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Research report

Fluoxetine improves the memory deficits caused by the chemotherapy agent 5-fluorouracil

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ABSTRACT

Cancer patients who have been treated with systemic adjuvant chemotherapy have described experiencing deteriorations in cognition. A widely used chemotherapeutic agent, 5-fluorouracil (5-FU), readily crosses the blood–brain barrier and so could have a direct effect on brain function. In particular this anti mitotic drug could reduce cell proliferation in the neurogenic regions of the adult brain. In contrast reports indicate that hippocampal dependent neurogenesis and cognition are enhanced by the SSRI antidepressant Fluoxetine. In this investigation the behavioural effects of chronic (two week) treatment with 5-FU and (three weeks) with Fluoxetine either separately or in combination with 5-FU were tested on adult Lister hooded rats. Behavioural effects were tested using a context dependent conditioned emotional response test (CER) which showed that animals treated with 5-FU had a significant reduction in freezing time compared to controls. A separate group of animals was tested using a hippocampal dependent spatial working memory test, the object location recognition test (OLR). Animals treated only with 5-FU showed significant deficits in their ability to carry out the OLR task but co administration of Fluoxetine improved their performance. 5-FU chemotherapy caused a significant reduction in the number of proliferating cells in the sub granular zone of the dentate gyrus compared to controls. This reduction was eliminated when Fluoxetine was co administered with 5-FU. Fluoxetine on its own had no effect on proliferating cell number or behaviour. These findings suggest that 5-FU can negatively affect both cell proliferation and hippocampal dependent working memory and that these deficits can be reversed by the simultaneous administration of the antidepressant Fluoxetine.

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1. Introduction

Systemic adjuvant chemotherapy is used in the treatment of many cancers including breast cancer. For some time breast cancer patients have reported that chemotherapy can have negative effects on cognition, a phenomenon which has led to the use of the terms chemobrain or chemofog [1,2]. This together with the realisation that many chemotherapy agents can cross the blood brain barrier has led to cross sectional and longitudinal studies of patient cognition during and after chemotherapy has been carried out. The majority of these studies have shown a mild to moderate cognitive deficits as reviewed in Collins et al. [3]. In particular four meta analyses of the literature have concluded that working, visual and verbal memory appear to be consistently affected in patients who have completed a course of chemotherapy [2,4–6]. Animal studies using a variety of chemotherapy drugs have produced mixed results on the cognitive effects of drug treatment. Most studies have shown cognitive impairment after chemotherapy treatment [7–12]. While some have shown no effect or an improvement in cognition [13,14]. In the present study we have sought to investigate the effects of the commonly used chemotherapy agent 5-fluorouracil (5-FU) which has been associated with chemobrain in reports by breast cancer survivors [4,15]. Chemotherapy drugs target proliferating cells and 5-FU works by blocking the synthesis of thymidine required in DNA replication [16] and easily crosses the blood–brain barrier by simple diffusion [17,18]. It has been suggested that the cognitive problems associated with chemotherapy could be associated with a reduction in the proliferation of cells in neurogenic brain areas including the dentate gyrus of the hippocampus [9,19,20,10,21]. The subgranular zone (SGZ) on the inside of the dentate gyrus contains continuously proliferating neural stem cells which contribute new neurons to

Abbreviations: 5-FU, 5-Fluorouracil; OLR, object location recognition; CER, conditioned emotional response; BDNF, brain derived neurotrophic factor; PI, preference index.

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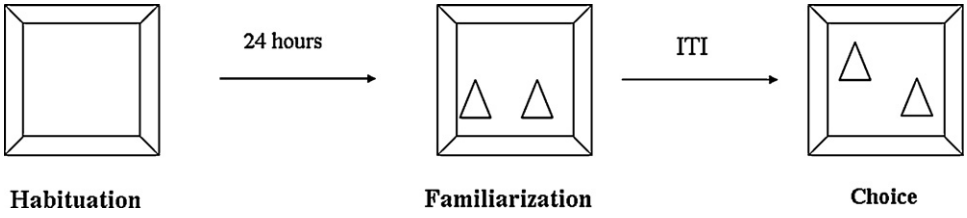


Fig. 1. The object location recognition test protocol was carried out over two days. On the first day, animals were allowed to habituate to the test arena for 1 h. On the following day, two identical objects were placed in different locations of the box with the animal to explore for 3 min (Familiarization trial). Animals were then removed outside the box for 5 min inter-trial interval (ITI) after which the animal was put in the same box after changing the objects location for 3 min (choice trial).

the dentate gyrus itself throughout life [22]. This neurogenesis is required for spatial working memory, a particular function of the dentate gyrus [23].

A number of human studies have found that treatment with the SSRI antidepressant, Fluoxetine, can improve cognition and memory in patients suffering from mild to moderate cognitive impairment [24–28]. The improvement in memory appears to be independent of depressive symptoms and the original causes of cognitive decline. Animal studies have confirmed these results and shown that antidepressants can increase cell proliferation in the SGZ [29–31].

In the present study we show, with two behavioural tests, that 5-FU, causes impairments in spatial and contextual memory and reduces the number of the proliferating cells in the SGZ. Co-administration of Fluoxetine however reduces the impact of these cognitive deficits and prevents the decrease in cell proliferation in the SGZ. Both chemotherapy and antidepressants may be altering hippocampal function and an understanding of this may provide a mechanism for improving cognition during cancer treatment.

2. Materials and methods

2.1. Animals, drug administration and behavioural testing

All experiments and animal care were in accordance with UK Animals (Scientific procedures) Act 1986 and in accordance with Ethics committee guidelines of the school of Biomedical Sciences, University of Nottingham. All experiments were on male Lister hooded rats (Charles River UK) of 150–170 g at the start of experiments and were weighed daily. Animals were allowed to habituate for two weeks before treatment and housed in groups of 3 or 4 under a standard condition of 12 h light–12 h dark cycle (from 8.30 a.m. to 8.30 pm) with free access to food and water. Behavioural testing of all animals was performed one day after treatment between 8.30 am and 2 pm. Doses given of 5-FU are within the range which reduces tumour load in rats [73,74], and are the standard doses used to investigate the effects of 5-FU which are within the human dose range [32]

20 rats, (10 controls and 10 5-FU treated) were randomly assigned to control and drug treated groups for the conditioned emotional response test (housed in groups of 3 or 4). Animals received 5 i.v. (tail vein) injections every other day over two weeks of either saline or 5-FU (25 mg/kg) (Mayne Pharma PLC) with leucovorin (25 mg/kg/day) (Teva UK Ltd.) under isoflurane anaesthesia. 5-FU is often administered to patients in combination with calcium folinate (leucovorin), which potentiates its action [33]. The contextual conditioned response (CER) was tested by association of foot shock with location using a previous protocol [34]. On day one, each animal was put individually in the CER box (25 cm × 24 cm × 25 cm) with a metallic grid floor through which 10 shocks (0.04 mA) one per minute, each lasting for 1 s were given. The box was cleaned between trials with 20% alcohol. On day two, each animal was put in the same box for 10 min, without shocks, and the freezing behaviour recorded by video camera. Freezing behaviour was defined as absence of movement during which the animal adopts a characteristic tense posture.

In a separate experiment 45 rats were randomly assigned to 4 groups (Saline (n = 12), 5-FU (n = 12), Fluoxetine (n = 11) and combined 5-FU and Fluoxetine treated (n = 10)). Animals were housed in groups of 4 or 3. 5-FU treated animals received 6 intravenous (i.v) bolus injections of 5-FU 20 mg/kg/day (MAYNE Pharma PLC) together with leucovorin, 20 mg/kg/day (TEVA UK LTD) under isoflurane anaesthesia over two weeks. This is a similar total dose to that given in the previous experiment but spread over 6 injections to reduce toxicity. Saline treated control animals (n = 12) or Fluoxetine treated (n = 11) received an identical sequence of injections. Animals receiving Fluoxetine (PINEWOOD Healthcare Colonel, Ireland) were given the drug in the drinking water with a dose of 10 mg/kg/day over three weeks being calculated from the amount of daily water intake [35]. The concentration of Fluoxetine given in drinking water was calculated to give the animals 25 mg/5 ml of water consumed. Water intake decreases with Fluoxetine treatment but dosages were recalculated every other day depending on water consumption and animal weights. Drinking water containing Fluoxetine was freely available to the animals. The solution of Fluoxetine was changed every other day.

2.2. Object location recognition task

This was modified from a previous protocol [36] and was recorded by video camcorder as done previously in our laboratory [9]. The test apparatus consisted of plastic boxes (39 × 23.5 × 30 cm). The procedure consisted of habituating the animals for 1 h in the box on the day prior to testing. The following day, two identical objects (water bottles) were placed in separate locations in the box and the animals allowed 3 min to explore (Familiarization trial). Animals were returned to their home cage for 5 min (inter-trial interval) during which the box was cleaned with 20% ethanol. For the choice trial the animals were returned to the box for 3 min where one object remained in its original position (familiar location) while the other object was moved to a new position (novel location) see (Fig. 1).

Exploration of the object was scored when the animal sniffed, licked, chewed or directed its nose at a distance ≤1 cm from the object [9]. Exploration was scored as the total time spent on each object (familiar and novel locations) and a discrimination index was calculated as the difference between the time spent on each object divided by the total exploration time [36,37].

2.3. Brain tissue preparation

On the following day after the OLR test, rats were put down by rapid stunning followed by decapitation. 29 brains from all groups were randomly chosen from animals undergoing behavioural testing. These brains were divided sagittally; one half was cryoprotected in a 30% sucrose solution for 3 h at 4 °C. before embedding in OCT, Embedding medium for frozen tissue specimens (VWR International Ltd., Lutterworth, UK) and stored at −80 °C. Frozen brains were serially sectioned (20 μm) in the coronal plane chosen from animals undergoing behavioural testing.from Bregma point −2.12 to −6.08 mm [38] to include the entire hippocampus using a Leica CM 100 cryostat (Leica Microsystems, Knowlhill, UK). Sections were mounted onto 3-aminopropyl-methoxysilane (APES) – coated slides and stored at −80 °C. for immunohistochemistry.

2.4. Immunohistochemistry

A systemic random sampling technique [39] was used to choose every 21th section throughout the length of the dentate gyrus (overall 10 sections). Sections were fixed with 0.5% paraformaldehyde (PFA) for 3 min, before incubation with polyclonal rabbit Ki67 (1:250); Vector Laboratories, Orton Southgate, UK) for 1 h, followed by 1 h incubation with Alexa 488 (Invitrogen/molecular Probes, UK) goat anti-rabbit secondary antibody (1:300) according to the manufacturer's protocol. Propidium Iodide 1:3000 (PI, Sigma) was used as a counterstain before mounting the sections in glycerol and viewing at X40 on a Nikon EFD-3 fluorescence microscope. Ki67 positive cells were counted within the SGZ, defined as within three cell diameters of the inner edge of the dentate gyrus [9]. Counts from all sections of one dentate gyrus were averaged to provide a number per section [9].

2.5. Statistical analysis

All statistical parameters were calculated using Graph pad Prism 4.0 software, USA. Paired Student t-test (two-tailed) and repeated-measures of ANOVA were used to analyse data of the OLR task, unpaired Student t-test was used to analyse data of the CER test and one way ANOVA with Bonferroni's post hoc test was used to analyse data of the proliferating cell count, the preference index and the mean velocity of animals. A probability level of $P < 0.05$ was considered statistically significant

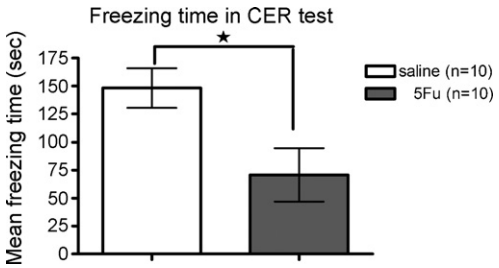


Fig. 2. The 5-FU treated group freezing time in the CER test is significantly less than the saline treated ($P = 0.01$). The analysis used was unpaired Student t-test (two-tailed).

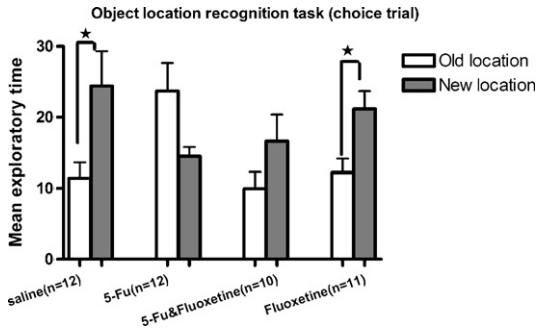


Fig. 3. The figure shows that the saline treated animals explored the new location (dark shaded) significantly more than the old location (white shaded) ($P = 0.01$). Whereas the 5-FU treated animals failed to discriminate between the new and the old objects ($P = 0.06$). There was a non significant increase in the exploration time of the new object than the old one in the combined (5-FU and Fluoxetine) group ($P = 0.1$). Concerning Fluoxetine treated rats, their exploration of the new location was significantly more than the old location ($P = 0.02$). The analysis was done using paired T test (two-tailed) to compare between the mean exploratory time of the old and the new location in the choice trial after treatment within each group.

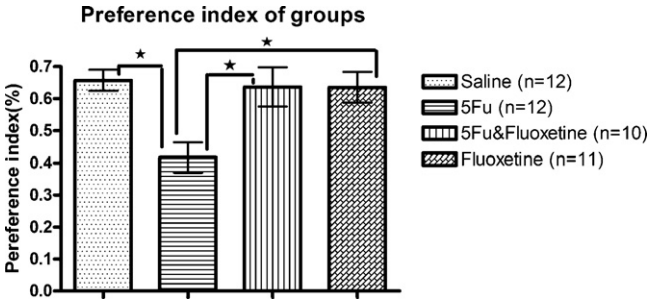


Fig. 4. The preference index is defined as the calculated exploration time of the new location, represented as a percentage of the combined exploration time of both novel and familiar locations of an object. The preference index showed a significant decrease in the 5-FU treated rats in comparison to the other three groups. The analysis used was one way ANOVA with Bonferroni's post hoc test.

3. Results

3.1. Effect of different treatments on weights of animals

Animals were weighed daily (data not shown). Animals on 5-FU showed a weight loss during drug administration and although they showed improved weight gain after the end of drug treatment they were still significantly below control weights. Animals on Fluoxetine or Fluoxetine and 5-FU were significantly lighter than controls during drug administration but recovered to control weights by the end of the experiment. This effect is a well-known side effect of 5-FU attributed to effects on the gastrointestinal tract lining [70]. Animals on 5-FU showed a 24 h drop in weight after each injection which was reversed on the following day. Animals treated with Fluoxetine also put on weight less than controls as found in previous studies [40]. It is possible that absorption of Fluoxetine could have been affected by 5-FU treatment.

3.2. Behavioural effects of 5-FU

In separate experiments the behavioural effects of i.v. injection of 5-FU was tested using the OLR and CER tests.

For the CER test, freezing behaviour was recorded 24 h after conditioning when animals were returned to the test box. Vehicle treated animals spent an average of 148.2 s in this behaviour. In contrast 5-FU treated animals spent an average of only 70.8 s exhibiting this type of behaviour, which was significantly less than controls ($P = 0.01$) see Fig. 2.

The OLR test measures interactions with objects either in familiar or novel locations within a test arena. During the familiarization trial, when animals explore two identical objects, both vehicle and drug treated groups showed no preference for either object or the total exploration time (data not shown). In addition there was no significant difference in speed of movement (mean velocity) between the groups (Fig. 5).

Following a 5 min inter-trial interval, one object is moved to a new location (choice trial) and object preference is recorded.

Saline injected controls and animals receiving Fluoxetine in their drinking water both explored the novel object significantly more than the old one ($P = 0.01$ and 0.02 respectively) (Fig. 3). However, animals treated with 5-FU by i.v. injection failed to show discrimination between objects in familiar and novel locations ($P = 0.06$). Surprisingly 5-FU treated animals spent more time examining the familiar object although this was not significantly different from the time spent on the novel object.

Animals which had received Fluoxetine in their drinking water throughout the experiment as well as i.v. injections of 5-FU spent more time exploring the novel object but this was not significantly different from the time spent on the familiar object. Further analysis using the preference index (PI) was done to compare between the four groups, see Fig. 4. Comparing the PI between the control and the 5-FU treated group demonstrated a significant decrease after treatment with the chemotherapy drug ($P < 0.01$). Similarly animals treated with Fluoxetine on its own showed a significantly higher preference index to 5-FU treated animals ($P < 0.05$). Comparing the PI of animals treated with 5-FU on its own and that of animals simultaneously given Fluoxetine and 5-FU showed that co- administration of Fluoxetine significantly improved preference compared with animals on 5-FU alone ($P < 0.05$). These findings indicate that animals receiving 5-FU show deficits in spatial memory which are improved by simultaneous administration of Fluoxetine.

3.3. Effect of treatments on proliferating cell counts

As shown in Fig. 6A. A there was a significant reduction in the total number of Ki67 positive cells of the 5-FU treated group compared to saline treated ($P < 0.05$). This difference was abolished by co-treatment with Fluoxetine ($P > 0.05$). Fluoxetine only didn't have any effect on the total number of Ki67 positive cells compared to controls ($P > 0.05$). Fig. 6B shows a representative image of Ki67 positive proliferating cells within the dentate gyrus counterstained with propidium iodide. Although 5-FU affected behaviour and reduced cell proliferation across the groups, there was no direct correlation between proliferating cell number and performance in the behavioural test between individual animals (data not shown).

4. Discussion

Reports by patients undertaking chemotherapy have indicated that systemic chemotherapy can produce a wide range of cogni-

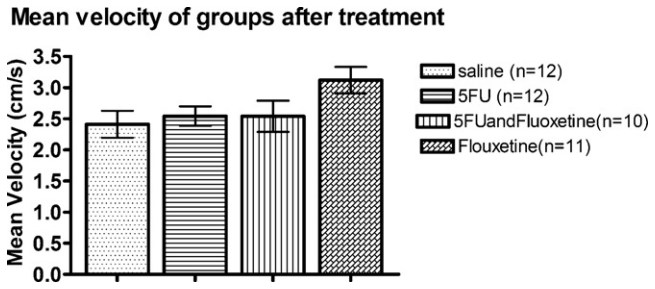


Fig. 5. There was no difference between all groups in the mean velocity after treatment during the object location recognition test (cm/s) ($P = 0.1$). The analysis used was one way ANOVA with Bonferroni's post hoc test.

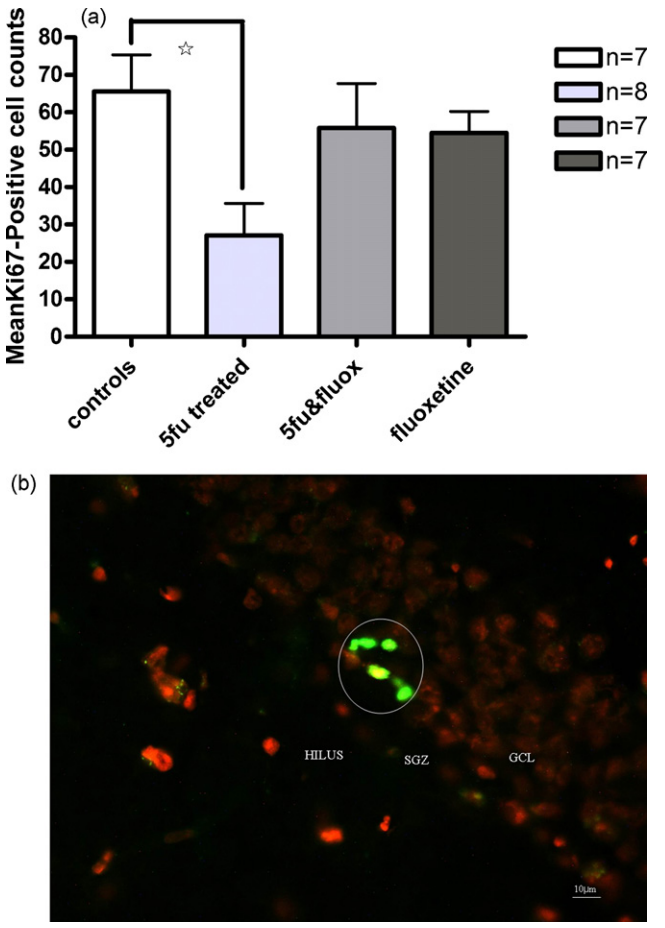


Fig. 6. (A) There was a significant reduction of the number of Ki67 positive cells of the 5-FU treated group compared to controls ($P < 0.05$) which was abolished after co-treatment (Fluoxetine and 5-FU) ($P > 0.05$). There was no difference in the proliferating cell count between controls and Fluoxetine only treated group ($P > 0.05$). The analysis used was one way ANOVA with Bonferroni's post hoc test. (B) Proliferating cells in cluster (circled) within the dentate gyrus (DG) are immunostained for Ki67 (green) and counterstained with Propidium iodine nuclear strain (red). Regions of the DG are highlighted: granular cell layer (GCL); subgranular zone (SGZ) and the hilus (HILUS) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

tive symptoms including difficulties in concentration, attention and memory which have become collectively known as "chemobrain" [41]. These effects can have a significant impact on patient quality of life and in their ability to return to work [42,43]. Psychometric testing of patients during and after systemic chemotherapy has confirmed patient reports and indicate that chemotherapy can produce a range of mild to moderate cognitive deficits which are dose dependent and can last for several years after the cessation of treatment [2,44,4,72]. The symptoms described by patients after chemotherapy treatment almost always include deficits in declarative memory. These involve difficulties in the recall of both semantic (factual) and episodic (temporal) aspects of memory for which the hippocampal formation is known to be required [45]. For this reason we have used two tests of hippocampal function as a means of assessing the cognitive effects of a therapeutically relevant dose of the commonly used chemotherapy agent 5-FU. Drug treated animals showed no difference in activity (total distance and mean velocity) (Fig. 5). The CER test as carried out here, measures recall of the context of an unpleasant stimulus after a 24 h interval and is a good measure of declarative memory [46]. Memory is quantified by the amount of freezing behaviour exhibited by the animal on being returned to the same arena (context) as the delivery of the unpleasant stimulus. Our results showed that treatment with 5-FU significantly reduced the amount of time animals spent in a freezing posture when returned to the arena compared to saline treated animals indicating a failure in the recall of the context of this experience.

Performance of the CER behavioural test where the stimulus is paired to a context requires the hippocampal formation [47,48] but also involves input and modulation from other brain structures notably the amygdala and anterior cingulate gyrus which are involved in the emotional and nociceptive aspects of the test [49–52]. In order to further test the hippocampal involvement in the memory impairment produced by 5-FU, a second behavioural test was carried out on a separate group of rats. This used the object location recognition task which has been shown to require an intact hippocampal dentate gyrus [53].

Confirming our previous results [9], these results show that 5-FU treatment caused deficits in this task. Control animals were readily able to discriminate between the novel and familiar locations of objects and spent significantly more time on the objects in the novel location. In contrast 5-FU treated animals showed no significant difference in the time spent on the two objects and in fact spent more time on the object in the familiar location. The total distance moved (cm) and the mean velocity (cm/s) performed by animals during the test were not different between all groups after treatment (Fig. 5).

As the OLR test requires an intact dentate gyrus and adult neurogenesis which contributes cells to this region is thought to be required for spatial memory, this test was chosen to look at the affects of the anti depressant Fluoxetine. Fluoxetine has been reported to increase hippocampal neurogenesis and improve spatial memory [54,55] making this a possible intervention to reverse the effects of chemotherapy.

Animals receiving both i.v. injections of 5-FU and Fluoxetine in their drinking water, although showing an improvement in their discrimination between familiar and novel object locations, still did not significantly discriminate between these positions (Fig. 3). However, calculation of the preference index, a measure of the percentage of the combined exploration time of both novel and familiar locations of an object [37] showed a significant improvement in selecting the object in the novel location over the 5-FU treated animals (Fig. 4).

Regarding the effect of 5-FU chemotherapy on memory, one possible cause could be disruption of adult hippocampal neurogenesis. The production of new neurons in the SGZ for incorporation into the dentate gyrus, is a well-characterized phenomenon in all mammals including humans [56,57]. Reductions in cell proliferation in the SGZ by pharmacological, environmental or genetic means are associated with deficits in memory [58,37,59]. In the present study, we showed that 5-FU chemotherapy significantly decreased the number of proliferating cells in the SGZ of the dentate gyrus using immunohistochemistry for the proliferative marker Ki67. No

regional effects (dorsal versus ventral hippocampus) were found when different groups were compared. Animals were put down four weeks after the start of drug treatment. This period is long enough for changes in neurogenesis to affect behaviour [60,61].

In line with previous investigations on changes in this process this was associated with a decline in the performance of memory specific tasks. Understanding the effects of chemotherapy on patients is complicated by other aspects of the disease and treatment and cancer patients can exhibit cognitive impairments prior to treatment [41]. The present study shows that 5-FU can produce cognitive decline in at least two behavioural tests and that this is associated with a decrease in proliferation in the neurogenic region of the hippocampus. These results are in line with animal studies of other chemotherapy agents which have found cognitive declines after treatment [7–10,12,13,19]. In a previous investigation we showed that 5-FU produced a non significant reduction in cell proliferation in the SGZ [9]. The present investigation increased the number of injections which were sufficient to significantly reduce cell proliferation.

Further, because our study was based on the whole dentate gyrus, future studies will have to determine if a reduction in hippocampal neurogenesis results in impaired performance in tasks that are specifically designed to assess differential functions associated with ventral vs dorsal areas of the hippocampus [75] although no regional differences (dorsal versus ventral hippocampus) were found when different groups were compared in the present study.

Clinically, it has been reported that antidepressants can improve memory function in patients suffering from a variety of conditions [26,25,28]. Similarly, in animal models, SSRI antidepressants have been reported to improve cognition after stroke or stress [55,62,63] but not in normal animals [35,64]. Some reports have found that antidepressants increase proliferation of cells in the SGZ [54] while others have failed to find this effect [65,66]. Our results show that co-treatment of Fluoxetine with 5-FU abolished the reduction in cell proliferation in the SGZ caused by 5-FU and improved performance in the OLR test. Fluoxetine on its own had no effect on proliferation or behaviour. It is unlikely that the effects of Fluoxetine are limited to neurogenesis in the hippocampus as several studies have shown positive effects on other brain regions associated with cognition notably frontal, cingulate and temporal lobes [67,68,69,71]. The action of fluoxetine on these regions as well as the hippocampus may therefore underlie its effects on cognition.

In conclusion, our results show that 5-FU treatment caused cognitive deficits which were associated with a reduction in cell proliferation in the SGZ of the dentate gyrus. These deficits were reduced when Fluoxetine was administered throughout the 5-FU treatment period.

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