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THE LONG-TERM IMPACT OF CHEMOTHERAPY ON NEUROGENESIS AND
THE POTENTIAL USE OF FLUOXETINE AS PREVENTATIVE TREATMENT

by

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A Thesis Submitted with Partial Fulfillment
of the Requirements for a Degree with Honors
(Psychology)

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Abstract

The present study attempts to investigate a potential underlying mechanism that contributes to cognitive impairments associated with chemotherapy treatment. In addition, it looks to investigate the potential preventative treatment using an antidepressant agent (Fluoxetine) to reduce the effects observed by chemotherapy treatment. One proposed explanation for Chemo Brain is a reduction in the proliferation of new brain cells. This study was designed to investigate this mechanism and was conducted in a series of two experiments. In experiment 1, 37 C57BL/6J male mice were administered saline or one of two chemotherapy agents; Cyclophosphamide or Doxorubicin over the course of eight days. Neural tissue was examined at 56 days and 6 months post chemotherapy administration to evaluate the effects of chemotherapy on cellular proliferation. A two-way ANOVA was performed on the 56 day and 6 month data. Analysis revealed no significant effect of treatment at either time point. A significant effect of day was observed.

In experiment two, 41 C57BL/6J male mice underwent treatment of saline, the chemotherapy agent 5-FU and saline, or an administration of Fluoxetine followed by an administration of 5-FU. Similarly, cellular proliferation in the neural tissue was measured at 56 days and 6 months post chemotherapy administration. A two-way ANOVA revealed no significant effect of treatment at either time point but a significant effect of time was observed. Overall, our results indicate that further research is needed to fully understand the long-term impacts of chemotherapy on neurogenesis and the potential use of neuroprotectant Fluoxetine as a preventative treatment. While the results of this study do not directly support the hypothesis that chemotherapy agents are capable of altering neurogenesis, more work will be required to identify the conditions under which such effects do or do not occur.

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INTRODUCTION

Barbara D. Wick is entering her 6th year of remission from breast cancer. She underwent extensive chemotherapy as treatment for her cancer and assumed after treatment termination she would soon return to her pre-cancer life. Her new reality post-treatment, however, is starkly different.

“I need to write everything down immediately,” Wick described during an interview with the New York Times in 2013 “including appointments, doctors’ comments, ideas of things to do, and using more than one modality – listening and writing – reinforces my ability to remember the information.”. Even six years following the termination of her treatment, Wick continues to report cognitive impairments, particularly with focus and retaining information that were not present previous to her undergoing chemotherapy treatment.

Wick is not alone in a growing number of cancer survivors. Over the last few decades, cancer treatment and survivability post-treatment have improved drastically. Between early detection and improved treatment methods, survivability rates have improved by about 20% between 1975 and 2006. The American Cancer Society and National Cancer Institute estimate there to be 13.7 million cancer survivors in the US alone, with a projected 18 million by 2022 (Siegel, 2016). In other words, the number of patients surviving post cancer treatment is substantial and continuing to grow.

One common approach to the treatment of cancer is the use of chemotherapy. Chemotherapy, by definition, works by stopping the growth and development of cells in

the body. While this can be successful in limiting cancer, it is not exclusive to cancer cells and can negatively impact healthy cells as well. Because of this, chemotherapy brings with it its own host of side effects that can drastically impact a patient's quality of life post treatment. Among the reported side effects is cognitive impairment, known as "Chemo Brain" or "Chemo Fog".

Chemo Brain: Patient Reports and Cognitive Performance

Cognitive impairment following chemotherapy treatment, often called Chemo Brain or Fog, largely impacts working memory, processing speed, focus and attention. Up to 75% of people indicate cognitive impairment while undergoing chemotherapy (Janelins et al., 2014). Research surrounding Chemo Brain originated with patients self-reporting cognitive impairments following chemotherapy treatment. Initially, these complaints were largely dismissed as being a psychological phenomenon rather than neurological in nature. In the 1980s, researchers began to explore the impact of chemotherapy on cognition.

Researchers began investigating the impact of chemotherapy on cognition by using standardized tests. Silberfar et al. (1980) had subjects who were undergoing chemotherapy perform neuropsychological testing before and after treatment to evaluate its potential impact. They found chemotherapy patients had significantly lower cognitive scores compared to controls. With this finding, researchers continued to evaluate the effects of chemotherapy on cognition using a variety of assessment methods.

Early studies focused on evaluating patient's cognitive abilities shortly following chemotherapy treatment, mostly 6 months post treatment or less. This approach has a

variety of issues. Firstly, during the first few months following chemotherapy treatment, increased stress and fatigue levels may negatively impact performance on standardized tests compared to normative controls (Ferguson & Ahles, 2003). For example, 95% of Komaki and colleagues (1995) subjects had impairments on standardized neuropsychological assessment shortly following treatment termination. Similarly, Wieneke and Dienst (1995) found 75% of their subjects performed two standard deviations below published norms 6 months post treatment. In a third test, Janelins et al. (2014) report up to 35% of patients having cognitive impairment in both patient reports and standardized testing years post treatment. With these results and observations, researchers began to investigate further the potential long-term impact of chemotherapy treatment on cognition.

One of the first long-term studies was conducted in 1999 by Schagen and colleagues who assessed chemotherapy patients two years post treatment. Those receiving chemotherapy treatment performed significantly worse on neuropsychological tests (used to evaluate cognitive abilities) than controls. Similarly, Wefel et al. (2010) found cognitive decline in the domains of learning and memory, executive function, and processing speed one year post-treatment. Other studies have noted decreased performance in information processing and deficiencies in the executive control network one-year post treatment (Chen et al., 2014). Recently, a study conducted by Stouten-Keperman et al. (2015) compared cognitive brain function in breast cancer survivors of similar illness severity who underwent chemotherapy treatment before treatment, two years following treatment and 11.5 years following treatment. The patients were separated into three groups; one group was comprised of individuals receiving high dose

chemotherapy, one group received conventional dosing chemotherapy, and one group did not receive any chemotherapy treatment. Researchers had each group undergo neuropsychological testing prior to chemotherapy treatment, two years post treatment, and 11.5 years post treatment. These long-term effects can have a profound negative effect on the quality of life of survivors. Today, it is largely accepted that the symptoms of Chemo Brain can be long lasting (greater than 10 years) and can negatively impact life post treatment for survivors.

Although research concerning Chemo Brain has increased significantly since the 1980's, limited progress has been made to understand the mechanisms behind cognitive impairment in Chemotherapy patients. Many roadblocks have slowed progress; lack of longitudinal studies, major differences in experimental designs (making it hard to compare results) and a lack of control in patient treatment techniques are just a few of the challenges that researchers face (Vardy et al., 2007). Although progress has been made, many questions still stand.

Chemotherapy

Chemotherapy, at a basic level, works to prevent cellular proliferation (the process by which there is an increase in the number of cells as a result of cellular growth and division). Chemotherapy is separated into a variety of drug classes, depending on the mechanism behind how they prevent cellular proliferation. These agents include Alkylating Agents, Antimetabolites and Topoisomerase Interactive Agents, among others. Because these agents work by distinct methods their impact on the body varies between classes. It is important to note that although chemotherapy agents are classified

into drug classes, the individual agents within a class may impact the body in unique ways.

Alkylating Agents:

Alkylating Agents interact with one or both DNA strands to interfere with cellular division (Han et al, 2008). In particular, the agent forms covalent bonds with a singular DNA strand or causes a crosslink between the two strands (Seigers et al., 2011). This suppresses DNA transcription and inhibits cellular division. One unique aspect to Alkylating Agents is they trigger apoptosis (or programmed cell death) and cellular damage independent of the cell cycle. Cyclophosphamide is a commonly prescribed Alkylating Agent that works by inducing apoptosis by forming DNA cross-links.

Antimetabolites:

Antimetabolites work by replacing necessary metabolites with nonfunctional substitutes, resulting in the disruption of cellular growth and development. This class of chemotherapy drugs is cell cycle dependent and is only active during the “S-phase” of the cell cycle. Among antimetabolites, 5-Fluorouracil (5-FU) is the commonly studied. 5-FU works by replacing pyrimidine, a key structure of DNA. It also blocks thymidylate synthase, an enzyme used in the assembly of thymidine, a crucial molecule of DNA synthesis (Seiger et al. 2011).

Topoisomerase Interactive Agents:

Topoisomerase is a crucial enzyme in the regulation of DNA replication. It works by cutting the coiled DNA strands during replication to allow for the DNA to be copied

(Seigers et al. 2011). These agents work by inhibiting or changing the action of Topoisomerase, thereby limiting or stopping replication. One common example of this agents is Doxorubicin.

Blood-Brain Barrier:

The blood brain barrier is a system within the central nervous system that only allows certain substances to cross into the CNS. Polar molecules and gases can fairly easily cross this barrier. The ability of chemotherapy agents to cross the barrier is agent specific. Doxorubicin, 5-FU and Cyclophosphamide has been seen to potentially have the ability to cross the barrier (Christie et al., 2012, Seigers et al., 2011). By having the ability to physically access the brain, the chemotherapy agents may be able to have a more profound impact.

Combination Therapy:

Often, a combination of various chemotherapy agents is used to treat cancer. This treatment could include multiple chemotherapy agents from the same or different classes. Because this is a common approach to treatment, many researchers argue further models should include a combination of drugs rather than a singular drug in isolation. For example, cyclophosphamide (an alkylating agent) and 5-fluorouracil (an antimetabolite) are often used in conjunction with methotrexate (an antimetabolite). When evaluating the role of chemotherapy on cognitive processing, it is important to remember common clinical practice in treatment. The first step for researchers, however, is to understand their individual roles.

An important step in understanding the cognitive impairments expressed in the patient population is understanding the effects of chemotherapy on the brain. The extent of chemotherapy's role and the underlying mechanisms behind Chemo Brain are an area of debate (Fremouw et al., 2011). Chemotherapy treatment has been associated with reduced neurogenesis and cell proliferation in the hippocampus, reduced or damaged white matter, reduced survivability of neurons, neuroinflammation and the development of Reactive Oxygen Species. These effects are destructive and could help explain cognitive impairments seen with Chemo Brain.

Decreased Neurogenesis and Cell Proliferation

Within the hippocampus is a region known as the dentate gyrus. This area is a site of neurogenesis and the integration of the new neurons into the brain. The process starts with a Pluripotent Neural Stem Cell (NSC), which has the ability to develop into Neuron Restricted Progenitor (NRP) Cells. This developmental period is known as cellular proliferation. Cells then go into a weeklong period of differentiation, beginning to form key neuronal structures known as dendrites and axons. After 2-3 weeks, neurons enter the “survival phase” of development and the new neurons begin to receive excitatory input from other axons. By 4-8 weeks, neurons are more fully integrated into the hippocampal circuit and begin to look and act like fully mature neurons (Aimone et al., 2014).

The integration of maturing neurons is key to regulating normative cognitive functioning. After development starts in the dentate gyrus, new neurons travel to the granule cell layer. Interrupted migration and integration has been identified in pathological cognitive conditions including neurodegenerative disease (Aimone et al.,

2014). During proliferation and in the developmental steps following proliferation, cells appear to be very vulnerable to chemotherapy agents (Dietrich et al., 2006).

The process of neurogenesis is crucial to positive cellular maturation and growth. Studies have indicated that across classes, chemotherapy agents negatively impacts neurogenesis in rodents; Alkylating Agents (Yang et al., 2012; Christie et al., 2012), Antimetabolites (Seiger et., 2008, Yang et al., 2012, Dietrich et al., 2006), Topoisomerase Interactive Agents (Christie et al., 2012, Yang et al., 2012) and a combination of Cyclophosphamide, Methotrexate, and 5-Fluorouracil (Briones et al., 2011).

Researchers are able to measure neurogenesis by marking specific proteins during the cell cycle, the process of cellular division that allows for the duplication of cells. Ki-67 is a protein that is present during the active phases of the cell cycle, including the G₁, G₂ S phase and mitosis, but is absent from resting cells. Because of this, Ki-67 is used as an indicator protein of cellular replication and allows researchers to observe neurogenesis levels in the hippocampus.

The mammalian brain contains two sites of continuous new neuron incorporation; the olfactory bulb and the dentate gyrus (DG) region of the hippocampus (Aimone et al. 2007). The olfactory bulb houses migrated immature neurons that originate in the subventricular zone. These neurons go on to integrate locally and serve as interneurons (Aimone et al. 2007). The dentate gyrus is the site of origination and integration of new neurons and plays a role in memory formation (Aimone et al. 2007). The subgranular zone of the dentate gyrus houses a subset of neuronal progenitor cells that develop into granule cells, the principle neuronal projections of the dentate gyrus (Aasebo et al. 2011).

These cells go through a variety of developmental stages before finally integrating into existing hippocampal circuitry (Aimone et al. 2007; Aasebo et al. 2011). It seems that the distinct properties of new neurons are critical to memory formation. Among these are the properties of plasticity and hyperactivity.

Plasticity is in reference to the nature of development of new neurons. It is evident within the first week of development, new neurons do not display dendritic spine arborization and thus are unlikely to have an effect on information processing (Aimone et al. 2007; Aasebo et al. 2011). The next period (14-21 days), known as the “survival phase”, is the time point at which granule cells have dendritic spine arborization and mossy axonal projections that have the ability to synapse with information retrieval portions of the hippocampus (Aimone et al. 2007; Aasebo et al. 2011). This period is crucial to neurogenesis and is often referred to as the “critical period”.

Hyperactivity refers to a new neuron’s ability to induce long-term potentiation faster and with less input than mature neurons (Aimone et al. 2007; Aasebo et al. 2011). This is largely driven by the role of GABA receptors in new neurons. In fully developed neurons, GABA receptors are inhibitory. In new neurons in the critical period, however, GABA receptors act in an excitatory role largely due to the high concentration of chloride ions present (Aimone et al. 2007). In fact, granule cells during this time point do not have GABA receptors that are inhibitory and because of this will generate an action potential well before a mature neuron.

It seems the integration of these new neurons could potentially play a role in memory. While mature neurons are seemingly good at representing past encoded information, new neurons are seemingly better at encoding novel events (Aimone et al.,

2014; Aimone et al., 2011). In other words, new neurons are good at combining the “what and where” of new experiences and then encoding them into memory. The combination of immature and mature neurons is crucial for proper memory encoding (Aimone et al., 2011). This ability is important for day-to-day cognitive functioning, and a decrease in neurogenesis following chemotherapy treatment could negatively impact memory abilities, potentially contributing to Chemo Brain.

Various chemotherapy agents decrease cellular proliferation in the dentate gyrus. The majority of these studies observe declines in proliferation levels shortly following chemotherapy administration. For example, Mustafa et al. (2008) and Yang et al. (2012) noted decreases in hippocampal neurogenesis levels 12 days following 5-FU administration. In another study, decreased hippocampal cellular proliferation was noted 7 days post administration of Methotrexate (an antimetabolite chemotherapy agent) (Seigers et al., 2009). Christie et al. (2012) as well as Janelins et al. (2010) noted decreases in hippocampal neurogenesis levels one week following administration of Cyclophosphamide or Doxorubicin.

Few studies have looked at longer time points. Those studies include Dietrich et al. (2006) who noted significantly decreased cell proliferation in adult mice one day post administration of BCNU or Cisplatin (both alkylating agents) but also saw a continued decrease 42 days following the administration of chemotherapy. Even after 42 days, cell proliferation levels in chemotherapy-administered mice did not reach controls levels. Another study was conducted by Han et al. (2008) evaluated the effect of 5-fluorouracil on cellular proliferation. Proliferating cells immediately following treatment were decreased in the subventricular zone and were continually decreased below control levels

at the 56 day and 6 month time point post administration. These results indicate the potentially extensive long-term effects chemotherapy can have and highlight the need for further study.

Reduced or Damaged White Matter

White matter, also known as glial cells, are considered the support cells that help neurons in the brain. Glial cells start as a pluripotent neural stem cell and develop into a progenitor cell known as glial restricted pre-cursor cells. These cells go on to become astrocytes or oligodendrocyte-type-2 astrocytes. If the cells become oligodendrocyte-type-2 astrocytes, they then go on to become oligodendrocytes, the key component of myelin in brain cells. Myelination is a crucial aspect to cognitive functioning, helping increase processing speed down an axon.

Studies have indicated chemotherapy can reduce the survivability of oligodendrocytes (Dietrich et al., 2006). Researchers evaluated the effects of various levels of Cytarabine (an antimetabolite chemotherapy agent) on oligodendrocytes as well as cancer cells. Even at small doses, oligodendrocyte survivability dropped from 100% to about 60%. This profound impact was more damaging to oligodendrocytes than to cancer cells. These effects were also detrimental to oligodendrocyte progenitor cells. In other words, chemotherapy could not only be impacting the oligodendrocyte themselves, but also limiting the generation of new oligodendrocytes.

Other studies have noted a long-term impact of chemotherapy on oligodendrocyte precursor cells. Researchers evaluated BrdU labeled cells as a way to evaluate the division of the progenitor cells in the hippocampus. One day following treatment of 5-fluorouracil (an antimetabolite chemotherapy agent) had reduced division

of oligodendrocyte progenitor cells compared to controls. Fifty six days post treatment, this reduction was still present. (Han et al., 2008).

Other studies have found myelin to be significantly reduced following chemotherapy treatment (Seigers et al., 2011). In addition, damage to myelin integrity has been noted. For example, researchers have noted a reduction in myelin thickness as well as damaged and degraded myelin sheaths following chemotherapy treatment (Han et al., 2008; Briones et al., 2014; Deprez et al., 2011). Similarly, other studies have noted a decrease in myelin basic protein following chemotherapy treatment (Han et al., 2008; Briones et al., 2014).

Neuronal Survivability

Others have evaluated the survivability of neurons following chemotherapy treatment. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling, or TUNEL+, labeling is used to measure cells undergoing apoptosis, or cell death. Systematic chemotherapy with cytarabine has lead to prolonged cell death, even 56 days post treatment (Dietrich et al., 2006; Han et al., 2008).

In addition, other studies have used TUNEL+ to evaluate cell death at even longer time points. Even at six months post chemotherapy treatment, the percent of TUNEL+ cells in the central nervous system was still higher than controls (Han et al., 2008).

Reactive Oxidative Species

One final mechanism by which chemotherapy impacts the brain is by the production of Reactive Oxygen Species, or ROS. ROS is naturally in the body as a byproduct of breathing, but at elevated levels it can be damaging to the body. The body counteracts ROS with antioxidants; however, chemotherapy targets antioxidants and

decreases their production (Angsutaraux, P et al., 2015). High ROS levels, in addition to the reduction of their destroyers (antioxidants), can cause extensive cell damage.

Preventative Treatments

Antidepressants:

One approach researchers have begun to investigate is the use of preventative treatments to potentially help limit the impact of chemotherapy on the brain. One of these treatment options is antidepressants, in particular Fluoxetine. Fluoxetine falls under the category of selective serotonin reuptake inhibitors (SSRI's). Serotonin is associated with cognitive function and may play a role in cognitive ability (Seigers et al., 2011). In addition to increasing serotonin levels, SSRI's are associated with increased levels of neurogenesis and proliferation (Surget et al., 2012). Studies have indicated mice treated with fluoxetine for 21 days had increased levels of hippocampal proliferation (Wang et al., 2008). By increasing proliferation levels prior to treatment, it is hypothesized that the treatment could potentially help protect against or reverse reductions seen as a result of chemotherapy. Treatment with antidepressants has been seen to reverse these decreases in proliferating in other studies (Dranovsky & Hen, 2006), indicating it could potentially work similarly to combat the effects seen in Chemo Brain.

In addition to promoting neurogenesis and cellular proliferation, antidepressant may also work as a neuroprotectant to combat cognitive impairment seen in patients

undergoing chemotherapy treatment. One study found that rats undergoing 5-fluorouracil treatment had reduced neurogenesis in the hippocampus, while animals that received 5-fluorouracil while also taking fluoxetine displayed no significant decreases in neurogenesis compared to controls (ElBeltagy et al., 2010). It is important to note that the fluoxetine administration was given at the same time 5-fluorouracil was given. Similarly, another study found success with using fluoxetine as a pre-treatment for rats undergoing treatment with the chemotherapy agent methotrexate. Animals just receiving methotrexate showed decreased cellular proliferation within the dentate gyrus of the hippocampus as well as decreased cell survivability. Animals who received methotrexate in addition to fluoxetine showed no decrease in survivability or proliferation following treatment (Lyons et al., 2011). By giving chemotherapy patients an agent that increases neurogenesis and cellular proliferation, such as an SSRI, it potentially could help prevent the noted decreases in neurogenesis following chemotherapy treatment.

Gaps in Literature

It is evident that at short-term time points, many chemotherapy agents can negatively impact levels of neurogenesis. Few studies have investigated the long-term impact of chemotherapy on neurogenesis. Although there have been noted decreases in neurogenesis levels at 42 days following BCNU or Cisplatin administration (Dietrich et al., 2006), many common chemotherapy agents such as Cyclophosphamide or Doxorubicin have yet to be studied at similar time points.

5-FU has been seen to impact neurogenesis levels at the longer time points of 56 day and 6 months following administration (Han et al., 2008). The administration of

fluoxetine with various chemotherapy agents, including 5-FU, has been noted to prevent decreases in neurogenesis compared to animals receiving the chemotherapy alone when neurogenesis levels have been measured shortly after chemotherapy administration (Lyons et al., 2011; ElBeltagy et al., 2010). Researchers have yet to investigate how neurogenesis levels are impacted by the use of fluoxetine as a pre-treatment to the administration of chemotherapy such as 5-FU at long time points post chemotherapy administration.

METHODS

Experiment 1

Aim of Experiment 1

Studies have shown that chemotherapy agents can reduce neurogenesis and cellular proliferation within the hippocampus a few weeks post treatment (Dietrich et al., 2006; Han et al., 2008; Mustafa et al., 2008; Seigers et al., 2008; 2009; Janelins et al., 2010; Christie et al., 2012; Yang et al., 2012). Few studies have investigated the effects of chemotherapy at long-term time points as well as the effects of different classes of chemotherapy agents on neurogenesis.

At 1 week post administration, studies have found the chemotherapy agents cyclophosphamide and doxorubicin can reduce neurogenesis following treatment (Janelins et al., 2010; Christie et al., 2012). The long-term effects of these compounds on neurogenesis is largely unknown. In addition, the potential differences in timing effects of the two compounds is also unknown. This study looks to evaluate neurogenesis 56 days and 6 months post cyclophosphamide and doxorubicin administration.

Cyclophosphamide (an alkylating agent) and doxorubicin's (a topoisomerase interactive agent) long-term impact on hippocampal neurogenesis will be evaluated. Using the proliferation marker Ki-67, the study will be able to assess neurogenesis. Neurogenesis is not specific to just proliferating neurons, but also white matter. In the region we are assessing, however, nearly 90% of the proliferating cells are neurons. Early and late effects of the chemotherapy treatment at 56 day and 6 months post administration will be evaluated. At both 56 day and 6 months, I hypothesize that animals treated with cyclophosphamide or doxorubicin will have decreased amounts of proliferating cells within the dentate gyrus of the hippocampus compared to controls.

Methods and Materials Experiment 1

Subjects: 37 C57BL/6J male mice aged 8-9 weeks were obtained from Jackson Labs in Bar Harbor, Maine. The animals were acclimated to our animal colony for approximately two weeks prior to treatment. Mice were socially housed with 3-4 mice per cage in a 70°F room using a twelve-hour light dark cycle (7:00 – 19:00) with food and water as needed. Animals were randomly assigned to one of three treatment conditions: doxorubicin, cyclophosphamide or saline controls.

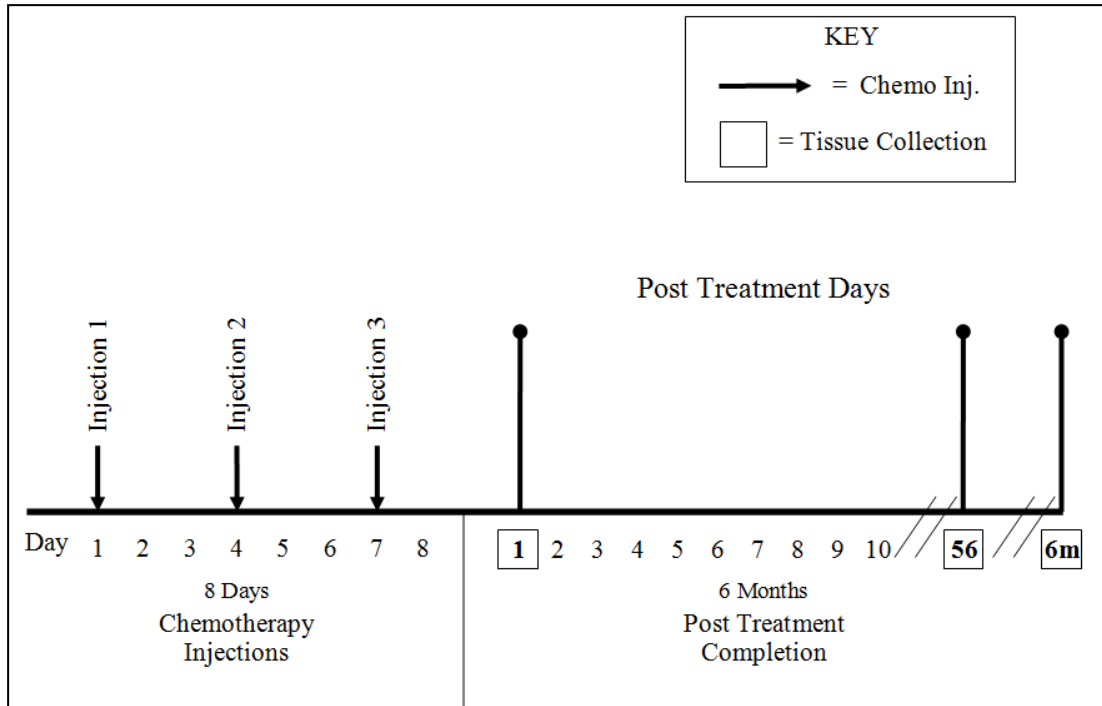


Fig. 1. Outlines methodology for experiment, including chemotherapy administration and tissue collection at specific time points.

Chemotherapy Treatment: Each mouse group received intraperitoneal injections over a period of 7 days (Figure 1). Cyclophosphamide (120mg/kg diluted in 0.9% saline; Acros Organics), doxorubicin (5mg/kg diluted in 0.9% saline; Fisher Scientific) or control saline (0.9% NaCl) were used (Figure 1). These injections were administered following the methods used in Janelson et al. (2010) on days 1, 4, and 7, totaling 3 injections for each animal. One major difference in our study, however, is the dosage amount. An increased dosage was used in an attempt to maximize potential long-term negative effects of the treatment on the brain. This amount was derived from tests with C57BL/6J mice and equaled the maximum amount the mice could survive in conjunction with typical patient dosages in humans. Because the effects to chemotherapy can vary as a function of circadian rhythm (Focan, 1995), mice were treated at approximately the same time, 8-9 hours post light onset. Weight was monitored daily for approximately 4 weeks, then

every other day until 56 days post treatment. For the 6 month group, mice were weighed once a week rather than daily after day 56. Teeth length was also monitored, trimming when necessary, as overgrowth can lead to weight loss.

Tissue Collection: Animals were sacrificed and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) at 56 days (6 saline controls, 6 cyclophosphamide, and 3 doxorubicin) and 6 months (8 saline controls, 9 cyclophosphamide, and 5 doxorubicin) following their last chemotherapy injection.

Brains were then extracted and submerged in PFA, then a 30% sucrose solution, then flash frozen. Six series of 40 μ m coronal sections were cut. Each single series tissue for an animal selection includes the entire hippocampus with approximately 10 section of tissue for each animal. The slices were then transferred into a 0.05% potassium phosphate buffered saline solution (KPBS), then moved into an antifreeze cryoprotectant and stored at -20°F.

Immunohistochemical Staining: An avidin/biotin-based peroxidase Vectastain Elite ABC Kit (Rabbit IgG) system (Vector Laboratories, Inc.) was used for immunohistochemical staining on prepared tissue. We utilized Ki-67 rabbit primary antibodies (Wako Pure Chemical Industries, Ltd.) with the Elite ABC kit for the detection of Ki-67 proteins. By using a titration series, we were able to identify the ideal Ki-67 antibody concentration for our tissues samples, following the Hoffman et al. (2008) recommended immunohistochemical staining method. After the tissue was selected, it underwent six, 10 minutes rinses with 0.05% KPBS to wash the preserved tissue. Afterward, it was placed in a 1% hydrogen peroxide, 0.05% KPBS solution for 15 minutes. Following this, the tissue was once again rinsed in 0.05% KPBS 4 times for 5

minutes each, 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 then was stored in the refrigerator (approximately 33°F) for 48 hours. Afterward, 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. After 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 underwent 3, 5 minute rinses each in 0.05% KPBS and then was rinsed again 3 times for 5 minutes each in 0.175% sodium acetate. The tissue then underwent the final 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 then air-dried mounted on microscope slides for 24 hours. Dried slides were dehydrated using graduated EtOH solutions (70-100%) and cleared with HistoClear (National Diagnostics Inc.). Following drying, the slides were then cover slipped using HistoMount (National Diagnostics Inc.) and dried again for 24 hours.

Tissue Analysis: The number of Ki-67 positive cells within 10 slices of the dentate gyrus of the hippocampus of each animal was counted by eye. This procedure follows standard unbiased stereological estimation protocol as defined by Noori & Fornal (2011).

Experiment 2

Aim of Experiment

This study is looking to evaluate the effects of the chemotherapy agent 5-FU on neurogenesis in the dentate gyrus of the hippocampus of mice. Han et al. (2008) found a decrease in neurogenesis in the SVZ region at 1 week, 56 days and 6 months post treatment, but only noted decreases in neurogenesis within the dentate gyrus at 14 days and 6 month. This study looks to repeat these findings evaluating neurogenesis levels in the dentate gyrus in mice at 56 day and 6 month following 5-FU treatment. In addition to evaluating the effects of chemotherapy alone, this study will also evaluate the effects of using fluoxetine as a pretreatment to 5-FU on neurogenesis levels. In the two time points the study will be evaluating the role the compound plays in preventing or protecting from deficits in neurogenesis following 5-FU treatment.

It is evident that Fluoxetine can boost neurogenesis (Ramirez-Rodriguez et al., 2011; Duman et al., 2000,2001; Malberg et al., 2000; Dranovsky & Hen, 2006; Czéh et al. 2007). Other studies have also found that fluoxetine can prevent or even restore deficits in neurogenesis when administered with 5-FU (ElBeltagy et al., 2010) at 1-2 weeks post treatment termination.

Research has yet to investigate the long-term potential protective abilities of neuroprotectants. Because of the given protective and restorative effects of fluoxetine, I hypothesize that at 56 day and 6 month post treatment, 5-FU administered that also receive a pretreatment of fluoxetine will have higher levels of cellular proliferation than those animals treated with 5-FU alone.

Methods and Materials Experiment 2:

Subjects: 41 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 (approximately 70°F) and light/dark controlled (7:00 – 19:00) environment with food and water as needed. Animals were randomly assigned to one of three treatment conditions: saline controls, 5-FU and Saline, or 5-FU and fluoxetine. Animals were administered fluoxetine through drinking water. The mice were housed by group to ensured they were receiving only their assigned treatment.

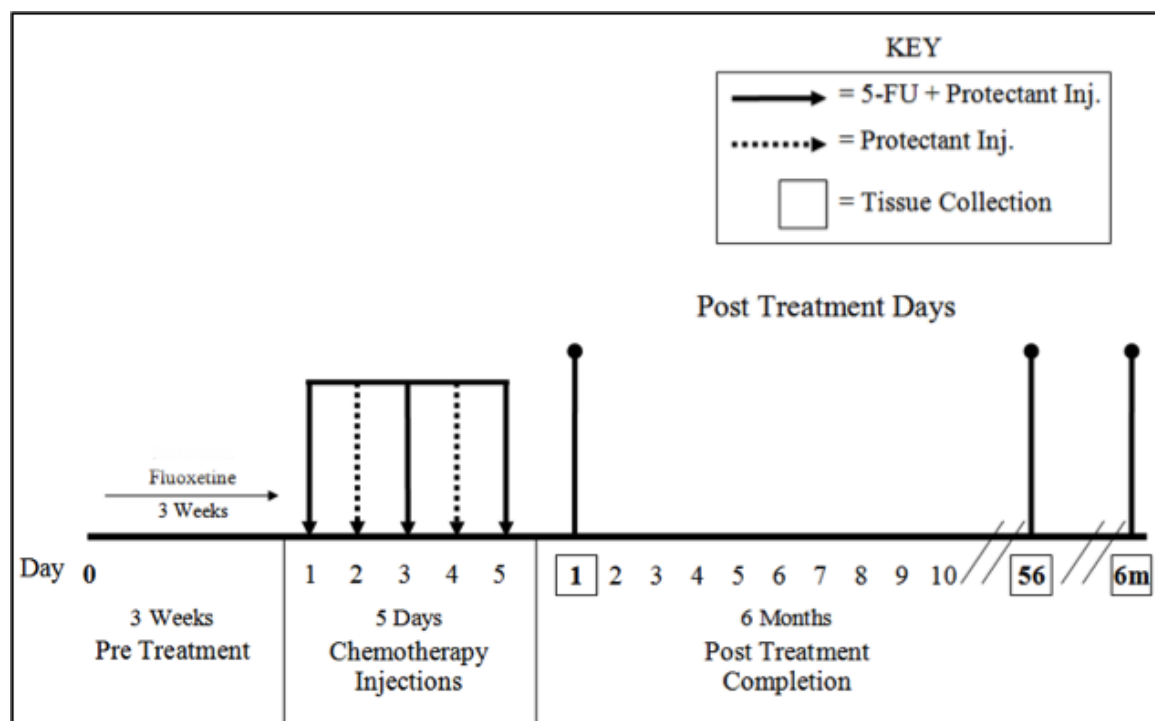


Fig. 2. Outlines methodology for experiment, including Fluoxetine pretreatment, chemotherapy administration and tissue collection at specific time points.

Neuroprotectant Water Treatment: As evident in Figure 2, the neuroprotectant fluoxetine was administered prior to the administration of 5-FU. The neuroprotectant was administered via drinking water, following the protocol outlined in ElBeltagy et al

(2010). There are a variety of benefits to administering fluoxetine via drinking water versus injection treatment. Firstly, it allows for long-term treatment, which allows our study to increase neurogenic levels prior to treatment with 5-FU. Secondly, it reduces unnecessary stress resulting from repeated injections. Every 4 days, water was weighted and changed as a way to measure water intake and ensure the target dose of fluoxetine was reached. The target dose for was calculated using previous studies that indicated peak neurogenic effects pretreatment from 10mg/kg to 15 mg/kg (Lyons et al., 2011; Lesemann et al., 2012). Other studies have utilized this 21-day timeline for water administration (ElBeltagy et al., 2012, Ramirez-Rodriguez et al., 2011; Dranovsky & Hen, 2006) prior to chemotherapy treatment. Water intake was calculated by the difference in averages of daily evaporation levels (3 control water bottles in empty cages were used) and bottle weight divided by average water consumed by amount of days and animal/cage. Figure 2 further illustrates the dosage timeline.

Chemotherapy and Neuroprotectant Treatment: Intraperitoneal (i.p.) injections were administered to mice in each group over 5 days (see Figure 2). Following procedure outlined in Han et al., (2008), 5-FU (70mg/kg diluted into 0.9% saline; Sigma-Aldrich) or control saline (0.9% NaCl) injections were administered on days 1, 3, and 5. Our study utilized maximally tolerated dosages for C57BL/6J mice calculated by previous dosage studies done within our lab. Due to the effects of circadian rhythm on toxicity and tolerance (Focan, 1995), our study treated mice at a similar approximate time (8-9 hours after light onset).

Although our study originally gave neuroprotectant treatment via water prior to chemotherapy treatment, during chemotherapy treatment we turned to injection

treatments for administration. Animals treated with chemotherapy often decrease their water intake as a result of their treatment. Because of this, our lab could not ensure the mice were receiving consistent amounts of neuroprotectants and we turned to injection treatment. Neuroprotection injections of fluoxetine (15mg/kg diluted into 0.9% saline; Sigma-Aldrich), or control saline (0.9% NaCl) were delivered 20 minutes prior to chemotherapy and once every 24 hours for a total of 5 injections per animal. Treatment timelines for the neuroprotectant and 5-FU is outlined in Figure 2. For 4 weeks, weight was measured daily. Afterward, mice were weighed every other day until day 56. Post day 56, the mice were weighed weekly. Teeth were trimmed when necessary for prevention of overgrowth which can result in 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 1

A two-way ANOVA revealed there was not a significant effect of treatment on mean proliferating cell amount [$F_{(2, 27)} = .067$, $p = .936$]. A two-way ANOVA indicated there was a significant effect of day on mean proliferating cell amount [$F_{(1, 27)} = 13.861$, $p = .001$] and there was no significant effect of the interaction of condition and day on mean proliferating cell amount [$F_{(2, 27)} = 1.232$, $p = .308$].

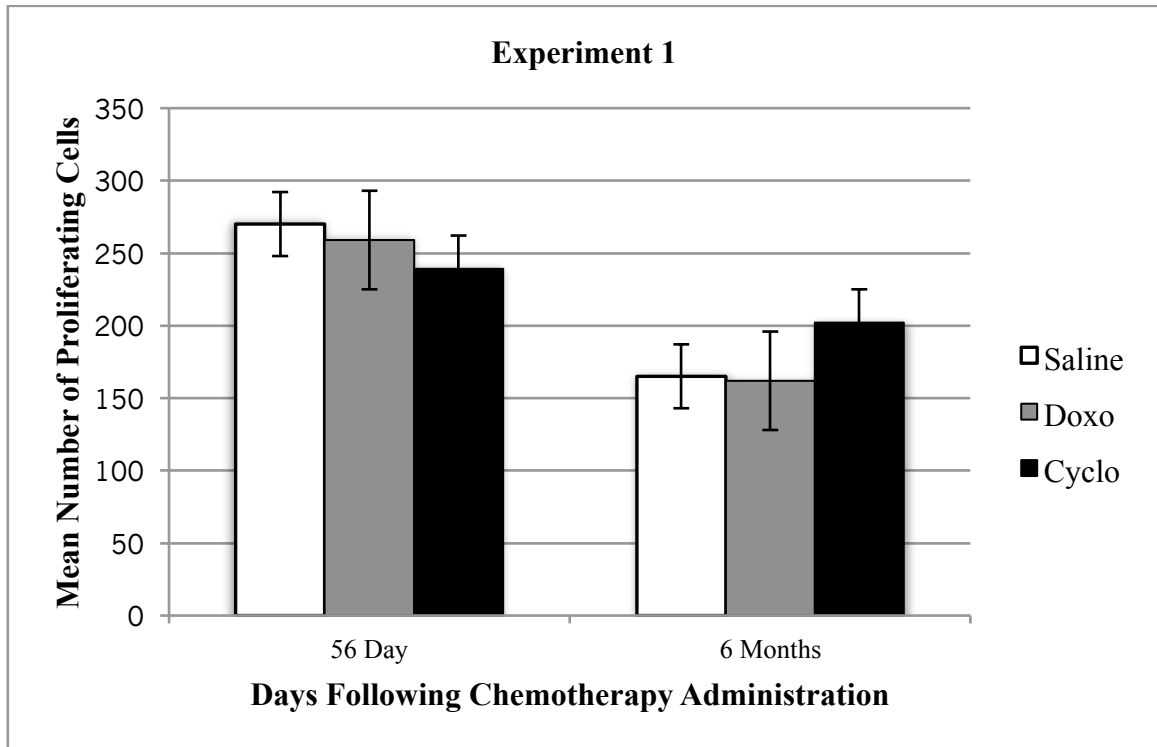


Fig. 3: Figure 3 shows the mean number of proliferating cells for 10 slices of the dentate gyrus of the hippocampus per animal as a function of treatment and days following chemotherapy administration. Error bars represent ± 1 standard error of the mean.

Experiment 2

A two-way ANOVA found no significant effect of treatment on mean proliferating cell amounts [$F_{(2, 33)} = .423$, $p = .659$]. There was a significant effect of day on mean proliferating cell amounts [$F_{(1, 33)} = 88.334$, $p = .000$] and no significant effect of treatment by day interaction [$F_{(2, 33)} = 2.238$, $p = .123$].

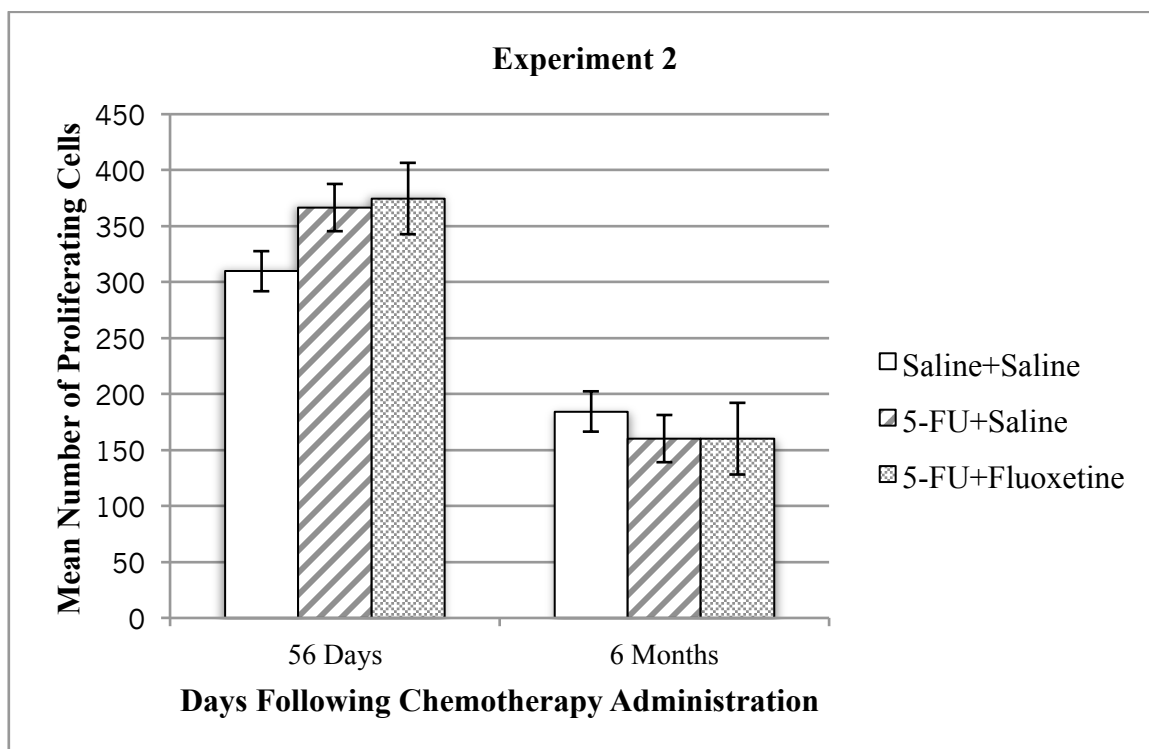


Fig. 4. Figure 4 shows the mean number of proliferating cells for 10 slices of the dentate gyrus of the hippocampus per animal as a function of treatment and days following chemotherapy administration. Error bars represent ± 1 standard error of the mean.

DISCUSSION

In the present study, two experiments explored the long-term impact of chemotherapy on neurogenesis as well as the potential use of a preventative treatment method. In the first experiment, a mouse model was used to investigate the impact of Cyclophosphamide and Doxorubicin on neurogenesis at 56 day and 6 months following administration termination. In the second experiment, the study looked to explore the impact of the protective treatment of Fluoxetine given prior to the administration of 5-FU on cellular proliferation in mice at 56 day and 6 months following treatment termination.

In experiment 1, I hypothesized observing a difference in mean number of proliferating cells as a function of Cyclophosphamide or Doxorubicin treatment, potentially seeing differences between the treatment methods. Although there was not a significant effect of Cyclophosphamide or Doxorubicin treatment on the amount of proliferating cells, there was a significant effect of day (56 days versus 6 months). These results are consistent with other studies which show a decrease in neurogenesis as part of the aging process. Studies have found that the ageing rodent brain has an exponential decrease in dividing cells between the age of 1 and 9 months (Seib&Martin-Villalba, 2015). The present study's results also indicate a decrease in neurogenesis over time, indicating our immunohistochemistry methods were largely successful and are not the source of why the study did not find an effect of chemotherapy on neurogenesis.

In experiment 2, I hypothesized seeing an increase in cellular proliferation levels in mice treated with protective treatment in addition to 5-FU compared to mice treated with 5-FU alone. Results indicated there was not a significant effect of the addition of protective treatment with the administration of 5-FU as compared to controls. Like experiment 1, there was a significant effect of day on neurogenesis levels (56 day versus 6 months). Although the results did not find a significant effect of treatment on the 5-FU animals, this alone cannot definitely say that 5-FU doesn't have an impact on neurogenesis at 56 day and 6 month time points. Other studies have noted decreases in neurogenesis following 5-FU treatment at similar time points, indicating the compound's potential to negatively impact the process (Han et al. 2008).

Our results measured how cellular proliferation was effected as a result of chemotherapy treatment. Although Ki-67 marking is used as a hypothetical measure of

neurogenesis, it only has the ability to identify actively dividing cells. Because of this, the use of an additional stain is necessary to adequately measure neurogenesis. One of the most commonly used stains is Doublecortin, which is nearly exclusively expressed in developing neurons and continually expressed for 2-3 weeks as the cells mature. The present study was unable to conduct double staining for this project due to time constraints but considers it an important aspect of future study on chemotherapy and its effects on neurogenesis.

Although these findings indicate there is a decrease in cellular proliferation as a result of time, they do not speak to the survivability of the newly generated cells. A key piece of survivability of neural cells is their integration into neuronal circuits.

Interruption of integration of these cells has been linked to disruptions in cognitive function (Aimone et al. 2014). It is possible that Cyclophosphamide and/or Doxorubicin could be more detrimental to the survivability of these cells rather than their generation. Other studies have noted a decrease in the survivability of developing neurons after undergoing chemotherapy treatment (Han et al., 2008; Dietrich et al., 2006). One way to potentially utilize a stain that measures the survivability of the newly generated cells.

It may also be beneficial in future study to better model the patient population. Studies could investigate the impact of having cancer while receiving chemotherapy treatment on neurogenesis levels. Furthermore, it may be beneficial to investigate the impact on female and elderly mice in future studies as well to better represent the patient population.

The study chose C57BL/6J mice due to their increased neurogenesis levels as compared to other strains (Kim et al., 2009). This allowed our study to more easily

identify changes in neurogenesis levels as a result of treatment. In comparison, other long-term studies such as Dietrich et al. (2006) and Han et al. (2008) used CBA strain mice. Although we did not observe any significant differences in cellular proliferation following treatment, it is possible that C57BL/6J mice are inherently better at protecting or repairing damages from processes that reduce proliferation. This strain could potentially not be effected by chemotherapy treatment in a way that other strains of mice with more normative levels of neurogenesis may be. This process of thinking can also be applied to the human population. It is possible that certain individuals are born with an ability to withstand damages to neurogenesis levels while others aren't. This could explain why only a subset of individuals experience symptoms of Chemo Brain. Further study of genetic differences among individuals and how this impacts neurogenesis is crucial to future study.

It is not likely that our methods of chemotherapy administration were the cause of our results. Dosages given to our mice were impacting the mice in such a way that it was evident the treatment was effective. In data that is not listed, mice in Cyclophosphamide, Doxorubicin and 5-FU groups from the present study all lost weight, a classic side effect of chemotherapy treatment. Mice that underwent cyclophosphamide administration in particular exhibited common side effects of greying hair and overgrowing of teeth. In addition to presenting side effects indicating the chemotherapy treatment was impactful, the study's dosages were consistent with other similar studies (Janelson et al. 2010).

The present study results are consistent with those recently published by Seigers et al. (2016). In their study, they found no significant effect of treatment of Cyclophosphamide, Doxorubicin or 5-FU on neurogenesis in the dentate gyrus of

C57BL/6J mice 3 weeks after treatment. These results are a shorter time frame, but are similar to our study's results indicating that chemotherapy agents may not significantly impact proliferation. The role of chemotherapy on neurogenesis and cellular proliferation may be less impactful than hypothesized.

It is possible that new neurons generated through neurogenesis do not play as big a role in the types of impairment that Chemo Fog patients display as originally thought. Although it is hypothesized that the combination of new and mature neurons are critical to working memory, it is entirely possible that memory processes are not majorly affected by losses in new neurons. Similarly, the integration of new neurons is often regarded as crucial to memory processes. It is possible that chemotherapy treatments do not affect the integration process, or effect neurogenesis at high enough levels to make a significant impact on memory.

Although many studies have researched the impact of chemotherapy agents on brain histology, few have investigated the time points that reflect the long-term or potentially permanent impairment the treatment can have. Although our results did not indicate significant effects of chemotherapy treatment on cell survivability, this is not to say that the chemotherapy agents in question do not affect cellular proliferation, neurogenesis, or survivability in all individuals or species. Looking ahead, it is necessary to utilize a double stain to better understand chemotherapy's specific impact on neurogenesis as well as its impact on survivability of neural cells. For example, one major difference in the present study and in Han et al. (2006) is the stains utilized to measure neurogenesis. Han et al. (2006) utilized a double stain of a stain known as Doublecortin and BrdU while we only used a Ki-67 stain. Further research is necessary to

understand how chemotherapy impacts the brain and how the symptoms of Chemo Brain can be prevented or treated.

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Author's Biography

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