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A clinically relevant dose of cyclophosphamide chemotherapy impairs memory performance on the delayed spatial alternation task that is sustained over time as mice age

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Abstract

Background—Cyclophosphamide chemotherapy is a mainstay of adjuvant breast cancer treatment. Unfortunately, this drug is associated with cognitive impairments in cancer patients that may accelerate cognitive aging. Memory is particularly affected in many patients. In order to better understand the precise cognitive impairments caused by this chemotherapy agent, we investigated a clinically relevant dose and administration paradigm on delayed spatial memory abilities in C57BL/6 mice. We utilized a delayed alternation paradigm similar to a delayed match to sample paradigm reported to be sensitive in neurotoxicology research.

Methods—A dose of 200 mg/kg cyclophosphamide was administered intravenously (at weekly intervals) for 4 weeks to C57BL/6 mice starting at 6 ½ months of age. Memory was tested in mice using a reward-based delayed spatial alternation paradigm with delay values of 1.5, 3, 6.1, 12.4 and 25 seconds presented randomly over 80 sessions (16 reinforcers per session), and testing began at the initiation of chemotherapy through 3 months.

Results—At the longest delay, i.e., that requiring the greatest memory, mice treated with chemotherapy exhibited a significant decline over time in proportion correct which leveled off compared to controls that continued to improve slightly.

Conclusions—Our clinically relevant model shows cyclophosphamide chemotherapy causes a slight decline in delayed spatial memories at the longest delay that is sustained over time as mice age.

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Keywords

cancer-related cognitive impairment; cancer treatments; “chemo brain”; memory; delayed alternation

Introduction

Cancer survivorship has increased substantially over the past 3 decades with over 13 million survivors currently living in the US today; treatment-related issues accompanying cancer survivorship produce a tremendous impact on an aging population during and after treatments (Ganz, 2005, 2008). Unfortunately, cancer treatments, particularly chemotherapy, leads to a host of side effects including cognitive impairments (Janelsins et al., 2014). Neuropsychological and self-report assessments during and following chemotherapy reveal deficits including memory lapses, trouble concentrating, difficulty in word association, confusion, trouble multi-tasking, and slow thinking and information processing (Vardy et al., 2007, Bower, 2008). Chemotherapy-related cognitive impairment (CRCI) is most common and most severe during and just after treatment (Wefel et al., 2004) and more pronounced in those who received high-dose chemotherapy, and importantly, these deficits can continue for several years following treatments (van Dam et al., 1998, Schultz et al., 2003). These observations suggest that managing the adverse neurobehavioral effects of chemotherapy during the course of treatment and early after treatment could prevent the onset or persistence of cognitive complications in cancer patients and survivors.

We need clinically relevant animal models that will help us understand the etiology of CRCI and to develop and test interventions to alleviate or prevent CRCI. Deficits in learning and memory are among the most frequently reported by cancer patients, and patients’ performance is reduced on a number of objective memory measures. Our group has interest in assessing CRCI using computerized batteries derived from experimental psychology which have shown that chemotherapy and other treatments impair cognitive performance in cancer patients (Capuron et al., 2001, Vardy et al., 2014, Vardy et al., 2015). In order to allow for forward and backward translation in research, we decided to assess the cognitive impact of a clinically relevant course of cyclophosphamide chemotherapy using a delayed spatial alternation paradigm that is similar to delayed spatial memory assessment paradigms used in clinical research that have shown to be sensitive in neurotoxicology research and have been used in investigations of cognitive effects in breast cancer patients (Fray and Robbins, 1996, Minton and Stone, 2012). Delayed spatial alternation allows for the assessment of learning and memory over multiple delay intervals and has been used in several experimental psychology models and has been suggested as a possible assessment tool for investigating chemotherapy toxicity (Cory-Slechta et al., 1991, Weiss, 2010). We hypothesized that chemotherapy, given in a clinically relevant manner at weekly intervals and in a human-to-mouse dose translatable fashion delivered intravenously, would lead to worse performance on the delayed alternation paradigm, particularly as delay values and thus required remembering time increased, as compared to controls administered equivalent volumes of saline.

Materials and Methods

Mice

Male C57BL/6 mice were purchased through Taconic (Germantown, NY) at 23-25g at 6-8 weeks of age. Upon arrival, all mice were allowed to acclimate to their home cage for two weeks prior to initiation of experiments. After this acclimation period, the mice were weighed and maintained at 80% of that weight via caloric regulation throughout the rest of the experiment. Mice remained in general good health throughout the experiment. The food reward used in behavioral testing food type was BioServe dustless precision pellets (20mg) rodent grain-based diet, and the mice were given supplemental food (Lab Diet 5001 rodent diet) if necessary to maintain the 80% weight.

All animal housing and procedures were performed in compliance with guidelines established by the University Committee of Animal Resources at the University of Rochester.

Behavioral Testing – Delayed Spatial Alternation

We followed a standard delayed alternation protocol adapted from Cory-Slechta et al. (Cory-Slechta et al., 1991). Behavioral testing was carried out in operant chambers, each of which contained two response levers, with a single light emitting diode over each lever, a houselight, and a pellet trough located midway between the two levers that was used to deliver food reward. Mice were first trained to lever press using an auto-shaping procedure developed in our laboratory that began with a variable time schedule during which food pellets were dispensed into the food trough at variable time (VT) intervals averaging 60 sec, independently of responding, for a period of 20 min. Any responses on either lever during that period produced additional food reward; if 10 responses occurred during that period, the VT component was terminated and a fixed ratio (FR) 1 schedule of reward was in effect in which a single response on either lever was required for food delivery. If 10 responses did not occur during the VT component, it terminated after 20 min and the FR component began. Sessions were terminated with the delivery of 50 food rewards. During autoshaping, lights above each lever were illuminated.

Following autoshaping, a response alternation training program was implemented in which mice were required to alternate responses on the two levers, with the houselight illuminated above the correct lever at the end of a constant delay interval. The correct lever press produced a pellet and the light was then extinguished, at which time the other lever became the “correct” lever and the next delay interval was started.

Following simple response alternation training, the full delayed alternation program was implemented. In this paradigm, alternating responses were again required, but no lever lights signaled which lever was correct; mice had to remember which lever they had pressed previously and subsequently move to the alternate lever. In addition, delays were imposed between the opportunities to alternate responses, using delay values that were sufficiently long to produce essentially chance responding. During the delayed spatial alternation procedure, mice were presented randomly within each session with 5 different delays at 1.5, 3, 6.1, 12.4 and 25 seconds allowing a delay by accuracy function to be generated for each

session. Responses on either lever during the delay reset the delay value and the correct alternate response was required before another trial began.

Training procedures were conducted for approximately 3 months to achieve stable delay functions, after which chemotherapy was implemented. Baseline data included the 10 sessions prior to the initiation of chemotherapy. Behavioral testing was carried out 5 days per week (Monday through Friday) on the delayed spatial alternation starting after the first chemotherapy administration and continuing for 3 months.

Chemotherapeutic Paradigms

The chemotherapeutic agent was purchased from Sigma Aldrich (St. Louis, MO). Cyclophosphamide (Cat # C-0768) was stored according to manufacturer's instructions until use, at which time it was diluted in sterile saline. The C57BL/6 mice were given four intravenous tail vein injections (approximately 6 ½ months of age) of chemotherapeutic agent (cyclophosphamide at 200 mg/Kg; n=5) or saline equivalent (n=4) on 4 consecutive Mondays. We followed the dose translation calculations of Reagan-Shaw et al (Reagan-Shaw et al., 2008). The clinically relevant dose our mouse equivalent dose was translated from is 600 mg/m^2 which is the dose that this chemotherapy agent is commonly administered in breast cancer therapy.

Statistical Analyses

MedAssociates software was used to collect data in the delayed spatial alternation which were then exported into RS1. Data were analyzed with R, SAS and JMP. Percent correct for each delay and number of responses per reinforce (i.e. reward) are reported for each day and delay. In all models, we assessed the cumulative effect of chemotherapy vs saline over time. To visualize the trajectories of the percent correct and responses/reward response over time, we plotted the data versus time, color coding by group. Then we superimposed nonparametric regression estimates of the average trajectories for each group. See Figure(s) 1 and 2. The LOWESS smoother (span=2/3, robust Tukey biweight with 3 iterations) was used to fit the nonparametric estimates (Cleveland, 1979). To test for statistically significant differences in trajectory shape between the groups, we used mixed models with shape (represented with a third-order polynomial) as the main effect, and the interaction between group and shape as the key measure of differences in shape over time. The mouse-specific random effect was also a third order polynomial. Maximum likelihood estimation was used, and a likelihood ratio test was used to evaluate the significance of the group by shape interaction. We found that third order polynomials gave a faithful representation of the curves visualized via the LOWESS smoother. This analysis was performed for each delay time. In order to combine delay times, we calculated the area under the percent correct (or responses/reward) versus delay time data (Area under the Curve, AUC) for each mouse using the trapezoidal rule. The AUC was then modeled using the same mixed modeling methodology as detailed above. All calculations were performed using R,(R Core Team, 2013) and the mixed models were fit using the nlme package (Pinheiro et al., 2015). See Figures 4 and 5. In all cases, $p \leq 0.05$ was considered significant.

Results

Baseline short delay percent correct values at 1.5, 3 and 6.1 seconds were 90%, 88%, and 83%, respectively. At delays of 1.5, 3.0, and 6.1 seconds, chemotherapy-treated mice generally had lower proportions of percent correct compared to saline-treated mice; however, there were no significant group or group × time interaction differences ($p>0.05$; Figure 1). At the 12.4 second delay, baseline percent correct values were 68%. Chemotherapy-treated mice initially started the study with slightly greater percent correct than saline-treated mice at this delay but during and following chemotherapy, chemotherapy-treated mice showed gradual decline in percent correct compared to saline-treated mice who gradually improved resulting in a lower percent correct in chemotherapy-treated mice compared to saline-treated mice and representing a significant group difference ($p<0.05$) as well as a significant group × time interaction ($p<0.005$; Figure 1). The 25 second delay value produced a baseline percent correct value of 57%, consistent with the fact that memory was most taxed at this long delay value. At this delay, while mice in both groups started off with similar percent correct values; the chemotherapy-injected mice continued to gradually decline over the course of the 3 months post-treatment, while the saline-injected mice continued to gradually increase over the post-treatment interval as evidenced by a statistically significant group × time interaction ($p<0.05$; Figure 1). We also performed an AUC analysis across percent correct on all delays, which revealed significant effects by group ($p<0.005$; Figure 2) and group × time interaction ($p<0.005$; Figure 2). To assess percent correct differences at key points in time between chemotherapy-treated and saline-treated mice we created forgetting functions (showing average percent correct as a function of delay on the same graph) for the baseline interval as well as the 2 and 4 weeks post-chemotherapy intervals (Figure 3). At baseline, mice in both groups performed similarly as a function of delay but in the post-treatment period, chemotherapy-treated mice performed less well than saline-treated mice as a function of delay.

The ratio of responses per reward was higher in chemotherapy-treated mice compared to controls at all delays; however the effects were only statistically significant at the 12.4 and 25 second delays ($p<0.05$; Figure 4). At the 12.4 second delay, the ratio of responses to reward delivery was higher in chemotherapy-treated mice compared to controls, and this difference was statistically significant ($p<0.05$; Figure 4) as well as the group × time interaction at both the 12.4 and 25 second delays ($p<0.05$; Figure 4) meaning that chemotherapy-treated mice made slightly more responses/reward over time and controls made slightly less responses/reward over time. The AUC analysis on all delays for responses/reward revealed a trend for overall group difference ($p=0.06$) and a significant group × time interaction ($p<0.05$; Figure 5).

There were no significant differences in weight in chemotherapy- and saline-treated mice by groups, and this lack of an anticipated difference is likely due to the caloric regulation required for motivation of responding ($p>0.05$). Overall, mice in both groups slightly increased in weights over the course of the experiment (from 19.4 to 20.4 on average), but did not differ more than 5% from their baseline.

Conclusions

Utilizing a delayed spatial alternation paradigm, we found, for what appears to be the first time, that chemotherapy leads to a subtle but sustained gradual memory decline over time in C57BL6 mice compared to controls receiving saline. These findings provide what may be a useful animal model of aspects of this phenomenon which is highly relevant clinically, as our paradigm uses a clinically relevant dose translated from the dosage given in breast cancer treatments as well as a clinically relevant route of administration. Moreover, the delayed spatial alternation task used is very similar to cognitive tests being used in clinical research (e.g. CANTAB delayed matching to sample and other similar tests) with cancer patients and other populations (Capuron et al., 2001, Egerhazi et al., 2007, Minton and Stone, 2012). Lastly, our results show a cumulative effect of chemotherapy treatments leading to a gradual and sustained subtle decline of cognitive function representing the long-term effects of treatment as mice age. The subtle nature of these effects with this model is somewhat akin to the subtle effects that are often observed on some clinical neuropsychological tests in humans.

The results at the 25 second delay support our hypothesis that chemotherapy-treated mice would perform less well at the longest delay (i.e. the delay where memory is most taxed), and it is interesting that the effects of treatment are gradual and sustained over time in our model. It is not surprising that chemotherapeutic treatment did not induce deficits at the shorter delays, since these delays involve less sustained memory compared to the longest delay. Thus, these shorter delays may not be sensitive enough to detect chemotherapy effects. It is less clear at present why the chemotherapy-treated mice initially performed better than saline-treated mice at the 12.4 second delay. However, with time post-treatment, as controls continued to show improvement at this delay, accuracy levels of chemotherapy-treated mice showed no improvement, but actually declined to levels similar to the performance curve at the 25 second delay. Correspondingly, the number of responses required for each reward earned was significantly higher. Interestingly, there is wide variation in the number of responses per reward illustrating the individual susceptibility of the mice to perform on this task upon chemotherapy exposure.

While our results provide novel data on long-term memory changes over time in a delayed spatial alternation paradigm, several other studies have shown behavioral deficits on other cognitive tasks in other chemotherapy paradigms. Primarily, aspects of spatial and non-spatial memory, as well as executive functions have shown to be impaired in rodents treated with chemotherapy (reviewed in (Dietrich et al., 2015) and (Seigers and Fardell, 2011).

It is still unclear whether the mechanisms leading to this memory decline are direct or indirect; however, several studies have shown that impaired neurogenesis in the hippocampus may be involved (summarized in (Dietrich et al., 2015)). Adult hippocampal neurogenesis—a process by which new neurons are generated from newly birthed neural precursors initiating from the subgranular zone (SGZ) of the dentate gyrus— is involved in maintenance of learning and memory as well as brain plasticity (Zhao et al., 2008). Mechanisms related to diminished neurogenesis are the most widely studied; however, evidence from other pre-clinical research (and clinical research) suggests that many

mechanisms play a role in the development of CRCI, including inflammation, hormonal changes, DNA damage, oxidative stress, mitochondrial dysfunction, and altered growth factor levels (Chen et al., 2006, Dietrich et al., 2006, Ahles and Saykin, 2007, Han et al., 2008, Janelsins and Gross, 2010, Kesler et al., 2013, Vichaya et al., 2016). Supporting the animal literature of cognitive impairments in chemotherapy models and brain inherent alterations in several molecular indicators of central nervous system abnormalities are an accumulating number of neuroimaging studies in cancer patients that show the impact of chemotherapy on brain structure and function, revealing white and gray matter loss, altered resting state metabolism changes and altered white matter integrity, and brain activation upon cognitive task challenge (Deprez et al., Inagaki et al., 2007, Silverman et al., 2007, Swayampakula et al., 2007, Abraham et al., 2008, de Ruiter et al., 2011, de Ruiter et al., 2012, Hosseini and Kesler, 2014, Dietrich et al., 2015). Lastly, the interactions between these factors and genetic factors (such as specific alleles in genes *APOE, TNF* and *COMT*) may exacerbate CRCI in the human condition and these relationships needs to be further investigated in animal studies (Ahles et al., 2003, Small et al., 2011).

Our study adds to the field by the usage of clinically relevant doses of chemotherapy and administration paradigms in a memory task not yet studied, as well as assessment of cognitive effects during chemotherapy and continuing over a long period of time after treatments have stopped and mice have aged.

Future studies need to address limitations to this work including assessing multiple agents simultaneously, assessing female mice and comparing young versus older mice. Additionally, conducting studies with multiple agents in relevant tumor models will allow us to differentiate cognitive effects of chemotherapy, aging, and cancer as well as to further elucidate the possible mechanisms discussed herein.

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Highlights

- Delayed spatial alternation detects subtle but sustained cognitive effects of cyclophosphamide chemotherapy
- Utilization of a chemotherapy model with clinically relevant features

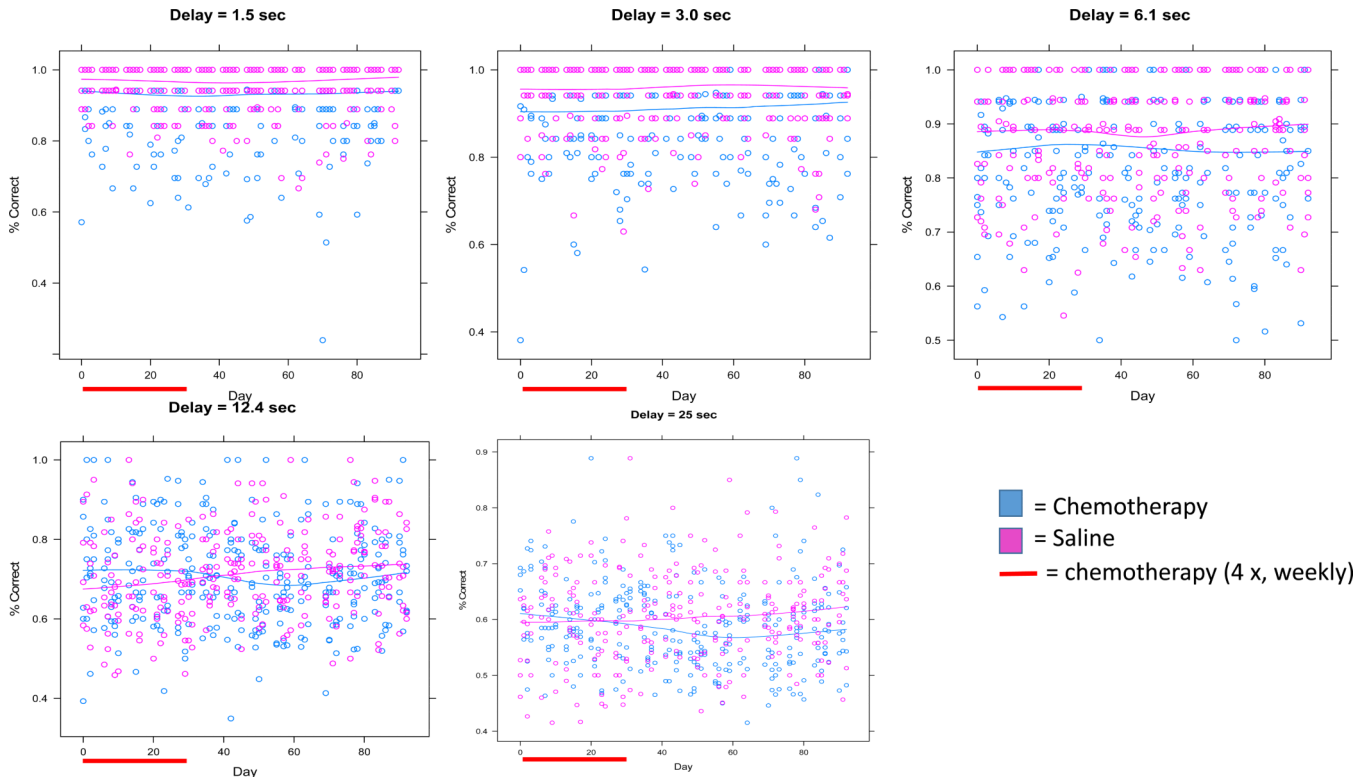

Figure 1. Percent Correct Across Delays

Each figure represents the percent correct for each delay with each day of delayed spatial alternation. Individual data points represent individual mice..The red bar represents the period of chemotherapy or saline administration (1 × per week for each of 4 weeks). Lines represent fitted lines for chemotherapy-treated mice and controls. Significant effects between groups and group by time interactions are found at the 12.4 and 25 second delays as noted in the text.

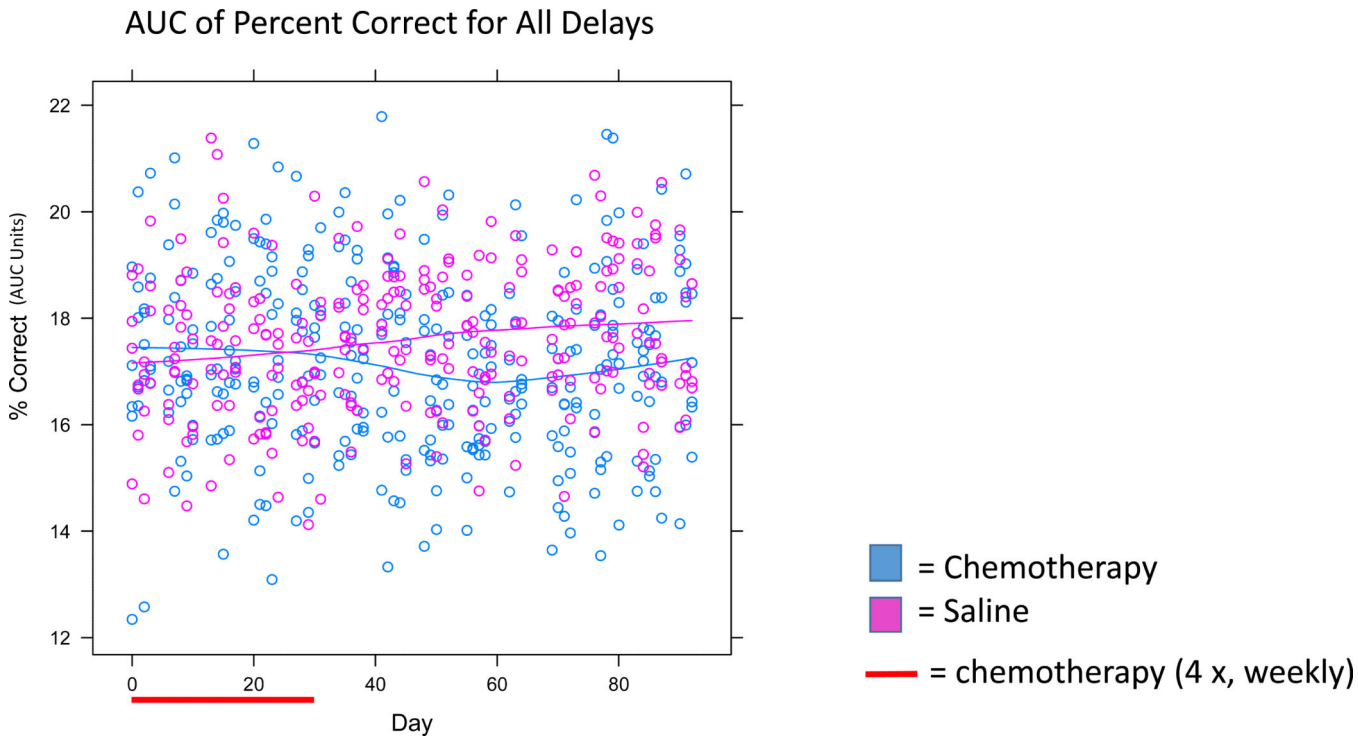

Figure 2. Area Under the Curve (AUC) Analysis for Percent Correct for All Delays

All data points from Figure 1 were combined together to represent an AUC. Individual data points represent individual mice. Significant effects were seen between groups as noted in the text.

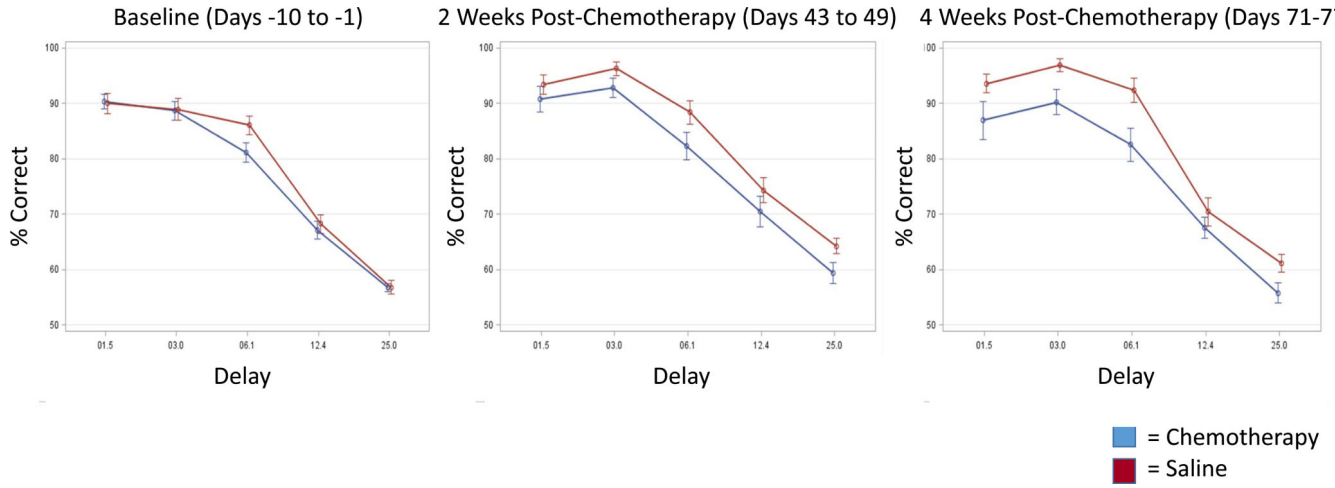

Figure 3. Forgetting functions of Percent Correct Across Delays

Forgetting functions of percent correct × delay were created for key time intervals including 10 days of baseline (day −10 to day −1), 2 weeks post-chemotherapy (Days 43-49) and 4 weeks post-chemotherapy (Days 71-77) as an alternate way of viewing the data in Figures 1 and 2. Data points represent averages across the data interval. Error bars are +/− SEM.

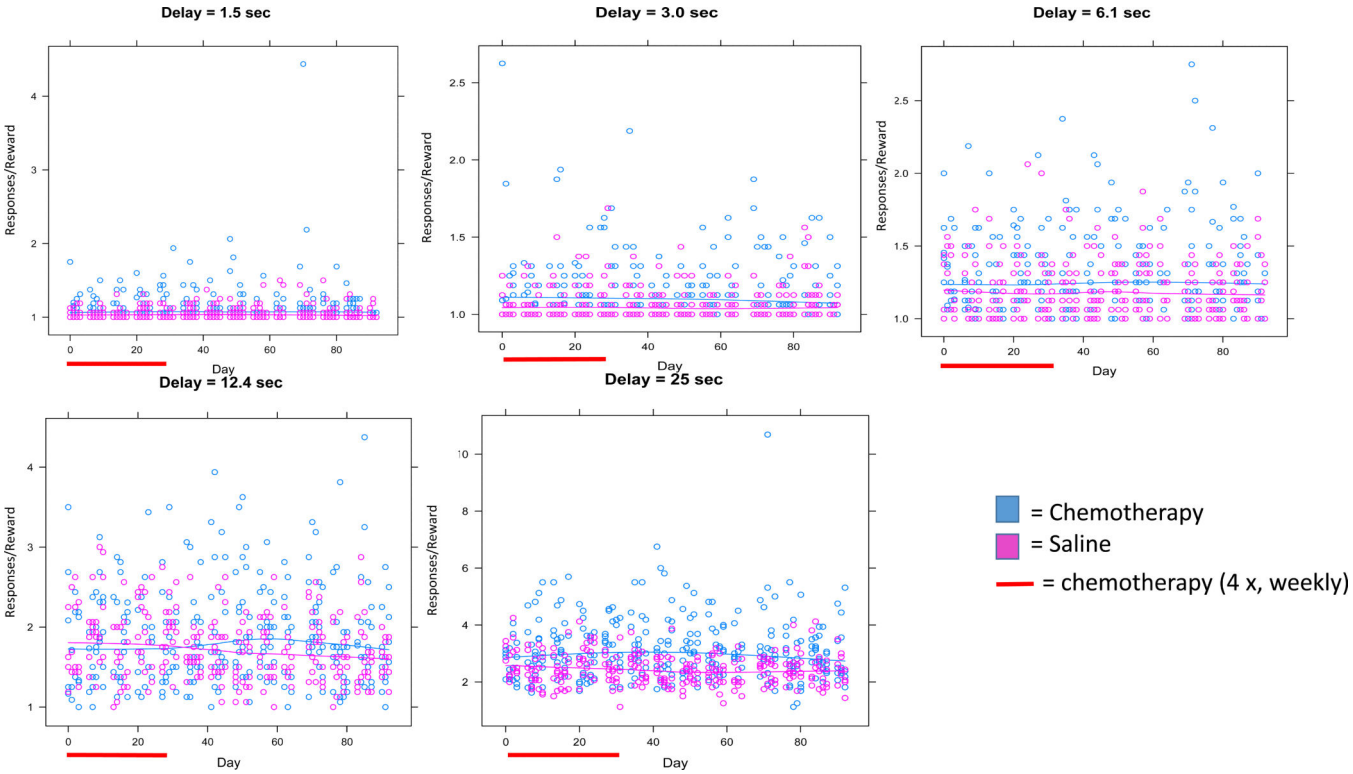

Figure 4. Responses/Reward Across Delays

Each figure represents the responses per reward for each delay for each day of delayed spatial alternation. Individual data points represent individual mice..The red bar represents the period of chemotherapy or saline administration (1 × per week for each of 4 weeks). Lines represent fitted lines for chemotherapy-treated mice and controls. Significant effects between groups and group by time interactions are found at the 12.4 and 25 second delays as noted in the text.

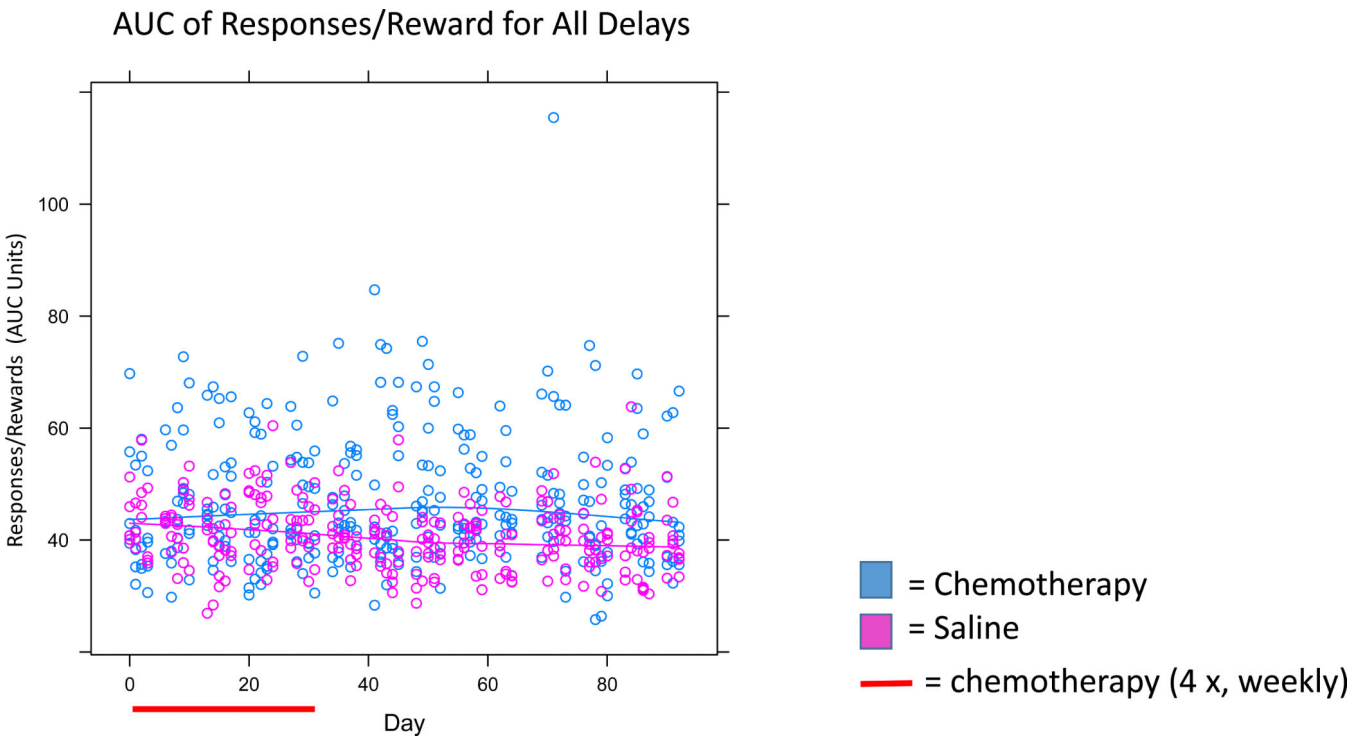

Figure 5. AUC Analysis for Responses/Reward for All Delays

All data points from Figure 4 were combined together to represent an AUC. Individual data points represent individual mice. Significant effects were seen between groups as noted in the text.