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14. ABSTRACT The primary objective of this project was to develop an animal model to measure possible cognitive deficits resulting from chemotherapy. We have established a rat model of multidose, human-equivalent combination chemotherapy using cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) that recapitulates the physical symptoms of chemotherapy treatment. We investigated the effects of this regimen on learning and memory, as well as on the production and survival of new neurons in the hippocampus (neurogenesis). We found no statistically significant differences between control and CMF animals on any measure of learning or memory, nor with respect to neurogenesis.					
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INTRODUCTION

As cancer diagnostics and treatments improve, patients live longer and healthier lives, but not necessarily free of potentially debilitating complications. Many chemotherapy recipients report difficulty with attention and memory (known generally as “chemobrain”) that can affect their work and home lives for indefinite periods of time. Our goal has been to develop the first comprehensive animal model (using the rat) of human-equivalent combination chemotherapy for breast cancer, and to assess subsequent cognitive, behavioural and neurological changes. Such a model could then be employed, not only to highlight potential mechanisms or markers for cognitive changes that could be followed in human patients, but also to test potential therapeutics to prevent or alleviate the symptoms of “chemobrain”.

BODY

The primary goal of the work was to develop the first comprehensive rat model of systemic chemotherapy, adapting common regimens used in breast cancer treatment. Our particular interest in such a model was to assess changes in the structure and function of the brain (cognitive and neurological changes) resulting from chemotherapy use. However, a model of this kind could have much broader applications for assessing many toxicologic and physiologic changes, as well as potential concurrent therapies.

In the original proposal for this award, we outlined three objectives, such that developing a rat model of combination chemotherapy would “enable us to assess 1) the type and degree of damage done to brain cells, and 2) the effects on learning and memory which may occur as a result of the standard chemotherapy treatments given for breast cancer”. Our further goal was to “then 3) evaluate a proven neuroprotective peptide in our model to assess cognitive deficit prevention”. An extension of this is the development or testing of therapeutics that can reduce or reverse any neurotoxicity which might be caused (and hopefully definitely demonstrated by success in establishing a rat model of) combination chemotherapy.

The Statement of Work established a primary Task 1 to this project (development of the animal model), and a secondary Task 2 which was contingent upon the Outcome of Task 1.

In Task 1 we were able to produce a model, and analyze the effects of chemotherapy on multiple behaviours as well as brain pathology (specifically, neurogenesis). In this respect, we met the first 2 of our 3 objectives. However, lack of any significant differences between control and chemotherapy-treated animals by the behavioural measures we employed, or at the level of neurogenesis, led us to postpone and ultimately forego Task 2 of the study, and focus more closely on analysis of the Task 1 animals. For practical and ethical reasons, there was no point to testing the neuroprotective peptide on new animals without significant indices by which to grade cognitive deficits resulting from chemotherapy.

A critical component to this work was whether or not we could establish the model, using common cocktails of chemotherapeutic drugs. If that were a success, then the two principal questions which we sought to address were:

1. Does chemotherapy affect learning and memory in rats?
2. Does this correlate with neurogenesis, the integration of new neurons, or brain pathology?

Chemobrain: is it real?

The most common treatment for breast and ovarian cancer is multi-dose, combination chemotherapy. While highly effective at addressing the tumour(s), chemotherapy often carries a number of adverse side effects. Time and again clinicians and varying reports in the literature indicate that subsets of patients display symptoms of “chemobrain”, an inability to concentrate or learn new tasks and reduced memory capacity. These symptoms can last for years following therapy and in some cases may be irreversible, making chemobrain a potential burden on the individual’s ability to contribute to their family and community. A recent workshop on cognitive effects of chemotherapy identified 5 priorities for this field, two of which were: (1) “development of animal models ... to address mechanisms that might cause cognitive impairment associated with chemotherapy”, and (2) “development of interventions to alleviate these problems” (Tannock et al., 2004).

Initial reports of cognitive deficits in patients indicated significant declines in a subset of the population treated with adjuvant chemotherapy. However, the argument was made that these studies failed to evaluate cognitive status prior to treatment. Subsequent studies have revealed these declines in cognitive function are significantly related to treatment, leading to a 3.3-fold increase in the proportion of patients who underperform (Stewart et al., 2007)

The types of learning and memory reportedly most affected by chemotherapy are declarative memory, which rely upon the hippocampal formation. This is also one of the two regions in which neurogenesis has been identified in adult animals and in humans (Eriksson et al., 1998). The other is the subventricular zone (SVZ), from which progenitors travel along the rostral migratory stream to the olfactory bulb (Curtis et al., 2007). Recent reports suggest that the SVZ may in fact be the source of all neural progenitors in the adult brain, and that a founder population of quiescent neural progenitors from the SVZ establishes itself in the Granule Cell Layer (GCL) of the hippocampal dentate gyrus (DG), to then generate amplifying neural progenitors for the hippocampus (Encinas et al., 2006). The fact that new neurons are produced in brain regions responsible for learned behaviour and for scent has led many to pursue studies that would link neurogenesis itself directly to learning, however this remains unproven.

We sought to remove the subjective component of cognitive assessment that is inherent in many human measures, by employing an animal system to model cognition during chemotherapy.

Materials and Methods

All animal behavioural studies and immunohistochemistry were performed by blinded observers to ensure no bias. Statistical analysis was performed using the two tailed T-test, with a significance if $p < 0.05$.

Animals

All animal experimentation was carried out under approval from the University of Auckland Animal Ethics Committee (under applications P397 and R493), and under approval from the USAMRMC Animal Care and Use Review Office (ACURO). Female Sprague-Dawley rats (11-21 weeks old, 200 - 300 g) were obtained from and kept in the University of Auckland Vernon Jeffries Unit. They were housed 2 per cage, kept on a 12 hour light-dark cycle, and fed standard chow and water *ad libidum*. All animals were euthanized by an overdose of anaesthetic (pentobarbital), and perfused by cardiac puncture with saline, followed by 4% formalin. Brains were immediately harvested and stored in 30% sucrose prior to processing tissue for analyses.

Chemotherapy Protocol

In designing our chemotherapy protocol, we tried to model human-equivalent doses and scheduling. Much of the preliminary work that suggested cognitive deficits in patients used older data sets, from which the CMF regimen (cyclophosphamide, methotrexate, 5-Fluorouracil) was the predominant therapy. Many countries have since supplanted use of methotrexate in lieu of adriamycin (doxorubicin). We therefore wished to evaluate both CMF and CAF cocktails.

There was sufficient evidence in the literature to suggest the chemotherapy drugs, when administered intraperitoneal (i.p.), would penetrate into the brain and have potential toxic effects on the brain regions implicated in learning and memory.

Methotrexate has been shown to cross the blood brain barrier (BBB) when given peripherally to rats (Cosolo and Christophidis, 1987). In male rats, repetitive low dose administration of methotrexate has been shown to damage cells in the hippocampus (but this was only studied 24 hours following the 3rd dose) (Gregorios et al., 1989). Studies using cyclophosphamide in young and old rats demonstrated it too crosses the BBB when administered peripherally, and caused toxicity within the dentate gyrus of the hippocampus (but was only studied at much higher doses than we use, and only checked within 24 hours of injection) (Rzeski et al., 2004). 5-Fluorouracil (5-FU) has been shown to penetrate the BBB in animals, including primates (Bourke et al., 1973). There is less evidence of direct effects of adriamycin on the brain, as most animal studies

have focused on the cardiotoxic effects (Suzuki et al., 2001). Reports of maximum tolerated doses of adriamycin, however, provided baselines for its application in our model (Linden, 1990).

We administered combination CMF or CAF at 65/6.5/65 mg/kg i.p. to Sprague-Dawley females aged 80-150 days and weighing 170-300 grams. These doses were scaled to human equivalent doses (HEDs) by the toxicology conversion formula used by the U.S. Food and Drug Administration (FDA, 2005), whereby a human dose in mg/m² is the equivalent of 6 times a rat dose in mg/kg. A typical human CMF or CAF regimen of 500 mg/m² cyclophosphamide, 50 mg/m² methotrexate (or adriamycin), and 500 mg/m² 5-Fluorouracil would translate to (500/6 = 83.3 mg/kg) 83/8.3/83 mg/kg C/M/F or C/A/F. Based on the reported cardiotoxicity of adriamycin, and literature indicating its use alongside 5-FU may minimize toxicity (Stathopoulos et al., 1998), we opted to lower the dosage somewhat so as to reduce the chances of excessive toxicity, but not to eliminate drug efficacy. We therefore applied 65/6.5/65 mg/kg doses, anticipating a full regimen of 4 cycles (injections every 21 days: Day 1, 22, 43, 64).

Animals were evenly distributed by age and assigned to either Control or CMF groups (n=12 per group). On Days 1, 22, and 43 each animal received 3 separate injections intraperitoneally (i.p.). CMF animals received 65 mg/kg cyclophosphamide (Cytosan, Bristol-Myers Squibb, Princeton, NJ; 50 mg/mL), 6.5 mg/kg methotrexate (methotrexate BP, Mayne Pharma, Melbourne, Australia; 2 mg/mL), and 65 mg/kg 5-FU (Fluorouracil Injection BP, Mayne Pharma, Melbourne, Australia; 50 mg/mL). Control animals received saline (0.9% NaCl) injections of equivalent volumes to each of the 3 drugs (as calculated per kg body weight).

An additional group of 12 animals were assigned to the CAF group. These received 2 rounds (Days 1 and 22) of 3 injections each of 65 mg/kg cyclophosphamide, 65 mg/kg 5-FU, and 6.5 mg/kg adriamycin (doxorubicin hydrochloride, Mayne Pharma, Warwickshire, UK; 2 mg/mL). Following the first round of injections, some of these animals exhibited signs of toxicity (weight loss, porphyrin staining around the eyes), but recovered within several days. Immediately following the second round of injections, several animals developed ascites and signs of severe toxicity, and all 12 CAF animals were euthanized for humane reasons.

Labeling Proliferating Neurons with BrdU

Bromodeoxyuridine (BrdU) is a thymidine analogue which becomes incorporated into proliferating cells during S-phase (DNA synthesis). 10 weeks after the final chemotherapy injections, animals received 2 injections of BrdU (Sigma, St. Louis, MO), reconstituted to 20 mg/mL in saline plus NaOH to pH~7. Each BrdU

injection was 200 mg/kg administered i.p., 48 hours apart. This dose (200 mg/kg) has been established as a saturating concentration which labels most, if not all S-phase cells in the DG during the approximately 2-hr period of bioavailability (Cameron and McKay, 2001; Eadie et al., 2005). A recent review notes that doses of BrdU up to 300 mg/kg have “no physiological side effects like weight loss or behavioural changes in adult rats, and no apparent toxic effects on dividing cells in the DG” (Taupin, 2007). Drugs which breach the blood-brain barrier (BBB), including chemotherapy drugs, could affect permeability and the subsequent labeling efficiency of proliferating neuronal progenitors. An important advantage to the use of BrdU is that it penetrates the BBB, providing equal availability to the proliferative marker in both treated and non-treated animals regardless of any effects from chemotherapy on BBB permeability (Taupin, 2007).

Another consideration for our studies is the potential compound influence of hippocampal damage and behavioural testing itself on rates of neurogenesis. This issue has been addressed in previous work, whereby levels of neurogenesis in animals suffering stroke were shown to be unaffected by behavioral testing (i.e. neurogenesis was the same in stroke only animals and in stroke + behavioural testing animals) (Briones et al., 2005)

Brain Pathology

Perfused and formalin-fixed brains were sectioned on a cryostat to provide 40 um thick coronal slices throughout the hippocampus (see Fig 1).

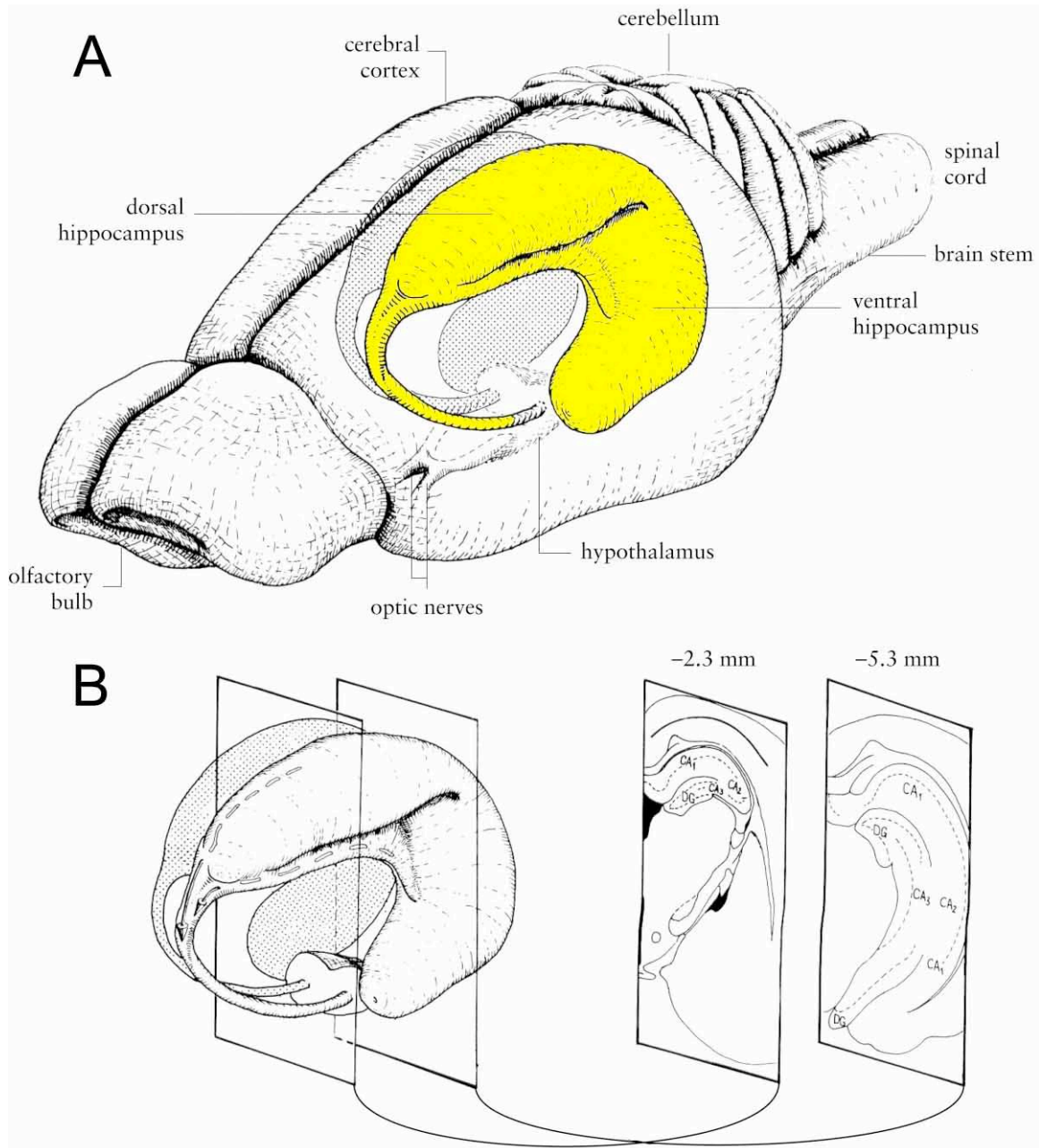


Figure 1 The rat hippocampus. A) The characteristic horn-shape of the hippocampus, with the left hemisphere highlighted in yellow. B) Coronal sections 40 μ m thick (from approximately bregma -2.3 to -5.3 mm) were cut and processed for immunohistochemistry. The dentate gyrus (DG) within the hippocampus is the region where neurogenesis takes place. Figure adapted from (Cheung and Cardinal, 2005).

Immunohistochemistry Procedures

BrdU Histology Protocol

For peroxidase BrdU-immunolabeling, methodology was adapted from Parent (Parent et al., 1997), based on the work of Kuhn (Kuhn et al., 1996). Briefly, brain sections were denatured prior to treatment with anti-BrdU antibody and peroxidase exposure, mounted on slides, and analyzed under the microscope to identify BrdU-positive cells in the hippocampal dentate gyrus.

Free-floating coronal brain sections 40 μ m thick were denatured by treatment with 50% formamide/2xSSC (0.3M NaCl, 0.03M sodium citrate) at 65°C for 2 hrs, rinsed 10 min in 2xSSC, then treated for 30 min at 37°C in 2N HCl, neutralized for 10 min in 0.1M Boric acid (pH 8.5), then washed 3x5 min at room temperature in phosphate buffered saline (PBS). Any remaining endogenous peroxidases were blocked by a 30 min wash in 50% methanol/1% H₂O₂ and washed 3x5 min in PBS.

Sections were blocked at room temperature for 5-12 hrs in immunobuffer consisting of PBS + 0.2% Triton-X100 (PBST) + 4% horse serum. Following blocking, primary antibody mouse anti-BrdU (cat. no. 1170 376; Boehringer Mannheim) was applied at 1:200 in immunobuffer for 24 hrs, then rinsed 3x5 min in PBST. Secondary anti-mouse IgG-Biotin (Sigma) at 1:200 in immunobuffer was applied for 3 hrs, then the sections were again rinsed 3x5 min in PBST. Sections were then incubated with Extravidin Peroxidase (Sigma) at 1:250 in immunobuffer for 2 hrs, washed 3x5 min in PBST, and the conjugated peroxidase activity reacted with diaminobenzidine (DAB) for approximately 5 min (until sufficient brown coloration arose).

Sections were rinsed in PBST, and stored overnight at 4°C in PBS prior to mounting on polylysine-coated microscope slides (Esco). Slides were air-dried 24-36 hrs at room temperature. Some slides were counterstained by using 0.5% cresyl violet for 2 hrs prior to all slides being dehydrated in graded ethanols, cleared with Xylene, and cover-slipped under Cytoseal 60 mounting medium (Richard-Allan Scientific).

Other Immunohistochemistry

Briefly, other primary antibodies used were against glial fibrillary acidic protein (GFAP), NeuN, proliferating cell nuclear antigen (PCNA), and others. TUNEL staining (using Chemicon ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit S7111) was performed on some sections, as was Fluorojade B staining, to

detect overall cell death or neuronal degeneration/death, respectively. For confocal immunofluorescence work, free-floating sections were denatured (as above), and incubated in mouse anti-BrdU mixed with other primary antibodies at 4°C overnight. Secondary antibodies conjugated to fluorescent dyes (AlexaFluor 488, Cy3, Cy5) were used, and microscopy performed on Leica TCS SP2.

General Animal Observations

Weight Loss

While several observations were carried out regularly to monitor the wellbeing of the animals, weight gain and loss were by far the most informative indicators of overall animal health. As depicted, rats receiving CMF chemotherapy tolerated the first round (of 3 injections) quite well (Fig 2). A day following chemotherapy, animals appeared normal. Within 48 hours, weight loss became evident and the animals began showing obvious signs of fatigue or lethargy by 72 hours. Some of the chemotherapy animals began to show signs of pink skin pigmentation on the snout, ears, in streaks behind the eyes, and on the extremities of the limbs. Some also began to show signs of rough coat (hair standing on end) by 4-5 days post-injections. Generally, following the first round of injections, the rats began to put weight back on at the same or greater rate than controls injected with vehicle (saline) alone.

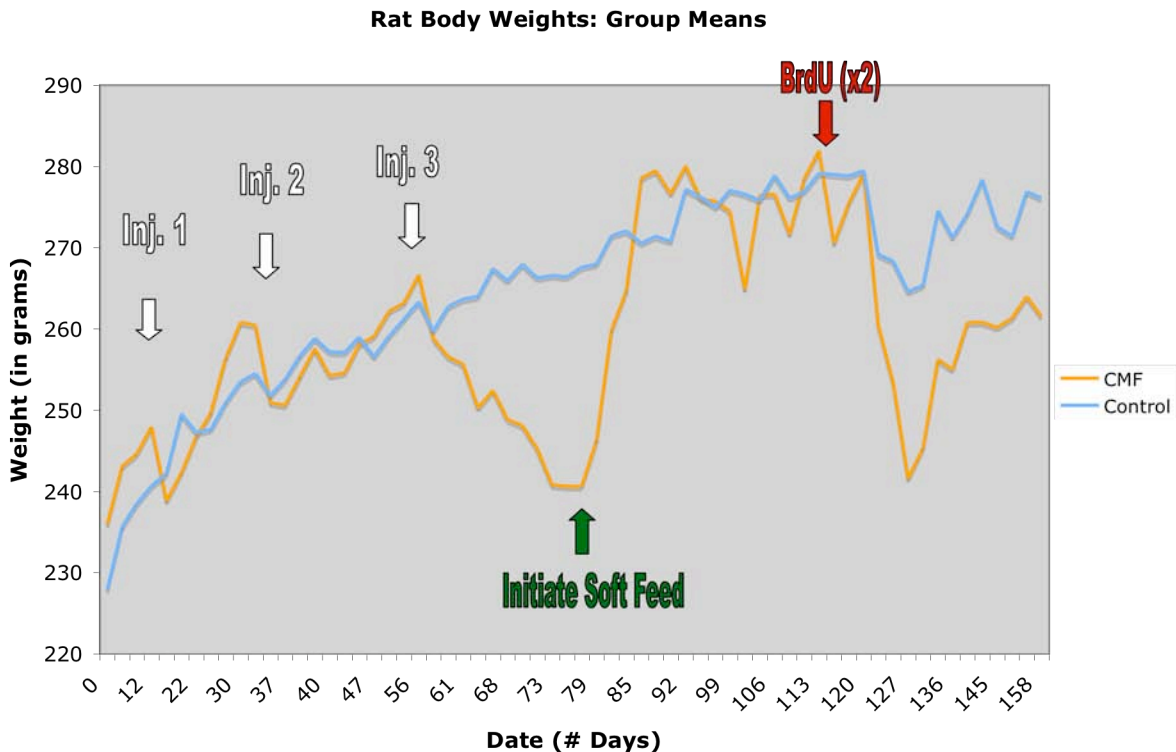


Figure 2 Group average body weights of rats receiving injections of saline (Control) or combination chemotherapy (CMF), n = 12 per group. Many CMF animals lost weight following the 3rd injection (Inj. 3) of chemotherapy, which was found to be the result of difficulties with hard feed pellets. All CMF animals regained weight once moistened pellets (soft feed) were introduced, with no effect on Control rats. The accidental removal of softened feed following BrdU injections resulted in another rapid drop in body weight, which had slower recovery once supplemented, suggesting possible toxicity from BrdU following chemotherapy.

The same pattern was recapitulated with the second round of chemo injections 3 weeks later, with the side effects being more severe and persisting several days longer. Some treated animals began to lose some hair, principally from the rump. The pink pigmentation to some skin areas intensified, and a hunched appearance was evident. Recovery, as marked by a halt of weight loss and some degree of return to weight gain, was slower following the second round than it was after the first round.

However, following the third round of injections, rats showed signs of toxicity that persisted for several days, and in a few exceptional cases they continued to lose weight gradually over the course of 3 weeks. For this reason, we chose not to continue with the scheduled fourth round of injections, and the animals were monitored more closely.

Because these animals continued to act normally, we hypothesized the weight loss may be due to factors other than toxic effects on the internal processing of nutrients or loss of desire to feed. As the standard rat chow consists of relatively large, hard and dry feed pellets, we decided to try softening the feed by pre-soaking it in water for several minutes. Those animals that demonstrated the most weight loss immediately approached the softened food dishes and ate well. We then decided to introduce dishes of this “soft feed” to all cages (Control and CMF alike), and the treated animals that had lost weight quickly put on weight over the subsequent days. Upon autopsy, we discovered those rats which had demonstrated the greatest weight loss all exhibited problems with their teeth and gums (including loss of teeth) which would have prevented them from feeding normally on the large pellets.

Another important observation from this figure is the weight loss which accompanied the two injections of BrdU. As discussed in the Materials and Methods section (above), injections of BrdU do not normally have physiological effects on rats. The rapid weight loss we observed is likely due, in most part, to the lack of supplementation with soft feed that occurred by accident over the course of the weekend following BrdU. However, given that these animals did not rapidly return to their pre-BrdU weights over the following month (during which behavioural testing took place) as they had previously (following the initial introduction of soft feed) suggests other factors are involved.

The stress of behavioural testing in of itself is likely a factor in the lower overall body weights, as Control animals also exhibited a drop, followed by a slow recovery at the same rate as treated rats. The compound effects of chemotherapy + BrdU must also be considered. As an analogue of thymidine, BrdU is incorporated into replicating DNA. 5-FU is also an analogue of thymidine, though the timespan between administration of the 2 drugs is sufficient to ensure no competition. The other chemo agents used in this study also function by interfering with DNA replication. While relatively little is known about the rat cellular machinery responsible for maintaining DNA integrity in this context, some degree of fatigue in DNA damage responses may have contributed to an overall deficit in the ability of these animals to recover completely following BrdU treatment.

Neurogenesis

Studies of neurogenesis focus on two principal features: 1) rates of cell proliferation and 2) survival of new cells. Different conditions, such as exercise, aging, or seizures can alter the rate at which new neural progenitors are generated in the dentate gyrus (DG). It is believed these cells must mature and become functionally integrated in order to survive, otherwise they are lost within 1 – 2 weeks (Overstreet-Wadiche and Westbrook, 2006). This process leads many to suggest that the act of learning itself may be carried out at the level of the cell through the integration of new neurons, enabling establishment of new synaptic connections into existing circuitry and hence consolidating memory.

BrdU Labeling (Cell Survival)

Cell counts were conducted for every sixth brain section (each 40 um thick) from bregma -2.5 to -5.0 (Paxinos and Watson, 1986), totaling 10 sections per brain. For each section BrdU-positive cells were counted in both left and right hemispheres of the hippocampus from within both the upper and lower blades of the DG: either at the border of the SGZ, within the granule cell layer, or within 2 cell widths outside the GCL (Fig 3).

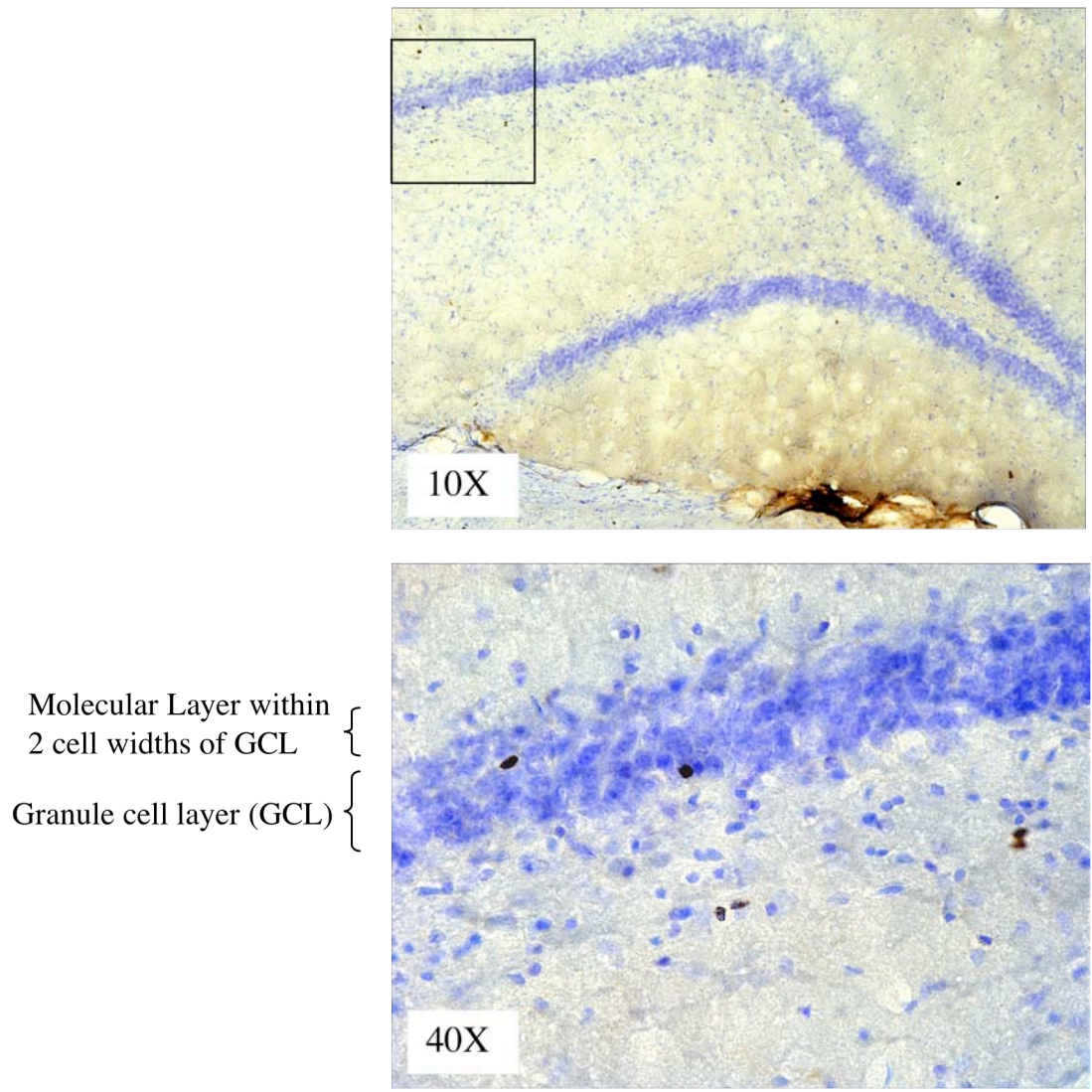


Figure 3 Survival of proliferating cells in the rat hippocampus after 30 days. Following 69 days recovery from chemotherapy, animals were injected i.p. with 2 x 200 mg/kg BrdU (48 hrs apart). Following behavioural testing, animals were killed 30 days later and brains processed for immunohistochemistry. 10 x 40 um sections were treated with anti-BrdU antibody per animal, representing every 6th section between bregma -2.5 and -5 mm. A representative section, counterstained with cresyl violet, is depicted under the light microscope with a 10X objective (top), and the boxed area in greater detail with a 40X objective (bottom). The numbers of BrdU+ cells in the Granule Cell Layer of the Dentate Gyrus, and/or within 2 cell widths of the GCL, were recorded to evaluate survival and functional integration of proliferating cells.

There were no statistically significant differences between group mean numbers of BrdU+ cells (Fig 4). However, a strong trend suggested several animals in the CMF group had fewer surviving cells 30 days post-BrdU injection.

Control	# BrdU+	CMF	# BrdU+
C1	11	M1	12
C1*	15	M1*	8
C2	38	M2	19
C2*	13	M2*	47
C3	24	M3	25
C3*	27	M3*	46
C4	32	M4	24
C4*	27	M4*	5
C5	62	M5	11
C5*	26	M5*	31
C6	30	M6	10
C6*	30	M6*	13
AVE	27.92	AVE	20.92
SD	13.43	SD	14.21
SE	4.05	SE	4.28

All BrdU counts in DG (Group Ave's)

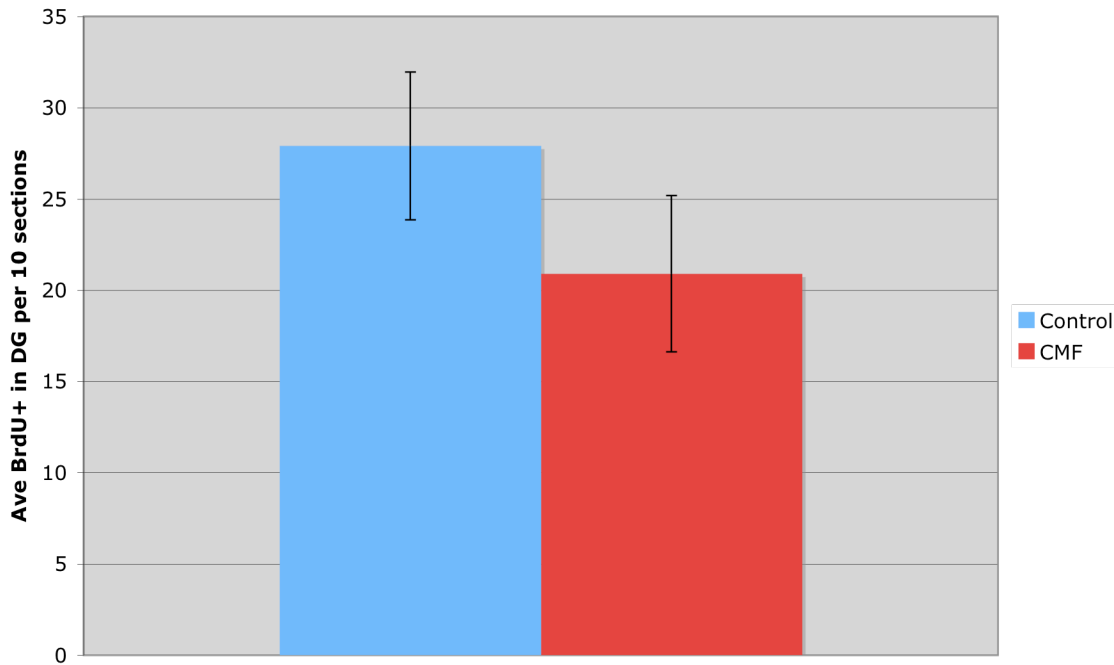


Figure 4 Numbers of surviving (BrdU-labeled) cells in the rat hippocampus after 30 days. Following 69 days recovery from chemotherapy, animals were injected i.p. with 2 x 200 mg/kg BrdU (48 hrs apart). Following behavioural testing, animals were killed 30 days later and brains processed for immunohistochemistry. 10 x 40 um sections were treated with anti-BrdU antibody per animal, representing every 6th section between bregma -2.5 and -5 mm. The total number of BrdU+ cells per 10 sections per animal is shown in the table (top), and the group means depicted in the graph (below). There were no significant differences between groups regardless of whether the counts included the 2 cell width region outside the GCL (data not shown).

Neuronal Differentiation

While BrdU labeling for cell survival was our principal measure of possible neurological effects of chemotherapy, we also briefly investigated several other conditions using immunohistochemistry. Confocal microscopy was employed to confirm the differentiated state of the BrdU-positive cells in several animals (Fig 5). These findings suggested that in both control and chemotherapy-treated rats, the majority of proliferating cells in the DG mature into neurons within the 30 day period between BrdU injections and animal sacrifice. While comprehensive co-labeling studies were not conducted for all animals in this manner, our observations from several brain sections on 3-4 animals per group confirm the general consensus from many other studies which compare different treatments (such as exercise or seizures) and their effects on both proliferation and differentiation, wherein the overall numbers of proliferating and surviving cells may change, but the relative proportions of mature neurons, astrocytes, and glia remain the same (Overstreet-Wadiche and Westbrook, 2006).

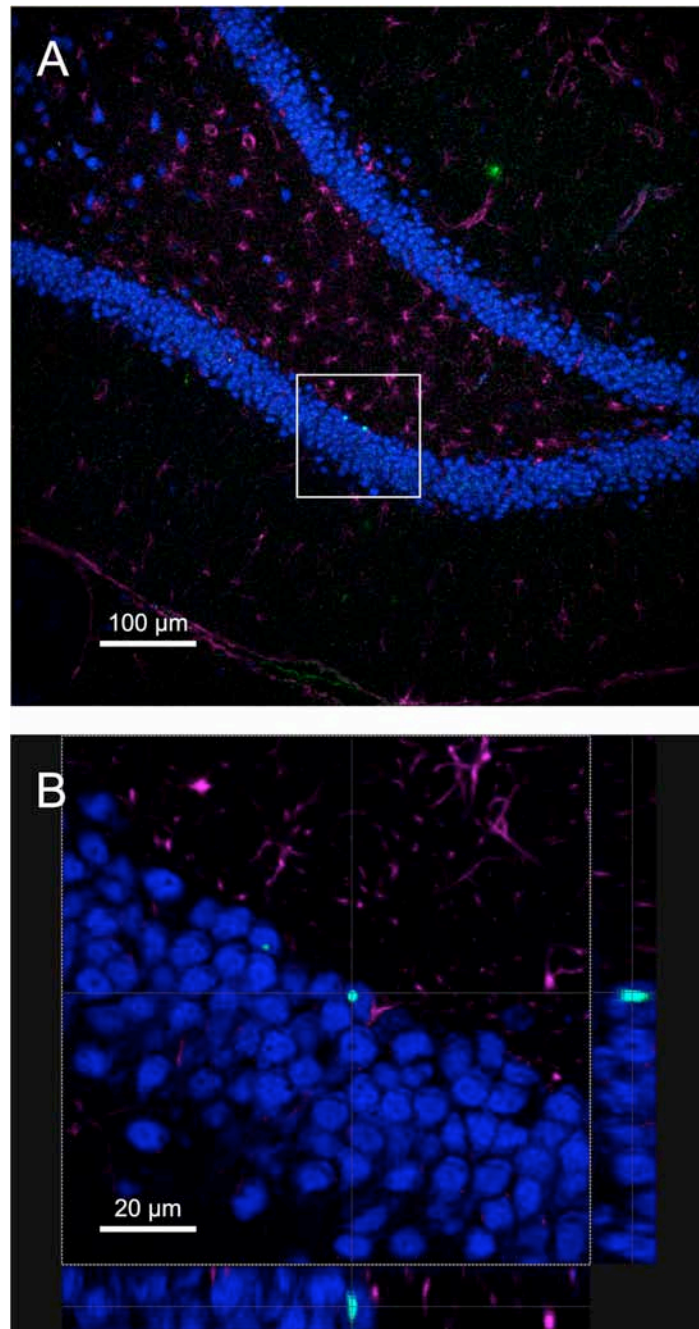


Figure 5 Proliferating cells in the hippocampus of chemotherapy-treated rats mature primarily into neurons within 30 days. Confocal microscopy was performed with triple labeling for BrdU (green), mature neuronal marker NeuN (blue), and astrocyte marker GFAP (magenta). A) 10X objective used to visualize two BrdU-positive cells in the subgranular zone of the hippocampal dentate gyrus, the region where neurogenesis takes place. B) 40X objective used to magnify the region boxed in A. Below and to the right of the image are stacked composites of optical sections, verifying the BrdU-positive signal is colocalized with a NeuN-positive mature neuron.

Behavioural Tests

All testing took place during the active (dark) cycle. All apparatus were cleaned with 70% ethanol between each animal, to remove scent cues (with the exception of the Morris Water Maze pool, from which feces were cleared between animals, and the pool entirely emptied and refilled daily).

Open Field

Test Methodology: Open Field

The open field test evaluates spontaneous locomotor activity and anxiety. This provides a baseline to exclude the possibility that differences exhibited in the other learning and memory tests are the result of motor deficits or anxiety, rather than impaired ability to learn. An empty circular tank is divided radially into segments of equal size, and concentrically into regions (see Fig 6). Each rat is placed in the center of the tank and allowed 5 min of free movement, during which a record is made of the number of times the rat's two front feet cross a line separating two segments. The more lines crossed, the more active the animal. The more time the animal spends interior to the outer ring indicates less anxiety, as rats naturally prefer enclosed spaces. Thigmotaxia is assessed by analysis of the fraction of time the rat spends within 10 cm of the wall of the tank (the outer ring). Each animal receives 1 trial only. Between each trial the tank is wiped with 70% ethanol.

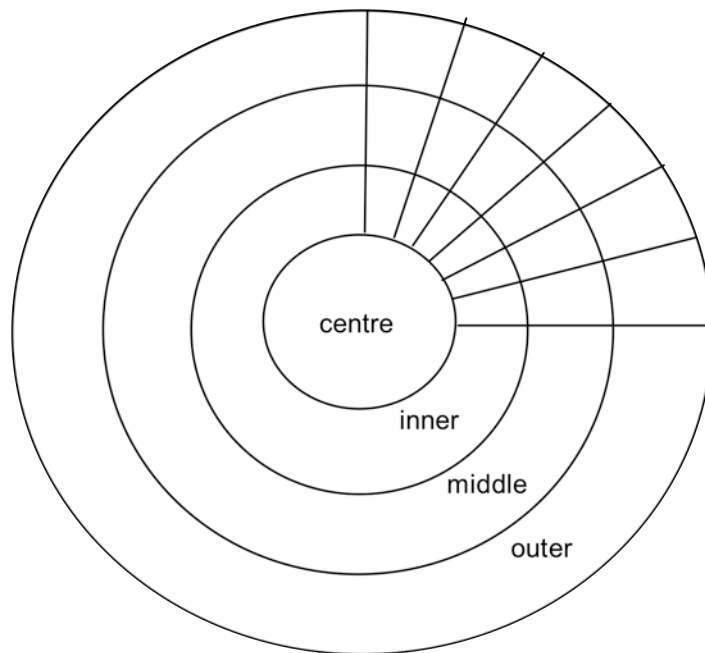
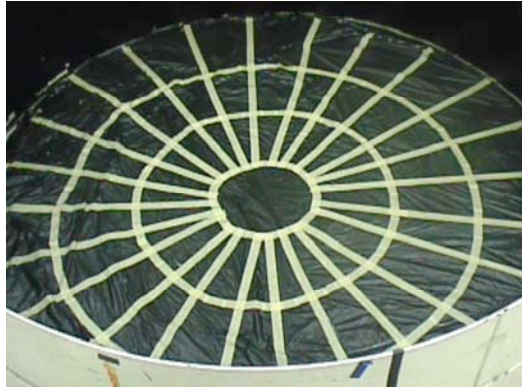
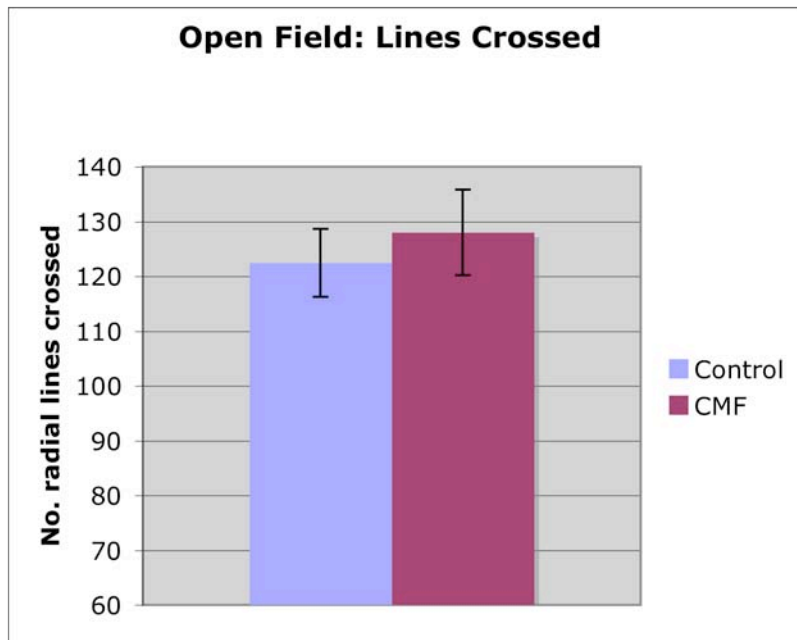


Figure 6 The Open Field apparatus. The emptied pool (top) with floor marked by concentric rings and radial lines (bottom). Rats are given 5 min to explore freely, and the number of lines crossed and time per ring recorded to establish activity and anxiety, respectively, prior to learning and memory tests.

Results: Open Field

There were no statistically significant differences between Control and CMF animals in the Open Field. Both groups spent approximately 95% of their time within 10 cm of the outer wall (in the outer ring), and crossed the same number of radial lines in the 5 min period (Fig 7). This indicates no differences between overall physical activity or fear of open spaces (a measure of anxiety) between control and chemotherapy-treated animals. As such, we expected both Control and chemotherapy-treated animals to be equally capable of physically performing the subsequent learning and memory tasks, with no differences in inherent stress levels.



Open Field: Times (Group Means)

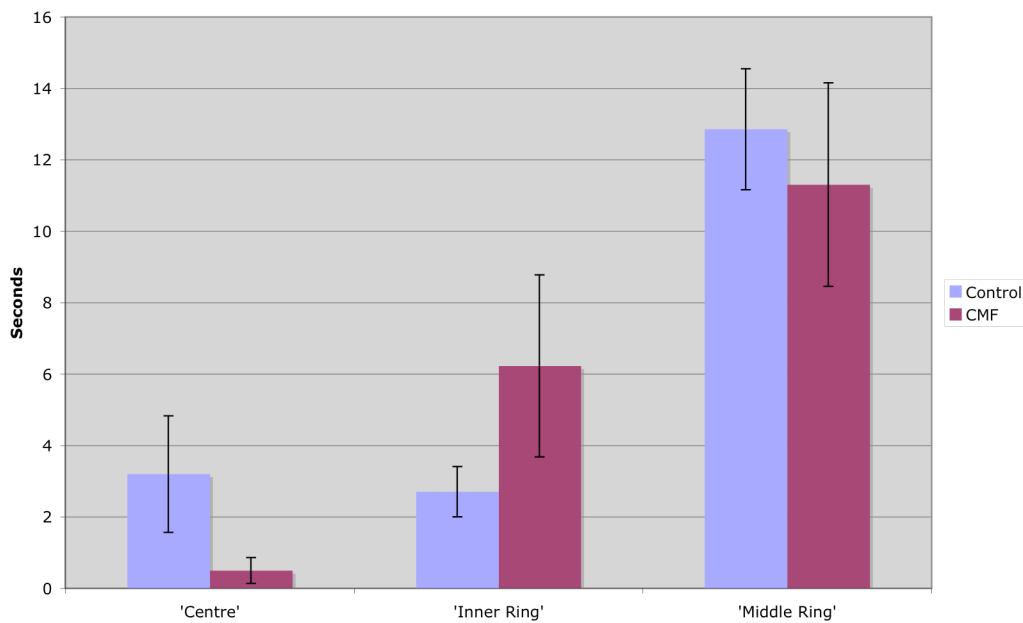


Figure 7 The Open Field test results. Top) No differences in overall physical activity levels were observed between control and chemotherapy-treated rats, as shown by the same number of radial lines crossed in the 5 min test period (group means). Bottom) No differences in anxiety were observed between groups, as shown by a lack of statistically significant differences in times spent in each ring. Time in outer ring not shown, for scale.

Novel Object Recognition (NOR)

Test Methodology: NOR

The rat's ability to distinguish between a new object and one previously seen before is measured by the amount of time spent exploring each. When objects are identical, the amount of time spent exploring each object is generally the same. However, the ability to remember which object was previously seen and which is new is reflected by more time being spent with the novel object than the old one. This provides a significant assessment of the ability to form and recall memories, in a hippocampal-dependent manner.

On Day 1 an individual rat is taken from their home cage and placed in an empty box, without any objects, for 15 minutes to familiarize itself with the arena before being returned to the home cage. On Day 2 the box is set up with 2 copies of the same object (such as a toy, can or block), one at each side of the chamber. The rats are left to explore the objects for 3 minutes before being returned to the home cage. One of the toy objects is removed and replaced with a different object, and the test re-administered 15 minutes, 4 hours, and 24 hours later (each time with 1 familiar and 1 new object). The amount of time the rat's nose/legs/body is within 2 cm of each object is scored. The box and objects are wiped down with 70% ethanol between trials.

Results: NOR

CMF-treated animals do not display any significant difference in preference for a novel object over a familiar object compared to control animals. Both Control and CMF rats show equal preference for the two identical objects at time=0 (the acquisition trial), as expected (Fig 8). When tested 15 min later, both groups show a preference for the novel object (implying they can recall the previous identical objects, and hence spend more time with a new object). However there is no statistically significant difference between the groups for their degree of preference for the new object. Even at 4 hrs, both groups explore another new object more than the original object, but again with no difference between groups. By 24 hrs both groups have returned to spending equal time exploring the original and another new object, indicating an equal degree of extinction of the memory of the original object.

NOR: % time spent with Novel Object

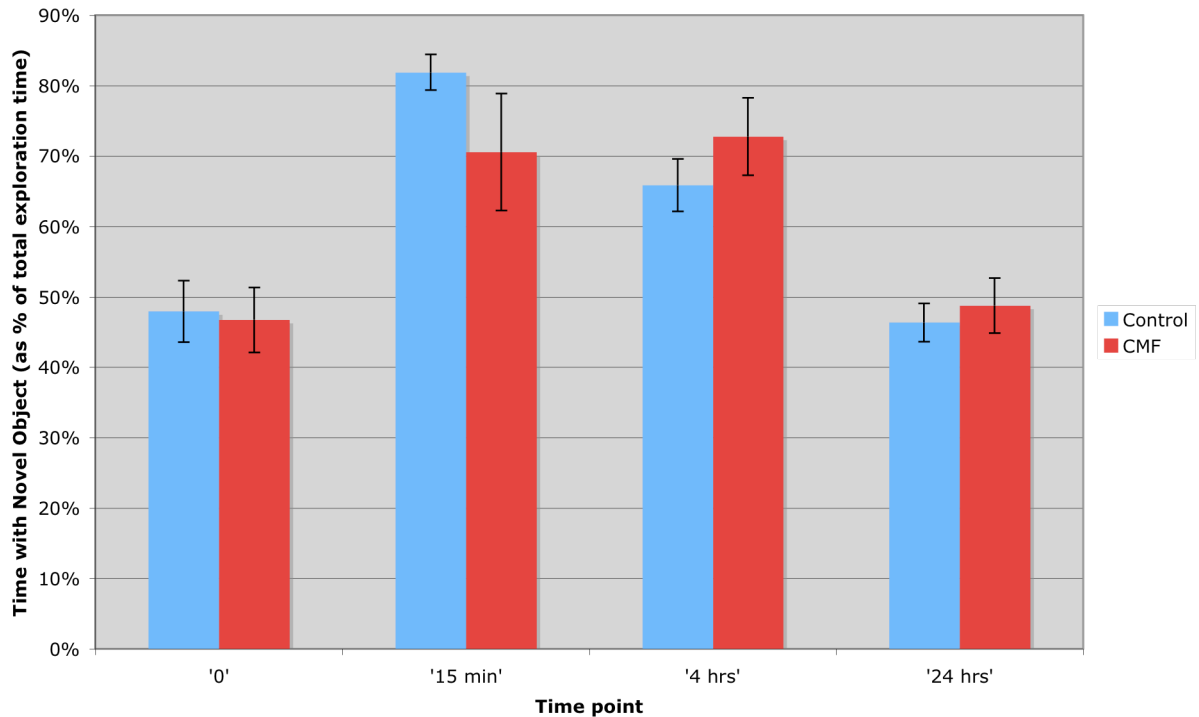


Figure 8 Proportion of exploration time (group means) spent with the new object in the Novel Object Recognition test.

Although chemotherapy did not seem to alter the working memory of rats in the NOR test, it did affect their exploratory behaviour. There is a clear trend demonstrating that chemotherapy-treated animals spend more time overall exploring all objects (Fig 9), although this only reaches statistical significance at the 24 hrs time point (two tailed t-Test; $p=0.028$). This increase in total exploration time in CMF animals could be the result of hyperactivity, heightened interest in the environment, or less inhibitions or fears. Hyperactivity would not be suspected, given the results of the Open Field test which indicated neither increased activity nor increased anxiety between groups.

Total Exploration Times

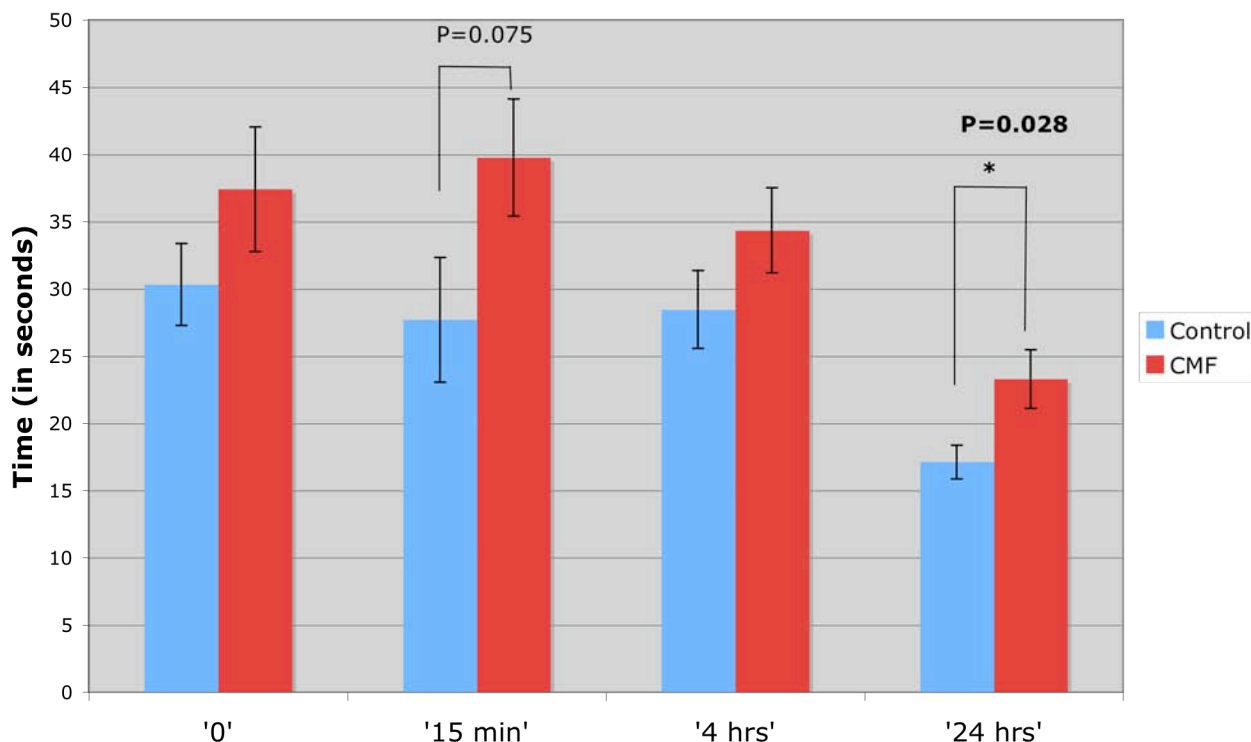


Figure 9 Total time (group means) spent exploring in the Novel Object Recognition test. At time=0, each rat was given 180s to familiarize itself with 2 identical objects, and then was tested at 3 subsequent intervals (15 min, 4 hrs, and 24 hrs later) where they were presented with 1 of the familiar objects and a different new object each time. While the trend suggests chemotherapy-treated rats spent more time exploring, this was only statistically significant (two-tailed t-Test, $P < 0.05$) at the 24 hrs time point.

There were no differences in NOR performance when animals were grouped by age and treatment group, nor by weight loss severity and treatment group either (data not shown).

An important consideration for multiple-time point retesting in the NOR is the degree of inherent interest of an object. A factor which we had not previously considered, and which we have not encountered in the literature, is that some objects may in of themselves be more interesting to a rat, and could potentially lead animals with deficits in recall to spend more time with an object. As such, use of this test would benefit from employing naïve animals of the required strain to assess potential objects for their degree of inherent interest, prior to introducing these objects to test animals. In our case, the Novel Object Recognition test does not reveal either an improvement or deficit in working memory or short-term recall following chemotherapy.

Morris Water Maze (MWM)

Test Methodology: Morris Water Maze

The Morris Water Maze (MWM) is the gold standard behavioural test for spatial learning and memory in rodents. Performance of this task is primarily dependent on the hippocampus. Though much more labor intensive than other tests, we chose to perform MWM to assess functional damage to the hippocampus which might result from chemotherapy treatment, as damage to the hippocampus is known to impair performance in this task (Moser et al., 1993).

A circular pool is filled with opaque water, and a platform is submerged just below the water surface. Spatial cues are provided (generally large unique shapes placed on each wall of the room, beyond the pool) to orient the position of the platform. Rats are competent swimmers, but naturally seek an escape from the water. A rat is placed in the pool repeatedly over several days for acquisition training, in which the rat learns of the existence of the platform. The position of the platform is fixed throughout the training. Later, the rat is tested for its ability to remember the location of the platform and swim to it. The expectation is that rats which are better learners and can remember the location of the platform will navigate and climb onto the platform faster. During the final probe trial, the platform is removed and the time spent in the quadrant of the pool where the platform had been is an index of the rat's spatial memory.

Apparatus:

The water maze is a circular pool (1.8 m diameter and 0.6 m height) which is filled to a depth of 27 cm with $26\pm 1^{\circ}\text{C}$ water. The escape platform is 17cm in diameter and is submerged 2cm below the surface of the water. A small amount (5ml) of non-toxic black tempura paint is added to the maze water to camouflage the platform, and provides a contrast which enables computer tracking of the albino rat. The position of the rat in the pool is monitored in 4 quadrants of the pool using a video tracking system.

Acquisition testing measures the ability to learn an optimal search strategy to find and remember the location of a hidden platform. The hidden platform is placed into the centre of one of the quadrants where it remains for the entire testing procedure. A trial consists of a rat placed in the water at the perimeter of the pool, facing outwards. The position of entry for the animal is varied at each trial such that each of the 4 cardinal start sites is used in each block of 4 trials, and the order altered pseudo-randomly each day. The rat is allowed 90 sec to find the platform. If the rat fails to find the platform in this time it is gently guided to it. Upon finding or being placed on the platform the rat remains there for 30 sec. Between each pair of trials the rat is placed in a covered holding cage for 1 min. Animals received 4 trials per day for 5 consecutive days.

The probe trial was carried out on Day 6, the day after acquisition testing. This trial involved removing the platform and allowing the rat to have a free swim for 120 sec. The probe trial measures the time and distance swum in the quadrant that previously contained the platform, and the number of times the rat crossed into a 30 cm area (15 cm radius) from the centre of the location where the platform was previously positioned. The probe trial gives a measure of the rat's knowledge of the precise location of the platform and the strength of memory retention.

On Day 8, a Visible Platform test is conducted to ensure no differences in vision, physical capacity, or motivation would influence the rat's ability to find the platform. The pool is filled several centimeters less, such that the platform is now visible above the waterline. 4 trials are again performed, as during acquisition, wherein each time the platform is moved to a different quadrant. The rat's path and time to locate and mount the platform is recorded.

Results: Morris Water Maze

There were no statistically significant differences between control and chemotherapy-treated animals by any measure in the MWM. There were no differences when animals were grouped by age and treatment group, nor by weight loss severity and treatment group either (data not shown).

Both groups learned the location of the platform at the same rate over the 5 day acquisition period (Fig 10). A trend emerged on Day 3 that suggested the CMF animals may have been acquiring faster than controls, however this was not statistically significant, and normalized the following day.

MWM Trial Averages (Acquisition)

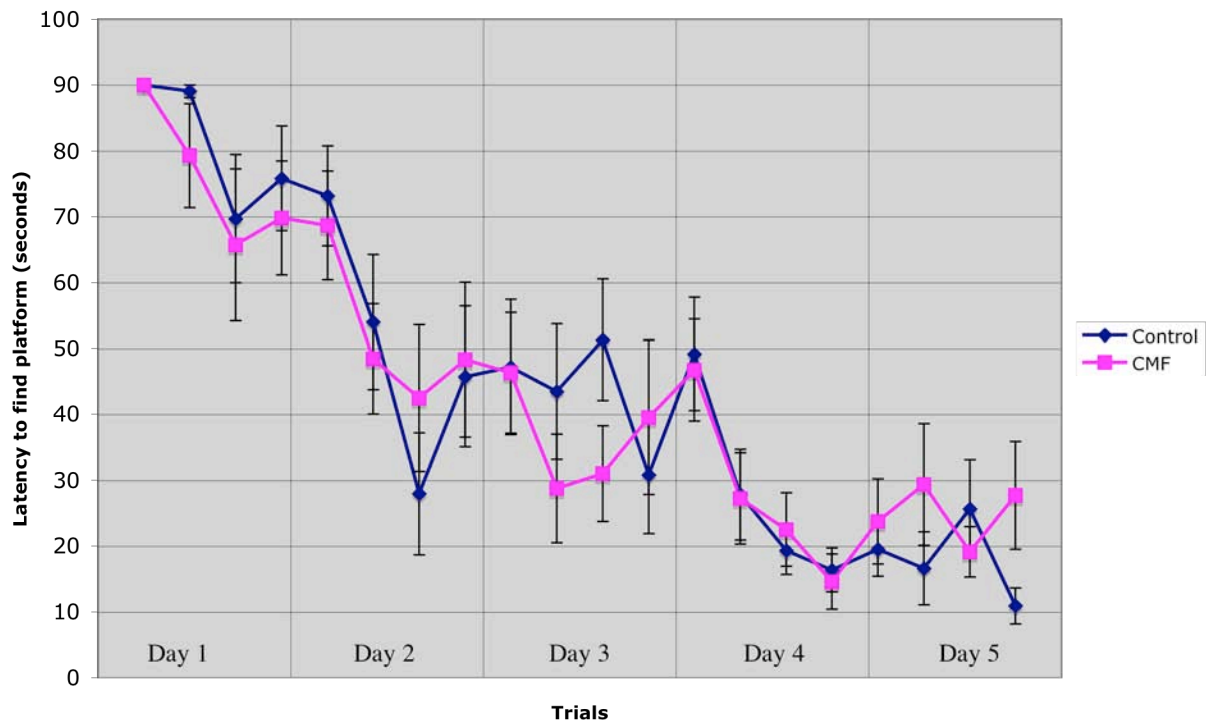


Figure 10 Acquisition training in the Morris Water Maze (group means). The time it took for rats to find the hidden platform in the pool was recorded on 4 trials per day over 5 days.

Testing for recall of the platform location on Day 6 took place during a 120 sec probe trial, wherein the platform was removed from the pool. There were no differences between the groups in the proportion of time spent in the quadrant where the platform had been (data not shown). A more specific measure than simply quadrant location is to record the time spent within a target zone, and the number of crosses into that zone (Fig 11).

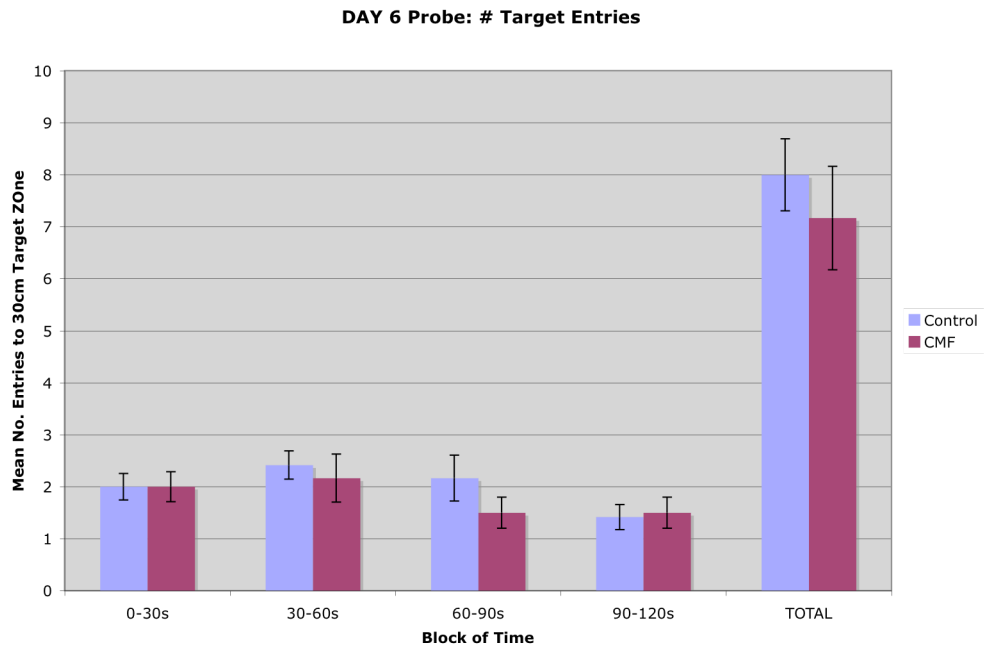
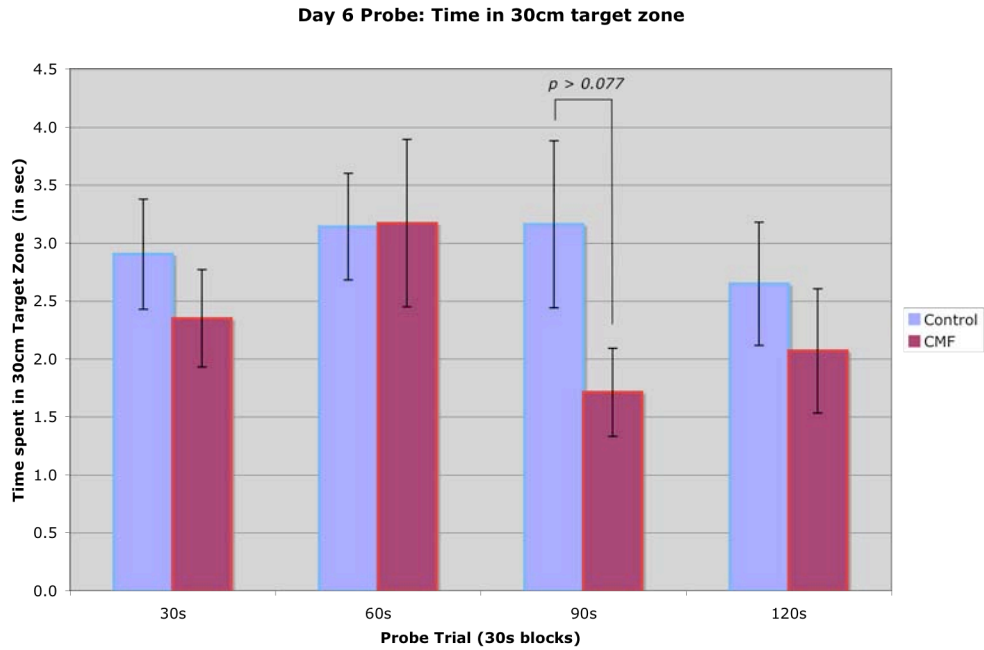


Figure 11 Morris Water Maze probe trial: time and number of entries into target zone (group means). A 30 cm diameter zone (invisible to the rats) emanating from the centre of the location where the platform had previously been was established and monitored by video capture. Top) The total time spent in this target zone was recorded in 30 sec blocks. The difference in the 60.1 – 90 sec block is not statistically significant ($p > 0.077$). Bottom) The number of times the rat's head crossed into the target zone in 30 sec blocks.

While there were no significant differences between groups in the probe trial, there was a trend that suggested that, following the first 60 sec, CMF animals began to spend less time in the target zone and quadrant. Though a difficult measure to interpret, it has been suggested that changes in strategy (the way in which the rat seeks out a platform, or alternate escape route) is better assessed by this kind of time block analysis (Vorhees and Williams, 2006). Changing strategies may reflect different levels of anxiety or recall, but this is a complex condition which did not reach significant difference in our study.

The visible platform trials on Day 8 suggested no differences between control and chemotherapy-treated rats with respect to their ability to detect and reach the platform, or motivation to exit the water (Fig 12).

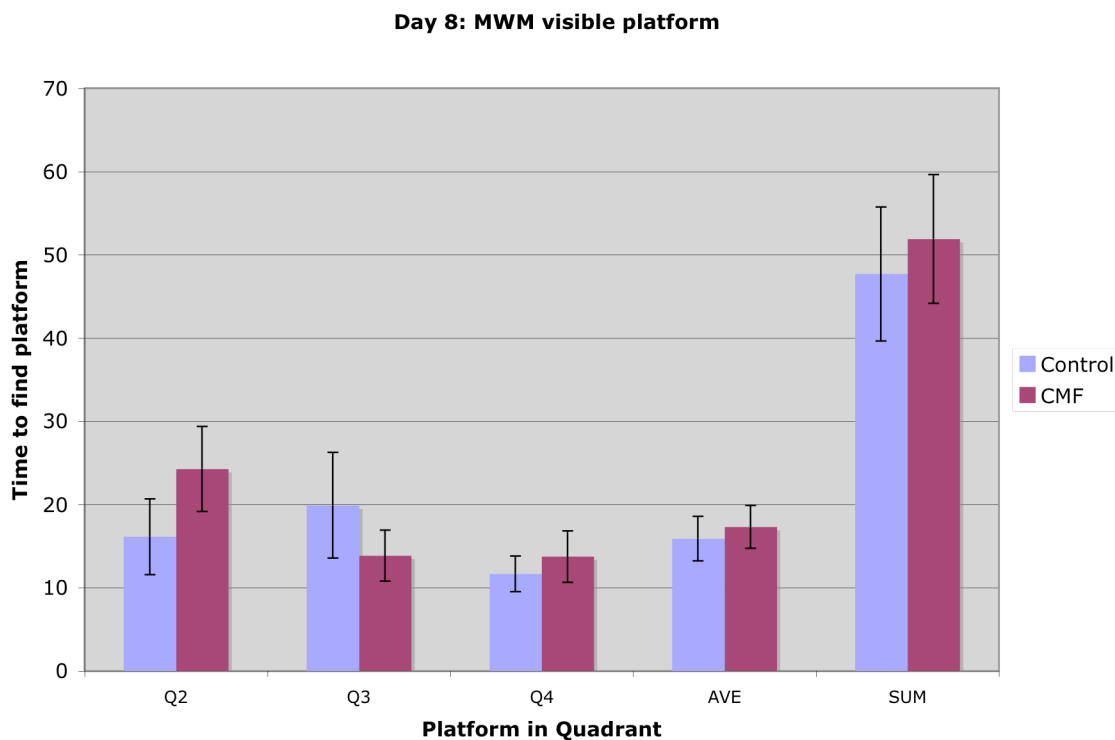


Figure 12 Morris Water Maze visible platform test (group means). Following the MWM testing, rats were returned to the pool 2 days later (Day 8) to verify their ability and motivation to get out of the water if presented with a platform slightly above the waterline. The platform was moved after each trial to a new quadrant, to ensure no preference or discouragement from particular regions of the pool arena.

Contextual and Cued Fear Conditioning

Test Methodology: Fear Conditioning

Fear conditioning is a simple one-trial learning paradigm which tests associative learning. Contextual fear conditioning is a means of testing memory by associating a stimulus with a particular situation or place. This is a behaviour dependent upon both the hippocampus and amygdala. Rats learn to recall and associate a stimulus with the context in which it was received - in this case, a chamber in which the stimulus (a mild foot shock) is received (Phillips and LeDoux, 1992). Rats that recall this stimulus will adopt an immobile or “frozen” stance. The strength of the association developed is measured by the amount of freezing when the rat is re-exposed to the test chamber.

For this test each rat is placed in a metal operant chamber (Med Associates) for 2.5 min exploration. A tone is sounded for 30 sec with 0.7 mA, 24 V shock administered for the last 1 sec. Rats are returned to their home cages 30 sec after the foot shock. 24 hours later, rats are returned to the chamber and scored for the number of 10 sec intervals spent frozen over a 6 min period. This test is conducted only once. Assessment at this time-point will give a measure of mid- to long-term contextual memory, to evaluate effects of the chemotherapy treatments on hippocampal function.

Additionally, amygdala-dependent but mostly hippocampal-independent behaviour can also be tested by cued fear conditioning. Two hours after the contextual fear test, the rat is placed in a novel context (a new chamber with no familiar stimuli). As with the contextual fear schedule, each rat is scored every 10 sec as freezing or moving, over a 3 min period (to assess adjustment to the new context). They are then re-exposed to the 30 sec tone only (which had preceded the foot-shock in the contextual fear test), but not the shock, and their freezing behaviour recorded again every 10 sec for 3 min. This trial provides us with an assessment of hippocampal involvement in a test which has another control component built-in: involvement of the amygdala. Due to the stresses caused by this test, fear conditioning was performed last, just prior to animal euthanasia.

Results: Fear Conditioning

While there was no statistically significant difference between control and chemotherapy-treated rats in the contextual fear test (24 hrs post-training), there was a slight trend which suggested some improvement in hippocampal function in the CMF animals (Fig 13). As with the other tests, we would not assume anxiety or ability to move plays a factor in the animal's freezing behaviour, since both groups performed equally in the Open Field test.

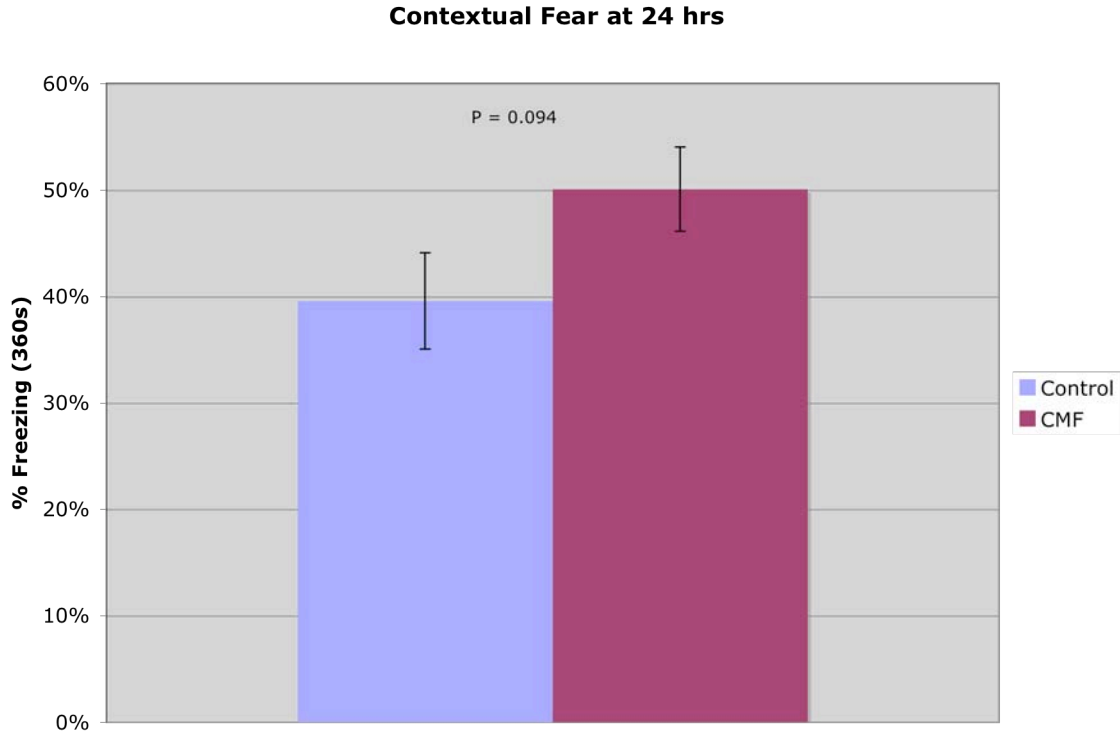


Figure 13 Contextual Fear testing 24 hrs following the tone/shock pair training. Rats were trained with a mild footshock in one context, and placed back into the same context (same operant box) 24 hrs later. Freezing (as assessed by the absence of all movement other than that required for respiration) was recorded every 10 sec over 6 minutes. Differences in freezing behaviour between groups were not statistically significant ($p > 0.09$).

The Cued Fear test also revealed no differences between groups, either in their baseline fear of a novel context nor in their response to the replayed tone (Fig 14). This indicates that the amygdala-hippocampal pathways of learning and memory are equally intact as the primarily hippocampal-dependent modes.

Cued Fear at 26 hrs

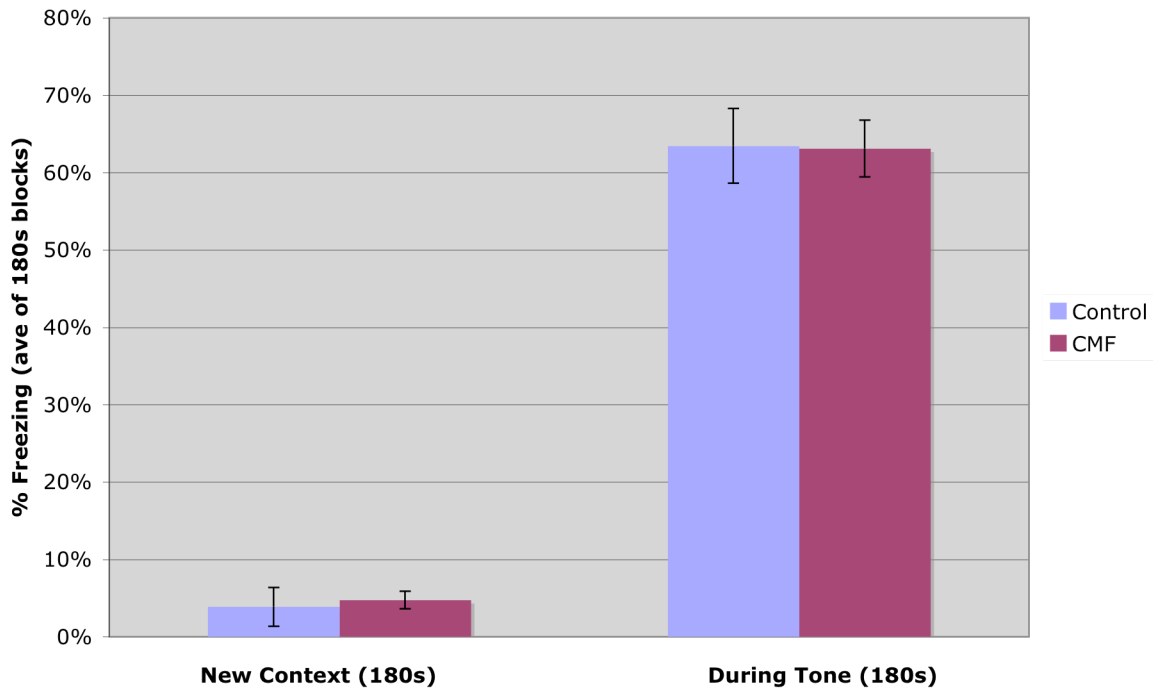


Figure 14 Cued Fear testing 26 hrs following the tone/pair training. Rats were trained with a mild footshock (preceded by a tone) in one context, and placed back into a different context (a new operant chamber) 26 hrs later. Freezing (as assessed by the absence of all movement other than that required for respiration) was recorded every 10 sec over 3 minutes. The tone was then replayed for 30 sec, during which freezing was again recorded (and for an additional 150 sec). Differences in freezing behaviour between groups in either situation were not statistically significant.

We also considered that the timing of the behavioural testing following chemotherapy may be important to the model. It is possible that no behavioural differences were observed in our model because of the ~100 day period between the last round of chemo and the end of testing. To address this, we performed a pilot study to evaluate acute effects of a single round of CMF treatment, on a pre-learned context. In this, 12 new animals received a 1 day conditioning session in the Contextual Fear apparatus (as described above). The next day they were split into 2 groups and administered a single round of saline or CMF injections (n = 6 per group). There were clear signs of fatigue and transient weight loss, suggestive of an acute toxicity and efficacy of the chemotherapy (as previously observed). These animals were allowed to recover for 10 days, after which they were tested by being replaced into the same context, and their degree of freezing scored. As with the long-term study, the animals in this group showed no significant differences in freezing behaviour (data not shown). These animals were then administered BrdU and sacrificed the next day – however these brain tissues were not processed.

Discussion of Findings

We have described the development of an animal model which mimics the physiological effects of human equivalent dose combination chemotherapy (as evidenced by weight loss, alopecia, and fatigue). We utilized a battery of behavioural tests to assess hippocampal-dependent learning and memory, in an effort to evaluate any cognitive deficits that may be associated with this type of chemotherapy. Our findings suggest no significant impairment in these measures of cognition. The only significant difference we discovered was an increase in time spent exploring objects.

We also investigated what effects this chemotherapy regimen might have on the survival of proliferating cells in the hippocampus. There was a trend which suggested some reduction in cell survival 30 days after labeling with BrdU (a total of 100 days following the last of the 3 chemo rounds), however this was not statistically significant.

Our results suggest that chemotherapy may directly affect neurogenesis. However, whether or not modified levels of neurogenesis resulting from chemotherapy will have any effect on cognition remains complex and uncertain. Environmental enrichment and exercise increase neurogenesis in the DG of rats, but only if there is no other trigger of neurogenesis such as ischemia (Olson et al., 2006). In fact, injury in non-enriched brains has been observed to boost neurogenesis up to the same levels as that seen in both the injured and non-injured enriched animals (Briones et al., 2005). When an insult occurs, natural mechanisms can be activated to increase proliferation in the DG. Chemotherapy-induced insults, however, may have longer lasting effects on this compensatory capacity.

Neurogenesis is not always entirely beneficial, either. In fact, blocking neurogenesis in the DG was recently shown to improve working memory in mice, when repetitive information is presented in a single day (Saxe et al., 2007).

Another possible reason for the lack of significant cognitive deficits in our model is the degree of hippocampal damage incurred. While hippocampal lesions have been shown to affect spatial memory before an object recognition test, the deficits only manifested when about 50% of the hippocampus had been lost, and nearly 75% hippocampal cell loss was needed before object recognition deficits emerged (Broadbent et al., 2004). Our partial analyses of cell death (through TUNEL and Fluoro-Jade staining) did not suggest anywhere near such high degrees of cell loss anywhere in the brain.

KEY RESEARCH ACCOMPLISHMENTS

Bulleted list of key research accomplishments emanating from this research.

- Establishment of a rat model of multi-dose, human-equivalent combination chemotherapy using cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) that recapitulates the physical symptoms of chemotherapy treatment
 - Timing and quantity of repeated doses

REPORTABLE OUTCOMES

Animal Models

The principal outcome of this work has been the establishment of a rat model for multi-dose combination chemotherapy. This model has employed different doses of cyclophosphamide and 5-fluorouracil with either methotrexate (CMF) or adriamycin/doxorubicin (CAF), along with behavioural testing for deficits in learning and memory, and pathological studies of the brain to assess levels of neurogenesis in the hippocampus.

CONCLUSION

We have established a working model of multi-dose chemotherapy with cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) in the rat. This model incorporates a battery of behavioural tests to evaluate different aspects of learning and memory.

Despite the emergence of several trends, we found no statistically significant differences in learning and memory between control and CMF rats, as measured by: Open Field test, Novel Object Recognition (NOR), Morris Water Maze, Contextual Fear or Cued Fear Conditioning. Behaviourally, the only significant difference between groups was an increase in the time CMF rats spent exploring objects in the NOR test.

We also replaced methotrexate with adriamycin to evaluate the CAF regimen (a more current therapy in clinical practice). However, in combination with other drugs, the dose of adriamycin used was excessively toxic and these animals were euthanized before any testing took place.

Following behavioural testing, control and CMF rats were euthanized and their brains were processed for immunohistochemical analyses. Preliminary analyses by double- and triple-labeling confocal microscopy, TUNEL and Fluorojade staining, and anti-PCNA, indicated no differences between group with respect to cell proliferation, differentiation, or death in the region of the hippocampus. More detailed analysis for BrdU+ cells after 30 days (an indicator of cell survival), suggested a trend towards decreased neurogenesis in CMF animals, but this was not statistically significant.

Future Work

This model could be further refined by providing easily ingested feed (crushed or moistened chow) to all animals from the outset, ensuring improved overall health. This could permit further rounds of chemotherapy, less time between treatments, and/or less time between chemotherapy and behavioural testing – all of which may increase the likelihood of detecting greater differences both behaviourally and pathologically. A decrease in the dose of adriamycin also may warrant retesting of this paradigm.

The animal model of combination chemotherapy we have described holds promise for testing therapeutics to combat the side effects of cancer treatments. This includes the neuroprotective peptide our group has reported previously, which we were not able to apply in this instance (During et al., 2003). All known antidepressant drugs induce neurogenesis, so the concomitant use of antidepressants and chemotherapy to modulate neurogenesis is one such potential therapy (Sahay and Hen, 2007).

An important consideration for future applications of this model would be to compare the timing of application of these therapeutics. To continue with the example of antidepressants, knowing when to pharmacologically activate neurogenesis during the course of chemotherapy could make a crucial difference. Activating the proliferation of progenitor cells in the brain at a time when chemo agents are present may cause greater damage to these cells, and hence create lasting impairments. In contrast, timing the activation of neurogenesis so as to replenish cells lost by chemo as quickly as possible following treatment, may prevent loss or permanent damage to the circuitry of the hippocampus and alleviate problems with learning and memory. Given that a significant proportion of cancer patients are already prescribed antidepressants to help deal with the emotional stresses of battling cancer, it is crucial that we establish windows of risk, safety, and benefit for combining neurotoxic chemotherapy with neurogenic prescriptions.

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APPENDICES

None