THE UNIVERSITY of ADELAIDE

Understanding the Acute Effects of Chemotherapeutic Agents, Methotrexate and 5-Fluorouracil in a Rat Model of Chemotherapy-Induced Cognitive Impairment

HONOURS DEGREE of BACHELOR OF HEALTH AND MEDICAL SCIENCES In The Discipline of Physiology

Adelaide Medical School

The University of Adelaide

by Olivia Haller

November 2020

Abstract

BACKGROUND

Around 75% of cancer survivors report symptoms of chemotherapy-induced cognitive impairment (CICI) lasting one month to 20 years. The underlying mechanisms remain largely unknown and the symptoms reported can be significant. Animal models are used to investigate CICI, however there is no gold standard model in terms of the time-point or chemotherapeutic agents investigated, which reflects the clinical scenario. This study investigated an acute time-point of 72 hours post-treatment of methotrexate (MTX) or 5-Fluorouracil (5-FU) in a rat model, as there is limited knowledge on early time course pathogenesis of CICI.

METHODS

Female Sprague Dawley rats received two injections of MTX (37.5mg/kg), 5-FU (75mg/kg) or saline intraperitoneally. Animals were assessed in the puzzle box and novel object recognition (NOR) to examine hippocampal and prefrontal cortex (PFC) functioning. Western blot analysis was conducted on PFC and hippocampal tissue to examine astrocytic activation through GFAP expression.

RESULTS

MTX treatment revealed impaired long-term memory in the puzzle box, however no significant differences were found in NOR. Both 5-FU and MTX increased GFAP expression in PFC significantly. Additionally, 5-FU increased hippocampal GFAP expression. This suggests impairment may occur in the hippocampus later on for MTX, or 5-FU has more extensive severity for damage.

CONCLUSION

These results demonstrate that MTX and 5-FU treatment produce an inflammatory response at 72 hours post-treatment in the PFC and hippocampus, whereas cognition is partially affected. This could potentially provide insight as to when and how to inhibit CICI from occurring.

Introduction

Chemotherapy treatment is among the most common cancer treatment utilised today. Due to medical advances, chemotherapeutic agents have produced relative success shown through an increase in cancer survivor numbers[1]. However, with the use of such agents comes adverse side effects, including neurotoxicity, often resulting in deficits in attention, concentration, speed of information processing and decision making[2]. This is a condition termed chemotherapy-induced cognitive impairment (CICI) or 'chemobrain.' As many as 75% of patients report symptoms during treatment[1, 3, 4], and 35% continue to report them post-treatment[3]. Studies suggest that these impairments can last between 6 months[5] to 20 years[6] and varies in terms of severity and duration.

The mechanisms driving CICI are largely unknown, however several mechanisms have been proposed. These include the ability of chemotherapeutic agents to alter the permeability of the blood brain barrier (BBB) and promotion of oxidative stress and neuroinflammation[1]. Neuroinflammatory mechanisms have recently been recognised as a key contributor driving CICI. The brain's innate immune system can become activated due to inflammatory challenges, and cellular and molecular changes occur as a result, as shown in CICI studies[7, 8]. Astrocytes are key mediators in the immune response in the central nervous system (CNS)[8]. In terms of chemotherapy treatment, neuroinflammation may be an outcome of peripheral inflammatory signalling due to the effect on the tumour, such as gut-brain activation. Or it could be a direct consequence of the agents localising to CNS cells causing a cascade of neuroinflammatory events and neurotoxicity, resulting in cognitive decline[9].

Methotrexate (MTX) and 5-Fluorouracil (5-FU) are commonly used cytotoxic agents to treat patients with various forms of cancer, such as Non-Hodgkin's lymphoma, acute lymphoblastic leukaemia[10], breast and colorectal cancer[11]. Both agents have been implicated in many clinical and pre-clinical CICI studies, suggesting a possible negative effect on cognition[1, 2, 12-16]. MTX has been shown to impair performance on object recognition in rats, affecting hippocampal function[15], whereas

5-FU has the ability to cross the BBB, impairing cognition and supressing hippocampal cell proliferation[17].

Several clinical studies have investigated CICI, examining dose amount, drug type, route of administration and administration either alone or in combination[18]. However, many confounding variables must be accounted for, such as cancer type, treatment regime, age and sex, which can all result in conflicting outcomes. Therefore, animal models are widely utilised, as they are more effective at controlling for these variables[1]. However, methodological differences exist across animal models and there is no 'gold standard' model. As a consequence, studies utilise different rodent strains, sex, chemotherapeutic agents, route of administration, dose frequency and behavioural tests[1]. A further challenge is the lack of simple behavioural tests of cognition available to assess more subtle cognitive changes, as might be expected in CICI.

Previous animal studies investigating CICI have focused on both acute and chronic time points with MTX and/or 5-FU treatment with inconsistent results[1]. The time-point at which impairment occurs is largely unknown as the reported cognitive impairment and duration often vary. This is of particular importance as the duration and prevalence of CICI reported in humans also varies. Cognitive impairment has been identified in rodent studies investigating CICI with MTX and/or 5-FU treatment at 1 to 3 days and 1 week to 5 weeks post treatment[2, 13, 15, 16, 19, 20]. Conversely, no significant impairment has been found at 3 days, 1 week and 3 weeks up to 15 weeks[12, 13, 21]. A limited number of studies have explored an acute time-point of 72-hours while simultaneously investigating both behavioural and neuroimmune expression changes. This highlights the importance for a study like this one to continue to chart the time frame in which CICI may be occurring, and the possible underlying neuroimmune mechanisms.

As such, the primary aim of this study was to elucidate both hippocampal and PFC focussed cognitive impairment at 72 hours post chemotherapy treatment, along with the related astrocytic activation for these regions. The second aim was to validate the puzzle box test in a CICI rat model. It was hypothesised that rats administered either MTX or 5-FU would show greater cognitive

impairment in behavioural tests compared to saline-treated controls. Additionally, an increase in astrocytic activation in the PFC and hippocampus would occur, as determined through glial fibrillary acidic protein (GFAP) expression.

Ethics

The University of Adelaide's Animal Ethics Committee approved the animal housing and experimental procedures for this study (S-2019-019). It was conducted in accordance with the provisions of the Australian Code for the Care and Use of Animal for Scientific Purposes. The following study is reported in accordance with Animal Research: Reporting in vivo experiments: The ARRIVE guidelines[22].

Materials and Methods

Animals and Experimental Design

Female Sprague Dawley (Hsd: SD) rats (n=36) aged eight weeks, were purchased from a pathogen-free, barrier-maintained animal facility (*Laboratory Animal Services*, University of Adelaide). The sample size per treatment group was calculated to achieve 80% power based on previous data. The number of animals required was derived using a standard deviation of 3.5s, with an effect size of 0.74, providing a statistical power of 82.6%. Animals were group housed (six per cage) in open-top cages (415mm x 260mm x 145mm, *Tecniplast*, Exton, PA, USA) with clean bedding (1/4-inch Grit-ology Enriched Corncob, *Corn-cob-ology*, NSW, Australia). A room temperature of 21-23ºC and a standard 12-hour light/dark cycle with lights on at 7am and off at 7pm was maintained. Animals were acclimatised for five days prior to the commencement of the study. Animals were provided with a standard diet of chow (Teklad Irradiated Global SoyProtein-Free Extruded Rodent Diet 2920X, *Envigo*, Madison, WI) and acidified RO water *ad libitum* for the duration of the study. Animals were randomly assigned to three treatment groups (n=12), using an online random number generator (*Randomness and Integrity Services Ltd*, Dublin, Ireland).

Animals received an intraperitoneal injection of MTX (37.5mg/kg; *Accord Healthcare Pty Ltd*, Melbourne, Australia), 5-FU (75mg/kg; *Hospira Australia Pty Ltd*, Victoria, Australia) or Saline (0.9% Sodium Chloride Inj.B. *Braun Australia Pty Ltd*), once weekly for two consecutive weeks prior to behavioural testing on days -7 and 0 (Figure 1). These chemotherapeutic agents were chosen due to their clinical relevance[23] and dosages selected based on previous findings showing cognitive impairment with minimal adverse systemic effects[20, 24]. During chemotherapy treatment, animals additionally received Nutrigel (High-energy dietary supplement, *Troy Laboratories Pty Ltd*, NSW, Australia) and DietGel®31M (*ClearH$_2$0*, Portland, USA) to enhance nutrient intake.

Animals were monitored daily, and weight recorded using the disease activity index (DAI) clinical record sheet. Puzzle box testing was conducted on days 0 to 3 post treatment and NOR was conducted on day 2 following the puzzle box trials for that day. Behavioural testing was conducted during the light cycle in the animal facility in a separate room to their housing. Animals were humanely euthanised on day 3 after behavioural testing by CO_2 asphyxiation (20% of chamber volume per minute by rising fill). Animals remained in the chamber until they were flaccid and no longer respiring, confirmed via eye blink response and pedal reflex tests. PFC and hippocampal tissue were collected, snap frozen and stored at -80^{O}C for western blot analysis (Figure 1).

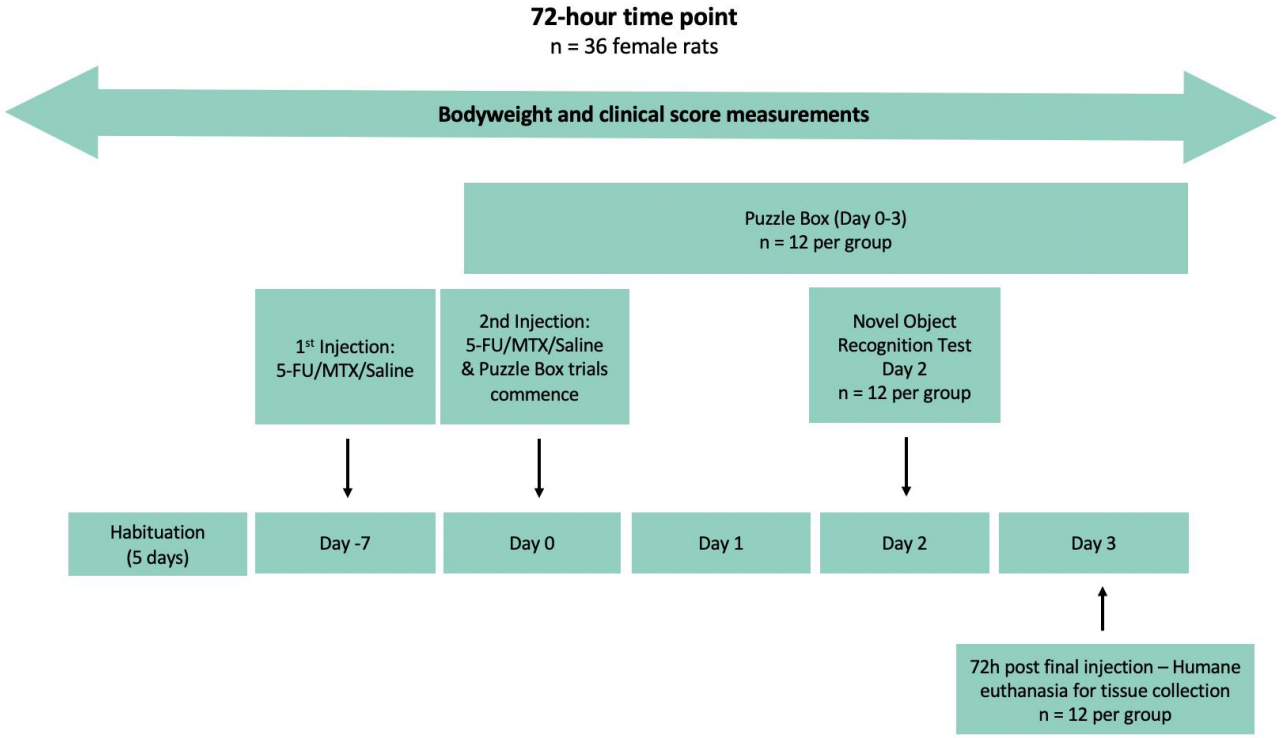

Figure 1. *Overview of experimental timeline.*

Behavioural Testing

1. Puzzle Box

The protocol for the puzzle box was adapted from previously published protocols by Abdallah et al., (2011) and Shepard et al., (2017), with slight modifications made to obstacles to suit a rat model[25, 26]. The apparatus consisted of a rectangular acrylic box with a removable barrier to create two areas: a light area known as the open-field zone and a dark area known as the goal box (Figure 2).

Figure 2. *Diagram of puzzle box.*

Animals were firstly habituated to the puzzle box before receiving their final treatment, during which the door was removed (H1) and unobstructed (H2) for five minutes per trial. The test trials commenced after animals received their final treatment. During these trials, animals were faced with three different obstacles blocking entrance to the door, which increased in complexity to remove. A total of three trials were conducted per obstacle to test executive function, short-term memory (STM) and long-term memory (LTM). The first trial tested executive function to determine whether the animal could develop strategies to move past the obstacle. The second tested STM by allowing a 15-minute gap in-between the first two trials and the third was conducted the following day to test LTM. Testing order was randomly assigned using an online random number generator (*Randomness and Integrity Services Ltd*, Dublin, Ireland).

On day 0 for T1 the door was unobstructed. The door was then obstructed with clean bedding for the following two trials, requiring animals to burrow through to the door (T2-T3). On day 1, animals underwent the same trial again (T4). Following this, the door was obstructed with a soft rectangular plug consisting of a medium density foam, requiring animals to pull it away (T5-T6). On day 2 animals firstly underwent T7 for the final soft plug trial. A hard, wooden plug was then placed through the door requiring animals to push or pull it (T8-T9). T10 was finally conducted on the following day (Table 1).

Day	Trial Number	Trial Condition
-1	H1	No barrier
	H2	Barrier present, open entrance
0	T1	Barrier present, open entrance
	T2	Burrowing (EXECUTIVE)
	T3	Burrowing (STM)
1	T4	Burrowing (LTM)
	T5	Soft plug (EXECUTIVE)
	T6	Soft plug (STM)
2	T7	Soft plug (LTM)
	T8	Hard plug (EXECUTIVE)
	T9	Hard plug (STM)
3	T10	Hard plug (LTM)

Table 1. *Puzzle Box timeline with day, trial number and trial condition, indicating whether executive function, STM or LTM was tested.*

The trials were conducted under homogeneous light. They commenced once the animal was placed into the open-field zone at the furthest point away from the goal box, with its body parallel to the sidewalls and nose facing away from the barrier hole to prevent any bias. The apparatus and materials were cleaned using 70% ethanol between animals to reduce olfactory cues. Each trial was video recorded using a Panasonic video camera (HC-V180, *Panasonic Corporation*, Kadoma, Osaka, Japan) placed above the apparatus. Videos were later analysed blindly using CowLog (Research Center for Animal Welfare and Department of Agricultural Sciences, University of Helsinki, Finland), with the latency to move into the goal box recorded. On analysis, latency time commenced once all four paws were in contact with the floor, while completion of the trial occurred either when all four paws entered the goal box or after five minutes had elapsed, indicating a failure.

2. Novel Object Recognition Test (NOR)

NOR was conducted on day 2 following the puzzle box trials for that day. The test was performed using the protocol developed by Bevins and Besheer (2006)[27]. The basis for the test being that rodents have a natural preference to explore novel objects[28]. Animals were placed in an enclosed arena measuring 50cm x 50cm x 50cm under a brightly lit area. The test consisted of three phases; habituation, where no objects were present, a familiar (T1) and a novel (T2) trial, each conducted for five minutes with a one hour break in-between. Each object was different in colour and shape but was of similar mass. To prevent coercion to explore the objects, animals were placed against the centre of the wall facing to the side of the objects. Each animal was randomised both in terms of the familiar and novel objects assigned to them and also the position (left or right) that the novel object was placed using an online random number generator (*Randomness and Integrity Services Ltd*, Dublin, Ireland).

T1 was conducted with two identical objects affixed to middle of the arena slightly apart from each other. T2 was similar to T1, however one of the objects was replaced with an unfamiliar, novel object (Figure 3). The surface and objects were cleaned using 70% ethanol between animals to reduce olfactory cues. Each trial was video recorded using a Panasonic video camera placed above the arena, as previously described. Videos were later blindly analysed using ANY-maze (ANY-maze UK, IL, USA) and object interaction time was recorded. Animals were removed from analysis if they did not spend a minimum of ten seconds exploring the objects in T1[29]. Object interaction time was scored based on the length of time spent investigating the objects when in direct contact. The degree of object recognition was determined using a preference index score, where the time spent investigating the novel object was divided by the sum exploration of the novel and familiar objects[30].

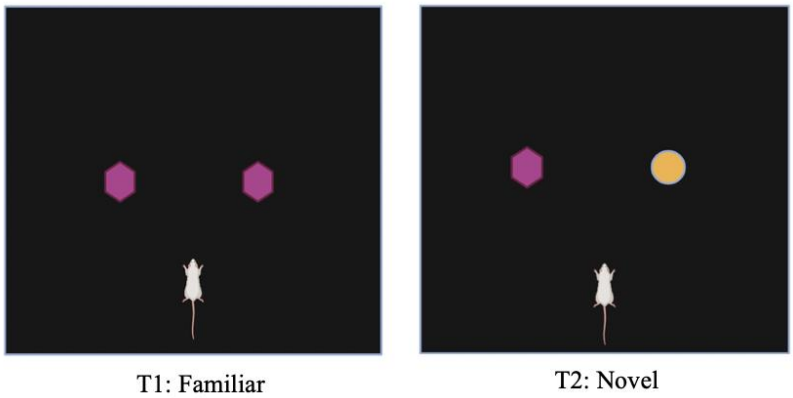

Figure 3. *NOR set-up; T1 with two identical objects and T2 with one familiar object from T1 and a novel object.*

Disease Activity Index (DAI)

DAI was recorded at four time points; baseline (prior to receiving final treatment), 24, 48 and 72-hours post treatment. DAI was measured based on overall appearance, weight loss, stool consistency and rectal bleeding. Animals were scored at each time-point on a severity scale from 0-3 (Table 2).

Parameters	Change	Score
Weight Loss	No weight loss	0
	0-5%	1
	5-10%	2
	>10%	3
Stool Consistency	Normal	0
	Loose	1
	Mild diarrhoea	2
	Overt diarrhoea	3
Rectal Bleeding	No visible blood	0
	Blood spots	1
	Blood in faeces	2
	Gross bleeding	3
Overall Appearance*	Normal	0
	Mild	1
	Moderate	2
	Severe	3

* Overall appearance based on pre-determined criteria; dull/ruffled coat, poor posture/hunched, pale/sunken eyes, behaviour change, dehydration, squealing when handled and reluctant to move.

Table 2. *Scoring criteria for DAI.*

Western Blot

Fresh, snap frozen PFC and hippocampal tissue were homogenised in standard RIPA buffer, centrifuged and the supernatant collected. A BCA assay kit (Pierce BCA, *Thermo Fisher Scientific Inc*, #23227) was used for protein estimation. Gel electrophoresis was performed using Bolt 4-12% Bis-Tris Plus gels (*Thermo Fisher Scientific Inc*) at 150V for 1.5 hours. Gels were transferred to a PVDF membrane using the iBlot 2 Dry Blotting System (*Thermo Fisher Scientific Inc*). Membranes were stained in ATX Ponceau S staining solution for five minutes and washed with tris buffered saline plus tween (TBST) until the Ponceau was removed. 5% skim milk solution (5g skim milk powder, 100ml TBST) was used to block the membranes at room temperature for two hours. Following this, membranes were washed another three times and incubated for two hours in 2% skim milk solution (1g skim milk powder, 100ml TBST) overnight on a rotating device with primary antibodies (GFAP; #ab7620, *Abcam*, Cambridge, UK) and housekeeper protein (GAPDH; #ab8245 Abcam, Cambridge, UK). Membranes were washed a further three times and incubated with secondary antibodies (LICOR 800CW (green) donkey/anti-rabbit 1:10,000; LICOR 800W (green) donkey/anti-mouse, 1:10,000) for two hours at room temperature in 2% skim milk solution on a rotating device covered with aluminium foil. Western blots were imaged using the Odyssey Infrared Imaging System (model 9120; software version 3.0.21) (*LI-COR*, Inc). Analysis of band signals were performed using ImageJ version 1.49 (Wayne Rashband, National Institutes of Health, USA).

Data Analysis

Data were checked for normality and heterogeneity utilising the Shapiro Wilk test and analysed parametrically or non-parametrically as appropriate. Data were considered significant if $p<0.05$. Statistical analyses were performed using MegaStat Excel Add-In (Version 10.3 Release 3.1.6, *McGraw-Hill Higher Education*, New York, NY, USA) and SPSS (*SPSS Inc*., Chicago, IL, USA). A statistician was consulted for analysis of puzzle box data. Data are presented as mean ± standard error of the mean (SEM), unless otherwise stated.

Results

Behavioural Tests

1. Puzzle Box

A two-way repeated measures ANOVA was conducted across ten time points between three treatment groups for both group and within-subject factors. This test was chosen as previous literature analysed their puzzle box data using this method[25, 26, 31]. Significance was found between treatment groups, ($F(2,33)=4.302$, $p=0.022$). A Tukey HSD post-hoc test with a Bonferroni correction for multiple comparisons found time-point differences between MTX and saline treatment, specifically at T7, ($p=0.026$) and T10, ($p=0.035$) (Figure 4 and 5). A number of animals did not complete several trials within the allocated time, hence the little variability shown, particularly for the hard plug trials.

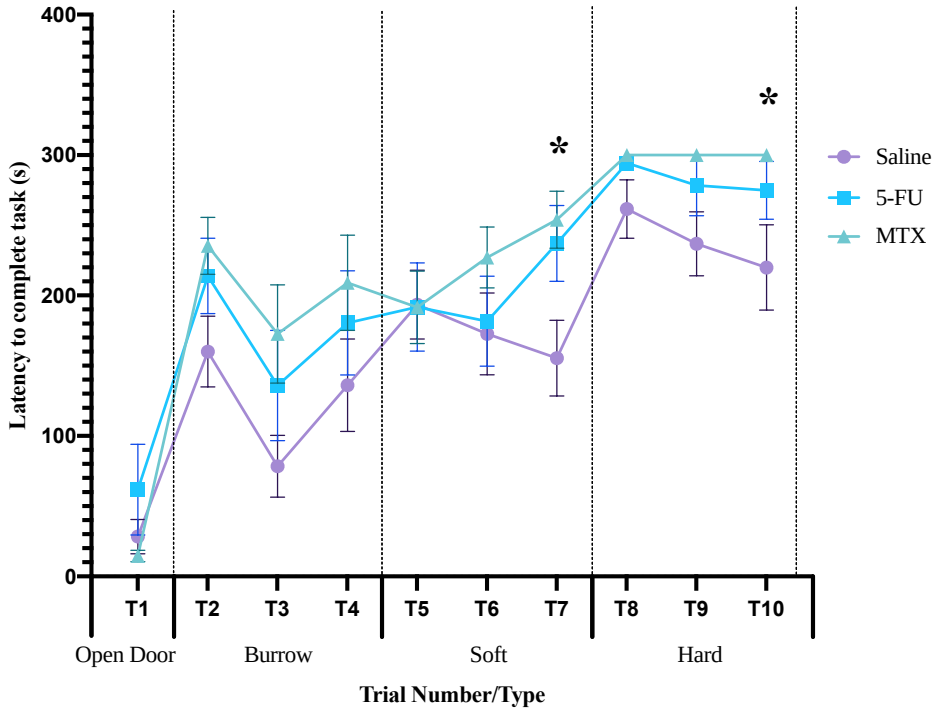

Figure 4. *Puzzle box latency to complete task (s).*

** indicates significance found at T7 (p=0.026) and T10 (p=0.035) between MTX and saline treatment. Data presented as mean ± SEM.*

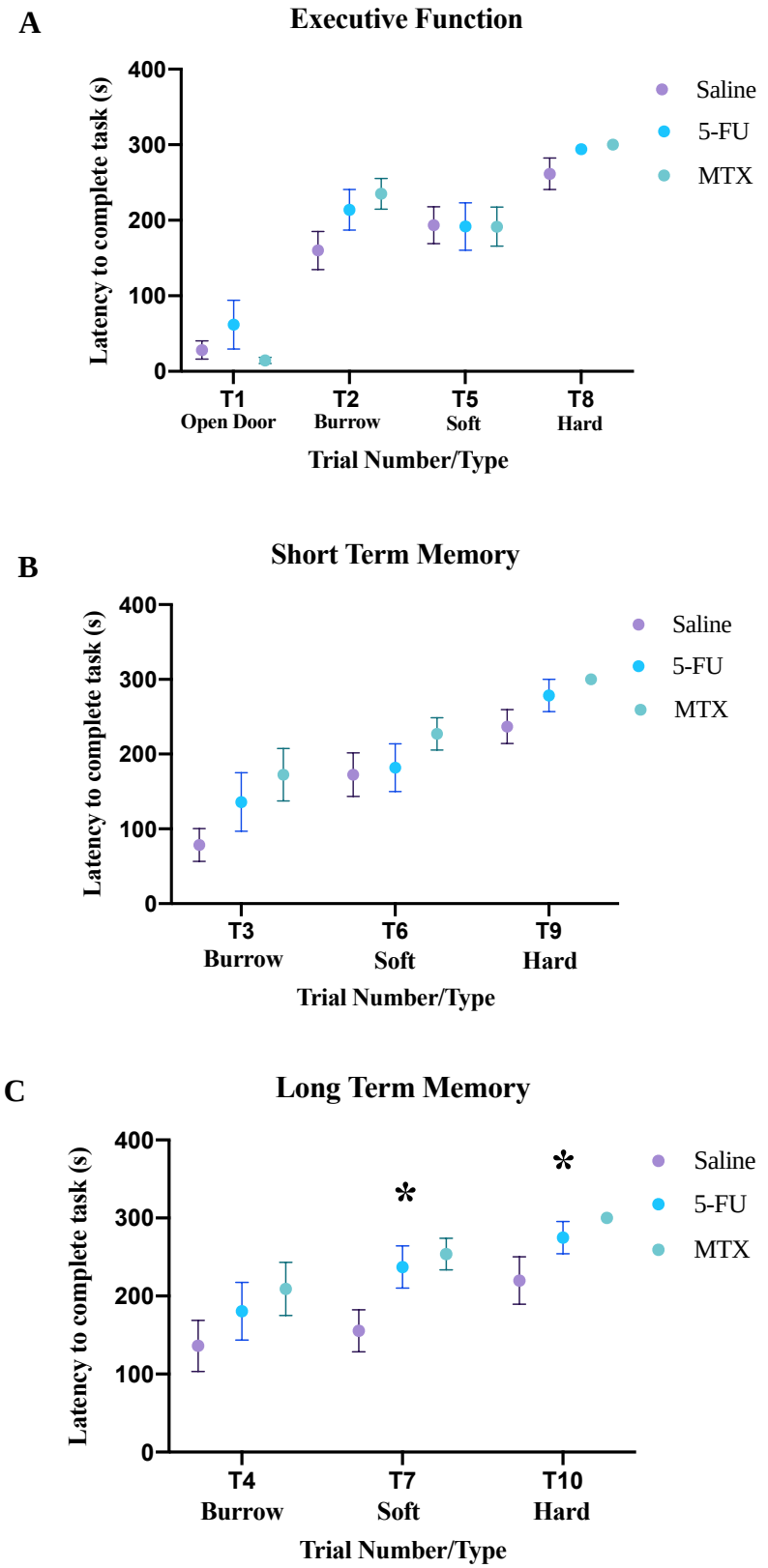

Figure 5. *Puzzle box latency to complete task (s); executive function (A), STM (B) and LTM (C) testing trials. * p<0.05 at T7 and T10 for LTM testing between MTX and saline treatment. Data presented as mean ± SEM.*

2. Novel Object Recognition (NOR)

A total of six animals were excluded from analysis, as they did not meet the required ten second minimum spent exploring the objects in T1 (5-FU=2, MTX=2 and Saline=2). A one-way ANOVA was conducted on preference index results and no significance was found, ($F(2,27)=0.46$, $p=0.6358$) (Figure 6).

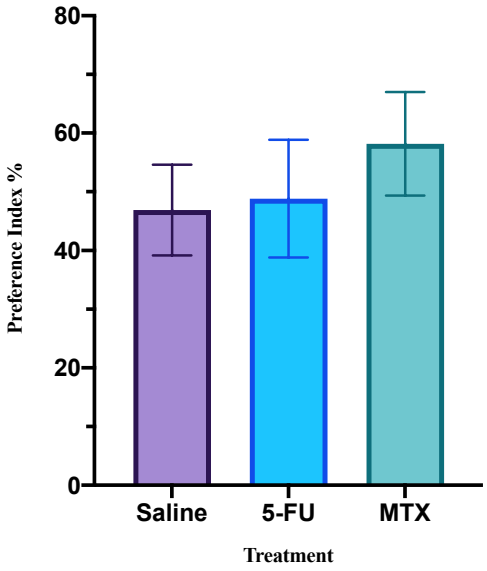

Figure 6. *NOR preference index scores %. n=10 per treatment group. No significance found, (p=0.6358). Data presented as mean ± SEM.*

Disease Activity Index (DAI)

A Friedman's test was conducted on DAI scores within groups, across time at baseline, 24, 48 and 72 hours. Scores increased significantly over time ($X^2(3)=15.160$, P=0.002). Post-hoc analysis using the Wilcoxon Signed Rank test found significance between MTX and saline treated animals across time between baseline and 72 hours, and 48 and 72 hours, (p=0.014 and p=0.016), respectively. A Kruskal Wallis H test was conducted to analyse between-group differences at individual time-points. Significance was found between treatment groups, (H(2)=12.757, p=0.002). Post-hoc analysis using a Mann-Whitney U test found significance at 72-hours between MTX and 5-FU (p=0.13) and MTX and saline (p=0.002) (Figure 7).

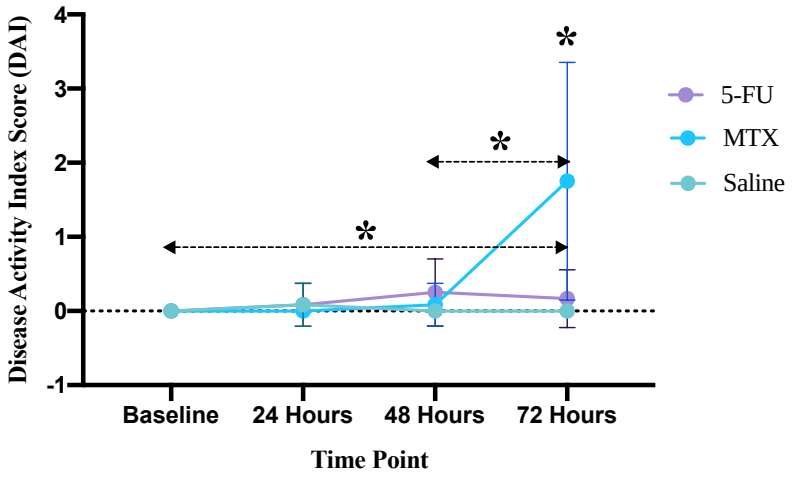

Figure 7. *DAI scores at four time points; baseline, 24, 48 and 72 hours.*

** p<0.05 across time between baseline and 72 hours, and 48 and 72 hours between MTX and saline. p<0.05 at 72 hours between MTX and saline and MTX and 5-FU. Data presented as median with range.*

Western Blot

A one-way ANOVA was performed on PFC and hippocampal relative density. Significance was found for both the PFC, (F(2,15)=5.351, p=0.018), and hippocampus, (F(2,15=12.861, p=0.001). A Tukey HSD post-hoc test found significant differences between 5-FU and saline (p=0.027) and MTX and saline (p=0.037) in the PFC. Significance was also found in the hippocampus between 5-FU and saline (p=0.001), and 5-FU and MTX (p=0.006) (Figure 8).

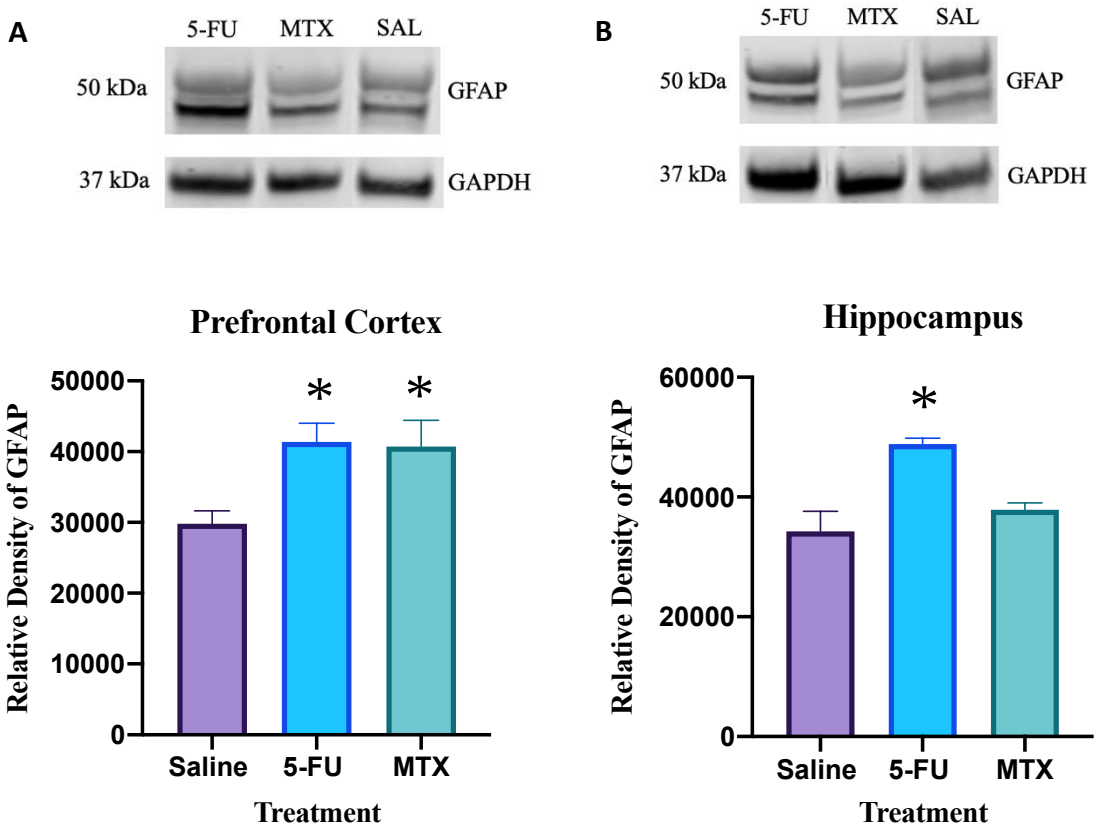

Figure 8. *Analysis of GFAP relative density within PFC (A) and hippocampal (B) tissue. n=6 per treatment group. * p<0.05 between 5-FU and saline and MTX and saline for the PFC, and 5-FU and saline and MTX and 5-FU for the hippocampus. Data presented as mean ± SEM.*

Discussion

CICI is a debilitating condition greatly impacting a person's life by affecting their ability to complete everyday tasks[3, 9, 32]. The aim of this study was to investigate the effects of MTX and 5-FU treatment on cognition at an acute time-point of 72 hours in a rat model, as displayed through performance in behavioural tests. Furthermore, the study aimed to provide evidence of a neuroimmune response occurring in the hippocampus and PFC at this time point. An additional aim was to validate the puzzle box test in a CICI rat model. The results from this study demonstrate that minimal cognitive impairment occurred at 72 hours post chemotherapy treatment. As shown through behavioural testing, impairment was only evident in the hard plug LTM testing trials, but not in others. However, glial activation in the PFC and hippocampus was found to be significantly different in both treatment groups.

The puzzle box was utilised as a novel PFC and hippocampal based task in a rat model of CICI. Differences observed occurred in trials testing for LTM for the hard plug obstacle, indicating that LTM may be affected in MTX treated animals at 72 hours following chemotherapy treatment. However, no differences were found in other trials that tested executive function and STM. Memory impairment is a common symptom reported in human cancer survivors. A longitudinal study of breast cancer survivors found that 61% reported memory impairment[33]. Similarly, animal studies have observed impairment in multiple memory tests in rodents, particularly with MTX and 5-FU treatment[14, 16]. However, it is important to be cautious about drawing conclusions based on results from this study alone. In this study, several animals did not complete trials in the allocated time, as they were not able to move past the obstacle into the goal box. This was particularly evident in the last few trials that utilised the hard plug obstacle. This could indicate that animals found the trials too difficult, or they had reduced motivation. However, as little significance was found between control and treatment groups in behaviour, it is unlikely that motivation towards completing the trials was the main reason as to why several animals did not enter the goal box. DAI scores indicated that animals were experiencing toxic effects from the chemotherapy treatment and therefore they may

have felt too unwell to complete the task. Previous studies utilising the puzzle box to assess cognitive function have done so in mice. This is the first CICI study to utilise it in rats, however further validation is required to ensure the obstacles chosen are correct. The puzzle box has been investigated in one CICI study, where mice treated with the chemotherapeutic agent cisplatin, took significantly more time to enter the goal box during the more difficult trials, indicating treated mice were cognitively impaired[34]. The test has also been utilised in other rodent studies, through investigating the efficacy of environmental enrichment[31], as well as diseases such as schizophrenia, where all mice showed altered executive functions[25], and dementia, where STM was found to be impaired in mice[35]. However, motivation is less likely to be impaired in these models compared to CICI models. This indicates that there is potential for the puzzle box test to be investigated further in other diseases such as CICI, as it can detect subtle cognitive impairment and requires no prior training.

NOR is a well validated behavioural test of cognition, however there is great variability in results seen in CICI research. This study did not observe any differences, which is similar to other studies where mice treated with 5-FU performed on par with controls after one week[12]. Furthermore, MTX treated rats showed no difference in novel object exploration compared to controls after three and seven days[13]. However, a study found cognitive impairment was occurring in rats administered with MTX after three weeks as they failed to distinguish the novel object[15]. This suggests that cognitive impairment could be occurring at a later time-point. NOR only tests the hippocampus for recognition memory, whereas CICI may be primarily affecting the PFC at this time-point[36]. NOR may also not be detecting more subtle cognitive changes. This could be due to only the hippocampus being tested, or the impairment induced being less severe at this time. Therefore, the use of more sensitive tests to detect subtle cognitive changes may be required. CICI does not only affect one brain region, it can impair cognition in the prefrontal, frontal, temporal and parietal regions[1]. Multiple behavioural tests that focus on different brain regions may be needed to detect the full extent of impairment and highlight why the puzzle box assessing both PFC and hippocampal function could be useful.

DAI scores increased over the four time points in chemotherapy treated animals. Taken with the behavioural findings, this suggests that gastrointestinal toxic effects from the chemotherapeutic agents are more severe than neurotoxic effects at 72 hours following chemotherapy treatment. MTX and 5-FU elicit mucositis rapidly[37-39] within 72 hours following chemotherapy treatment in rats[37-39]. Due to these rapid toxic effects, it is challenging to determine whether the behavioural tests are a true representation of the cognitive impairment incurred, or are instead a result of illness and reduced motivation to perform the tests[40]. It is proposed that chemotherapy exposure influences the gut-brain axis via different mechanisms which include the fact that gastrointestinal peripheral effects precede CNS effects[41]. Therefore, 72 hours may be too early to see cognitive changes occur[41] if toxic effects are occurring in the gut prior to the CNS.

Astrocytic activation was analysed through GFAP expression. Increased astrocytic activation was found in the PFC and hippocampus in MTX and 5-FU treated animals. However, whilst 5-FU treated animals were affected in both the PFC and hippocampus, MTX treated animals were only affected in the PFC. This suggests that the PFC may be impacted first by chemotherapy, with hippocampal damage occurring later, or that 5-FU has an increased neurotoxic effect compared to MTX. Both MTX and 5-FU and are antimetabolites, however 5-FU is able to cross the BBB[14] and may therefore cause potential damage to specific brain regions sooner, whereas MTX may have a more indirect effect through being unable to cross the BBB[42]. This could explain why it could potentially take longer to affect these brain regions. Similarly, a study examining mice administered with chemotherapeutic agents; docetaxel, adriamycin and cyclophosphamide, saw an increase in GFAP levels in the PFC, but not the hippocampus[43]. Astrocytes are involved in the maintenance and permeability of the BBB, forming a selective barrier for entry of immune cells into the CNS[8]. The release of inflammatory mediators such as cytokines and astrocytes is necessary to perform normal physiological functions, however when inflammation becomes excessive, this can lead to alterations in the structure and function of the CNS[8]. Future studies could investigate inflammatory cytokine

expression at this time-point in these regions, along with markers for microglial activation, such as IBA1, to produce a more comprehensive picture of the neuroimmune response.

The puzzle box is a relatively new behavioural assay, and to the best of our knowledge is the first study to investigate in rat models of CICI. It has potential to provide an easy method to assess PFC and hippocampal functioning, however it has not been well validated thus far, therefore making it a limitation to the study. The challenge was finding an appropriate behavioural test for a 72-hour time-point. An alternative test such as the five-choice serial reaction time test, is a PFC based task that allows for the investigation of cognitive impairment beyond the hippocampus. However, this would not have been possible to conduct as animals are required to undergo extensive prior training. Future studies could therefore look to develop an improved protocol for the puzzle box, such as validating the obstacles.

In conclusion, this study aimed to examine both hippocampal and PFC cognitive impairment at 72 hours post chemotherapy treatment, along with the related astrocytic activation for these regions. The second aim was to validate the puzzle box test in a CICI rat model. The results demonstrate that no significant impairment was observed in cognition through behavioural testing. Additionally, glial activation occurs in the PFC and hippocampus in rats treated with MTX and 5-FU at 72-hours following chemotherapy treatment. Therefore, no explicit link between the response and cognitive changes has been identified. However, this could be a precursor to the cognitive changes that may be occurring. Future studies are therefore vital to chart the time course of behavioural and neuroimmune reactivity marker expression changes over multiple time-points, in an effort to target and inhibit CICI from occurring in cancer survivors.

Professional & Funding Acknowledgements

Special thanks to my supervisor Dr Alexandra Whittaker, co-supervisor Rebecca George and lab members Ines Semendric and Jane Morphett for guidance, laboratory, technical and protocol support this year. Additionally, special thanks to Assoc. Prof. Lyndsey Collins-Praino for guidance and laboratory use. This study was funded by the Neurosurgical Research Foundation.

References

1. Matsos A & Johnston IN (2019). Chemotherapy-induced cognitive impairments: A systematic review of the animal literature. *Neuroscience and Biobehavioral Reviews* **102**, 382-399.
2. Foley JJ, Raffa RB & Walker EA (2008). Effects of chemotherapeutic agents 5-fluorouracil and methotrexate alone and combined in a mouse model of learning and memory. *Psychopharmacology* **199**, 527-538.
3. Janelsins MC, Kesler SR, Ahles TA & Morrow GR (2014). Prevalence, mechanisms, and management of cancer-related cognitive impairment. *International Review of Psychiatry* **26**, 102-113.
4. Shi DD, Huang YH, Lai CSW, Dong CM, Ho LC, Wu EX, Li Q, Wang XM, Chung SK, Sham PC & Zhang ZJ (2019). Chemotherapy-Induced Cognitive Impairment Is Associated with Cytokine Dysregulation and Disruptions in Neuroplasticity. *Molecular Neurobiology* **56**, 2234-2243.
5. Collins B, Mackenzie J, Stewart A, Bielajew C & Verma S (2009). Cognitive effects of chemotherapy in post-menopausal breast cancer patients 1 year after treatment. *Psychooncology* **18**, 134-43.
6. Koppelmans V, Breteler MMB, Boogerd W, Seynaeve C, Gundy C & Schagen SB (2012). Neuropsychological performance in survivors of breast cancer more than 20 years after adjuvant chemotherapy. *Journal of Clinical Oncology* **30**, 1080-1086.
7. DiSabato DJ, Quan N & Godbout JP (2016). Neuroinflammation: the devil is in the details. *Journal of Neurochemistry* **139**, 136-153.
8. McLeary F, Davis A, Rudrawar S, Perkins A & Anoopkumar-Dukie S (2019). Mechanisms underlying select chemotherapeutic-agent-induced neuroinflammation and subsequent neurodegeneration. *European Journal of Pharmacology* **842**, 49-56.

9. Vichaya EG, Chiu GS, Krukowski K, Lacourt TE, Kavelaars A, Dantzer R, Heijnen CJ & Walker AK (2015). Mechanisms of chemotherapy-induced behavioral toxicities. *Frontiers in Neuroscience* **9**.

10. Koźmiński P, Halik PK, Chesori R & Gniazdowska E (2020). Overview of dual-acting drug methotrexate in different neurological diseases, autoimmune pathologies and cancers. *International Journal of Molecular Sciences* **21**.

11. Raffa RB & Tallarida RJ (2010). Chemo fog: cancer chemotherapy-related cognitive impairment. Preface. *Adv Exp Med Biol* **678**, vii-viii.

12. Fremouw T, Fessler CL, Ferguson RJ & Burguete Y (2012). Preserved learning and memory in mice following chemotherapy: 5-Fluorouracil and doxorubicin single agent treatment, doxorubicin-cyclophosphamide combination treatment. *Behavioural Brain Research* **226**, 154-162.

13. Li Y, Vijayanathan V, Gulinello ME & Cole PD (2010). Systemic methotrexate induces spatial memory deficits and depletes cerebrospinal fluid folate in rats. *Pharmacol Biochem Behav* **94**, 454-63.

14. Mustafa S, Walker A, Bennett G & Wigmore PM (2008). 5-Fluorouracil chemotherapy affects spatial working memory and newborn neurons in the adult rat hippocampus. *European Journal of Neuroscience* **28**, 323-330.

15. Seigers R, Schagen SB, Beerling W, Boogerd W, van Tellingen O, van Dam FS, Koolhaas JM & Buwalda B (2008). Long-lasting suppression of hippocampal cell proliferation and impaired cognitive performance by methotrexate in the rat. *Behav Brain Res* **186**, 168-75.

16. Winocur G, Vardy J, Binns MA, Kerr L & Tannock I (2006). The effects of the anti-cancer drugs, methotrexate and 5-fluorouracil, on cognitive function in mice. *Pharmacology Biochemistry and Behavior* **85**, 66-75.

17. Lyons L, Elbeltagy M, Bennett G & Wigmore P (2012). Fluoxetine counteracts the cognitive and cellular effects of 5-Fluorouracil in the rat hippocampus by a mechanism of prevention rather than recovery. *PLoS ONE* **7**.

18. Selamat MH, Loh SY, Mackenzie L & Vardy J (2014). Chemobrain experienced by breast cancer survivors: A meta-ethnography study investigating research and care implications. *PLoS ONE* **9**.

19. Gandal MJ, Ehrlichman RS, Rudnick ND & Siegel SJ (2008). A novel electrophysiological model of chemotherapy-induced cognitive impairments in mice. *Neuroscience* **157**, 95-104.

20. Briones TL & Woods J (2011). Chemotherapy-induced cognitive impairment is associated with decreases in cell proliferation and histone modifications. *BMC Neuroscience* **12**.

21. Seigers R, Loos M, Van Tellingen O, Boogerd W, Smit AB & Schagen SB (2016). Neurobiological changes by cytotoxic agents in mice. *Behav Brain Res* **299**, 19-26.

22. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, Emerson M, Garner P, Holgate ST, Howells DW, Karp NA, Lazic SE, Lidster K, MacCallum CJ, Macleod M, Pearl EJ, Petersen OH, Rawle F, Reynolds P, Rooney K, Sena ES, Silberberg SD, Steckler T & Würbel H (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *Experimental Physiology* **105**, 1459-1466.

23. Coates AS, Tattersall MHN & Swanson C (1984). Combination therapy with methotrexate and 5-fluorouracil: A prospective randomized clinical trial of order of administration. *Journal of Clinical Oncology* **2**, 756-761.

24. Winocur G, Wojtowicz JM, Merkley CM & Tannock IF (2016). Environmental enrichment protects against cognitive impairment following chemotherapy in an animal model. *Behavioral Neuroscience* **130**, 428-436.

25. Ben Abdallah NMB, Fuss J, Trusel M, Galsworthy MJ, Bobsin K, Colacicco G, Deacon RMJ, Riva MA, Kellendonk C, Sprengel R, Lipp HP & Gass P (2011). The puzzle box as a

simple and efficient behavioral test for exploring impairments of general cognition and executive functions in mouse models of schizophrenia. *Experimental Neurology* **227**, 42-52.

26. Shepard R, Beckett E & Coutellier L (2017). Assessment of the acquisition of executive function during the transition from adolescence to adulthood in male and female mice. *Developmental Cognitive Neuroscience* **28**, 29-40.
27. Bevins RA & Besheer J (2006). Object recognition in rats and mice: A one-trial non-matching-to-sample learning task to study 'recognition memory'. *Nature Protocols* **1**, 1306-1311.
28. Ennaceur A & Delacour J (1988). A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behavioural Brain Research* **31**, 47-59.
29. Antunes M & Biala G (2012). The novel object recognition memory: Neurobiology, test procedure, and its modifications. *Cognitive Processing* **13**, 93-110.
30. Gaskin S, Tremblay A & Mumby DG (2003). Retrograde and anterograde object recognition in rats with hippocampal lesions. *Hippocampus* **13**, 962-969.
31. O'Connor AM, Burton TJ, Leamey CA & Sawatari A (2014). The use of the puzzle box as a means of assessing the efficacy of environmental enrichment. *Journal of Visualized Experiments*.
32. Bagnall-Moreau C, Chaudhry S, Salas-Ramirez K, Ahles T & Hubbard K (2019). Chemotherapy-Induced Cognitive Impairment Is Associated with Increased Inflammation and Oxidative Damage in the Hippocampus. *Molecular Neurobiology* **56**, 7159-7172.
33. Argyriou AA, Assimakopoulos K, Iconomou G, Giannakopoulou F & Kalofonos HP (2011). Either called "chemobrain" or "chemofog," the long-term chemotherapy-induced cognitive decline in cancer survivors is real. *Journal of Pain and Symptom Management* **41**, 126-139.
34. Ma J, Huo X, Jarpe MB, Kavelaars A & Heijnen CJ (2018). Pharmacological inhibition of HDAC6 reverses cognitive impairment and tau pathology as a result of cisplatin treatment. *Acta neuropathologica communications* **6**, 103.

35. Van der Jeugd A, Vermaercke B, Halliday GM, Staufenbiel M & Götz J (2016). Impulsivity, decreased social exploration, and executive dysfunction in a mouse model of frontotemporal dementia. *Neurobiology of Learning and Memory* **130**, 34-43.

36. Kovalchuk A & Kolb B (2017). Chemo brain: From discerning mechanisms to lifting the brain fog—An aging connection. *Cell Cycle* **16**, 1345-1349.

37. Vanhoecke B, Bateman E, Mayo B, Vanlancker E, Stringer A, Thorpe D & Keefe D (2015). Dark Agouti rat model of chemotherapy-induced mucositis: Establishment and current state of the art. *Experimental Biology and Medicine* **240**, 725-741.

38. Bowen JM, Gibson RJ & Keefe DMK (2011). Animal Models of Mucositis: Implications for Therapy. *Journal of Supportive Oncology* **9**, 161-168.

39. Sangild PT, Shen RL, Pontoppidan P & Rathe M (2018). Animal models of chemotherapy-induced mucositis: Translational relevance and challenges. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **314**, G231-G246.

40. Whittaker AL, Leach MC, Preston FL, Lymn KA & Howarth GS (2016). Effects of acute chemotherapy-induced mucositis on spontaneous behaviour and the grimace scale in laboratory rats. *Laboratory Animals* **50**, 108-118.

41. Bajic JE, Johnston IN, Howarth GS & Hutchinson MR (2018). From the bottom-up: Chemotherapy and gut-brain axis dysregulation. *Frontiers in Behavioral Neuroscience* **12**.

42. Cooper I, Last D, Guez D, Sharabi S, Elhaik Goldman S, Lubitz I, Daniels D, Salomon S, Tamar G, Tamir T, Mardor R, Fridkin M, Shechter Y & Mardor Y (2015). Combined local blood-brain barrier opening and systemic methotrexate for the treatment of brain tumors. *Journal of Cerebral Blood Flow and Metabolism* **35**, 967-976.

43. Shi DD, Huang YH, Lai CSW, Dong CM, Ho LC, Li XY, Wu EX, Li Q, Wang XM, Chen YJ, Chung SK & Zhang ZJ (2019). Ginsenoside Rg1 Prevents Chemotherapy-Induced Cognitive Impairment: Associations with Microglia-Mediated Cytokines, Neuroinflammation, and Neuroplasticity. *Molecular Neurobiology* **56**, 5626-5642.