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Single high dose treatment with methotrexate causes long-lasting cognitive dysfunction in laboratory rodents

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ABSTRACT

Clinical studies have suggested that cognitive impairment due to chemotherapy persists long after treatment cessation. While animal studies have similarly found impairments in cognition due to chemotherapy, these studies are limited as they only assess the acute or extremely short-term effects of chemotherapy on cognition (e.g. within 1 month of treatment). Male hooded Wistar rats (N = 22) received either a high dose of methotrexate (MTX: 250 mg/kg i.p.) or physiological saline. Cognitive performance was evaluated acutely at 2 weeks, and up to 8 months post injection using the Morris water maze, Novel object recognition task, and an instrumental go/no-go task to assess discrimination learning. MTX-treated rats displayed impaired novel object recognition compared to controls at 11, 95, and 255 days after treatment. MTX rats were able to learn the hidden spatial location of a platform 22 days after treatment. When tested again after a 95-day retention interval, MTX rats showed impaired spatial memory compared to controls, but were subsequently able to re-learn the task. Finally, MTX-treated rats showed considerable difficulty learning to inhibit their behaviour in an instrumental discrimination task. These results show that chemotherapy produces persistent but subtle cognitive deficits in laboratory rodents that vary with time post treatment.

1. Introduction

Patient complaints of cognitive impairment persisting long after adjuvant chemotherapy treatment for cancer (Schagen et al., 1999) have led to studies investigating whether patients have long term impairment on objective measures of neurocognitive function due to chemotherapeutic treatment (Vardy and Tannock, 2007). Emerging consensus from this research suggests a subset of cancer-chemotherapy survivors experience impairments in verbal and visual memory, attention/concentration and processing speed (Vardy and Tannock, 2007). However, the duration of these impairments has been difficult to ascertain as the majority of published studies have been cross sectional in design with testing performed at varying time points post chemotherapy (Vardy et al., 2008). While some researchers reported cognitive impairment persisting 5–10 years post treatment (Ahles et al., 2002), others have shown that patients who presented with cognitive impairments at 2 years post treatment for breast cancer (Schagen et al., 1999; Van Dam et al., 1998) showed no impairment when re-tested 4 years after treatment (Schagen et al., 2002). Further, some groups have found long term functional changes in the central nervous system (CNS); Kreukels et al. (2005) found that breast cancer patients had altered event-related potentials and impaired performance on an information-processing task when tested 5 years post cyclophosphamide, methotrexate (MTX) and 5-Fluorouracil (5-FU) chemotherapy, when compared to cancer survivors who received surgery alone. In addition, Silverman et al. (2007) found cancer survivors who received adjuvant chemotherapy during treatment had altered frontocortical, cerebellar, and basal ganglia activity on brain imaging relative to healthy controls 5–10 years after chemotherapy cessation.

Animal models offer a unique opportunity to investigate the association between chemotherapy and cognition as the causal role of chemotherapy can be evaluated in the absence of cancer and other anti-cancer treatments, enabling identification of key agents responsible for the observed impairment. This approach has demonstrated that rats or mice treated with MTX (Madhyastha et al., 2002; Seigers et al., 2008, 2009), 5-FU (Mustafa et al., 2008), MTX in combination with 5-FU (Foley et al., 2008; Gandal et al., 2008; Winocur et al., 2006), doxorubicin (Liedke et al., 2009), cyclophosphamide in combination with doxorubicin (Konat et al., 2008; MacLeod et al., 2007), and cytosine arabinoside (Li et al., 2008) have impaired performance in tasks requiring short or long term memory or rule learning. However, there is a paucity of animal studies that address the long term impact of chemotherapy, as all but one study (Lee et al., 2006) investigated the behavioural effects of chemotherapy within 1 month of treatment (Boyette-Davis and Fuchs, 2009; Foley et al.,

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2008; Gandal et al., 2008; Konat et al., 2008; Li et al., 2008, 2010; Liedke et al., 2009; MacLeod et al., 2007; Madhyastha et al., 2002; Mustafa et al., 2008; Reiriz et al., 2006; Seigers et al., 2008, 2009; Winocur et al., 2006). Yet it is likely that the cognitive impairment observed shortly after chemotherapy persists for longer. Noble and colleagues assessed the impact of several chemotherapy agents on neural progenitor cells and dividing and non-dividing oligodendrocytes at 6 months post treatment (Dietrich et al., 2006; Han et al., 2008). Their studies show mice given 5-FU had long-lasting and significant suppression of neural progenitor cells in the subgranular ventricular zone of the dentate gyrus up to 6 months post treatment. Analysis of auditory brain stem responses corroborated these findings and indicated myelin damage at 56 days post treatment (Han et al., 2008). However, no behavioural data was reported for 6 months post treatment.

This study aims to address this significant gap in the literature by investigating the long term impact of chemotherapy in three animal models of cognition. First, the novel object recognition (NOR) task was used to assess the effects of MTX on short-term recognition memory and familiarity at 11, 95 and 254 days post MTX. Second, the Morris water maze (MWM) evaluated the effects of MTX on recent or remote long term memories. And finally, the rats were trained in a go/no-go discrimination task 6 months after MTX to assess whether MTX produced long term effects on rule learning.

2. Methods

2.1. Subjects

Twenty-two male hooded Wistar rats (University of Sydney, School of Psychology breeding program), weighing between 303 g and 460 g, with an average weight of 348.95 g ± 7.61 SEM were used. Rats were approximately 8 months old at experiment commencement and had been used previously in an unrelated behavioural experiment using Pavlovian flavour conditioning. This previous experiment was non-aversive, assessing simple appetitive classical conditioning and unconditioned-stimulus pre-exposure. It was thought not to interfere with the complex cognitive tasks employed in the present experiment. Animals were housed in groups of 4 in large acrylic tubs, with continuous access to both food and water. The home cages were kept in a colony room maintained at 21 °C ± 1 °C and a 12:12 h light:dark cycle, with lights on at 08:00. All experimentation was approved by the Animal Ethics Committee, University of Sydney.

2.2. Drug administration

Rats were weighed daily for 7 days prior to treatment and assigned to either a single intraperitoneal (i.p.) injection of MTX (n = 11; 250 mg/kg, 100 mg/ml, Sigma-Aldrich, St. Louis, MO) or physiological saline (vehicle; n = 11) by matching for weight. There was no weight difference between the groups prior to experiment start (MTX 349.91 g ± 8.982 SEM; vehicle 348.00 ± 12.743 SEM; $t(20) = 0.122$, $p > .05$). Following a procedure similar to that of Seigers et al (2008), a single (i.p.) injection of leucovorin (20 mg/kg, 10 mg/ml, Sigma-Aldrich, St Louis, MO), a rescue remedy used with human chemotherapy patients, was administered 18 h post MTX injection. Rats were observed everyday post injection for a total of 14 days, after which rats were weighed twice weekly.

2.3. Behavioural testing

2.3.1. Novel object recognition

Novel object recognition (NOR) testing took place in an opaque black plastic circular arena (80 cm diameter × 30 cm height). The day preceding testing, rats were habituated to the testing arena for 5 min in the absence of any objects, for exploration. The following day rats received a paired trial consisting of a sample trial and a test trial. During the sample trial rats were individually placed in the arena for 3 min in the presence of two identical objects, spaced 35 cm apart. The rat was then returned to its home cage, and after a 1 hour delay the test trial was conducted. During the test trial the rat was placed in the arena for 3 min with one object the same as in the sample trial and a new, "novel" object that was different in shape/size and texture. All objects were either fixed to the floor of the arena or weighted so that they could not be moved around. Between each trial, the arena and objects were cleaned with 50% ethanol solution and air-dried, ensuring no odors were present or associated with any of the objects or arena. NOR testing was carried out at 11, 95, and 254 days post MTX injection. On day 255 post treatment an additional 2 hour delay was employed. Each testing time point employed a different pair of objects and was counterbalanced for order across rats. The paired objects were: a glass bottle and aluminium cylinder; a ceramic mug and plastic rectangular prism; a large plastic tooth and capped conical glass flask; a plastic serrated cylinder and small tin cylinder.

2.3.2. Spatial Morris water maze

Training and testing performance on the spatial version of the Morris water maze (MWM) task took place in a circular pool (200 cm diameter × 60 cm height) surrounded by salient visual cues (e.g. large black square) that the rats could use to navigate. These spatial cues remained in a fixed location for both the training and testing. The pool was filled with water (20–23 °C) and contained a clear Perspex Atlantis platform (20 cm diameter, Med Associates Inc, VT) below the surface (water height 48 cm) for the rats to escape to. The water was made opaque by the addition of a non-toxic acrylic paint (ROPAQUE™ ULTRA E Opaque Polymer, Rohm and Hass). A small closed circuit camera was mounted directly above the centre of the tank and the rats were tracked with a custom made Labview program (v8.2, National Instruments).

During training, magnets on the base of the platform kept the platform submerged 20 cm below the surface of the water. Once the rat passed over the platform location, the platform was raised to 1 cm below the surface of the water by the tracking software to provide an escape for the rat. For the spatial version of this task the platform remained fixed in the same position from trial to trial, while the start location varied from trial to trial. If the rat failed to swim to the platform within 2 min it was gently guided to the platform by the experimenter. Once the rat was on the platform it was allowed to remain there for 10 seconds (s). The rat was then removed and dried with a towel by the experimenter and placed in a dry cage in front of a heater to await the next trial. The inter-trial-interval was approximately 5 min. Training commenced 22 days after treatment, and took place over 3 consecutive days, with rats completing 4 trials per day.

A probe trial was conducted on day 25. During the probe trial the platform was kept submerged on the pool bottom and the rats were allowed to freely swim for 60 s. After 60 s the platform became "active"; once the rat swum over the platform location the computer triggered the platform to rise to 1 cm below the water surface and the rat could use it to escape from the water. As in training, if the rat failed to find the platform within 2 min it was guided to the platform by the experimenter. The rat was allowed to remain on the platform for 10 s before being removed, dried and placed in the dry cage. This was done to avoid extinction of the spatial location for subsequent remote memory testing. Throughout training and probe trials, latency to cross the platform location was measured. During the probe trial the amount of time spent in each quadrant, and number of incorrect entries to each quadrant was also recorded.

At 4 months (120 days) post MTX treatment, rats completed another probe trial to assess long term retention of the location of the escape platform. This probe trial was followed by 2 further training trials. Twenty-four hours later another probe trial was conducted.

2.3.3. Go/no-go discrimination task

Training and testing took place in 7 operant chambers, measuring 30×26×31 cm, housed in sound- and light-resistant shells. Each chamber was fitted with a fan that provided masking noise. Each chamber had Plexiglas sidewalls, aluminium ceiling and end walls and stainless steel rod flooring. A magazine located in the centre of one of the end walls had liquid reinforcer (20%v/v sucrose solution) delivered from a 0.1-ml dipper cup. On the same wall two 48-mm wide retractable levers (MED Instruments Inc.) were located either side of the magazine aperture; only the right-hand lever was used in this experiment. This lever was 65 mm from the bottom of the chamber and the edge of the magazine, and projected 19 mm into the chamber. The chambers were controlled by a custom built Labview (v7.1, National Instruments).

Rats were habituated to the chambers for 10 min, and magazine trained for 2 days. Magazine training consisted of 20 trials with sucrose delivered every 60 s. In total, rats were in the operant chambers for 30 min and had the opportunity to receive 20 reinforcers. A second magazine training session was conducted on the following day that lasted approximately 20 min.

Lever press acquisition began the following day and continued until rats had achieved over 80% press criterion. Each acquisition session consisted of 20 discrete trials where the lever was inserted for 15 s separated by a variable inter-trial-interval (ITI) set at 60 ± 10 s. If the rat pressed the lever during the 15 s insertion the lever retracted and sucrose reinforcement was delivered to the magazine. If the rat failed to press the lever during the 15 s insertion, the lever retracted and no reinforcement was delivered.

Go/no-go discrimination training commenced 174 days after MTX injection. Training sessions consisted of both go and no-go trials. During go trials, a discriminative stimulus (S^D: audible tone/static chamber light) was presented for 15 s; 5 s after the commencement of the S^D the lever was inserted into the chamber for 15 s. If the rat pressed the lever during this time the lever retracted and sucrose reinforcement was delivered to the magazine chamber, otherwise the lever retracted as before. In no-go trials the lever was inserted into the chamber for 15 s, without any additional stimuli. If the rat pressed the lever during this time the lever retracted and no reinforcement was delivered to the magazine. If the rat withheld the lever press response during the 15 s lever insertion, sucrose reinforcement was delivered upon lever retraction. Each training session consisted of 20 or 21 trials, dependant on the specific ratio of go/no-go trials, separated by a 60 s ± 10 s ITI, with a maximum of 3 go or 3 no-go trials presented consecutively. For days 1–14 of go/no-go training, 10 go trials and 10 no-go trials were presented (1:1 ratio) and days 15–28 a ratio of 1:2 trials was employed to prevent prepotent go responding.

2.4. Statistical analysis

2.4.1. Weight

The rat's body weight was converted to percent weight gain relative to initial weight for the first 14 days post treatment, and analysed using a repeated measure ANOVA to partition variance into the main effects of: days since treatment (days 1 to 14), drug treatment (vehicle vs. MTX), and their interactions.

2.4.2. NOR

Investigation of objects was quantified as nose either touching or within 0.5 cm of the object. Standing next to, or sitting on the object was not considered investigation. Time investigating the objects was converted to a novel object preference score by the following formula: (time investigating novel object [s])/(time investigating novel object [s] + time investigating familiar object [s])*100. Independent t-tests were carried out on the preference score at each time test point comparing MTX and control rats' relative level of novel object preference.

2.4.3. MWM

Escape latencies during initial training (days 22–24) were averaged over the day and were analysed by repeated measures ANOVA to partition variance into the between subjects drug treatment main effect, the within subjects training day effect and their interactions. Similarly, the escape latencies for the 2 training trials conducted on day 120 were averaged and potential differences between the treatment groups were assessed by an independent samples t-test.

For the probe trials on days 25, 120 and 121, the dependent variables were; latency to cross the platform location, time spent searching in the target quadrant (expressed as a percentage of total probe time), number of platform location crossings, and number of errors (number of incorrect quadrant entries). These were analysed by independent t-tests at all time points. Due to computer mishap, data was lost for one rat (MTX) at the 4-month probe test.

2.4.4. Go/no-go discrimination task

Number of bar presses made during lever press acquisition sessions were analysed with a repeated measures ANOVA with group as a between subjects factor. For the go/no-go discrimination task go trials were analysed separately to no-go trials. There was no difference between go trials that employed an audible tone or static light discriminative stimuli (data not shown), so the results were assessed across discriminative stimuli. For go trials the number of correct hits was analysed by a repeated measures ANOVA, with group as the between subjects factor and training session (1–28) as the repeated within subjects measure. Similarly, for the no-go trials number of correct rejections was analysed by repeated measures ANOVA.

3. Results

3.1. Weight

Fig. 1 shows the percent weight gain post treatment. Rats treated with MTX lost a maximum of 5% of their initial body weight on days 4 and 5 post treatment, and then began to recover on day 6 post treatment. The main effect of day post treatment was significant ($F(13,260) = 16.469$, $p < .001$), as was the main effect of group ($F(1,20) = 15.118$, $p = .001$). In addition, the interaction between group and day was significant ($F(13,260) = 6.261$, $p < .001$).

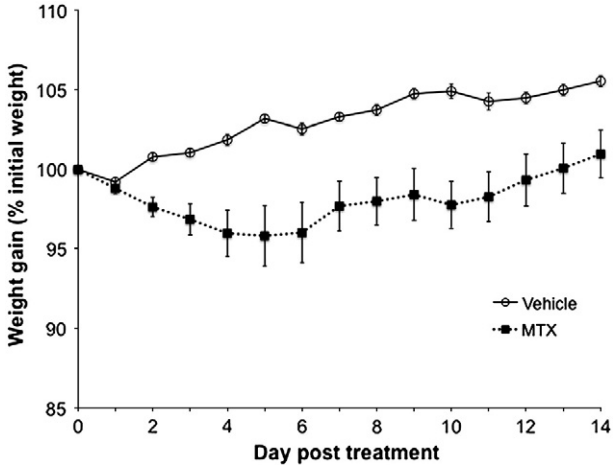

Fig. 1. Mean body weight gain post treatment with either saline (open circles) or MTX (closed squares). Body weight is expressed as a percentage of the pre-treatment weight (day 0). The bars represent ± standard error of the mean.

3.2. NOR

Fig. 2 shows the preference scores for the novel object when tested 11, 95, 254 and 255 days after treatment. Control rats generally spent more time investigating the novel object than the familiar object during testing, as reflected in higher preference scores for the novel object. When rats were tested with a 1 hour delay between sample and test trials, control rats spent more time investigating the novel object and had significantly higher preference scores than rats treated with MTX at 11 ($t(20)=6.779$, $p<.001$) and 95 days ($t(20)=5.043$, $p<.001$), but not at 254 days post treatment ($t(20)=.996$, $p>.05$). However, when tested with the more difficult 2 hour delay between sample and test on day 255, MTX-treated rats spent less time investigating the novel object compared to the familiar, and as such had significantly lower preference scores for the novel object than control rats ($t(20)=5.701$, $p<.001$). There were no differences in total object investigation time between MTX and control rats at all time points ($p>.05$, data not shown).

3.3. MWM

Fig. 3 shows the latency to find the hidden platform during training in the MWM. Latency to reach the platform reduced across training sessions on days 22–24, supported by a significant main effect of training day ($F(2,20)=6.305$, $p<.01$). There was no main effect of group or group by training day interaction (largest $F(1,20)=2.980$, $p>.05$). Consistent with the initial training results, when rats were tested for memory of the spatial location 24 h after the last training trial (day 25) no differences were found between control and MTX-treated rats in latency to cross the platform location, time spent searching the target quadrant (Fig. 3), time spent searching in the platform location, number of platform crossings and number of errors committed (largest $t(20)=1.527$).

On re-testing in the MWM at 4 months post treatment, rats treated with MTX performed worse than controls: rats treated with MTX made fewer platform location crossings (mean entries (SEM): vehicle 3.64 (0.54), MTX 2.10 (0.43), $t(19)=2.180$, $p<.05$), and spent less time searching in the target quadrant (Fig. 3, $t(19)=2.531$, $p<.05$). No other significant differences were found between control and MTX-treated rats (largest $t(19)=1.723$), though there was a trend for MTX-treated rats to take longer to cross the platform location (mean latency (SEM): vehicle 74.33 s (11.52), MTX 100.80 s (9.94), $t(19)=1.723$, $p=.101$).

During re-training at 120 days post treatment, rats treated with MTX appeared to perform worse than control rats, with MTX rats taking longer to find the escape platform (mean latency (SEM), vehicle 42.33 s (11.76), MTX 72.83 s (10.38), $t(18)=2.026$, $p=.058$; Fig. 3); however during probe testing 24 h after re-training, there were no significant differences between MTX-treated rats and controls on any dependent variables (largest $t(18)<1$).

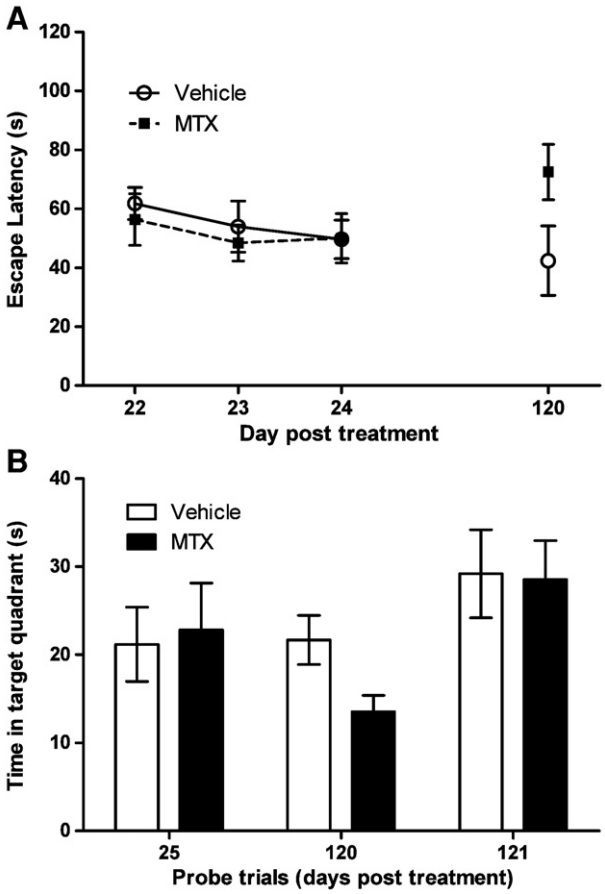

Fig. 3. (A) Morris water maze mean escape latencies during training. The bars represent ± standard error of the mean. No differences were found between MTX (coloured squares) and vehicle (open circles) rats during initial training (days 22–24), however during re-training (day 120) MTX rats appeared to perform worse than control rats ($p=.058$). (B) During initial probe testing (day 25) no difference was found between MTX and control rats. When re-tested 95 days later (day 120) MTX rats spent less time in the target quadrant than the vehicle rats in probe 2 ($p<.05$). After re-training on day 120 no differences were found between MTX and saline rats during probe test 3 (day 121; $p>.05$).

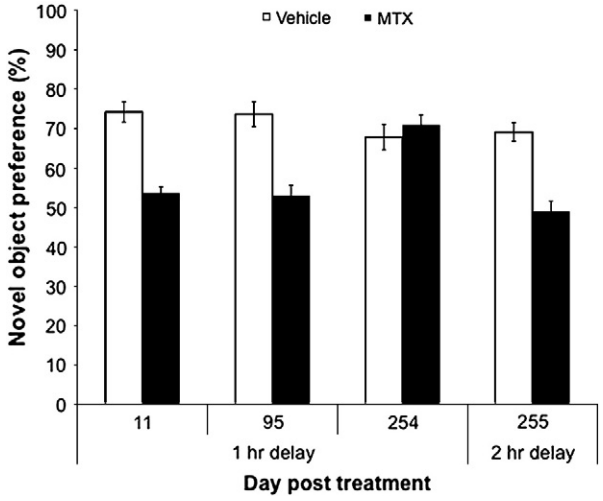

Fig. 2. Novel object recognition results displayed as a preference score for the novel object. The columns represent the amount of time spent investigating the novel object relative to the total object investigation for MTX rats (coloured) and vehicle-treated rats (open). The bars represent ± standard error of the mean. A 1 hour retention period between sample and test trials was tested on days 11, 95, 254, and a 2 hour retention period was tested on day 255 post treatment. MTX rats performed worse than vehicle rats during testing at 11, 95, and 255 days post treatment ($p<.001$), but not day 254.

3.4. Go/no-go discrimination

All rats acquired lever press response, with rats increasing the number of lever presses made across training sessions ($F(14,210)=21.811$, $p<.001$). There was no difference between MTX-treated rats and vehicle controls, and no significant interaction between group and training session ($Fs<1$; data not shown), indicating no difference in the rate of lever press acquisition between MTX-treated rats and vehicle controls.

Fig. 4 shows the number of correct hits and correct rejections. There was no difference in the number of correct hits rats made according to treatment, and no significant group by training session interaction ($Fs<1$). There was a significant effect of training session, with rats in both groups increasing the number of correct

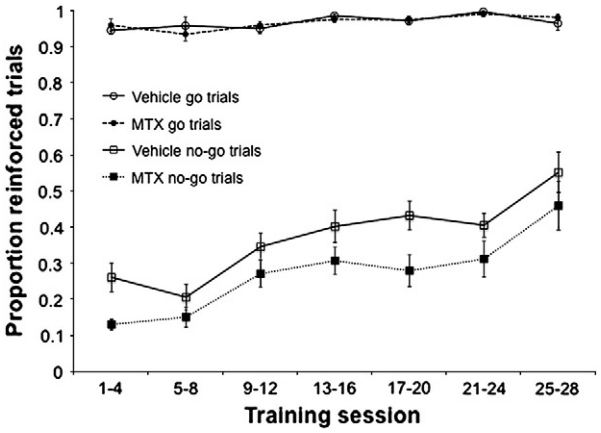

Fig. 4. Go/no-go discrimination task results. Go/no-go training commenced 174 days post treatment with MTX (coloured bullets) or vehicle (open bullets). No difference was found between MTX- and vehicle-treated rats during go trials (circles) ($p>.05$), while MTX-treated rats performed worse than vehicle-treated rats during no-go trials (squares) throughout training ($p<.05$).

hits across sessions ($F(27,513)=2.398$, $p<.001$). While there was significant effect of training session on the number of correct rejections ($F(27,513)=9.303$, $p<.001$), with rats improving with training, rats treated with MTX performed significantly worse than controls on the number of correct rejections ($F(1,19)=7.060$, $p<.05$). There was no significant group by training day interaction ($F<1$), suggesting that MTX-treated rats maintained the poor performance relative to controls across all 28 training sessions.

4. Discussion

The present study demonstrated that a single high dose of MTX had significant effects on rats' behaviour in tasks that require short- and long term memory, and rule learning. Moreover, this study demonstrated that these impairments were enduring and persisted for at least 8 months after treatment. First, rats treated with MTX showed clear impairments in short-term novel object recognition memory when tested 11, 95 and 255 days post treatment. However, the impairment at 255 days was not as pronounced as at the earlier time points and only became evident when the task was made more difficult. Second, the rats showed evidence of a subtle impairment in long term memory for a spatial location when trained 22–24 days post treatment. While, there was no difference between MTX-treated and control rats when tested within 24 h of training, MTX-treated rats showed an impaired ability to find the escape platform location compared to controls when tested 95 days after training completion. This suggests that while MTX-treated rats could readily learn and re-learn the platform location, treatment with MTX affected long term retention or recall of a spatial memory. Finally, the MTX-treated rats showed impaired performance in an instrumental discrimination task. While MTX treatment had no effect on acquisition of a lever press response and the rate of lever-pressing in the presence of a discriminative stimulus (S_D), rats treated with MTX failed to learn to withhold the lever press response in the absence of the S_D.

To date, all but one (Lee et al., 2006) of the animal studies investigating the cognitive effects of chemotherapy have been limited to examining the acute and short-term effects on cognition post treatment cessation. Cognitive impairment after chemotherapy has been found within 24 h (Foley et al., 2008; Konat et al., 2008; Liedke et al., 2009; Reiriz et al., 2006), 2 days (Konat et al., 2008); (Foley et al., 2008) and 3 days (Li et al., 2010). In the short-term, cognitive impairment at 1 week (Li et al., 2010; Liedke et al., 2009; MacLeod et al., 2007; Reiriz et al., 2006) and 3–5 weeks (Gandal et al., 2008; Li et al., 2008; Seigers et al., 2008; Winocur et al., 2006) due to chemotherapeutic treatment has been found. In contrast, Lee et al. (2006) found improved performance in the MWM and the Stone maze when the rats were trained 7 and 9 weeks respectively after treatment with cyclophosphamide or 5-FU.

The major result of the present study is that MTX treatment induces a clear impairment in the NOR at 11 and 95 days after treatment. This is consistent with Seigers et al. (2008) who also observed impairment in NOR 4 weeks after treatment. Further, the present results show that the cognitive impairment was still present 8 months post treatment, but was only apparent under more difficult testing conditions. In contrast, Gandal et al. (2008) found no effect of 5FU and MTX treatment on NOR in mice when tested 2 weeks later. However procedural differences may account for the discrepancy in the results; here rats were administered a single high dose of MTX at 250 mg/kg, whereas Gandal et al. gave their mice 37.5 mg/kg MTX with 75 mg/kg 5-FU once a week for 4 weeks and employed a 24-hour delay between sample and test trials.

MTX chemotherapy caused a subtle deficit in long term memory: rats treated with MTX learned to find a hidden platform in the MWM just as quickly as the control rats when trained 22 days after treatment, and performed equally well as control rats in a probe test 24 hours after the last training trial. In contrast, Winocur et al. (2006) found mice trained in the MWM 9 days after MTX and 5-FU treatment completion made more errors and were slower to find the platform location, though these effects had disappeared by the end of training. Like the results reported here, Seigers et al. (2008) also found that MTX did not affect training in the MWM 3 weeks after treatment, but they observed that MTX-treated rats took longer to find the platform location in a probe test 24 hours after training. Notably, the rats in the present study showed an impaired ability to find the platform location when they were re-tested 95 days after training. These findings are consistent with those of Li et al. (2008) who also failed to see an effect of cytosine arabinoside during MWM training or probe testing 24 hours after chemotherapy, but saw evidence of impairment when tested 30 days after training. Further to Li et al.'s (2008) findings, despite the impaired recall or retention of a spatial location observed here, MTX-treated rats re-learned the platform location with two training trials and retained this information for 24 hours. Given this, it appears that MTX does not induce a global impairment in spatial learning, but rather it seems that chemotherapy has a temporally graded effect on long term memory, and specifically affects memories for relatively remote events more than for recent ones. Thus, it appears re-training or reminding may ameliorate some of the recall deficits observed. However, one procedural difference between the present study and Seigers et al (2008) worth mentioning is a difference in the water temperature of the MWM; here the water was between 20–23 °C similar to that of Winocur et al (2006), while Seigers et al (2008) maintained the water temperature between 25–27 °C. Lower water temperatures have been shown to facilitate learning in the MWM (Sandi and Pinelo-Nava, 2007). Thus the lower temperature employed here may limit the dissociative properties of the test and lead to the more subtle effects of MTX on spatial learning and memory observed here and in the results of Winocur et al (2006).

The final important aspect of the present study is the demonstration that chemotherapy produced long term impairment in instrumental discrimination learning. Chemotherapy-treated rats showed no impairment in learning to press a lever with reinforcement. Nor was there any impairment in learning to press the lever when a S_D signalled that lever-pressing would be reinforced. In contrast, the MTX rats showed a specific impairment learning to withhold responding in the absence of the S_D. These results are consistent with those of Winocur et al. (2006), who observed that mice treated with MTX and 5-FU made more errors while learning a non-matching to sample task in the MWM. The source of the impairment here is not clear, but at least three interpretations are possible: MTX-treated rats may have an impaired ability to learn simple rules; MTX-treated rats

may have attentional deficits and fail to notice the presence or the absence of the S_D on each trial; or MTX may impair executive function and the ability to inhibit inappropriate responses. Unfortunately, the present data does not allow for differentiation between these possibilities. However, regardless of the exact nature of the impairment, it is important to note that the impairment was observed approximately 6 months after treatment and persisted throughout training.

In several previous studies cognitive impairments due to chemotherapy treatment have been found to be associated with hippocampal dysfunction. Specifically, MTX has been shown to diminish neurogenesis (Seigers et al., 2008, 2009), reduce serotonin, dopamine and noradrenalin (Madhyastha et al., 2002), and affect blood supply and microglial function (Seigers et al., 2010) in the hippocampus. However, the role of the hippocampus in the present results is not clear. First, learning and remembering the spatial location of the hidden platform in the MWM requires an intact hippocampus (D'Hooge and De Deyn, 2001), yet chemotherapy-treated rats readily learn to find a hidden platform in the MWM. Second, there is considerable controversy about the role of the hippocampus in the NOR. For instance, damage to the hippocampus has been found to impair MWM performance while sparing NOR, yet damage to the perirhinal cortex impairs NOR while sparing MWM performance (Winters et al., 2004). Third, the present data suggests that the impairment may occur in systems important for the retention and/or retrieval of remote memories rather than those important for recent memories. Moscovitch et al. (2005) argued that, in humans, the hippocampus is important for the retention and retrieval of remote autobiographical memories but not remotely learned semantic knowledge. Consistent with this, mice recalling recent fear conditioning show increased metabolic activity in the hippocampus, however the frontal and the anterior cingulate cortices become more active when recalling remotely learned information (Frankland et al., 2004). Finally, while the exact nature of impairment seen in the go/no-go discrimination task is not well defined, the failure of rats treated with MTX to inhibit a behavioural response would suggest both frontal cortical and sub-cortical dysfunction (Aron et al., 2007; Robbins and Arnsten, 2009). Therefore the locus of impairment may not be in the hippocampus per se but in diverse cortical areas important for the retrieval processes for remote memories at test, in the processes that transfer memories from a hippocampal-dependent recent memory system to non-hippocampal remote memory systems, or both. Indeed, this analysis is supported by both patient neuropsychological data and imaging results. In neuropsychological testing, cancer-chemotherapy patients consistently show impairments in verbal/visual memory, attention/concentration and processing speed after chemotherapeutic treatment (Vardy and Tannock, 2007). Further, metabolic activity is altered in frontocortical and cerebellar areas during short-term memory tests (Silverman et al., 2007), and widespread changes in gray and white matter volumes in the brains of chemotherapy-treated patients have been found (Inagaki et al., 2007).

Little work has been conducted on the effects of chemotherapy on aged animals; the majority of studies employ mice aged between 6–10 weeks (e.g. Winocur et al., 2006), and rats between 2–4 months old (e.g. Seigers et al., 2008). However it is possible that treatment with chemotherapy interacts with increasing age to induce cognitive deficits. Two studies have included older rats, though no conclusions were made regarding the impact of age and chemotherapy and cognition (Konat et al., 2008; Lee et al., 2006). For example, Lee et al. (2006) found cyclophosphamide treatment did not significantly impair cognitive performance of either young (7 months old) or aged (18 months old) rats, despite evidence of toxicity (e.g. weight loss) relative to saline controls. Further, no direct comparisons in performance were made between young and aged animals, and young and old animals received different treatment regimes; 5 injections of 100 mg/kg versus 5 injections of 80 mg/kg respectively. Konat et al (2008) found cyclophosphamide and doxorubicin treatment impaired passive avoidance learning in 10 month old female rats. While not yet old, the rats used in the present study were 8 months at commencement of experimentation, making them close to 1.5 years old at the completion of experimentation. However, in both the present study and that of Konat et al (2008), no young control group was available for comparison, making our conclusions tentative: it is possible that older age interacts with chemotherapeutic treatment to accelerate the aging process, producing subtle and diffuse effects on cognition and aging as observed here. In non-clinical populations cognitive aging is associated with decrements in processing speed, and this change in processing speed is one of the strongest predictors of performance across a range of cognitive tasks (Eckert et al., 2010). Further, changes in processing speed are associated with age-related decreases in white matter integrity, with this decreased white matter in prefrontal regions mediating the relationship between perceptual motor speed and episodic memory (Bucur et al., 2008). These results relate well to those of Noble et al and Seigers et al who have shown suppression of oligodendrocites and decreased myelination in the corpus callosum of mice and rats treated with either carmustine, cisplatin, cytosine arabinoside or MTX (Dietrich et al., 2006; Han et al., 2008; Seigers et al., 2009). Together this suggests that chemotherapy and age may increase the normal rate of white matter degradation associated with age leading to cognitive impairment as seen in cancer-chemotherapy patients (c.f. Inagaki et al., 2007).

In conclusion, laboratory rats given a single high dose of MTX demonstrated long-lasting subtle and pervasive impairments in short- and long term memory, and rule learning in the absence of cancer. The results reported here validate cancer survivors' reports of persistent cognitive impairment long after chemotherapy treatment. Moreover, improvement in object recognition at 8 months post treatment suggests some recovery of function. This recovery process may offer targets for potential treatments. The present results also indicate extra-hippocampal areas are affected by chemotherapy, and further research is needed to examine the contribution of these areas to the observed cognitive impairment. Finally, if animal models are to be used successfully to assess if chemotherapeutic agents cause long term cognitive impairment, it is imperative that the animals are tested at longer durations post treatment.

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