the familiarisation trial may also be an explanation for their improved performance in the choice trial compared to the controls. However, no difference was seen between total exploration time in the choice trial

($F_{3,44}$=3.244, one-way ANOVA, Fig. 3.8b). No significant difference between mean locomotor velocity (recorded during the habituation period) ($F_{3,43}$=2.70, Fig. 3.9) was found between the groups indicating that the different treatments did not impair activity of the rats.

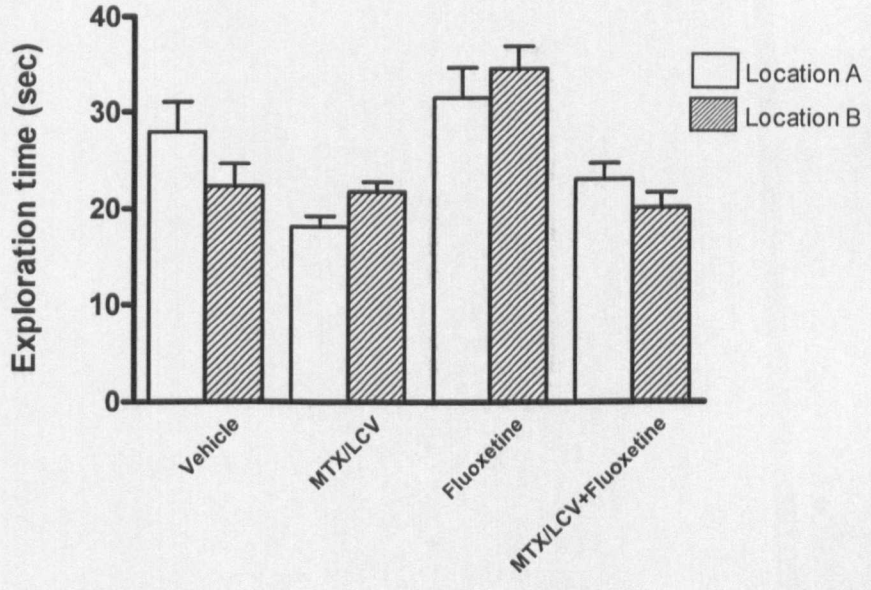

Figure 3.5 Exploration times (mean ± SEM, n=11-12) of the rats for each object in the familiarisation trial of the novel location recognition (NLR) task. None of the groups spent a significantly different time exploring either of the two objects (Student's paired *t*-test).

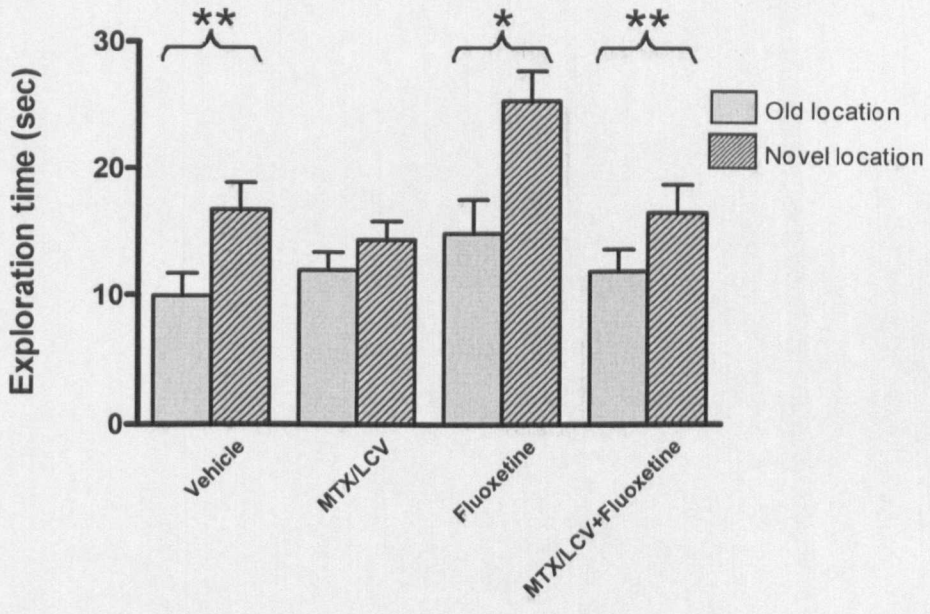

Figure 3.6 Exploration times (mean ± SEM, n=11-12) of the rats for each object in the choice trial of the novel location recognition (NLR) task. Vehicle-treated, fluoxetine-treated and MTX/LCV administered with fluoxetine-treated rats spent significantly longer exploring the object in the novel location compared with the familiar location (*$p<0.05$, **$p<0.01$, Student's paired *t*-test), whereas MTX/LCV failed to show a similar significant discrimination (Student's paired *t*-test).

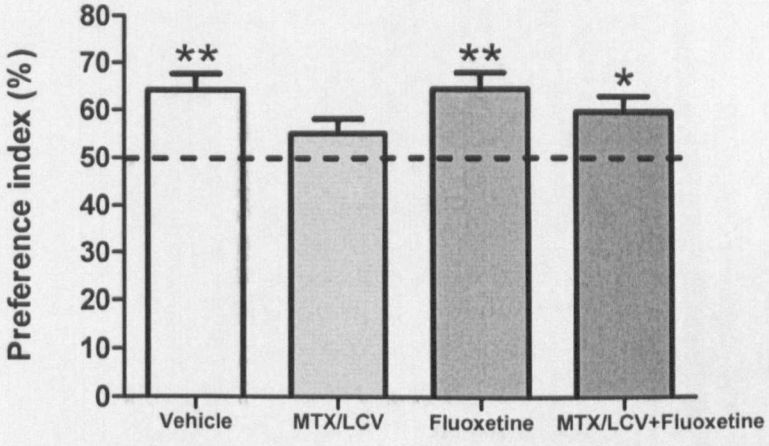

Figure 3.7 Preference indices (PI) of each treatment group (mean ± SEM, n=11-12) were created by expressing time spent exploring the object in the novel location as a percentage of the sum of exploration time of novel and familiar locations in the choice trial (Bruel-Jungerman et al. 2005). Compared to 50% chance, mean PI of the vehicle, fluoxetine and MTX/LCV+fluoxetine groups were significantly different (*p<0.05, **p<0.01, one-sample t-test), whilst the MTX/LCV group was not. PI were not significantly different between groups (one-way ANOVA).

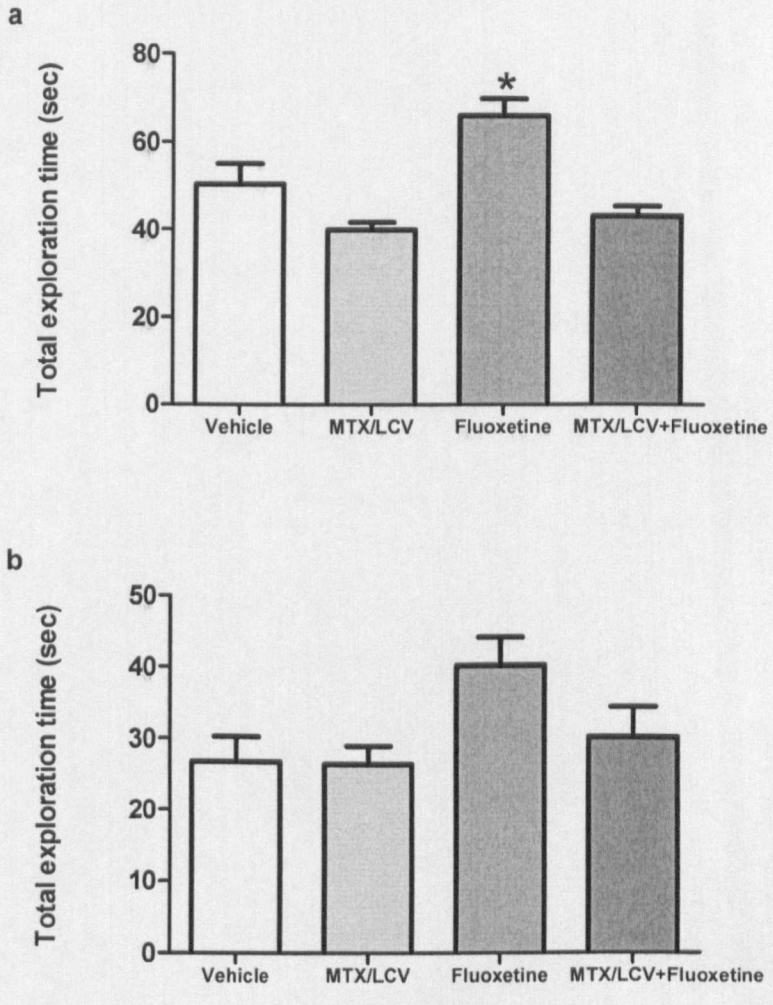

Figure 3.8 The total exploration time (mean ± SEM, n=11-12) was significantly higher for the fluoxetine group compared to the control group ($F_{3,44}$=12.11, p<0.05, one-way ANOVA, n=11-12) in the familiarisation trial (a). No significant difference ($F_{3,44}$=3.244, one-way ANOVA, n=11-12) was found between groups in the choice trial (b).

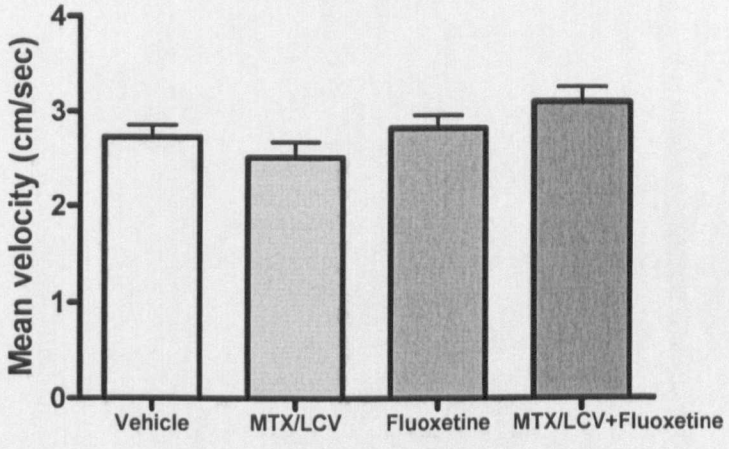

Figure 3.9 Velocity (mean ± SEM) of rats (n=11-12) was not significantly different between groups (one-way ANOVA).

3.3.3 MTX and fluoxetine do not alter spontaneous alternation in the T-maze

The spontaneous alternation in the T-maze task was also used to assess the effects of MTX/LCV and fluoxetine on cognition. No significant difference was found between percentage of correct turns for any of the groups (one-way ANOVA, Fig. 3.10), indicating that neither MTX nor fluoxetine influence cognition in this task.

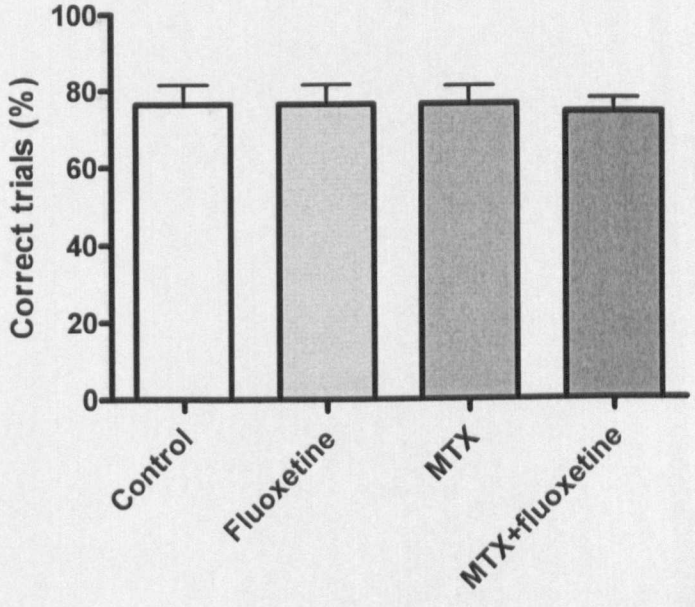

Figure 3.10 Percentage of correct turns (mean ± SEM, n=11-12) in spontaneous alternation in the T-maze. No significant difference was found between any of the groups (one-way ANOVA).

3.3.4 Fluoxetine prevents the reduction in cell proliferation in the SGZ caused by MTX

Ki67 was used to quantify the numbers of dividing cells in the SGZ of the dentate gyrus in all treatment groups at the end of the experiment (Fig. 3.11). A one-way ANOVA showed significant differences between mean Ki67-positive cell counts ($F_{3,24}$=11.58, p<0.001, Fig. 3.11). Further analysis with a Bonferroni post hoc test revealed that rats treated only with MTX/LCV had a significantly reduced number of Ki67-positive cells (p<0.05). In contrast, rats treated only with fluoxetine had more Ki67-positive cells compared with the controls, a significant increase (p<0.05). Rats treated with both MTX/LCV and fluoxetine showed no significant difference in from the controls, but had significantly more Ki67-positive cells than the group treated with MTX/LCV alone (p<0.05) indicating that the fluoxetine had prevented the reduction in cell proliferation caused by MTX/LCV.

3.3.5 Fluoxetine reverses reduction in new-born hippocampal cell survival caused by MTX

BrdU was administered to rats on the first day of either saline or MTX/LCV injections to label cells undergoing division at the start of treatment. BrdU-positive cells were quantified at the end of the experiment to determine the survival of these cells (Fig 3.12a-c). A one-way ANOVA showed significant differences between mean Ki67-positive cell counts ($F_{3,20}$=24.34, p<0.001, Fig. 3.12d). Bonferroni post hoc test revealed that MTX/LCV treatment significantly reduced the number of BrdU-positive cells (p<0.001) compared to vehicle-treated controls indicating that the chemotherapy, as well as reducing cell proliferation was reducing the survival of cells dividing at the start of treatment. Treatment with fluoxetine alone significantly increased BrdU-positive cell numbers (p<0.05). Animals co-treated with MTX/LCV and fluoxetine showed no significant difference in the BrdU-positive cell numbers from the control animals, but had significantly more BrdU-positive cells than the group treated with MTX/LCV alone (p<0.001). These results suggest that fluoxetine can protect newly dividing precursors in the dentate gyrus from MTX-induced loss.

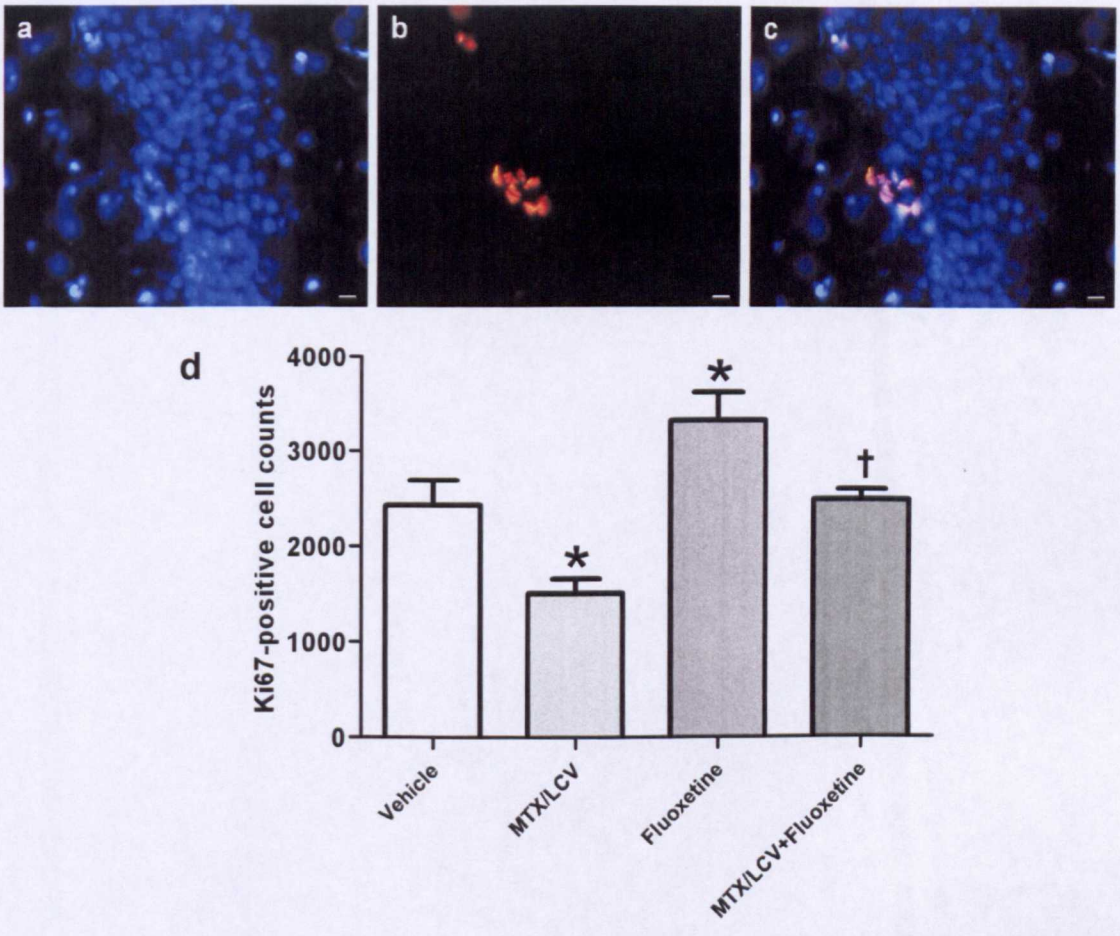

Figure 3.11 Representative photographs of the nuclei of cells in the SGZ of the dentate gyrus from the vehicle group (blue, a), Ki67-positive cells (red, b) and the photos merged (c). Bar scales indicate 20μm. Total numbers of Ki67-positive cells (mean ± SEM, n=11-12) in the dentate gyrus were estimated from cell counts (d). Rats receiving MTX/LCV had significantly fewer Ki67-positive cells ($p<0.05$, one-way ANOVA) in the subgranular zone (SGZ) and rats receiving fluoxetine had significantly more ($p<0.05$, one-way ANOVA) than the saline-treated control group. Rats treated with both MTX/LCV and fluoxetine did not significantly differ from the controls but had significantly more Ki67-positive cells than the MTX/LCV group ($^{\dagger}p<0.05$, one-way ANOVA).

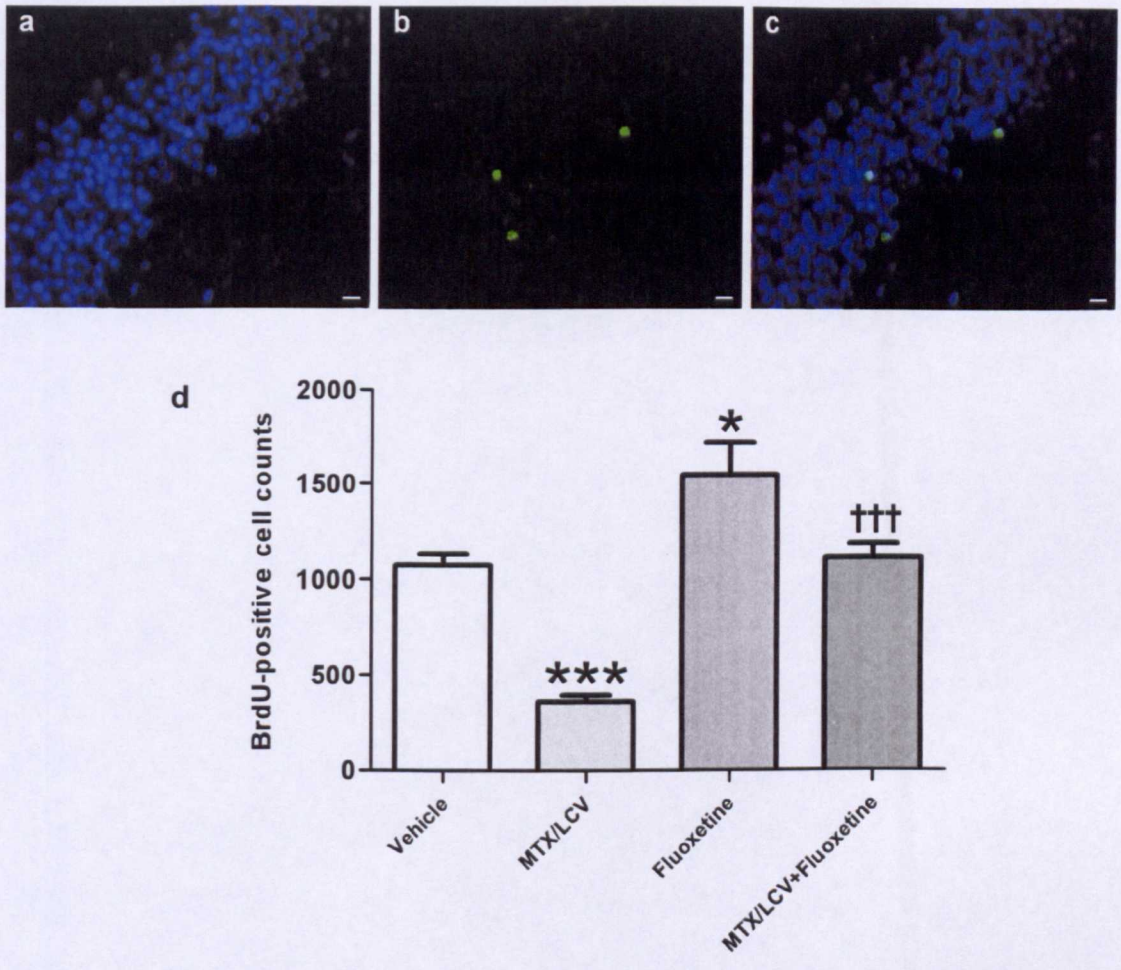

Figure 3.12 Representative photographs of the nuclei of cells in the dentate gyrus from the vehicle group (blue, a), BrdU-positive cells (green, b) and the photos merged (c). Bar scales indicate 20μm. Total numbers of BrdU-positive cells (mean ± SEM, n=11-12) in the dentate gyrus were estimated from cell counts (d). Rats receiving MTX/LCV had significantly fewer BrdU-positive cells (***$p<0.001$, one-way ANOVA) in the SGZ and rats receiving fluoxetine had significantly more (*$p<0.05$, one-way ANOVA) than the saline-treated control group. Treatment groups receiving both MTX/LCV and fluoxetine did not significantly differ from the controls but had significantly more BrdU-positive cells than the MTX/LCV group ($^{\dagger\dagger\dagger}p<0.001$, one-way ANOVA).

3.4 Discussion

In the present study, the effects of MTX chemotherapy on spatial working memory and the effects of the chemotherapy agent on cell proliferation and survival in the dentate gyrus of the adult hippocampus were determined. Both the NLR task and spontaneous alternation in the T-maze task were used to assess spatial working memory. These tasks were chosen as they are both based upon rats' spontaneous preference towards novelty as opposed to positive and negative reinforcements and animals are impaired in both tasks after hippocampal lesions (Deacon and Rawlins 2005; Lalonde 2002; Lee et al. 2005; Mumby et al. 2002). In addition, it was demonstrated that the SSRI antidepressant, fluoxetine, could counteract the behavioural and cellular effects of MTX in a rodent model.

The SGZ of the dentate gyrus is one of the brain regions where the formation of new neurons continues throughout life (Ehninger and Kempermann 2008). Newly formed dentate gyrus neurons have been shown to be preferentially used in spatial learning tasks (Kee et al. 2007) and reductions in dentate gyrus neurogenesis cause deficits in the ability of animals to perform these tasks (Imayoshi et al. 2008). It has been suggested from previous work in our laboratory and elsewhere that chemotherapy may reduce hippocampal neurogenesis and cause deficits in the cognitive domains dependent upon this process (Dietrich et al. 2006; ElBeltagy et al. 2010; Han et al. 2008b; Mustafa et al. 2008; Seigers et al. 2009). It was previously considered that systemic chemotherapy does not cross the blood brain barrier and so would have little effect on the brain, however, it has now been shown that many chemotherapy agents, including MTX when administered in high doses, can access the brain in significant concentrations (Lassman et al. 2006).

In the present study, animals receiving a 2 week course of MTX/LCV treatment were unable to recognise an object in a novel location from an object in an old location in the NLR behavioural test, 5 weeks after treatment had ended. However, animals treated with both MTX/LCV and fluoxetine were able to distinguish the objects. This indicates that MTX

causes a decline in spatial working memory compared to vehicle-treated controls or fluoxetine alone, which is counteracted by the co-administration of fluoxetine. A PI was derived from the exploration data for each animal in the choice trial to produce a single value for comparison between groups. Although the PI of each group other than MTX treated animals significantly differed from chance, consistent with the specific analysis of the group differences, no significant variation was found between groups by ANOVA, indicating that the memory deficit seen is subtle. Conversely, no difference was seen between any of the groups in their ability in the spontaneous alternation in the T-maze task. Both the T-maze and the NLR task are tasks of spatial working memory, and require an intact hippocampus to be performed (Deacon and Rawlins 2006; Dere et al. 2007). Therefore this difference in ability to perform the two tasks was unexpected, possibly because the cognitive impairment caused by MTX treatment was too subtle for the T-maze task to detect, and hence the task was modified to be more difficult in the study presented in the subsequent chapter.

The data from the NLR task supports previous reports that chemotherapy can cause spatial memory deficits in both patients (Ahles and Saykin 2002; Matsuda et al. 2005) and animals (ElBeltagy et al. 2010; Gandal et al. 2008; Mustafa et al. 2008; Yang et al. 2010). In particular high doses of MTX have been shown to produce spatial memory deficits in rats as measured in the Morris water maze, novel object recognition and an instrumental go/no-go task (Fardell et al. 2010; Seigers et al. 2007; Seigers et al. 2009). Furthermore, Fardell et al. (2010) demonstrated that these effects were both short and long-term, continuing for 8 months after treatment, suggesting that MTX has a lasting effect on behaviour.

The present work is consistent with these findings and in addition shows that lower doses are able to produce significant impairments in the NLR test 5 weeks after termination of the treatment. These results are comparable to clinical observations of breast cancer sufferers showing memory deficits after chemotherapy (Matsuda et al. 2005). Most patient studies which have detected cognitive effects of chemotherapy have looked at breast cancer survivors

who have received a combination of chemotherapy agents including MTX (Brezden et al. 2000; Wieneke and Dienst 1995). LCV, used here as a recovery drug for the toxic effects of MTX, has been shown to have subtle cognitive benefits in aged rats with a folate deficiency in the hippocampal-dependent T-maze task, but not in psychomotor and motor activity and Morris water maze spatial learning tasks. However LCV is thought not to have a cognitive effect on rats with sufficient folate (Lalonde et al. 1993) and Seigers et al. (2009) found it had no effect on neurogenesis when used alone. This allows us to attribute the deficits in memory and neurogenesis seen in the present study to the MTX chemotherapy.

As spatial learning is thought to depend on both the production and the survival of new-born neurons in the dentate gyrus (Dupret et al. 2007), the effect of MTX on both were investigated. In the present study, MTX/LCV reduced the number of dividing cells in the SGZ of the dentate gyrus compared to the control group. Survival of cells dividing at the time of the first MTX/LCV injection was also severely reduced at this time point. These results suggest that the action of MTX on neurogenesis and new neuron survival provides a mechanism for the long-term cognitive effects produced by this chemotherapy agent.

Fluoxetine is known to improve memory in patients with impaired cognition (Gallassi et al. 2006; Levkovitz et al. 2002; Mowla et al. 2007) and to increase the number of neurons produced during adult neurogenesis in the rodent hippocampus (Kodama et al. 2004; Marcussen et al. 2008). For these reasons co-administration of fluoxetine with MTX was tested to see if it could prevent the changes produced by MTX chemotherapy. The results show that both the behavioural and cellular deficits were prevented when fluoxetine was given for 40 days. Fluoxetine alone had no effect on PI, or average velocity, but increased total exploration time of animals. Work in our group has previously shown that fluoxetine can prevent the cognitive deficits produced by 5-FU (ElBeltagy et al. 2010), while the present study is the first to demonstrate a reversal of the cognitive deficit induced by MTX, suggesting that fluoxetine may be able to prevent the cognitive deficits induced by a range of

chemotherapy drugs. The mechanism by which fluoxetine is having these effects is unclear. Fluoxetine may have little effect on cognition or neurogenesis in healthy animals (Huang et al. 2008a; Monleon et al. 2007), but has been shown to be neuroprotective after injury (Chiou et al. 2006b; Jin et al. 2009) and to up-regulate hippocampal neurogenesis when it is reduced (Duman 2004; Duman et al. 2001a; Feldmann et al. 2007). Fluoxetine has also been shown to reduce apoptosis in adult rat dentate gyrus (Egeland et al. 2011) and inhibit apoptosis in stem cell cultures derived from the adult rat hippocampus (Chen et al. 2007; Chiou et al. 2006a). Interestingly, unlike the results shown in the present study, previous work from our group found that fluoxetine did not increase proliferation of cells within the dentate gyrus when administered without chemotherapy (ElBeltagy et al. 2010). This could be attributed to the increased length of time that the animals received the antidepressant (from 21 days in the previous study to 40 in the present) highlighting the importance of the period of time for which fluoxetine is administered.

Further work is needed in both animals and humans to examine the impact of other antidepressants and to determine the effects of different chemotherapy agents. In the following chapter another chemotherapy agent from the CMF combination, 5-FU, is investigated and its effects on spatial working memory and hippocampal changes are demonstrated. Furthermore, as work in the present chapter has shown that chronic chemotherapy treatment still affects cognition and cell proliferation in the hippocampus after treatment has terminated, the time course of when it would be beneficial to administer antidepressants to reverse these deficits was examined in the following study.

In addition, fluoxetine has been reported to increase levels of BDNF (Alme et al. 2007; Duman and Monteggia 2006), a neurotrophic factor which regulates neurogenesis (Alme et al. 2007; Duman et al. 2001a). Further work to quantify levels of BDNF within the hippocampus may help to understand the pathways by which fluoxetine acts in these situations and this was investigated in the subsequent chapter.

In conclusion, this study reveals MTX chemotherapy causes cognitive impairments and a reduction in both the proliferation and survival of neural precursors in the hippocampus. Furthermore, these impairments were reversed by the co-administration of the SSRI antidepressant, fluoxetine, suggesting that it has a neuro-protective effect. If the effects of the fluoxetine demonstrated in the present study can be translated to patients it could provide a relatively simple means to alleviate the cognitive effects experienced by some cancer patients. This finding not only has potential to improve the quality of life for cancer survivors, but provides further information on chemotherapy-induced cognitive deficits and methods to counteract it. This work has been recently published (Lyons et al. 2011b).

Chapter 4

Fluoxetine counteracts the cognitive and cellular effects of 5-fluorouracil in the rat hippocampus by a mechanism of prevention rather than recovery

4.1 Introduction

CMF (CP: cyclophosphamide, MTX: methotrexate and 5-FU: 5-fluorouracil) is a chemotherapy combination used clinically for the treatment of breast cancer and has been reported to cause cognitive impairment in patient studies (Kreukels et al. 2008; Schagen et al. 2002a) (see section 1.1.2, Chapter 1). In the previous chapters of this thesis the cognitive effects of CP and MTX were determined along with their effects on the generation of new cells in the adult hippocampus (see Chapters 2 and 3). Furthermore it was shown that the negative effects on memory and hippocampal cell proliferation and survival caused by MTX were counteracted with the SSRI antidepressant, fluoxetine, when it was co-administered with the MTX (Chapter 3). In the study presented in the present chapter, the effects of 5-FU, the final chemotherapy agent in the CMF combination, on cognition and hippocampal cell survival and proliferation were determined. This is a continuation of studying the effect of 5-FU and fluoxetine in our group (ElBeltagy et al. 2010). In the present study however, various time periods of fluoxetine administration were compared in order to determine the most beneficial period to administer fluoxetine to reverse the cognitive and cellular effects of 5-FU.

Although chemotherapy combinations including the antimetabolite, 5-FU, have been associated with impairment in cognition in patients (Kreukels et al. 2008; Schagen et al. 2002a), there is no clinical evidence that it has this effect when it is administered alone. Its ability to cross the blood-brain barrier by passive diffusion enables 5-FU to directly affect the brain when given systemically (Bourke et al. 1973). In the majority of previous studies, performed in our laboratory and elsewhere it was found that 5-FU impaired cognition and suppressed hippocampal cell proliferation in rodents (ElBeltagy et al. 2010; Mustafa et al. 2008; Walker et al. 2011; Wigmore et al. 2010).

Consequently, the cytotoxic effect of chemotherapy on the proliferation of neural stem and precursor cells required for adult hippocampal neurogenesis has been considered as a possible mechanism for chemotherapy-induced cognitive impairment (ElBeltagy et al. 2010; Mustafa et

al. 2008; Seigers et al. 2009). The SGZ of the dentate gyrus is one of a limited number of regions where neurogenesis persists throughout adulthood (Lledo et al. 2006b). Memory formation and spatial memory are both functions of the hippocampus (see section 1.5.2, Chapter 1) and the proliferation and integration of the neuronal precursors into existing circuits is thought to play a functional role in this process (Ehninger and Kempermann 2008; Zhao et al. 2008).

Fluoxetine, has been shown to increase cell proliferation in the hippocampus in both rodents (Kodama et al. 2004; Marcussen et al. 2008) and humans (Boldrini et al. 2009) and improve memory in patients with impaired cognition (Gallassi et al. 2006; Levkovitz et al. 2002; Mowla et al. 2007). Furthermore, recent rodent investigations in our group showed that fluoxetine can reverse the impaired spatial memory and reduced proliferation of hippocampal cells caused by treatment with 5-FU (ElBeltagy et al. 2010) and MTX (see Chapter 3) when administered before, during and after chemotherapy treatment.

In the present study, we utilised a rat model to confirm that co-administration of fluoxetine during 5-FU treatment counteracts the cognitive deficit and the reduction in proliferation of cells found in the SGZ and their survival caused by the chemotherapy. To investigate whether the mode of action of fluoxetine prevents cognitive decline or whether fluoxetine enables recovery from the cognitive decline and reduced neurogenesis caused by 5-FU, fluoxetine was given for 3 different time periods (Fig. 4.1); before and during 5-FU administration (preventative), after 5-FU treatment (recovery), and for both of these periods combined (throughout). These studies have demonstrated that the action of the antidepressant fluoxetine, in chemotherapy treatment, prevents the 5-FU induced cognitive deficits and cellular changes but has no effect in recovery.

4.2 Materials and methods

4.2.1 Animals and treatment

Male Lister-hooded rats (175-200g; Charles River, UK, total n=72) were randomly allocated to vehicle, 5-FU, fluoxetine, 5-FU + fluoxetine (throughout), 5-FU + fluoxetine (preventative) or 5-FU + fluoxetine (recovery) groups (each, n=12). Rats were housed as described in section 2.2.1.1, Chapter 1 and allowed to habituate for 1 week prior to drug administration.

Rats were administered 5-FU (25 mg/kg, 5 i.p. doses, each 3 days apart, at a volume of 2.5ml/kg, dissolved in 0.9% sterile saline; Medac, Germany) or 0.9% sterile saline at an equivocal volume. 3 BrdU injections were administered to all groups, 24 h apart starting 2 days prior to their first 5-FU/saline injection (100mg/kg, i.p., at a volume of 4ml/kg, pH 7.0; Sigma Aldrich, UK).

Mean water consumption and mean animal weight were determined every 3 days to estimate a dose of 10mg/kg/day of fluoxetine (Pinewood Healthcare, Ireland, oral solution) diluted in drinking water, for the fluoxetine groups of rats. Drinking water treated with fluoxetine was administered to the 5-FU + fluoxetine (preventative) group starting 5 days before the first BrdU injection and to the 5-FU + fluoxetine (recovery) group starting the day of the last 5-FU/saline injection, both for 20 days. The fluoxetine and the 5-FU + fluoxetine (throughout) groups received it for both these periods for 40 days (Fig. 4.1).

Throughout the experiment, rats were maintained in a 12 h light/dark cycle (7.00/19.00 h) and food and water (some fluoxetine-treated) was provided *ad libitum* and weighed every 3 days or daily during 5-FU administration. All procedures were in accordance to UK Home Office Guidance regulations and with local ethical committee approval.

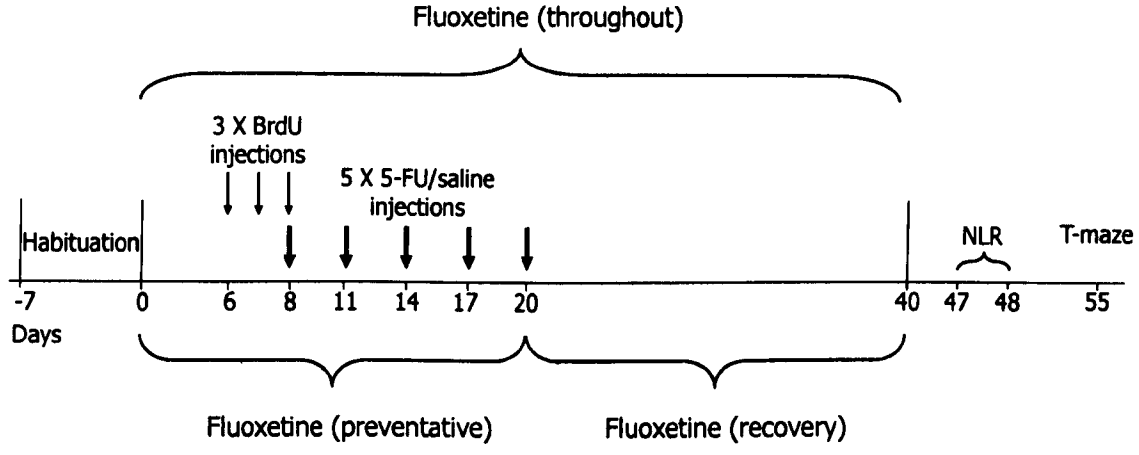

Figure 4.1 Time line showing protocol of drug administration and behavioural testing. Arrows represent single i.p. injections of 100mg/kg BrdU (fine) and 5-FU (25mg/kg)/saline (thick). Brackets represent the period of time for which fluoxetine was administered in the drinking water. The day after novel location recognition (NLR) behavioural testing, animals were killed and their brains were removed.

4.2.2 Behavioural testing

4.2.2.1 NLR task

The NLR test was carried out (as described in section 2.3.2.4.1, Chapter 2) 7 days after fluoxetine treatment ended. Animals were video recorded during the behavioural testing and EthoVision 4.1 was used to measure the mean velocity of the rats during the 30 min habituation, 24 h prior to testing. Exploration times of both objects and trials were recorded blind twice and averaged using a stopwatch from digitised recordings, so no observer was in the room during the trials. The exploration data of all animals was included in the analysis.

4.2.2.2 Spontaneous alternation in the T-maze

Spontaneous alternation in the T-maze was carried out with modifications, 6 days after completion of the NLR task. The methods were as described in section 3.2.2.2, Chapter 3, but the amount of time the rat was trapped in each arm was reduced to 5 sec (previously 25 sec) and the delay between trials was increased to 1 min (previously 30 sec), to make the task more difficult. The percentage of correct trials was recorded for each animal. The T-maze data of all animals was included in the analysis.

4.2.3 Brain tissue preparation

The day after behavioural testing was completed rats were put down by concussion and immediate cervical dislocation. Brains were prepared (as described in section 2.3.2.3, Chapter 2) and sections were mounted on to APES-coated slides and stored at -20°C until used for immunohistochemistry.

4.2.4 Ki67 and BrdU immunohistochemistry

Ki67 and BrdU immunohistochemistry, microscopy and quantification of Ki67 and BrdU-positive cells were carried out as described previously (section 2.3.2.4, Chapter 2). All counting was performed blind.

4.2.5 Western blot analysis of DCX and BDNF expression in hippocampus and frontal cortex

4.2.5.1 Sample preparation, Lowry assay and protein separation

Preparation of hippocampus and frontal cortex tissue samples, determination of protein concentration utilising the Lowry assay and protein separation were as described in section 2.3.2.4.1, Chapter 2, other than 6µl of the samples (n=10) rather than 15µl was loaded and they were run on purchased 4-20% SDS-polyacrylamide gels (Fisher Scientific, UK). 1-2µl recombinant BDNF (rBDNF) was also loaded in 2 wells. After protein transfer was confirmed with Ponceau solution, the membrane was cut to separate DCX and BDNF bands.

4.2.5.2 Immunodetection, quantification of protein and data analysis

Immunodection of DCX, BDNF and β-actin utilised the SNAP i.d. Protein Detection System (Millipore, UK). The nitrocellulose membrane was placed in a blot holder and blocked with 15ml of fish skin gelatine (1.5% in TBST), driven through the membrane by use of a vacuum. All antibodies were diluted in TBST with 1.5% fish skin gelatine. 1ml of polyclonal rabbit anti-DCX (1:1000; Cell Signalling) and mouse anti-β-actin (1:1000; Cell Signalling) primary antibodies or 1ml of rabbit anti-BDNF (1:100) covered the membrane for 10 min and was then vacuumed through. 3 washes of 15ml TBST were vacuumed through, then 1ml of IRDye 800CW goat anti-rabbit and IRDye 700CW goat anti-mouse or just 800CW goat anti-rabbit (both 1:20000; LI-COR Biosciences) covered the membrane for 10 min and was vacuumed through. The membrane was scanned and protein concentration was quantified as described in section 2.3.2.4.2, Chapter 2.

4.2.6 Statistical analysis

Student's paired *t*-tests were used to compare exploration times of animals in the familiarisation and choice trials. Preference indices (PI) were created by expressing time spent exploring the object in the novel location as a percentage of the sum of exploration time of novel and familiar locations in the choice trial, to create a single value to compare between

groups. PI were compared to 50% chance using a one-sample *t*-test. One-way ANOVA was used to compare PI, total exploration time and average velocity of the animals, the percentage of correct trials in the T-maze, cell counts and DCX and BDNF expression from Western blotting. Two-way repeated measures ANOVA was run to determine difference in animal weight between treatment groups. When ANOVA was significant Bonferonni post-hoc test was performed. Statistical analysis and graphs were created using GraphPad Prism 5 and significance was regarded as $p<0.05$.

4.3 Results

4.3.1 5-FU and fluoxetine reduce weight gain and fluid intake

Both treatment and time had a significant effect on body weight ($F_{5,1848}=11.50$, $F_{28,1848}=2040$, $p<0.001$ respectively, two-way repeated measures ANOVA, Fig. 4.2a). Both 5-FU and fluoxetine significantly reduced weight gain which is attributed to disruption of intestinal absorption by 5-FU (Huang et al. 2002) and fluoxetine (Freeman et al. 2006). Treatment and time significantly affected the amount of water drunk ($F_{5,169}=17.93$, $F_{14,168}=52.09$, $p<0.001$ respectively, two-way repeated measures ANOVA, Fig. 4.2b). However, by the end of the experiment no significant difference was seen (one-way ANOVA). The animals remained in good health throughout the study and never lost more than 10% of their maximum body weight.

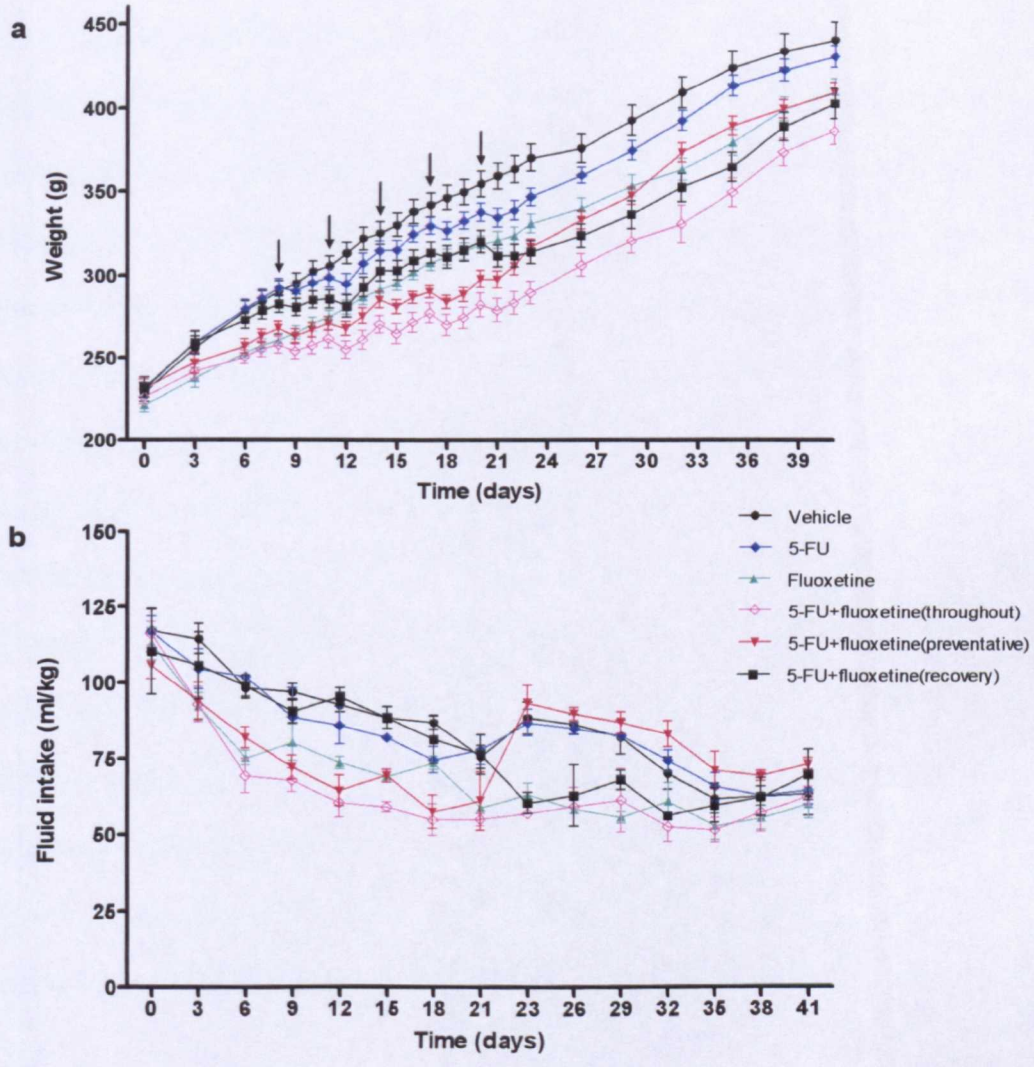

Figure 4.2 Body weights of rats (a) and their fluid intake (b) during fluoxetine treatment period (mean ± SEM, n=12). Arrows indicate 5-FU (25mg/kg)/saline injections. Fluoxetine was given in drinking water (10mg/kg/day) from day 1 to day 20 for the 5-FU + fluoxetine (preventative) group, from day 21 to day 40 for the 5-FU + fluoxetine (recovery) group and from day 0 to day 40 for the 5-FU + fluoxetine (throughout) and the fluoxetine alone groups. Both treatment and time had a significant effect on body weight ($F_{5,1848}$=11.50, $F_{28,1848}$=2040, $p<0.001$ respectively, two-way repeated measures ANOVA). 5-FU and fluoxetine significantly reduced weight gain. Treatment and time significantly affected the amount of fluid intake ($F_{5,169}$=17.93, $F_{14,168}$=52.09, $p<0.001$ respectively, two-way repeated measures ANOVA). At the end of the experiment there no significant difference between groups in fluid intake (one-way ANOVA).

4.3.2 Fluoxetine reverses NLR spatial memory deficits caused by 5-FU when administered in prevention but not recovery

The NLR test makes use of the preference of rats for novelty to measure the ability of rats to discriminate between objects in novel and familiar locations. In the familiarisation trial, the rats explored 2 identical objects and no group showed a significant difference in exploration time for either object (Student's paired *t*-test, Fig. 4.3) indicating no preference for either object's location. During the choice trial (Fig. 4.4) however, the groups of vehicle treated rats, rats receiving only fluoxetine and rats receiving both 5-FU and fluoxetine throughout or for prevention were able to perform the memory task, namely spending significantly longer exploring the object in the novel location compared to the object in the familiar location (all $*p<0.05$, $**p<0.01$, Student's paired *t*-test). In contrast, rats treated with 5-FU only or 5-FU with fluoxetine after chemotherapy (recovery) showed no object preference, namely no significant difference in exploration time of either object (Student's paired *t*-test), indicating impairment in memory. Conversion of raw exploration times showed the means of the PI of these 2 groups did not differ from a level of 50% chance, whereas the mean PI of the other groups was significantly different ($*p<0.05$, $**p<0.01$, $***p<0.001$, one-sample *t*-test, Fig. 4.5).

The total exploration time in the familiarisation trial (Fig. 4.6a) was significantly higher for the 5-FU treated group compared to the control group ($F_{5,66}=3.475$, $p<0.05$), indicating that the 5-FU treated animals may have been more inquisitive. This could give an advantage in performance of the task as the animals spent longer learning the locations of the objects, although this was not the case in the present experiment as 5-FU treated animals performed worse in the choice trial than the control group. No difference was seen between the total exploration times of any of the groups in the choice trial ($F_{5,66}=2.777$, one-way ANOVA, Fig. 3.8b). No significant difference was found between groups for mean velocity (Fig. 4.7) indicating none of the groups were impaired in their locomotor activity.

4.3.3 5-FU and fluoxetine do not affect spontaneous alternation in the T-maze

The spontaneous alternation in the T-maze task was also used to assess the effects of 5-FU and fluoxetine on cognition. No significant difference was found between any of the groups (one-way ANOVA, Fig. 4.8), indicating that neither 5-FU nor fluoxetine effected cognition for this task.

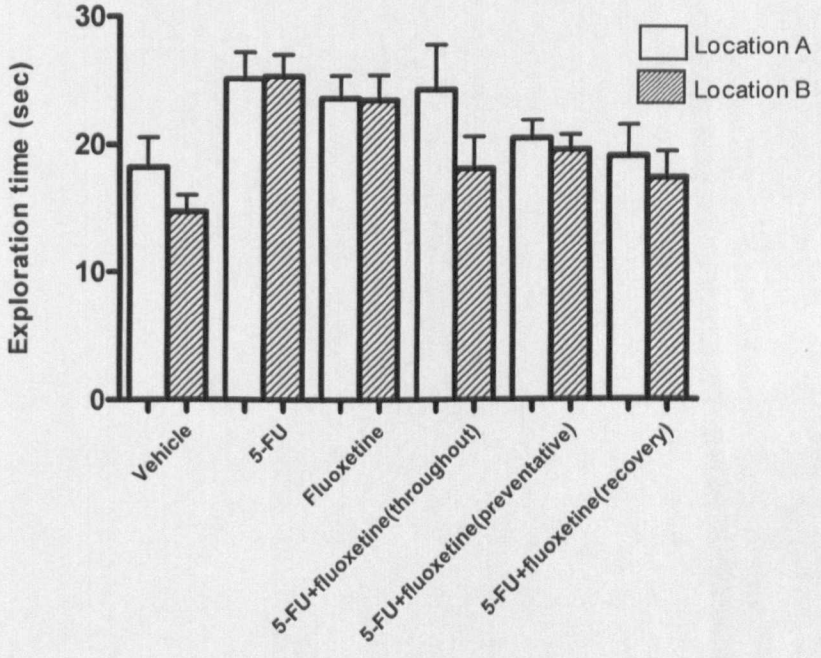

Figure 4.3 Exploration times (mean ± SEM, n=12) of the rats for each object in the familiarisation trial in the familiarisation trial of the novel location recognition (NLR) task. There was no significant difference in exploration time of either object for any group in the familiarisation trial (Student's paired *t*-test).

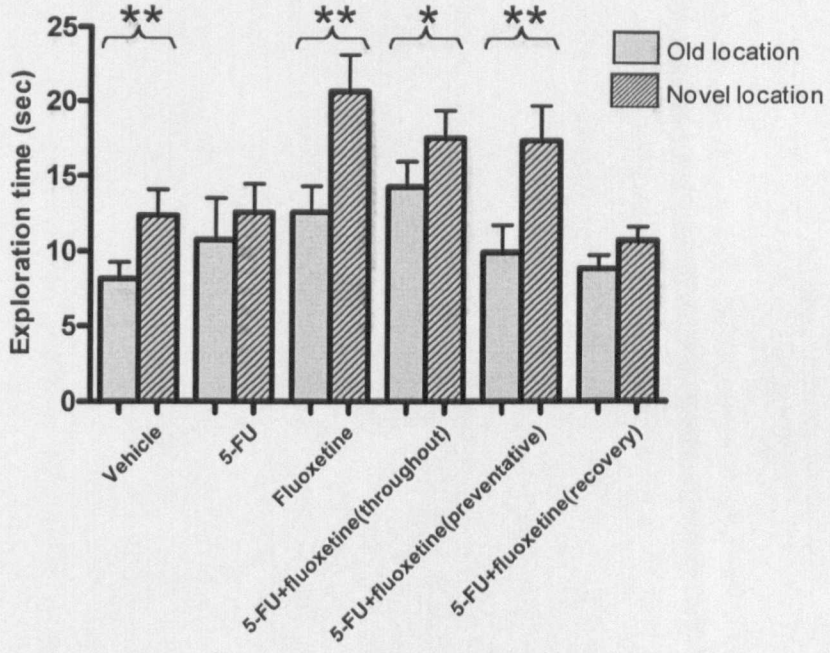

Figure 4.4 Exploration time times (mean ± SEM, n=12) of the rats for each object in the choice trial of the novel location recognition (NLR) task. There was no significant difference in exploration time of either object for any group in the familiarisation trial (Student's paired *t*-test). In the choice trial, all groups spent significantly longer exploring the object in the novel location (*p<0.05, **p<0.01, Student's paired *t*-test), except the groups receiving 5-FU alone or 5-FU with fluoxetine in recovery (Student's paired *t*-test).

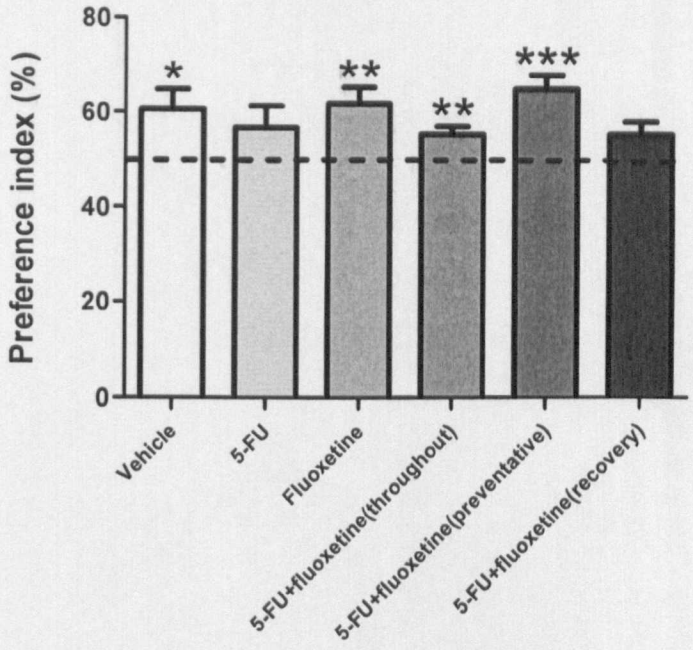

Figure 4.5 Preference indices (PI) of each group (mean ± SEM, n=12) were created by expressing time spent exploring the object in the novel location as a percentage of the sum of exploration time of novel and familiar locations in the choice trial (Bruel-Jungerman et al. 2005). Compared to 50% chance, PI of all groups was significantly different (*p<0.05, **p<0.01, ***p<0.001, one-sample t-test), other than the groups receiving 5-FU alone or 5-FU with fluoxetine in recovery (one-sample t-test). PI were not significantly different between groups (one-way ANOVA).

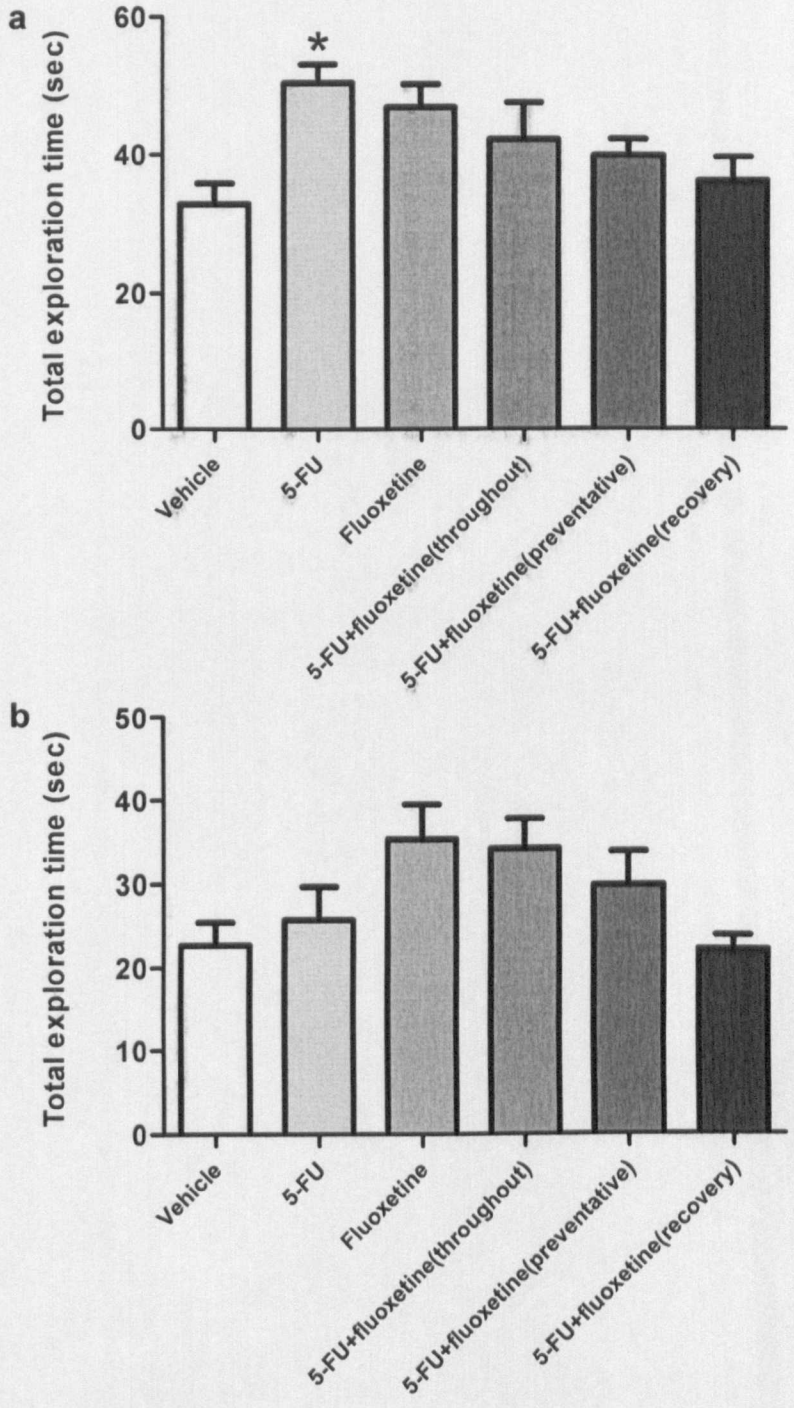

Figure 4.6 The total exploration time (mean ± SEM, n=12) was significantly higher for the group treated with 5-FU compared to the control group ($F_{5,66}$=3.475, p<0.05, one-way ANOVA, n=12) in the familiarisation trial (a). No significant difference ($F_{5,66}$=2.777, one-way ANOVA, n=12) was found between groups in the choice trial (b).

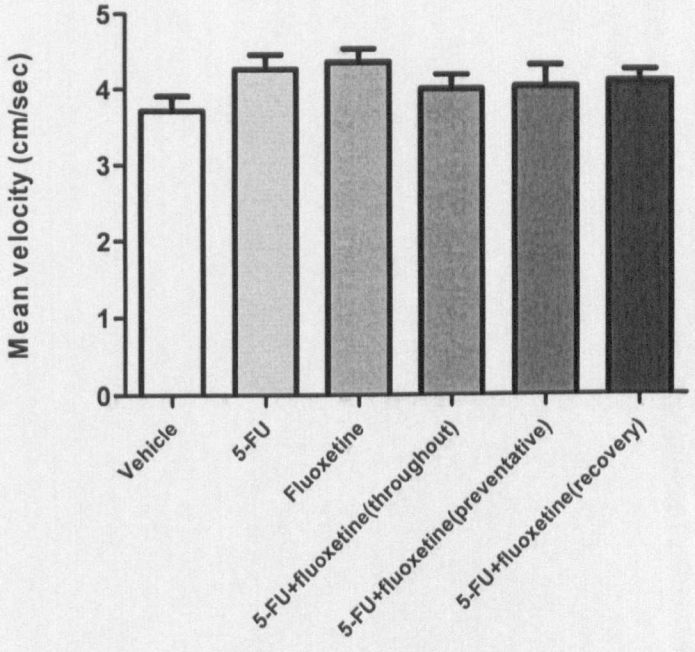

Figure 4.7 Mean velocity (mean ± SEM, n=12) of rats recorded during the habituation period using EthoVision 4.1. No significant difference (one-way ANOVA) was found between the groups.

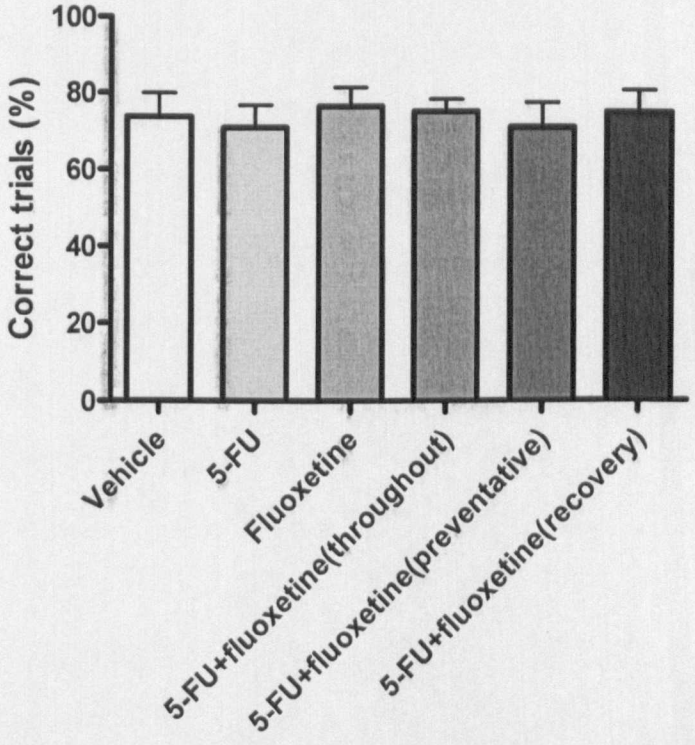

Figure 4.8 Percentage of correct turns (mean ± SEM, n=12) in spontaneous alternation in the T-maze. No significant difference (one-way ANOVA) was found between each group.

4.3.4 Fluoxetine reverses the reduction of cell proliferation in the dentate gyrus caused by 5-FU when administered in prevention but not recovery

Cell proliferation in the SGZ at the end of the experiment was quantified using Ki67 (Fig. 4.9a-d). Rats which received only 5-FU had a significantly lower number of Ki67-positive cells ($p<0.01$, one-way ANOVA) and rats which received only fluoxetine had a significantly larger number compared to the vehicle-treated controls ($p<0.05$, one-way ANOVA). The number of Ki67-positive cells in rats treated with both 5-FU and fluoxetine did not significantly differ from the controls when fluoxetine was administered throughout and in prevention (one-way ANOVA). Although rats treated with 5-FU and subsequently given fluoxetine (recovery) did not have significantly fewer Ki67-positive cells compared to the controls (one-way ANOVA), there does appear to be a reduction of Ki67-positive cells. Furthermore, the groups which received both 5-FU and fluoxetine in the prevention phase or throughout had a significantly larger number of Ki67-positive cells than the group receiving 5-FU alone ($^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$, one-way ANOVA) whereas the group receiving 5-FU and fluoxetine in prevention did not. These results indicate that 5-FU has a long-term effect (at least 30 days) in reducing cell proliferation in the SGZ. This effect can be counteracted by fluoxetine only if it is administered before and during the 5-FU treatment period, but the deleterious effects of 5-FU chemotherapy are not counteracted if fluoxetine is only administered after chemotherapy treatment.

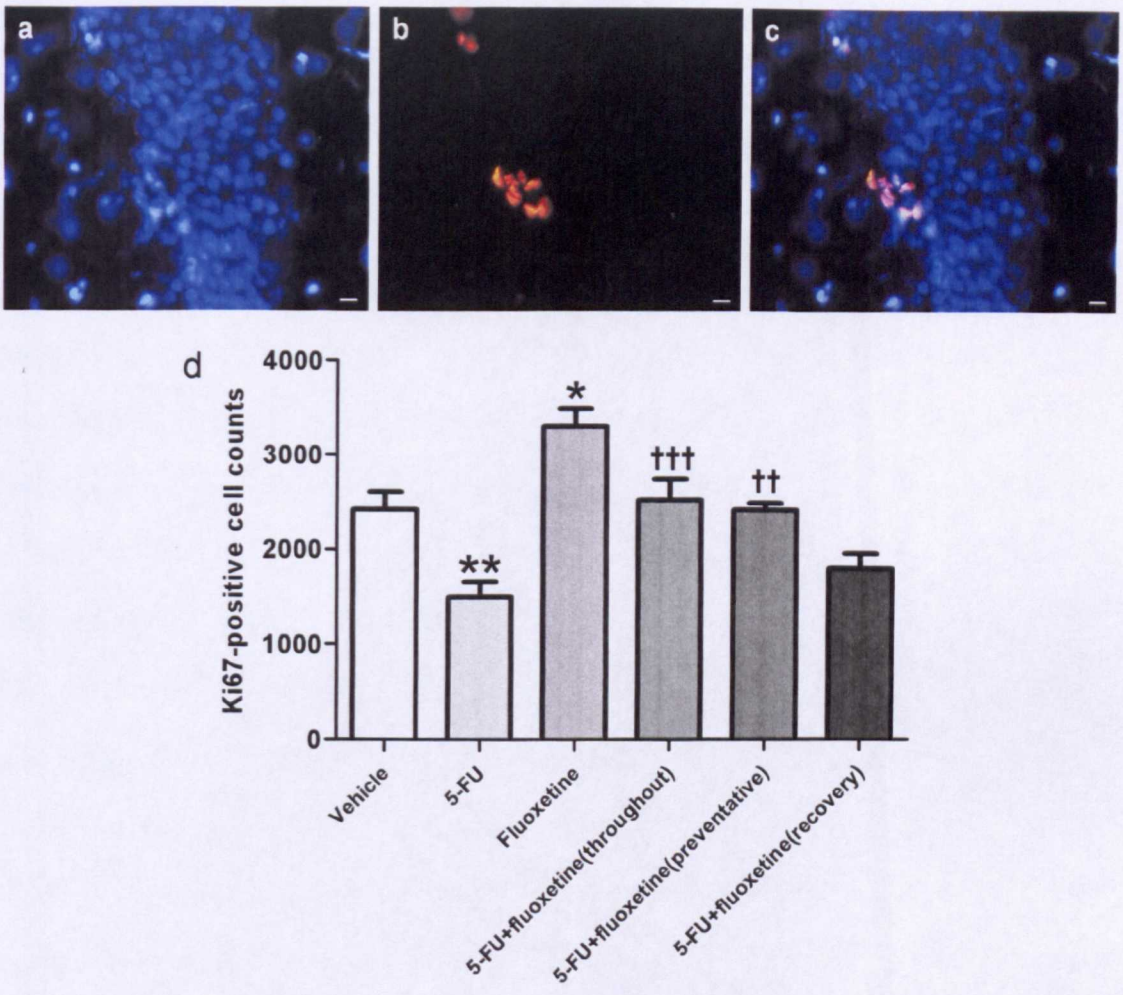

Figure 4.9 Representative photographs of the nuclei of cells in the SGZ of the dentate gyrus from the vehicle group (blue, a), Ki67-positive cells (red, b) and the photos merged (c). Bar scales indicate 20μm. Total numbers of Ki67-positive cells (mean ± SEM, n=12) in the dentate gyrus were estimated from cell counts (d). Rats receiving 5-FU had significantly fewer Ki67-positive cells (**$p<0.01$, one-way ANOVA) in the subgranular zone (SGZ) and rats receiving fluoxetine had significantly more (*$p<0.05$, one-way ANOVA) than the saline-treated control group. Groups receiving both 5-FU and fluoxetine in the prevention phase or throughout had a significantly larger number of Ki67-positive cells than the group receiving 5-FU alone ($^{††}p<0.01$, $^{†††}p<0.001$, one-way ANOVA) whereas the group receiving 5-FU and fluoxetine in prevention did not.

4.3.5 Fluoxetine reverses the reduction of cell survival in the dentate gyrus caused by 5-FU when administered in prevention but not recovery.

A course of 3 BrdU injections was given to the rats preceding and on the first day of 5-FU or saline injections, to be recruited into cells at the beginning of the experiment. The BrdU-positive cells were counted in the dentate gyrus after the experiment was completed to quantify the survival of these cells (Fig. 4.10a-d). There were significantly more BrdU-positive cells in rats treated with only fluoxetine ($p<0.001$, one-way ANOVA) compared with the control group and significantly fewer in rats treated with 5-FU ($p<0.01$, one-way ANOVA). The rats treated with both 5-FU and fluoxetine did not have a significantly different number of BrdU-positive cells than the controls when fluoxetine was administered throughout and in prevention (one-way ANOVA), but when fluoxetine was only administered in recovery, the rats had a significantly smaller number ($p<0.001$, one-way ANOVA). Furthermore, the groups which received both 5-FU and fluoxetine in prevention phase or throughout the study had a significantly larger number of BrdU-positive cells than the group receiving 5-FU alone ($p<0.001$, one-way ANOVA) whereas the group receiving 5-FU and fluoxetine in prevention did not. These results suggest that when administered before and during, but not after 5-FU treatment, fluoxetine can protect neural precursors from cell loss induced by 5-FU.

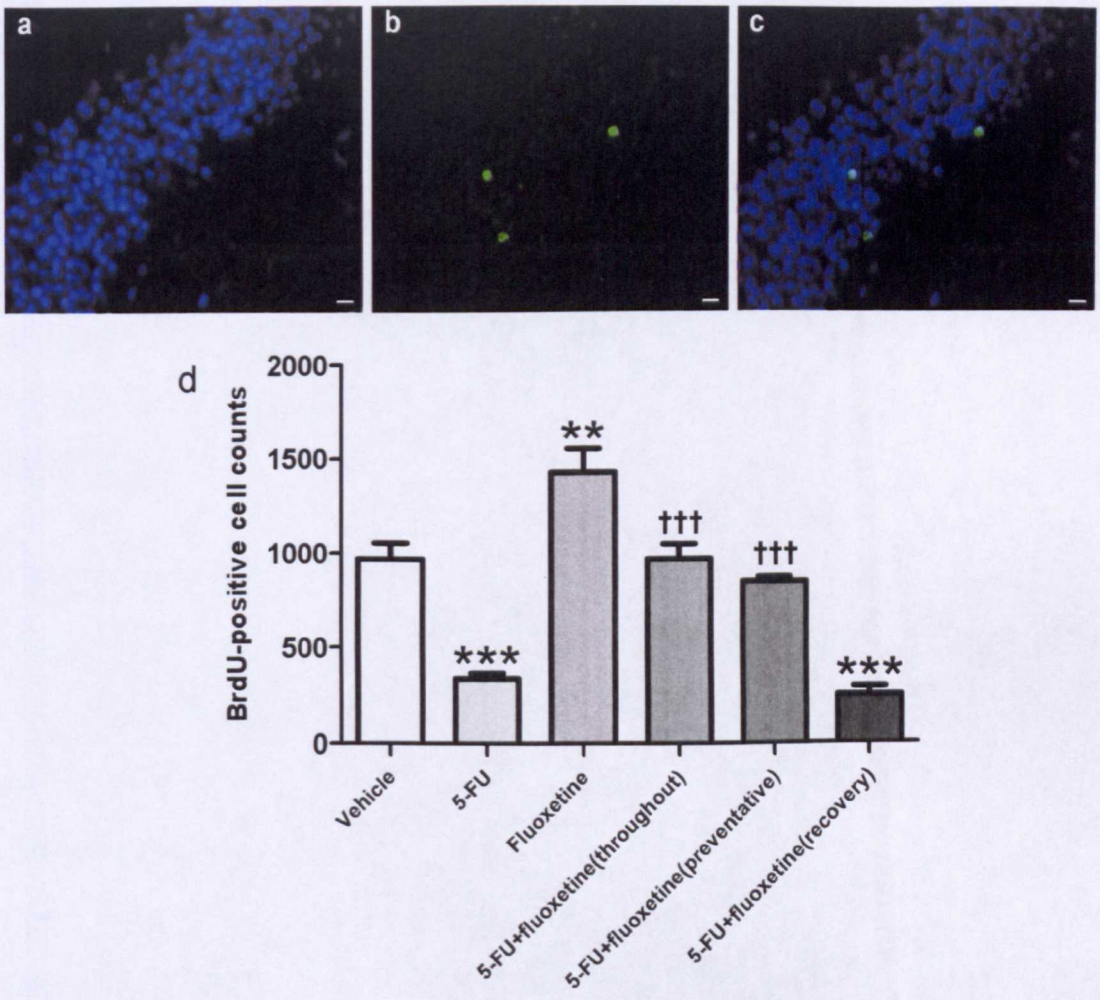

Figure 4.10 Representative photographs of the nuclei of cells in the dentate gyrus from the vehicle group (blue, a), BrdU-positive cells (green, b) and the photos merged (c). Bar scales indicate 20μm. Total numbers of BrdU-positive cells (mean ± SEM, n=12) in the dentate gyrus were estimated from cell counts (d). Rats receiving 5-FU had significantly fewer BrdU-positive cells ($p<0.001$, one-way ANOVA) in the SGZ and rats receiving fluoxetine had significantly more ($p<0.01$, one-way ANOVA) than the saline-treated control group. Treatment groups receiving both 5-FU and fluoxetine throughout and in prevention did not significantly differ from the controls but had significantly more Ki67-positive cells than the group receiving 5-FU alone ($^{\dagger\dagger\dagger}p<0.001$, one-way ANOVA). The group receiving 5-FU with fluoxetine in recovery had significantly fewer BrdU-positive cells than the controls ($^{***}p<0.001$, one-way ANOVA) but did not differ from the group receiving 5-FU alone.

4.3.5.1 Expression of DXC

Western blotting analysis was performed to determine the effects of 5-FU and fluoxetine on levels of DCX in the hippocampus and frontal cortex (Fig. 4.11). Whole brain was used as a positive control for DCX, but it is not shown on this immunoblot. Protein levels of DCX were normalised in comparison with β-actin loading control. A one-way ANOVA showed no significant difference in DCX expression between any of the treatment groups in either the hippocampus (one-way ANOVA , Fig. 4.12a) or the frontal cortex (one-way ANOVA, Fig. 4.12b).

4.3.5.2 Expression of BDNF

Expression of BDNF levels in the hippocampus and frontal cortex were also quantified by Western blotting analysis (Fig. 4.11). Whole brain was used as a positive control for BDNF, but is not shown on this immunoblot. Protein levels of BDNF were normalised in comparison with β-actin loading control. No significant difference in BDNF expression was found between any of the treatment groups treated with 5-FU, fluoxetine, or both in combination in either the hippocampus (one-way ANOVA, Fig. 4.13a) or the frontal cortex (one-way ANOVA, Fig. 4.13b).

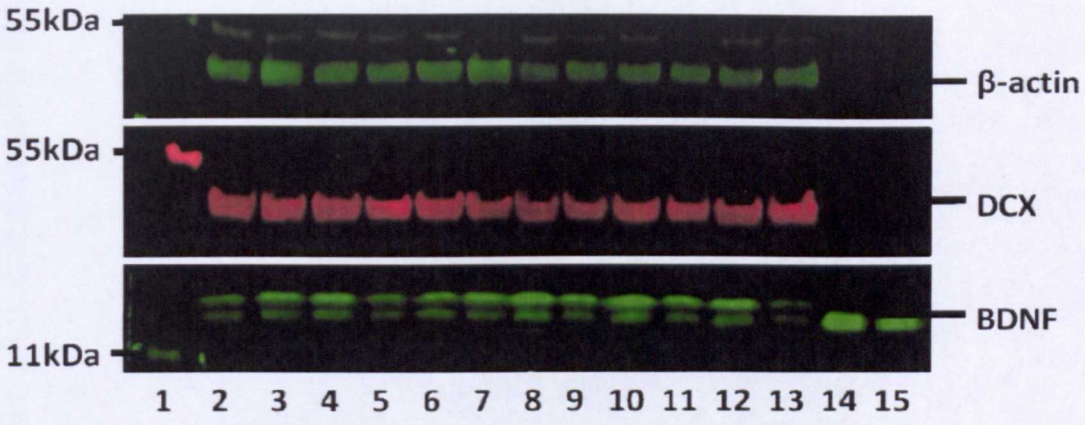

Figure 4.11 Example photomicrograph of immunblot bands from Western blotting showing expression of β-actin (green, at 43 kDa), DCX (red, at 47 kDa) and BDNF (green, at 14 kDa). Samples from hippocampus and frontal cortex were loaded at 6µl into the lanes as follows:

Lane 1: Molecular weight marker (1µl, Fermentas, USA)

Lane 2: Vehicle group, hippocampus

Lane 3: Vehicle group, frontal cortex

Lane 4: 5-FU treated group, hippocampus

Lane 5: 5-FU treated group, frontal cortex

Lane 6: Fluoxetine treated group, hippocampus

Lane 7: Fluoxetine treated group, frontal cortex

Lane 8: 5-FU + fluoxetine (throughout) treated group, hippocampus

Lane 9: 5-FU + fluoxetine (throughout) treated group, frontal cortex

Lane 10: 5-FU + fluoxetine (preventative) treated group, hippocampus

Lane 11: 5-FU + fluoxetine (preventative) treated group, frontal cortex

Lane 12: 5-FU + fluoxetine (recovery) treated group, hippocampus

Lane 13: 5-FU + fluoxetine (recovery) treated group, frontal cortex

Lane 14: 2µl recombinant BDNF (rBDNF)

Lane 15: 1µl rBDNF

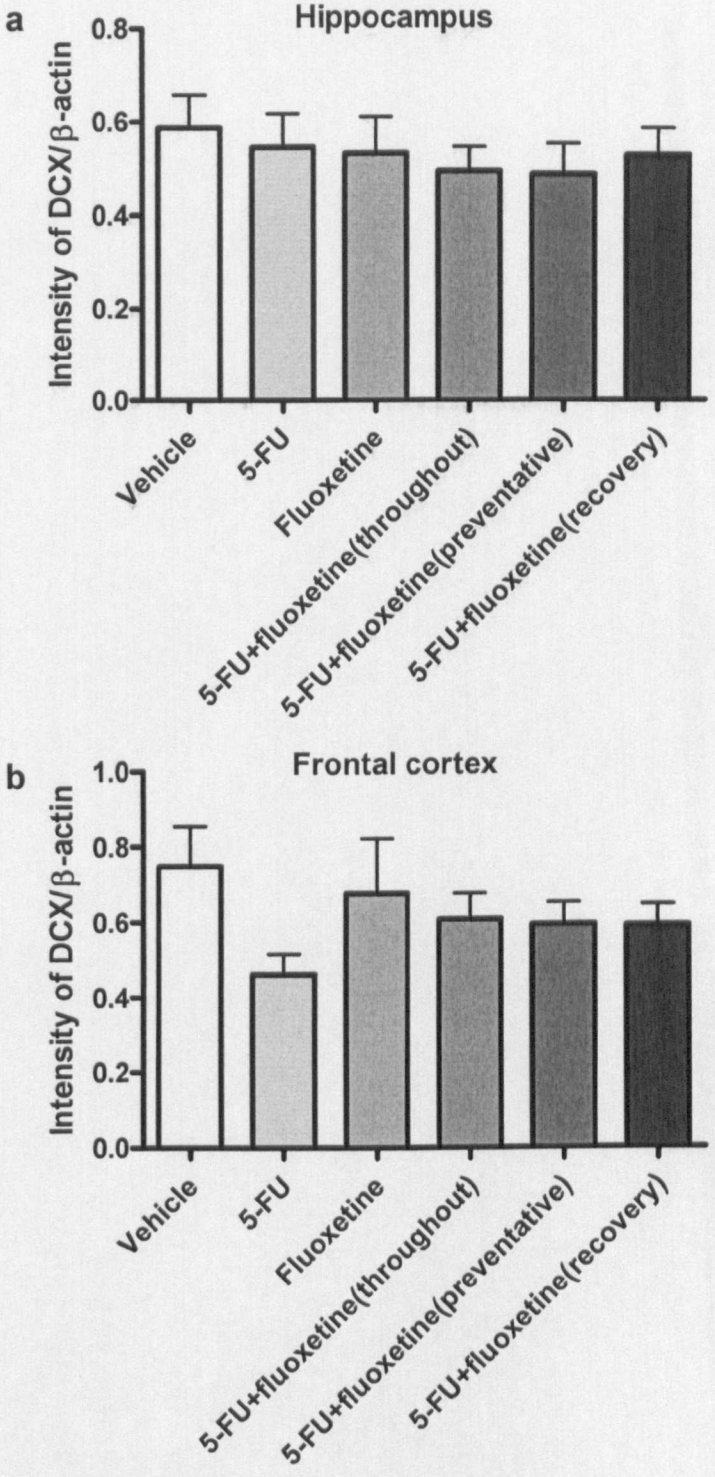

Figure 4.12 Levels of DCX (mean ± SEM, n=10) in the hippocampus (a) and frontal cortex (b) of all groups of animals, normalised by β-actin loading control, were not significantly different (one-way ANOVA).

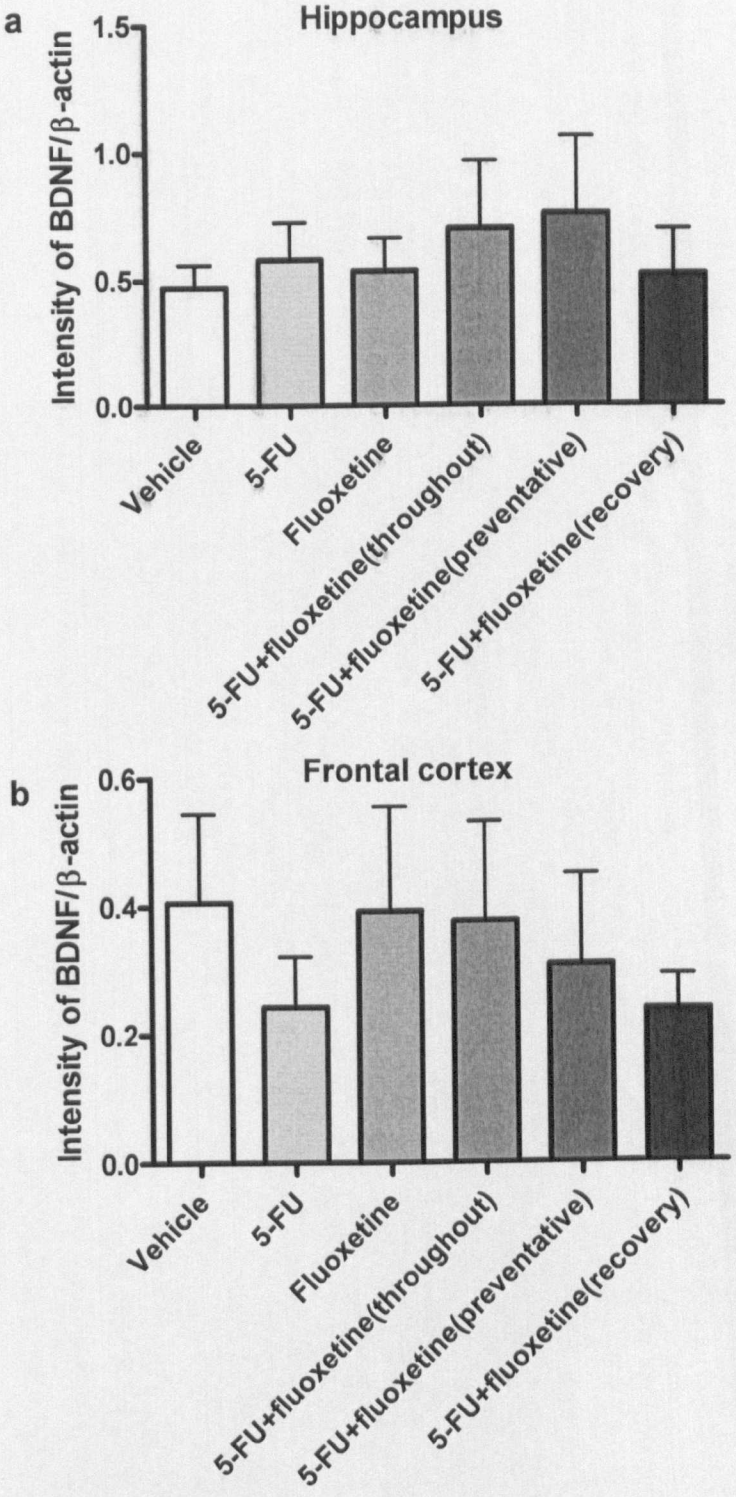

Figure 4.13 Levels of BDNF (mean ± SEM, n=10) in the hippocampus (a) and frontal cortex (b) of all groups of animals, normalised by β-actin loading control, were not significantly different (one-way ANOVA).

4.4 Discussion

The present study showed that the chemotherapy agent, 5-FU, caused a memory impairment measured by the NLR task which was associated with the reduction in both the proliferation and survival of neural precursors in the SGZ of the dentate gyrus. These effects were counteracted when the SSRI antidepressant fluoxetine was co-administered before and during (preventative) but not after (recovery) 5-FU treatment.

Similar to the study in the previous chapter, the NLR and spontaneous alternation in the T-maze memory tasks were chosen in the present study to assess spatial memory. The spontaneous alternation in the T-maze task was modified in an attempt to make it more difficult, however as the control groups' ability to perform the task did not differ in the T-maze tasks in the previous and present chapters, it did not appear that this was successfully achieved. In the present study, 5-FU-treated animals were unable to recognise an object in a novel as opposed to a familiar location, suggesting a memory deficit compared to saline treated controls, supporting clinical observations of chemotherapy-induced cognitive impairment in patients. The dose of 5-FU chosen was 25mg/kg administered chronically to the animals as it is clinically relevant and it is comparable to the 20mg/kg dose used previously in our laboratory which caused cognitive impairment in rats (ElBeltagy et al. 2010). In the previous chapter fluoxetine was shown to have cognitive benefits for rats treated with chemotherapy when given for a total of 40 days, before, during and after chemotherapy treatment. In the present study, the 5-FU-induced cognitive impairment was prevented when fluoxetine was administered 40 days before, during and after the period of 5-FU treatment. This positive effect on cognition remained when the co-administration of fluoxetine was before and during the 5-FU treatment for 20 days. However, the rats remained cognitively impaired when fluoxetine was only administered after the final 5-FU injection for 20 days. This indicates the importance of the timing of the fluoxetine administration, indicating a mechanism of prevention and not recovery.

Conversely, none of the treatment groups significantly differed in their ability to perform the spontaneous alternation in the T-maze task. It is possible that this could be due to the task not being able to detect the subtle difference in cognitive impairment induced by 5-FU, despite modifications to make it more difficult from the task used in the previous chapter by amending the time the animal was trapped in the arm and between trials (see section 4.4.2 of the present chapter).

Fluoxetine treated animals initially had a lower fluid intake than controls, which is likely to be due to a taste aversion to fluoxetine (Prendergast et al. 1996). However, by the end of the experiment, all groups had the same fluid intake. It is unlikely that differences in fluid intake would have affected their performance in the NLR task. Fluoxetine concentration in the drinking water was adjusted to take account of the animal's weight and differing levels of water consumption.

Disruption of neurogenesis is a possible mechanism by which 5-FU causes cognitive impairment (ElBeltagy et al. 2010). Production and survival of new neurons in the hippocampus is thought to be essential for spatial memory and learning (Ehninger and Kempermann 2008; Zhao et al. 2008) and cognitive impairments are seen when neurogenesis is disrupted by irradiation (Madsen et al. 2003; Snyder et al. 2005), chemotherapy drugs (ElBeltagy et al. 2010; Lyons et al. 2011b; Seigers et al. 2008) or genetic manipulation (Dupret et al. 2007). In the present study, the effects of 5-FU and fluoxetine on the production and survival of new hippocampal neurons were examined. The 5-FU treatment had a significant negative effect on both the survival of newly born hippocampal cells in the SGZ of the dentate gyrus and their proliferation as measured 30 days after the completion of treatment, suggesting that 5-FU has a long-term effect on the newly-born cells. Rats administered with both 5-FU and fluoxetine in the present study did not significantly differ from the controls when fluoxetine was administered for the "preventative" and "throughout" periods. When fluoxetine was only administered after the

final 5-FU injection, the rats still had significantly reduced survival of newly-born neurons and a subtle reduction in proliferation was also evident. Rats administered with fluoxetine alone also showed increased proliferation and survival as reported in Chapter 3.

The results in the present study are the first to examine the time course of the effects of fluoxetine on the response to the chemotherapy agent, 5-FU. The cognitive effects of 5-FU found here are consistent with earlier work from our group (ElBeltagy et al. 2010; Mustafa et al. 2008) and other studies which show that 5-FU affects memory 2 days (Foley et al. 2008) to 5 weeks (Gandal et al. 2008) after drug administration. 5-FU has also previously been shown to have a negative effect on hippocampal cell proliferation (ElBeltagy et al. 2010; Mustafa et al. 2008). However one study showed no effect of this chemotherapy agent on hippocampal cell proliferation (Mignone and Weber 2006) but this may be because the effects of 5-FU were examined the day following treatment, not allowing enough time for the drug to affect the population of newly born cells. The proliferation, survival and apoptosis of neural progenitors are all involved in memory formation and the stage of growth of newly born neurons when learning and memory takes place is an important factor (Dupret et al. 2007). In the present study, memory ability and neurogenesis were affected 4 weeks after the 5-FU chemotherapy treatment period indicating that 5-FU had a chronic effect on both these features. The association between the decline in cognition and neurogenesis provides further evidence that these processes are linked and that the impact of chemotherapy on neurogenesis is a likely mechanism for the changes in cognition. The present results show that fluoxetine alone increased the survival of cells and that it exhibits properties which enable it to protect the population of new neurons from 5-FU. However once 5-FU has a negative impact, fluoxetine is not able to replenish the population of neurons.

The mechanisms by which fluoxetine exhibits its neuroprotective properties are unclear. However, fluoxetine has been shown to increase levels of BDNF which influences the

regulation of neurogenesis (Alme et al. 2007; Duman and Monteggia 2006) and increases proliferation of neural precursors in the dentate gyrus (Pinnock et al. 2010) (see section 1.4.3, Chapter 1). In addition, fluoxetine has been shown to directly increase proliferation of hippocampal derived-neural stem cells and to prevent lipopolysaccharides-induced apoptosis *in vitro* (Chiou et al. 2006a). However, results from Western blotting in the present study indicate that there was no significant difference in levels of BDNF in the hippocampus or frontal cortex between any of the treatment groups. This suggests that neither fluoxetine nor 5-FU alters levels of BDNF. However, it is also a possibility that the Western blotting technique was not sensitive enough to detect subtle concentration changes of BDNF across whole brain regions.

Furthermore, levels of the microtubule associated protein DCX in the hippocampus or frontal cortex did not significantly differ between any of the treatment groups. DCX is present in young neurons so it is perhaps surprising that there was no difference in hippocampal levels considering that the present study also revealed that the proliferation and survival of hippocampal cells was significantly altered by fluoxetine and 5-FU. An explanation for this could be that the newly generated cells detected were not indeed neurons. However this is unlikely as it has been reported that more than 80% of dividing cells in the SGZ in the adult rat become neurons (Snyder et al. 2009). This could therefore provide evidence that the Western blot quantification techniques used in the present study may not be sensitive enough to detect subtle chemical changes. Further studies to more precisely quantify BDNF and DCX would be necessary to enable conclusions to be drawn. It would also be interesting to quantify apoptosis and in the hippocampus to provide further information to help understand the detailed mechanism of how fluoxetine is acting against 5-FU.

The period of administration for fluoxetine was at least 20 days as this is sufficient to have anxiolytic effects (Conley and Hutson 2007) and increase hippocampal neurogenesis

(Kodama et al. 2004) in rats. Behavioural testing was carried out a week after termination of fluoxetine treatment, as fluoxetine and its primary metabolite norfluoxetine have a long half-life and take 3 days to wash out of the system (Caccia et al. 1990). Although levels of serum fluoxetine and norfluoxetine were not measured in the present study, a comparable study by Thompson et al. showed that Wistar rats treated with 6-7ml/kg/day of fluoxetine in drinking water for 37 days had fluoxetine and norfluoxetine serum levels of 281±44 and 1209±123 nmol/l respectively (Thompson et al. 2003) . These levels are comparable to previous studies when the drug has been administered by injection (Caccia et al. 1990). Clinical reports have suggested that studies of chemotherapy-induced cognitive impairment in patients could be confounded by stress (Wefel et al. 2004). In the present study, stress to the animals was kept to a minimum, by giving fluoxetine in drinking water rather than injection, regular handling and group housing of the animals. Furthermore, administering fluoxetine in the drinking water is clinically relevant as it is administered to patients orally.

The results of the present study show that fluoxetine exhibits effects which can protect newly born hippocampal neurons from the cytotoxic effects of 5-FU which would subsequently cause cognitive decline. Furthermore, this is the first time to show that these effects are only demonstrated when fluoxetine is given before and during the 5-FU treatment and that fluoxetine cannot initiate recovery of the chemotherapy-induced reduction in neurons and cognitive ability in animals after it has occurred. It is premature to estimate the extent to which these results are translatable to humans, but if similar effects of prevention of chemotherapy-induced memory deficits by fluoxetine are apparent in patients, these results may offer a relatively simple way to counteract cognitive impairment and suggest that the antidepressant should be delivered before and during chemotherapy treatment to prevent cognitive impairment.

Chapter 5

The effect of 5-fluoruracil, fluoxetine and norfluoxetine on the viability and proliferation of neural stem cells isolated from the adult mouse hippocampus

5.1 Introduction

Adjuvant chemotherapy is reported to negatively affect cognition in cancer survivors for years after treatment has ended (Janelsins et al. 2011). There is evidence to suggest that this may be at least in part due to the effects of certain chemotherapy agents on hippocampal neurogenesis (ElBeltagy et al. 2010; Lyons et al. 2011b; Seigers and Fardell 2011). In the previous chapter presented in the thesis, it was shown that treatment with 5-fluorouracil (5-FU) can cause an impairment in the rats' ability to perform the novel location recognition (NLR) spatial memory task and reduces both the proliferation and survival of newly generated cells in the dentate gyrus of the hippocampus. Furthermore, these negative cognitive and cellular effects are reversed when the selective serotonin reuptake inhibitor (SSRI) antidepressant, fluoxetine, is co-administered for a period of time before and during the 5-FU treatment regimen, but the mechanism by which fluoxetine has this neuroprotective effect remains unclear. In the present study the direct effects of 5-FU, fluoxetine and its metabolite norfluoxetine on mouse hippocampal neural stem cells (NSC) were investigated *in vitro*.

5-FU is an antimitotic agent which has been shown to decrease cell proliferation by inhibition of the enzyme thymidine synthetase (Longley et al. 2003) (TS; see section 1.2.3.1, Chapter 1) and has been shown to be cytotoxic to human HB1.F3.CD NSC cancer cell lines *in vitro* and *in vivo* (Joo et al. 2009). Furthermore, previous work in our laboratory and work from the present thesis has shown that 5-FU reduces proliferation of non-cancerous hippocampal cells in the subgranular zone (SGZ) of the rat dentate gyrus. However, the effects of 5-FU on viability and proliferation on primary hippocampal NSC *in vitro* have not yet been investigated and are demonstrated in the present chapter.

Conversely, work in our laboratory has shown that fluoxetine upregulates the proliferation of cells in the adult rat hippocampus (see Chapters 3 and 4). In addition, fluoxetine increases the proliferation of isolated rodent NSC *in vitro* (Chen et al. 2007; Manev et al. 2001; Zusso

et al. 2008). Fluoxetine has also been previously shown to increase cell proliferation *in vitro* after administration *in vivo* following chronic stress (Hitoshi et al. 2007). In the present study the effects of different concentrations of fluoxetine on both the viability and proliferation of mouse primary hippocampal NSC *in vitro* were investigated. *In vivo*, fluoxetine is metabolised by the liver to norfluoxetine. Therefore, the direct effect of norfluoxetine on viability of isolated hippocampal NSC was determined in the present study.

5.2 Materials and methods

5.2.1 Isolation and propagation of adult mouse hippocampal NSC

Although rats were used in the previous *in vivo* experiments of this thesis, in the present experiment NSC from the hippocampus of mice were used due to unavailability of rat NSC and the amount of time available. Three brains of male and female adult 129 and ME1 mice (Biomedical Service unit (BMSU), University of Nottingham) were dissected and immersed in ice cold PSB. The hippocampi were dissected, diced (into $<1mm^3$ pieces), pooled in a 30ml universal tube and the PBS was removed. 1ml of accutase (0.5mM in PBS, Millipore, USA) was added to the tube and incubated for 45 min at 37°C to digest the tissue. To facilitate dissociation, the tissue was gently pipetted using a P1000. 10ml of PBS was added and the tube was shaken to ensure complete dissociation of the tissue. The cells were pelleted by centrifugation (5 min, 200G), PBS was removed and the tissue was resuspended in NSC medium [Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12), neurobasal media, N2 supplement, B27 supplement, Pen/Strep, epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2)] (see Appendix II). 2ml of the suspension was placed in a 6 well plate and cultured at 37°C in 5% CO_2. The next day the cells were collect and passaged (see below) and placed in T25 flasks.

5.2.2 Culturing and passaging of hippocampal NSC

Mouse hippocampus NSC were maintained in sterile conditions at 37°C, with 5% CO_2, in NSC medium in a T25 flask and passaged every 4-7 days. To passage the NSC, the medium

and cells were centrifuged (5 min, 200G) to pellet the cells. They were resuspended in 0.5ml accutase, incubated for 5 min at 37°C and gently pipetted with a P1000 to dissociate the neurospheres and again pelleted by centrifugation in 10ml of PBS. The cells were resuspended in 1ml NSC medium and transferred into new T25 flasks with 5ml of fresh NSC medium.

5.2.3 Proliferation of hippocampal NSC

After dissociation of the neurospheres, the cells were plated in sterile 6 well plates at a density of 5×10^5 per well in NSC medium treated with 0, 0.1, 1 or 10μM of fluoxetine. Cells were incubated at 37°C, with 5% CO_2 for 1, 3 and 7 days. The neurospheres were then centrifuged and dissociated (as described in section 5.2.2 of the present chapter) and the density of single cells was assessed using an Improved Neubar Hemocytometer (see Appendix III). Quantification of NSC was performed in triplicate.

5.2.4 MTT assay

The MTT [3- (4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] assay was used to test the effects of a range of concentrations of 5-FU, fluoxetine and norfluoxetine on the viability of hippocampal NSC. This is a colourimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt MTT to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and thus of cell viability (Mosmann 1983).

After dissociation of the neurospheres, the cells were plated out in sterile 96 well plates at a density of 5×10^3 in 200μl of MTT NSC medium [DMEM/F12 and FBS](see Appendix II) per well, 8 days prior to the MTT assay. 24 h, 4 or 7 days prior to the MTT assay, the medium was carefully removed with an aspirator and cells were treated with different concentrations (0, 0.01, 0.1, 1, 10 and 100μM) of 5-FU, fluoxetine or norfluoxetine (n=8) in fresh MTT NSC medium and incubated at 37°C. 2 h prior to the end of the incubation, 20μl MTT (2.5mg/ml in PBS) was added to each well. At the end of incubation NSC media was

removed and 200µl of dimethyl sulfoxide (DMSO) was added to each of the wells and gently agitated. The optical density of each well was read at 570nm wavelength using a Dynex MRX Model 96 Well Plate Reader (MTX Lab Systems Inc., USA). Cell counting required to seed a specific number of cells was performed using an Improved Neubar Hemocytometer (see Appendix III).

5.2.5 Statistical analysis

Statistical analysis and graphs were created using GraphPad Prism 5 and significance was regarded as $p<0.05$. Cell viability is presented as the optical density as a percentage of the control. For cell proliferation, the number of cells per ml was estimated from a 10µl sample. Cell viability and proliferation were analysed using two-way ANOVA. When ANOVA was significant Bonferonni post-hoc test was performed.

5.3 Results

5.3.1 5-FU reduces viability of adult mouse hippocampal NSC

MTT assays were performed to assess viability of NSC after treatment with a range of concentrations of 5-FU (0, 0.01, 0.1, 1, 10 and 100µM), following an incubation of 1, 4 or 7 days. Optical densities were read and normalised against the control (0µM 5-FU). None of the concentrations of 5-FU had a significant effect on cell viability after 1 day (two-way ANOVA, Figure 5.1a). All concentrations of 5-FU caused significantly fewer viable NSC after 4 or 7 days exposure compared to the control ($*p<0.05$, $**p<0.01$, $***p<0.001$, two-way ANOVA, Figure 5.1a) with the exception of 4 day exposure to 0.01µM 5-FU, which did not significantly differ from the control.

5.3.2 Fluoxetine reduces proliferation of adult mouse hippocampal NSC

In the present study hippocampal NSC were quantified to investigate the direct effect of various concentrations of fluoxetine on cell proliferation after incubations of 1, 3 and 7 days (Figure 5.2 and 5.3). Counts were analysed using a two-way ANOVA. After 1 day, none of

the concentrations (0.1, 1 or 10μM) of fluoxetine significantly reduced the number of cells, compared to the control. After 3 days incubation, cells treated with 0.1μM of fluoxetine did not differ from the control group, but cells treated with 1 and 10μM showed a significant decrease in proliferation ($p<0.001$). After 7 days of incubation, all the concentrations of fluoxetine caused a significant decrease in cell number ($*p<0.05$, $***p<0.001$).

5.3.3 Fluoxetine and norfluoxetine reduces viability adult mouse hippocampal NSC

The direct effects of fluoxetine and its major active metabolite on NSC cell viability were also assessed using the MTT assay. A range of concentrations of either fluoxetine or norfluoxetine were used (0, 0.01, 0.1, 1, 10 and 100μM), after an incubation of 1, 4 or 7 days. 0.01μM of fluoxetine had no significant effect on the viability of NSC after 1 day (two-way ANOVA, Figure 5.1b), but significantly reduced the number of viable cells after an incubation of 4 and 7 days ($p<0.001$, two-way ANOVA, Figure 5.1b). All the other, higher concentrations of fluoxetine reduced the number of viable cells compared to the control (0μM fluoxetine), after each incubation period ($p<0.001$, two-way ANOVA, Figure 5.1b).

Norfluoxetine did not significantly affect the viability of NSC at a concentration of 0.01μM compared to the control group (0μM norfluoxetine) at any time point (two-way ANOVA, Figure 5.1c). The higher concentrations (0.1-100μM) all significantly reduced the NSC viability after each incubation period ($*p<0.05$, $**p<0.01$, $***p<0.001$, two-way ANOVA, Figure 5.1c). These results show that both fluoxetine and norfluoxetine have a dose-dependent negative effect on viability of adult mouse hippocampal NSC.

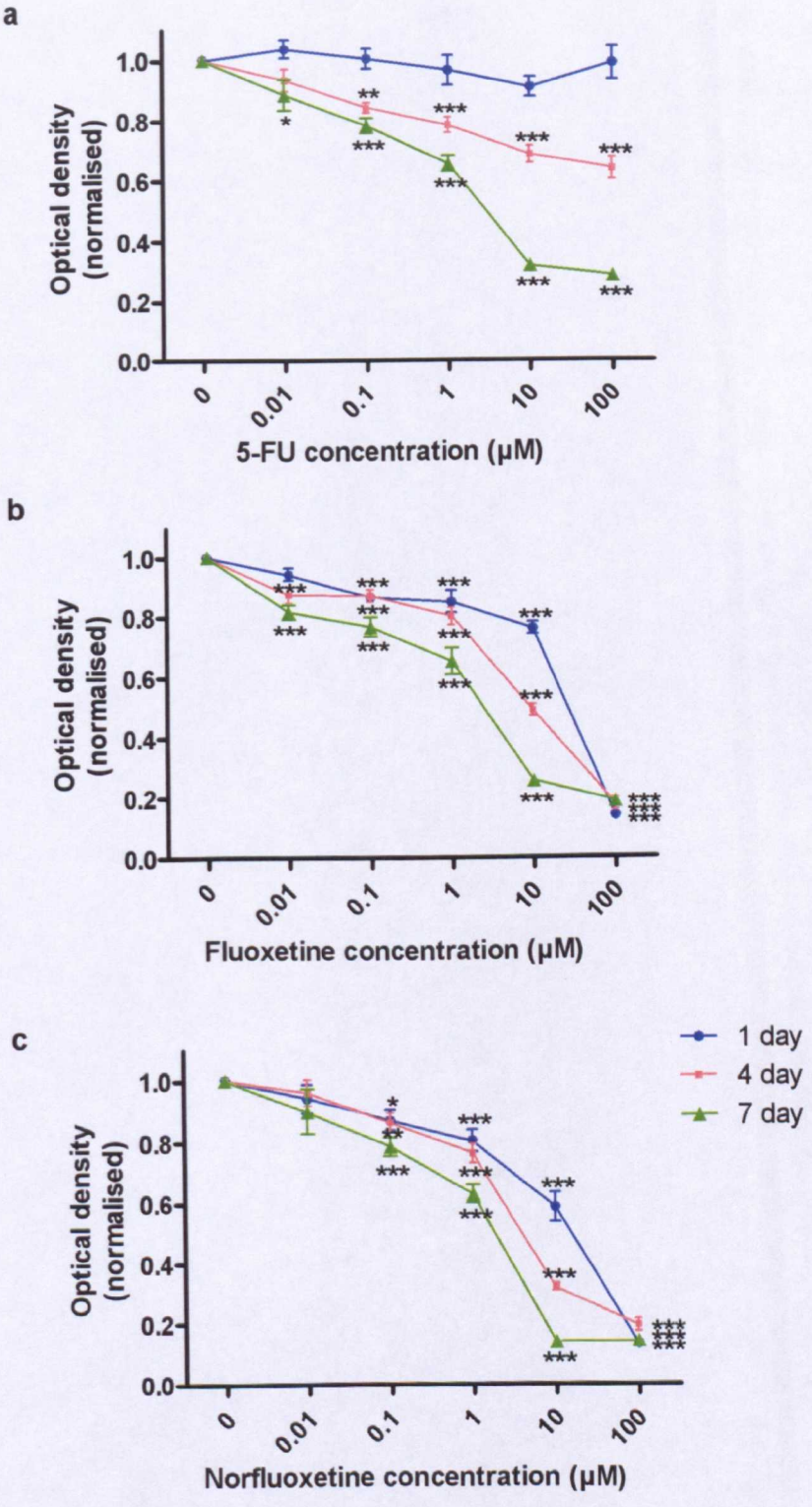

Figure 5.1 Viability of adult mouse hippocampal NSC assessed by optical density at 570nm wavelength (normalised as a proportion of the control (0μM of 5-FU/fluoxetine/norfluoxetine, n=8) from the MTT assay after exposure to a range of

concentrations (0, 0.01, 0.1, 1, 10 and 100μM) of 5-FU (a), fluoxetine (b) or norfluoxetine (c). Cell viability was not affected by any 5-FU concentration (0.01 - 100μM) after an exposure time of 1 day compared to the control group (two-way ANOVA). After an exposure time of 4 days, concentrations of 0.1-100μM 5-FU significantly decreased NSC viability (**p<0.01, ***p<0.001, two-way ANOVA) but cells treated with 0.01μM 5FU did not differ from the controls. At 7 days exposure time, all 5-FU concentrations (0.01-100μM) reduced NSC viability (*p<0.05, ***p<0.001, two-way ANOVA). All concentrations of fluoxetine (0.01-100μM) significantly reduced the viability of the NSC at each exposure time (1, 4 and 7 days), (p<0.001, two-way ANOVA) with the exception of 1 day's exposure to 0.01μM fluoxetine, which did not differ from the control group. Exposure to 0.01μM norfluoxetine did not significantly differ from the controls at any exposure time. Exposure to higher concentrations of norfluoxetine (0.1 - 100μM) decreased the viability of NSC at all exposure times (1, 4 and 7 days), (*p<0.05, **p<0.01, ***p<0.001, two-way ANOVA).

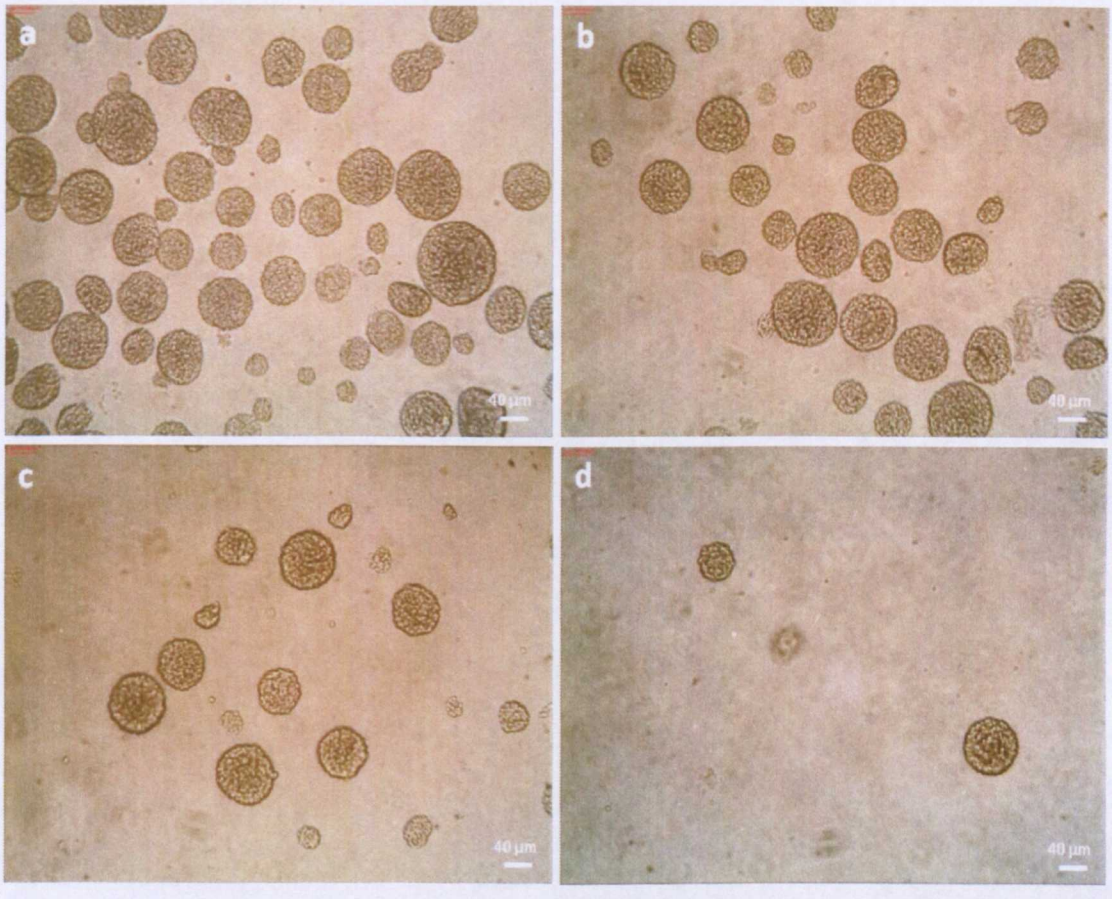

Figure 5.2 Photographs showing the effect on the formation of neurospheres from adult mouse hippocampal NSC after an incubation time of 1 week in different concentrations of fluoxetine: 0 (a), 0.1 (b), 1 (c) or 10 (d) µM.

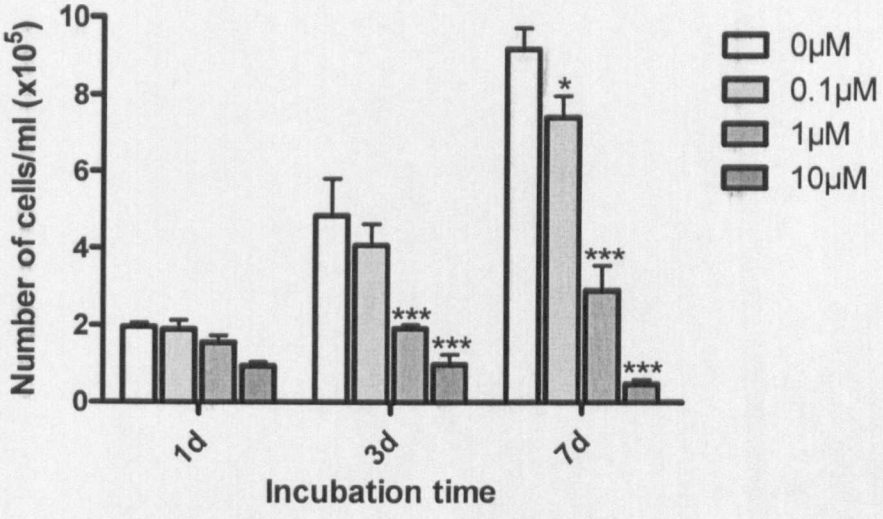

Figure 5.3 The number (mean ± SEM, n=3) of adult mouse hippocampal NSC (dissociated neurospheres) per ml grown in culture after incubations of 1, 3 and 7 days in different concentrations of fluoxetine (0.1, 1 or 10μM). At 1 day, none of the concentrations of fluoxetine reduced the number of cells/ml (two-way ANOVA) compared to the control group (0μM fluoxetine). After 3 days incubation in 0.1μM fluoxetine did not differ from the control group, but concentrations of 1 and 10μM fluoxetine significantly reduced the number of cells/ml (p<0.001, two-way ANOVA). At 7 days of incubation, all concentrations of fluoxetine (0.1, 1 or 10μM) significantly reduced the number of cells/ml compared to the control group (*p<0.05, ***p<0.001, two-way ANOVA).

5.4 Discussion

The aim of the present study was to establish an *in vitro* model of adult hippocampal NSC proliferation and viability in response to 5-FU, fluoxetine and its active metabolite norfluoxetine to further understand the mechanisms of chemotherapy and antidepressants *in vivo*. In the previous chapters presented in this thesis, it was shown that chronic 5-FU treatment reduced cell proliferation and survival in the hippocampus of the adult rat. Administration of fluoxetine to the rats had the reverse effect, promoting the proliferation and survival of hippocampal cells and preventing the negative cognitive and cellular effects of 5-FU when it was co-administered before and during 5-FU treatment. In the present study, the direct effects of 5-FU, fluoxetine and norfluoxetine on viability of hippocampal NSC isolated from the hippocampus of the adult mouse were determined.

The viability of hippocampal NSC, shown by the MTT colourimetric assay, was not affected by exposure to 5-FU for a single day at any of the concentrations used (0.1 - 100μM). However, at 4 days exposure to the drug, the cell viability decreased as the concentration of 5-FU increased and the effect was even more pronounced at 7 days, demonstrating that 5-FU has a dose and exposure time dependent effect on the hippocampal NSC. The concentrations used in the present study cover the range of 0.3-71μM found in the CSF of primates 5 to 60 min after i.v. infusion of 5-FU, so are therefore clinically relevant. The results from this study indicate that *in vitro* hippocampal NSC could be a good model for what is happening in the hippocampus *in vivo* as 5-FU administration has been shown to reduce cell proliferation in the hippocampus in animal models (ElBeltagy et al. 2010) (results from Chapter 4).

There has not yet been a dose-dependent study in an animal model investigating the effects of 5-FU on neurogenesis. However, 5-FU has previously been shown to reduce the viability of human colon cancer cell lines, COLO-205 and HT-29 *in vitro* at concentrations of 3.2μM and 1.3μM (Wiebke et al. 2003) and also mouse brain melanoma cell line F3.CD by 0.5 μM

of 5-FU (Joo et al. 2009). Furthermore, 5-FU has a similar effect on primary NSC from the mouse CNS *in vitro* reducing viability of oligodendrocytes at concentrations of 0.5-5μM, although, unlike the present study, this effect was seen after an exposure time of just 24 hours. 5-FU is toxic to cancerous cells by inhibiting the production of thymidine (Longley et al. 2003). Since 5-FU is able to cross the blood-brain barrier (Patel et al. 1998), it is probable that it enters the hippocampus and causes the proliferating NSC to apoptose by a similar mechanism to its cytotoxicity to cancerous cells, by inhibition of the enzyme TS (Longley et al. 2004).

Conversely, the effects of fluoxetine and norfluoxetine treatment on the hippocampal NSC did not accurately reflect the result seen *in vivo*. When administered to adult rats in the studies shown in previous chapters, fluoxetine increased the proliferation and survival of hippocampal cells. However, the *in vitro* study performed in the present chapter showed that fluoxetine significantly reduced the viability (shown by the MTT assay) of adult mouse hippocampal NSC at all concentrations (0.01-100μM) at all exposure times (1-7 days) with the exception of exposure to 0.01μM fluoxetine for 1 day. The reduction in viability was also demonstrated to be dose and time dependent. Furthermore, the rate of proliferation of the cells assessed by cell counts was reduced with concentrations of 1 and 10μM fluoxetine at 3 days and 0.1-10μM at 7 days. Fluoxetine's primary active metabolite, norfluoxetine, also decreased the viability of cells at concentrations of 0.1-100μM at all exposure times (1-7 days) but showed no effect at 0.01μM. The concentration range used for these drugs in the present study covered therapeutic plasma concentrations (Ayelli Edgar et al. 1999; Thompson et al. 2003).

The present results were unexpected, firstly because they do not correlate with the earlier *in vivo* work presented in this thesis and secondly because it is not in accordance with other literature investigating the affects of antidepressants on primary NSC. One previous study used NSC derived from the hippocampus of the adult rat and found that their proliferation

rate was up-regulated after 7 days incubation in 20μM fluoxetine (Chen et al. 2007). In another experiment the proliferation of adult rat hippocampal NSC was decreased by 48 h exposure to 1μM fluoxetine after they had been *in vitro* for 1 day, but increased after the same exposure time and fluoxetine concentration when the cells had grown for 10 days *in vitro* (Manev et al. 2001). Similar results have also been shown in isolated rat cerebellum NSC showing that 1μM fluoxetine up-regulating cell proliferation (Zusso et al. 2008; Zusso et al. 2004).

One reason for the decrease in proliferation of the hippocampal NSC in the present study may be the comparatively short exposure time to fluoxetine, compared to previous *in vivo* studies of this thesis. *In vivo* fluoxetine takes up to 4 weeks to alter hippocampal neurogenesis and have beneficial cognitive effects (Conley and Hutson 2007; Kodama et al. 2004). However, the results from the present study show that cell viability still decreased between 4 and 7 days exposure to both fluoxetine and norfluoxetine, which suggests that if fluoxetine induces any increase in proliferation *in vitro*, a much longer time period would be necessary to see these effects.

Alternatively, the species from which the NSC are derived could have an impact on the proliferation of the isolated NSC as, unlike the present experiment, none of the previous studies investigating the effect of fluoxetine on NSC *in vitro* derived the cells from mice. Fluoxetine has been shown to increase the rate of hippocampal neurogenesis *in vivo* in the adult rat (Chen et al. 2007; Manev et al. 2001; Zusso et al. 2008; Zusso et al. 2004), but not in adult mice (Holick et al. 2007; Huang et al. 2008b). Furthermore, it has been reported that *in vivo* that neurons from the adult rat hippocampus are more numerous, differentiate faster and have a higher survival rate than neurons in the mouse hippocampus (Snyder et al. 2009) and it is possible that this may also effect cell proliferation and viability for cultured hippocampal NSC.

The results from the present study suggest that isolated mouse hippocampal NSC grown *in vitro* is not a suitable model to investigate the effects of fluoxetine on cell proliferation *in vivo*. It is perhaps not possible to create an accurate model *in vitro* due to the complex *in vivo* factors excluded from cell culture methods. For example, fluoxetine has been shown to protect against caspase-mediated apoptosis by activating anti-apototic proteins such as the cellular FLICE-inhibitory protein, Bcl-2 and Bcl-x in culture studies where NSC have been isolated from the adult rat hippocampus (Chen et al. 2007; Chiou et al. 2006a). This could indicate that fluoxetine protects against the apoptotic effects of 5-FU, but does not increase proliferation alone. Moreover, the process of neurogenesis *in vivo* is highly regulated by BDNF, in a complex feedback and feed-forward mechanism (Castrén and Rantamäki 2010; Duman et al. 2001b; Scharfman et al. 2005) (Chapter 1.2).

It is possible, that the mechanism by which fluoxetine stimulates neurogenesis *in vivo* involves the CREB, BDNF pathway (Duman et al. 2001b). BDNF is produced *in vivo* by mature granule cell neurons (Conner et al. 1997), glia and endothelial cells (Linnarsson et al. 2000), and therefore may not be present in immature NSC grown in culture and is likely not be stimulated by fluoxetine directly, which could be an explanation as to why it does not increase their proliferation and survival *in vitro*. In the present study 5-HT was not within the growth medium but the expression of 5-HT and the serotonin reuptake transporter, SERT, in the NSC was not investigated. The expression of SERT and 5-HT has been revealed in NSC isolated from the rat cerebellum (Zusso et al. 2008) and in 10 week old NSC isolated from the midbrain region of embryonic mice (Ren-Patterson et al. 2005). Therefore, it is likely that SERT would be expressed in the NSC of the present study and they would release 5-HT. However the pharmacological mechanism by which fluoxetine acts in this model remains to be determined but would help to understand the *in vitro* effect of fluoxetine and norfluoxetine on hippocampal NSC.

In conclusion, although isolated adult mouse hippocampal NSC appear to be a potential model of the effects of 5-FU on cell proliferation in the rat hippocampus, they are not a suitable model of the long-term *in vivo* effects of fluoxetine. In future studies it would be interesting to directly compare the effects of fluoxetine and 5-FU on both rat and mouse hippocampal NSC to see the extent of variation between species. To create a viable model of work presented in this thesis, it would also be necessary to observe the effects of antidepressants and chemotherapy in combination to investigate if antidepressants exhibit a neuroprotective effect to hippocampal NSC directly exposed to antidepressants and 5-FU. It remains important to establish a cell culture model of hippocampal neurogenesis to help determine the mechanisms of action of the drugs involved and in particular how fluoxetine is able to prevent chemotherapy induced-cognitive impairment.

Chapter 6

General discussion

Cognitive impairment is a common experience of patients following chemotherapy treatment (see section 1.1, Chapter 1). The principle aim of the studies in the present thesis was to develop a model of chemotherapy-induced cognitive impairment using the adult rat and apply this to understanding the causes and treatments of this condition. This model was used to investigate the effects of the individual chemotherapies, from the CMF breast cancer treatment combination, on hippocampal dependent spatial memory and to determine if changes in adult hippocampal neurogenesis may underlie the observed behavioural deficits. In the second part of the thesis, the potential effects of the SSRI antidepressant, fluoxetine, in counteracting the cognitive and cellular deficits induced by chemotherapy, were investigated. In particular, the optimum time period to administer fluoxetine was determined which not only indicates how this treatment might be applied clinically, but also provides an insight into the possible mechanism of action of fluoxetine.

To investigate if the effects of chemotherapy and fluoxetine on NSC could be reproduced *in vitro*, NSC were isolated from the hippocampus of the adult mouse and their viability and proliferation was determined after direct treatment with 5-FU, fluoxetine or its active metabolite norfluoxetine.

6.1 Principal findings

The principal findings of the present project are summarised and presented in Table 6.1 and 6.2. In the preliminary study (section 2.2, Chapter 2) the NOR and NLR tasks were used to test the effect of CP on working memory in the adult rat. However, the animals in the control group failed to show a preference for the novel stimuli in both tasks, indicating that higher numbers of animals were needed per group to make the tasks valid. In the second experiment (section 2.3, Chapter 2) the parameters of the NLR task were altered resulting in validation of the task. CP treated animals in the revised NLR task were not impaired in spatial memory. The NOR task was not used in this study to avoid confounding results from recognising the objects or locations between tasks. Animals treated with CP did not have

reduced levels of cell proliferation but had reduced cell survival in the dentate gyrus, or reduced levels of DCX in the hippocampus or frontal cortex compared to the control group. This work has been published in Lyons et al. 2011a (The effect of cyclophosphamide on hippocampal cell proliferation and spatial working memory in the rat. PLoS ONE 6(6)).

The next experiment (Chapter 3) showed that in contrast to CP, MTX significantly reduced spatial memory performance in the NLR task, but not in the spontaneous alternation in the T-maze task compared to the control group. The memory deficit was reversed when fluoxetine was co-administered before, during and after MTX treatment. Furthermore, MTX significantly decreased the proliferation and survival of hippocampal cells, effects which were also reversed by co-administration of fluoxetine. This work has been published in Lyons et al. 2011b (Fluoxetine reverses the memory impairment and reduction in proliferation and survival of hippocampal cells caused by methotrexate chemotherapy. Psychopharmacology 215: 105-115).

Treatment with 5-FU reduced the animals' ability to perform the NLR task, but did not alter their behaviour in the spontaneous alternation in the T-maze task. The behavioural effects of 5-FU were associated with decreased hippocampal cell proliferation and cell survival compared to the control animals (Chapter 4). The behavioural and cellular deficits seen after 5-FU treatment were prevented when fluoxetine was co-administered before and during the 5-FU treatment regimen (preventative) but not when fluoxetine was administered only after 5-FU treatment had finished (recovery). Levels of DCX and BDNF in the hippocampus and frontal cortex were not altered by 5-FU or fluoxetine. This work has been submitted for publication in PLoS ONE.

The MTT assay was used to assess the viability of NSC isolated from the hippocampus of the adult mouse *in vivo* (Chapter 5). 5-FU, fluoxetine and norfluoxetine all caused cell viability to decrease with increasing concentrations (0 to 100μM) and exposure time (1 to 7

days). Fluoxetine also reduced cell proliferation, as quantified by cell counts, in a dose (0 to 10μM) and time (1 to 7 days) dependent manner.

Experiment (chapter)	Treatment used	NOR	NLR	T-maze	Hippocampal cell survival	Hippocampal cell proliferation	DCX levels	BDNF levels
CP, preliminary study (2)	**CP:** 30mg/kg, 4 i.v. injections 3-4 days apart	n-number too low for conclusions	n-number too low for conclusions	n/a	n/a	n/a	n/a	n/a
CP (2)	**CP:** 30mg/kg, 7 i.v. injections 3 days apart	n/a	Not affected	n/a	Significant deficit by CP	Not affected	Not affected	Not affected
MTX/LCV and fluoxetine (3)	**MTX:** 75mg/kg, 2 i.v. injections a week apart followed by LCV rescue **Fluoxetine:** 10mg/kg in drinking water for 40 days	n/a	Significant deficit by MTX/LCV, counteracted by fluoxetine co-treatment	Not affected	Significant deficit by MTX/LCV, counteracted by fluoxetine co-treatment. Increased by fluoxetine alone.	Significant deficit by MTX/LCV, counteracted by fluoxetine co-treatment. Increased by fluoxetine alone.	n/a	n/a
5-FU and fluoxetine (4)	**5-FU:** 25mg/kg, 5 i.p. injections 3 days apart **Fluoxetine:** 10mg/kg in drinking water for 20 days "preventative" or "recovery" or for 40 days "throughout"	n/a	Significant deficit by MTX/LCV, counteracted by fluoxetine co-treatment when "preventative" or "throughout" but not "recovery"	Not affected	Significant deficit by MTX/LCV, counteracted by fluoxetine co-treatment when "preventative" or "throughout" but not "recovery". Increased by fluoxetine alone.	Significant deficit by MTX/LCV, counteracted by fluoxetine co-treatment when "preventative" or "throughout" but not "recovery". Increased by fluoxetine alone.	Not affected	Not affected

Table 6.1 Summary of results from the *in vivo* experiments presented in this thesis. Abbreviations; CP: cyclophosphamide, MTX: methotrexate, LCV: leucovorin, 5-FU: 5-fluorouracil, NOR: novel object recognition, NLR: novel location recognition.

Drug	Effect on viability of adult hippocampal rat NSC	Effect on proliferation of adult hippocampal rat NSC
5-FU	Dose and time dependent reduction	n/a
Fluoxetine	Dose and time dependent reduction	Dose and time dependent reduction
Norfluoxetine	Dose and time dependent reduction	n/a

Table 6.2 Summary of results from the *in vitro* experiment presented in Chapter 5 of this thesis. Abbreviations; 5-FU: 5-fluorouracil, NSC: neural stem cell

6.2 Cognitive effects of chemotherapy

Evidence from clinical studies has indicated that a number of chemotherapy treatment combinations are able to induce long-term deficits in memory, attention and cognitive ability in cancer survivors (reviewed in section 1.1.2, Chapter 1). This has led to several studies in rodents to test the effect of various chemotherapy drugs, used individually or in combination on different behavioural paradigms that model learning and memory (reviewed in section 1.1.3, Chapter 1). Furthermore, the use of animal models has provided insight into the mechanisms by which chemotherapy agents may cause cognitive deficits including their effects on adult hippocampal neurogenesis (reviewed in Fardell et al. 2010). Proliferation and survival of neurons within the dentate gyrus have been reported to be involved in certain types of hippocampal-dependent memory formation (section 1.5, Chapter 1) and previous work within our group and elsewhere, has shown correlations between chemotherapy-induced cognitive impairment and a reduction in hippocampal neurogenesis (ElBeltagy et al. 2010; Fardell et al. 2010).

Studies in the present thesis investigated the effects of three chemotherapy drugs from the CMF treatment combination; CP, MTX and 5-FU, on working memory and cellular changes in the hippocampus in the adult rat. Animals treated with MTX or 5-FU showed a reduction in their ability to perform the NLR spatial working memory task which is consistent with much of the previous literature which reports chemotherapy-induced cognitive decline in animal models of chemotherapy (as described in section 1.1.3, Chapter 1). Furthermore, findings from the present studies provide evidence that it is the chemotherapy itself causing the cognitive impairment, rather than factors such as stress, depression or fatigue which have been suggested to confound clinical studies, in patients, of these parameters (Hermelink et al. 2007; Jenkins et al. 2006). However, the ability of animals to perform the spontaneous alternation in the T-maze task was not altered by treatment with MTX or 5-FU in the experiments carried out in the present thesis. This may be because the task assesses a different cognitive domain from the NLR task and this domain is not affected by MTX or 5-

FU, even though both tasks require spatial working memory and an intact hippocampus to be performed (Deacon and Rawlins 2005; Dere et al. 2007; Mumby et al. 2002). An alternative explanation could be that the ability to perform the spontaneous alternation in the T-maze task is not sensitive enough to detect the subtle cognitive impairment in spatial memory produced by MTX and 5-FU.

One of the interesting findings of the present study was that CP did not cause the same spatial memory deficit in animals that was induced by MTX and 5-FU, as measured by the NLR task using exactly the same parameters. Although, the dose of CP and the behavioural task used cannot be discounted as a reason for this, it may indicate that CP does not have a substantial effect on spatial cognition. Previous studies in rodents have shown varied results in the cognitive effects of CP administered alone (Lee et al. 2006; Reiriz 2006; Yang et al. 2010) (see Table 1.2), but results from the present study would suggest that even if CP does cause impairment in hippocampal-dependent memory, it is not as substantial as that induced by MTX or 5-FU.

When chemotherapy-induced cognitive impairment has been investigated clinically, it is rarely discussed with respect to particular chemotherapy agents or combinations which may contribute to the cognitive defects (Table 1.1). However, it is evident from the results in the present study that the effects of individual chemotherapies need to be taken into account. Furthermore, there is evidence from animal studies which reports that the cognitive deficits induced by chemotherapy agents are potentiated when administered in combination (Foley et al. 2008; Walker et al. 2011).

6.3 Effects of chemotherapy on adult hippocampal neurogenesis

All three of the CMF chemotherapy drugs are able to cross the blood-brain barrier when delivered in high doses (Lassman et al. 2006; Patel et al. 1998; Perry 2008), allowing them to have a direct effect on the brain. Studies from the present thesis showed that MTX and 5-FU decreased the number of dividing cells in the neurogenic region (SGZ) of the dentate

gyrus in the hippocampus and furthermore, reduced the survival of the new dentate gyrus cells which were proliferating at the beginning of the drug administration period. This is consistent with previous work in our laboratory and elsewhere reporting that MTX and 5-FU can inhibit neurogenesis (see Table 1.2).

In contrast to MTX and 5-FU, CP showed no significant effect on the proliferation of hippocampal cells. These results are interesting because they correlate with the effects of the CMF drugs on spatial memory and provide evidence that a reduction in neurogenesis may play a role in the memory impairment observed. However, it remains unclear why CP does not alter neurogenesis and cognition to the same extent as MTX and 5-FU. CP is an alkylating agent whereas MTX and 5-FU are antimetabolites (see section 1.2, Chapter 1). It is possible that this difference in mechanism of cytotoxicity is responsible for the differences seen between the drugs' effects, although a previous study found that another alkylating agent, thioTEPA impaired the ability of mice to perform the NLR and NOR tasks and also reduced the proliferation of hippocampal cells (Mondie et al. 2010). In the present study, CP significantly reduced the survival of newly formed cells involved in hippocampal neurogenesis suggesting that it has a toxic effect on these cells. This reduction could possibly lead to long-term cognitive effects which may become more evident with higher doses. However, the present findings suggest that CP is less neurotoxic than the other drugs in the CMF combination with which it is used.

6.4 Effects of fluoxetine on cognition and neurogenesis in the hippocampus

Despite the amount of evidence supporting chemotherapy-induced cognitive impairment, very little work has been carried out into potential methods to prevent or counteract it. In the present project it was demonstrated that the spatial cognitive deficits induced by MTX and 5-FU are reversible by the co-administration of the SSRI antidepressant, fluoxetine. This is in line with previous literature which showed that fluoxetine is beneficial when non chemotherapy-induced cognitive impairments are present in rodents (El Hage et al. 2004;

ElBeltagy et al. 2010; Li et al. 2009; Monleon et al. 2007) and humans (Gallassi et al. 2006; Levkovitz et al. 2002; Mowla et al. 2007; Vythilingam et al. 2004). If the results from the present project are translatable to humans, this could explain some of the variation in chemotherapy-induced cognitive impairment observed in clinical studies, since the co-administration of antidepressants to patients undergoing chemotherapy has not been previously taken into account (see Table 1.1).

Moreover, it has been reported in previous studies that fluoxetine increases hippocampal neurogenesis and this may be the basis of the reversal of the cognitive deficit (ElBeltagy et al. 2010). The upregulation of hippocampal cell proliferation and survival was clearly demonstrated in the present studies. However, it is still not fully understood how fluoxetine exhibits these neuroprotective and behavioural effects. One way this question was addressed in the present project was to investigate if fluoxetine acted by a mechanism of prevention or reversal. Fluoxetine counteracted the spatial memory deficit and cellular alterations in the hippocampus when it was administered before and during 5-FU treatment, but not when it was only administered after treatment, indicating a prevention process. This indicates that once 5-FU has affected neurogenesis and cognition, it cannot be reversed by later administration of fluoxetine. This needs to be considered if clinical trials to prevent chemotherapy-induced cognitive impairment by fluoxetine are to be carried out, since it would be important to start the administration of fluoxetine before the onset of chemotherapy treatment.

A further approach to understanding fluoxetine's neuroprotective effect was to directly expose cultured NSC isolated from the adult mouse hippocampus to fluoxetine and its primary active metabolite, norfluoxetine *in vitro*. However, like the cytotoxic agent, 5-FU, both fluoxetine and norfluoxetine reduced the viability of the NSC and fluoxetine also reduced their proliferation. Other studies have shown that fluoxetine increases proliferation *in vitro* of NSC from rats (Chen et al. 2007; Manev et al. 2001; Zusso et al. 2008; Zusso et

al. 2004), but this effect was not mirrored in the present study using NSC from mice. It has been reported that there are significant species differences *in vivo* between rats and mice in the cytokinetics of the cell proliferation involved in hippocampal neurogenesis (Snyder et al. 2009). The work from the present study highlights the importance of considering species when modelling this type of process *in vitro*. It would be beneficial to complete further studies investigating the direct effects of chemotherapy *in vitro* on NSC cultures isolated from the rat hippocampus to establish a cell culture model for chemotherapy-induced cognitive impairment. It would be interesting to directly compare the effects of different chemotherapy agents and fluoxetine on NSC isolated from both species and to ideally create a model where fluoxetine prevents the 5-FU induced reduction in proliferation, to further understand the mechanisms by which fluoxetine acts.

6.5 Experimental critique and future considerations

6.5.1 The use of a larger range of behavioural tasks

In clinical studies, a range of cognitive domains have been reported to be affected by chemotherapy (see Table 1.1) including working memory and visuospatial ability in which the hippocampus is thought to play a role (Baddeley et al. 2011; Loureiro et al. 2011; Sharma et al. 2010). One approach to further investigate the effects of 5-FU and MTX on hippocampal-dependent memory would be to use the MWM spatial memory task (Sharma et al. 2010). Previous studies in rodents have reported that MTX impairs ability in this task (Seigers et al. 2008; Seigers et al. 2009), but 5-FU was shown to improve animals' performance in the MWM in one study (Lee et al. 2006). When 5-FU and MTX were administered together, they also impaired animals' ability in this task (Winocur et al. 2006). Patient studies have also shown that other types of memory which are thought to be more reliant on brain regions other than the hippocampus such as executive function and language (Lum et al. 2011) are also affected (see Table 1.1). For this reason it would be interesting to investigate the effects of MTX and 5-FU on the ability of animals to perform non-

hippocampal dependent tasks to determine if the cognitive deficit observed extends to other brain regions.

6.5.2 The use of female animals

The majority of clinical studies investigating chemotherapy-induced cognitive impairment have been carried out on breast cancer patients, who are therefore females (see Table 1.1). However, the majority of studies in rodents including those in the present thesis were carried out in males (see Table 1.2). This was done to avoid any influence of the oestrogen cycle, which can affect neurogenesis in rodents (Galea et al. 2008; Mazzucco et al. 2006; Tanapat et al. 1999) and cognitive behaviour in humans and rodents (Galea et al. 2008; Maki and Dumas 2009; Sundermann et al. 2010). Although chemotherapy-induced cognitive impairment is not exclusive to females and has been reported in survivors of testicular cancer (Fung and Vaughn 2011), more studies using female rodents need to be considered for future work to make the results more relevant to breast cancer patients.

6.5.3 Drug interactions and the use of other antidepressants

It is important when administering more than one drug *in vivo*, to consider interactions between them. Fluoxetine inhibits cytochrome P450 enzymes (Alfaro et al. 2000) which are involved in the metabolism of a number of anti-cancer agents including 5-FU (Komatsu et al. 2000; Yamazaki et al. 2001) and CP (Kivisto et al. 1995; Rae et al. 2002). Indeed, the majority of SSRI antidepressants are reported to affect cytochrome P450 enzymes (Nemeroff et al. 1996). Therefore the efficacy of chemotherapy agents needs to be monitored when fluoxetine is co-administered. It would also be beneficial to examine the effects of other antidepressants, as they are also likely to affect cognition and hippocampal cell proliferation, when co-administered with chemotherapy. Researchers have already shown that cognitive performance and neurogenesis can be upregulated by antidepressants other than fluoxetine or SSRIs, including tianeptine (Akyurek et al. 2008; Kasper and

McEwen 2008) and imipramine (Hitoshi et al. 2007). A likely common factor is the action of antidepressants in increasing BDNF levels.

6.5.4 Alternative potential methods to counteract chemotherapy-induced cognitive impairment

Antidepressants are not unique in being able to act as neuroprotectants and a number of growth factors including CTNF and IGF-1 have been shown to act this way (Cool et al. 2005; Grunbaum-Novak et al. 2008; Janelsins et al. 2010; Lee and Son 2009; Shimazaki et al. 2001). It would be interesting to see if these agents can prevent the cellular and cognitive effects of chemotherapy and provide alternative strategies in helping patients with this treatment. In the present study we investigated the effects of the chemotherapy on BDNF and this is discussed in the next section (6.5.5).

A recent study demonstrated that donepezil, a cognitive-enhancing drug normally used for the treatment of Alzheimer's disease, was effective in reversing the cognitive impairments caused by a combination of MTX and 5-FU in a battery of memory tests in mice (Winocur et al. 2011). Although this finding needs confirmation, as only a single study has been carried out, it provides a promising alternative to fluoxetine as a potential pharmaceutical treatment for chemotherapy-induced cognitive impairment.

Another potentially useful approach to counteract the effects of chemotherapy-induced cognitive impairment could be to increase exercise. Physical activity has been shown to enhance hippocampal dependent cognition (Creer et al. 2010) and neurogenesis (Kim et al. 2010; Uda et al. 2006) in rodents and has been reported to be beneficial to sufferers of depression (Babyak et al. 2000). One study also reported that running had a greater effect on neurogenesis when compared to fluoxetine (Marlatt et al. 2010). Although it would be unlikely that patients undergoing chemotherapy would be able to perform vigorous exercise, it would be interesting to investigate if gentle exercise could positively enhance their cognition.

6.5.5 The role of BDNF

Measurement of the levels of BDNF and the microtubule associated protein, DCX, in the hippocampus and frontal cortex carried out in the present study did not show any effect of 5-FU or fluoxetine given alone or in combination. This was surprising, as DCX expression has been reported to reflect neurogenesis in the adult mammalian brain (Couillard-Despres et al. 2005). It is possible that the results found in the present study do indicate that there is no difference in hippocampal levels of DCX and BDNF. However it is also possible that the Western blotting technique used was not sensitive enough to detect the subtle concentration differences of these proteins when using whole brain structures. The availability of BDNF is regulated by fluoxetine (Duman et al. 2001b; Merz et al. 2011) and could be involved in the mechanism by which fluoxetine protects new hippocampal neurons. It would therefore be interesting to use more sensitive quantitative techniques such as real time polymerase chain reaction or immunohistochemistry to clarify if levels of DCX and BDNF, and the locations of the cells containing these proteins, are altered by chemotherapy or fluoxetine. These techniques could also be used in future studies to better determine the levels of other growth factors which are reported to influence neurogenesis (see Table 1.3).

6.5.6 The effect of stress on cognition and neurogenesis

Stress can affect both neurogenesis and cognition (reviewed in Dranovsky and Hen 2006; Samuels and Hen 2011) (see section 1.4.1, Chapter 1). Therefore, in the present study, measures were taken to minimise the stress experienced by the animals. However, as many of the animals were administered with chemotherapy agents or fluoxetine and all animals received multiple injections, it is possible that they experienced some stress or anxiety during the experiments. It would have been interesting to monitor stress or anxiety levels in the experiments by behavioural tests such as the open field, forced swim or tail suspension tests. However, it is likely that these tests in themselves may have caused a large amount of stress and affected the animals' behaviour and neurogenesis.

An alternative method would have been to measure blood levels of corticosterone which are elevated in stressed animals (Heine et al. 2004). This would have allowed comparison of stress levels between treatment groups to determine the stress induced by chemotherapy. Corticosterone can effect plasticity in the hippocampus (Maggio and Segal 2010) and it would therefore it would be beneficial to investigate the effect of stress in future studies.

6.5.7 Immunohistochemistry of the hippocampus

In the present studies, cell proliferation was quantified by counting Ki67-positive cells and cell survival by quantifying the number of BrdU-positive cells after the end of treatment. Both Ki67 and BrdU antibodies are markers for dividing cells, however they are not specific to neurons (Kempermann 2006; Scholzen and Gerdes 2000). Therefore, it is likely that some of the proliferating cells detected were destined to become glial cells, although it has been estimated that 89% of dividing cells in SGZ of the adult rat hippocampus become neurons (Snyder et al. 2009). It would be useful in future studies to double label the cells with glial and neuronal markers, to see the exact extent of the effects of chemotherapy and fluoxetine on neurogenesis and whether these treatments are affecting the ratio of neurons to glia generation.

Furthermore, it would be beneficial to determine the regional specificity of chemotherapy and fluoxetine within the hippocampus as distinct regions of the hippocampus are involved in different behaviours (Bannerman et al. 2004; Bast et al. 2003). The dorsal hippocampus has connections to the prefrontal cortex, amygdala and hypothalamus and is associated with emotional state and anxiety (Kjelstrup et al. 2002), whereas the temporal hippocampus is primarily involved in spatial learning, receiving connections from the dorsolateral entorhinal cortex (Fyhn et al. 2007; Moser and Moser 1998). Neurons in the intermediate region of the hippocampus encode rapid place learning and behavioural performance (Bast et al. 2009). Therefore it would be interesting to determine if chemotherapy primarily decreases

neurogenesis only in the temporal hippocampus or consistently throughout the structure and to observe the protective effects of fluoxetine in different regions.

6.6 Conclusion

The findings from the present project revealed that the chemotherapy agents, 5-FU and MTX, but not CP, negatively affect hippocampal dependent spatial learning. In addition, this reduction in cognitive ability correlated with a decrease in the proliferation and survival of new cells in the dentate gyrus of the hippocampus. This provides evidence that adult neurogenesis may be involved in the mechanism of chemotherapy-induced cognitive impairment. Furthermore, it was demonstrated that the behavioural and cognitive deficits observed could be counteracted by the SSRI antidepressant, fluoxetine, when it was co-administered before and during chemotherapy treatment but not when administered only after. However, the exact mechanism by which fluoxetine has neuroprotective effects needs further work and clarification.

In conclusion, the findings of the present thesis highlight the importance of considering individual agents when investigating the effects of chemotherapy on cognition. Furthermore, these findings provide insight into the mechanisms underlying chemotherapy-induced cognitive impairment and if the results shown in the present animal model are translatable to humans, they have the potential to offer a relatively simple method to alleviate the cognitive problems experienced by cancer survivors.

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Appendix I

Reagents for Western blotting

(All materials for Western blotting are from Sigma-Aldrich, USA, unless otherwise indicated).

Lysis buffer

20mM Tris (Invitrogen, USA)	12.1g
1mM Ethylene Glycol Tetraacetic Acid (EGTA) (Sigma-Aldrich, USA)	1.9g
320mM Sucrose (BDH Laboratory supplies, UK)	51.7g
1% Triton X100	500µl
1mM NaF	0.021g
10mM Beta glycerophosphate	1.08g

The above reagents were dissolved in 500ml of dH_2O and the solution was adjusted to pH 7.6. One mini protease inhibitor tablet (Roche, Germany) was dissolved in 10ml of lysis buffer prior to use.

2X Solubilisation buffer

0.5M Tris	2.5ml
Glycerol	2.0ml
10% SDS	2.0ml
Beta mercaptoethanol	1.0ml
2.5% Bromophenol Blue	40µl
dH_2O	500ml

Lowry solution A

100mM NaOH (Fisher Chemicals, UK)	2g
7mM SDS	1g
187mM $NaCO_3$	10g
dH_2O	500ml

Lowry solution B

1.0% $CuSO_4$ (Fisons Scientific Apparatus, UK)	100µl
2.0% NaK Tartrate (BDH Laboratory supplies, UK)	100µl
dH_2O	40ml

10X Electrophoresis buffer

250mM Tris (Invitrogen, USA)	30.3g
1.92 Glycine	144g
35M SDS	10g

The above components were dissolved in 1ltr of dH_2O. To prepare a working solution of electrophoresis buffer, 50ml of 10X buffer was diluted in 450mh dH_2O.

Transfer buffer

25mM Tris (Invitrogen, USA)	30.3g
1.92M Glycine	144g
dH_2O	8Ltr
Methanol	2Ltr

The transfer buffer was stored below 4°C.

TBST 0.1%

25mM Tris (Invitrogen, USA)	30.29g
125mM NaCl (Fisher chemicals, UK)	73.12g

The above reagents were dissolved in 1ltr dH_2O and adjusted to pH 7.6 then made up to 10ltr with dH_2O and 10ml Tween 20 added.

One 10% SDS-polyacrylamide gel is comprised of the following:

4% Stacking gel	
30% Acrylamide	3.12ml
0.5M Tris-HCl	6ml
10% SDS	0.24ml
10% Ammoniumpersulphate (APS)	0.12ml
Tetramethylethylenediamine (TEMED)	0.024ml
dH_2O	14.6ml

10% Resolving gel

30% Acrylamide	10.12ml
Resolving gel-buffer 1.5M Tris-HCl	8ml
10% SDS	0.32ml
10% Ammoniumpersulphate (APS)	0.16ml
Tetramethylethylenediamine (TEMED)	0.032ml
dH_2O	13.12ml

Appendix II

Reagents for cell culture

(All materials for cell culture are from Sigma-Aldrich, USA, unless otherwise indicated).

Growth medium

Dulbecco's Modified Eagles Medium (DMEM)	500ml
FBS	50ml
L-glutamine	10ml
Pen/Strep	10ml

Serum free medium with 0.3mM dibutyryl cAMP

Dulbecco's Modified Eagles Medium (DMEM)	15ml
L-glutamine	300µl
Pen/Strep	300µl
30mM dibutyryl cAMP	150µl

Neural stem cell (NSC) medium

Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12 1:1, Invitrogen, USA)	48ml
Neurobasal media (Invitrogen, USA)	48ml
N2 supplement (Invitrogen, USA)	1ml
B27 supplement (Invitrogen, USA)	2ml
0.5% Pen/Strep	0.5ml

The above components were kept as stock and EGF and FGF (both 20ng/ml) were added fresh prior to use).

NSC medium for MTT assay

Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12 1:1, Invitrogen, USA)	9ml
FBS	1ml

Appendix III

Haematocytometry

To count the cells, firstly the cell suspension was thoroughly mixed. A 10μl sample was taken from the suspension and transferred of the chamber of an Improved Neubauer Hemocytometer where it was drawn under the cover slip from the pipette by capillary action. The fluid should run to the edge of the grooves bordering the chamber but not over. The viable cells, with a bright round appearance, within the 5 diagonal squares within the central $1mm^2$ were counted. The viable cell concentration was counted calculated using the following equation.

$$c = (n \times d)/v$$

c was the cell concentration (cells/ml)

n was the average number of cells counted (from each square)

v was the volume counted (ml)

d was the dilution of the cell suspension

The depth of the chamber was 0.1mm and v was $0.1mm^2$, or $1 \times 10^{-4}ml$.

The total number of cells = sample concentration (cells/ml) × original volume of cell suspension from which the sample was taken.