Spring 5-13-2017

An Analysis of Neurogenesis in a Mouse Model of Chemotherapy Related Cognitive Impairment

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AN ANALYSIS OF NEUROGENESIS IN A MOUSE MODEL OF CHEMOTHERAPY RELATED COGNITIVE IMPAIRMENT

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B.S. Whitman College 2009

A DISSERTATION

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

(in Psychology)

The Graduate School

The University of Maine

May 2017

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Cancer patients treated with adjuvant chemotherapy often experience cognitive decline following treatment. This phenomenon, often dubbed "chemo brain" or "chemo fog" is usually temporary, but for a subset of survivors, these cognitive impairments can be long-lasting (>10 years) and negatively affect patients' quality of life, career performance, and social fulfillment. While it is unclear what neurobiological mechanisms underlie chemotherapy related cognitive impairment, the majority of the animal literature has focused on adult neurogenesis. One process important for neurogenesis is the proliferation of new neurons within the dentate gyrus of the hippocampus. It is evident that many chemotherapy agents can negatively impact levels of neurogenesis shortly after treatment. However, only a few studies have investigated the long-term impact of chemotherapy on neurogenesis. The present studies explore the long-term impact of three commonly used chemotherapy agents on neurogenesis utilizing immunohistochemistry in a male C57BL/6J mouse model. EXP 1: The effects of cyclophosphamide or doxorubicin on neuronal proliferation were evaluated at 1 day, 56

days and 6 months post-treatment. Results indicated that neither cyclophosphamide nor doxorubicin treatment altered proliferation rates across either short-term or long-term intervals. EXP 2: The effects of 5-FU (alone or in combination with either the antioxidant melatonin or the antidepressant fluoxetine) on neuronal proliferation were evaluated at 1 day, 56 days and 6 months post-treatment. The results indicated that there was no effect of 5-FU or neuroprotectant treatment at any time point. The current studies suggest that neither cyclophosphamide, doxorubicin, nor 5-FU affect neurogenic proliferation in C57BL/6J mice directly after injection or up to 6 months post injection. As such, impaired neurogenic proliferation is an unlikely cellular mechanism for chemotherapy related cognitive impairment detected within this strain.

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INTRODUCTION

The American Cancer Society estimates 1.7 million new patients will be diagnosed with cancer in the US in 2017. Fortunately, advancements in early detection and treatments have led to steadily increasing 5 year survival rates from 48.7% in 1975 to 68.5% in 2006 (National Cancer Institute, based on data from SEER 9 Incidence & U.S. Mortality 1975-2013). Survival rates are particularly high for some cancers. For example, the 5 year survival rate for breast cancer increased from 75.2% in 1975 to 90.6% in 2008. As the number of surviving patients dramatically increases, research regarding the long-term side effects of different cancer treatment strategies has become progressively more important; patients who would not have survived their cancer in previous generations now present their treatment specialists and doctors with a new set of problems as the long-term side effects of cancer treatments become evident. One major source of these long-term side effects is treatment with chemotherapy compounds.

Cognitive impairment is one of the most commonly reported long-term side effects of chemotherapeutic treatment (Boykoff et al., 2009). Complaints regarding problems in mental function are referred to as chemotherapy related cognitive impairment (CRCI) in the literature, but are also often dubbed chemo-brain or chemo-fog (Farrell at al., 2013). Research conducted in humans regarding CRCI focuses primarily on those reported in breast cancer patients. These patients generally have a better prognoses and higher rates of survival that allow for easier recruitment and long-term cognitive assessment when compared to patients with other types of cancer. As a result, many of the first reported complaints of cognitive decline following chemotherapy treatment were detected in breast cancer patients.

Cognitive problems detected in breast cancer patients varies from study to study, but the most common complaints seem to be associated with decreased memory, difficulty focusing, and trouble multitasking. These cognitive problems are typically reported as mild to moderate when compared to relative normative ranges or healthy controls in neuropsychological evaluation (Ahles & Saykin, 2001; Staat & Segatore 2005). A meta-analysis conducted by Vardy et al. (2007) examined the evaluation of cognitive function associated with chemotherapy related impairment. Deficits were reported across a diverse range of processes, including: working memory, attention, processing speed, concentration, and executive functions. Cognitive problems were noted to occur intermittently, making it difficult to obtain objective measures of cognitive impairment using traditional neuropsychological assessment (Vardy et al., 2007). While severity of impairments are often categorized as mild to moderate, Staat and Segatore (2005) suggest that clinical presentation of CRCI can include increased levels of stress, serious detriments in the ability of patients to multitask, and poorer performance when both acquiring new skills and returning to environments with high-level cognitive demands. Furthermore, Olin (2001) concluded that once patients successfully survive the diagnosis and treatment of cancer, the realization of ongoing mild cognitive impairment and inability to return to pre-treatment levels of functioning can be devastating. Even mild impairments may lead to a diminished quality of life when capacity to carry out daily activities or future occupational and educational potential is adversely impacted.

Human Literature

In 1980, Peter Silberfarb and colleagues investigated the cognitive outcomes of patients given various chemotherapy treatments. This study is one of the earliest to

recognize reduced scores on standard neuropsychological tests in patients that received chemotherapy that had not been directed at the central nervous system (CNS). Silberfarb et al. (1980) reported that systemic chemotherapy treatment was significantly associated with poor cognitive test scores, regardless of the primary site of the cancer and whether or not there was metastasis to the brain. Prior to this discovery, many of the complaints of cognitive decline following chemotherapy treatment were dismissed by the medical community as psychological rather than neurological. It was thought that many of the agents used to treat cancer could not penetrate the blood-brain barrier (BBB) and thus were unlikely to cause neurotoxicity when administered systemically (Raffa & Tallarida, 2010). With Silberfarb et al.'s (1980) findings, interest in the cognitive impairment seen following chemotherapy has increased exponentially (Raffa & Tallarida, 2010).

Prevalence and Assessment Variables

Reported rates of CRCI in patient populations vary drastically from study to study. Some studies report rates as low as 17% (Schagen et al., 2001) while others have reported rates up to 95% (Komaki et al., 1995). Most researcher agree that only a subset of patients treated with chemotherapy develop long-lasting (>10 years) cognitive impairment that can severely impact quality of life (Ahles & Saykin 2001; Raffa & Tallarida, 2010). There is an important distinction between cognitive impairments detected during or shortly after chemotherapy treatment and those impairments that are maintained or return long after treatment cessation. Although it is quite common for patients treated with chemotherapy to experience cognitive problems immediately following treatment, for many, these problems subside after recovery (Ahles & Saykin, 2001; Ferguson & Ahles 2003). Discrepancies in the prevalence of CRCI within the

literature are at least in part due to conflating those patients experiencing only short-term CRCI with those that develop long-lasting impairments. In addition, a variety of experimental design factors may also contribute to variability across study results. These factors include: timing of the assessment, type of assessment (e.g. self-report, neuropsychological testing, brain imaging), and the comparison group chosen (e.g. healthy controls, normative ranges, use of matched non-chemotherapy controls, pre-chemotherapy baseline) (Castellon et al., 2005; Vardy et al., 2007; Farrell at al., 2013; Evenden, 2013).

Type of Assessment: A variety of assessment methodologies have been used within the CRCI research literature. Typically the highest rates of impairment are generated from subjective self-reports. In 2006, Hurria and colleagues found that over half (51%) of their sample of 45 patients completing the Squire Memory Self-Rating Questionnaire before treatment and 6 months post-treatment perceived a decline in memory function post-chemotherapy. In particular, patients reported a worsening of the ability to learn new information suggesting hippocampal involvement. Although many patients exposed to chemotherapy treatment complain of cognitive problems in self-reported questionnaires, these subjective impairments are often not associated with actual problematic performance on neuropsychological evaluations in studies that have been conducted using both measures (van Dam et al., 1998; Castellon et al., 2004, 2005). A review by Hutchinson et al. (2012) showed that relationships between objective and subjective measures of impairment reach significance in only 8 out of 24 studies examined. Furthermore, of the 8 studies in which a relationship was found, most of the correlations were restricted to memory tests rather than overall or index scores on

neuropsychological batteries. The questionable accuracy of subjective self-reports has driven more recent efforts within the research field to favor more formal, objective neuropsychological testing which typically indicate lower rates of CRCI. Typical neuropsychological testing within the literature include multiple cognitive domains, but deficits are most often detected in tests of the following: verbal memory (e.g. Wechsler Memory Scale – Logical Memory, Memory Scanning Test, Hopkins Verbal Learning Test, Rey Auditory Verbal Learning Test), visual memory (e.g. Wechsler Memory Scale – Visual reproduction, Wechsler Memory Scale – Non-Verbal Memory, Rey Osterreith Complex Figure), visuospatial function (e.g. Wechsler Adult Intelligence Scale – Block Design, Rey Complex figure – Copy Trial, Road Map Sense Test), processing speed (e.g. Wechsler Adult Intelligence Scale – Digit symbol, Monroe Sherman Reading Comprehension, Zazzo's Attention Test– Speed), and attention (e.g. Paced Auditory Serial Addition Test, Stroop Test, Trail Making, Automated performance test system – Sternberg Test, Simple Reaction Time) [as reported in Anderson-Hanley et al., 2003; O'Shaughnessy, 2003; Castellon et al., 2004, 2005; and Vardy et al., 2007].

Timing of Assessment: The timing of cognitive assessment relative to completion of chemotherapy treatment is critically important in determining the extent of CRCI. Early studies such as those conducted by Komaki et al. (1995) or Wieneke and Dienst (1995) reported that 75 – 95% of their sample exhibited cognitive impairment shortly following treatment. These high rates of CRCI are common in studies that assess patients within a time period of less than 6 months after cancer treatment. According to Janelsins and colleagues (2014), up to 75% of patients report some form of chemotherapy related cognitive impairment during treatment. However, only 35% of these patients experience

cognitive impairments that last for more than a few months. Ferguson and Ahles (2003) suggested that one reason for the high level of impairment during or shortly after chemotherapy is that elevated levels of stress and fatigue may make neuropsychological assessment more difficult and contribute to an inflation of poorer performance on standardized measures, especially when comparing data to normative scores in the general population. These factors most likely contributed to the high rates of detected impairment in Komaki et al. (1995) and Wieneke and Dienst (1995).

Assessment intervals that extend past 6 months commonly report much lower rates of CRCI (Schagen et al., 1999; Schagen et al., 2001; Ahles et al., 2002). For instance, when neuropsychological assessments were conducted 2 years after treatment, Schagen et al. (2001) found only 17% of patients exhibited signs of CRCI. van Dam et al. (1998) found similar results. In their study, 32% of patients treated with high dose chemotherapy exhibited CRCI, while 17% of the standard dose group showed signs of CRCI 2 years after treatment. In another study, Wefel et al. (2004) conducted comprehensive neuropsychological evaluation in a sample of breast cancer patients before treatment, at 6 months after treatment, and 1 year after treatment. The results indicated that while 61% of patients exhibited cognitive decline 6 months post-chemotherapy, only 30% of the total sample still exhibited impairment 1 year post-treatment.

Taken together, these studies suggest that assessment of patients relatively soon after chemotherapy treatment may inflate rates of cognitive impairment if one is interested in understanding how many patients develop long-lasting CRCI; patients experiencing short-term CRCI and those that end up with long-lasting and potentially permanent CRCI are conflated in studies that use shorter assessment intervals. Questions

raised by these early studies became the impetus for more rigorous longitudinal studies that favor designs with multiple time points over those that only assess cognitive function immediately following treatment.

Control and Comparison Groups: Another factor important for the classification and detection of CRCI within the human literature is the selection of a control or comparator population. Given its medical efficacy in the treatment of cancer, ethical limitations within the human literature make it difficult for researchers to obtain comparator groups that are identical to the treatment group aside from chemotherapy. Given that most of the CRCI research has been conducted in breast cancer patients and most women with breast cancer who participate in CRCI studies are often highly educated, it has been argued by Vardy et al. (2007) that test scores from this population may appear normal when compared to standard population scores even when these scores may represent a significant loss of function. The selection of an appropriate comparator is thus an important factor within the CRCI literature and may play a role in the rates and degrees of impairment detected between studies.

One common method that many studies have utilized to evaluate the cognitive outcomes of patients treated with chemotherapy has been to compare patients receiving only local cancer treatments (e.g. surgery or regional radiation therapy) with those patients receiving systemic chemotherapy. Studies that compare rates of cognitive impairment between local therapy controls rather than healthy controls generally report lower rates of CRCI. For example, in 2002 Ahles and collogues compared scores obtained from chemotherapy patients to a control group receiving local treatment rather than to normative population scores on neuropsychological tests, as in earlier studies.

The samples consisted of 35 chemotherapy and 36 local therapy long-term survivors (minimum of 5 years after diagnosis) with an average post-treatment assessment time interval of 10 years. Testing consisted of a neuropsychological battery containing nine testing domains and several psychological tests including a test of depression, fatigue and anxiety. Results indicated that patients treated with chemotherapy scored significantly lower than those that received local treatment on tests of verbal memory, psychomotor function, spatial ability and visual memory, even when controlling for age and education. Furthermore, 39% of chemotherapy patients compared with 14% of the local therapy patients were categorized as overall 'low performing' on neuropsychological tests (defined as the lower quartile on the neuropsychological performance index). Chemotherapy treated patients also self-reported greater problems with working memory utilizing the Squire Memory Self-Rating questionnaire. Similar results have also been obtained by Schagen et al. (1999) when comparing groups of cancer survivors that received chemotherapy or local treatment only, with assessments done approximately 2 years after completing treatment. Neuropsychological testing in the study revealed that 28% of the chemotherapy group compared to 12% of the local therapy group scored two standard deviations below the mean of the local therapy control. Consistent with previously discussed problems associated with self-report data, while interviews indicated that patients treated with chemotherapy complained of more concentration and memory related problems, no significant correlation was found between reported complaints and composite scores for either global or domain specific neuropsychological tests. Similar results were obtained by Schagen et al. (2001), in which cognitive scores were compared across three groups: high dose chemotherapy, standard dose

chemotherapy and local therapy only. Results indicated that 2 years after treatment, chemotherapy treated patients scored worse: 32% of the high-dose group, 17% of the standard-dose group and 9% of the local therapy group scoring under two standard deviations below the mean of the control group in neuropsychological examinations. These studies suggest that when cognitive scores are compared to a local therapy groups, a subset of individuals rather than the majority of those treated with chemotherapy show long-term impairment.

The studies discussed so far highlight three major factors that seem to impact the detected rates of CRCI in any given sample. These factors are: 1) type of assessment used, 2) timing of assessment relative to completion of treatment, and 3) the particular type of comparison group selected. Regardless of the discrepancies present in the literature, there seems to be a general agreement that rates of CRCI should reflect long-term cognitive dysfunction, such as the rates found in studies that examine patients at time points greater than 6 months after completion of treatment. In addition, there is a growing consensus that the use of established neuropsychological batteries for the primary assessment of CRCI provides the clearest indicators of declining function. Studies that meet these criteria typically report that 17-34% of assessed patients demonstrate clear cognitive impairment following chemotherapy treatment. (Ferguson & Ahles 2003; Ahles & Saykin, 2007; McDonald, Saykin & Ahles, 2008). Thus, only a subgroup of patients treated with chemotherapy suffer cognitive impairments that persist for at least a year after completion of treatment.

While research efforts exploring the cognitive effects of chemotherapy have increased since the late 1990s, it can be argued that limited progress has been made

elucidating the exact longitudinal aspects of CRCI, the extent to which various chemotherapy compounds differ in their capacity to induce CRCI, and in the identification of vulnerable brain structures and cellular mechanisms that underlie CRCI. Human studies are often limited to cross sectional experimental designs, have limited longitudinal access to patients, and have no control over the individualized and often complicated medical regimens of patients that include a large array of pharmaceutical compounds and therapy techniques. These factors exemplify just a handful of confounds and methodological challenges that have been blamed for the slow progress within the field (Castellon et al., 2005; Vardy et al., 2007; Farrell at al., 2013; Evenden, 2013). Experimental challenges are further potentiated when comparisons are made across studies with different experimental designs, making data difficult to combine or compare results within the field. As a result, many of the more recent achievements in the field have been in the development of new tools, experimental designs, and evaluation standards with which to overcome many of the confounding factors that have stifled progress. In 2006, many of the key researchers in the field formed the International Cognition and Cancer Task Force (ICCTF) in order to establish guidelines for streamlining study methodologies to increase comparability across studies and to minimize the effect of confounds within the field in general (Vardy et al., 2008; Wefel et al., 2011). As a result of the ICCTF's efforts, more recent work has centered on research approaches that examine patients longitudinally with longer post-treatment time intervals, that make comparisons with multiple control groups (healthy controls, local therapy, baseline measurements, and normative data) with agreed upon core neuropsychological tests of learning and memory, processing speed, and executive function [Hopkins Verbal

Learning Test-Revised, Trail Making Test, and the Controlled Oral Word Association (COWA) of the Multilingual Aphasia Examination] (Wefel et al., 2011).

Neuroimaging: Initial Insult, Possible Recovery, and Permanent Decline

Many recent attempts within the human literature to explore CRCI have also focused on combining new more powerful neuroimaging techniques like structural magnetic resonance imaging (MRI), functional magnetic resonance imaging (fMRI) and diffusion tensor imaging (DTI) with performance on neuropsychological batteries and cognitive tests recommended by ICCTF.

Structural Imaging: In 2010, McDonald and colleagues conducted one of the first longitudinal imaging studies to evaluate structural alterations in patients' gray matter density shortly after receiving chemotherapy and throughout the course of recovery. MRI imaging utilized voxel-based morphometry to measure gray matter density differences among groups (chemotherapy treated patients, non-chemotherapy patients, and healthy controls). Participants were assessed before treatment, one month, and one year after treatment. Results indicated that chemotherapy treated patients exhibited decreased gray matter density in parts of bilateral frontal, temporal, and cerebellar regions relative to baseline measurements when assessed one month after treatment. Data collected one year after treatment indicated that recovery had occurred in some of these regions, including superior areas of both frontal and temporal regions, but decreases were still detected in cerebellar regions and those more medial areas of both frontal and temporal regions suggesting that long-term structural changes took place. This unique pattern of gray matter alteration was only found in the chemotherapy treated group.

Although McDonald et al. (2010) did not run a neuropsychological battery, they argue that the structural changes detected were consistent with the types of cognitive dysfunction and the pattern of complaints observed in previous studies of chemotherapy patients examined along the same time intervals. Structural changes in the brain observed by McDonald et al. (2010) and cognitive deficits detected in other studies find the most pronounced deficits during and shortly after chemotherapy treatment. These cognitive and structural effects abate for some during a recovery period in the following months to a year, with a subset of patients reporting persistent long-term cognitive problems that are consistent with alterations in gray matter density detected a year after chemotherapy treatment. This study produced the first results indicating structural changes in the brain following chemotherapy treatment that were consistent with previous behavioral data, suggesting that structural changes in the brain may be related to reported cognitive problems.

More recent studies utilizing MRI techniques to investigate structural changes include a cohort study conducted by Koppelman et al. (2012) in which total brain volume and gray matter volume were measured in a sample of 184 cancer survivors with an average of 21 years post-treatment. Data collected from this sample was compared to an aged-matched cancer-free reference pool consisting of 368 subjects. Comparisons indicated that chemotherapy treated patients exhibited significantly smaller total brain volume and gray matter volume than reference subjects. These results suggest that structural changes may arise from chemotherapy treatment and may represent permanent alterations in the macrostructure of the brain, although the lack of a proper control group makes this conclusion tentative at this point. Koppelman et al. (2012) argue that the

volume differences detected by the study were comparable to the effect of approximately 4 years of age on gray matter loss. Interestingly, Conroy and colleagues (2013) detected a positive correlation between the duration of the post-chemotherapy interval (with a sample that was evaluated at an average of 6 years post-treatment) and gray matter density in the frontal cortex. While these results appear to contrast with the findings of Koppelman et al. (2012), the difference in post chemotherapy interval between the studies may be critical. Studies that utilize longer intervals may more accurately assess and report long-lasting, possibly permanent structural changes within the brain of patients treated with chemotherapy, while those studies using shorter intervals may produce data more influenced by periods of recovery, like those detected by McDonald et al. (2010), that occur shortly after treatment. These results seem to suggest that cancer survivors treated with chemotherapy exhibit temporal patterns of both cognitive changes and alterations in brain macrostructure. Some of these changes have similarities with normal aging and have been described as an acceleration of the natural aging process.

Functional Imaging: In a review of neuroimaging studies, Simo et al. (2013) summarized the findings of 8 CRCI fMRI studies. These fMRI studies identify both a subtle pre-treatment impairment in working memory, characterized by higher levels of activation within the frontoparietal attentional network (FPN), specifically in the prefrontal region, and two temporally distinct periods of altered fMRI activity following completion of chemotherapy. Studies have identified an early period (a few months to 1-2 years) and a later period (3 years or greater) after treatment in which both fMRI activation is altered and impairments on working memory and verbal memory recall are detected (McDonald et al., 2012; Kesler et al., 2009). In the early period, altered

neuronal activation is characterized by decreased bilateral frontal activation especially in the inferior frontal cortex, which returns to baseline after 1-2 years. A longitudinal study conducted by McDonald and colleagues (2012) provides evidence for this early period, in which most early changes in neuronal function detected via fMRI return to baseline after 1 year. In addition to this early period of change, other long-term follow-up studies have identified a later period of altered functioning that can last for more than 3 years and is characterized by hypoactivation of several brain regions including the prefrontal cortex (Ruiter et al., 2011), inferior frontal cortex (Kesler et al., 2009; 2011), medial temporal lobe (Ruiter et al., 2011), and the posterior parietal cortex (de Ruiter et al., 2011; McDonald et al., 2012). Simo et al. (2013) suggest that this pattern of results may be interpreted by an initial alteration in activation that may be compensatory in nature soon after treatment, followed by more long-term brain hypoactivation that may be related to long-lasting cognitive deficits.

White Matter Imaging: Recent studies utilizing both DTI and neuropsychology testing have provided evidence that cognitive decline following chemotherapy treatment may be related to changes in white matter tracts throughout the brain. Longitudinal data collected by Deprez et al. (2012) prior to chemotherapy and 3-4 years after treatment indicates that DTI is sensitive enough to detect disrupted microstructural properties of white matter when the corpus callosum and various cortical regions including parietal, frontal and occipital regions were compared. Interestingly, these alterations in white matter, characterized by decreased fractional anisotropy, were positively correlated to changes in neuropsychological test scores of attention and verbal memory (Deprez et al.,

2012). This suggests that chemotherapy induced damage to white matter may be partially responsible for cognitive impairments detected in patients.

Summarizing the neuroimaging literature, there appears to be more evidence for wide spread decreases in both white matter and grey matter throughout the brain following chemotherapy treatment than for specific targets of damage. Nevertheless, several studies argue that a handful of cortical areas including: middle frontal gyrus (MFG), medial temporal lobe (MTL), and lateral posterior parietal cortex (LPPC), may be particularly sensitive to the effects of chemotherapy treatment when utilizing region of interest analyses on both functional and structural neuroimaging measures (Inagaki et al., 2007; Deprez et al., 2011; de Ruiter et al., 2012). Interestingly, studies that have specifically examined the hippocampus for grey matter (Yoshikawa et al., 2005) or white matter (Ferguson et al., 2007) alterations following chemotherapy treatment have failed to detect any differences when compared to controls.

Neuroimaging studies like the ones discussed above have certainly helped to identify new areas for future research and have provided more objective evidence to corroborate patients' subjective complaints of CRCI. Although these studies highlight structural changes within the brain following chemotherapy treatment, the mechanisms behind these structural alterations are unclear, as is the time course. Interestingly, it appears that chemotherapy treatment may induce both transient and fairly long-lasting structural changes within the brain and that some of these changes may represent alterations related to recovery processes. Many researchers within the human literature have begun to recognize the importance of establishing animal models of CRCI in order

to identify potential underlying physiological and cellular mechanisms (Ahles et al., 2007; Evenden, 2013).

Animal Behavioral Models

Animal models that are able to further pinpoint factors associated with chemotherapy related cognitive deficits have been recognized as necessary for the development of the field. Behavioral models of learning and memory in rodents have already been established and are relatively well understood (e.g. autoshaping, contextual fear conditioning, Morris water maze, novel object recognition, etc.). Thus, rats and mice provide researchers with an opportunity to reexamine the cognitive decline associated with chemotherapy treatment without many of the confounding variables and problems discussed throughout the human scientific literature regarding this phenomenon. In particular, animal models have provided researchers with additional opportunities for determining which chemotherapy compounds (in isolation and in combination) seem to cause cognitive impairment and to elucidate the mechanisms by which these compounds may interact with the brain and body to induce cognitive impairment. By combining behavioral study with direct physiological brain and cellular analysis, animal studies are providing insight into the behavioral deficits and biological mechanisms associated with specific chemotherapy regimes. While animal models of CRCI were once scarce, the number of publications has grown in recent years.

Differences in Drug Class

One important factor to consider when investigating CRCI is that chemotherapy agents vary drastically in chemical structure and physiological properties. Each class of

the following chemotherapy compounds are effective in treating cancer, but do so via different molecular mechanisms with the potential to cause different types and degrees of cognitive dysfunction. Although all of following chemotherapy compounds achieve their anti-cancer efficacy by interfering with processes involved in cell division and proliferation, each drug class does this via a different mode of action. As such, the ability to develop animal models to study these compounds in isolation and in combination allows researchers to understand the effects of these different drug classes.

Alkylating Agents: Alkylating agents are a class of chemo drug that have the ability to alkylate electron-rich atoms such as DNA bases to form covalent bonds. These bonds can result in both intrastrand and interstrand DNA cross-links that prevent the transcription and replication of DNA. Cells that attempt to replicate or repair DNA that contain these cross-links usually lead to DNA fragmentation which triggers a state of programmed cell death referred to as apoptosis. Of the alkylating agents, cyclophosphamide is the most commonly studied. Thiotepa is also a commonly prescribed alkylating agent used to treat cancer. Both compounds utilize their ability to induce apoptosis in cells via the formation of DNA cross-link produced by covalent bonds. It should be noted that unlike other anti-cancer drugs, alkylating agents are cell cycle-independent and can trigger cell death and cause DNA damage regardless of the particular cell cycle stage. This ability may cause alkylating agents to achieve broader levels of cellular toxicity that may not be limited to proliferating cells.

Platinum-based Agents: Platinum-based agents are referred to as "alkylating-like" as they permanently interfere with DNA, like alkylating agents, but do so by allowing a platinum atom to bind to DNA bases instead of an alkyl group. These bonds interfere

with DNA in much the same way that alkylating agents do, causing problems with DNA transcription and replication and are capable of forming DNA cross-links. Cisplatin, carboplatin and oxaliplatin are all platinum-based chemotherapy agents that are commonly prescribed to treat cancer. Unlike alkylating agents, platinum-based compounds are known to cause peripheral neuropathy and their main dose-limiting side effect when used as a treatment for cancer is recognized as neurotoxicity. Several studies have examined the effect of platinum-based agents on neurological processes. These studies indicate that platinum-based agents like cisplatin are potentially more toxic to neural progenitor cells and oligodendrocytes than they are to cancer cells (Dietrich et al., 2006) and induce apoptosis (Dietrich et al., 2006; Gopal et al., 2012). In 2012, Gopal and colleagues conducted a study to quantify the neurotoxicity of cisplatin in vitro and found cisplatin induced rapid increases in neuronal firing followed by cell death. These results indicate that platinum-based agents like cisplatin are toxic to neurons and capable of inducing excitotoxic alterations.

Antimetabolites: Antimetabolites are a class of chemical compounds that inhibit normal physiological functioning by mimicking the structure of normal human metabolites and replacing them with nonfunctional alternatives. For example methotrexate, which is an antifolate drug, competitively inhibits the enzyme dihydrofolate reductase by mimicking the structure of folate. This reduces the synthesis of folic acid which in turn slows the production of the nucleoside thymidine, essential for synthesis of DNA and proteins. In most cases, antimetabolites disturb biosynthesis and interfere with the production of DNA and RNA causing cell cycle arrest and apoptosis. Unlike alkylating agents, antimetabolites are cell cycle dependent and are only effective during DNA synthesis

phase (S-phase) of the cell cycle. Of the antimetabolites used to treat cancer methotrexate and 5-fluorouracil are the most studied. 5-fluorouracil is a pyrimidine analog which is incorporated into DNA and RNA of a cell and inhibits the cells ability to synthesize DNA and can cause apoptosis. In addition to these, cytarabine, a cytosine deoxyribose analogue, has been studied by a few groups and is also an antimetabolite that inhibits the synthesis of DNA. Studies examining the in vitro and in vivo effects of antimetabolites like cytarabine (Dietrich et al., 2006) and 5-fluorouracil (Han et al., 2008) indicate that these drugs reduce cell division within the CNS when administered systemically, and are more toxic to neural progenitors and oligodendrocytes than to cancer cells when directly exposed to cultured cells.

Topoisomerase Interactive Agents: Topoisomerase interactive agents are a class of chemical compounds that inhibit or alter normal topoisomerase activity. In general topoisomerases are enzymes that participate in the regulation of DNA topology by cutting the phosphate backbone of the DNA, to release tension within the DNA double helix structure that occurs during normal DNA replication, which can then be resealed. While it is widely used and researched in combination with other drugs, doxorubicin is one of the more commonly studied topoisomerase interactive agents. Doxorubicin does not readily cross the blood-brain barrier and acts by inhibiting the resealing mechanism of the enzyme topoisomerase II, by intercalating DNA once it has been cut by the enzyme, and subsequently stabilizes the topoisomerase II complex that prevents future DNA replication. In addition to doxorubicin, etoposide is also a topoisomerase interactive agent that similarly disrupts the function of topoisomerase II causing DNA strands to break.

Antimicrotubule Agents: Antimicrotubule agents are a class of chemical compounds that interfere with normal microtubular function which is required to form the mitotic spindle necessary for the separation of replicated DNA, a critical component of mitosis. Similar to the topoisomerase interactive agent, doxorubicin, many of the commonly prescribed antimicrotubule agents do not readily cross the blood-brain barrier. Of the antimicrotubule agents, paclitaxel and docetaxel have been examined within the CRCI literature.

Combination Treatments: It is fairly common for clinicians to use combinations of various chemotherapy compounds to treat breast cancer and other types of cancer. Therapeutic strategies for the treatment of cancer that use different classes of compounds, or even two slightly different drugs from within the same class, are generally thought to be more effective in disrupting cancer cells by utilizing multiple drug mechanisms while allowing lower doses that reduce toxicity (Raffa & Tallarida, 2010). Several animal studies have opted to examine the behavioral effects of commonly used clinical regimens instead of focusing on particular drugs in isolation. Many of these researchers argue that it may be more important to model and test the effects of these clinically relevant combination treatments rather than focusing on individual drugs or class of drug. Two of the most commonly used combination treatments examined within the animal literature include a combination of cyclophosphamide and doxorubicin or the widely used clinical combination chemotherapy treatment, CMF (cyclophosphamide, methotrexate and 5-fluorouracil).

Current Gaps in the literature

A summary of recent animal behavioral findings regarding CRCI is provided in Table 1. Although inconsistency exists across behavioral results presented in Table 1, these studies demonstrate that CRCI occurs in animal models across a variety of different chemotherapeutic agents in isolation and combination. In general, these studies have provided evidence that all of these compounds are capable of producing behavioral deficits in tasks that evaluate cognitive functions related to memory and learning. However, the bulk of the animal behavioral data collected to date has focused on relatively short time periods, with only 6 of the 37 studies listed in Table 1 utilizing assessment intervals greater than 3 months following treatment. Furthermore, most of these studies have only used behavioral tasks that are sensitive to spatial memory dysfunction, prioritizing traditionally hippocampal dependent tasks. This focus on hippocampal function has created a rift between animal models of CRCI and the human literature, which has emphasized impairments related to disrupted frontal cortical function (Evenden, 2013).

Table 1: A summary of recent animal behavioral findings regarding CRCI

Drug	First Author and Year	Dosing Regimen	Animals	Behavioral Assessment	Tasks in Which Deficits Detected	Tasks in Which No Deficits Were Detected
Alkylating cyclophosphamide	Acharya 2015	weekly x 4 weeks 100mg/kg, i.p. + some got hippocampal stem cell grafts	Rats (M) Athymic nude	NOR, NOR temporal order, NOL, CFC, (all 9-11 weeks a.t.)	NOR, NOR temporal order, CFC (impairment for contextual memory)	NOL, CFC (cue memory) *Animals with hippocampal stem cell grafts performed like saline treated animals
cyclophosphamide	Lee 2006	1 every 4 weeks x 18 weeks, 100mg/kg, i.p.	Rats (F) Fischer, 7 or 18 months old	MWM (7 or 29 weeks a.t.), Stone Maze (9 weeks or 31 a.t),	None	MWM, Stone Maze
cyclophosphamide	Reiriz 2006	single 8, 40, or 200mg/kg, i.p.	Mice (M) CD1	OF, PA (24 hrs or 1 week a.t.)	PA (Impairment, 24 hrs r.i.)	OF, PA (Lower dose [8mg/kg] or 1 week r.i)
thiotepa	Mondie 2010	daily x days, 10mg/kg, i.p.	Mice (M) C57BL/6	FST (3,6,8,13,21 weeks a.t.), TST (8,13,21 weeks a.t.), NOR (2,4,8,12,20,30 weeks a.t.), NOL (2,4,13,20,30 weeks a.t.)	NOR (impairment 8 + 12 weeks a.t.) NOL (impairment 20 weeks a.t.)	FST, TST, NOR (all other time points), NOL (all other time points)
cyclophosphamide	Yang 2010	Single 40mg/kg, i.p.	Mice (M) ICR	OF (12hrs and 10 days a.t.), PA (12hrs and 10 days a.t.), NOR (12hrs and 10 days a.t.)	PA (impairment 12 hrs a.t.), NOR (impairment 12 hrs a.t.)	OF, PA (10 days a.t.), NOR (10 days a.t.)
cyclophosphamide	Christie 2012	weekly x 4 weeks, 50mg/kg, i.p.	Rats (M) Athymic nude	NOL (1 week a.t.), CFC (2 weeks a.t.)	NOL (impairment 5 min r.i.) CFC (impairment for contextual memory)	NOL (24 hrs r.i.), CFC (cue memory)
Platinum-based oxaliplatin	Fardell 2012	single 12mg/kg, i.p.	Rats (M) Wistar	NOR (14 days a.t., 1 hr r.i.), MWM (21 days a.t.), CFC (28 days a.t.)	NOR (impairment 14 days a.t.), CFC (impairment for contextual memory)	MWM, CFC (cue memory)

Table 1: Continued

oxaliplatin	Sharpe 2012	single 12mg/kg, i.p.	Rats (M) Sprague-Dawley	CFC (10 days a.t.), CFC extinction (13 days a.t.), CFC renewal (15 days a.t.) *new context	CFC renewal (impairment for renewal in novel context)	CFC (No Impairment in cued or contextual tests, conditioning or extinction)
Antimetabolites methotrexate	Seigers 2008	single 250mg/kg, i.v.	Rats (M) Wistar	MWM (3 weeks a.t.), NOR (4 weeks a.t., 1 hr r.i)	MWM (impairment 3 weeks a.t) NOR (Impairment 4 weeks a.t., 1hr r.i.)	MWM (all other measures)
methotrexate	Seigers 2009	single 250mg/kg, i.v.	Rats (M) Wistar	MWM (training prior to treatment tested 1 week a.t.), CFC (training prior to treatment tested 1 month a.t.)	MWM (impairment 1 week a.t. for time spent in target quadrant) CFC (impairment 1 month a.t.)	None
methotrexate	Fardell 2010	single 250mg/kg, i.p.	Rats (M) Wistar	MWM (2 weeks + 8 months a.t.), NOR (11,95,254 days a.t.,1 hr r.i + 255 days a.t. with 2 hrs r.i.), Go-No/Go Task (174 days a.t.)	MWM, (impairment 8 months a.t.), NOR, (11+95 days a.t. with 1hr r.i., at 255 days a.t. with 2hrs r.i,) Go-No/Go Task (impairment learning)	MWM, (2 weeks a.t.), NOR, (254 days a.t. with 1hr r.i.)
methotrexate	Li 2010	Acute: single 250mg/kg i.p; Chronic: weekly x 10 weeks, 1mg/kg, i.p.	Rats (M) Long Evans	OF (3 days a.t.), NOL (1 week a.t., 5 or 10min r.i.), NOR (1 week a.t., 2hrs r.i.)	NOL (impairment 1 week a.t., 5min r.i. for both acute and chronic)	OF, NOL (week a.t.,10min r.i.), NOR
methotrexate	Lyons 2011	weekly x 2, 75mg/kg, i.v.	Rats (M) Listar	NOL (3 weeks a.t., 15min r.i.)	NOL	None
methotrexate or 5-fluorouracil	Foley 2008	single i.p. 15mins prior to task, varied dose: MTX (1 to 32mg/kg) or 5-FU (3 to 75mg/kg)	Mice (M) Swiss Webster	Autoshaping task (involved learned rewarded response patterns)	Autoshaping task,(high dose of 5-FU only during retrieve a previously learned response 2 days a.t.)	MTX and lower doses of 5-FU failed to cause impairments on task acquisition or retention/retrieval

Table 1: Continued

methotrexate or 5-fluorouracil	Walker 2011	weekly x 3 weeks. MTX (3.2 or 32mg/kg) or 5-FU 75 mg/kg, i.p. last injection 15mins prior to task	Mice (M) Swiss-Webster	Autoshaping task	5-FU alone (impairment on acquisition and retention)	MTX (no impairments)
methotrexate or cytarabine	Bisen-Herch 2013	3 i.p. injections PD 14 15 and 16, of MTX (1 or 2 mg/kg) or Ara-C (10 or 20 mg/kg)	Mice (M+F) Swiss-Webster	Autoshaping task, NOR (1hr r.i.), Conditional discrimination task, (all done 19 days a.t.)	Autoshaping task* (impairment for retention and acquisition), NOR*, Conditioned Discrimination (impairment in acquisition), *only found with higher doses	no significant differences were found between M and F mice, Lower doses
methotrexate	Yang 2012	Some Tumor-Bearing* single 40mg/kg, i.p.	Mice (F) C3H/HeN	OF, TST, PA (all done 24 hrs a.t.)	TST (more depressive like behavior for tumor-bearing as well MTX alone or in addition), PA (impairment caused by MTX 24hrs a.t.)	OF
5-fluorouracil	Mustafa 2008	5 over 12 days, 20mg/kg, i.v.	Rats (M) Listar	NOL (5 min r.i.)	NOL (impairment in mean exploratory times of novel location but not in preference index scores)	None
5-fluorouracil	ELBeltagy 2010	6 over 2 weeks, 20mg/kg, i.v. + some animals received 10mg/kg/d Fluoxetine in drinking water	Rats (M) Listar	NOL (1 day a.t.), CFC (1 day a.t.)	NOL (1 day a.t.), CFC (1 day a.t.)	NOL (animals treated with 5-FU+Fluoxetine showed no impairment)
5-fluorouracil	Krynetskiy 2013	3 over 24hrs, 75mg/kg, i.p.	Mice (M) Swiss Webster	Autoshaping task (1 day a.t., 24hrs r.i.)	Autoshaping task*(impairment for retention and acquisition on 24 hrs r.i),	Autoshaping task (initial acquisition was unaffected)

Table 1: Continued

5-fluorouracil	Mahoney 2013	daily x 5 days, low (20mg/kg), medium (40mg/kg), or high (60mg/kg) i.p.	Mice (M+F) C57BL/6 and MCP-1 -/- Mice	Measure of Fatigue: Voluntary Wheel Running (collected for 15 days post-treatment), Grip strength (days 5 and 14 a.t.)	Dose dependent reductions in voluntary Wheel Running	Grip strength
5-fluorouracil	Fremouw 2012	weekly x 3 weeks, 100 mg/kg, i.p.	Mice (M) C57BL/6	CFC (1 weeks a.t.), CFC remote (2 weeks a.t., 40 days r.i.), NOR (2 weeks a.t., 1 hr r.i.)	None	All testing: CFC + CFC remote, NOR
cytarabine	Fremouw 2012	daily x 5 days, 275mg/kg, i.p.	Mice (M) C57BL/6	MWM Recent (42 days a.t., 24 hrs r.i.), MWM Remote (42 days a.t., 30 days r.i.)	None	All testing: MWM recent + remote memory, Acquisition,
cytarabine	Li 2008	daily x 5 days, 400mg/kg, i.p.	Rats (M) Sprague-Dawley	MWM (1 week a.t., 24 hrs r.i. and 30 days r.i.),	MWM remote memory (impairment on 30 days r.i.)	MWM recent memory (no impairment on 24hrs r.i.)
Topoisomerase Interactive doxorubicin	Christie 2012	weekly x 4 weeks, 2mg/kg, i.p.	Rats (M) Athymic nude	NOL (1 week a.t.), CFC (2 weeks a.t.)	NOL (impairment 5 min r.i.) CFC (impairment for contextual memory)	NOL (24 hrs r.i.), CFC (cue memory)
doxorubicin	Fremouw 2012	weekly x 3 weeks, 4mg/kg, i.p.	Mice (M) C57BL/6	CFC (1 weeks a.t.), CFC remote (2 weeks a.t., 40 days r.i.), NOR (2 weeks a.t., 1 hr r.i.)	None	All testing: CFC + CFC remote, NOR
doxorubicin	Liedke 2009	single 0.5, 2 or 8mg/kg, i.p. either 30mins before training or after 30mins	Rats (M) Wistar	PA treatment before training (3hrs, 24hrs and 7 days r.i.) PA treatment after training (3hrs, 24hrs and 7 days r.i.)	PA treatment before training (24hrs and 7 days r.i.)	PA treatment before training, (no impairment in 3hrs r.i.), PA treatment after training
etoposide	Wood 2006	Some Tumor-Bearing* 5 over 2 weeks 50mg/kg, i.p.	Mice (F) C57BL/6	Voluntary Wheel Running (before and during treatment)	Reductions in voluntary Wheel Running, due to chemo and/or tumor	None
Antimicrotubule paclitaxel	Boyette 2009	1 every other day x 12 days 1mg/kg i.p.	Rats (M) Long Evans	MPW (tested throughout treatment for 20 days), 5CSRTT (tested during treatment for 20 days)	"peripheral neuropathy" MPW (threshold decreased within 24hrs)	5CSRTT (no impairments)

Table 1: Continued

docetaxel	Fardell 2013	weekly x 3 weeks, 6mg/kg or 10mg/kg i.p.; or single 10mg/kg i.p.	Rats (M) Wistar	MWM (2 weeks a.t, 24hrs or 100 days r.i.), NOR (1 or 2 weeks a.t., 2hrs or 100 days r.i.), MPW (12 or 120 days a.t)	NOR (trends for impairment at both time points), MPW (impairment 12 days a.t)	MWM (no impairment but poor control performance) MPW (no impairment 120 days a.t)
Combination Treatments methotrexate and 5-fluorouracil	Gandal 2008	weekly x 4 weeks, High: MTX (37.5mg/kg)+5-FU(75mg/kg), i.p. Low: MTX (18.75mg/kg)+5-FU(37.5mg/kg), i.p.	Mice (M) C57BL/6	NOR (2 weeks a.t., 24hrs r.i.), CFC (28 day a.t.), EEG auditory gating (during treatment)	EEG Auditory Gating (Less gating during treatment)	NOR, CFC
doxorubicin and cyclophosphamide	Fremouw 2012	weekly x 3 weeks, 4mg/kg Dox + 80mg/kg Cyclo i.p.	Mice (M) C57BL/6	CFC (1 weeks a.t.), CFC remote (2 weeks a.t., 40 days r.i.), NOR (2 weeks a.t., 1 hr r.i.)	None	All testing: CFC + CFC remote, NOR
doxorubicin and cyclophosphamide	Konat 2008	weekly x 4 weeks Dox (2.5mg/kg) + Cyclo (25mg/kg), i.p.; one group given NAC 200mg/kg 3 per week x 4 weeks	Rats (F) Sprague-Dawley	OF, PA (2 days a.t, 24hrs r.i.)	PA (impaired 24hrs memory)	OF, PA (*NAC treatment fully prevented)
doxorubicin and cyclophosphamide	Macleod 2007	weekly x 3 weeks Dox (4mg/kg) + Cyclo (40mg/kg) i.v.	Rats (F) Ovariectomized Sprague-Dawley	CFC (1 week a.t., 24hrs r.i.)	CFC (impairment for contextual memory)	CFC (cue memory)
cyclophosphamide, methotrexate and 5-fluorouracil	Briones 2011	weekly x 4 weeks CMF: Cyclo (40mg/kg) + MTX (37.5mg/kg) + 5-FU (75mg/kg), i.p.	Rats (F) Wistar	MWM (2 weeks a.t.), MWM cued discrimination task	MWM (impaired acquisition and memory), MWM cued discrimination task (more errors)	None

Table 1: Continued

cyclophosphamide and 5-fluorouracil	Long 2011	3 monthly Cyclo (50mg/kg) + and 5-FU (75mg/kg) last 2 doses: [Cyclo (75mg/kg) + 5-FU (120mg/kg)] i.p.	Rats (M) Fischer-344	Remote CFC (trained b.t., tested 120 days a.t.), MWM (130 days a.t.), Stone Maze (150 days a.t.)	None	Remote CFC, MWM, Stone Maze
methotrexate and cytarabine	Bisen-Herch 2013	3 i.p. injections PD 14 15 and 16, of (1 mg/kg MTX + 10mg/kg Ara-C) or (2mg/kg MTX and 20mg/kg Ara-C)	Mice (M+F) Swiss-Webster	Autoshaping task, NOR (1hr r.i.), Conditional discrimination task, (all done 19 days a.t.)	Autoshaping task (impairment for retention and acquisition), NOR, Conditioned Discrimination (impairment in acquisition *and memory with higher dose),	no significant differences were found between M and F mice
oxaliplatin and 5-fluorouracil	Fardell 2012	Single OX 8mg/kg + 5-FU 75mg/kg i.p., [half got running wheels for 4 weeks]	Rats (M) Wistar	NOR (14 + 40 days a.t., 1hr + 2hrs r.i.), MWM (21 days and 54 a.t.), CFC (28 days a.t.)	NOR (14 days a.t., and 40 days a.t., 2hr*), MWM (54 days a.t.*) CFC (impairment for contextual memory)	MWM (21 days a.t.), NOR (40 days a.t., 1hr), CFC (cue memory) *Running Wheels prevented impairments in MWM + NOR
methotrexate and 5-fluorouracil	Walker 2011	weekly x 3 weeks. 3.2 or 32mg/kg MTX + 7 mg/kg 5-FU, i.p.; last injection 15mins prior to task	Mice (M) Swiss-Webster	Autoshaping task	Autoshaping task (impairments in acquisition and retention) *lower dose of MTX with 5-FU was more impairment	*Higher doses of MTX caused less impairment
methotrexate and 5-fluorouracil	Winocur 2006	weekly x 3 weeks MTX 37.5mg/kg + 5-FU 75mg/kg, i.p.	Mice (F) BALB/c	Modified MWM tests 1 week a.t.: Standard, Cued Memory, NMTS, DNMTS (60,120,240 sec r.i.), Black-White Discrimination	NMTS (small increase in errors), DNMTS (240 sec r.i.),	MWM standard test, MWM test Cued Memory, NMTS (latency), DNMTS (60, 120 sec r.i.), Black-White Discrimination

Table 1: Continued

methotrexate and 5-fluorouracil	Winocur 2011	weekly x 4 weeks MTX 50mg/kg + 5-FU 75mg/kg, i.p.; some given Donepezil 3mg/kg/d in water during treatment	Mice (F) BALB/c	Modified MWM tests 1 week a.t.: Standard, Cued Memory, NMTS, DNMTS (60,120,240 sec r.i.),	Standard (impaired spatial memory) NMTS, DNMTS (all delays)	MWM test Cued Memory, Donepezil reduced the deficits in all tasks
methotrexate and 5-fluorouracil	Winocur 2012	weekly x 3 weeks MTX 37.5mg/kg + 5-FU 50mg/kg, i.p.	Mice (F) BALB/c	Modified MWM tests 24hrs and 3 months a.t.: Standard, Cued Memory, NMTS, DNMTS (60, 120, 240 sec r.i.), Conditioned associative learning, Brightness discrimination learning	Standard (impaired spatial memory at both time points), NMTS (both time points), DNMTS (both time points all delays), Conditioned associative learning (impaired learning), Brightness discrimination learning (impaired learning)	MWM test Cued Memory,
methotrexate and 5-fluorouracil	Winocur 2015	weekly x 3 weeks MTX 37.5mg/kg + 5-FU 50mg/kg, i.p.	Rats (F) Long Evans	Modified water T maze test: low inference, high inference,	high inference (memory impairment)	high inference (no learning impairment), low inference (no learning or memory impairment)

Abbreviations – Ara-C: cytarabine; Cyclo: cyclophosphamide; CMF: combination of cyclophosphamide, methotrexate and 5-fluorouracil; Dox: doxorubicin; MTX: methotrexate; NAC: N-acetyl cysteine; OX: oxaliplatin; 5-FU: 5-fluorouracil; CFC: contextual fear conditioning; 5CSRTT: five-choice serial-reaction time task; FST: forced swim test; NOL: novel object location; NOR: novel object recognition; MPW: mechanical paw withdrawal threshold; MWM: Morris water maze; OF: open field test; PA: passive avoidance conditioning; NMTS: non-matching to sample; and DNMTS: delayed non-matching to sample; TST: tail suspension test; a.t.: after treatment; i.p.: Intraperitoneal injection; i.v.: Intravenous injection; r.i.: retention interval.

Only 8 of the 37 studies listed in Table 1 have used tasks that assess animal behaviors that may be related to frontally mediated functions such as response inhibition, behavioral spontaneity, and habituation. These tasks include: autoshaping paradigms (Foley et al., 2008; Walker et al., 2011; Bisen-Herch et al., 2013; Krynetskiy et al., 2013), the 5-choice serial reaction time task (5CSRTT) (Boyette et al., 2009), and a non-matching-to-sample (NMTS) variant of the Morris water maze (Winocur et al., 2006; 2011; 2012). Thus, the current animal behavior literature concerned with CRCI faces two major problems in further elucidating the phenomenon: too much focus on a single cognitive system, and insufficient long-term designs. These problems have made it difficult for researchers to make progress understanding the cognitive and neurological deficits caused by chemotherapy treatment and have limited the translational insight that the current animal literature can provide to the human condition of chemo-brain.

Behavioral Tasks: Much of the animal behavior literature has focused on tasks thought to be dependent on hippocampal function. The initial emphasis on these hippocampal based tasks was driven by the knowledge that chemotherapy agents disrupt proliferating cells, and as such, may disrupt neuronal structures in which neurogenesis takes place, like the hippocampus. Furthermore, behavioral models of learning and memory in rodents that are sensitive to hippocampal damage are relatively well established. Commonly studied hippocampal dependent behavioral tasks in the literature include: novel object location, Morris water maze, contextual fear conditioning, and passive avoidance conditioning. These tasks are commonly used because they are thought to assess an animal's ability to successfully learn contextual spatial cues and access spatial memory during training and testing.

For instance, novel object location relies on rodents' natural tendency to approach and explore novelty in order to assess whether a rodent remembers the previous location of an object that has been moved to a novel location. Tasks like the Morris water maze, contextual fear conditioning, and passive avoidance conditioning, all rely on rodents' desire to escape or avoid an aversive stimulus by correctly remembering spatial and contextual cues. Chemotherapy compounds appear capable of producing deficits in both learning and memory across all of these tasks when tested shortly after treatment and, as such, are thought to impair hippocampal function, potentially by interfering with neurogenesis related processes (e.g., Konat et al., 2008; Seigers et al., 2009; Yang et al., 2010; Briones et al., 2011; Fardell et al., 2012; Christie et al., 2012)

There have only been 8 attempts to date, within the animal literature to explore cognitive domains outside spatial memory. Of these studies, those that utilize autoshaping paradigms are the most common and produce the most reliable, albeit subtle, deficits in acquisition and latency to retrieve previously learned responses (Foley et al, 2008; Walker et al., 2011; Bisen-Herch et al., 2013; Krynetskiy et al., 2013). In addition, Boyette and colleagues (2009) ran the only study within the field that attempted to evaluate CRCI in animals with the 5-choice serial reaction time task (5CSRTT). The 5CSRTT is an operant paradigm in which animals must attend to a randomly presented visual stimuli and quickly respond (within 0.5 sec.) with nose-poke response in order to receive a reinforcement. The task is thought to be sensitive to changes in attention systems, executive function and information processing speed. Finally, Winocur et al. (2006; 2011; 2012) argue that they have been able to detect impairments using a modification of the standard Morris water maze, in which animals must learn a NMTS

rule in order to correctly use a visual cue to escape the maze. The addition of the NMTS rule is posited by Winocur and collegeues (2006) to be "highly sensitive to frontal-lobe dysfunction but not typically affected by damage to the hippocampus" (p. 68).

Together these studies represent a small minority of the tasks used within animal behavior models of CRCI. When examined as a whole, the behavioral tasks presented in Table 1 demonstrate that the animal behavior literature currently suffers from a lack of variety. The overwhelming focus on hippocampal function has resulted in a failure to investigate the effects of chemotherapy compounds on other cognitive systems in animals, including those that have already been highlighted within the human literature.

Lack of Long-Term Designs: 31 of the 37 studies listed in Table 1 have examined the effects of chemotherapy compounds only out to 3 months after treatment, with the vast majority of these only investigating effects up to 1 week after treatment. Although short-term impairments are meaningful, they provide little insight into the possible long-term effects of these compounds, recognized as so important in the human literature. Furthermore, deficits detected shortly after treatment are confounded with sickness like effects that are known to occur in rodents shortly after chemotherapy administration (Wood et al., 2006). Of the studies listed in Table 1, only 6 have investigated the behavior of animals more than 3 months after receiving treatment and only half those studies (Fardell et al., 2010; Mondie et al., 2010; and Winocur et al., 2012) found any detectable deficits while the other half failed to identify any impairments in any behavioral measures (Lee et al., 2006; Fardell et al., 2013; Long et al. 2011). The only interesting commonality seen in 2 of the 3 studies that found long-term deficits were that both Fardell et al. (2010) and Winocur et al. (2012) found deficits in the Morris water

maze after animals were administered methotrexate. This suggests that methotrexate may be a particularly effective disruptor of hippocampal function at longer time intervals compared to other agents and combinations of agents that were not able to produce these effects. Furthermore, these results stress the need for better extended profiling of these compounds and their long-term capacity to disrupt cognitive function. More long-term designs are needed to confirm these limited cognitive findings and to help determine if CRCI detected shortly after treatment, may persist for longer or reflect permanent loss of function.

Inconsistent Results: A cursory glance at the results in Table 1 reveal a considerable amount of inconsistency across the behavioral findings of the studies listed. For instance, when comparing studies that examined the effects of methotrexate on rats assessed with novel object recognition, Seigers et al. (2008) found impairment while Li et al. (2010) did not. Both studies administered a single dose of 250 mg/kg methotrexate to male rats that were approximately 12 weeks of age and both studies assessed memory function with a novel object recognition test. While these studies are quite similar they were conducted with different rat strains, as Seigers et al. (2008) used Wistar rats and Li et al. (2010) used Long Evans rats, and the exact timing of assessment differed with Seigers et al. (2008) testing animals 4 weeks after treatment while Li et al. (2010) assessed animals 1 week after treatment. The contrasting results of these two studies demonstrate the sensitivity of behavioral tests like novel object recognition to experimental variability.

There are numerous theoretical sources for this variance within the behavioral data. In the studies listed in Table 1, the exact timing and dose used during treatment can vary greatly even when examining studies using the same drug, within the same species.

While it is common to justify the choice of dosing regimen, these differ based on the goals unique to each study. Dosing regimens differ based on desire to mimic clinical paradigms (Long et al., 2011), the use of a maximally tolerable dose determined by pilot data (Seigers et al., 2008), the use of a reference dose based on pharmacokinetics to find dose-dependent effects (Liedke et al., 2009), or even the use of a specific dose that replicates previous studies (Fardell et al., 2010). Regardless of the reason, these decisions can drastically alter the behavioral results making comparisons and interpretations of contrasting results difficult. Typical variations seen within the literature include: the use of multiple low dose injections, single high dose injections, multiple injections over a long period (month or greater), and multiple injections over a short period (several days to a week). Furthermore, the animal literature regarding CRCI has generally not addressed the issue that toxicity and tolerance of chemotherapy agents can vary drastically as a function of circadian rhythm, further compounding variance among studies (Focan, 1995; Borniger et al., 2017; although see Fremouw et al., 2012a and 2012b). While these problems are not unique within the field of neuropsychopharmacology, it represents a significant barrier when determining which chemotherapeutic agents may cause cognitive dysfunction in animal models.

Suggested Biological Targets and Mechanisms

One strength of animal models is the ability to design experiments that directly explore the neurobiological mechanisms that are responsible for CRCI. This approach allows researchers a different perspective that focuses on the neurobiological changes following chemotherapy treatment. By understanding and mapping out these changes researchers may be able to make sense of the inconsistent behavioral results obtained in

animal models to date. The identification of cellular targets and processes that underlie these negative alterations are important for the translation of research accomplishments in the field into clinical application. Once these mechanisms have been identified and understood, clinicians will be better able to reduce or even prevent the cognitive impairments seen in patient populations.

Potential Cellular Targets

White Matter Damage: While neuronal damage and related processes have been the primary subject of many research efforts, researchers in both the animal and human literature have recognized the importance of white matter changes following chemotherapy treatment. White matter plays a critical role in regulating neuronal impulse conduction and synchronizing communication across neuronal networks (Madden et al., 2012). In addition to these functions, myelinating glia appear to have a variety of other activities that support normal neuronal activity, including the production of proteins that constrain nervous system plasticity (Fields et al., 2008). Many researchers have suggested that white matter tracts throughout the CNS may be vulnerable to chemotherapeutic insult following treatment through direct toxicity, damage via oxidative stress, or inflammatory disturbance.

Interestingly, recent human research has suggested that age-related cognitive difficulties may be due to differences in structural integrity of white matter (Madden et al., 2012). In a review by Madden et al. (2012), it is suggested that normal cognitive decline associated with aging may be related to white matter integrity and a decrease in efficiency of communication among networks important for fluid cognitive abilities.

Some have made the theoretical leap that the effects of aging on cognitive systems may be similar to the effects of chemotherapy treatment. In other words, chemotherapy may cause accelerated aging of cognitive systems and negatively impact white matter integrity throughout the CNS. As previously discussed, evidence in support of this theory does exist within the human literature. For example, the DTI studies of Deprez et al. (2011, 2012, and 2013) suggest that chemotherapy compounds can disrupt white matter integrity and result in cognitive impairments in attention, psychomotor speed, and memory that can last up to 5 months. de Ruiter and colleagues (2012) have even reported that changes in white matter integrity following chemotherapy treatment can be detected up to 10 years after treatment when compared to non-chemotherapy treated patients.

Within the animal literature, only a handful of studies have examined the role of white matter and chemotherapy related insult. Han et al. (2008) found that 5-fluorouracil is toxic to both immature and mature oligodendrocytes in vitro. Furthermore, when administered in vivo, 5-fluorouracil resulted in loss of myelin basic protein and cellularity within the corpus callosum up to 56 days after completion of chemotherapy treatment. In addition to these findings, Han reported increased auditory brainstem response (ABR) inter-peak latencies detected at 14 and 56 days following completion of chemotherapy treatment. The authors argue that these results represent compromised functional integrity of white matter tracts within the CNS due to chemotherapy treatment. Recent follow-up experiments within our lab have revealed similar short-term deficits (up to 14 days after treatment) in ABR inter-peak latencies following treatment with 5-fluorouracil, cyclophosphamide or doxorubicin in mice. ABR analysis of white matter function at 56 days and 6 months post-treatment failed to detect any impairment.

Subsequent tissue analysis was conducted utilizing black gold II, an aurohalophosphate complex, which is selectively absorbed into myelinated axons and used to produce high contrast images of myelin in tissue samples. Results of histochemical tissue analysis with black gold II were consistent with the ABR data, showing no deficits in myelin integrity at 56 days and 6 months post-treatment. However, black gold II staining of tissue collected 14 months post-treatment suggested long-term, possibly permanent demyelination had occurred in animals treated with either 5-fluorouracil or a combination of cyclophosphamide and doxorubicin. Unfortunately, ABRs could not be collected from animals at this point due to hearing loss across the entire sample. Taken together, these studies provide evidence that white matter tracts may be susceptible to chemotherapeutic insult shortly after treatment and, for some individuals, may reemerge after initial recovery as a persistent loss in white matter integrity with age. This pattern of damage to white matter detected in animal models is consistent with human imaging studies that indicate similar early transient impairments, followed later, by more persistent alterations in brain structure and function (Simo et al., 2013; Deprez et al., 2012).

While animal research investigating the association between white matter integrity and chemotherapy related cognitive impairment is limited, there are many well established animal models designed to explore the role of white matter damage in cognition, in particular its function in multiple sclerosis (MS). These models provide both behavioral and physiological benchmarks by which chemotherapy related white matter damage may be compared to and assessed. The most prominent rodent models are experimental autoimmune encephalomyelitis (EAE) and cuprizone-induced toxic demyelination. Both of these established experimental animal models are thought to

approximate and produce many of the same pathological features of MS including: inflammation, demyelination, and axonal loss (Skripuletz et al., 2008; Constantinescu et al., 2011; Kurkowska-Jastrzębska et al., 2013). Furthermore, several researchers have documented deficits in learning and memory following demyelination using these techniques in tasks such as the Morris water maze (D'Intino et al., 2005; Kurkowska-Jastrzębska et al., 2013) and the Y maze (Xiao et al., 2008; Makinodan et al., 2009). Both the EAE and cuperizone models may provide useful comparison data to determine if chemotherapy treatments cause similar toxicity induced demyelination and white matter damage.

Currently it is unclear whether significant differences in susceptibility to chemotherapy related damage exist between white matter and neurons. Gliogenesis, glial inflammation, and oxidative stress could all represent mechanisms by which white matter may be damaged. It is currently unknown to what degree white matter damage may explain CRCI. Many researchers posit that white matter damage may be a key mechanism responsible for the phenomenon. While extended studies that investigate long-term changes in white matter integrity following chemotherapy treatment are still lacking, results from several animal models and human DTI studies seem to indicate that white matter damage may persist long after chemotherapy treatment has ended. Chemotherapy induced damage to white matter seems to represent a plausible cellular target for CNS damage that maps on well to the both the types of cognitive problems being reported and the persistent nature of some of these side effects.

Proliferative Cell Vulnerability: Cellular processes involved in mitosis and the proliferation of cancer cells are the primary targets of the majority of chemotherapeutic

compounds used to treat cancer. Unfortunately these compounds are not selective and can theoretically affect proliferative cells in both the PNS and CNS. In addition, populations of both neurons and glial cells within the CNS are known to proliferate. As such, they may both be vulnerable to insult from chemotherapeutic compounds or their metabolites. The process of neuronal proliferation referred to as neurogenesis is known to occur within the hippocampus and other select regions of the adult brain. New neurons within the subgranular zone (SGZ) of the dentate gyrus (DG) that originate from dividing populations of adult neural stem cells may eventually migrate to the granule cell layer and integrate into existing neural networks after several weeks (Song et al., 2012). While some of these newborn neural progeny successfully mature, a significant percentage are eliminated through normal processes of apoptosis and microglia-mediated phagocytosis (Song et al., 2012). Many researchers suspect that proliferating neurogenic cells within the CNS may be particularly susceptible to insult and a likely target for cellular damage within the brain following chemotherapy treatment.

A Variety of Chemotherapy Drugs Decrease Neurogenesis: Research conducted in vitro suggests that a variety of chemotherapy compounds may be more toxic to neural progenitor cells and oligodendrocytes than to cancer cells when administered directly to the cells (Dietrich et al., 2006; Han et al., 2008). In addition to being toxic, chemotherapy compounds that enter the brain may cause significant disruptions to the cellular processes that underlie neurogenesis, including proper developmental differentiation and successful maturation. Several studies have investigated neurogenesis levels following chemotherapy treatment and have found that a variety of chemotherapy compounds can negatively impact neurogenesis within the hippocampus of rodents

including: cyclophosphamide (Yang et al., 2010; Janelsins et al., 2010; Christie et al., 2012), thiotepa (Mondie et al., 2010), BCNU (Dietrich et al., 2006), 5-fluorouracil (Han et al., 2008; Mustafa et al., 2008; Janelsins et al., 2010), methotrexate (Seigers et al., 2008; 2009; Lyons et al., 2011; Yang et al., 2012), cytarabine (Dietrich et al., 2006), doxorubicin (Christie et al., 2012; Janelsins et al., 2010), paclitaxel (Janelsins et al., 2010), cisplatin (Dietrich et al., 2006), and a clinical combination CMF (Briones et al., 2011). Taken together, these studies demonstrate that every chemotherapy drug class has the capacity to decrease neurogenesis. To quantify decreases in neurogenesis, studies employ immounohistochemical methodology and either Ki-67 or BrdU antibodies as markers for cellular proliferation. Ki-67 is an endogenously produced protein that is strictly associated with cellular proliferation. Unlike Ki-67, BrdU is an exogenous marker of proliferation. BrdU is a synthetic nucleoside and a structural analog of thymidine that can be incorporated into the DNA during replication as a thymidine substitute, and can later be detected via BrdU specific antibodies. Antibodies for Ki-67 and BrdU can be used to create high contrast immounohistochemical staining to label proliferating cells within the hippocampus. While results from these studies report significant reductions in Ki-67 and BrdU positive cells following chemotherapy treatment, the causes of these reductions remains unclear.

When examining the results of studies that have assayed both apoptosis and proliferation, different patterns of results emerge suggesting that some chemotherapy compounds, even compounds from within the same drug class, may induce apoptosis in these new neurons while others reduce proliferation without causing cell death. For instance, Janelsins et al. (2010) found decreases in BrdU positive cells within the DG of

animals 2 days after completion of treatment with cyclophosphamide, doxorubicin, 5-fluorouracil, or paclitaxel without significant increases in apoptotic BrdU labeled cells. In contrast, Dietrich et al. (2006) found that animals treated with BCNU or cisplatin demonstrated decreases in BrdU positive cells within the hippocampus and corresponding increases in apoptotic cells 1 day after treatment. Interestingly, while both BCNU and cyclophosphamide are alkylating agents, comparing the results reveals that even though each compound reduces proliferation, cyclophosphamide does so without causing increases in cell death, while BCNU treatment increase rates of cell death. The discrepancy between these results suggest that underlying mechanisms that lead to compromised levels of neurogenesis may differ across chemotherapeutic agents, and may be unique to each compound. Regardless of differences in the exact nature of neurogenic disruption, it is clear that chemotherapy agents induce reductions in neurogenic proliferation following treatment.

Lack of Long-Term Data: Most of the studies that have assayed neurogenesis levels in animals treated with chemotherapy compounds have assessed animals within a relatively short time following treatment, typically ranging from several days to weeks. While it is often claimed that deficits in neurogenic proliferation shortly following chemotherapy treatment contribute to the cognitive problems seen shortly after chemotherapy administration in animal models, this seems unlikely given the fact that newborn neurons take approximately 6 weeks to display similar morphological and functional characteristic as fully mature cells within the DG (Song et al., 2012). It is more likely that behavioral deficits detected within a few weeks after treatment relate to the sickness and fatigue effects of treatment or reflect toxicity induced cell death within

the CNS rather than to any reductions in neurogenic proliferation. For instance, Yang and colleagues (2010) used both Ki-67 and doublecortin (DCX), a protein expressed in immature neurons, to assess neurogenesis in mice treated with cyclophosphamide. They detected a sharp decline in both Ki-67 and DCX positive cells during the first 24 hours after treatment, but these reductions normalized over the next 10 days, suggesting recovery. Interestingly, concurrent behavioral tests indicated transient deficits that corresponded to the neurogenesis data, with animals exhibiting impairment 12 hours post injection in both a passive avoidance task and in a novel object recognition task. When these behavioral tasks were reexamined 10 days after treatment no deficits were detected (Yang et al., 2010). While Yang and colleagues (2010) suggest that the cognitive deficits detected are potentially due to decreases in neurogenesis, this seems unlikely given the time it takes for new neurons to mature and become functionally integrated. I believe these results are the product of sickness related side effects of chemotherapy treatment.

When examining the handful of studies that have assessed the long-term effects of chemotherapy compounds on neurogenesis, all of them argue that their data support the idea that chemotherapy compounds can cause long-term alterations in neurogenesis. Dietrich et al. (2006) and Han et al. (2008) both present data with statistically significant reductions in neurogenic proliferation detected at the longest time point of assessment (42 days in Dietrich et al., 2006 and 6 months in Han et al., 2008) for at least some of the chemotherapy compounds used. Furthermore, Mondie et al. (2010) found that chemotherapy treatment can induce multiple episodes of decreased cellular proliferation within the DG that can be detected up to 12 weeks post treatment, but appear to be transient. While these results are promising, they represent the only attempts within the

literature to address the long-term aspects of neurogenic proliferation alterations following chemotherapy treatment.

Investigation of long-term changes in neurogenic proliferation following chemotherapy treatment is needed to address gaps within the research literature. Thus, it remains to be determined if the effects of chemotherapy on neurogenesis could explain long-lasting CRCI within the human literature. Without further investigation it will be difficult to determine to what extent populations of neurogenic cells within the hippocampus may be vulnerable to various chemotherapeutic agents and to what degree any disruptions detected may persist long-term.

Timing Profiles of Neurogenesis Reductions Differ by Drug: Although long-term data regarding decreases in neurogenesis following chemotherapy treatment is scarce, there is limited evidence that timing profiles for these decreases may differ across chemotherapy compounds. For instance, Dietrich and colleagues (2006) examined the long-term neurogenic proliferation profiles of mice treated with cytarabine, BCNU, or cisplatin. Levels of BrdU labeled cells were assessed in the subventricular zone (SVZ) and the dentate gyrus of the hippocampus 1 or 42 days after treatment (cytarabine was tracked up to 56 days). Although mice treated with cytarabine exhibited significant decreases in proliferative cells within SVZ immediately following treatment, which was also detected 56 days after treatment, significant decreases within the DG were only detected on day 56. A somewhat similar pattern was seen with BCNU administration which led to immediate decreases in proliferative cells in the SVZ that were maintained at day 42, but decreases detected in the DG never reached statistical significance. In

contrast, cisplatin administration reduced proliferation immediately following treatment within both the SVZ and DG, but these reductions were not detected at day 42.

In a related study conducted by the same group, Han and colleagues (2008) found that mice treated with 5-fluorouracil exhibited a different timing profile. Results indicated that mice treated with 5-fluorouracil demonstrated both an immediate short-term deficit in cell proliferation within the SVZ, followed by a rebound to control levels at 7 and 14 days after treatment. Reductions in proliferation within the DG were detected on day 14, followed by a rebound to control levels on day 56. Interestingly, while neurogenesis level was found to return to baseline approximately 2 weeks after treatment in the SVZ and at day 56 in the DG, this recovery was transient, as reemergence of these deficits occurred with long-lasting suppression of proliferating cells detected in both the SVZ and the DG when animals were examined at 6 months post-treatment.

Finally, Mondie et al. (2010) conducted a study that examined the long-term effects of thiotepa on neurogenesis within mice. Animals were given BrdU 30 minutes before tissue collections done at weekly time points throughout the experiment including: weeks 0, 1, 2, 3, 4, 6, 12, and 30 following chemotherapy treatment. Result indicated an initial 50% decrease in BrdU positive cells within the DG immediately after treatment. This decrease was followed by a recovery in proliferation levels over the next three weeks. However, significant decreases remerged when examined on weeks 4, 6 and 12 post-chemotherapy treatment. In addition to these proliferative assays, Mondie et al. (2010) found that when animals received BrdU at the completion of chemotherapy treatment, and were assessed over the following 4 weeks, no labeled BrdU positive cells were detected past week 0. This suggests thiotepa may be capable of inducing decreases

in neuronal proliferation up to 12 weeks after treatment and drastically inhibits the survival of cells born during and immediately following chemotherapy treatment. Mondie et al. (2010) argues that natural age related reductions in neurogenesis may be responsible for a floor effect at 30 weeks making it difficult to detect any reductions in neurogenic proliferation due to chemotherapy treatment. Interestingly, this pattern of results may represent an accelerated aging effect, with chemotherapy treated animals demonstrating early, age-like reductions in neurogenesis that occur before control animals. Like other chemotherapy agents, these disruptions within the DG may represent a unique long-term profile.

Thus, chemotherapeutic agents can cause significant decreases in proliferation levels, and negatively impact the survivability and integration of newborn cells in the CNS. The literature discussed above suggest that chemotherapy drugs may not only cause decreases in hippocampal cellular proliferation, but that each chemotherapy compound may have a unique timing profile during which these induced alterations in neurogenesis occur. These unique profiles provide further support that subtle differences present within the current neurogenesis CRCI animal literature may be related to different underlying physiological mechanisms. Further research is required to understand to what degree each chemotherapy compound may disrupt neurogenesis over time in order to understand how these proliferative changes relate to and may be responsible for CRCI. Human imaging research has suggested that structural and functional changes within the brain occur at two distinct time periods, one early (months to 1-2 years) and another later (3 years or more) following chemotherapy treatment (McDonald et al., 2012; Kesler et al., 2009). It is unclear to what degree these detected difference may be caused by

chemotherapy induced neurogenic disruption. Identifying which chemotherapy compounds can produce long-term suppression of neurogenesis and the time course of these effects is essential. This information will elucidate to what degree neurogenesis mechanisms may relate to structural and functional changes detected within the brain following chemotherapy treatment.

Theoretical Impact of Neurogenesis on Cognition: Unfortunately, while there is evidence that various stages of neurogenesis can be negatively affected by a variety of chemotherapy compounds, the link between neurogenesis and its role in learning and memory is not well understood. The lack of clarity regarding the extent to which adult neurogenesis may contribute to learning and memory function makes it difficult to evaluate cognitively within CRCI models. However a variety of supported theories exist suggesting different ways in which hippocampal neurogenesis may be involved with learning and memory including: pattern separation, temporal encoding, and memory resolution (Aimone et al., 2014).

Briefly, pattern separation refers to the ability of neurons or networks of neurons to respond differently to different, yet very similar possible cortical input. Theoretically this ability would allow for the optimal formation of distinct memories even when events and information share a high degree of similarity. The large number and high density of the neurons within the dentate gyrus, relative to other medial temporal structures, make it uniquely suited for continual processing and storing information distinctly (Aimone et al., 2014).

According to theories regarding temporal encoding, ongoing proliferation of neurons within the dentate gyrus allows cortical networks within the structure to allocate different populations of newborn cells to different episodic events (Aimone et al., 2014). Depending on temporal proximity of experienced events, information will either be encoded by the same or different populations of young neurons. Thus, events that occur in close temporal proximity are related in long-term episodic memory while those events that occur later are encoded by a new set of cells. It is theorized that this process enhances the effectiveness of temporal separation for memory of events far apart in time and increases temporal integration for events that occur around the same time.

Theories regarding the role of the dentate gyrus and neurogenesis in memory resolution incorporate aspects of both pattern separation and temporal encoding. Physiological properties of dentate gyrus cells change as they mature, allowing more broadly tuned immature neurons to assist with pattern integration and more mature neurons to be tightly tuned and assist with pattern separation (Deng et al., 2010). As the dentate gyrus continually produces new sets of neurons, there is a constant supply of both young and newly matured cells that allow for a combination of sparse and distributed coding systems to act simultaneously. It is theorized that this feature allows the dentate gyrus to assist in high resolution memory formation, encoding detailed memory representations that link experiential information for what, where and when (Aimone et al., 2014). Interestingly, Frankland et al. (2013) suggests the same proprieties that may make the dentate gyrus important for memory resolution may make it important for processes involved with forgetting. Continuous neurogenesis within the dentate gyrus may represents a decay process that works to persistently degrade and clear memories

from the hippocampus further ensuring that pattern separation within memory traces is achieved.

Unfortunately the behavioral evidence regarding these various theories is complex and widely debated, making it difficult to provide a definitive answer regarding the role of hippocampal neurogenesis in learning and memory. Even if a clear link between hippocampal neurogenesis and learning and memory can be established, it does not necessarily mean that the cognitive deficits observed after chemotherapy treatment are caused by deficits in hippocampal neurogenesis. In fact, Evenden et al. (2013) suggests that focus on neurogenesis and hippocampal based tasks within the animal literature has begun to create a divergent rift between animal researchers and the human literature, where studies tend to emphasize frontal cortical deficits involving working memory in which neurogenesis has traditionally been viewed to have little suggested influence.

It is important to note, however, that there is evidence to suggest that while working memory and long-term memory systems are independent, when tasks traditionally thought of as short-term memory start to exceed working memory capacity or when rehearsal mechanism are inhibited, medial temporal lobe structures may be recruited (Shrager et al., 2008). This suggests that in situations in which there is an abundance of complex stimuli presented simultaneously, medial temporal lobe structures including the hippocampus may assist in memory processing and maintenance over short time intervals. If chemotherapy treatment has compromised the function of these systems by interfering with neurogenesis, it may lead to a failure of long-term memory systems in these "working memory" like situations.

When viewed as a whole, the literature concerning the impact of neurogenesis on cognition suggests that these new cells are likely important, especially in cognitively demanding situations. While these continually renewing neurons within the DG have been shown to be related to traditional hippocampal functions, the potentially complex role these cells play in cognition is still unclear. Given this nuanced role, it makes sense to confirm the cellular impact of chemotherapy compounds on neurogenic zones within the DG before attempting to quantify cognitive deficits that my result from these insults. Furthermore, establishing long-term temporal profiles of potential neurogenesis related decreases following chemotherapy treatment in animal models will provide critical evidence either supporting the theory that CRCI is associated with disrupted adult neurogenesis or that it is unlikely to be an important factor in cognitive declines detected in patient populations.

Potential Cellular Mechanisms

Neuroinflammation: One possible mechanism by which chemotherapeutic agents may induce cognitive deficits and damage throughout the CNS is through neuroinflammation. Inflammation begins as a defense mechanism initiated in part by microglial cells, which are considered to be the primary resident immune system cells of the CNS. Microglia actively monitor their external environment, and act quickly upon the detection of an insult to neutralize it and restore normal structure and function. Acute inflammation of nervous tissue is characterized by rapid activation of microglia, during which these cells quickly alter their genetic expression and morphology, and initiate and mediate the inflammatory response (Garden, 2013). This response includes the release of a variety of pro-inflammatory cytokines that include, but are not limited to: tumor necrosis factor

alpha (TNFα), Interlukin-1 (IL-1), and Interlukin-6 (IL-6). These cytokines act as chemical messengers that both trigger and organize the inflammatory response by recruiting other cells and pro-inflammatory processes to the site of damage. Typically these responses are tightly regulated, but if microglia remain chronically active, this can result in a self propagating and deleterious process marked by chronic inflammation and cytokine dysregulation within the CNS (Block & Hong, 2005; Ahles et al., 2007). Dysregulation of cytokine release can promote oxidative stress leading to an escalating cycle of damage with greater levels of neuroinflammation leading to higher levels of oxidative stress and vice versa (Wilson et al., 2002). Chemotherapy agents can cause cytokine release in the periphery that may subsequently induce inflammation, cytokine release and activation of microglia within the CNS, even in the absence of direct contact between chemotherapy agents and the CNS. Sentinel cells in the periphery such as macrophages, monocytes, dendritic cells, and Kupffer cells are sensitive to toxic substances and respond to potentially harmful agents by releasing pro-inflammatory cytokines (Wood et al., 2006). Significant communication between cytokines within and outside the CNS exists via transport across the blood brain barrier or through the vagus nerve (Ahles et al., 2007). The release of pro-inflammatory cytokines like IL-1β and TNFα rapidly respond and peak following an immune challenge, and are thought to support the mobilization of the immune response initiating a cascade of cytokine signaling. If these pro-inflammatory cytokines are transported across the BBB they can then quickly activate microglia within the CNS to mount an immune response. Another cytokine, IL-6, is an inflammatory-responsive cytokine that has both pro-inflammatory and anti-inflammatory properties (Wood et al., 2006). IL-6 production is triggered by

release of IL-1β and TNF-α, but subsequently down-regulates the activity of IL-1β and TNF-α once it accumulates causing an attenuation of the inflammatory response. Unlike IL-1β and TNF-α that peak rapidly, IL-6 peaks gradually, and can remain elevated for several hours following its release. IL-6 is particularly important within the CNS where it is thought to play a role at the hypothalamus, triggering increases in body temperature, and like other cytokines, may interact with P38 mitogen-activated protein kinase (p38-MAPK). This protein is thought to be responsive to environmental stressors, to induce fatigue and sickness-like behavior, alter cell differentiation, and induce apoptosis.

Wood et al. (2006) examined the ability of the chemotherapeutic agent etoposide to induce pro-inflammatory cytokine release in blood of mice. Results suggest that in addition to reduced voluntary wheel-running activity, mice exposed to etoposide showed significant increases in the level of IL-6 found in blood collected outside the CNS (Wood et al., 2006). In addition, follow-up in vitro application of etoposide to murine macrophages caused an increase in IL-6 gene expression, IL-6 protein release and activation of p38-MAPK within cultured media (Wood et al., 2006). Interestingly, pre-treatment of macrophages with the p38-MAPK inhibitor ML3403, completely blocked both p38-MAPK activation and increases in IL-6, suggesting that IL-6 cytokine production may be dependent on p38-MAPK activation. Other studies have suggested that increased p38-MAPK activity is required for some chemotherapeutic agents to induce cytotoxicity. For instance, Elsea et al. (2008) found that p38-MAPK blockade selectively diminished cytotoxicity associated with administration of etoposide, 5-fluorouracil, and doxorubicin, but not docetaxel, in murine macrophages.

Christie et al. (2012) demonstrated that while either cyclophosphamide or doxorubicin could induce behavioral deficits seen with rats assessed in both novel object recognition and contextual fear conditioning tasks, only animals treated with cyclophosphamide had increased levels of activated microglia (ED1-positive cells) throughout the hippocampus. This suggests that cyclophosphamide may induce behavioral deficits through microglia mediated neuroinflammatory mechanisms while doxorubicin may either not rely on an inflammatory mechanism or do so without overtly activating microglia. Furthermore, Borniger et al. (2017) found that the timing of changes in the expression of pro-inflammatory genes in mice administered a combination of cyclophosphamide and doxorubicin varied by tissue type. This suggests that inflammatory mechanisms activated following chemotherapy exposure may be under circadian influence and more toxic to certain tissues at particular circadian intervals.

In a related study, Briones and Woods (2014) examined the effects of cyclooxygenase (COX-2) inhibitor, NS-393, to block the inflammatory response in female rats treated with a combination of cyclophosphamide, methotrexate, and 5-fluorouracil given weekly for 4 weeks. COX-2 is a key enzyme responsible for the synthesis of prostaglandin E2, a ubiquitous central pro-inflammatory mediator. Injections of 10 mg/kg NS-393 were given to animals 1 hour after receiving the first chemotherapy treatment followed by daily injections for 28 days. Results indicated that rats treated with chemotherapy had elevated levels of pro-inflammatory cytokine IL-1β, TNFα, and COX-2. These differences persisted for 4 weeks after treatment. Rats treated simultaneously with the COX-2 inhibitor NS-393 showed attenuated chemotherapy induced neuroinflammation. Behavioral results from the same study show a similar

pattern of results with chemotherapy treated animals demonstrating impairment on two variants of the Y maze tests, one using temporal discrimination and the other using object placement, compared to saline controls and rats treated with NS-393 and chemotherapy. Together these studies suggest that chemotherapy compounds can induce increases in pro-inflammatory cytokines and microglia activity that persist up to 1 month after treatment. This neuroinflammation may be related to detected cognitive impairments.

Recent research suggests that neuroinflammation associated with chemotherapy treatment may induce both hippocampal and cortical deficits that are associated with both behavioral impairment and compromised neuronal architecture (e.g., reduced dendritic arborization and decrease spinogenesis) (Acharya et al., 2015). Acharya and colleagues further found that intrahippocampal transplantation of human neural stem cells can resolve both cognitive impairments, and cellular insult associated with chemotherapy treatment. The study found rats treated with cyclophosphamide produced behavioral deficits on a variety of object recognition tasks when tests were conducted between 5-7 weeks after completion of treatment. Tissue analysis revealed an approximate 2 fold increase in the number of activated microglia, indicated using a CD68 antibody marker, in the DG, CA1 and CA3 regions of the hippocampus. This neuroinflammation was not detected in saline treated controls, and was suppressed in animals grafted with human neural stem cells after completion of chemotherapy treatment. Significant decreases in neuronal architecture and ultrastructure measures in chemotherapy treated animals relative to controls were detected and included: dendritic complexity, total dendritic length, total dendritic volume, and spine density of neurons within the DG, CA1 and CA3 regions of the hippocampus. Chemotherapy induced decreases in all of these measures

were not detected in animals that received stem cell transplantation following chemotherapy; the authors suggest that the capability of grafted stem cells to protect animals from neurocognitive chemotherapy related insult may depend on the ability of grafted stem cells to attenuate activation of microglia, suppress cytokine signaling, and reduce neuroinflammation within the hippocampus (Acharya et al., 2015).

While neuroinflammation has become one of the more popular candidates of mechanisms leading to CRCI, there have been some inconsistencies within the literature regarding the ability of chemotherapeutic agents to induce neuroinflammation. For example, Seigers et al. (2010) examined the effects of methotrexate on neuroinflammation in rats. Results indicated that while microglia activation as measured by cell morphology was elevated in the hippocampus 3 weeks after administration, multiplex analysis of various cytokine levels within hippocampal tissue, assayed 5 days and 20 days after treatment, revealed no significant differences compared to controls. While it is not yet clear exactly how neuroinflammation may be linked to CRCI, it certainly represents a plausible mechanism by which proliferative cell populations and white matter tracts within the CNS may be damaged.

Oxidative Stress: Oxidative stress results from an imbalance between systemic creation of reactive oxygen species (ROS) and both endogenous and exogenous compounds and systems that detoxify and repair oxidative damage. Reactive oxygen species (ROS) are a natural byproduct of the normal metabolism of oxygen, in which they are mainly produced within the respiratory chain of mitochondria (Wang et al., 2006). If an imbalance between antioxidant mechanisms and ROS generation is created, elevated levels of ROSs can lead to mutations in mitochondrial DNA. These mutations can lead to

a cycle where mitochondrial DNA coded proteins have more errors, causing altered electron transfer. This can eventually lead to more ROS generation (Seigers et al. 2011). If the pace of ROS generation surpasses the body's ability to detoxify them, significant cell damage can occur including: DNA damage, lipid peroxidation, damage to proteins, and inactivation of certain enzymes. In particular, oxidative stress has been suggested to be one of the most common causes of DNA damage within neurons (Ahles et al., 2007). DNA damage within cells including neurons can lead to apoptosis.

Since most chemotherapeutic agents are designed to disrupt DNA, many believe that mitochondrial DNA may be a potential target of chemotherapy treatment induced ROS formation and contribute to increased levels of oxidative stress. According to Ahles et al. (2007), treatment with chemotherapeutic agents is associated with both increased levels of free radicals (not involving oxygen) and reduced antioxidant capacity. Both can contribute to increased oxidative stress and subsequent DNA damage. Cells within the CNS, including both neurons and oligodendrocytes may be particularly vulnerable to increased levels of oxidative stress as the brain consumes large amounts of energy and oxygen.

Potential Neuroprotectants

Understanding the neurological dysfunction and cellular mechanisms that underlie CRCI are current goals within the research literature. While basic research continues to be important, current research emphasis is also interested in translational efforts understanding and impacting patient's clinical outcome. Preventing and repairing damage caused by chemotherapy treatment has become a priority. As such, research into

compounds that can act as neuroprotectants throughout the duration, or potentially even after a course chemotherapy treatment, have become more common. The exact processes involved in CRCI have yet to be determined, but a variety of compounds have been suggested to be beneficial in reducing the negative effects associated with chemotherapy treatment. The following neuroprotectants may be effective in preventing or repairing damage caused by chemotherapy treatment and their efficacy or lack thereof may be informative when elucidating cellular mechanism underlying chemotherapy related damage.

Antidepressants: It is well established that long-term antidepressant treatment can up-regulate expression of brain BDNF and cAMP response element binding protein (CREB), inducing changes in both synaptic plasticity and neuronal adaptation (Nibuya et al., 1996; Thome et al., 2000). A variety of antidepressant treatments including: serotonin selective reuptake inhibitors (SSRI), norepinephrine selective reuptake inhibitors, monoamine oxidase inhibitors and induced electroconvulsive seizures are known to increases levels of neurogenesis within the adult hippocampus (Dranovsky & Hen, 2006; Duman et al., 2000, 2001; Malberg et al., 2000). In a study conducted by Wang et al. (2008), mice treated chronically with fluoxetine (an SSRI) for 28 days showed not only increases in hippocampal proliferation but increased rates of neuronal maturation and dendritic arborization following treatment. Wang et al. (2008) also examined the effects of chronic fluoxetine on the novelty-suppressed feeding test, which is a behavioral task that measures latency to begin eating as an index of antidepressant/anxiety-like behavior. Results revealed beneficial effects of chronic fluoxetine exposure that could be reversed with ablation of neurogenesis with x-irradiation. Results from several other studies

indicate that while chronic stress down-regulates genes important for proliferation and plasticity within the hippocampus, treatment with antidepressants can reverse these changes in gene expression suggesting a common mechanism (Alfonso et al., 2004; Dranovsky & Hen, 2006; Mahar et al., 2014). While the neurogenesis boosting effects of fluoxetine have been detected following at least 1 week of treatment, most reports indicate the strongest effects of fluoxetine on neurogenesis after 3-4 weeks of treatment (Malberg et al., 2000; Miller et al., 2008). Interestingly, the temporal delay (approximately a month) in the efficacy of antidepressants in the treatment of depression in humans and animal models of chronic stress seems to match the time course required for proliferating neurons to become functionally integrated (Ge et al.2007; Jacobs et al., 2000; Mahar et al., 2014). These converging lines of experimental evidence suggest that antidepressants alter and promote proliferative cell populations within the hippocampus.

The effect of antidepressants on proliferating cells is not limited to neurons within the hippocampus. In a study conducted by Czéh et al. (2007), chronic fluoxetine treatment was associated with an increased number of BrdU positive cells indicative of cellular proliferation in both the dentate gyrus of the hippocampus and the medial prefrontal cortex (mPFC). In addition to BrdU, phenotypic analysis was done using double immunofluorescence labeling with NeuN (a neuronal marker) or NG2 (a glial marker). Results indicated that a majority (70-77%) of BrdU positive cell within the DG expressed NeuN, while the majority (63-80%) of BrdU positive cell within the mPFC expressed NG2. These results suggest that chronic fluoxetine treatment can boost levels of both neurogenesis and gliogenesis. Interestingly, the detrimental effects of chronic social stress on both proliferation and survival of new neurons in the hippocampus and

new glial cells in the mPFC were reversed after 4 weeks of fluoxetine treatment. This study illustrates that fluoxetine not only boosts neurogenesis, but can boost gliogenesis as well.

There is evidence that antidepressants may have neuroprotectant properties within animal models of chemotherapy related cognitive impairment. For instance, ELbeltagy and colleagues (2010) found that rats treated with 5-fluorouracil (5-FU) injections over two weeks exhibited impaired performance on a novel location recognition task (1 day after treatment) and had fewer Ki-67 positive cells in the dentate gyrus compared to controls (3 days after treatment). In contrast, animals that received 5-FU while also receiving a dose of 10 mg/kg/day fluoxetine via drinking water over three weeks, showed no significant impairment on the novel location task and no significant decrease in neurogenesis compared to controls.

Similar results were detected by Lyons et al. (2011). They found a beneficial effect of 10 mg/kg/day fluoxetine pre-treatment in rats treated with methotrexate. While animals treated with methotrexate in the absence of fluoxetine showed impaired performance on a novel location recognition task and exhibited decreases in both cell survival and proliferation within the dentate gyrus when examined at approximately a week following treatment. Animals that received fluoxetine were protected from chemotherapy insult in both behavioral and cellular measures. These studies suggest that antidepressants may be an effective neuroprotectant which may prevent short-term decreases in neurogenesis associated with chemotherapy treatment. The timing and long-term capacity of antidepressants to produce and maintain these beneficial neurogenesis related effects remains unknown and represent an opportunity for further research.

Antioxidants: The generation of reactive oxygen species is a natural byproduct of normal respiration, but if left uncontrolled these reactive molecules can cause damage to cells within the CNS by damaging proteins, lipids, mitochondria, and DNA. It is thought that during periods of oxidative stress, and progressively over time, ROSs may build up and overwhelm homeostatic endogenous antioxidant defenses within the CNS, causing damage to both neurons and glial cells (Andersen, 2004; Dröge & Schipper, 2007). The 'free-radical theory of aging' (Harman, 1992) hypothesizes that the aging brain and body are susceptible to increasing levels of oxidative stress and that antioxidant supplementation may increase both lifespan and lead to better functional outcomes. While the details regarding the exact role oxidative stress may play in aging is still debated, it is fairly well established that antioxidants can have positive effects on the cognitive outcomes associated with age and age-related diseases. In a cross-sectional, prospective study of dementia, Zandi et al. (2004) found that antioxidant and vitamin supplements were associated with reduced prevalence and incidence of Alzheimer disease (AD) in their sample of 4740 elderly (65 years or older) residents of Cache county, Utah. Krikorian et al. (2010) found similar results when they evaluated the effects of consuming concord grape juice, known to contain high levels of antioxidants, in older adults for 12 weeks. Results from the California Verbal Learning Test indicated that individuals that had consumed grape juice had significantly higher rates of item acquisition compared to those consuming the placebo, indicative of better verbal memory. Similar results have also been reported in animal models where the effects of foods rich in antioxidants such as blueberries (Joseph et al.1999), strawberries (Joseph et al.1998), concord grapes (Shukitt-Hale et al., 2006), and red wine (Anekonda, 2006) have

been shown to have both preventative and restorative effects on age-related cognitive and motor dysfunction. Together these studies suggest that antioxidant supplementation may be a useful tool in combating oxidative stress mechanisms that lead to cognitive impairments.

Melatonin: Melatonin is an endogenously produce indoleamine that is known to play a significant role in circadian rhythms and synchronicity (Kennaway & Wright, 2002; Reiter, 1993). Melatonin and its metabolites are direct free radical scavengers (Reiter, 2000; Tan et al., 2002; Hardeland, 2005), known to stimulate antioxidative enzymes (Rodriguez et al., 2004; Barlow-Waldon et al. 1995), chelate transition metals which normally participate in redox reactions (Limson et al., 1998) and reduce free radical formation (Hardeldand et al., 2005; Leon et al. 2004; López et al., 2009). Melatonin is able to enter both lipid and aqueous environments, unlike other antioxidants such as vitamins C and E, which allow for increased free radical scavenger efficiency (Reiter et al., 2009). Melatonin is also known to reduce toxicity, ototoxicity and tinnitus due to the use of aminoglycosides and cisplatin without causing attenuation of functional efficiency of either drug (for review see Reiter et al., 2011). Melatonin has also been demonstrated to promote survival and dendritic maturation in adult neurogenesis (Ramirez-Rodriguze et al., 2011), to effectively protect developing oligodendrocytes following white matter damage in neonatal rats (Olivier et al., 2009; Villapol et al., 2011), and can reduce glia-mediated inflammatory responses (Wu et al., 2011). Together these properties make melatonin an ideal neuroprotectant compound capable of supporting a wide range of positive reparative and protective responses to CNS insult.

The primary goals of research efforts regarding chemo-brain are to identify the source of CRCI and ultimately to minimize and possibly reverse these negative side effects. Each of the neuroprotectants discussed above provide a possible method for protecting against damage caused by chemotherapy treatment. Furthermore, it is possible that these compounds may even assist cellular repair mechanisms following chemotherapy insult, accelerating recovery, and lead to better clinical outcomes for cancer survivors. By investigating the effects of these neuroprotectant compounds on cognitive and cellular models of CRCI, research based achievements can begin to translate into clinical applications.

Current Study Justification

Summarizing the research literature discussed, there is clear evidence that a variety of chemotherapeutic compounds negatively impact proliferative cell populations within the CNS. In addition, limited data suggests that white matter tracts may also be vulnerable to chemotherapy treatment. While several studies suggest that chemotherapy compounds are toxic to cells within the CNS and disrupt neurogenic processes within the hippocampus following treatment, the mechanisms and time course of these processes are not well understood. Most of the studies conducted to date have focused on the effects of chemotherapy within the CNS during and shortly after treatment. Only 3 studies have examined the long-term effects (greater than 1 month post-treatment) of chemotherapy on hippocampal proliferation (Dietrich et al., 2006; Han et al., 2008; Mondie et al., 2010).

Given that laboratory mice typically have an average life-span of 600 days (Festing, 1998), deficits detected 30 days post chemotherapy treatment should not be

considered long-term or permanent without subsequent extended testing. Thus, the bulk of the current animal literature, in which decreases in neurogenesis have been detected shortly after treatment, is unable to elucidate reports of long-term cognitive decline in patients that are detected up to 10 years after treatment (Raffa & Tallarida, 2010).

The current study attempts to expand upon knowledge within the current literature regarding the time course of CRCI. By evaluating both immediate and delayed effects of chemotherapy treatment on proliferative processes within the CNS, the current study may elucidate to what degree disrupted hippocampal neurogenesis may account for CRCI detected in patient populations. The long-term nature of this study provides a better understanding of the initial deficits, any recovery periods, and possible persistent problems in neurogenesis capacity that may appear following chemotherapy treatment.

Finally, the current study attempted to evaluated the effectiveness and establish long-term profiles of two different neuroprotectants: the antidepressant fluoxetine and the neurohormone melatonin. Evaluating the effectiveness of each of these compounds to prevent and possibly restore normal structure and function within the CNS following chemotherapy treatment expands the understanding of potential clinical interventions.

METHODS AND RESULTS

Experiment 1

Aim of Experiment 1

Several studies have indicated that chemotherapy compounds can negatively impact processes involved in neurogenesis and inhibit proliferating cells within the hippocampus a few weeks following treatment (Dietrich et al., 2006; Han et al., 2008; Mustafa et al., 2008; Seigers et al., 2008; 2009; Janelsins et al., 2010; Mondie et al., 2010; Yang et al., 2010; Briones et al., 2011; Lyons et al., 2011; Christie et al., 2012; Yang et al., 2012). It is unclear however, whether or not these effects persist at later time points and to what degree administration of different classes of chemotherapeutics differ in their long-term capacity to impact neurogenesis.

Results obtained by Janelsins et al. (2010) and Chrisite et al. (2012) have indicated that both cyclophosphamide and doxorubicin administration can disrupt neurogenesis shortly after treatment (2 days to 4 weeks). However, it is currently unknown whether these effects represent long-term, persistent decreases in neurogenic potential and to what degree these two chemotherapy agents may produce unique timing profiles with regard to decreases in neurogenic proliferation. To evaluate the potential long-term effects and establish long-term timing profiles for these compounds, the present study examined neurogenesis 1 day, 56 days, and 6 months after treatment to monitor for long-term changes in proliferation rates similar to those detected with BCNU, cisplatin, 5-FU and thiotepa (Dietrich et al., 2006; Han et al., 2008; Mondie et al., 2010).

The long-term design chosen for this experiment allowed for assessment of neurogenesis utilizing the endogenous proliferative marker Ki-67. Comparisons of potential early and late effects of chemotherapy treatment on cellular proliferation within the DG of the hippocampus were assessed. Given that Dietrich et al. (2006) has already demonstrated that chemotherapy compounds can vary with regard to their capacity to disrupt neurogenesis over time, the goal of Experiment 1 was to determine the degree to which cyclophosphamide and doxorubicin may affect neurogenesis rates and the timing profiles of these effects.

Materials and Methods Experiment 1:

Subjects: 62 C57BL/6J male 10-11 week old mice were used for the study. Mice arrived from Jackson labs and were acclimated to our animal colony for approximately 2 weeks before treatment began. Animals were housed socially (3-4 mice per cage) in a temperature (~70°F) and light/dark controlled (7:00am – 7:00pm) environment with food and water ad libitium. Animals were randomly assigned and counterbalanced for initial weight across 3 treatment conditions: saline controls, cyclophosphamide, or doxorubicin.

Figure 1: Time-line for Experiment 1

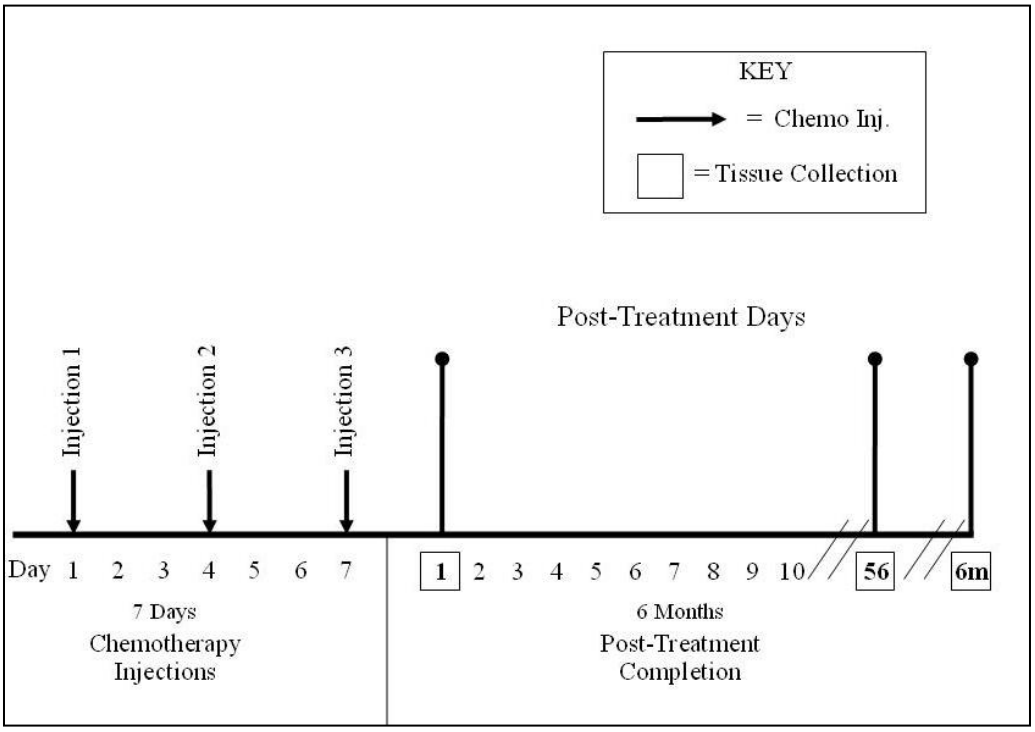

Figure 1: Animals received injections on days 1, 4, and 7 with saline (0.9% NaCl). cyclophosphamide (120mg/kg) or doxorubicin (5mg/kg). Animals were perfused 1 day, 56 days or 6 Months after the last injection.

Chemotherapy Treatment: Mice in each group received intraperitoneal (i.p.) injections over a period of 7 days (time-line shown in Figure 1). Cyclophosphamide (120mg/kg diluted in 0.9% saline; Acros Organics), doxorubicin (5mg/kg diluted in 0.9% saline; Fisher Scientific), or saline (0.9% NaCl) injections were administered on days 1, 4, and 7, for a total of 3 injections per animal, as detailed in Janelsins et al. (2010). However, the injection dose used for cyclophosphamide in this study is higher than the 50mg/kg dose used in Janelsins et al. (2010). This higher dose was used in an attempt to maximize any long-term neurobiological effects that might be detected. Because toxicity and tolerance to chemotherapeutic compounds can vary drastically as a function of circadian rhythm (Focan, 1995), all mice were treated at approximately the same time, 8-9 hours after light

onset. Weight was monitored daily for approximately 4 weeks, then every other day until 56 days post-treatment. Thereafter, mice were weighed weekly. In addition to weight, teeth were monitored regularly and trimmed when necessary in order to prevent overgrowth which can lead to weight loss.

Tissue Collection: Animals were sacrificed and transcardially perfused with 0.9% saline followed by 4% paraformeldehyde (PFA) at 1 day (6 saline controls, 6 cyclophosphamide, and 6 doxorubicin), 56 days (6 saline controls, 6 cyclophosphamide, and 3 doxorubicin) and 6 months (7 saline controls, 7 cyclophosphamide, and 4 doxorubicin) following the last chemotherapy injection. Brains were extracted and submerged in PFA. Brains were then moved to a 30% sucrose solution and allowed to sink prior to being flash frozen. Six, approximately identical series of 40 µm coronal sections were cut into a 0.05% potassium phosphate buffered saline solution (KPBS), moved to an antifreeze cryoprotectant, and stored at -20°F until they were needed for immunohistochemistry.

Immunohistochemical Staining: An aviden/biotin-based peroxidase Vectastain Elite ABC Kit (Rabbit IgG) system (Vector Laboratories, Inc.) was used to perform high sensitivity, low background immunohistochemical staining on prepared tissue. For detection of Ki-67 proteins, Ki-67 rabbit primary antibodies (Wako Pure Chemical Industries, Ltd.) were used in conjunction with the Elite ABC kit. A titration series was conducted to determine the optimal Ki-67 antibody concentration to use with our prepared tissue samples in accordance with the recommended immunohistochemical staining method suggested by Hoffman et al. (2008). For Ki-67 immunohistochemical staining, a single series of tissue for each animal was selected that encompassed the entire

hippocampus (10 sections of tissue for each animal) for staining. After the tissue was selected, it was initially rinsed in 0.05% KPBS 6 times for 10 minutes each to wash the cryogenically preserved tissue after which the tissue was incubated in a 1% hydrogen peroxide 0.05% KPBS solution for 15 minutes. Following this incubation, the tissue was rinsed again in 0.05% KPBS 4 times for 5 minutes each, and then incubated in a (1:4,000) Ki-67 rabbit primary antibody diluted in 0.05% KPBS/0.4% Triton X-100 solution for 1 hour at room temperature. The tissue was then stored in the refrigerator at approximately 35°F for 48 hours. Following this incubation period, the tissue was thoroughly rinsed in 0.05% KPBS 10 times for 6 minutes each, and then incubated in a (1:500) biotinylated, affinity-purified anti-immunoglobulin secondary antibody (Vector Laboratories, Inc.) diluted in 0.05% KPBS/0.4% Triton X-100 solution at room temperature for 1 hour. Following another series of 5, 10 minute rinses in 0.05% KPBS the tissue was incubated in an avidin biotinylated enzyme complex (ABC) diluted in 0.05% KPBS/0.4% Triton X-100 solution at room temperature for 1 hour. Following this incubation, the tissue was rinsed 3 times for 5 minutes each in 0.05% KPBS and then rinsed 3 times for 5 minutes each in 0.175% sodium acetate. The tissue then went through incubation in a nickel enhanced DAB chromogen solution (2.5% Ni + 2% DAB) for 25 minutes. After this final incubation, the tissue was rinsed 3 times for 5 minutes each in 0.175% sodium acetate to stop the chemical reaction, followed by two final rinses in 0.05% KPBS for 10 minutes each. Stained tissue slices were then floated in .0125% KPBS and mounted on microscope slides and air-dried for at least 24 hours. Dried slides were dehydrated using graduated EtOH solutions (70-100%) and cleared with HistoClear (National Diagnostics Inc.). Prepared slides were then coverslipped using HistoMount

(National Diagnostics Inc.) and allowed to dry for 24 hours. Bright-field images were taken using a Nikon Eclipse E200 microscope equipped with an Accu-Scope Excelis HD camera (See Appendix A for an example of captured tissue images).

Tissue Analysis: Following standard unbiased stereological estimation protocols as outlined in (Noori & Fornal, 2011), the number of Ki-67 positive cells within 10 slices of the DG of the hippocampus of each animal were counted by eye under 400X magnification by a blind rater. These counts provided an indication of the number of actively proliferating cells at the time of sacrifice.

Results Experiment 1

Animal Deaths

Doxorubicin induced toxicity resulted in several animal deaths prior to assigned perfusion dates in Experiment 1: 4 deaths occurred in the day 56 group and 7 deaths occurred in the 6 month group (often these animals were euthanized following our animal care and use protocol). No deaths occurred within the day 1 group or in any of the animals injected with cyclophosphamide. Any data collected for animals that died prior to perfusion were excluded from all analyses, including weight loss analyses. (See Appendix B for mortality data regarding Experiment 1)

Neurogenesis Analysis Experiment 1

Figure 2 shows the average number of Ki-67 positive cells detected in all 10 tissue slices of the hippocampus for Experiment 1 across all three collection time points (day 1, day 56, and 6 months). As shown in Figure 2, younger animals perfused at day 1

had higher rates of cellular proliferation compared to older animals perfused at 6 months regardless of treatment condition. While not statistically significant, this trend is also seen when comparing animals perfused at day 1 to those perfused at day 56 and those perfused at day 56 to those perfused at 6 months. A 3 x 3 factorial ANOVA [Treatment × Day] indicated that there was a significant main effect of day, ($F_{2, 42} = 5.10$; $p = .01$, $\eta_p^2 = .20$), with no other significant effects detected. Post hoc analysis (Tukey HSD) indicated that animals perfused at day 1 had significantly more Ki-67 positive cells than those animals perfused at 6 months ($p < .01$). No other pairwise comparisons were significant (*p's* >.20). These results indicate that neither cyclophosphamide nor doxorubicin administration lead to any significant differences in proliferation rates within the DG compared to saline injected animals. Given prior evidence that both cyclophosphamide and doxorubicin are capable of reducing cellular proliferation within the DG of treated animals shortly after treatment, exploratory one-way ANOVA's were conducted at each time point and confirmed there were no statistically significant differences in the number of Ki-67 positive cells across treatment conditions at any time point (p's > .29).

Figure 2: Experiment 1: Tissue Analysis

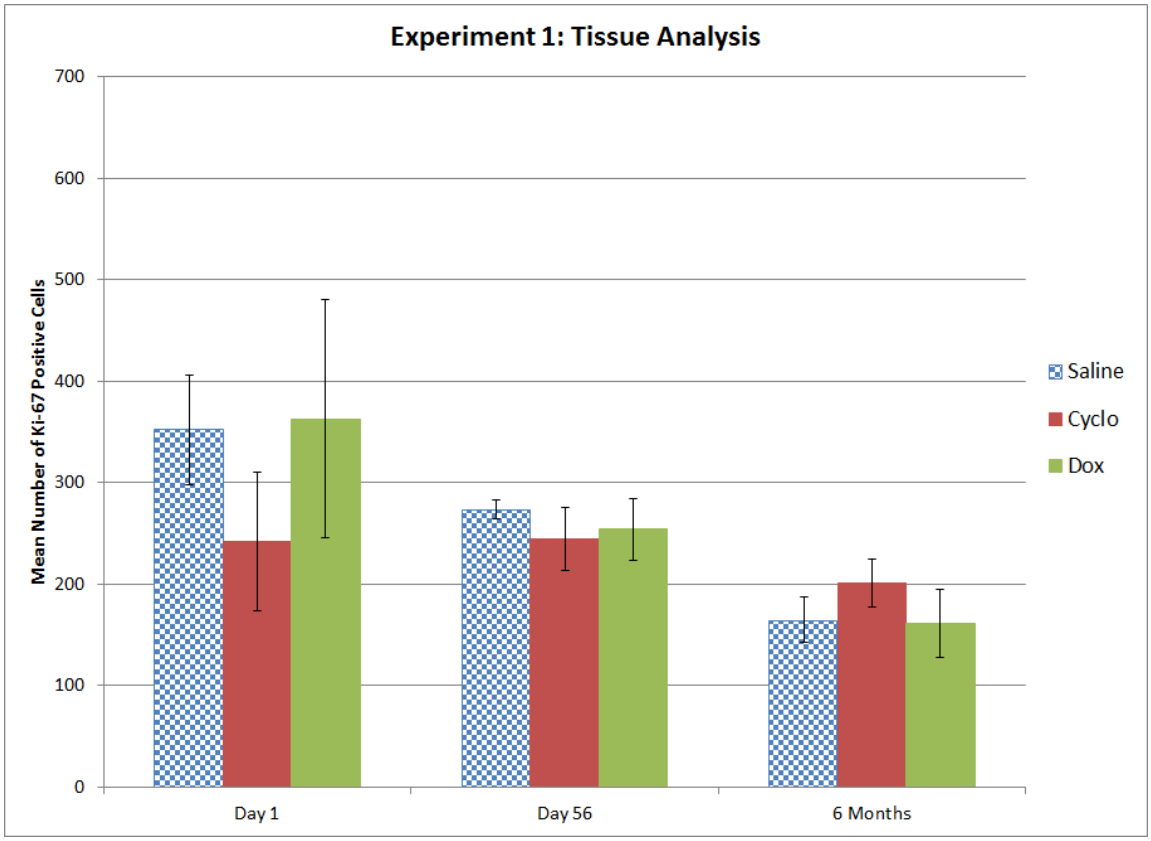

Figure 2: Mean number of Ki-67 positive cells detected in tissue collections at day 1, day 56 and 6 month, for animals treated with saline, cyclophosphamide, and doxorubicin. Error bars represent ±1 standard error of the mean. A factorial ANOVA indicated a main effect of day, (p = .01) with post-hoc analysis (Tukey HSD) indicating animals perfused at day 1 had significantly more Ki-67 positive cells than those animals perfused at 6 months (p < .01). No significant treatment effect was detected.

Weight Data Experiment 1

Weight Loss Analysis: Previous data collected within my lab (Fremouw et al., 2012a) and others (Janelsins et al., 2010; Seigers et al., 2008) suggests that a variety of chemotherapy compounds reliably induce weight loss in rodents during and a few days after injections have ceased. Given the lack of any detectable treatment effect on neurogenesis, weight loss analysis was conducted to provide evidence that the chemotherapy doses and the injection protocol used produced expected toxicity levels.

To consistently assess weight loss relative to pre-treatment weight within the experiment, two-factor mixed-design ANOVAs were used across the injection protocol (from the day following the first injection to the first day following the last injection) for each batch of animals. While longer time intervals could have been used with animals perfused at day 56 and 6 months, the shorter window allowed for consistency in analysis and mapped on to weight loss data in previous studies. In addition, one way ANOVAs and follow-up post hoc analysis with Tukey HSD were run on weight data collected on day 1, following the end of the injection protocol, to confirm expected weight differences.

Weights Day 1: Figure 3, depicts percent weight (relative to pre-treatment weight) as a function of day, starting from the day of the first injection to the day after the last injection, for the animals that were perfused on day 1. As shown in Figure 3, animals treated with either cyclophosphamide or doxorubicin lost weight during the 7 day injection protocol, while control animals receiving saline gained weight. A two-factor mixed-design ANOVA [Treatment × Day] was conducted to analyze weight differences between the treatment groups for the first 7 days of the experiment (starting from the day following the first injection to the first day following the last injection). There was a significant main effect of treatment, ($F_{2, 15} = 21.13$; $p < .0001$, $\eta_p^2 = .74$) and a significant treatment by day interaction ($F_{4.3, 32.5} = 6.77$; $p < .001$, $\eta_p^2 = .48$), Greenhouse-Geisser corrected). A one-way ANOVA confirmed that weight change differed by the end of the injection protocol ($F_{2, 15} = 15.67$; $p < .0001$, $\eta_p^2 = .68$). Post hoc analysis (Tukey HSD) indicated that the saline treated group had lost less weight than cyclophosphamide or doxorubicin treated groups (*p's* < .001).

Figure 3: Experiment 1: Weight Data - Day 1

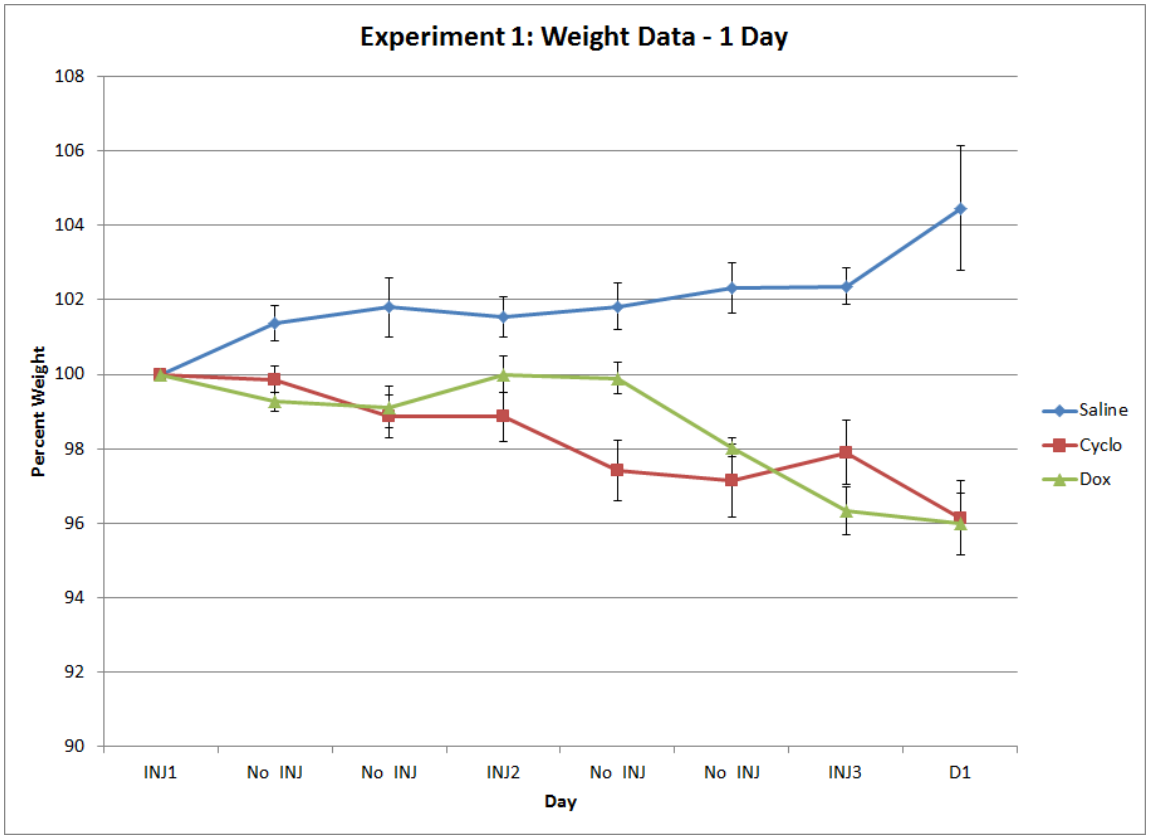

Figure 3: Mean % body weight (relative to pre-treatment weight) as a function of day for animals perfused on day 1. Mice received IP injections of either saline (0.9% NaCl; n = 6), cyclophosphamide (120 mg/kg; n = 6), or doxorubicin (5 mg/kg; n = 6), on days 1, 4, and 7 (INJ1, INJ2 and INJ3). Error bars represent ±1 standard error of the mean. A two-factor mixed-design ANOVA indicated a significant main effect of treatment ($p < .0001$), and treatment by day interaction ($p < .001$).

Weights Day 56: Figure 4 depicts percent weight as a function of day, starting from the day of the first injection to the day after the last injection for the animals that were perfused on day 56. As shown in Figure 4, animals treated with either cyclophosphamide or doxorubicin lost weight during the injection protocol while control animals appear to have maintained weight. A two-factor mixed-design ANOVA [Treatment × Day] was conducted to analyze weight differences between the treatment groups for the first 7 days of the experiment (starting from the day following the first injection to the first day

following the last injection). There was a significant main effect of treatment, ($F_{2, 12}$ = 13.58; $p < .001$, $\eta_p^2 = .69$), a significant main effect of day, ($F_{2.7, 32.7} = 18.16$; $p < .0001$, $\eta_p^2 = .60$, Greenhouse-Geisser corrected), and a significant treatment by day interaction, ($F_{5.5, 32.7} = 10.64$; $p < .0001$, $\eta_p^2 = .64$, Greenhouse-Geisser corrected). Thus, the animals perfused at day 56 followed a similar pattern of weight loss to those animals perfused at day 1; the saline group maintained or increased weight over the first 7 days of the experiment while animals injected with cyclophosphamide or doxorubicin lost weight. A one-way ANOVA confirmed that weight change differed by the end of the injection protocol ($F_{2, 12} = 20.05$; $p = .0001$, $\eta_p^2 = .77$). Post hoc analysis (Tukey HSD) indicated that the saline treated group had lost less weight than cyclophosphamide or doxorubicin treated groups (*p's* < .001).

Figure 4: Experiment 1: Weight Data - Day 56

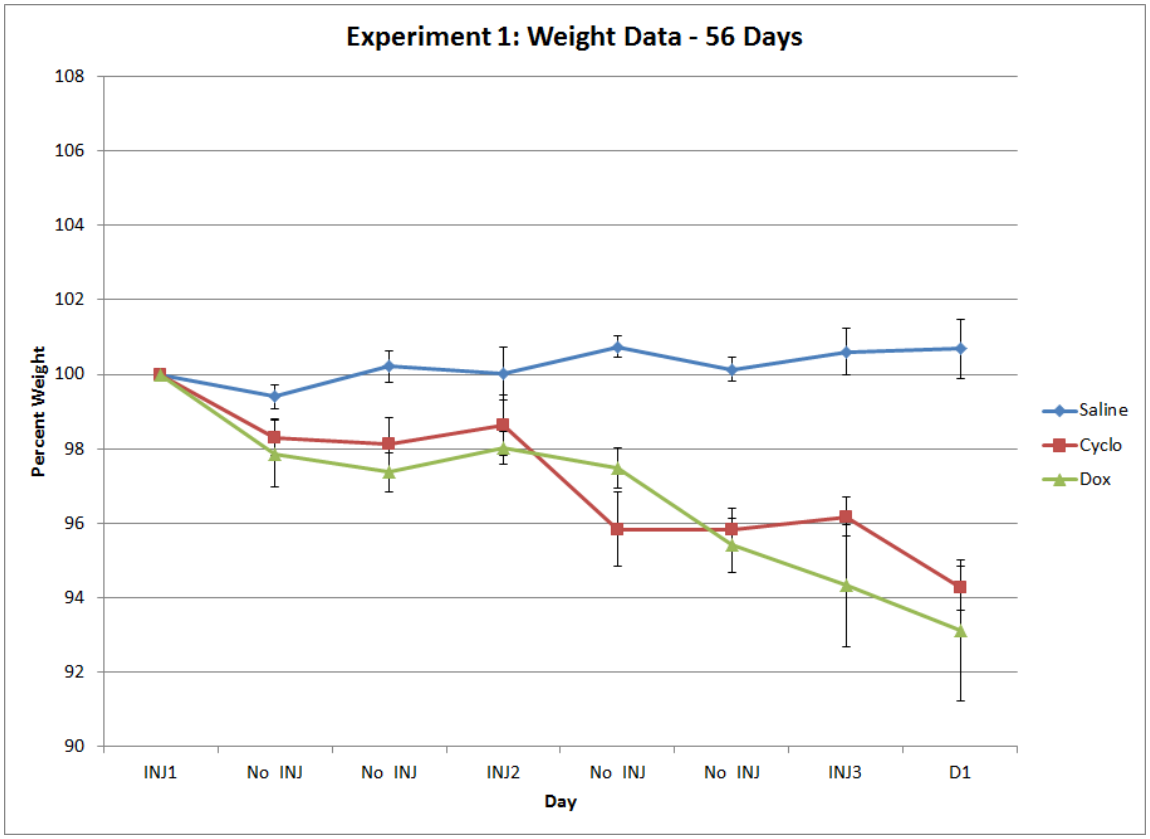

Figure 4: Mean % body weight (relative to pre-treatment weight) as a function of day for animals perfused on day 56. Mice received IP injections of either saline (0.9% NaCl; n = 6), cyclophosphamide (120 mg/kg; n = 6), or doxorubicin (5 mg/kg; n = 3), on days 1, 4, and 7 (INJ1, INJ2 and INJ3). Error bars represent ±1 standard error of the mean. A two-factor mixed-design ANOVA for the first 7 days of the experiment, indicated a main effect of treatment ($p < .001$), a main effect of day ($p < .0001$), and a treatment by day interaction ($p < .0001$).

Animals perfused on day 56 that were injected with either cyclophosphamide or doxorubicin lost weight until approximately 2 days after the last injection, at which point the cyclophosphamide injected animals began to regain weight while the doxorubicin injected animals' weight loss plateaued (see Appendix C for a figure of the extended weight loss data).

Weights 6 Month: Figure 5 depicts percent weight as a function of day, starting from the day of the first injection to the day after the last injection, for the animals that were perfused at 6 months. A similar pattern of weight change was detected. A two-factor mixed-design ANOVA [Treatment × Day] was conducted to analyze weight change between the groups over the first 7 days of the experiment (starting from the day following the first injection to the first day following the last injection). As with the 56 day animals, both main effects of treatment, ($F_{2, 15} = 25.56$; $p < .0001$, $\eta_p^2 = .77$), and day, ($F_{2.7, 40.4} = 9.14$; $p = .0001$, $\eta_p^2 = .38$, Greenhouse-Geisser corrected), were detected. There was also a significant treatment by day interaction, ($F_{5.4, 40.4} = 6.87$; $p < .0001$, $\eta_p^2 = .48$, Greenhouse-Geisser corrected). Following a pattern similar to those animals perfused on day 1 and 56, the weight of saline injected animals perfused at 6 months tended to increase slightly over the first 7 days of the experiment while both the cyclophosphamide and doxorubicin injected animals lost weight. A one-way ANOVA confirmed that weight change differed by the end of the injection protocol ($F_{2, 15} = 16.13$; $p = .0001$, $\eta_p^2 = .68$). Post hoc analysis (Tukey HSD) indicated that the saline treated group had lost less weight than cyclophosphamide or doxorubicin treated groups (*p's* < .03).

Figure 5: Experiment 1: Weight Data - 6 Months

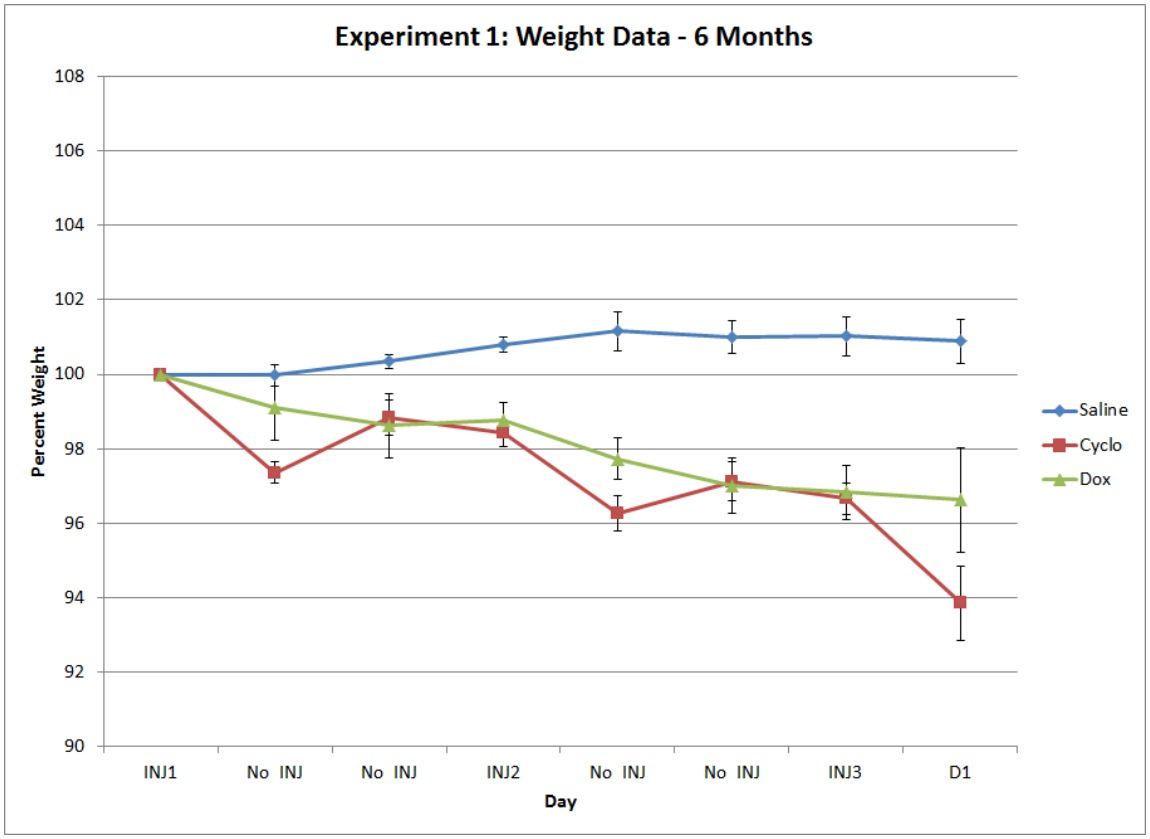

Figure 5: Mean % body weight (relative to pre-treatment weight) as a function of day for animals perfused at 6 months. Mice received IP injections of either saline (0.9% NaCl; n = 7), cyclophosphamide (120 mg/kg; n = 7), or doxorubicin (5 mg/kg; n = 4), on days 1, 4, and 7 (INJ1, INJ2 and INJ3). Error bars represent ±1 standard error of the mean. A two-factor mixed-design ANOVA for the first 7 days of the experiment indicated a main effect of treatment, a main effect of day, and a treatment by day interaction (all *p*'s < .0001).

Animals perfused at 6 months followed the same general pattern of weight loss after the injection protocol that was detected in animals perfused on day 56. Animals that were injected with either cyclophosphamide or doxorubicin lost weight until approximately 2 days after the last injection, at which point, the cyclophosphamide injected animals began to regain weight while the doxorubicin injected animals' weight loss plateaued (see Appendix D for a figure of the extended weight loss data). The weight data, as a whole, suggest the most significant weight loss occurs within the first

few days following the last injection of cyclophosphamide or doxorubicin which is consistent with previous data collected in my lab (Fremouw, et al., 2012a).

Discussion Experiment 1

Treatment with cyclophosphamide or doxorubicin has been shown to reduce levels of proliferating cells within the DG of mice immediately following treatment (Yang et al. 2010; Janelsins et al., 2010; 1 or 2 days after the last chemotherapy injection respectively). In contrast to these previous studies, the results of the present study found no significant treatment effect at any of the assessment time points, including tissue collected at 1 day after the last chemotherapy injection.

As previously discussed, the current research literature suggests that each chemotherapeutic compound may have a unique temporal profile regarding the capacity to reduce neurogenic proliferation. For instance, Dietrich et al. (2006) detected deficits in neurogenic proliferation within the DG of mice 56 days after cytarabine administration, while animals treated with BCNU showed no treatment effects within the DG at this same time point. Given the present study's results, cyclophosphamide and doxorubicin appear to have no effects on proliferation rates within the DG of mice at any of the time intervals examined.

It is unclear why cyclophosphamide or doxorubicin treatment failed to reduce proliferation in tissue collected at day 1. The high level of variability in Ki-67 positive cells detected in tissue collected at day 1 makes comparison between treatment groups difficult to interpret and may have obscured any possible treatment effect at this time point. The discrepancies between the current study's results and previous studies may

suggest that reductions in proliferation rates within the DG shortly following treatment with these compounds may not be as consistent or robust to experimental parameters as previously thought. For instance, while many of the experimental parameters in the present study were the same as those used by Janelsins et al. 2010, (e.g. mouse strain and injection schedule) it appears that subtle differences in a variety of factors including: age (9 weeks vs. 13 weeks), dose (50 mg/kg vs. 120 mg/kg cyclophosphamide), and assessment timing (2 day vs. 1 day after the last injection), may have contributed to the different findings despite the similarities across the studies.

A recent study conducted by Seigers et al. (2016) provides experimental evidence that supports the present study's findings. Seigers et al. (2016) was unable to detect any reductions in proliferation within the DG of C57BL/6J mice treated with either a single injection of cyclophosphamide (150 mg/kg) or doxorubicin (5 mg/kg), when assessed utilizing Ki-67 for neurogenic proliferation at either 3 weeks or 16 weeks following treatment.

In summary, Experiment 1 showed that neither cyclophosphamide nor doxorubicin caused a decrease in proliferation rates at any of the three time points within the study. While unexpected, the lack of any deficits detected at day 1 is not completely inconsistent with the current literature which highlights the degree to which reported neurogenesis deficits may differ across studies. The results of tissue analysis at the later time points suggest that neither cyclophosphamide nor doxorubicin treatment cause a significant long-term disruption in proliferation rates within C57BL/6J mice.

Experiment 2

Aim of Experiment 2

The aim of Experiment 2 was to build upon the results obtained by Han and colleagues (2008) who examined the effect of 5-FU administration on neurogenesis in mice. While Han et al. (2008) found decreases in neurogenesis within the SVZ at 1 day, 7 days, 56 days, and 6 months after 5-FU administration, decreases detected within the DG only reached significance at 14 days and 6 months after treatment. In addition to these results, Janelsins et al. (2010) has detected deficits in neurogenesis 2 days after treatment with 5-FU. The current study was designed to replicate these findings by examining neurogenesis levels in mice 1 day, 56 days and 6 months following 5-FU treatment. In addition, these same time points were used to evaluate the effectiveness of two different neuroprotectant compounds, fluoxetine and melatonin, to prevent, protect, and/or repair deficits in neurogenesis following chemotherapy treatment.

The long-term nature of this study provides a timing profile for decreases in neurogenesis following treatment with 5-FU and information regarding the effect of either melatonin or fluoxetine to alter levels of neurogenesis when co-administered with 5-FU. These neuroprotectans were chosen based on prior research indicating that both melatonin (Ramirez-Rodriguze et al., 2011) and fluoxetine can boost neurogenesis levels (Duman et al., 2000, 2001; Malberg et al., 2000; Dranovsky & Hen, 2006; Czéh et al. 2007). Furthermore, animal models of CRCI have already demonstrated that fluoxetine can be used to prevent or restore decreases in neurogenesis following 5-FU (ELBeltagy et al., 2010) or methotrexate treatment (Lyons et al., 2011), when neurogenesis is assessed within 1-2 weeks after treatment. In addition, melatonin is a potent antioxidant that has

been shown to reduce oxidative stress in addition to its proliferative boosting effects (Reiter et al., 2000; Tan et al., 2002; Hardeland, 2005; López et al., 2009). Given these established benefits, the effectiveness of these neuroprotectants to alter the impact of 5-FU treatment on neurogenesis was examined.

Materials and Methods Experiment 2:

Subjects: 83 C57BL/6J male 10-11 week old mice were used for the study. Mice arrived from Jackson labs and were acclimated to our animal colony for approximately 2 weeks before treatment began. Animals are housed socially (3-4 mice per cage) in a temperature (~70°F) and light/dark controlled (7:00am – 7:00pm) environment with food and water ad libitium. Animals were randomly assigned and counterbalanced for initial weight across 4 treatment conditions: saline controls, 5-FU and saline, 5-FU and melatonin, or 5-FU and fluoxetine. Due to the administration of fluoxetine and melatonin through drinking water, animals were housed by group, to ensure they were receiving only their assigned treatment.

Figure 6: Time-line for Experiment 2

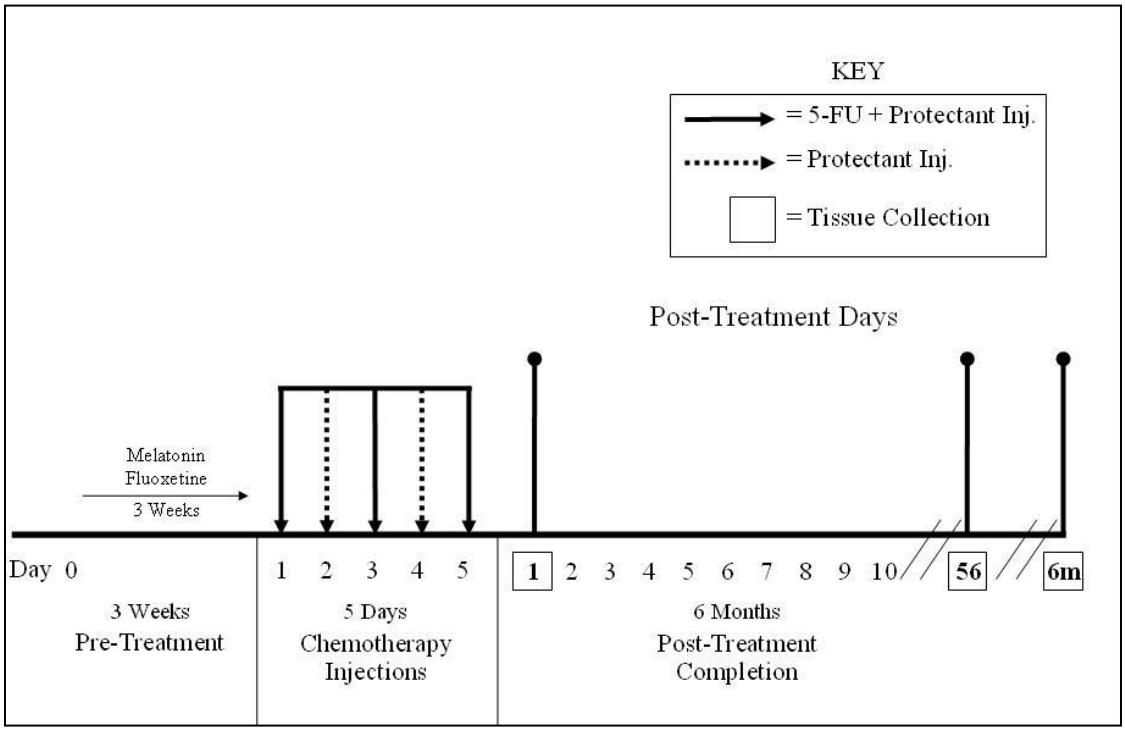

Figure 6: Access to water pre-treatment with melatonin or fluoxetine began 21 days prior to the first injection. Animals received protectant injections on days 1-5 with saline (0.9% NaCl), melatonin (25mg/kg) or fluoxetine (12mg/kg). Animals received additional injections on days 1, 3, and 5 with saline (0.9% NaCl) or 5-FU (70mg/kg). Animals were perfused 1 day, 56 days or 6 Months after the last injection.

Neuroprotection Water Treatment: Prior to initial chemotherapy administration, mice received neuroprotective treatment administered through the drinking water, as detailed in ELBeltagy et al. (2010). Water administration allows for long-term treatment, preemptively boosting neurogenic levels and antioxidant levels in an attempt to prevent 5-FU induced reductions, without increasing stress levels unnecessarily through repeated injections. Water bottles were covered with aluminum foil to prevent light exposure to the melatonin treated water (light-sensitive) and this was controlled across groups. Water was weighed and changed every 4 days in order to calculate water intake and adjust the melatonin and fluoxetine concentration accordingly to maintain the target dose. Water

intake was estimated by calculating the difference between average daily evaporation levels (calculated from 3 control water bottles in empty cages) and bottle weight and then dividing the amount of water consumption by number of days, and number of animals per cage. Melatonin dosing was determined based on previous studies showing boosts in neurogenic potential, antioxidant effects, and white matter protection following pre-treatment with 8mg/kg melatonin (Ramirez-Rodriguze et al., 2011) or 10mg/kg (Manda et al., 2009). The chosen fluoxetine dose reflects a range of doses from 10mg/kg to 20mg/kg used in studies that have found neurogenesis benefits following administration of fluoxetine in drinking water (Lyons et al., 2011; Lesemann et al., 2012) or delivered orally (Czeh et al., 2007). Target doses of 12mg/kg melatonin (MP Biomedicals) and 15mg/kg fluoxetine (Sigma-Aldrich) were diluted into drinking water with access to drinking water starting 21 days before chemotherapy treatment. This timeline was chosen primarily to match the water administration time-line used by ELBeltagy et al. (2012). For a visual indication of water pre-treatment relative to chemotherapy treatment see Figure 6.

Chemotherapy and Neuroprotection Treatment: Mice in each group received intraperitoneal (i.p.) injections over a period of 5 days (time-line shown in Figure 6). 5-FU (70mg/kg diluted into 0.9% saline; Sigma-Aldrich) or saline (0.9% NaCl) injections were administered on days 1, 3, and 5, for a total of 3 injections per animal, as detailed in Han et al. (2008). Dosing of 5-FU was based on maximally tolerated doses for C57BL/6J mice determined by previously piloted dosing studies done within our lab. Han et al. (2008) used the same approach to determine the maximally tolerated dose of 5-FU in CBA mice. Because toxicity and tolerance to chemotherapeutic compounds can vary

drastically as a function of circadian rhythm (Focan, 1995), all mice were treated at approximately the same time, 8-9 hours after light onset.

Animals treated with chemotherapy have a tendency to temporarily decrease their water intake following treatment, making it difficult to ensure neuroprotectant levels are maintained throughout treatment when administered via drinking water. As a result, injections of neuroprotectants were administered to ensure that levels of protectants were maintained and maximally effective at the time of chemotherapy administration. Neuroprotection injections of melatonin (25mg/kg diluted in 0.9% saline; MP Biomedicals), fluoxetine (12mg/kg diluted into 0.9% saline; Sigma-Aldrich), or saline (0.9% NaCl) were delivered 20 minutes prior to chemotherapy and once every 24 hours for a total of 5 injections per animal. As a result, mice received chemotherapy and neuroprotectant on days 1, 3, and 5, and a single neuroprotectant injection on days 2 and 4 (see Figure 6). Weight was monitored daily for approximately 4 weeks, then every other day until 56 days post-treatment. Following day 56, mice were weighed weekly. In addition to weight, teeth were monitored regularly and trimmed when necessary in order to prevent overgrowth which can lead to weight loss.

Tissue Collection: The same tissue collection procedure used in Experiment 1 was used.

Immunohistochemical Staining: The same immunohistochemical staining procedure used in Experiment 1 was used.

Tissue Analysis: The same tissue stain analysis protocol used in Experiment 1 was used.

Results Experiment 2

Animal Deaths

The only 5-FU induced animal deaths in Experiment 2 occurred within the day 56 group. No animal deaths occurred within the day 1 or 6 month groups in Experiment 2. Within the day 56 group, 1 animal injected with 5-FU, 2 animals injected with 5-FU + melatonin, and 2 animals injected with 5-FU + fluoxetine died prior to perfusion. Any data collected for animals that died prior to perfusion were excluded from all analyses, including weight loss analyses (See Appendix E for mortality data regarding Experiment 2).

Neurogenesis Analysis Experiment 2

Figure 7 shows the average number of Ki-67 positive cells detected in tissue for Experiment 2 across all three tissue collection time points (day 1, day 56, and 6 months). As shown in Figure 7, younger animals perfused at day 1 or day 56 had higher rates of cellular proliferation compared to older animals perfused at 6 months regardless of treatment condition. Furthermore, pre-treatment with melatonin or fluoxetine did not alter the detected levels of neurogenic proliferation at any time point within the study. A 4 x 3 factorial ANOVA [Treatment × Day] indicated that there was a significant main effect of day, ($F_{2, 66} = 17.65$; $p < .0001$, $\eta_p^2 = .35$), with no other significant effects detected. Post hoc analysis (Tukey HSD) indicated that animals perfused at day 1 had significantly more Ki-67 positive cells than animals perfused at 6 months ($p < .0001$) and a trend for animals perfused at day 56 ($p < .07$). Cell counts obtained from animals perfused at day 56 were significantly higher than those seen in animals perfused at 6

months ($p < .005$). Together these results suggest a similar pattern to that obtained in Experiment 1. While, 5-FU injections did not lead to any detectable differences in proliferation rates within the DG compared to saline injected animals, there was a decrease in proliferation over time as the animals aged. Additional exploratory one-way ANOVA's confirmed there were no statically significant differences in the number of Ki-67 positive cells across treatment conditions at any of these time points.

Figure 7: Experiment 2: Tissue Analysis

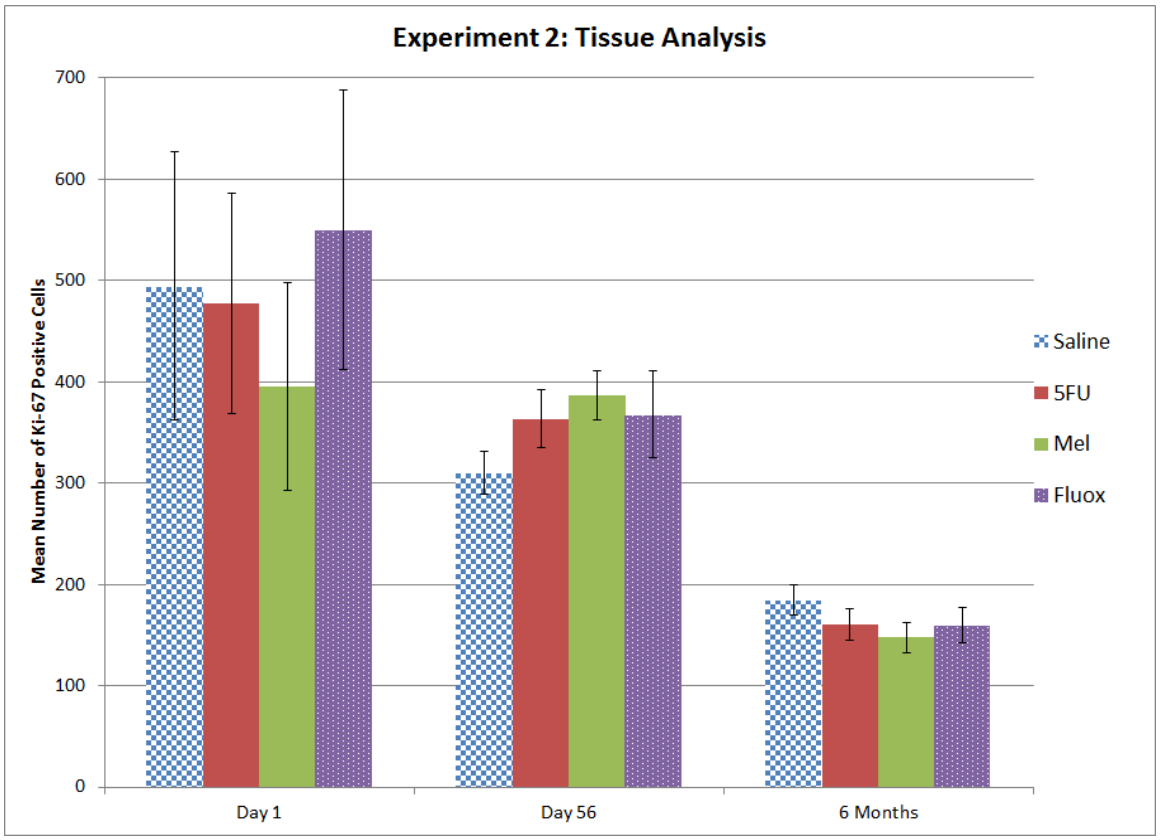

Figure 7: Mean number of Ki-67 positive cells detected in tissue collections at day 1, day 56 and 6 month, for animals treated with saline, 5-FU, 5-FU + melatonin, and 5-FU + fluoxetine. Error bars represent ±1 standard error of the mean. A factorial ANOVA indicated a main effect of day, ($p < .0001$), with post-hoc analysis (Tukey HSD) indicating animals perfused at 6 months had significantly less Ki-67 positive cells than those animals perfused at day 1 ($p < .0001$) and at day 56 ($p < .005$). No significant treatment effect was detected.

Weight Data Experiment 2

Weight Loss Analysis: Weight data was analyzed following the same procedures used in Experiment 1.

Weights Day 1: Figure 8 depicts percent weight (relative to pre-treatment weight) as a function of day, starting from the day of the first injection to the day after the last injection, for the animals that were perfused on day 1. As shown in Figure 8, animals treated with 5-FU, regardless of protectant, all lost weight during the 5 day injection protocol, while control animals receiving saline maintained or gained weight. A two-factor mixed-design ANOVA [Treatment × Day] was conducted to analyze weight change between the treatment groups for the first 5 days of the experiment (starting from the day following the first injection to the first day following the last injection). Results indicated main effects of treatment, ($F_{3, 24} = 4.16$; $p < .017$ $\eta_p^2 = .33$), and day, ($F_{4, 96} = 17.53$; $p < .0001$, $\eta_p^2 = .35$), as well as a significant treatment by day interaction, ($F_{12, 96} = 12.53$; $p < .0001$, $\eta_p^2 = .58$). This result follows the same pattern as Experiment 1, as weight for controls injected with saline increased during the injection protocol (the first 5 days of the experiment) while 5-FU injected animals lost weight. A one-way ANOVA confirmed that weight change differed by the end of the injection protocol ($F_{3, 24} = 11.24$; $p < .0001$, $\eta_p^2 = .58$). Post hoc analysis (Tukey HSD) indicated that the saline treated group had lost less weight than 5-FU only, 5-FU + melatonin, and 5-FU + fluoxetine treated groups (*p's* < .002).

Figure 8: Experiment 2: Weight Data - Day 1

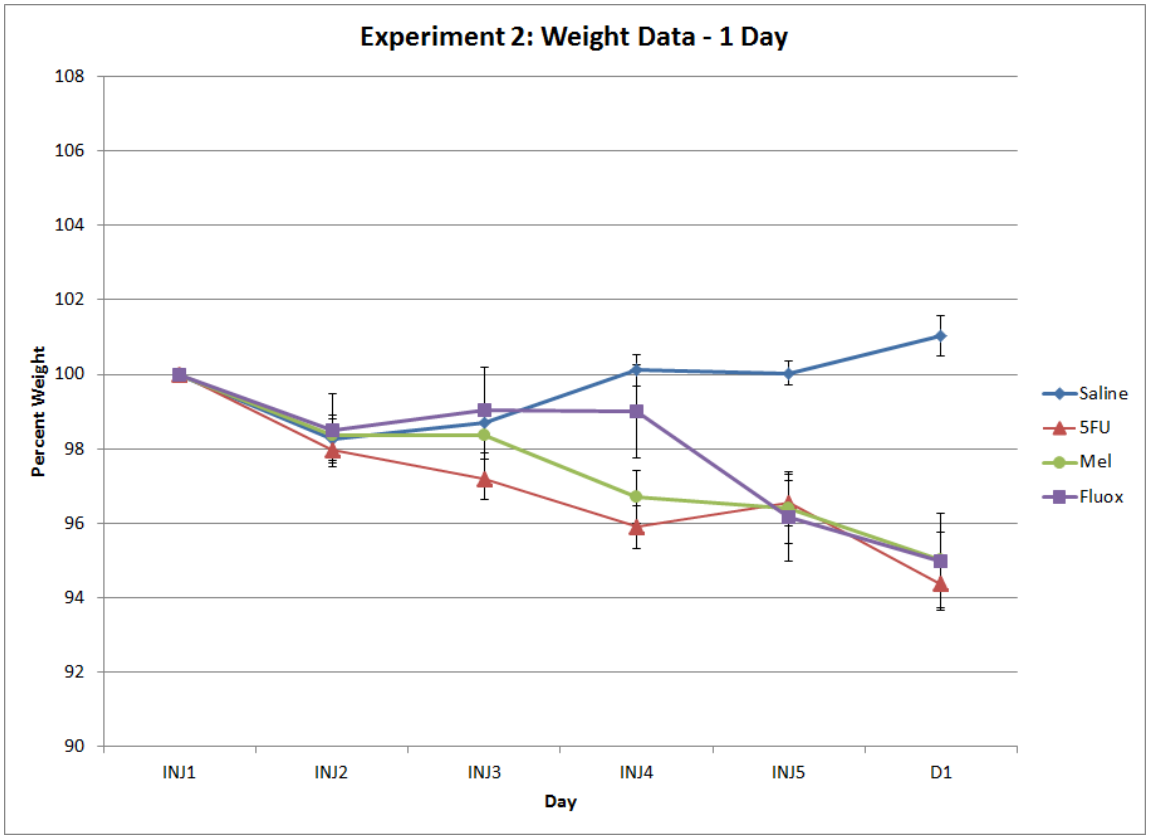

Figure 8: Mean % body weight (relative to pre-treatment weight) as a function of day for animals perfused on day 1. Mice received IP injections of either saline (0.9% NaCl; n = 7), 5-FU (70 mg/kg; n = 7), 5-FU + melatonin (25 mg/kg; n = 7), or 5-FU + fluoxetine (12 mg/kg; n = 7) on days 1-5 (protectants on all 5 days, 5-FU on days 1, 3, and 5). Error bars represent ±1 standard error of the mean. A two-factor mixed-design ANOVA indicated a main effect of treatment ($p < .017$), a main effect of day ($p < .0001$), and a treatment by day interaction ($p < .0001$).

Weights Day 56: Figure 9 depicts percent weight as a function of day, starting from the day of the first injection to the day after the last injection, for the animals that were perfused on day 56. As shown in Figure 9, animals injected with 5-FU, regardless of protectant, all lost weight during the 5 day injection protocol, while control animals receiving saline maintained or gained weight. To assess differences in the extent of weight lost between the treatment groups across the protocol a two-factor mixed-design ANOVA [Treatment × Day] was conducted to analyze weight loss between the treatment

groups for the first 5 days of the experiment (starting from the day following the first injection to the first day following the last injection). Results indicated main effects of treatment, ($F_{3, 19} = 13.15$; $p < .0001$, $\eta_p^2 = .68$), and day, ($F_{2.5, 46.8} = 90.81$; $p < .0001$, $\eta_p^2 = .83$, Greenhouse-Geisser corrected), as well as a significant treatment by day interaction, ($F_{7.4, 46.8} = 10.20$; $p < .0001$, $\eta_p^2 = .62$, Greenhouse-Geisser corrected). These results suggest that while weight increased over the first 5 days of the experiment for saline injected controls, 5-FU injected animals lost weight regardless of protectant. A one-way ANOVA confirmed that weight change differed by the end of the injection protocol ($F_{3, 19} = 25.43$; $p < .0001$, $\eta_p^2 = .80$). Post hoc analysis (Tukey HSD) indicated that the saline treated group had lost less weight than 5-FU only, 5-FU + melatonin, and 5-FU + fluoxetine treated groups (*p's* < .002). Additionally, there was a trend suggesting that the 5-FU + melatonin group had lost less weight than the 5-FU only group ($p = .071$).

Figure 9: Experiment 2: Weight Data - Day 56

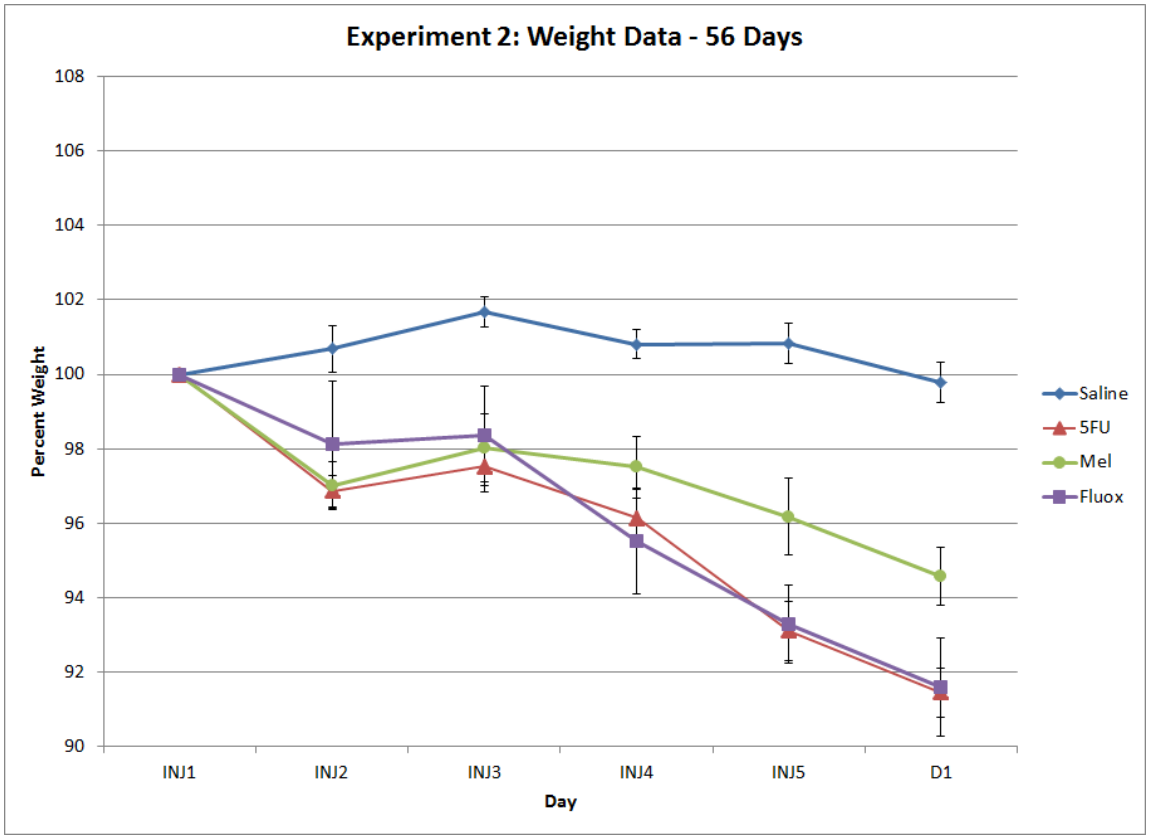

Figure 9: Mean % body weight (relative to pre-treatment weight) as a function of day for animals perfused on day 56. Mice received IP injections of either saline (0.9% NaCl; n = 7), 5-FU (70 mg/kg; n = 6), 5-FU + melatonin (25 mg/kg; n = 5), or 5-FU + fluoxetine (12 mg/kg; n = 5) on days 1-5 (protectants on all 5 days, 5-FU on days 1, 3, and 5). Error bars represent ±1 standard error of the mean. A two-factor mixed-design ANOVA indicated a main effect of treatment, a main effect of day and a treatment by day interaction (all *p's* < .0001).

Another interesting feature of the weight data for Experiment 2 is the pattern of weight loss seen over the first month of the experiment. Consistent with patterns of weight lost detected in previous experiments (Fremouw, et al., 2012a) and pilot testing, animals injected with 5-FU appear to go through two bouts of weight loss. One of these occurs earlier, lasting through the 5 day injection protocol, while the second occurs following a transient period of weight gain and recovery (see Appendix F for a figure of the extended weight loss data).

Weights 6 Month: Figure 10 depicts percent weight as a function of day, starting from the day of the first injection to the day after the last injection, for the animals that were perfused at 6 months. As shown in Figure 10, a similar consistent pattern of weight lost was observed in the 6 month animals when compared to those animals perfused on day 56. Again, animals injected with 5-FU, regardless of protectant, all lost weight during the 5 day injection protocol, while control animals receiving saline maintained or gained weight. A two-factor mixed-design ANOVA [Treatment × Day] was conducted to analyze weight loss between the treatment groups for the first 5 days of the experiment (starting from the day following the first injection to the first day following the last injection). Results indicated main effects of treatment, ($F_{3, 23} = 25.78$; $p < .0001$, $\eta_p^2 = .77$), and day, ($F_{1.7, 39.8} = 53.93$; $p < .0001$, $\eta_p^2 = .70$, Greenhouse-Geisser corrected), as well as a significant treatment by day interaction, ($F_{5.2, 39.8} = 7.50$; $p < .0001$, $\eta_p^2 = .49$, Greenhouse-Geisser corrected). Consistent with the weight data obtained for the day 1 and 56 animals, these results suggest that animals injected with saline alone maintained or increased their weight over the first 5 days of the experiment while 5-FU injected animals lost weight regardless of protectant. A one-way ANOVA confirmed that weight change differed by the end of the injection protocol ($F_{3, 23} = 24.76$; $p < .0001$, $\eta_p^2 = .76$). Post hoc analysis (Tukey HSD) indicated that the saline treated group had lost less weight than 5-FU only, 5-FU + melatonin, and 5-FU + fluoxetine treated groups (p's $< .001$).

Figure 10: Experiment 2: Weight Data - 6 months

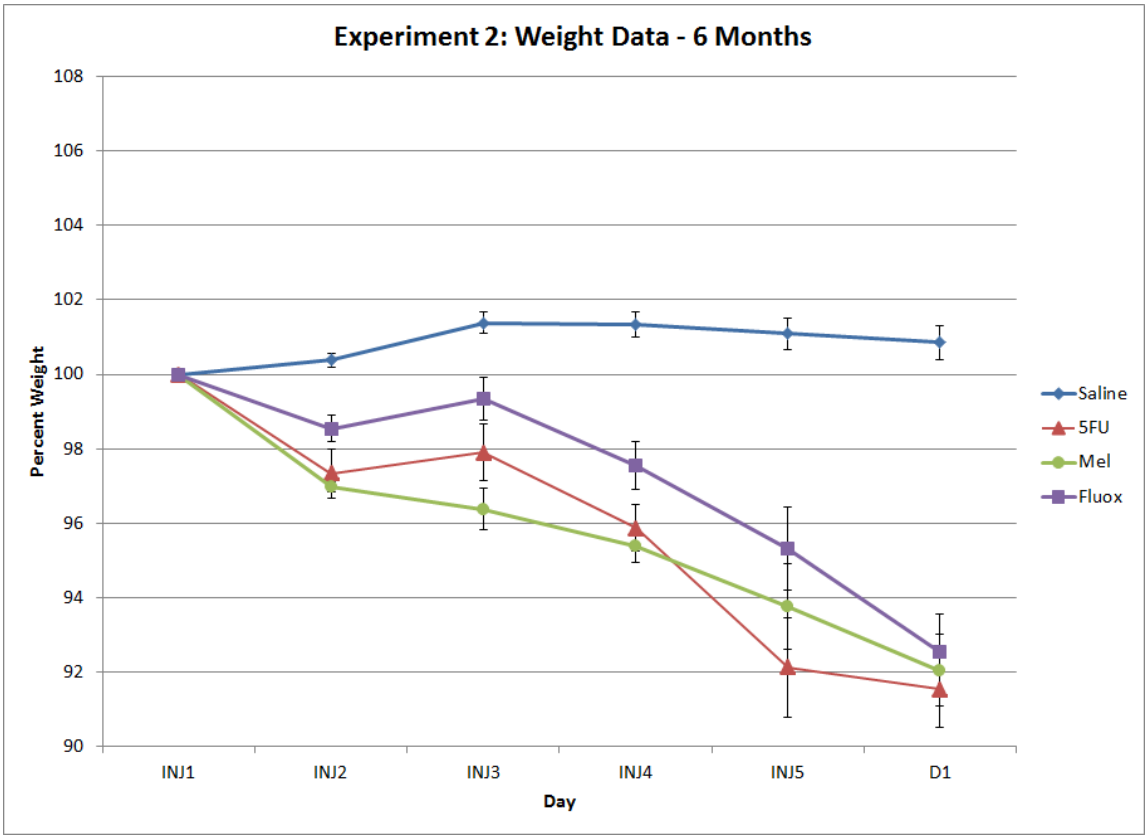

Figure 10: Mean % body weight (relative to pre-treatment weight) as a function of day for animals perfused on day 56. Mice received IP injections of either saline (0.9% NaCl; n = 7), 5-FU (70 mg/kg; n = 7), 5-FU + melatonin (25 mg/kg; n = 6), or 5-FU + fluoxetine (12 mg/kg; n = 7) on days 1-5 (protectants on all 5 days, 5-FU on days 1, 3, and 5). Error bars represent ±1 standard error of the mean. A two-factor mixed-design ANOVA indicated a main effect of treatment, a main effect of day and a treatment by day interaction (all *p's* < .0001).

Animals perfused at 6 months demonstrated the same pattern of two bouts of weight loss as detected in the animals perfused at day 56 (see Appendix G for a figure of the extended weight loss data).

Discussion Experiment 2

Treatment with 5-FU has been shown to reduce levels of proliferating cells within the DG of mice both shortly following treatment (Janelsins et al., 2010; 2 days after the

last chemotherapy injection) and up to 6 months after treatment completion (Han et al., 2008). In contrast, the results of the present study found no significant effect of 5-FU on proliferating cells within the DG at any of the assessment time points, including tissue collected at 1 day. As with Experiment 1, the results from Experiment 2 conflict with these earlier reported reductions of neurogenesis following chemotherapy treatment in mice.

It is unclear what the cause of theses discrepancies are. It may suggest that reductions in proliferation rates within the DG shortly following treatment with 5-FU in rodents may not be as consistent or robust to experimental parameters as previously thought. For instance, while many of the experimental parameters used in the present study were similar to those used by Han et al. 2008 (e.g. injection schedule, assessment windows) it appears that differences in a variety of factors including mouse strain and dose may have contributed to the different findings despite the similarities across the studies. Considering that Han et al. (2008) had only been able to detect a statistically significant reduction in proliferation within the SVZ at 56 days after treatment while failing to detect a significant reduction in DG at this time point, the present study's lack of treatment effect at 56 days can be considered consistent with previous data. This may suggest that populations of proliferative neurons within the DG may be less vulnerable to 5-FU insult when compared to populations of neurogenic cells within the SVZ of rodents. When comparing the present study to the Janelsins et al. (2010) in which the same mouse strain and similar dose of 5-FU was administered, the present study failed to reproduce the early deficits in proliferation which had previously been reported.

When comparing results across the present study, Han et al. (2008), and Dietrich et al. (2006) the data appear to support not only the theory that each chemotherapeutic compound may exhibit a unique timing profile of neurogenic suppression but particular populations of these cells may be more vulnerable. Taken together the complete lack of any 5-FU related long-term reduction in proliferation was unanticipated but highlights the fact neurogenesis assays may be susceptible to the same kinds of inconsistency present within the animal behavioral literature regarding CRCI.

Again the recent study conducted by Seigers et al. (2016), provides experimental evidence that supports the present study's findings. As Seigers et al. (2016) was unable to detect any reductions in proliferation within the DG of C57Bl/J6 mice treated with a single injection of 5-FU (75 mg/kg), when assessed utilizing Ki-67 for neurogenic proliferation at either 3 weeks or 16 weeks following treatment. At both time points, 5-FU treatment failed to produce any detectable differences in proliferation rates compared to saline treated controls.

Finally it is difficult to evaluate the effectiveness of pre-treatment with melatonin or fluoxetine given that 5-FU did not cause a decrease in proliferation. While previous studies in rats have indicated chronic fluoxetine treatment can boost proliferation rates within the DG (Duman et al., 2001), and co-administration of fluoxetine can prevent deficits in proliferation detected at less than one week following treatment with 5-FU (ELbeltagy et al., 2010) or methotrexate (Lyons et al., 2011), it is less clear if fluoxetine can boost levels of proliferation in otherwise healthy mice. In a recent review, Miller and Hen (2015) report inconsistencies within the rodent literature regarding the capacity of fluoxetine to boost levels of adult hippocampal neurogenesis within healthy control mice.

They suggest that the most consistently detected increases in neurogenic proliferation following fluoxetine treatment occur in rodents that are stressed and/or animals displaying depression or anxiety-like behaviors. This may suggest that fluoxetine would not be able to boost levels of proliferation within the DG of mice without an initial insult. Thus, it may be that fluoxetine was unable to induce any detectable differences in proliferation rates within collected tissue as 5-FU failed to disrupt normal rates of neurogenic proliferation. The same may be true when considering the role of melatonin. While it was initially suspected that melatonin may have been a more powerful protectant against 5-FU related deficits, given both its neurogenic boosting and antioxidative effects in mice (Ramirez-Rodriguze et al., 2011) this was not supported given the lack of any treatment effects within Experiment 2.

In summary, Experiment 2 found no treatment effects of 5-FU or any clear effects of pre-treatment with fluoxetine or melatonin on proliferation rates at any of the three time points within the study. While unexpected, the lack of any significant differences detected between groups, as with Experiment 1, suggest that both short and long-term reported neurogenesis deficits may differ across studies and may be the result of variability across experimental parameters used.

GENERAL DISCUSSION

Chemotherapy Toxicity

Given the lack of any treatment effects detected in either experiment, especially in tissue collected at day 1, the first potential explanation would be that chemotherapy dosing in the present study was insufficient to induce the deficits detected in previous studies. While possible, this is highly unlikely given the decreases in weight seen in animals treated with chemotherapy. The detected weight loss across both experiments indicates that the dosage used for each compound achieved the typical level of toxicity seen within the literature. Furthermore, the dosing in the current study was equal to or higher than doses used in previous studies in which deficits have been detected at time points shortly after chemotherapy administration. Given that in Experiment 1 and 2 a few animals died after receiving their chemotherapy, it seems likely that our chemotherapy doses where close to or slightly higher than the maximum tolerated dose.

Animal deaths that occurred across both experiments were minimal in most cases and unlikely to mask any treatment effects. In particular, no animals died in any groups at 1 day and still no differences in neuronal proliferation were detected. However, there was a portion of animals treated with doxorubicin in Experiment 1 that were lost prior to perfusions on day 56 and at 6 months. As a result, it is difficult to interpret any potential treatment effects of doxorubicin within these groups without running additional animals.

Age-Related Effects

It is clear from both experiments that proliferation rates decreased as the animals aged. Tissue collected 1 day following completion of chemotherapy administration

exhibited the highest rates of Ki-67 positive cells regardless of the treatment condition. At later time points, rates of neurogenesis decreased, with the lowest detected rates of Ki-67 positive cells observed in tissue collected at the 6 month time point. This pattern of decreasing neurogenesis with increasing animal age was consistent across both experiments. Furthermore, recent data collected by Seigers et al. (2016) demonstrated a similar reduction in proliferation within the DG of C57BL/6J mice between 3 weeks and 16 weeks regardless of treatment with any of the chemotherapeutic compounds (cyclophosphamide , doxorubicin, or 5-FU) used in the present study.

The most likely explanation for these detected decreases in neurogenesis is natural aging. While limited, studies show that adult mammalian hippocampal neurogenesis naturally undergoes significant decreases in rats (Kuhn, Dickinson-Anson, and Gage, 1996; Cameron and McKay, 1999; Bizon and Gallagher, 2003) and in mice (Kempermann et al., 1998; Harrist et al., 2004, Kronenberg et al., 2006). A few studies have attempted to map out the time course of these age-related declines in neurogenesis (Seki and Arai, 1995; Rao, Hattiangady, and Shetty, 2006). In one such study, Nada and Colleagues (2010) examined the time course of natural reductions of neurogenesis in C57BL/6J mice at 1-5, 7 and 9 months of age. Utilizing Ki-67, Nada et al. (2010) observed an exponential decrease in proliferating cells within the subgranular zone of the dentate gyrus with increasing age. An approximate 40% relative decrease in the number of proliferating cells was detected at each interval in the study. The bulk of these decreases in neurogenesis occur within the first 6 months at which point they begin to plateau and then stabilize several months after.

It seems likely that the progressive decrease in Ki-67 positive cells seen in the present study as the mice aged is the product of the natural age-related declines in neurogenesis. Interestingly none of the treatment conditions seemed to significantly alter this natural age-related decline in either direction. Importantly, the decrease in neurogenesis with age seen in the present study provides evidence that the Ki-67 immunohistochemistry protocol was successful in labeling proliferating cells within the DG. Thus the lack of any group differences in neuronal proliferation is unlikely to be caused by Ki-67 staining issues.

Strain Differences

There are numerous, relatively well established phenotypic differences present between commonly used inbred strains of mice. A limited number of these studies have identified variation in the rates of neurogenesis between the 9 most commonly used mouse strains, including C57BL/6J's (Kempermann, Kuhn and Gage 1997; Kempermann and Gage, 2002; Hayes and Nowakowski, 2002; Kim et al., 2009; Snyder et al., 2009). Understanding these differences may be critical when comparing rates of neurogenesis detected across multiple CRCI studies that have used these different strains of mice.

In the present study C57BL/6J mice were used. Several studies have demonstrated that C57BL/6J mice have an unusually high rate of adult neurogenic proliferation within the hippocampus compared to other commonly used laboratory inbred strains (Kempermann, Kuhn and Gage 1997; Hayes and Nowakowski, 2002; Kim et al., 2009; Snyder et al., 2009). It appears this increase in proliferation is coupled with a decrease in cell survival such that when those newly born cells are assessed 4 weeks

after birth, the number of these cells that survive in the C57BL/6J generally do not differ from those strains which previously had fewer cells present during proliferation (Kempermann, Kuhn and Gage, 1997; Hayes and Nowakowski, 2002). It is unclear, if and how a higher basal rate of proliferation may affect detected rates of proliferation following chemotherapy administration. Additionally, apoptotic assays used to assess DNA damage and cell death within the DG of C57BL/6J mice may be higher as a result of the strains inherent neurogenic process. These strain based differences highlight the way in which different cellular assays of chemotherapy related effects may be susceptible to metabolic and cellular properties of a particular animal strain. Whether researching the death of new cells or rates of cellular proliferation within the DG, attempts to characterize the effects of chemotherapy on neurogenesis may be unique to each chemotherapy compound and to each animal strain. Studies that compare effects across multiple inbred and outbred/wild strains may clarify these effects.

Taken together, these studies provide evidence that genetic variation between different inbred mouse strains can influence detected rates of neurogenesis. It should be noted that the neurogenic assay used in this study, a Ki-67 marker, can only be used to assess proliferation rates. Essentially, Ki-67 provides a snapshot of active proliferation at the time of tissue collection. Given that C57BL/6J mice have especially high rates of neurogenic proliferation relative to other strains, it is possible that this fact may have acted to protect against chemotherapy induced insult or at the very least may have made it more difficult to detect any difference at the longer time points. Conversely, it can be argued that by using C57BL/6J mice, the present study ensures the baseline level of neurogenic proliferation is high enough to detect any possible reductions due to

chemotherapy administration, thereby avoiding possible floor effects. Although it is possible that strain related factors may account for differences in experimental results between Han et al.(2008), Dietrich et al. (2006) and the present study at the longer time intervals, it seems unlikely to account for the differences between these studies at shorter intervals given that Janelsins et al. (2010) have shown that C57BL/6J mice show similar decreases in neurogenesis shortly after chemotherapy injections. Unfortunately, to date, I am not aware of any studies that directly compare rates of neurogenesis in C57BL/6J and CBA mice (which were used by both Han et al., 2008; and Dietrich et al., 2006) making it difficult explore strain related factors across studies. Regardless of the factors that using the C57BL/6J strain may have introduced in the present study, the results are consistent with data collected in recent study conducted by Siegers et al.(2016) in which the same strain was assessed for chemotherapy related decreases in neurogenesis using Ki-67, in which no effects were detected at either 3 or 15 weeks post-treatment.

Proliferation vs. Cell Survival

Marking for Ki-67 provides for a reliable assay of cellular proliferation. However Ki-67 is unable to provide any information regarding rates of cell death, cell survival, cellular differentiation, or the status of successful integration of these new cells into existing neural networks. It is possible that chemotherapy administration could spare proliferative processes while interfering with other processes involved in neurogenesis. It should be noted that while proliferation, migration, differentiation, integration and maturation are all known to be important for successful neurogenesis, the exact timing and the mechanisms behind each of these processes are not thoroughly understood and are still actively being researched (Deng et al., 2010).

The animal literature concerning CRCI has focused on neurogenesis as a potential underlying mechanism, but hasn't systematically differentiated the possible affects of chemotherapeutic compounds on neurogenic proliferation within the DG versus affects on the survival of these new neurons. Only 2 studies have attempted to assess the affect of chemotherapy on cell survival within the DG and both have indicated that chemotherapy treatment can disrupt neurogenic cell survival (Mondie et al., 2010; Lyons et al., 2011). While it is plausible that those cells actively proliferating would be the most vulnerable to the cytotoxic effects of chemotherapy exposure, it is unclear if a reduction in proliferation was detected, whether the loss of these proliferating cells during a brief injection protocol may represent significant damage or loss of function. It may be that any brief reduction in proliferation may be compensated for through subsequent cellular proliferation events such that the net level of new granule cells within the DG is unaffected by chemotherapy treatment. The ability of new granule cells to successfully survive and mature may be a critical factor rather than determining the number of proliferating cells at any one moment.

Assessing other factors in addition to proliferation, like those pertaining to cell survival and successful integration and maturation of granule cells into the hippocampus will be important for interpreting the contribution of chemotherapy induced neurogenesis deficits to CRCI. Certainly the studies discussed above suggest that neurogenesis is a dynamic multifactor phenomenon whose processes may differ in terms of both baseline function and resiliency to insult as a function of genetic variation and age.

Future Directions

The present study did not detect any reduction in proliferation rates within the DG of C57BL/6J mice treated with cyclophosphamide, doxorubicin, or 5-FU at 1 day, 56 days or 6 months after treatment. Additional animals may have been needed to overcome to the high variability of Ki-67 positive cells detected in tissue collected at day 1 in both experiments, to completely rule out any potential effects masked by this variability. However, it is fairly clear that there were no major alterations in proliferation rates within the DG in treated C57BL/6J mice at 56 days or 6 months compared to controls in both experiments. This pattern of results suggests that neurogenesis processes involved with proliferation of cells within the DG are unaffected in C57BL/6J mice treated with these chemotherapeutic compounds.

Within the human literature regarding CRCI it is well established that chemotherapy treatment seems to affect only a subset of individuals that undergo treatment for cancer. Not unlike the human CRCI phenomenon, it is possible that the C57BL/6J inbred mouse strain used in the present study may be resistant to some of the neurobiological effects of chemotherapy treatment that lead to reduced neurogenesis rates detected in other mouse strains. The lack of any alterations in neurogenesis rates in the current study are consistent with previous behavioral testing done in my lab that indicate no deficits in learning or memory following treatment with the same chemotherapy compounds (Fremouw et al., 2012a). To confirm potential strain effects follow-up studies should utilize a variety of mouse strains, including wild type, to systematically assess the role genetic factors, relating to particular inbred strains, may have on chemotherapy related reductions in neurogenesis. Furthermore, study of these strain

related factors may be able to identify particular genetic traits that may be protective and represent new treatment vectors.

As previously discussed, proliferation of new cells within the DG represents only a small part of the complicated processes that underlie neurogenesis. As a result, it will be necessary to use additional methodological tools to evaluate neurogenesis across each of these processes. Future studies, will utilize additional cellar markers to such as DCX, to assess levels of immature neurons, NeuN, to mark fully mature neurons and BrdU, to assess survival of newly generated neurons throughout neurogenesis. In addition, detection of cell death and DNA related apoptotic damage within the DG due to chemotherapy exposure should be assessed via terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assays. These assays and immnohistochemical markers can be used in conjunction with more sophisticated imaging software to assess morphological features such at dendritic complexity and length, within these neurons, and critically throughout the neurogenesis process.

Other neurobiological targets and potential mechanism that may relate to CRCI still need to be explored. Additional experiments done in my lab have already identified deficits in white matter following treatment with 5-FU or a combination treatment of cyclophosphamide and doxorubicin 14 months after treatment. Interestingly, preliminary data generated from the same animals show an increased expression of ionized calcium-binding adapter molecule 1 (iba1), indicative of upregulation of microglia activity and neuroinflammation within the CNS. These convergent results suggest that chemotherapy treatment in C57BL/6J mice may eventually induce long-term neuroinflammatory states and deficits in white matter integrity within the CNS.

The present study and others are needed to help identify and clarify the underlying mechanisms responsible for CRCI. Long-term experiments that examine neurogenesis and other cellular processes within the CNS that may be vulnerable to chemotherapeutic agents are needed within the CRCI literature. The goal of these experiments should be to systematically establish unique profiles for each chemotherapeutic agent, that address the cellular targets and mechanisms involved as well as the timing of any detected effects. These studies will facilitate research being done in patient populations that explore factors related to cognitive dysfunction following chemotherapy treatment and how to mitigate, prevent and repair damage done to patients who receive chemotherapy treatment.

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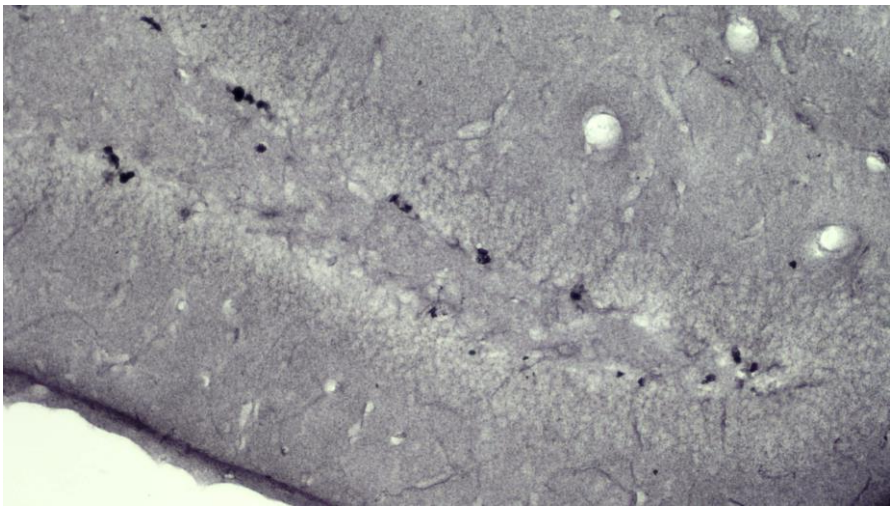
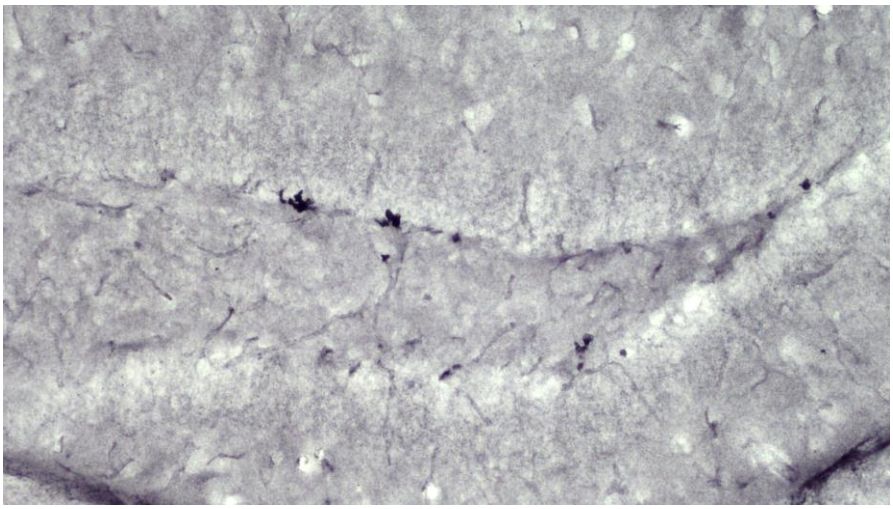
Figure 11: Representative Bright-Field Micrographs

A



B



C

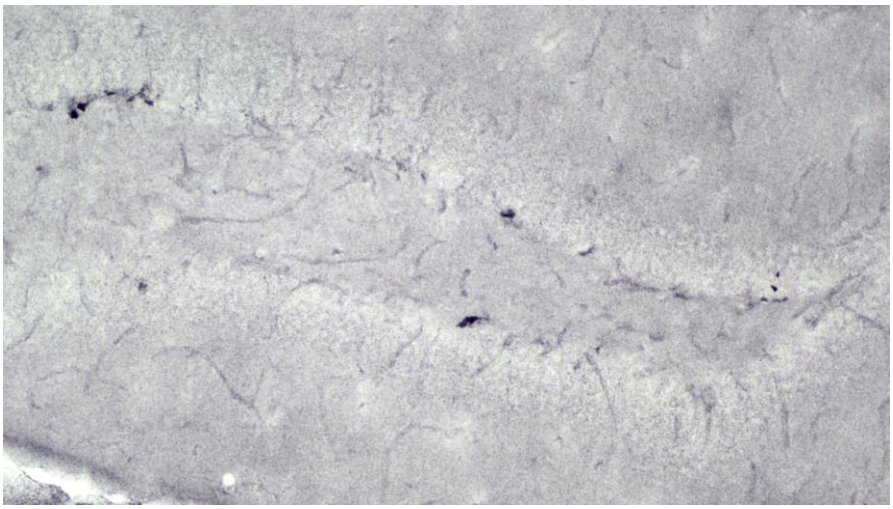


Figure 11: Representative bright-field micrographs of control animals perfused at day 1 (A), day 56 (B) and 6 Months (C)

APPENDIX B. MORTALITY DATA EXPERIMENT 1

Figure 12: Mortality Rates: Experiment 1

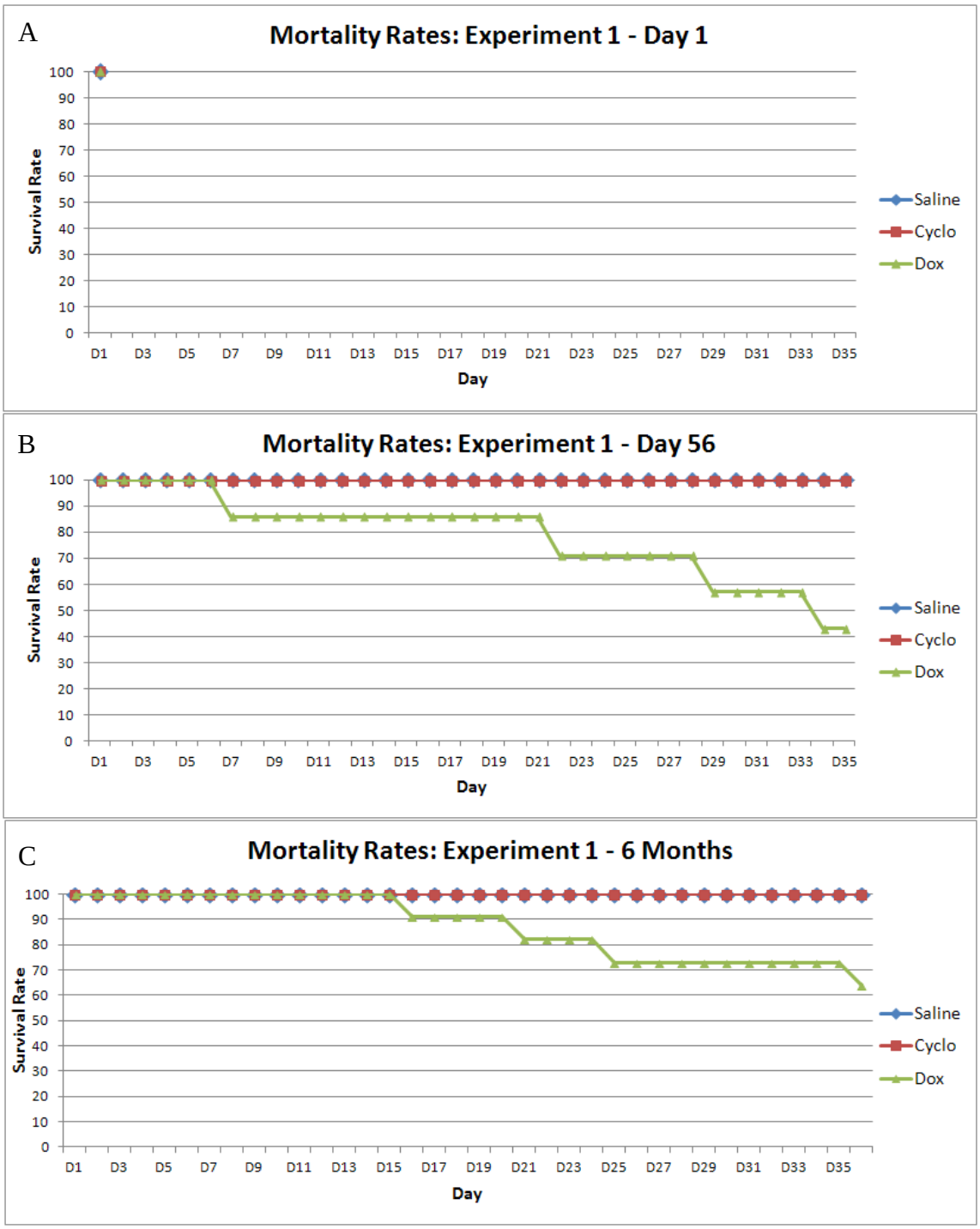


Figure 12: Graphs of the % of animals surviving as a function of day for animals in experiment 1 from day 1 to day 35. A indicates % survival of day 1 animals. B indicates % survival of day 56 animals. C indicates % survival of 6 month animals.

APPENDIX C. EXPERIMENT 1: EXTENDED WEIGHT DATA - 56 DAYS

Figure 13: Extended Weight Data Experiment 1 - Day 56 Animals

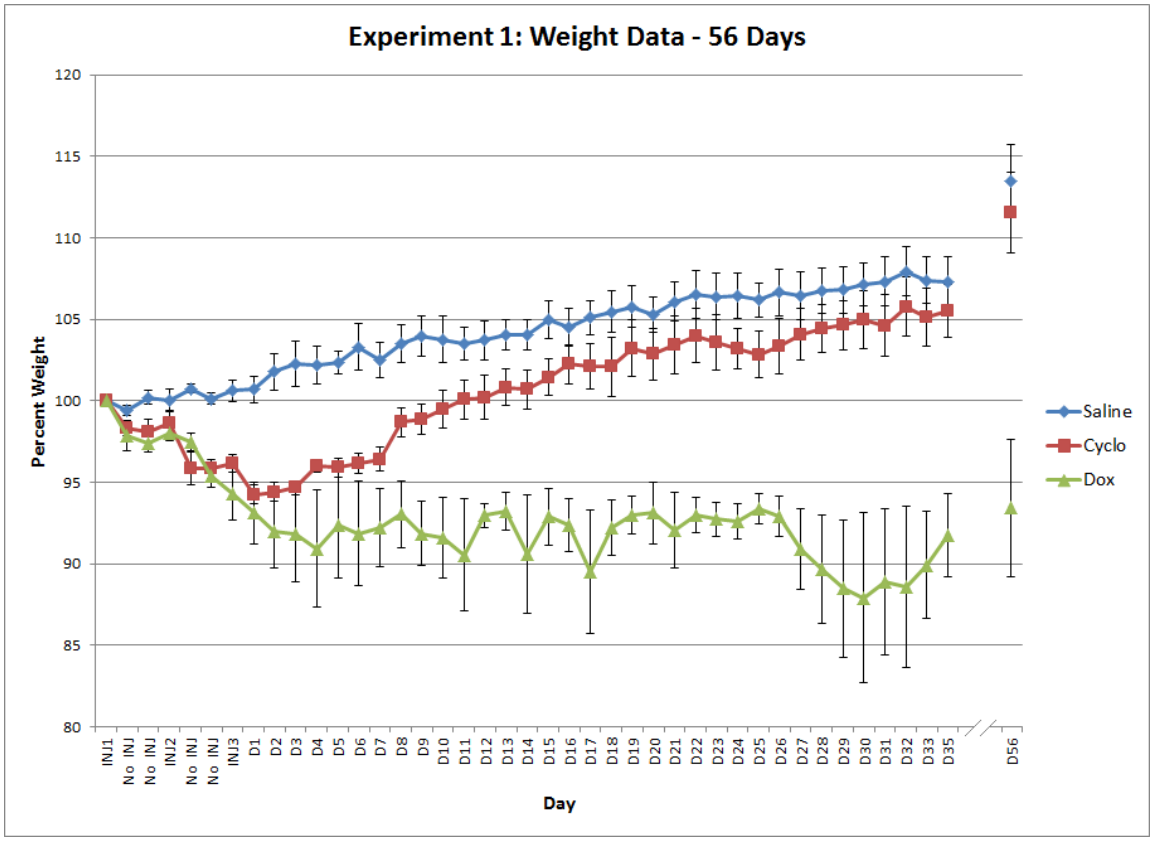


Figure 13: Mean % body weight (relative to pre-treatment weight) as a function of day for animals perfused at day 56 in experiment 1. Mice received IP injections of either saline (0.9% NaCl; n = 7), cyclophosphamide (120 mg/kg; n = 7), or doxorubicin (5 mg/kg; n = 4), on days 1, 4, and 7 (INJ1, INJ2 and INJ3). Error bars represent ±1 standard error of the mean.

APPENDIX D. EXPERIMENT 1: EXTENDED WEIGHT DATA - 6 MONTHS

Figure 14: Extended Weight Data Experiment 1 - 6 Month Animals

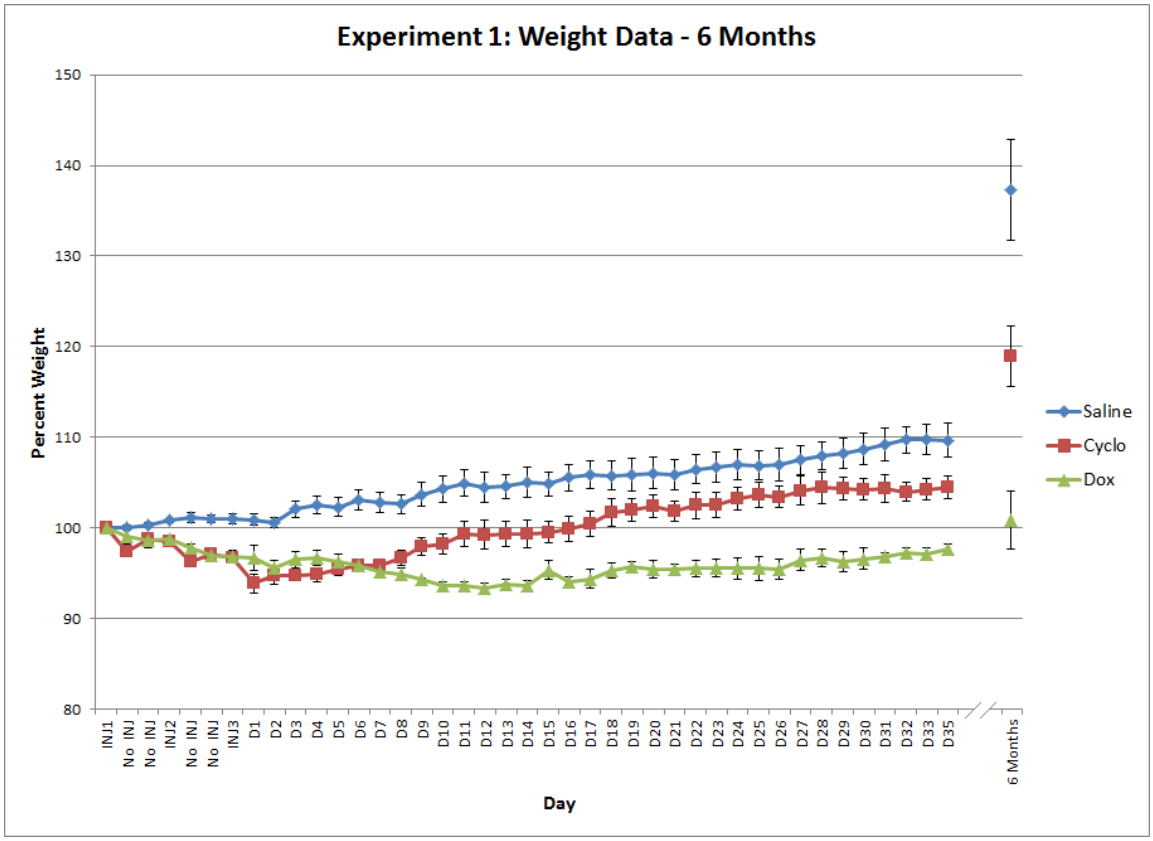


Figure 14: Mean % body weight (relative to pre-treatment weight) as a function of day for animals perfused at 6 months in experiment 1. Mice received IP injections of either saline (0.9% NaCl; n = 7), cyclophosphamide (120 mg/kg; n = 7), or doxorubicin (5 mg/kg; n = 4), on days 1, 4, and 7 (INJ1, INJ2 and INJ3). Error bars represent ±1 standard error of the mean

APPENDIX E. MORTALITY DATA EXPERIMENT 2

Figure 15: Mortality Rates: Experiment 2

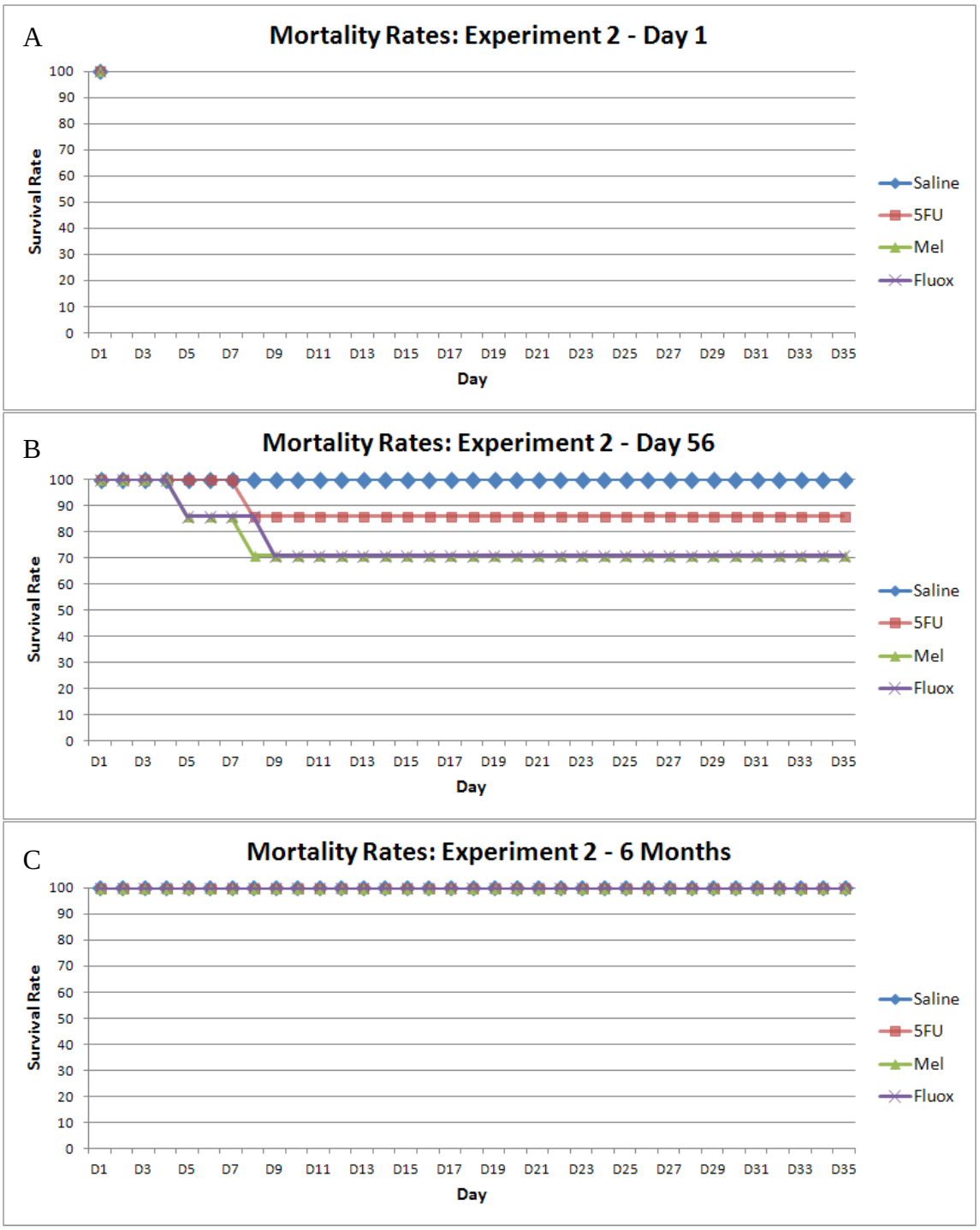


Figure 15: Graphs of the % of animals surviving as a function of day for animals in experiment 2 from day 1 to day 35. A indicates % survival of day 1 animals. B indicates % survival of day 56 animals. C indicates % survival of 6 month animal

APPENDIX F. EXPERIMENT 2: EXTENDED WEIGHT DATA - 56 DAYS

Figure 16: Extended Weight Data Experiment 2 - Day 56 Animals

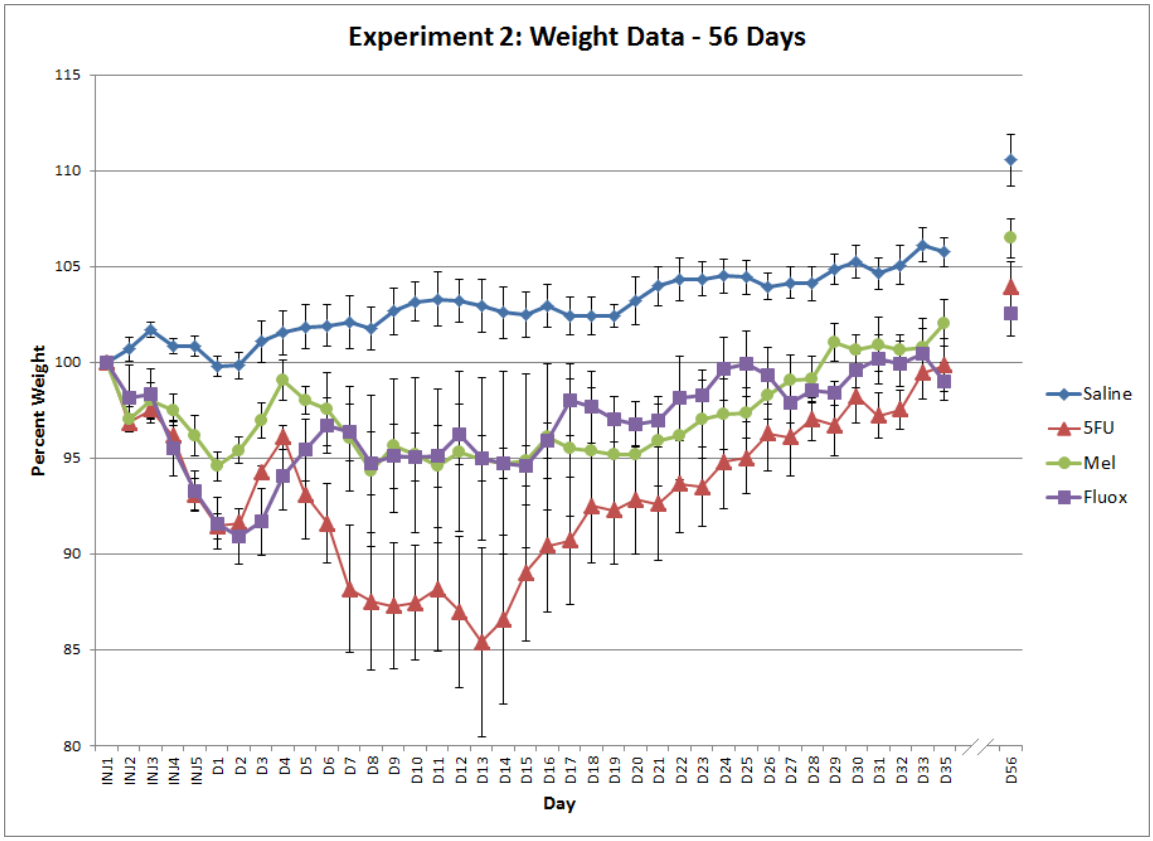


Figure 16: Mean % body weight (relative to pre-treatment weight) as a function of day for animals perfused on day 56 in experiment 2. Mice received IP injections of either saline (0.9% NaCl; n = 7), 5-FU (70 mg/kg; n = 6), 5-FU + melatonin (25 mg/kg; n = 5), or 5-FU + fluoxetine (12 mg/kg; n = 5) on days 1-5 (protectants on all 5 days, 5-FU on days 1, 3, and 5). Error bars represent ±1 standard error of the mean.

APPENDIX G. EXPERIMENT 2: EXTENDED WEIGHT DATA - 6 MONTHS

Figure 17: Extended Weight Data Experiment 2 - 6 Month Animals

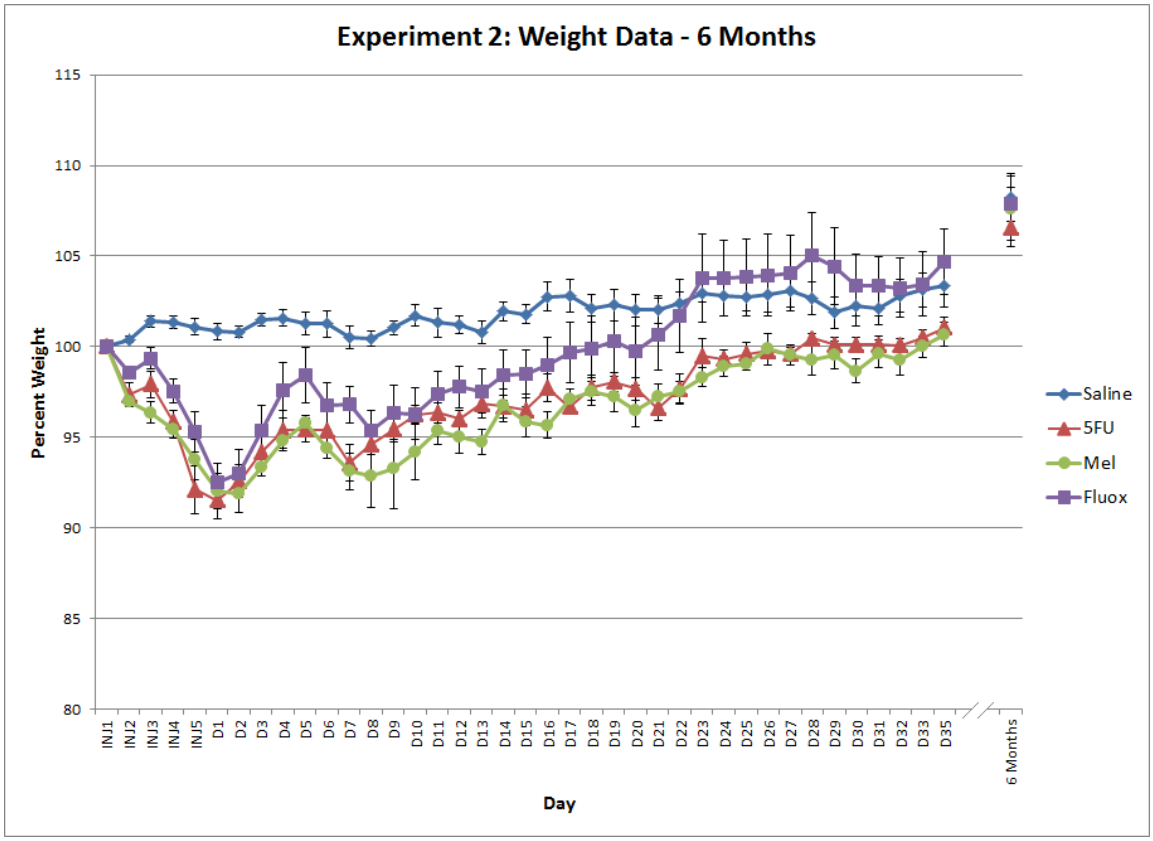


Figure 17: Mean % body weight (relative to pre-treatment weight) as a function of day for animals perfused at 6 months in experiment 2. Mice received IP injections of either saline (0.9% NaCl; n = 7), 5-FU (70 mg/kg; n = 7), 5-FU + melatonin (25 mg/kg; n = 6), or 5-FU + fluoxetine (12 mg/kg; n = 7) on days 1-5 (protectants on all 5 days, 5-FU on days 1, 3, and 5). Error bars represent ±1 standard error of the mean.

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