5-2014

Long Term Effects of Chemotherapy on Cognition, Preventative Potential of Antidepressants

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LONG TERM EFFECTS OF CHEMOTHERAPY ON COGNITION;
PREVENTATIVE POTENTIAL OF ANTIDEPRESSANTS

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

The Honors College
University of Maine
May 2014

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Abstract

Each year, over 1.6 million people in the U.S. alone will be diagnosed with some form of cancer. With advances in treatment, survival rates have risen to nearly 65%. While remission and survival are the ultimate goals of treatment, it has become clear that many cancer survivors (estimates range from 15% to 70%) treated with chemotherapy experience significant, long-lasting cognitive impairment. This chemotherapy associated cognitive impairment is often called "Chemo Fog" or "Chemo Brain." For some, the effects are mild, such as having difficulty with focusing, concentrating, and speed of processing. For others, the cognitive impairments can be significant and may dramatically alter their day-to-day lifestyles, even preventing some from ever returning to work. Growing evidence suggests these impairments are sometimes permanent.

The present study attempts to identify the underlying mechanisms of Chemo Fog that contribute to impairment in learning, memory and processing speed, associated with chemotherapy administration. This study also investigates a potential antidepressant treatment that, when co-administered, may reduce the effects of chemotherapy drugs. Two proposed explanations as to why such changes follow chemotherapy treatment are; a reduction in the proliferation of new brain cells, and a decrease in the myelination of axons. A B6 strain of mice was used as a model for this investigation. Animals were administered the chemotherapy agent 5-Fluorouracil (5-FU) and five a known neuroprotectant (fluoxetine) over a period of 5 days. Neural tissue was examined to assess the effects of the treatment at 1 day, 14 days and 6 months post-treatment. A chi-squared test was used to analyze data from day 1 and day 14 tissue. A One-way Anova test was used to analyze data from 6-month tissue. Analysis revealed a significant
reduction in myelin at 1 and 14 days but not 6 months for individuals that received only 5-FU as compared to controls. Analysis also showed that animals treated with fluoxetine and 5-FU had myelin that was consistent with control animals for short-term time points (1+14 days) suggesting myelin preservation. Statistical tests showed no myelin protection for the animals treated with fluoxetine at the 6-month time point as their tissue was not statistically different from animals receiving 5-FU alone.
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INTRODUCTION

The issue of pharmacologically induced cognitive impairment associated with the administration of chemotherapy is one of great complexity. For decades, the neurological implications of cytotoxic drugs have been largely unknown, and only technological advances of the recent years have allowed scientists to begin the arduous process of unraveling the intricacies of the biochemical mechanisms involved. To progress from a comprehension of symptoms, to causes, to treatment requires a considerable breadth of knowledge, spanning many disciplines of biology, chemistry and psychology. This paper offers an overview of relevant background information, from the pathology of cancer, to animal behavioral testing, as well as analysis of research currently being conducted, which aims to delineate underlying processes as well as test potentially preventative treatments.

1. Cancer

Cancer, as defined by the National Institute of Health, is a disease in which abnormal cells divide rapidly and without control. These cells can invade other healthy tissues by traveling through the lymphatic and circulatory systems. Cancer is caused by a disruption in cellular mechanisms that generally regulate cell replication. Damage to DNA induces mutations that lead to changes in the way a cell functions. Often these mutations are deleterious and result in cell death. Other times these mutations interfere with the process of apoptosis, which is intended or programmed cell death. When this is
the case, cells do not die when they are old, damaged or obstructive. Instead they replicate continuously, forming masses, or tumors. Evidence suggests that adult stem cells in particular play an important role in carcinogenesis (Sell 2004).

Cancer is the second leading cause of death in the United States (cdc.gov). Each year, more than 1.6 million people will receive a cancer diagnosis, and the probability of an individual developing cancer throughout their lifetime is 40% (cancer.org). The most common forms of cancer are those of the prostate and breast. Although cancer is a prominent health concern, national trends are encouraging. Deaths associated with cancer have been declining since 2000 and current survival rates are near 65%. While treatment success is in part attributable to advances in technology, much can be accredited to public health education and the avocation of screening and early detection for at least some forms of cancer.

There are a variety of methods used in the treatment of cancer and often the exact regimen is dependent both on the type and location of the cancer, as well as characteristics of the patient. However, the most common methods of cancer treatment include surgery, radiation therapy or chemotherapy (Seigers et al. 2011). Frequently, the most effective treatments employ a combination of these three strategies. Most cancer treatments involve surgery at some point, particularly when tumorous cells are localized. When this is the case, individuals have an increased likelihood of curing their cancer.

Radiation is the most common form of chemotherapy treatment. Radiation therapy works to damage or destroy cancer cells by exposing them to high levels of energy originating from an exterior source, often in the form of x-rays. This is accomplished through damaging portions of the DNA associated with replication. Radiation affects
healthy cells as well, so it is the goal of this therapeutic technique to be as precise as possible. Unlike radiation therapy, chemotherapy works from within the body through drugs administered to disrupt cell replication.

2. *Chemotherapy*

Roughly one-half of cancer patients receive chemotherapy at some point during their treatment. Chemotherapy is administered intravenously and circulates throughout the body. The use of drugs as part of cancer treatment has its origins in the early decades of the 20th century (Papac 2002). The introduction of chemical weapons during the First World War provided some unexpected insight into the potentially therapeutic uses of mustard gases (sulfuric and nitrogenous). It was reported that individuals exposed to these gases had atrophies of lymphatic, testicular and bone marrow tissues, as determined postmortem (Papac 2002). In addition, cases of mustard gas poisoning produced individuals with lower levels of red and white blood cells (Papac 2002). A subsequent study in 1929 revealed that sulfur mustard was “anti-carcinogenic” (Papac 2002). These findings eventually lead to animal, and then clinical testing of nitrogen mustard on the suppression of tumors in 1942, the results of which showed unparalleled success. Following the incorporation of nitrogen mustard into clinical use, other alkylating chemotherapy agents have been developed, but the principals behind many of the current chemotherapy drugs have remained the same (Papac 2002). Contemporary cytostatic drugs can be broken up into a number of categories: alkylating agents, platinum-based, anthracyclines, and antimetabolites (Seigers et al. 2011). Variation in classification exists across different disciplines, however these categories are consistently accepted. Regardless of the mechanisms, traditional chemotherapy drugs function to disrupt
cellular growth by preventing cell division.

2.1 Alkylating agents:

The advent of chemotherapy began with alkylating agents as both nitrogen and sulfur mustards fall under this category (Litterman et al, 2013). Alkylating agents work to interfere with cellular division by interacting with one or both strands of DNA (Seigel et al. 2011). These drugs induce electron rich atoms to form covalent bonds with a single DNA strand in the case of monofunctioning alkylating agents, and cross links between the two strands in the case of bifunctioning alkylating agents (Seigel et al. 2011). Alkylating agents also result in the methylation of DNA bases (Litterman et al. 2013). The cytotoxic consequence results from the accumulation of these DNA lesions and while these drugs act non-specifically, they have their greatest anti-neoplastic effects on cells that proliferate rapidly (Litterman et al. 2013). The most well documented alkylating agent having effects on cognitive performance is cyclophosphamide (Seigel et al. 2011). Cyclophosphamide has been shown to reduce contextual memory and passive learning in mice (Seigel et al. 2011).

2.2 Platinum-based agents:

Platinum-based chemotherapy agents are often used in the treatment of epithelial malignancies including those of the lungs and bladder (Terada et al. 2013). Similar to alkylating agents, it is thought that cisplatin and other platinum-based agents form both interstrand and intrastrand cross-bridges within cellular DNA (Seigel et al. 2011). It is also believed that these drugs induce apoptosis through the disruption of
mitochondrial pathways by means of protein activation and increased oxidative stress (Terada et al. 2013). Little has been studied as to cisplatin induced cognitive changes (Seigers et al. 2011)

2.3 Anthracyclines:

Anthracyclines have a cytotoxic effect by inhibiting topoisomerase enzymes (Seigers et al. 2011). These enzymes are responsible for creating upstream conformational changes in unwinding DNA strands to reduce torsional strain resulting from supercoiling (Seigers et al. 2011). Blocking these enzymes results in accumulated DNA damage and cessation of replication. Anthracyclines do not readily cross the blood brain barrier. The blood brain barrier is a system within the CNS that works to allow only certain substance to cross from the circulatory system into the central nervous system. Typically molecules that are polar, as well as gases, can enter the brain relatively easily as they can pass through the polar lipid bilayer of both the capillary walls and the assisting astrocytes. Molecules are precluded from going in between these cells by “tight” junctions, a defining feature of the blood brain barrier. Therefore any molecule that is not a gas or is non-polar must be actively transported across these membranes, requiring a specific transporting protein to do so. Without this, molecules have very little success exiting the capillaries within the CNS. Anthracyclines are able to pass into the endothelial cells that line the capillaries in the brain, but are not able to go further as they are a substrate that is actively transported out of the CNS by p-glycoprotein (P-gp) mechanisms (as are many common cytotoxic drugs)(Christie et al. 2012). The most well documented anthracycline having cognitive effects is doxorubicin, associated with
impaired performance on the novel place recognition task and contextual memory tasks in rats (Christie et al. 2012). Doxorubicin is also associated with increased oxidative stress.

2.4 Antimetabolites:

Chemotherapy drugs of this kind interact with nucleic acids in the DNA sequence and interfere with DNA and RNA synthesis (Seigers et al. 2011). One of two most often studied antimetabolites in the context of cognition is 5-FU (Seigers et al. 2011). This compound is a pyrimidine analogue (Kareem et al. 2012), meaning its structure is similar a class of nucleic acids, pyrimidines. 5-FU works primarily by blocking thymidylate synthase, an enzyme responsible for assembling thymidine, a crucial molecule for DNA synthesis (Seigers et al. 2011). 5-FU, along with another antimetabolite methotrexate, have been shown to reduce performance in spatial MWM as well as delayed and non-delayed non-matching to sample learning (Winocur et al. 2006). 5-FU is one of several drugs that are able to pass through the blood brain barrier with at least some degree of success. 5-FU is structurally similar to a molecule that is actively transported into the CNS and is therefore able to enter the brain.

Traditional cytostatic drugs are nonspecific and effect cells that proliferate rapidly (Littermen et al. 2013), including lymphocytes, epithelial cells lining the gastrointestinal tract, and hair cells. This helps to explain some of the adverse side effects associated with chemotherapy. Nearly all chemotherapy treatment is associated with immune suppression in part due to reduced white blood cell production within the bone marrow,
but also due to its effects on lymphatic tissues. Cytostatic agents have been found to reduce the magnitude and quality of T and B-lymphocytes to an antigen (Littermen et al. 2013). T-cells and B-cells work cooperatively in conjunction with other facets of the immune system to protect the body from foreign substances. B-cells are referred to as signaling lymphocytes, as they produce specific antibodies that mark antigens for destruction (Littermen et al. 2013). T-cells can be differentiated and serve several functions, one of which is to destroy antigens marked by antibodies. These cells proliferate in the presence of antigens (Littermen et al. 2013) and a reduction of this proliferation as a result of cytostatic drugs would reduce the body's ability to respond to external pathogens and viruses. Other common side effects of chemotherapy include nausea/vomiting and hair loss, both again in part to the anti-neoplastic mechanisms of chemotherapy agents.

3. Chemo-fog

In addition to the physical side effects of chemotherapy, cognitive side effects are widely reported and well documented. Clinical research and self-reports suggest that anywhere from 15%-75% of individuals receiving chemotherapy treatment experience some sort of cognitive impairment (Christie et al. 2012) and that for a subset of those effected (17%-34%), impairments are persistent and long lasting (Ahles et al. 2007). This phenomenon is often referred to as “chemo-fog” or “chemo-brain” and effects can manifest over a range of cognitive functions. Most commonly affected are the domains of working memory, executive functioning, processing speed and attention. Changes in these functions are typically mild and do not dramatically interfere with cognitive tasks.
(Ahles et al. 2007). In other instances, impairment can be detrimental, substantially altering daily function and significantly reducing an individual’s quality of life (Fremouw et al. 2011). It is important to note that while working memory is impaired, the retrieval of stored memories remains unaffected (Ahles et al. 2007) and that this could provide a clue as to the pathways disturbed. The extent to which chemotherapy alone is responsible for cognitive changes in cancer survivors continues to be an area of debate, as too are the underlying biological mechanisms that could bring about chemotherapy induced cognitive alterations (Fremouw et al. 2011). It is likely that extraneous factors play a role in the impairment of cognition for those receiving chemotherapy. Emotional stresses associated with diagnosis, treatment and disease affliction as well as the presence of cancer have been suggested to depress cognitive abilities (Fremouw 2011). Additional variables such as genetics, environment and development further confound the origins of cognitive decline in cancer patients. Despite ambiguity in clinical settings, several hypotheses have been developed to explain the cognitive effects that may be attributable to chemotherapy, and subsequent animal research has begun to shed light on processes that have previously been poorly understood.

Recent studies as cited by Ahles et al. 2007 have been able to show structural changes in the brain that correlate with cognitive impairment in human patients. Furthermore these changes appear in areas (such as the prefrontal cortex) that are known to mediate the process of working memory as well as structures associated with processing speed (white matter) (Ahles et al. 2007). Functional magnetic resonance imaging has allowed researchers to identify decreased activation in associated pathways, potentially resulting in an impairment of functions dependent on such structures.
Additionally, immunohistochemical staining of post-mortem patient tissue has shown damage to areas of the hippocampus following radiation and chemotherapy treatment (Monje et al. 2012). Unfortunately, the same contemporary studies have shown no structural abnormalities in subsets of patients that exhibit significant cognitive impairment (Monje et al. 2012). This suggests that more subtle processes must be affected that do not necessarily propagate physical alterations but induce a significant physiological change.

In an attempt to investigate the molecular mechanisms at play in the impairment of cognition, a substantial amount of research has utilized a rodent model in experimental construction. By doing so, researchers can avoid many of the confounds associated with human subjects as well as the experimental constraints imposed by human testing. Rodents allow for the investigation of chemotherapy without the presence of cancer, while additionally giving experimenters the ability to control for genetic factors, normalize environmental conditions and analyze tissue postmortem at desired time points. The translation between animal research and clinical application is not entirely straightforward however. In humans, much of the cognitive impairment experienced by chemotherapy treated patients is associated with changes in cortical regions, while research using rats and mice has found the hippocampus as being the major structure that mediate the processes of learning and working memory (Evenden 2013). While less is known about the role of the hippocampus in working memory tasks in humans, there is some clinical evidence that suggests that it not only play a role in memory consolidation, but also in working memory (Squire 1992), in particular the processing of verbal, spatial and recognition memory (Mustafa et al. 2008). This has directed much of the focus of
animal chemotherapy research to investigate the hippocampus. In addition to being involved in the formation of memories, the hippocampus is a structure of interest because it is the site of life long neuronal cell regeneration referred to as neurogenesis. Research has identified a population of neuronal stem cells within the hippocampus (specifically the dentate gyrus) that contribute to the formation and integration of new neurons in this location (Monje et al. 2012). In addition, it is possible migrating neurons developed from hippocampal neurogenesis may become integrated in forebrain structures (Monje et al. 2012). Neurogenesis in the hippocampus seems to be directly related to memory. Studies have shown that animals with decreased neurogenesis perform worse on hippocampal-mediated tasks and exhibit learning impairment (Seigers et al. 2011). Shor et al. 2001 demonstrated that a reduction in newly generated neurons leads to impairment on trace memory in rats, and that a subsequent recovery in cell production restores performance. In addition, animals reared in conditions thought to increase neurogenesis, such as high levels of exercise, or an enriched developmental environment exhibit an increased capacity for learning (Monje et al. 2012).

In addition to hippocampal neurogenesis, research suggests that a reduction in myelin also contributes to cognitive impairment following chemotherapy treatment. Recent studies with breast cancer survivors who received chemotherapy as part of their treatment regimen found an association between reported cognitive impairment and reduced integrity of myelin structures (de Ruiter et al. 2012). The role of myelin in support of proper cognitive function is further substantiated by literature investigating the effects of aging on the brain. Researchers in Madden et al. 2012 reported that age-related decreases in white matter integrity contributes to a reduction in communication
efficiency between networks, leading to general impairment in cognitive abilities.

In both human and animal studies, there are two methods of assessing the cognitive effects of chemotherapy. One is through behavioral quantification. In humans, this can be administered as a questionnaire to assess changes in cognition, or clinical testing in which participants abilities are actively tested. In animal studies, behavioral measures of cognition (particularly those mediated by the hippocampus) are often quantified through the administration of tests such as the Morris water navigation test or the object location recognition test.

The second method for detecting the effects of chemotherapy on cognition is through the analysis of histological data. Human studies frequently make use of imaging techniques such as FMRI and DTI that allow researchers to externally analyze brain structures of interest while patients are still alive. Comparatively, rodent studies often employ methods that apply stains directly to relevant tissue post-mortem. In this study, we utilized two such stains to quantify histological changes in neurogenesis and myelin density.

4. Staining

As mentioned, research that investigates the phenomena of chemotherapy induced cognitive impairment can do so in two ways, behaviorally and histologically. However research is most complete when behavioral changes in the presence of chemotherapy can be associated with anatomical changes in the areas of the brain responsible for coordinating such processes. In the case of the present study, two such staining techniques were employed to investigate changes in myelin and neurogenesis.
**Myelin:** There are several different methods of staining myelin, but perhaps the most popular and straightforward approach involves the use of gold salt compounds (Schmued et al. 2008). The most contemporary gold based salt compound, and the one utilized in this study is Black Gold II. When dissolved in solution and applied to white matter tissue, Black Gold II is taken up by the myelin surrounding axons. Though it is not known why this occurs, the result is a permanent reddish brown staining of axon bundles that can be clearly seen under an illuminating field (Schmued et al. 2008).

**Neurogenesis:** In contrast to myelin staining using Black Gold II, the staining for neurogenesis is much more time consuming and labor intensive as it involves immunohistochemical processes. The most fundamental component of immunohistochemistry is the specificity of anti-bodies to antigens. The process begins by isolating a certain protein that you wish to amplify. In the case of neurogenesis, one such protein is Ki67. Ki67 is a nuclear protein expressed transiently, during the G₁, G₂ and S phases of cell division and is therefore an indicator protein of cellular replication. Once the protein has been isolated, it is transposed into a host organism (often rabbits are used). The host animal’s immune system recognizes the injected protein as a foreign substance, and the subsequent response produces antibodies that are specific to that protein. These antibodies can then be isolated and extracted, and placed back into the original animal. When the induced antibodies are injected into the animal from which the antigen originated, these antibodies will bond to those proteins in the animal. In the case of Ki67, antibodies will only bind to cells that are actively dividing, as they are the only ones expressing that protein. Once antigens signal the presence of the protein of interest,
phosphorescent indicators can be injected. These indicators have previously been engineered to bind to the antibody-signaling molecule, and can be seen using phosphorescent scanning technology. Through this process, it is possible to stain for cells that are actively dividing and allow research to observe levels of neurogenesis within the hippocampus.

5. **Chemotherapy and cognition**

It has been well established that chemotherapy is related to a decline in cognitive function for both humans and rodents. Additionally, we have established two different methods at identifying these effects. That being said, to truly justify our motivation for this study, it is necessary to further understand the modes by which chemotherapy brings about these changes, thus supporting the association between cognitive impairment and chemotherapy. Because chemotherapy drugs are cytotoxic, preferentially targeting cells that are actively dividing, it is to be expected that they would have an effect on neurogenesis and oligogenesis if given direct access. Indeed these predictions are supported in a number of research studies. Seigers et al. 2011 found that the administration of Methotrexate (MTX) in mice significantly decreased levels of cell division in the subgranular zone of the denate gyrus for as long as 3 weeks after treatment. Dietrich et al. found similar results using other common cytotoxic agents, increasing cell death in the corpus callosum and denate gyrus up to 10 days after treatment, and decreasing cell division up to 6 weeks post treatment. MTX is a chemotherapy agent known to cross the blood brain barrier, however doses used by Seigers et al. 2011 (meant to represent those used in adjuvant chemotherapy treatment for
breast cancer) are thought to not cross the blood brain barrier successfully. This, combined with clinical evidence of cognitive impairment following treatment with chemotherapy agents unable to cross the blood brain barrier, and sustained cognitive impairment after treatment cessation, suggests that additional mechanisms disrupting neurogenesis must be present, other than direct cytotoxic effects. In addition, cognitive impairment is not seen in 100% of patients who receive chemotherapy. This requires further consideration when hypothesizing the underlying mechanisms, taking into account genetic variability.

6. Mechanisms

Despite the accumulation of research over the last several decades, a consensus as to the precise mechanisms propagating cognitive change remains elusive. The most robust theories attribute decline to a number of potential mechanisms that interact to cause an effect. Though many chemotherapy drugs have little success crossing the blood brain barrier, two common therapeutics agents, 5-FU and methotrexate, appear to be exceptions (Ahles et al. 2007). In the case of these two drugs, it is likely that their main impact results from direct anti-mitotic activity, as is their effect systemically. While other chemotherapy drugs commonly used such as cisplatin, BCNU and paclitaxel are not thought to pass through the blood brain barrier, positron emission tomography (PET) studies have shown low (but detectable) levels of these drugs in the CNS following intravenous administration (Ahles et al. 2007). So, while these drugs are not effective at treating tumors within the brain, their presence at low levels may still effect populations of dividing neuronal and glial cells (Ahles et al. 2007). If this is the case, drugs not
thought to enter the CNS by the criteria of being ineffective at treating cerebral tumors, may in fact be present at very low concentrations, and these concentrations may be enough to have direct cytotoxic effects on neurogenesis and oligogenesis. As mentioned before, many common chemotherapy drugs cannot enter the brain as they are substrates of the P-gp protein pump, which is responsible for pumping out exotoxins from the endothelial cells of the CNS capillaries of the blood brain barrier (Ahles et al. 2007). Included among these are cisplatin, BCNU and paclitaxel (Ahles et al. 2007), suggesting there must be variability in the effectiveness of this particular efflux protein pump. In fact, several polymorphisms of the gene that codes for the P-gp have been identified, and of these, individuals possessing the T allele show lower levels of P-gp expression and are more susceptible to long term cognitive impairment (Ahles et al. 2007). This suggests that individuals who have lower levels of P-gp expression are exposed to higher levels of chemotherapy drugs not thought to pass the blood brain barrier. This has been supported by animal studies conducted by Muramatsu et al. 2004 in which P-gp deficient mice had greater cerebral levels of the chemotherapy agent vincristine following peripheral administration as compared to controls.

While many chemotherapy drugs are designed to cause DNA damage to replicating cells as treatment for cancer, they are also associated with increased levels of unbound iron, free radicals (H+ protons) and decreased antioxidant activity (Ahles et al. 2007). These changes, in addition to exogenous toxins and endogenous metabolic by-products increase levels of oxidative stress. Similar to the antimitotic effects of some chemotherapy, oxidative stress induces DNA damage through the breaking of single and double strand bonds (Ahles et al. 2007). In addition to bond breakage, oxidative stress
(along with chemotherapy and genetic variation) accelerate the shortening of telomeres. Telomeres can be thought of as protective caps on the ends of chromatids. Under normal conditions, telomeres are shortened by roughly 20-200 nucleotides following each replication (Ahles et al. 2007). Generally, telomere shortening is believed to play a role in the process of aging. While most neuronal cells do not replicate, and therefore are not susceptible to telomere shortening, those involved in neurogenesis and glial genesis would be susceptible (Ahles et al. 2007). The shortening of telomeres offers an additional mechanism by which cognition is impaired by chemotherapy as well as a candidate mechanism for long-term effects, either by its direct effect on cerebral cell telomere length, or by a general acceleration of the aging process.

Further adding to the variability with which cognition is affected by chemotherapy are genetic differences in DNA damage repair mechanisms (Ahles et al. 2007). It is likely that at least a portion of cognitive impairment can be attributed to reduction in neural cell proliferation induced by DNA damage. Polymorphisms in the ability to successfully repair DNA damage caused by chemotherapy and oxidative stress, would likely lead to some individuals with a reduced capacity to recover from the consequences of CNS exposure to cytotoxic drugs (Ahles et al. 2007). Interestingly, these same individuals may have a predisposition to developing cancer in the first place as they would also have a reduced ability to repair random DNA mutations that are the initial cause of cancer (Ahles et al. 2007).

A final mechanism by which chemotherapy may impair cognition is by way of cytokine deregulation and neuroinflammation. Cytokines are signaling molecules released as part of an immune response, triggered by a number different events, among
them DNA damage induced by chemotherapy. Cytokines play a role in the regulation of certain neuronal processes, including glial function, repair, and metabolism of certain neurotransmitters (Ahles et al. 2007). However, deregulated, prolonged elevation of cytokine levels is associated with cognitive impairment via neurotoxicity or the induction of fatigue and depression (Ahles et al. 2007). The implications of cytokine mediated cognitive decline are crucial for several reasons.

One, cytokines offer an alternative explanation as to how drugs that are thought to not pass through the blood brain barrier still have an effect on cognition. Though some studies suggest that agents other than methotrexate and 5-FU can enter the CNS (Ahles et al. 2007), it is possible that they do so in concentrations too low to affect cerebral cell proliferation directly. If this is the case, their effect must originate peripherally. Ahles et al. 2007 suggests that cytokine levels outside of the CNS can have an effect on levels within the CNS, either by transport across the blood brain barrier, or through the central release of cytokines by way of stimulation of the vagus nerve by peripheral cytokines (Ahles et al. 2007). This suggests that high circulating levels of cytokines as a response to chemotherapy induced cell damage in areas outside the CNS, could elevate levels within the CNS despite the absence of the agent.

The second crucial implication of cytokine dependent cognitive decline is the potential explanation of long-term cognitive impairment. DNA damage resulting from chemotherapy induces an immune response of cytokine release. Whether this damage is in the CNS or not, communication between peripheral cytokines and central cytokines raises levels within and outside the CNS, while creating system-wide inflammation. Elevated levels of cytokines within the brain, as well as prolonged neuroinflammation,
produce neurotoxic effects on dividing cells directly through glutamate receptor-mediated damage or by increasing oxidative stress (Ahles et al. 2007). These effects further increase DNA damage (or initiate if they’re a response to peripheral cell damage) in dividing neuronal cells, which in turn sustains centrally elevated cytokine levels even after treatment is discontinued.

Though all aforementioned mechanisms provide potential explanations as to how chemotherapy can affect neurogenesis and white matter integrity, it is likely that the true process involves a combination of these and others yet to be hypothesized. A number of factors must be taken into account including the type of agent, genetic variations and age when considering the overall affect of chemotherapy on cognition as it pertains to neurogenesis and oligogenesis.

To complete our motivation for this study, we must consider not only how chemotherapy affects neurogenesis and myelin integrity in the brain, but also why affecting these aspects of cranial anatomy would impair cognition.

### 7. Neurogenesis and Memory Formation

Neurogenesis appears necessary for normal hippocampal functioning and certain types of memory formation as demonstrated through the use of anti-proliferating agents that decrease neurogenesis and cause memory impairment (Mustafa et al. 2008). Less clear however, is the way in which neurogenesis facilitates memory formation. In the mammalian brain, new neurons are continuously incorporated at two locations (Aimone et al. 2007). The first is the olfactory bulb, to which immature neurons born in the sub-ventricular zone migrate. These developing neurons integrate locally and serve as
interneurons (Aimone et al. 2007). The second location, the denate gyrus (DG) region of the hippocampus, is home to both the origination and integration of new neurons and it is this location that is involved in memory formation (Aimone et al. 2007). Within the subgranular zone of the denate gyrus, a population of neuronal progenitor cells divide, giving rise to granule cells (GC), which act as the principle neuronal projections of the denate gyrus (Aimone et al. 2007, Aasebo et al. 2011). Granule cells undergo several stages of development before being integrated into existing hippocampal circuitry (Aimone et al. 2007, Aasebo et al. 2011). The proportion of developing neurons to those that make up the denate gyrus is very low however, roughly 3% (Aasebo et al. 2011). This further complicates the role of neurogenesis in memory formation, as it is counterintuitive to accept that such a small percentage of neurons can have a robust effect on memory. To fully understand the function of the denate gyrus and the hippocampus in memory formation, it is necessary to reject the notion that these structures are static. Instead, these subcortical regions must be viewed as fluid and that their role is dependent on the constant addition of maturing neurons. Part of solution lies in unique properties of maturing neurons compared to those already incorporated into neuronal pathways. Perhaps most importantly among them are the properties of hyperplasticity and hyperactivity (Aimone 2007, Aasebo 2011).

Hyperplasticity: Maturing granule cells in the denate gyrus undergo several stages of development that are gradient rather than distinct (Aimone et al. 2007). In the rodent brain, new neurons do not show dendritic spine arborization and are believed to have little effect on information processing during the first week after their birth (Aimone et al.
Aasebo et al. 2011 further support this notion as they found that ablation of neurons <7 days of age showed no effect on behavioral measures of memory. In a period that spans the next 14-21 days, maturing granule cells exhibit robust dendritic spine arborization as well as mossy axonal projections that begin to synapse with information retrieval portions of the hippocampus (ca1 and ca3 regions) (Aimone et al. 2007, Aasebo et al. 2011). It is this period that appears to be key in the function of neurogenesis and has thus been labeled the “critical period”. In addition to rapid arborization, granule cells in the critical period show transient expression of NR2B- NMDA glutamate receptors (Deng et al. 2010). This occurs as dendritic projections reach the molecular layer of the dentate gyrus and are exposed to a glutamatergic environment (Deng 2010). It is unclear whether this glutamate receptor sub-type plays an instructive or permissive role in the maturation of neurons, however they are associated with neuronal plasticity during the critical period of development.

Hyperactivity: In addition to the property of hyperplasticity, granule cells in the critical period of maturation are also characterized as being hyperactive. This means that these neurons can be induced to show long-term potentiation (LTP) faster and with less input than fully mature neurons (Aimone et al. 2007, Aasebo et al. 2011). This primarily comes from the reversed role of GABA receptors in these neurons (Aasebo et al. 2011). During the critical period, GABA receptors act in an excitatory role due to the high concentration of chloride ions in the young neurons (Aimone et al. 2007) until about the third week (Aasebo et al. 2011). In adult neurons, GABA receptors are inhibitory. Generally, neurons are excited through the culmination of thousands of input signals,
which can be either excitatory or inhibitory depending on what neurotransmitter is released at the synapses, and which receptors are activated. If incoming signals activate more excitatory receptors than inhibitory receptors, an action potential will be generated. Because granule cells in the critical period do not have GABA receptors that are inhibitory, they will generated an action potential well before an adult neuron receiving identical input. This characteristic is retained even after GABA receptors transition from excitatory to inhibitory at around 3 weeks (Deng et al. 2010), due in part because this transition coincides with the initiation of glutamate (an excitatory neurotransmitter) exposure to dendrites as they reach the molecular layer of the dentate gyrus (Deng et al. 2010).

These properties of young neurons appear to be intimately related to the process of memory formation and the encoding of information. As mentioned previously, certain environmental conditions and behaviors are thought to increase neurogenesis and improve dependent memory functions. Initially, experience and information processing appear to support maturing neurons and distinguish those that will survive to be incorporated, from those that die off (Aasebo et al. 2011). This is thought to be mediated through the activation of the NMDA-glutamate receptors, which then propagate further glutamate synapse and dendrite spine formation within activated neurons (Aasebo et al. 2011). The notion that experience and information input regulate the maturation of neurons helps to explain not only why proper memory formation requires the survival of only some new neurons, but also how new neurons facilitate this process.

The exact process by which the hippocampus encodes memories and the role of
the denate gyrus/neurogenesis in this process is still up for debate. One potential factor could be the location of the denate gyrus in relation to hippocampal pathways (Deng et al. 2010). Traditionally, the denate gyrus is considered the “gateway to the hippocampus, receiving informational from the entorhinal cortex and sending projections to the Ca3 region of the hippocampus (Deng et al. 2010). What remains to be discovered is the role neurogenesis plays at this memory formation switchboard. Aimone et al. 2007 proposes that two processes, pattern separation and pattern integration facilitate the proper encoding of events. Pattern separation, suggests Aimone, results from the relatively few number of neurons in the denate gyrus that are activated for any one given experience. Statistically, the likelihood that these same neurons would be activated for a following event is low, providing an anatomical basis for the separation of events as a function of time. This is not an intrinsic property of granule cells in the critical period, but a general function of adult neurons in the denate gyrus. According to Aimone et al. 2007, young neurons assist in the formation of memories by providing pattern integration. Due to their hyperactive property, new neurons preferentially respond to incoming stimuli (Aimone et al. 2007) and it is therefore more likely that a single new neuron can be activated by different events. Young granule cells do not dominate this response however, as they still represent only a small portion of the denate gyrus neuronal population, preserving the pattern separation effect. In combination, these two processes simultaneously allow for the ability to distinguish events, yet associate them. Incoming information activates mature neurons discretely, with each new event activating distinct mature populations. Simultaneously, each event activates the same population of highly excitable granule cells that are in their critical period. All this encoded information is
sent downstream to the ca1 and ca3 retrieval locations of the hippocampus.

In addition to pattern integration, critical period neurons also assist in pattern separation (Aimone et al. 2007, Aasebo et al. 2011). One proposed mechanism is that waves of new neurons incorporate incoming information as they develop (Aimone et al. 2007). As they reach adulthood, they lose this hyperactive property, and a new wave of critical period granule cells replaces them. Thereby, events occurring on the same day are all encoded by a common population of critical period neurons, but events separated by several days are encoded by two distinct populations of immature neurons (Aimone et al. 2007).

While a significant portion of immature neurons successfully develop and become incorporated within the neuronal circuitry, a percentage of them die off (Aimone et al. 2007). Enriched environments increase the rates of survivability of developing neurons by activating a greater proportion of them. It is in this way that greater experience increases neurogenesis, and neurogenesis facilitates the encoding of these experiences.

With the survival and integration of a large portion of immature neurons, the question then becomes of their long-term function and the consequence of continuous addition of new neurons to the already existing circuitry. While granule cells born of postnatal neurogenesis closely resemble mature neurons of the dentate gyrus by 8 weeks of age, they still retain a distinct and elevated level of plasticity as late as 6 weeks after birth (Aimone et al. 2007, Deng et al. 2010). This property leads to the conclusion that neurogenesis facilitate the formation of new memories by providing neurons that are not completely specific to older memories, and thus available to encode new information (Aimone et al. 2007). Current studies suggest that new neurons do not simply replace old
ones, which would require that new information replace old information (Aimone et al. 2007, Aasebo et al. 2011). Instead, maturing neurons are additively integrated into hippocampal pathways (Aimone et al. 2007). This model of neurogenesis integration has two important implications. First, the formation of new memories, as facilitated by adult born granule cells, prevents the destruction of old memories to make way for the encoding of new information (Aimone et al. 2007). It also prevents an overload of information that theoretically could occur if additional information was encoded by the dentate gyrus without the addition of new neurons, or with a systematic addition and deletion model of integration (Aimone et al. 2007).

The second implication proposed by Aimone et al. 2007 through their computational model, is that immature neurons that are integrated into the hippocampal circuitry as they encode new information may be activated preferentially by the same information in the future. This provides a long-term function for neurons originating from postnatal neurogenesis. If young neurons are specialized, or imprinted with certain events as they are integrated, than exposure to the same stimuli at a later point in time may utilize these already integrated pathways. Doing so may streamline the processing of the hippocampus, increasing the efficiency of memory encoding.

8. **Oligodendrocytes and Oligogenesis**

Neurogenesis in the hippocampus offers not only an explanation as to the formation of new memories, but it also allows for a reasonable hypothesis as to how chemotherapy could impair memory. However, survivors receiving chemotherapy also report issues with processing speed and attention. To tackle the cause of these impairments, it
becomes necessary to investigate another process likely disrupted by chemotherapeutic agents.

In addition to neurons, progenitor cells in subcortical areas of the brain also give rise to another class of CNS cells. These ‘support’ cells are known as glial cells, and can be further delineated according to their function. Perhaps of most relevance to the process of cognition are oligodendrocyte glial cells. These cells are responsible for encapsulating neurons of the central nervous system in a myelin sheath. Due to the high lipid content of myelin, it serves to increase the speed at which electrical signals are propagated down the length of an axon. This is thought to allow information processing to occur normally, and subsequent damage to myelin would likely decrease processing speed. In addition, the lipid component of myelin gives axons a white appearance. Within the CNS, white matter therefor reflects areas that consist of mostly myelinated axons. At birth, large portions of the CNS remain unmyelinated (Catts et al., 2013), however by early adulthood, it is believed that a majority of neuronal myelination has occurred. That being said, oligodendrocyte genesis continues throughout life in both cortical and subcortical areas. The role of newly derived oligodendrocytes may be to either myelinate neurons for the first time, or repair damaged myelin in both the brain and spinal cord (Mctigue et al. 1998). Because these cells are continuously produced throughout adulthood, they are also susceptible to the effects of chemotherapy due their proliferative characteristic.

Chemotherapy has been shown to affect the levels of myelin, specifically in the Corpus Callosum (Seigers et al. 2011). In addition these changes have been associated with decreased myelinating cells and oligodendrocyte precursor cell expression following treatment with 5-FU in rats (Han et al. 2008). While decreased expression of the
precursor cells suggest that cognitive impairments are a result of a reduction in new glial cell production, research also indicates that the functionality of adult oligodendrocytes may also be impaired (Dietrich et al. 2008). In one study, Dietrich et al. 2008 investigated whether 5-FU had an effect on adult oligodendrocytes in the corpus callosum. This was achieved through marking and labeling of Olig2, myelin basic protein (MBP) and CC1, all proteins expressed by functional myelinating cells. Researchers found a significant reduction in the expression of Olig2 and MBP 56 days after treatment in animals that received 5-FU. Expression of CC1 protein was consistent between the control and chemotherapy groups at day 56. This suggests that there was not a reduction in the amount of adult oligodendrocytes, but there was a significant impairment in their function. In addition to somewhat long-term effects suggested by Dietrich et al. 2008, short-term effects of chemotherapy on myelin have also been documented. Researchers in Han et al. 2008 suggested the presence of myelin damage 14 days following chemotherapy treatment through the use of auditory brain response (ABR) analysis. ABR is a technique that measures the latency of auditory information signaling. Increased latency in auditory processing indicates damage to myelin tracts in auditory pathways (Ito et al., 2004). In accordance with Hans et al. 2008, unpublished results from our lab suggest the presence of myelin damage following treatment with 5-FU 1 day and 14 days after administration as indicated by ABR.

Animal literature has also shown pharmacologically induced myelin damage to negatively impact cognition. In studies that replicate myelin damage seen with multiple sclerosis, researchers have found impairments in spatial learning and memory in rats at both long term (90 days) and short-term time points (Xiao et al. 2008, D’Intino et al.
This animal data supports clinical studies in which neuroimaging has shown decreased amounts of white matter in patients following chemotherapy treatment (Ahles et al. 2007). In addition, it is also well documented that lesions to white matter areas of the brain have a negative impact on cognition (Frisoni et al., 2007). These observations of white matter damage can also be correlated with self-reported impairment in attention and processing speed. In a study by Deprez et al. (2011) researchers found that reports of cognitive impairment by patients 3-6 months after chemotherapy treatment, negatively correlated with white matter integrity as determined using diffusion tensor imaging (DTI). DTI is a technique that measures the ability of water molecules to diffuse into white matter tissue. The directionality of this movement is quantified using Fractional Anisotropy, and the highest FA values are recorded when white matter is healthy (Madden et al. 2012). Additionally, reports of more severe symptoms were associated with the identification of more widespread white matter damage.

Research into the effects of aging on cognition support the notion that white matter integrity is associated with cognitive performance. In studies that investigate white matter changes as people age, researchers have found that cognitive decline associated with both normal aging and disorders such as dementia, is often congruent with microstructural decreases in white matter integrity (Vernooji et al., 2009). Furthermore, Bucur et al. 2008 was able to demonstrate that reduced processing speed resulting from white matter damage in the corpus callosum and pericallosal frontal cortex was initially mediating the impairment of explicit memory retrieval, rather than aging effects. As a whole, the human literature suggests that (i) cognitive decline, such as that associated
with aging, is at least in part mediated by reduction in white matter integrity and (ii) changes in white matter can be induced by processes other than aging (such as chemotherapy).

9. **Neuroprotectants**

Much of the recent research surrounding chemotherapy and cognitive impairment has focused on developing an understanding of the biochemical mechanisms responsible for inducing histological changes in the brain, and how these changes would lead to cognitive decline. However, some studies have also investigated potential treatments that could help alleviate cognitive symptoms. Seigers et al. 2011 reviews a host of potential remedies that each address a proposed mechanism. Among them are; treatment with antioxidants that reduce the effects of oxidative stress, administration of steroids to mediate the effects of pro-inflammatory cytokine release, an exercise, which is thought to have widespread beneficial effects.

In addition to those treatments mentioned above, some studies have investigated the potential of selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine to mitigate chemotherapy induced cognitive decline, as serotonin is one of several neurotransmitters that appear to influence cognitive function (Seigers et al. 2011). A study in which Madhyastha et al. 2002 administered methotrexate to mice, found lower levels of dopamine, norepinephrine and serotonin in the hippocampus and that these changes were associated with cognitive impairment by way of conditioned avoidance task.

By administering a reuptake inhibitor like fluoxetine, it is possible to induce
greater extracellular concentrations of neurotransmitters. This is achieved by fluoxetine via noncompetitive inhibition. Normally when a neurotransmitter is released into the synaptic cleft from the presynaptic membrane, excess neurotransmitter is pumped back into the presynaptic cell by transporter proteins. Inhibitors like fluoxetine bind to an allosteric site on these transporters, inducing a conformational change in the protein, thus lowering its affinity for the neurotransmitter and preventing their proper transport back into the presynaptic neuron.

Though the mechanisms are not entirely understood, studies indicate that administration of fluoxetine and other SSRI’s results in increases levels of neural cell genesis (Surget et al. 2011). While these studies often investigate the function of antidepressants in the context of depression, their effect on neurogenesis and oligogenesis supports the notion that they may be useful in treating cognitive impairment partially caused by a reduction in neuronal cell proliferation.

To review, the objectives of the present study are; (i) to replicate the reduction of neurogenesis and white matter integrity through the administration of the chemotherapy agent 5-FU, (ii) to show that these changes are not only acute, but persist well after treatment cessation, and (iii) identify if the antidepressant drug Fluoxetine (a SSRI) can prevent or reduce cognitive impairment when co-administered. Contemporary research across many disciplines provides motivation for the goals of this study. There is a host of literature to suggest that chemotherapy has negative effects on tissue in the brain, particularly on proliferating cells in the hippocampus and the integrity of white matter in the corpus callosum. In addition, there is significant evidence that indicates these two
aspects of cranial anatomy mediate working memory and normal processing speed respectively. Finally, while investigation into potential remedies is novel, there is evidence to suggest that certain substances that are known to increase cell proliferation may be used to prevent or mitigate the negative effects of chemotherapy on cognition. To accomplish these goals, animals will be subject to three separate conditions. The first will consist of animals that will receive only saline treatment. This provides a control to which the other groups can be compared. The second group will include animals that only receive 5-FU injections. The final group will consist of animals who receive both 5-FU and fluoxetine treatment. The two experimental groups allow for the study to independently investigate the effects of only chemotherapy, and the effects of chemotherapy in the presence of a neuroprotectant.

METHODS

1. **Animals**

Sixty male C57BL/6J mice were obtained from Jackson Laboratories in Bar Harbor, Maine. Animals were housed four or five to a cage and given access to food and water at their discretion. A twelve-hour light dark cycle (7:30 A.M. – 7:30 P.M.) was used for the duration of the experiment. Animals were routinely weighed as well as checked for teeth and coat quality. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maine and conducted in accordance with “The Principals of Laboratory Animal Use and Care” (NIH publication No. 86–23, revised 1985).
2. *Treatments*

All mice began treatment at approximately 10-11 weeks of age. Mice were assigned randomly to 1 of 3 conditions: Control, 5-FU, or 5-FU + Fluoxetine. Because increases in cell proliferation via fluoxetine administration take 2-3 weeks to occur, mice in the 5-FU + Fluoxetine group underwent a pretreatment period for 21 days during which fluoxetine was administered via their drinking water (attempted dose: 16mg/kg). Actual dose as assessed by weighing the water bottles every three days varied between 12 and 15 mg/kg. Following the pretreatment period, animals were given a total of 3 intraperitoneal injections (an injection every other day for 5 days) of either the chemotherapy agent (5-Fluorouracil) or saline as a control as depicted in Figure. In addition, the 5-FU + Fluoxetine animals received neuroprotectant injection (fluoxetine) every day over that five day period. The control and 5-FU animals received an injection of saline every day over that five day period as a control for the 5 injections of fluoxetine that the 5-FU + Fluoxetine animals received in addition to their 3 injections of 5-FU. Concentrations and dosages are displayed below. The chemotherapy and neuroprotectant agents were dissolved in 0.9% saline.

**Intraperitoneal injections**

- Saline (0.9%) + Saline (0.9%)
- 5-fluorouracil (70 mg/kg) + Saline (0.9%)
- 5-fluorouracil (70mg/kg) + Fluoxetine (12mg/kg)
3. **Slicing**

At 1 and 14 days and 6 months following the cessation of treatment, animals from each condition were transcardially perfused with 4% paraformaldehyde (PFA). Brains were removed from the animals and placed in a PFA solution followed by a 30% sucrose solution, before being flash frozen. Tissue was kept in freezers until they were ready to be sliced. 40-micrometer coronal sections were sliced, starting at the anterior of the cortex. Slices were placed in a potassium phosphate buffer saline solution and later transferred to an antifreeze cryoprotectant solution and stored at -20 F.

4. **Myelin Staining**

Staining began by mounting tissue on a series on slides, with each slide containing a brain from five different conditions (Saline, 5-FU, 5-FU + Fluoxetine and 5-FU + two other neuroprotectants not included in the scope of this study. Slides were mounted using a .0125% potassium phosphate buffer solution to float the tissue into position and then left to dry. Slides were chosen based on their position (anterior – posterior) by
identifying where the Corpus Callosum first came together, and then choosing the 4\textsuperscript{th} slide following in that series. The position of the tissue from each condition was rotated for each of the slides prepared.

Slides were rehydrated through a series of washes (30% ethanol and double distilled water for two minutes each) before being placed in a black gold II solution heated to 63 degrees centigrade. Tissue was submerged in the BGII solution for 8-10 minutes, with periodic monitoring of stain uptake and quality. Following the BGII solution, slides were again washed starting with double distilled water (2 min), a thiosulfate solution (3 min), tap water (3 times, 5 min each), 70% ethanol solution (2.5 minutes), 95% ethanol solution (2.5 minutes), 100% ethanol solution (2x 2.5 min) and Histoclear for 5 minutes. Cover slips were mounted using paramount gel and slides were left to air-dry.

5. \textit{Images and Rating}

After slides were thoroughly air-dried, images of each brain were taken using camera-mounted Zeiss Axio Imager Z1 microscope, with 400x magnification. The specific location of images focused on the lateral corpus callosum at consistent anterior/posterior locations as indicated by figure 2. This location was chosen for two reasons. First, the corpus callosum is a widely accepted model of global myelin health. Secondly, more medial sections of the corpus callosum have a tendency to curl up during staining, resulting in an inflation of myelin density in this region. Rating of short term and long-term data was conducted separately, using different rating techniques, as damage occurring in tissue collected shortly following treatment was distinctly unique than in tissue collected 6 months post treatment. ImageJ rating software was used to analyze
tissue from the 6-month conditions. This analysis works through calculating the intensity of each pixel in the image. Pixels are rated on a 0-256 scale following the conversion to a grey scale, with 0 being black and 256 being white. The median value for all pixels in the image was calculated and coding was inverted (intensity value was subtracted from 256) to make it so that more myelin was represented by higher values and less myelin by lower values. Figure 3. Illustrates different myelin densities increasing from image A-D.

Tissue from days 1 and 14 exhibited myelin damage that was not conducive to analysis by ImageJ software. Damage from these two time points manifested as missing fibers rather than the widespread decrease in density as seen at 6 months. If ImageJ were used, areas of white created by missing tissue would have impacted results. Given this, a different method for tissue analysis was required. Instead of rating tissue on a density scale, tissue was identified as either having significant myelin damage or not having significant myelin damage, based on the degree of missing fibers. Figure 4 depicts tissue with myelin damage and figure 5 depicts tissue with no myelin damage.
Figure 2.

Figure 3.
RESULTS

As seen in Figure 6, myelin damage at day 1 was rare in the control and 5-FU + fluoxetine groups, but fairly common in the 5-FU group.

Day 1 data:

<table>
<thead>
<tr>
<th>Condition</th>
<th>No myelin damage</th>
<th>Myelin damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>5-fluorouracil</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>5-fluorouracil + fluoxetine</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 6.
A chi-square test indicated that the groups did differ \( \chi^2 (2, N = 20) = 6.29, p < 0.05 \). Follow-up testing further analysis revealed this difference to be between the control group and 5-FU groups \( \chi^2 (1, N = 13) = 4.95, p < 0.05 \). This statistical analysis shows significantly more animals in the 5-FU condition had tissue that exhibited myelin damage as compared to the control group 1 day following treatment.

As seen in Figure 2, myelin damage at day 14 was rare in the control and 5-FU + fluoxetine groups, but common in the 5-FU group.

Day 14 data:

<table>
<thead>
<tr>
<th>Condition</th>
<th>No myelin damage</th>
<th>Myelin damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>5-fluorouracil</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>5-fluorouracil + fluoxetine</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

Figure 7.

A chi-square test indicated that the groups did not differ \( \chi^2 (2, N = 19) = 4.25, p = 0.12 \).

Because our main hypothesis was that the control and 5-FU groups would differ, we compared those two groups directly as well. A chi-square test indicated that more animals given 5-FU showed tissue indicative of myelin damage than the control animals \( \chi^2 (2, N = 13) = 3.90, p < 0.05 \).
Given the relative symmetry of the day 1 and day 14 data, these sets were combined to increase statistical power. Figure 3 shows the combined Day 1 and Day 14 data.

Day 1 and 14 combined data:

<table>
<thead>
<tr>
<th>Condition</th>
<th>No myelin damage</th>
<th>Myelin damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>5-fluorouracil</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>5-fluorouracil + fluoxetine</td>
<td>10</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 8

A chi-square test indicated that the groups did differ $\chi^2(2, N = 39) = 10.03$, $p < 0.01$. Further analysis revealed more animals in the 5-FU condition had tissue that exhibited myelin damage than the control group $\chi^2(1, N = 26) = 8.55$, $p < 0.005$. Interestingly, further analysis also revealed that more animals in the 5-FU condition had tissue that exhibited myelin damage than the group that received 5-FU + fluoxetine $\chi^2(1, N = 27) = 4.64$, $p < 0.05$. 
6 Month data:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Median Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>77.00 +/- 3.4</td>
</tr>
<tr>
<td>5-fluorouracil</td>
<td>68.375 +/- 10.5</td>
</tr>
<tr>
<td>5-fluorouracil + fluoxetine</td>
<td>78.429 +/- 15.85</td>
</tr>
</tbody>
</table>

Figure 9.

A One way Anova of the data from figure 9 indicated that the groups did not differ in myelin density: F\(_{2, 19}\) = .262 N = 21 p = .722

DISCUSSION

The goals of this study were to investigate the effects of the chemotherapy agent 5-FU on neurogenesis and white matter integrity at both short term and long-term time points. In addition, this study aimed to test the effectiveness of the proliferating agent fluoxetine, at preventing chemotherapy-induced changes in the hippocampus and corpus callosum.
1. Myelin

The results indicate that the administration of 5-FU significantly decreased the integrity of white matter tissue in the corpus callosum at both 1 and 14 days post treatment as compared to controls. This is consistent with findings by Seigers et al. 2011 in which researchers identified decreased white matter thickness in the lateral corpus callosum in mice following methotrexate administration at one day, one week and three weeks post treatment. These decreases, along with decreases in neurogenesis, were associated with decreased cognitive performance at one week and four weeks post treatment.

These results may be indicative of either a reduction in new glial cell production, or damage to existing myelinating cells in the corpus callosum. Previous research by Han et al. 2008 has shown that 5-FU affects not only progenitor cells in the CNS, but also non-dividing oligodendrocytes. Results from this study indicated a significant reduction in progenitor cells as well as a possible increase in cell apoptosis in the corpus callosum one day following treatment. This study also showed a delayed effect of 5-FU. At 56 days post treatment, researchers identified a reduction not only in cell proliferation, but also a reduction in oligodendrocytes that produce protein Olig2+ (present in normal functioning oligodendrocytes), however there was no cell apoptosis present at this time point.

Our short-term results fit the observations by Han et al. 2008 concerning the types of damage seen in the corpus callosum. Previous research indicates that apoptosis may be present shortly after treatment but is absent at long term time points. Our staining and
analysis indicate a significant difference in the degree of fibers present shortly following chemotherapy for control animals and those receiving 5-FU. Given the loss of fibers, our data suggests that short-term myelin damage in the corpus callosum may be a result of oligodendrocyte apoptosis and a reduction in progenitor cell proliferation.

Our short-term data also indicates the fluoxetine may be effective at preventing myelin damage in the corpus callosum when co-administered. Animals that received both fluoxetine and 5-FU exhibited myelin that was similar to controls. Because fluoxetine is a proliferating agent, administering it concurrently with a chemotherapy drug may at least compensate for the decrease in oligogenesis. That being said, there is no literature that suggests proliferating agents would be effective in preventing cell apoptosis.

Results of data from tissue 6 months following treatment was unsuccessful in showing significant effects of 5-FU on white matter integrity in the corpus callosum. Animals from all three conditions showed similar levels of myelin density as determined by both imaging software and researcher ratings. This data would seemingly oppose other contemporary studies in which researchers suggested there to be myelin damage in the corpus callosum 6 months following chemotherapy treatment (Han et al. 2008). However, unpublished data from our lab indicates animals that received 5-FU exhibited significant decreases in myelin density 14 months following treatment. Furthermore, the levels of myelin density for animals 14 months following 5-FU injection were similar to the myelin density levels of 5-FU animals and 5-FU + fluoxetine animals 6 months following injection.
If we assume that levels of myelin density 6 months following treatment, for animals receiving 5-FU in this study are indicative of white matter integrity reduction (as values are consistent with chemotherapy-induced damage in other lab conditions), we can proceed cautiously with an analysis of long term results from this study that are consistent with contemporary literature.

Unlike tissue from short-term time points, tissue from the 6 months post treatment animals was quantified only by the density of black gold II stain (rather than degree of fibers), as there was no apparent difference in the amount of myelinated fibers present. Results indicate that both animals receiving 5-FU alone, and those receiving 5-FU and fluoxetine had reduced myelin density in the corpus callosum when compared to controls outside the condition. While short-term effects of chemotherapy seem to indicate chemotherapy-induced apoptosis and a reduction in proliferation, 6-month data indicates either a continued reduction in cell proliferation, a reduction in the amount of mature oligodendrocytes that are functioning properly, or both. Consistent levels of fibers across conditions at this time point suggest an absence of apoptosis, consistent with findings from Han et al. 2008. Interestingly, these results indicate that fluoxetine was not effective at preventing myelin damage induced by 5-FU 6 months following treatment.

Results from both the short term and long term data in this study suggest discrepancies in the way 5-FU effects white matter tissue in the corpus callosum over time. Tissue from animals treated with 5-FU collected shortly following the cessation of treatment exhibited a reduction in the amount of fibers present in the corpus callosum, where tissue collected from animals 6 months following treatment showed no difference in the amount of fibers present, but instead exhibited a reduction in myelin density.
While these results indicate that 5-FU has both acute and prolonged effects on white matter integrity, it also suggests that distinct processes contribute to each.

Given the reduction of fibers seen in 5-FU animals shortly following treatment and supporting research by Han et al. 2008, we can tentatively conclude that at least some of the myelin damage at this time point is indicative of oligodendrocyte apoptosis. It is more difficult to conclude the type of oligodendrocyte that is affected. Because apoptosis is not thought to occur well after stopping treatment, and we do not see a difference in the amount of fibers 6 months post treatment, it would appear that the reduction in fibers shortly following chemotherapy administration is a consequence of apoptosis. However, our results indicate that the presence of fluoxetine prevents a reduction in fibers caused by 5-FU shortly following treatment. Because fluoxetine is a proliferating agent, you would expect it to prevent myelin damage by increasing oligogenesis. If the reduction of fibers by 5-FU is a result of apoptosis targeting proliferative oligodendrocyte precursor cells, we would expect that concurrent fluoxetine administration would counteract this effect, thereby eliminating fiber reduction in the corpus callosum following chemotherapy treatment. This is not contradictory to our results that show no effect of fluoxetine 6 months following treatment. The reason for this calls upon the proposed mechanisms of the long-term effects of chemotherapy on cognition. Recall that chemotherapy administration is thought to increase neuroinflammation, oxidative stress and CNS cytokine levels. If levels of any of these remained high following the cessation of both chemotherapy and neuroprotectant treatment, you would expect unabated decreases in oligogenesis, resulting in prolonged reduction in white matter integrity.
What is difficult to explain is the absence of fiber reduction 6 months following chemotherapy administration. One possible explanation is that 5-FU induces apoptosis of both progenitor cells and adult oligodendrocytes. This would be surprising as 5-FU is thought to target only proliferating cells, however previous research shows that even non-dividing oligodendrocytes are susceptible to apoptosis induced by 5-FU, though it is not clear why (Han et al. 2008). If this were the case, not only would the administration of chemotherapy kill entire cells that are myelinating axons but it would also reduce the ability to replace these cells. Short-term tissue may reflect this through an absence of fibers since adult oligodendrocyte death could leave some axons completely unmyelinated. Fluoxetine could potentially mitigate this effect if it were to counteract the antiproliferating effects of 5-FU and hold oligogenesis rates normal, thereby replacing the apoptotic adult oligodendrocytes as they died. Following the initial wave of adult oligodendrocyte death initiated directly by 5-FU, it is possible that subsequent oligogenesis would preferentially myelinate those axons that were completely stripped. This may explain why there does not appear to be a reduction of fibers 6 months following 5-FU administration, but only a reduction in myelin density. Because chemotherapy exposure is thought to initiate antineogenic processes that continue after treatment is stopped, levels of oligogenesis may remain low following only chemotherapy administration, and drop once treatment with fluoxetine was discontinued. The myelin around a single axon is not static over time. As with most tissue in the body, it eventually breaks down and needs replacing. It is believed that one role of oligogenesis is to replace myelin that breaks down naturally. Research suggests that following chemotherapy; levels of oligogenesis remain suppressed as a result of
neuroinflammation. If this were the case, you would expect to see less myelin repair of the normal breakdown of myelin well after the cessation of chemotherapy treatment, and that decreased myelin density would be a reflection of this process.

Regardless of the exact mechanisms by which 5-FU acted to induce myelin damage, these results suggest that systematic administration of 5-FU decreases the quality of myelin in the corpus callosum at both acute and prolonged time points. This study, in conjunction with research affirming the necessity of healthy myelin for normal information processing, strongly supports the hypothesis that some symptoms (both acute and persistent) of cognitive impairment following chemotherapy are a result of myelin damage in the CNS. In addition, this study confirms the possibility that the co-administration of the antidepressant drug fluoxetine may help to prevent acute cognitive impairment caused by myelin reduction.

2. Neurogenesis and the Hippocampus

The assessment of neurogenesis in the hippocampus following 5-FU administration was a goal of this study for several reasons. First, the production of new neurons in the hippocampus has been linked to the process of memory formation. Secondly, memory impairment is not only one of the most pervasive cognitive symptoms experienced by chemotherapy patients shortly following and well after treatment, but ablation of hippocampal neurogenesis in animal research has consistently resulted in declined performance on hippocampal mediated memory tasks. Finally, given the nature of chemotherapy drugs as anti-proliferating agents, a reduction in the proliferation of
cells in the hippocampus provides a reasonable hypothesis to the mechanisms through which chemotherapy impairs memory.

Unfortunately, given the time constraints imposed by the deadline of this project, we were unable to prepare and analyze tissue for the presence of protein indicators of neurogenesis in the hippocampus. However, given the results of the data from the myelin staining, we can make inferences as to what the hippocampal neurogenesis results are likely to be. Persistently low levels of myelin density 6 months following chemotherapy treatment suggests that the brain is still limited in its ability to produce normal amounts of myelin. This must mean that the process of oligogenesis (the only way by which the brain can produce new myelin) must be affected some way, either through the actual production of new glia cells, or the successful survivability and integration of those produced. In either case, it is likely that if oligogenesis is affected by chemotherapy, so too will neurogenesis in the hippocampus.

While many studies have investigated the impact of a variety of chemotherapy agents on both brain histology and behavioral measures of cognition in animals, few have made experimental investigations at time points considered to reflect long term or permanent impairment in humans. This is one of several reasons why results from this study are being considered with cautious optimism; as providing potential evidence for the presence of well-documented, histological abnormalities in areas of the brain associated with learning, memory and processing speed, following chemotherapy administration, not only transiently, but well after the discontinuation of treatment. Before hard conclusions can be drawn from this study, not only will the analysis of
hippocampal neurogenesis need to be run, but all results will need to be substantiated through multiple replications. This is particularly necessitated by the small n-values for each condition, and the potentially imprecise assessment and determination of tissue quality. In addition, the use of nissle body staining will be necessary in determining identifying characteristics of oligodendrocytes in the corpus callosum.

Despite resource limitations and time constraints, this study was successful in accomplishing a majority of the goals initially presented. Analysis of tissue stained with Black Gold II indicated that the administration of the chemotherapy agent 5-fluorouracil resulted in a decrease in myelin quality at both acute (1 day, 14 days) and extended (6 months) time points following treatment. In addition, results indicated that fluoxetine has the potential to reduce the myelin damage caused by 5-FU, shortly following the treatment cessation.
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AUTHOR BIOGRAPHY

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