The Effect of Systemic Temozolomide on Learning, Emotional Behaviour, and Hippocampal Synaptic Plasticity: Implications for chemotherapy-induced cognitive impairment

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ABSTRACT

The Effect of Systemic Temozolomide on Learning, Emotional Behaviour, and Hippocampal Synaptic Plasticity: Implications for chemotherapy-induced cognitive impairment

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Patients who undergo chemotherapy often complain of a persistent ‘brain fog’ that can be present up to years after treatment ends. This fog is expressed as marked impairments in areas of learning, memory and mental health. As it stands, researchers have yet to determine the mechanism at fault for these impairments. The present experiment investigates if the neurogenesis that takes place in the subgranular zone of the hippocampus is suppressed as a result of chemotherapy treatment, and results in these impairments. In the following thesis, two models of chemotherapy are used to explore the treatment effects on Long-Evans rats. From here, three behavioural assessments and three measures of immunohistochemical techniques are used to explore the effects of Temozolomide on memory and anxious behaviour. Our findings support the current literature that suggests that Temozolomide suppresses adult hippocampal neurogenesis and results in cognitive and emotional impairments.

Keywords: Chemotherapy, Chemotherapy-Induced Cognitive Impairment, adult hippocampal neurogenesis, pattern separation, Long-Evans rats, contextual fear discrimination, shock-probe test, elevated plus maze, Temozolomide, EGR1, Dentate Gyrus, Synaptophysin, DCX

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Table of Contents

List of Figures

List of Abbreviations

AHN- Adult Hippocampal Neurogenesis

BBB- Blood-brain barrier

BrdU- Bromodeoxyuridine / 5-bromo-2'-deoxyuridine

CA- Cornu Ammons

CICI- Chemotherapy-Induced Cognitive Impairment

DCX- Doublecortin

DG- Dentate Gyrus

EC- Entorhinal Cortex

EGR1- Early Growth Response 1

EPM- Elevated Plus Maze

HPC- Hippocampus

QOL- Quality of Life

SGZ- Subgranular zone

SVZ- Subventricular zone

TMZ- Temozolomide

Chapter I: Introduction

Chemotherapy can be used as the primary treatment choice for various types of cancers. The goal of chemotherapy is to damage or kill cancer cells by interfering with the division and spread of these fast-dividing cells throughout the body (Khan et al., 2014). Advances in chemotherapy and other aspects of prevention, early detection, and treatment modalities have resulted in an increasing percentage of patients surviving cancer (Glimelius et al., 1996). There are currently over 7.6 million cancer survivors in Canada, a figure expected to reach over 9 million by 2030 (Brenner et al., 2022). For some types of cancer, chemotherapeutic drugs are allowing patients to live many years beyond their initial diagnoses (Glimelius et al., 1996). However, essentially no drug is devoid of adverse side-effects (Basak et al., 2021). The acute cytotoxic effects of chemotherapeutic drugs are well known and are often required for their therapeutic benefit. As such, there is a growing interest now being directed at the study of how cancer and its treatment affect the quality of life of the patient.

Accumulating evidence has highlighted a concerning link between chemotherapy and the emergence of adverse neurological symptoms after treatment (Yang & Moon, 2013; Gupta et al., 2022; Ren et al., 2019). This work has revealed that more than half of cancer patients experience difficulties with attention, learning, and memory after chemotherapy that can continue to linger for months or even years after treatment has ended (Dias-Carvalho et al., 2022; Dutta, 2011; Eide & Feng, 2020; Pendergrass et al., 2018; Raffa, 2013). This phenomenon, often called "chemobrain" or "chemo-fog" is usually transient, but for a subset of cancer survivors, these impairments continue to be an ongoing problem that negatively impacts their quality of life, vocational performance, and social fulfillment (Argyriou et al., 2011; Bolton et al., 2018; Boykoff et al., 2009; Pearre et al., 2018). Thus, as the survival rate of cancer patients continues

to increase globally, there is a greater interest being directed at examining the adverse consequences of chemotherapy and understanding the neurobiological mechanisms that underlie chemotherapy-induced cognitive impairment (CICI).

There are several chemotherapeutics available for use to treat brain cancers. One frequently used chemotherapeutic medication is Temozolomide (TMZ, Temodar), which is used for treatment of primary high grade malignant glioblastomas and anaplastic astrocytomas (Schreck & Grossman, 2018; Wesolowski et al., 2010). TMZ is frequently used together with radiotherapy as a part of the first-line treatment for high-grade gliomas (Schreck & Grossman, 2018; Wesolowski et al., 2010). This drug shows a favourable toxicity profile and is generally well tolerated at therapeutic doses, despite being known to produce some non-hematological effects such as nausea, vomiting, headache, fatigue, and constipation (Scaringi et al., 2013). However, evidence has also begun to emerge showing that there may be longer-lasting cognitive effects after TMZ treatment that could greatly impact the quality of life for patients (Dietrich et al., 2015; Egeland, 2017; Nokia et al., 2012; Tseng et al., 2004). Thus, the purpose of my Master's thesis is to develop an animal model to examine the impact of TMZ on learning and memory using a treatment regimen similar to that used in humans for chemotherapy and to determine possible neurobiological mechanisms that may underlie TMZ-induced cognitive deficits.

1.1 The History of Chemotherapy

The term 'Chemotherapy' was first coined by the German chemist Paul Ehrlich (DeVita & Chu, 2008). In its broadest sense, the term chemotherapy was defined as the use of synthetic chemicals that work together with the body's own defences to interfere with the metabolism of

infectious organisms (DeVita & Chu, 2008). Ehrlich was one of the first investigators to utilize animal models to screen novel compounds for their potential effectiveness in treating a disease, an accomplishment that had major ramifications for cancer drug development (DeVita & Chu, 2008). While Ehrlich's most significant contribution to drug discovery was the development of arsenicals for the treatment of syphilis, he was also the first to demonstrate the potential efficacy of alkylating agents, e.g., the use of nitrogen mustard for cancer treatment in animals (Bosch & Rosich, 2008; DeVita & Chu, 2008). However, it was not until the seminal work by Goodman and Gilman (Gilman et al., 1946; Goodman & Wintrobe, 1946) that the full therapeutic potential of alkylating agents was realized.

Building on initial observations that soldiers exposed to the warfare agent mustard gas during the First and Second World Wars experienced significant leukopenia (low white blood cell count), Goodman and Gilman (1946) showed that nitrogen mustard could be effective in producing regression of advanced lymphosarcoma in human patients. This led to the use of nitrogen mustard and nitrogen mustard analogs as the first chemotherapeutics for the treatment of lymphomas. In the following years, the rise of additional alkylating agents were synthesized to combat cancer, such as mechlorethamine, cyclophosphamide and chlorambucil (DeVita & Chu, 2008). This new anti-cancer arsenal helped fuel the development of folate antagonists such as aminopterin and amethopterin, which paved the way to the landmark discovery of methotrexate for the treatment of childhood leukemia (Farber et al., 1948). At the same time, these important findings helped change the public perception of cancer treatment, which, until this point, was largely viewed as incurable. By the late 1960s, with the introduction of a combination of chemotherapy agents like nitrogen mustard, vincristine, methotrexate, and prednisone (MOMP protocol), more patients had longer remission from cancerous diseases like lymphomas and leukemia, leading to more people living post-cancer and cancer treatment (DeVita & Chu, 2008).

1.2 Prevalence of Chemotherapy-induced cognitive impairment

In 2022, an estimated 233,900 Canadians were diagnosed with cancer (Statistics Canada, 2022). Assessment of cancer-specific endpoints have traditionally focused on evaluation criteria, such as response rate, being progress-free, and overall survival rate (Delgado & Guddati, 2021). However, with the improvement of cancer diagnosis and treatment, the survival rate of patients with cancer has increased and the mortality rate has decreased (Torre et al., 2016). Therefore, greater attention has now been given towards studying the impact of cancer treatment on the quality of life of the patient. As an important component of quality-of-life, there have been several reports demonstrating a diminution of cognitive functions in some cancer patients during and after adjuvant chemotherapy (Dias et al., 2014; Dietrich et al., 2015; Jansen et al., 2011; Hermelink et al., 2007; Hurria et al., 2006; Kanaskie & Loeb, 2015; Nokia et al., 2012; Selamat et al., 2014; Van Dyk & Ganz, 2017). This phenomenon has been found not only for patients receiving chemotherapy for brain cancer, but also for patients receiving chemotherapy for cancers in peripheral locations, such as breast, lung, liver, and other tissues (Hansen et al., 2008; Hermelink et al., 2007; Kanaskie & Loeb, 2015).

Although the prevalence of cognitive impairment associated with chemotherapy remains unclear, estimates suggest that roughly half of all cancer survivors will experience some level of chemotherapy-induced cognitive impairment (CICI) (Janelsins et al., 2012; Jean-Pierre et al., 2011). Common cognitive impairments associated with chemotherapy typically affect domains related to visuospatial skills, language, motor skills, concentration, attention, multitasking, and memory (Seigers & Fardell, 2011). However, estimates of the prevalence of CICI can vary widely with the duration of symptoms only lasting a short time or persisting for longer periods of time after cessation of chemotherapy (Ahles et al., 2002). For example, longitudinal studies in

cancer patients indicate that about a third of patients experience some level CICI prior to treatment, with up to 75% experiencing CICI during therapy and 35% experiencing ongoing chemo-related cognitive side effects for months or even years after treatment has stopped (Janelsin et al., 2014; Jansen et al., 2011; Mitchell & Turton, 2011; Ouimet et al., 2009; Seigers & Fardell, 2011; Vardy et al., 2008). In one previous study conducted from the time of diagnosis (prior to receiving chemotherapy) and after chemotherapy was complete, 45.2% of patients reported a diminishment in cognitive function compared with 10.4% of healthy people over a similar period (Janelsins et al., 2017). In addition, significantly higher risk of late (about 2 years after treatment) onset cognitive impairment, particularly in the areas of concentration and memory, in breast cancer survivor patients treated with six courses of chemotherapy than patients who received the same surgical and radiation therapeutic protocol but did not receive chemotherapy (Schagen et al., 1999). Importantly, the presence of these impairments was largely unaffected by factors such as anxiety, depression, fatigue, or self-reports of cognitive impairment suggesting that the negative consequences on CICI are not the result of either psychological or general systemic changes to health (Schagen et al., 1999). Finally, neuropsychological tests performed in breast cancer survivors found that women given adjuvant chemotherapy more than 20 years earlier continued to exhibit ongoing impairments across multiple cognitive domains compared to women with no cancer diagnosis (Koppelmans et al. 2012). Taken together, these findings suggest that chemotherapy can cause both acute, delayed, or even long-lasting (if not permanent) changes in cognitive function for some survivors, demonstrating just how impactful CICI can be on the quality-of-life of survivors (Ahles et al., 2012).

1.3 Assessment of Cognitive Impairment After Chemotherapy: Human Studies

Given that the vast majority of chemotherapeutics target rapidly dividing cells, including both cancer and non-cancer cells that are actively growing and dividing, the potential for toxic

side effects, such as hair loss, skin changes, gastrointestinal syndromes, and dysfunction of the bone marrow, among many others, would be expected (Hackbarth et al., 2007; Hauner et al., 2017; Miteva et al., 2007). However, it is now recognized that chemotherapy can also have unintended effects on brain function and behaviour (Dias et al., 2014; Hermelink et al., 2007; Hurria et al., 2006).

Cancer survivors have long reported cognitive dysfunction at various stages of the disease course with associated consequences upon well-being and functional independence (Pendergrass et al., 2018; Von Ah et al., 2016). Nonetheless, until relatively recently, chemotherapy-induced cognitive impairment (CICI) in patients with noncentral nervous system malignancies went unacknowledged or even ignored. This prevailing attitude was reinforced by the assumption that chemotherapeutic compounds were unable to cross the blood-brain barrier, thereby precluding the possibility of a direct neurotoxic effect from these cancer therapies (Ginsberg et al., 1981). Instead, subjective reports of cognitive impairment and declining mental function were considered a reflection of psychological processes and stress associated with the disease (Janelsins et al., 2014; Pendergrass et al., 2018; Wefel et al., 2010). However, a growing body of literature has verified the presence of CICI in cancer patients; research has now confirmed the presence of cognitive deficits following chemotherapy in humans with cancer (Eide & Feng, 2020; Jacobs et al., 2016; Jansen et al., 2011; Kanaskie & Loeb, 2015; Lindner et al., 2017; Mitchell & Turton, 2011; Pendergrass et al., 2018; Player et al., 2014; Walker et al., 2012).

To the individuals who are suffering with CICI, the impairments are often very disruptive to their daily life, despite being considered in the literature as ‘subtle’ (Kanaskie & Loeb, 2015).

Changes in attention, concentration, executive function, information and processing speed, language, motor function, visuospatial skill, learning and memory have all been areas of

cognition reported by survivors (Jansen et al., 2011; Kanaskie & Loeb, 2015; Myers, 2010). Despite how subtle the impact may be on some individuals, these side-effects can be very detrimental and impactful for their day-to-day functioning (Kanaskie & Loeb, 2015). Many survivors of cancer are working individuals, so these impairments can also actually inhibit their ability to re-enter their careers following treatment, sometimes with noticeable deficits lasting for up to several years after treatment is complete (Bender et al., 2006; Chen et al., 2007; Schagen et al., 1999).

With the complexity of the issues expressed following chemo treatment, it leaves researchers with the task of pinpointing exactly what is occurring at a neurobiological level. Memory loss is one of the most debilitating complaints of CICI, given that our ability to navigate our environment, complete daily tasks, and recall people and events all rely on our memory (Mitchell & Turton, 2011; Moscovitch, 2016). The ways in which memories are created also rely on the involvement of other cognitive processes (Aly & Turk-Browne, 2017; Chun & TurkeBrowne, 2007; Yonelinas et al., 2010). An impairment in attention can lead to an impairment at the recollection stage of the memory process, and both memory and attention are modulated in the hippocampus (Aly & Turk-Browne, 2017; Chun & Turke-Browne, 2007; Geddes et al., 2003; Yonelinas et al., 2010). The hippocampus is the area of the brain that is involved in working memory, spatial memory, acquisition and retrieval of stored memories, as well as consolidation of information for long-term memories (Yang & Moon, 2015). With both attention and memory being listed as some of the cognitive deficits chemotherapy survivors experience, it stands to reason that the mechanism behind these issues is found in the hippocampus, which is an area already known to be highly susceptible to infarcts (Aly & TurkBrowne, 2017; Geddes et al., 2003).

Testing the hypothesis that the hippocampus is being impaired by chemotherapy treatment, researchers have begun to explore different aspects of memory function in patients. In a study done using pediatric cancer survivors and fMRI, impairments in working memory were found and discovered to be associated with an impairment in memory manipulation and retrieval (Stefancin et al., 2019). Another research team conducted a similar study using adult patients and found that during a recognition task, there was a decrease in the recruitment of brain regions associated with working memory and memory recognition (Wang et al., 2015). They also found that the left-middle hippocampus showed decreased activity when compared to healthy controls (Wang et al., 2015). Another study focused on women who were diagnosed with breast cancer and had three groups of cancer patients (Bender et al., 2006). One group of women had cancer but did not receive treatment, one group received chemotherapy, and the last group received chemotherapy and tamoxifen (an estrogen blocker) (Bender et al., 2006). When cognitive function was assessed with the use of neuropsychological batteries at three different time-points (after surgery but before treatment began, 1 week after chemotherapy was complete, and 1 year after it was complete), it was found that the individuals who did not do the chemotherapy were able to show practice effects as their cognitive scores improved, but the group who did chemotherapy exhibited deteriorations in verbal working memory, and the group that received both the hormone blocker as well as the chemotherapy showed deterioration of visual and verbal working memory and had more memory complaints (Bender et al., 2006). This led researchers to infer that adjuvant chemotherapy in female breast cancer patients can be associated with deteriorations in memory that may persist over time (Bender et al., 2006). Other researchers have also shown an impairment in spatial memory impairment following breast cancer treatment (Cheng et al., 2017).

While research on the impairment of humans following chemotherapy has been important, it also has very clear limitations. Chemotherapy cannot ethically be given to people at random, as it is so invasive to the individual's health and wellbeing. This means that in every human chemotherapy study that is conducted, there is the confound of the cancer also being present. This is especially difficult to draw conclusions from when cancer is already known to cause cognitive issues and memory impairments (Anderson et al., 2019; Biegler et al., 2009). Therefore, to test my hypothesis that chemotherapy is suppressing the neurogenesis that occurs in the hippocampus and causing these cognitive impairments, the use of an animal model without cancer is a necessary method of research.

1.4 Assessment of Cognitive Impairment After Chemotherapy: Animal Studies

While chemotherapy studies in humans allow researchers to see what actually occurs when cancer patients are exposed to treatment, it is limited in the way the research can be conducted. For starters, much of the human research on cognitive dysfunction following chemotherapy is conducted via self-reports, which are a flawed metric by design with studies showing that the wording, format and context of questions can be leading, yielding a higher percentage of error (Schwarz, 1999). Further, the use of human studies have the limitation of being conducted with survivors, and there currently is no probe for human neurogenesis that is sensitive and specific enough to compare to that of animal research (Ho et al., 2013). Animal studies also provide the benefit of controlling all factors of the sample to isolate and standardize age, diet, stress, environment and all other external factors to independently explore the effects of chemotherapy itself. Further, conducting animal studies allows for the effects of chemotherapy to be explored in isolation with it being given to animals without cancer being present, a feat which

would be unethical in humans. Moreover, rodents in particular have been a popular choice for neuroscientists for several decades, and as such, the organization and function of the rat brain has been thoroughly investigated, mapped and recorded, making the rat an appropriate model to study chemotherapy and neurogenesis (Paxinos & Watson, 2007).

Prior to this study, researchers have explored the effects of various chemotherapeutics for their cytotoxic repercussions on proliferating cells and behavioural testing performance. One study showed that a single dose of the chemotherapeutic 'methotrexate' lead to dosagedependent suppression of Ki-67-labelled cells in the subgranular zone (SGZ) of male rats (Siegers et al., 2008). It also showed an impairment on the Morris water test (a model of spatial learning in rodents) following a single high dose, as well as anterograde memory loss and impairment of object recognition (Seigers et al., 2008). A study conducted using another chemotherapeutic, cisplatin, found that the drug yielded a suppression of DCX-labelled cells for a week after a single treatment in a rat model (Hinduja et al., 2015). Studies using the drug 'cyclophosphamide' found that it suppressed neurogenesis with only a single dose, and yielded a 40% reduction of DCX-labelled cells, and a 30-90% reduction of BrdU-labelled cells when treatment spanned four-weeks (Christie et al., 2012; Kitamura et al., 2015; Lyons et al, 2011). Cyclophosphamide-treated rats also showed impaired performance on the novel place recognition task, contextual fear conditioning and novel location recognition (Christie et al., 2012; Lyons et al., 2011); the likes of which assess a rodent's short-term, immediate-term and long-term memory, associative fear learning and memory, and short and long-term memory recognition, respectively (Antunes & Biala, 2012; Chesworth et al., 2018; Shoji et al., 2014).

TMZ, the chemotherapeutic commonly used to treat glioma that was used in this study, has also been shown to suppress neurogenesis in a rodent model (Egeland et al., 2017; Nokia et

al., 2012). Prior studies exploring its effect in animals have shown that it results in an impairment on tests of cognition such as the trace eyeblink conditioning and delay eyeblink conditioning (Nokia et al., 2012), providing evidence that TMZ affects declarative and associative learning (Woodruff-Pak & Disterhoft, 2008). These insights may parallel those of this study since previous findings by researchers Medina et al. (2002) indicate that eyeblink and fear conditioning uses a similar design to test the formation of new memories. Along with the effects on learning that TMZ has had, rodent studies with TMZ have exhibited behavioural and biochemical changes such as depressive mood, and increased anxiety associated with the drug and in relation to a suppression of neurogenesis (Egeland et al., 2017; Strokotova et al., 2024). Strokotova et al. (2024) found that the use of long-term TMZ treatment led to an increase of anxiety in adult rats, and Egeland et al. (2017) found a significant correlation between the level of ventral hippocampal neurogenesis and corticosterone response. These findings support that a TMZ-treated rodent model is an adequate research model for CICI and should yield findings that parallel those of TMZ-treated humans.

1.5 Chemotherapy's Effects on the Hippocampus

The hippocampus is the brain structure that moderates learning and memory, and is important for our innate ability to differentiate between different contexts (Schmidt et al., 2012). The hippocampus proper consists of five main parts: the DG, CA3, CA2, CA1 and hilus. In the feedforward projections, sensory input comes through the EC which projects to the DG, then down through the other portions of the hippocampus (O'Reilly & McClelland, 1994; Yassa & Stark, 2011). The DG, the pertinent structure for hippocampus-dependent learning and memory, is located in the posterior portion of the hippocampus (Hainmueller & Bartos, 2020). It's here in the DG where the hippocampus produces neural progenitor cells into adulthood (Altman, 1962).

Despite its great importance, the DG, and the hippocampus in entirety, is highly subject to hypoxic-ischaemic infarcts, as well as other traumas that can result in the suppression of these cells (Ceanga et al., 2021; Nokia et al., 2012; Niibori et al., 2012).

The hippocampus can be divided into two different poles: the ventral and the dorsal pole, which each contribute to learning and memory in different ways (Loureiro et al., 2012). The dorsal hippocampus is connected to the EC which receives visuospatial information, contributing to spatial memory acquisition (Loureiro et al., 2012). Spatial memory acquisition supports retrieval of the locations of objects and places in the environment, aiding navigation of the environment (Baram et al., 2019). Interestingly, spatial learning has actually been found to modulate the rate of neural proliferation, maturation, activation and survival (Hernandez, Mercado & Zepeda, 2022). At the other end, the ventral hippocampus is connected to the basolateral amygdala and projects onto the CA1 modulating stress, emotion and affect (Loureiro et al., 2012; Yang & Wang, 2017). It is the ventral hippocampus that is a key region for the generation of the anxiety response (Forro et al., 2022).

The results of studies conducted on CICI have overwhelmingly supported the theory that there is an impairment in learning and memory associated with chemotherapy treatment that seems to affect the hippocampus. The suppression of neurogenesis found in many studies is one indicator of this (Egeland et al.,2017; Nokia at al., 2012; Strokotova et al., 2024). Another source for this theory is the reports of memory and learning impairments from chemotherapy survivors (Janelsins et al., 2014; Pendergrass et al., 2018; Von Ah et al., 2016; Wefel et al., 2010) as well as parallel impairments found from animal studies (Egeland et al., 2017; Nokia at al., 2012; Strokotova et al., 2024). These findings support the theory that the hippocampus suffers from an impairment as a result of chemotherapy treatment. The mechanism by which this occurs

however, is not yet agreed upon. It is the theory behind this study that the mechanism of action is likely due to a suppression of the neural progenitor cells of the SGZ.

1.6 Neurogenesis

Adult neurogenesis, as first described in the novel findings by Joseph Altman (1962), is the process by which new neurons develop in the neocortex of the adult mammal from neural progenitor cells (NPCs). Only two areas of the neocortex continually produce new neurons into adulthood. Prior to the findings of Altman, it was believed that neurogenesis was a process which occurred only during the embryonic and early postnatal stages of life. However, we now understand that there are two areas that do express neurogenesis into adulthood; the subventricular zone (SVZ), where the neurons develop and travel towards the olfactory bulb, and the SGZ of the DG in the hippocampus (Sahay et al., 2011). In the hippocampus, the process of neurogenesis allows for neural circuit plasticity, and the constant evolution and integration of progenitor cells into the DG-CA3 circuit (Sahay et al., 2011). The generation of new granular cells in the SGZ into adulthood is referred to as ‘adult hippocampal neurogenesis’ (AHN). The number of cells that emerge in this area into adulthood does not vary in adult animals, meaning that in relatively healthy animals, we can expect the number of neural progenitor cells to be relatively equal in animals of the same species (Rapp & Gallagher, 1996). Therefore, any vast changes or suppressions of the generation of neurons in this area would indicate that an error has occurred in the generation of granular neurons.

The capacity for the hippocampus to store new memories is a direct result of the ongoing neurogenesis and apoptosis that occurs in the hippocampal circuitry as found in rat studies, and both decreases and increases in neurogenesis were found to impact the recovery of memory

capacity (Alam et al., 2018). When adult neurogenesis or synaptic integration of adult progenitor cells are impaired, the resulting effect is on the acquisition of new memories (Scott et al., 2021; Winocur et al., 2006). Further, the reduction of AHN has been found to disrupt functions related to executive functioning and mood (Lucassen et al., 2010; Eisch and Petrik, 2012). I expect that my study will replicate these findings and support the literature currently showing that chemotherapy has an impairment on the adult neurogenesis that occurs in the hippocampus that causes cognitive and mood impairments.

1.7 Pattern Separation

Pattern separation is a process by which the hippocampus separates highly similar experiences of episodic memory into divergent and non-overlapping experiences, which result in the ability of distinguishing between similar contexts (Bakker et al., 2008; Moscovitch et al., 2016). AHN is believed to be an important component of the process of pattern separation with this process being found to occur in the SGZ, with the pathway from the DG to CA3 responsible for creating pattern separation representations (Gschwend et al., 2015; Schmidt et al., 2012; Yassa & Stark, 2011). Previous research has shown that the firing rate and tempo of CA3 and DG neurons differ when encoding different stimuli (Niibori et al., 2012). In the DG, a change in context produces changes in the firing rate of the same population of active neurons, while in the CA3, the same change in context will instead alter which population of neurons fire (Niibori et al., 2012).

Previous research has shown that when AHN is suppressed, it also impairs the ability to distinguish between two highly similar contexts (Niibori et al., 2012). Lesioning studies have shown that when the DG is lesioned, memory retrieval is found to be impaired, while a lesion to

the CA3 impairs retrieval but not encoding (Yassa & Stark, 2011). This indicates that if the TMZ of this study successfully suppresses AHN in the DG or CA3, I can expect to find issues with memory retrieval or encoding when animals on the test of contextual fear discrimination.

1.9 Objectives and Hypothesis

The direction for my study was to examine if chemotherapy is capable of suppressing AHN, and affecting learning and memory. Previous research has supported the idea that AHN is important for the integration of new memories. Research has also shown that some chemotherapeutics are capable of crossing the BBB due to their small molecular size. I therefore believe that this phenomenon is related and the suppression of AHN is what is causing the impairment in learning and memory of cancer survivors. By exploring this theory with the use of animal models, I will effectively be able to remove the confound of the cancer, using healthy animals instead of animals with cancer, and be able to explore the brain tissue posthumously to examine what exactly occurred at a neurological level. My hope is that this research is able to pinpoint the mechanism of action that creates the cognitive impairment that is reported by cancer patients.

1.9.1 Objective 1

This thesis will be carried out in two parts. To begin, I intend to perform a dose-seeking study to determine the lowest effective dose of TMZ to cause suppression of AHN. I will use a small sample of Long-Evans rats and give them either a moderate (25 mg/kg), or large (50 mg/kg) dose of TMZ; or a 2ml dose of saline for control animals. Once the animals have received three days of treatment, they will be euthanized and their brain tissue will be examined

through BrdU staining measures to determine which is the lowest effective treatment dose. I intend to use the lowest dose to limit the stress on my animals, and keep in accordance with Canadian Council for Animal Care and Trent University Animal Care Committee guidelines. These findings will provide the basis for my primary project, in which I will use the smallest effective treatment dose to carry out my behavioural tests.

1.9.2 Objective 2

To assess the cognitive side-effects of chemotherapy in relation to learning and memory, I will build on my findings from the dose-seeking study, extending the treatment length to match that of a typical treatment course that a cancer patient would undergo before subjecting my animals to various behavioural tasks. Previous research has indicated that chemotherapy suppresses episodic memory in humans (Bradley-Garcia, Winocur & Sekeres, 2022), and leads to increased anxiety (Pandey et al., 2006). It is my belief that I may replicate these findings through our project design and be able to advance the literature and understanding surrounding the cognitive and emotional deficits of chemofog.

1.9.3. Objective 3

Once my main study is conducted, I will euthanize my animals to once again examine the brain tissue posthumously. I will do so by conducting three different staining techniques that explore different aspects of the hippocampus. This part of my study is conducted with the intention that I may uncover the mechanism of CICI. I hypothesize that when examining this tissue, my DCX stain will show a suppression in adult progenitor cells in the DG. Second, I will

use EGR1 as an activity-dependent neural marker to see how activation of this immediate early gene (IEG) compares between the control and TMZ group. I also chose to explore Synaptophysin to see if synaptic boutons were affected by the chemotherapy, and may be the cause of the cognitive impairments instead. My hope is that I will be able to identify the mechanism of action that results in chemotherapy resulting in CICI by exploring this brain tissue.

Chapter 2: Experimental Methodology

In this section, I will provide an overview of the experimental procedures and methods used in my research.

2. Materials and Methods

2.1. Animals

Male Long Evans rats (N=10), weighing between 200-300 g, were obtained from Charles River Laboratories (QC, Canada). All animals were initially group housed (2 rats per cage) upon arrival before being separated and housed individually in standard rectangular cages. The animals were housed in a temperature-controlled room (25 ± 2°C) at 60% humidity with ad libitum access to food and water. All experiments began after 1 to 2 weeks of handling. Experiments were performed between 0900 and 1800 h during the photophase of a 12-hour light/dark schedule (lights on at 0700 h). All procedures were approved by Trent University Animal Care Committee and followed the guidelines from the Canadian Council on Animal Care.

2.2 Drug Treatments

Temozolomide (TMZ, MedChem Express, CAS: 825622-93) is a DNA alkylating prodrug with high blood-brain barrier permeability. In humans, TMZ (2-5 mg/kg) has been effectively used to treat low grade glioblastoma (Laskari et al., 2011). TMZ (2.5 mg/ml) was freshly prepared each day by dissolving in ice-cold sterile 0.9% (w/v) saline (NaCl, pH 5.5-6.0). The TMZ solution was then vortexed (~ 30 s) and briefly sonicated (50 W, 5 s) in an ice bath. The TMZ solution was sterile filtered before use to remove remaining insoluble residues and was placed on ice during injections.

Bromodeoxyuridine (BrdU) is a thymidine analogue that is widely used to label proliferating cells in the CNS. BrdU (20 mg/ml) was prepared by dissolving in warm NaCl (50°C) and then sterile filtered (0.45 μm) before use.

2.3 Experiment 1 – Effect of TMZ on Hippocampal Neurogenesis

2.3.1. Procedure

The purpose of this experiment was to determine an effective dose of TMZ that could alter levels of hippocampal neurogenesis. In this experiment, subjects underwent two separate 3day cycles of TMZ treatment. This procedure involved subjects receiving an injection of TMZ at either 50 mg/kg/day (25 mg/kg TMZ injection with a minimum of 4 hrs between injections, n=4), 25 mg/kg/day (alternating doses of 25 mg/kg TMZ then saline every 4 hrs, n=4), or saline injections (n=4) for three consecutive days. Each 3-day treatment cycle was then followed by four days of no injections. Approximately four hours after receiving the last TMZ or saline injection, all subjects received a single dose of BrdU (200 mg/kg, 20 mg/ml in 0.9% saline, i.p., BA-5002 Sigma Aldrich) and were euthanized 24 hrs later to examine the impact of TMZ treatment on levels of hippocampal cell proliferation and neurogenesis.

2.3.2 Tissue Preparation and Immunohistochemistry

Twenty-four hours after receiving BrdU, rats were deeply anaesthetised with sodium pentobarbital (340 mg/ml, Euthansol, Merck Animal Health Care Canada) and then underwent transcardiac perfusion with 0.1 M phosphate buffered saline (PBS; pH=7.4) followed by ice-cold 4% (w/v) paraformaldehyde. The brains were extracted and post-fixed in the same fixative for 48 hrs at 4°C prior to being sectioned at 50 μm on a Leica VT-1000 vibratome. All sections were stored at 4°C in 0.01% (w/v) sodium azide in PBS. DCX was not used in this procedure because previous research with BrdU had already yielded a reduction in neurogenesis (Nokia et al., 2012), and our main goal was only to determine if a difference occurred between treatment groups at this stage and a more in-depth analysis was to be conducted in the second experiment.

Every 12th free-floating (50 um) section was processed using procedures previously described (Kalinina et al., 2019). Sections were washed several times in PBS and then placed in 2 N HCl at 45 °C for 50 min to expose the BrdU antigen. The sections were then incubated for 1 h at room temperature in a blocking solution comprised of 5% (v/v) normal horse serum, 1% (w/v) bovine serum albumin (BSA), and 0.3% (v/v) Triton X-100 dissolved in 1X PBS. After blocking, the sections were incubated with a mouse anti-BrdU monoclonal primary antibody (1:500, 4°C) diluted in the previously described blocking solution for two days. Following incubation with the primary antibody, the sections were washed several times in PBS and then placed in incubated with a secondary antibody (biotinylated horse anti-mouse, 1:500, 2 h, room temperature, Vector Laboratories) followed by avidin–biotin peroxidase (1:500, 1 h, room temperature, Vectastain ABC Elite, Vector Laboratories). Immunolabeling was visualized using 0.033% (w/v) 3,3′-diaminobenzidine (DAB) and 0.00786% (v/v) hydrogen peroxide diluted in PBS to yield a brown product.

The number of BrdU-labeled cells located in the subgranular layer were counted using a modified version of the optical fractionator method (West et al., 1991). Because BrdU-labeled cells are relatively rare, no guard zones were used, and all cells located within the region of interest were counted. BrdU-labeled cells were counted across a series of sections that systematically covered the entire dentate gyrus of each animal. The resulting numbers were summed and multiplied by section sampling fraction (12) to provide an estimate of the total number of BrdU-labeled cells.

2.4 Experiment 2 – Examination of temozolomide-induced behavioural impairment in a rodent model of chemobrain

2.4.1 Procedure

In the present study a multi-cycle (four week) treatment that has been used previously (Gathe et al., 2009; Xiang et al., 2012) was employed to examine the cyclical effects of TMZ treatment on neural progenitor cell expression and behavioural changes. Rats were injected twice daily with dosages of 25 mg/kg of TMZ (2.5 mg/ml, i.p.) for three consecutive days, followed by a four-day recovery period, for a total of six weeks. This chosen TMZ dose for rats closely resembles the common 200 mg/m^2 dose given to humans, with appropriate adjustments for weight and height (human weight, 65 kg; human height, 170 cm; rat weight, 0.3 kg, Oncological Tools: Dose Calculator - http://www.accessdata.fda.gov/scripts/cder/onctools/animalquery.cfm). Moistened rat chow and/or meal supplement replacement fluids (Ensure) was given to any animals that showed symptoms of adverse weight loss from TMZ treatment.

2.4.2 Behavioural Testing

One week after the last treatment, the impact of TMZ treatment on several behaviours was assessed using various behavioural tests, including contextual discrimination learning, elevated plus maze (EPM), and shock-probe test. An outline of the experimental design is presented in the Result section **(Figure 2)**.

2.4.3 Contextual Fear Discrimination

For context fear discrimination training, two operant conditioning chambers identical in shape and size (25.4 x 25.4 x 36.5 cm) housed in a sound attenuating box (54.3 x 46.4 x 55.1 cm) were used. Both contexts shared many common features, including a transparent front door and roof as well as an exposed stainless-steel grid floor. However, Context B was modified in that the chamber walls were covered with checkered plastic inserts (made from ethylene vinyl acetate). Context A was cleaned using Oxivir Five 16 Concentrate (1:16 dilution), whereas Context B was cleaned each with a 70% ethanol and lemon scent solution to provide distinct olfactory cues associated with each context.

Each rat was randomly assigned to receive shocks in either Context A or B, with the opposite context (Context B or A) being assigned as the no-shock/'safe' condition. Training occurred for 6 days with each rat receiving 2 exposures to each context (A or B) daily. For shock trials, subjects were placed into their designated contexts (context A or B), and after an acclimation period (120 s), a single footshock (2 s, 1.0 mA) was delivered. Subjects were removed from the chamber 60s later and returned to their home cage. For safe (no-shock) trials, subjects were placed into their designated contexts (Contexts B or A) for 180 s and then returned to their home cages. There were four trials per day with two trials (shock or safe) in the morning

(separated by 1-1.5 hrs) and another two trials (shock or safe) in the afternoon (separated 1-1.5 hrs). The interval between morning and afternoon sessions was approximately 3-4 hrs, and the order presentation of the shock and safe trials for each of these sessions was randomized each day using a Latin-square design. Defensive freezing, which was defined as the absence of observable movement except those necessary for respiration, was measured during all sessions using an automated freeze detection system (AnyMaze). The percentage of freezing that occurred during the first 120 s of exposure to context A or context B was used as a measure of conditioned fear. The first 120 s were analysed because this is the time frame prior to the administration of the shock, if the animals were to receive one. By using the 120 s prior to the shock, I am able to assess if the animal is displaying fear in anticipation to the shock, and not a response to the experience of having been shocked or not.

2.4.4 Elevated Plus Maze

Anxiety-like behavior was assessed using the EPM consisting of two open arms (50 cm x 10 cm) and two enclosed arms (50 cm x 10 cm x 40 cm), elevated 50 cm from the floor. The plus maze was placed in the center of a homogeneously illuminated room. Each rat was placed in intersection between the arms facing the open arm opposite to the investigator. Each session was video recorded for five minutes and the rat's position was determined by automatic video tracking (AnyMaze, Stoelting, Co.). The percentage of open arm and closed arm entries as well as time spent in the open and closed arms was recorded. The frequency of stretch attending posture, defined as a forward elongation of the head and shoulders into the open arms while the two hind paws remain in the closed arms or center region, was also calculated for each animal.

At the conclusion of the test, the rat was returned to their home cage, and the plus maze was cleaned with 70% ethanol and dried with paper towels before the next test.

2.4.5 Shock Probe Defensive Burying Test

The shock probe test was carried out using procedures slightly modified from those used by (Pesold & Treit, 1992). Briefly, animals were initially habituated to the shock-probe chamber for 15 minutes over four consecutive days. The chamber was made of Plexiglas and measured 40 cm long x 30 cm wide x 40 cm high, with 2 cm of bedding material covering the flooring. No shock probe was present during the habituation sessions.

For the shock probe defensive burying test, each rat spent 15 min in the shock-probe chamber. A wire-wrapped Plexiglas probe was inserted into the center of one of the walls, 3 cm above the floor bedding. The probe was constantly electrified (1.5 mA) during the duration of the training session. Each animal's test was recorded and analyzed using AnyMaze software (Stoelting, Co). The total number of contact-induced shocks as well as the amount of time spent burying (i.e., pushing bedding forward with the forepaws or snout towards the probe) was measured. In addition, the conditioning chamber was divided at its mid-point into distal and proximal segments relative to the position of the probe, and the amount of time spent as well as the percentage of freezing in each compartment was also recorded. After each test, the probe and the walls of the chambers were cleaned with Oxivir Five 16 Concentrate (1:16 dilution) and the bedding replaced for the next rat.

2.4.6 Tissue Preparation and Immunohistochemistry

Ninety-minutes after the completion of the shock probe test, rats were deeply anaesthetised with sodium pentobarbital (340 mg/ml, Euthansol, Merck Animal Health Care Canada) and then underwent transcardiac perfusion with 0.1 M phosphate buffered saline (PBS; pH=7.4) followed by ice-cold 4% (w/v) paraformaldehyde. The brains were extracted and postfixed in the same fixative for 48 hrs at 4°C prior to being sectioned at 50 μm on a Leica VT1000 vibratome. All sections were stored at 4°C in 0.01% (w/v) sodium azide in PBS.

To examine the impact of TMZ chemotherapy on markers of hippocampal neurogenesis and plasticity, sections were processed immunohistochemical staining using previously described procedures (Fournier et al., 2009; Horsey et al., 2020; Kalinina et al., 2019). The following primary antibodies were used: rabbit polyclonal anti-doublecortin (DCX; 1:2000, Cell Signalling), rabbit polyclonal Egr1 (1:1000, Cell Signalling), and rabbit polyclonal antisynaptophysin (1:5000, Cell Signalling Technology). All primary antibodies were diluted in the same blocking solution comprised of 5% (v/v) normal goat sera, 1% (w/v) bovine serum albumin, and 0.3% (v/v/) Triton X-100 diluted in PBS and incubated for 24 to 48 hrs at 4°C. Sections were ten treated with a biotinylated goat anti-rabbit secondary antibody (1:500, 2 hrs, room temperature, Vector Laboratories) followed by amplification with avidin-biotin complex (1:500, 1 hr, room temperature). Immunolabeled cells were visualized with a solution containing 0.02% (w/v) DAB, 2.5 % (w/v) nickel ammonium sulfate and 0.000083% (v/v) H_2O_2 diluted in 0.175 M sodium acetate (pH = 7.0) to yield to produce a blue-black product or a solution containing 0.033 % (w/v) DAB and 0.00786% (v/v) H_2O_2 diluted in PBS to yield a brown product. Sections were mounted onto glass slides and coverslipped with Entellan (Fisher Scientific) mounting medium.

2.3.7 Quantification

The total number of DCX+ immature neurons was estimated using the optical fractionator method (West et al., 1991). Every sixth section was examined at 100X (oil immersion) magnification on a Nikon Eclipse 80i microscope equipped with a motorized stage and a computerized stereology system (Stereologer). There were a total of 8 to 11 sections per subject. DCX+ cells found within the dentate granule cell layer and the SGZ were counted and the total number of DCX+ cells was estimated using the following formula: NTotal = $\sum Q$ = 1/ssf X A(x,y step)/ a(frame) X t/h; where $\sum Q$ is the number of cells counted; ssf is the section sampling fraction (1/6); A(x,y step) is the area associated with each x,y movement (200 μm); a(frame) is the area of the counting frame (5250 μm2); t is the weighted average section thickness; and h is the height of the dissector (12 μm). A guard zone of 2 μm was used to avoid sectioning artifacts. The coefficient of error was calculated and all values <0.15 were accepted, but there were no values that were greater than .15. To determine the impact of TMZ treatment on DCX+ cells across the septotemporal axis of the hippocampus, we extracted from the total number of DCX+ cells counted across the entire dentate gyrus from at least 3 sections corresponding to dorsal dentate gyrus (AP -2.92 to -3.600 mm) and ventral dentate gyrus (AP 4.92 to 5.64 mm), respectively. The experimenter was blind to the treatment condition of the subjects during quantification.

For quantification of Egr1 immunoreactivity, images were comprised of each of the hippocampal subfields (CA1, CA3, and dentate gyrus) collected at 10X magnification on a Nikon Eclipse T2 inverted microscope (Nikon Instruments, USA). All analyses were performed offline using ImageJ software. A threshold was then set so that the Egr1+ immunoreactive cells could be clearly identified. This analysis was used instead of unbiased stereology due to time constraints and impediments resulting from the Covid-19 pandemic, leaving me to resort to

another method to analyse my Egr1 and synaptophsin-stained tissue. The area of the immunoreactivity was then expressed as a percent of the total area in each hippocampal region. The mean immunoreactivity was measured across at least 3 sections. All quantifications were performed by an experiment that was blinded to the animal history.

Synaptophysin was quantified using a procedure adapted from a previously published protocol (Fournier et al., 2009). Briefly, images were captured at 8-bit resolution using a digital camera that was attached to a Nikon Eclipse T2 microscope (10x objective). Camera exposure and gain settings were held constant between animals. Using image-analysis software (ImageJ 1.35, National Institute of Mental Health), the mean relative optical density was calculated from digital images of three coronal sections (300 um distance between sections). We quantified synaptophysin immunoreactivity within the laminar boundaries of the CA3 subfield (stratum oriens, stratum pyramidal, stratum lucidum and stratum radiatum), dentate gyrus (hilus, lower and upper blade of the granule cell layer, the inner molecular layer, and the outer and middle molecular layer) and hilus. The dentate gyrus was further divided into the upper and lower blades, respectively. Background staining was controlled by calculating the average optical density levels from the corpus callosum and subtracting these values from each area of interest. Standardized values were calculated and were then expressed as a percentage change from controls. The experimenter was blind to each animal's condition.

2.4.8 Statistical Analyses

All analyses were performed using Statistical Package for the Social Sciences (v. 21.0) software. All data was examined for normality and homogeneity of variance. On most measures, comparisons between groups were examined using independent t-tests or one-way analysis of

variance (ANOVA). For contextual discrimination learning, the percentage of freezing during the first 120 s of the session was recorded for the shock and no shock (safe) contexts over the 6 days of training and was analyzed using a three-way repeated measures ANOVA with drug treatment (TMZ vs. saline) as the between subject factor and context (safe vs. shock) and day of training (days 1 through 6) as the within-subject factors. Significant differences were examined further using paired and independent t-tests, or one-way ANOVAs where appropriate. For the shockprobe test, the amount of time the animal spent in the distal and proximal segment of the conditioning chambers was examined using a two-way repeated measure ANOVAS with drug treatment (TMZ vs. saline) was the between subject factor and proximity to the probe (proximal vs. distal) as the within-subject factor. The criterion for statistical significance was set at $P < 05$. All data is presented as the mean ± standard error of the mean (S.E.M.).

Chapter 3: Results

3.1 Experiment 1: TMZ administration reduces adult hippocampal neurogenesis in rats without impairing general health

Temozolomide (TMZ) is an alkylating agent and is an approved drug for the treatment of glioblastoma multiforme in both adults and children (Agarwala & Kirkwood, 2000; Attarian et al., 2021; Yazici et al., 2016). TMZ exerts antiproliferation and cytotoxic effects through conversion to the active metabolite MTIC (monomethyl triazene 5,3-methyl triazen-1ylimidazole4-carboxamide) (Darkes et a., 2002). MTIC is a reactive methylating agent that introduces methyl groups to DNA guanine bases resulting in base-pair mismatch (Thomas et al., 2013). Unsuccessful engagement of DNA repair mechanisms leads to accumulation of DNA damage through breaks and permanent nicks which prevents mitotic division and facilitates apoptosis (Wesolowski et al., 2010).

TMZ can rapidly cross BBB and introduce direct toxicity to the brain through several mechanisms, including impairments in hippocampal neurogenesis (John et al., 2021; Sekeres et al., 2021). However, due to its cytotoxic nature, TMZ can also induce adverse secondary reactions which may have implications for cognitive performance. Thus, the primary goal of this study was to perform a dose-seeking experiment to establish a dose of TMZ that could effectively disrupt levels of hippocampal neurogenesis while minimizing potential adverse effects on the overall physiological health of our subjects.

3.2 Effect of TMZ cyclic treatment on hippocampal cell proliferation and neurogenesis

To assess the broad impact of TMZ, adult male Long-Evans rats received two cycles of TMZ treatment (3 days and 4 days recovery) at dosages of either 25 mg/kg/day, 50 mg/kg/day, or

saline (0.9%) (**see Figure 1a**). We regularly monitored subjects in their home cages throughout the duration of the study and did not observe any gross changes involving body weight loss, fur loss or body coat after TMZ treatment. As shown in Figure 1b, there was no effect of TMZ at 25 mg/kg/day or 50 mg/kg/day on body weight gain after two cycles (P=85,

Figure 1b) suggesting that TMZ treatment did not appear to produce significant side effects. To investigate the effect on TMZ on AHN and cell proliferation, rats were injected with a single dose of BrdU (200 mg/kg; 20 mg/ml; i.p.) four hours after receiving their last TMZ or saline injection and were sacrificed 24 hrs later. BrdU was administered at a single saturating dose of 200 mg/kg body weight (i.p.) as this has been previously shown to label all actively dividing neural progenitors in the rat dentate gyrus (Cameron & McKay, 2001; Eadie et al., 2005). The results showed a tendency toward a dose-dependent relationship after two weeks of TMZ treatment and hippocampal cell proliferation as measured by BrdU immunohistochemistry (One-way ANOVA, P<.054). As shown in Figure 1c, rats that had received 50 mg/kg/day TMZ for two cycles had a tendency towards significantly fewer BrdU+ cells (approx. 68% reduction) at the end of treatment compared to saline controls (Dunnett's, P=.054). Dunnett's was chosen for this test to compare the two treatment group results against the control. However, when this analysis was restricted to compare the number of BrdU+ cells across the dorsal and ventral dentate gyrus separately. Previous research has indicated that when examining the dorsal and ventral hippocampus, that it should be done at a 50/50 split (Bannerman et al., 1999); however, studies note that this is an arbitrary divide (Fanselow & Dong, 2010). As such, we used dorsal, middle and ventral sectioning for our estimates. There was a significant decrease in BrdU+ cells for the ventral dentate gyrus for the rats that received TMZ [t(6)=3.27, P<.031, **Figure 1d**]. There was no difference in the number of BrdU+ cells for the dorsal dentate gyrus for TMZ or saline groups (TMZ-50 vs. saline, P=.301; TMZ-25 vs. saline, P=.228]. These results suggest

that at TMZ at a dose of 50 mg/kg/day was necessary to significantly impact levels of hippocampal cell proliferation and that this effect appeared to differentially affect the ventral dentate gyrus. However, more importantly, treatment of TMZ at either dose or duration of treatment was not associated with an adverse effect on health as inferred by changes in body weight.

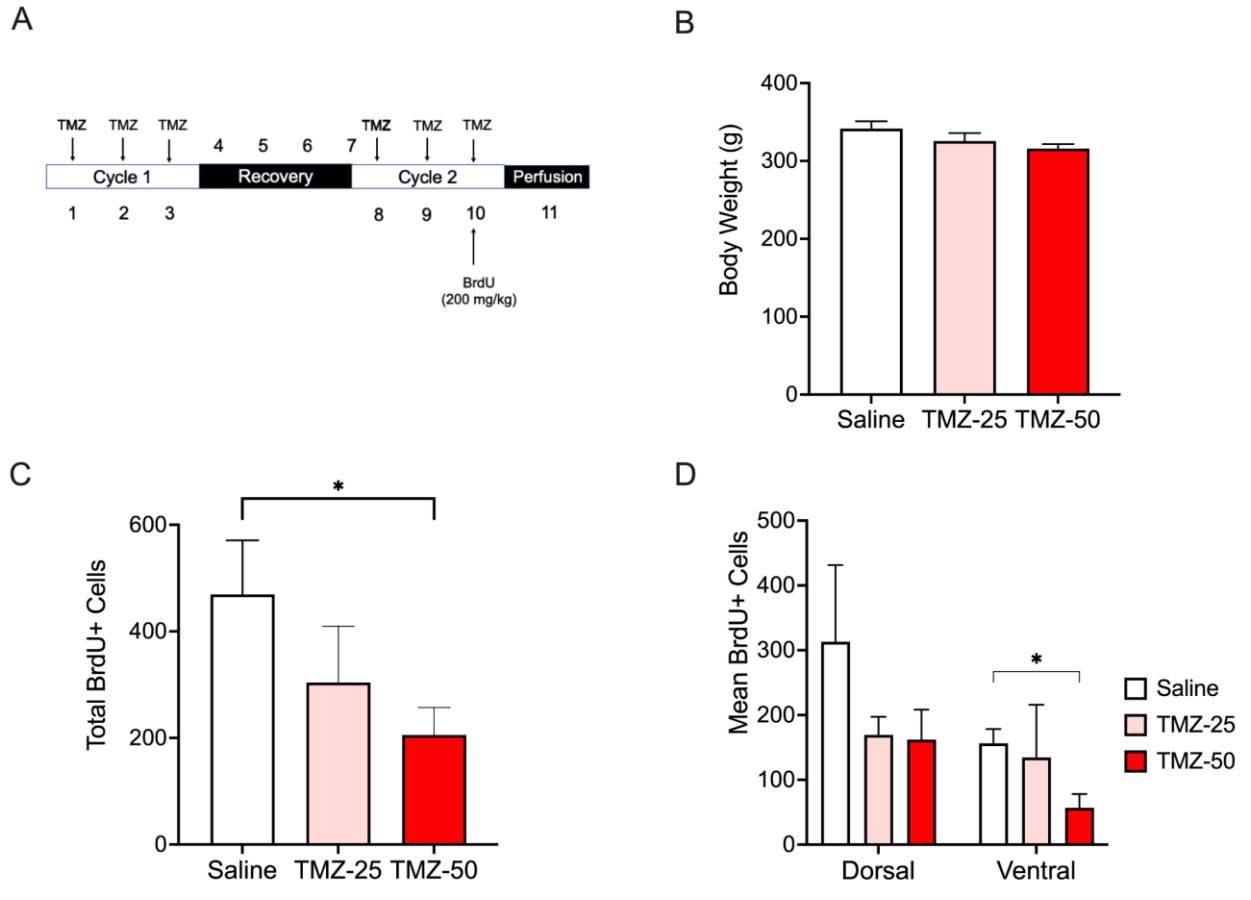


*Figure 1***.** Effects of dose-seeking experiment. (A) Dose-Seeking Experiment Timeline. Animals were subjected to two treatment cycles at three days each. Animals were given four days of rest in between and all animals were perfused on the 11th day of testing. (B) Effect of Dose-Seeking Experiment on Body Weight. Mean body weight of each group following the two cycles of treatment. (C) Total Mean BrdU+ Cell Count. Quantification of the total number of BrdU+ cells found in the SGZ after the dose-seeking experiment. Data is presented in mean ± SEM. *p < .05. (D) Dorsal and Ventral Mean BrdU+ Cell Count**.** Quantification of the mean number of BrdU+ cells found in either the dorsal or ventral hippocampus after the dose-seeking experiment. Data is presented in mean ± SEM.. *p < .05.

3.3 Experiment 2: Examination of temozolomide-induced cognitive impairment in a rodent model of chemobrain

Glioma/glioblastoma is one of the most prevalent and highly aggressive primary tumor originating from glial cells and it is the third most common cause of death of cancer patients 15 to 34 years of age. It is associated with an array of symptoms including memory impairments, persistent headaches, alterations in personality, seizures, speech disorders, and weakness or numbness experienced in the arms, legs, and facial regions. While the standard of care for glioblastoma includes surgery/radiation, several clinical trials have shown that the DNA alkylating agent TMZ is highly efficacious for the treatment of glioblastoma leading to its widespread clinical use. TMZ is readily permeable through the blood-brain barrier, and as a result, it can produce direct toxicity to the brain. However, only a few studies have directly examined whether chronic cyclic treatment with TMZ can adversely affect cognitive behaviours.

The hippocampus plays an important role in the formation and retention of episodic and spatial memories. A subregion of the hippocampus, the dentate gyrus, stands out as it is one of the few brain regions that is involved in the continuous production of new neurons throughout life in both rodents and humans. Accumulating evidence has shown that adult-born neurons mature and functionally integrate within the surrounding hippocampal circuitry, displaying a period of heightened plasticity and excitability that enable them to critically impact hippocampal functions. As we showed in Experiment 1, cyclic TMZ treatment can reduce levels of hippocampal cell proliferation and neurogenesis. Given that reduced neurogenesis has been linked to impairments in learning and memory functions, as well as changes in emotional function, we set out to examine whether chronic cyclic treatment of TMZ in rats could produce be associated with the development of behavioural symptoms, such as cognitive impairments and affective disorders that are often observed in patients that have underwent chemotherapy.

3.3.1 Results

Figure 2 shows the general experimental design of this study. Based on the most effective dose found in our dose-seeking study, all TMZ-treated animals were given a dose of 50mg/kg of TMZ per injection. Rats received either saline (N=6) or TMZ (50 mg/kg, N=6) for 4 cycles (3 consecutive treatment days followed by 4 rest days for each cycle). Seven days after the last TMZ treatment, all underwent a series of cognitive and behavioural tests starting first with contextual discrimination learning (six days), followed by EPM (one day), and finally, the shockprobe test (four days of habitution and one day of testing).

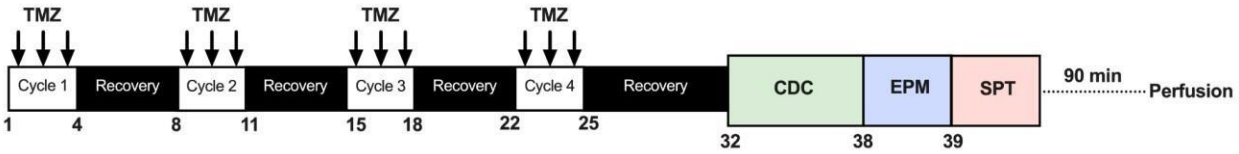


*Figure 2***.** General Experiment Timeline of Experiment 2. Animals (N=10) were randomly assigned to either a saline control (n=5), or 50 mg/kg TMZ treatment group (n=5). Injections lasted four cycles of three days, with four days of rest for a total of four weeks. Following the final injections, all animals were given a week of recovery before beginning behavioural testing. Context discrimination testing was conducted first, lasting a total of six days. Then animals did one EPM test, and finally all animals were subjected to the shock-probe test, beginning with four sessions of habituation and ending with one day of testing.

Two rats were removed from the analysis; one rat in the TMZ group was removed due to an adverse reaction to TMZ treatment (<30% weight loss during course of treatment), and one saline control was removed from the analysis due technical issue during behavioural testing. However, there was no difference in body weight between the TMZ group and the saline-treated controls at the end of the experiment. Thus, the final group composition was: 5 saline controls and 5 TMZ-treated rats.

3.3.2 Temozolomide impairs contextual discrimination learning

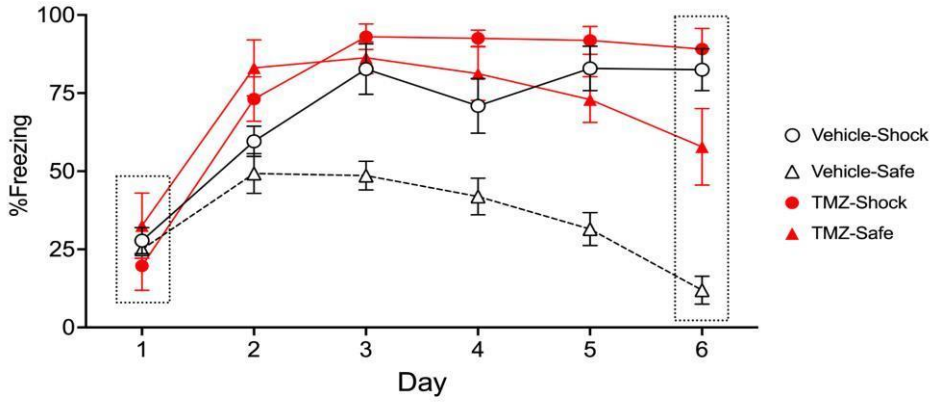
The purpose of this experiment was to examine whether the influence of TMZ on levels of AHN could disrupt performance on a behavioural pattern separation task. Here, I used contextual discriminative fear conditioning to probe at the putative function of adult-born neurons in the dentate gyrus and examine whether TMZ-induced depletion of neurogenesis could impact discriminative learning. Previous research indicated that adult neurogenesis in both the dorsal and ventral hippocampus were important contributors to memory acquisition and recall, and overall hippocampal function (Huckleberry et al., 2018). Pattern separation refers to the process in which experiences can be represented as distinct neural ensembles within the dentate gyrus, thereby minimizing the interference that can arise from recalling seemingly similar experiences. In this task, rats are tested in their ability to discriminate (based on their freezing behaviour) between a context paired with a footshock from a similar context that was not paired with the footshock. This procedure has been shown to correlate with levels of AHN.

The results of a three-way mixed design ANOVA with day (days 1 through 6) and context (shock and safe) as the within-subject factors and treatment (saline vs. TMZ) as the between subject factor was used to compare freezing levels during contextual discrimination learning. The analysis revealed significant two-way interactions for Day and Treatment [F(5,40)

= 5.36, P<.001] and for Context and Treatment [F(1,8) = 5.38, P<.049], respectively. The main effect of Treatment was also found to be significant [F (1,8) = 14.94, P<.005]. Further analysis showed while both TMZ and saline controls exhibited comparable mean levels of freezing to the safe/no-shock context on Days 1 and 2 of training, rats that received TMZ continued to display higher levels of freezing to the safe context on Day 3 of training with this difference persisting until the end of testing (All Ps <.008, **Figure 3a, b**). It was decided to analyse data this way as breaking it down by session as opposed to day did not add any extra insight into the findings, and breaking them down by day still showed clearly where the change in learning occurred. As anticipated, both the TMZ and saline controls exhibited high levels of freezing when exposed to the shock context throughout the training period (**Figure 3a**).

The results presented above show a significant group difference in freezing responses to the safe context, however, it is important to address if there was a change in freezing between the shock and safe contexts during training. To examine this, we performed a series of paired-tests comparing freezing to the shock and safe contexts for TMZ and saline controls separately. The results showed that saline controls displayed less freezing in the safe context than the shock context on Days 3 (P=.016), Day 5 (P=.008), and Day 6 (P=.006, **Figure 3a**) of testing indicating that this group could effectively discriminate between both conditioning contexts. In contrast, rats that received TMZ only showed less freezing to the safe context compared to the shock context on Day 5 only (P=.007) with a non-significant trend for Day 6 (P=.075, **Figure 3b**). These results suggest that while saline controls could effectively discriminate between the two contexts after 3 days of training, TMZ-treated rats continued to display marked impairments in context discrimination with some improvement occurring after 5 days of training.

A



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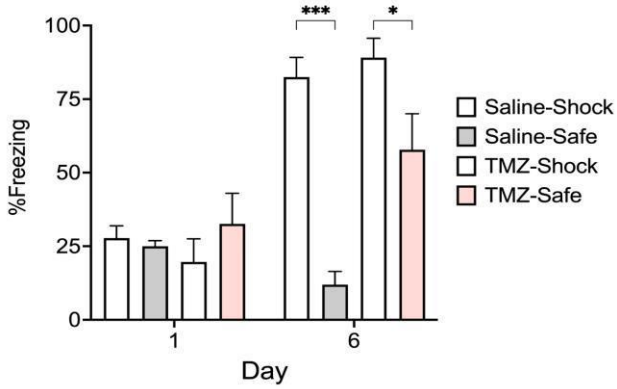

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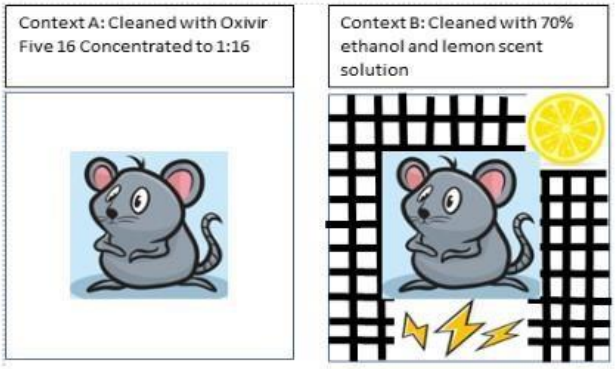

Figure 3. Results of the contextual discrimination test. (A) Freezing Effects during Contextual Discrimination. This figure shows the mean freezing percentages for each treatment group and each context. (B) Freezing Effects at First and Last Training Days during Contextual Discrimination. This figure shows the mean freezing percentages for each treatment group and each context from the first and last days of training. Data is presented in mean ± SEM. *p < .05. **p <.01. (C) The Contextual discrimination test apparatus. Context A is a plexiglass box cleaned with oxivir Five 16, while Context B had a checkered pattern on the walls and was cleaned with 70% ethanol and lemon scent.

3.3.3 Temozolomide increases anxiety-like behaviour and induces greater avoidance

Following the completion of contextual discrimination learning, we examined anxietylike behaviour in the EPM and shock-probe defensive burying test, two well-validated animal models of anxiety. Rats that received TMZ spent significantly less time in the open arms than salinetreated group [t(8)=2.52, P<.036, **Figure 5a**]. Additionally, the TMZ group also had higher frequency of closed arm entries [t(8)=2.47, P<.039, **Figure 5b upper**] and protected stretch attending postures [t(8)==2.66, P<.029, **Figure 5c**] compared to the saline group. These results suggest that prior cyclic TMZ treatment was associated with an elevation in open arm avoidance and risk-assessment behaviour.

Following the completion of EPM (Figure 4), rats underwent testing on the shock-probe defensive burying test. The number of contacts with the electrified probe was not different between the groups [P>.67]. As shown in Figure 4d., the TMZ-treated group spent more time immobile [t(8)=2.50, P<.037, **Figure 5d**] than the saline controls during 15-minute acquisition test. Additionally, the TMZ group spent significantly less time in the compartment area containing the probe [t(8)=2.64, P<.030, **Figure 5e**] suggesting that rats that underwent chronic cyclic treatment with TMZ engaged higher avoidant responses to the electrified probe. Finally, The TMZ group also showed a decrease in burying ratio compared to controls [t(8)=5.24, P<.001, **Figure 5f**].

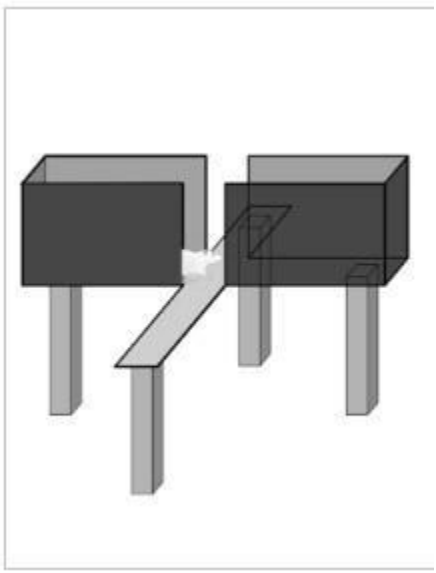

*Figure 4***.** The EPM apparatus. The cross-shaped maze is elevated 50 cm from the ground to create an anxiety-inducing scenario for the animals. The maze consists of two open arms 50 cm long and 10 cm wide, and two enclosed arms 50 cm long, 10 cm wide, and with 40 cm high walls.

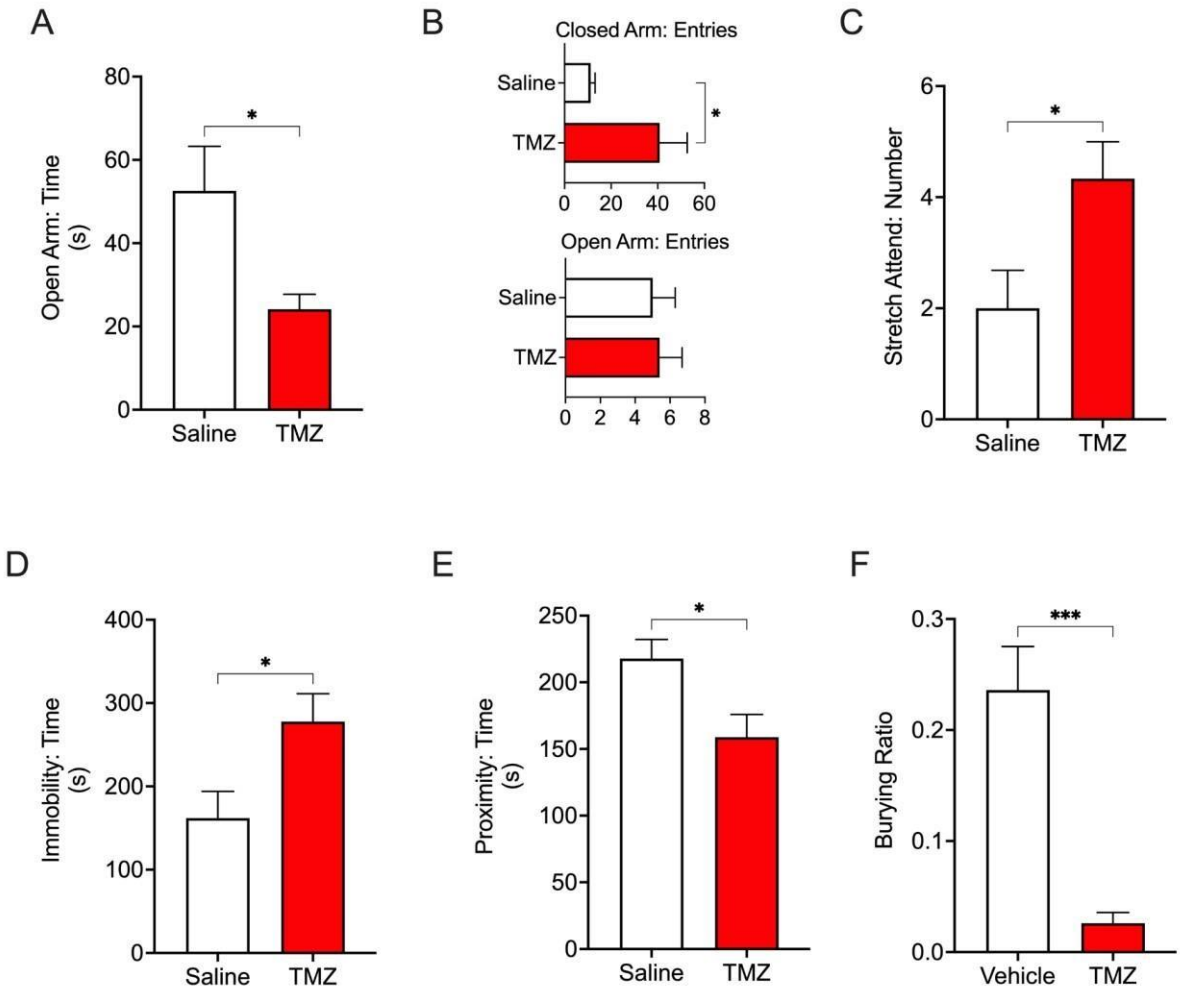

Figure 5. Results of the contextual discrimination test. (A) Time Spent in Open Arms in EPM. This figure shows the mean number of seconds spent in the open arms during the EPM. Data is presented in mean ± SEM. *p < .05. (B) Closed & Open Arm Entries in EPM. This figure shows the mean number of entries into the closed arms (upper); and the mean number of entries into the open arms (lower) of the EPM. Data is presented in mean ± SEM. *p < .05. (C) Number of Stretch Attending Postures in EPM. This figure shows the total number of stretch-attending postures performed by each treatment group during the EPM test. Data is presented in mean ± SEM. *p < .05. (D) Time Spent Immobile during Shock-Probe. This figure shows the average time (in seconds) each group spent immobile during the shock-probe test. Data is presented in mean ± SEM. *p < .05. (E) Time Spent in Proximity to Probe during Shock-Probe. This figure shows the average time in seconds that each group spent in proximity to the electrified probe during the shock-probe test. Data is presented in mean ± SEM. *p < .05. (F) Burying Ratio during Shock-Probe. This figure shows the ratio of burying completed by each treatment group during the shock-probe test. Data is presented in mean ± SEM. ***p <.001.

3.3.4 Temozolomide reduces adult neurogenesis and hippocampal structural plasticity

To confirm our earlier findings that TMZ impacted levels of hippocampal neurogenesis, we examined the immature neuron marker doublecortin (DCX). Consistent with prior studies and the results from Experiment 1, we found that the number of DCX+-immunoreactive cells was significantly lowered after 4 weeks of TMZ treatment compared to saline controls [$t(8)=3.61$, $P<.007$, **Figure 6a**]. Further analysis indicated this effect of TMZ was confined specifically to the ventral dentate gyrus only [dorsal dentate gyrus, $t(8)=1.16$, $P=.301$; ventral dentate gyrus, $t(8)=5.01$ $P<.001$, **Figure 6b, c**]. Together, these findings suggest that TMZ caused a decrease in levels of hippocampal neurogenesis, as measured by DCX immunolabeling, particularly within the ventral dentate gyrus.

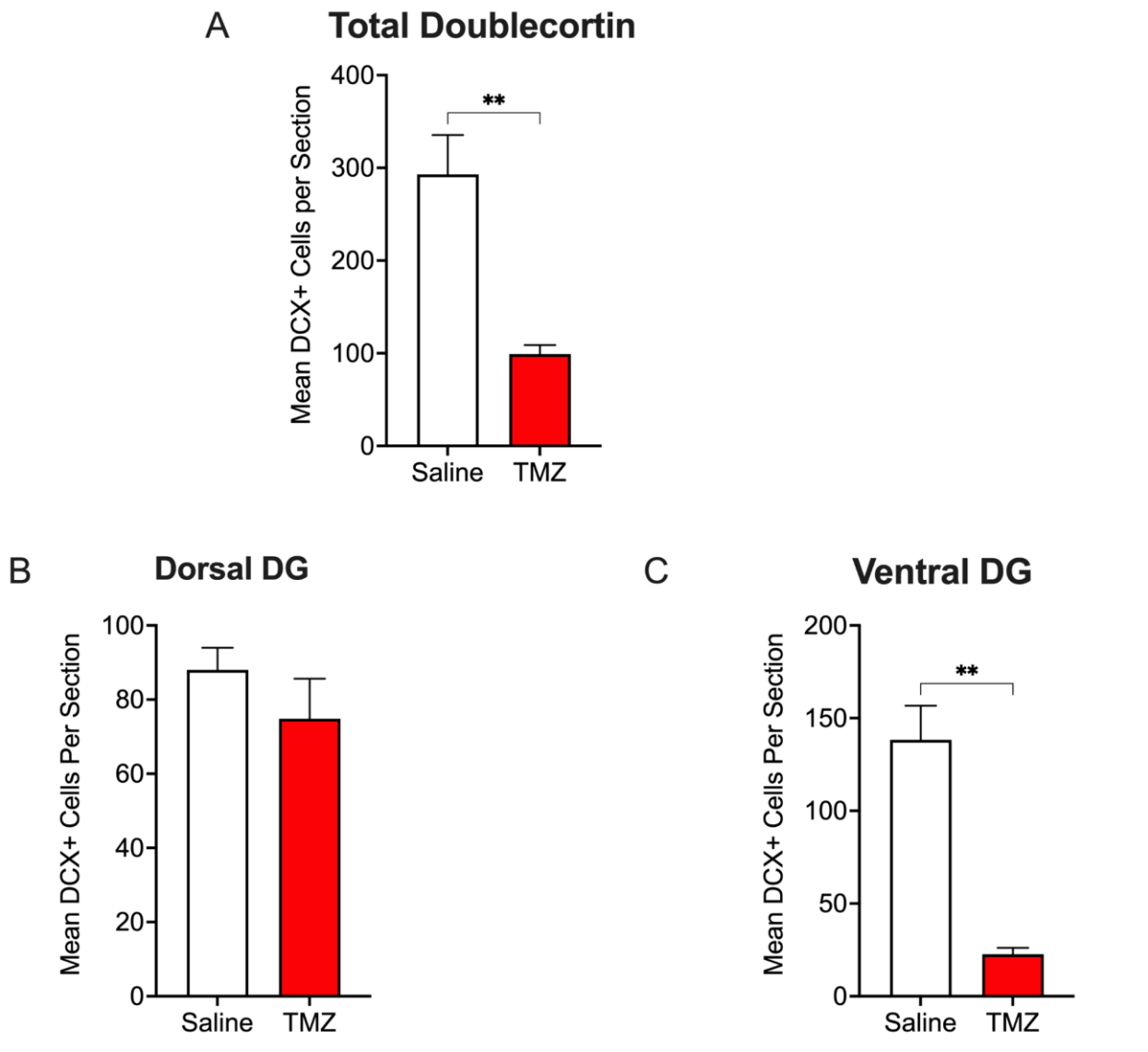

Figure 6. DCX+ cells following the perfusion of animals from the general experiment. (A) Mean DCX+ Cells per Section. This figure shows the mean number of DCX+ cells from the general experiment. Data is presented in mean ± SEM. **p <.01. (B) Mean DCX+ Dorsal DG Cells per Section. This figure shows the mean number of DCX+ cells found in the dorsal DG following the general experiment. This data was not found to be significantly different between groups. (C) Mean DCX+ Ventral DG Cells per Section. This figure shows the mean number of DCX+ cells found in the ventral DG following the general experiment. Data is presented in mean ± SEM. **p <.01.

To further address the impact of TMZ on an additional marker of structural plasticity, we quantified the expression of the presynaptic terminal marker synaptophysin within the hippocampus. Synaptophysin immunolabelling showed a classical punctate pattern that was primarily localized to the dendritic fields of CA1 and CA3 subfields and dentate gyrus with varying staining intensity. The results of synaptophysin labeling is presented in **Figure 7a**. Compared to saline controls, there was a significant reduction in synaptophysin primarily within the stratums oriens (SO) [$t(8)=3.17$, $P<.011$] and radiatum (SR) [$t(8)=2.73$, $P<.023$] of the CA1 subfield after 4 weeks of TMZ. Additionally, there was also decreased synaptophysin immunoreactivity was restricted to the CA3 SO only after TMZ [$t(8)=2.38$, $P<.041$]. No additional changes were observed in synaptophysin labeling for the other laminar regions of the hippocampus after TMZ treatment. There was no evidence that changes in synaptophysin expression after TMZ treatment differentially affected the dorsal or ventral hippocampus as a similar reduction was observed across the sections collected from regions.

The results presented suggested that there is a reduction in structural plasticity within the hippocampus after 4 weeks of TMZ treatment. To determine whether these structural changes might also relate to alterations in patterns of neuronal activation within the hippocampus during behavioural performance, we probed for the immediate early gene marker Egr1 in animals that were euthanized ninety-minutes after the shock-probe test. Immediate early genes are rapidly transduced in response to neuronal activity and their activity-dependent induction are critical for coordinating molecular signalling events important for learning, memory, and synaptic plasticity. We found that TMZ-treated rats showed a significant reduction in the number of Egr1+ cells in the dentate granule cell layer ($P<.031$) and CA1 subfield ($P<.036$, **Figure 7b**).

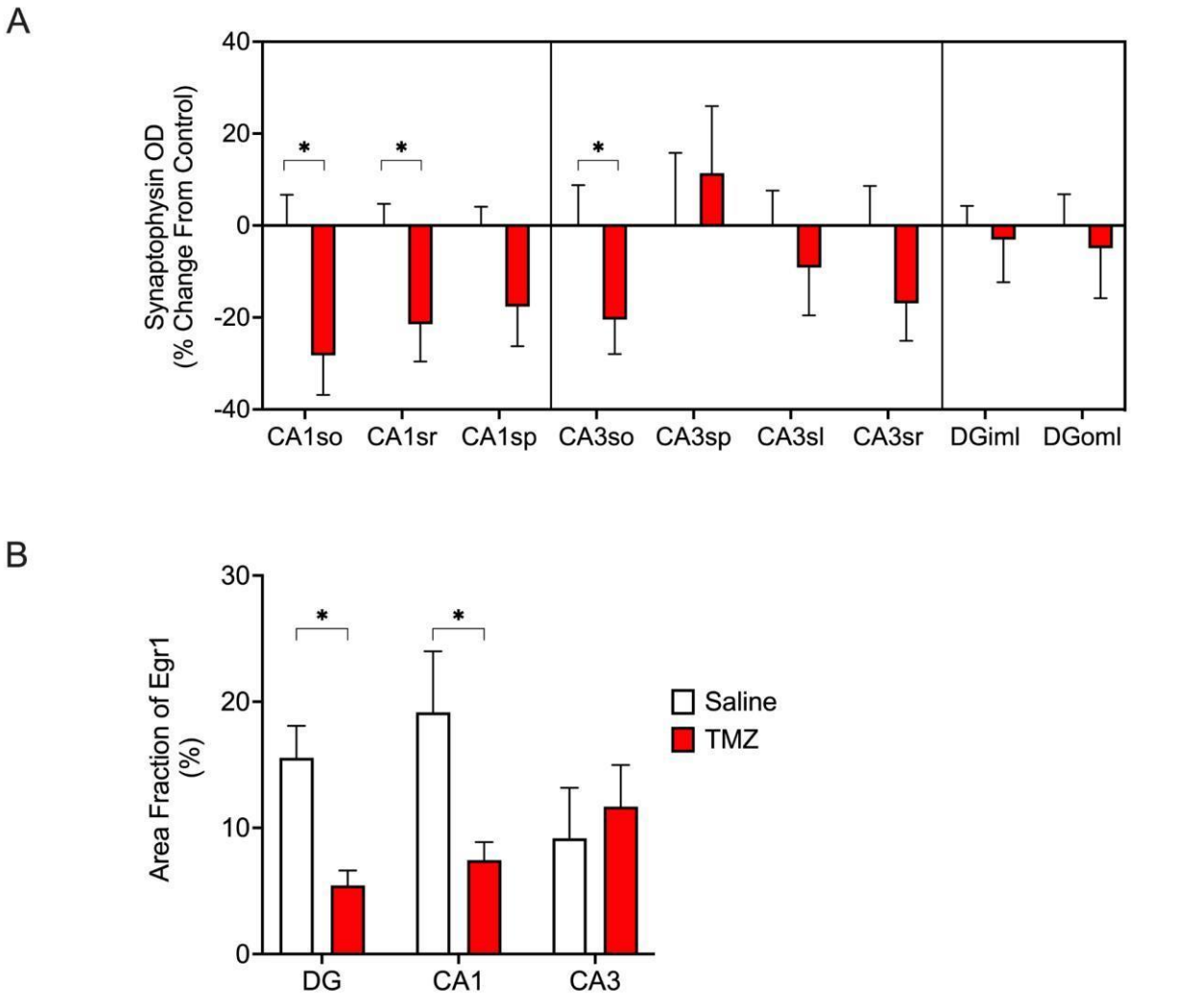

Figure 7. Synaptophysin and Egr1 cell quantification. (A) Synaptophysin-labeled cells. This figure shows the mean number of quantified Synaptophysin cells found in the subregions of the hippocampus. Data is presented in mean ± SEM. *p <.05. (B) EGR1+ cells in the CA1 Subfield. Quantification of Egr1 in the specific regions of the DG, CA1 and CA3. Data is presented in mean ± SEM. *p <.05.

Chapter 4: Discussion

4.1 A 50 mg/kg dose of TMZ is optimal for expression of SGZ cells during two cycles of treatment

TMZ has been shown to be an effective adjuvant oral therapy for the treatment of highgrade malignant glioma multiforme. The most common protocol involves TMZ administered for 5 consecutive days (between 75 mg/m^2 to 200 mg/m^2 per day) followed by a 23 day rest period with this pattern repeating for generally 4 to 6 consecutive treatment cycles. This type of multicycle treatment regimen reduces the risk of adverse physiological reactions associated with chronic TMZ on inflammatory responses and body weight changes in cancer patients. The intention of my dose-seeking study was to assess how well this dose worked in suppressing the neural progenitor cells of the AHN of a rat model. My findings concluded that TMZ does have an impact on SGZ cells. The 50 mg/kg dose was found to significantly decrease BrdU+ cells found in the SGZ, while the 25 mg/kg dose was trending towards significance but ultimately did not show a statistically significant difference in cell expression from the control group. The 50 mg/kg dose was chosen as the results of this study showed the greatest suppression, no adverse side effects on the animals following treatment, and no significant difference between the weight of the treatment groups following the chemotherapy. No noticeable changes in appetite and body weight suggest that there were no additional side effects confounding the findings.

Breaking down these results further for exploration, it was made clear that the ventral and dorsal poles of the DG experienced asymmetrical cell suppression following TMZ treatment. The dorsal and ventral poles are known to modulate behaviour differently, with the ventral pole being associated with emotionality and anxiety processes, the passive avoidance of painful stimuli, and burying behaviours on the shock-probe test; whereas the dorsal pole is important for the

processes of memory and spatial learning (Bertoglio, Joca & Guimaraes, 2006; Degroot & Treit, 2002; Fuster-Matanzo et al., 2011). The evidence from this experiment indicated that TMZ actually showed select inhibition of the DG, affecting the ventral pole of the DG disproportionately from the rest of the DG structure. These findings are consistent with earlier research (Egeland et al., 2017; Sekeres et al., 2021), suggesting that TMZ affects hippocampal progenitor cells, but does so significantly more in the ventral hippocampus than the dorsal hippocampus. Therefore, this asymmetrical suppression of AHN neurons should indicate a clear inhibition of performance of behavioural tests of fear and anxiety.

One of the goals of this study was to assess if TMZ caused a reduction in neurogenesis. BrdU is a marker of cell synthesis, regeneration and repair; and is not an exclusive marker of neurogenesis (Taupin, 2007). As such, the findings of this pilot study have shown that TMZ has an effect on hippocampal cells, but has not confirmed if there is an impact on neurogenesis yet. Therefore, to explore these treatment effects on neurogenesis, this study needed to be repeated with the additional exploration of neurogenesis-dependent tasks to draw definite conclusions.

4.2 TMZ causes cognitive impairment on tests of learning and memory

The contextual fear discrimination test is a test of associative learning, contextual memory acquisition, consolidation and retrieval (Besnard et al., 2020; Curzon et al., 2009; Dos Santos Corrêa et al., 2019; Ji & Maren, 2008). This test measures freezing response following the pairing of an unconditioned stimulus (foot shock) to a conditioned stimulus (environmental context), and the ability for the animals to differentiate between the shock and safe contexts are assessed by examining their freezing rates (Curzon et al., 2009). This test was chosen because the relationship between AHN cells and contextual discrimination are well-known in the research

community, with studies showing that an enhancement of neurogenesis leads to better performance on the contextual discrimination test, and a suppression leads to an inhibition of performance (Besnard & Sahay, 2021; Tronel et al., 2010). The findings from this test suggest that there was a robust effect of discrimination learning on day 2 and 3 where the controls were able to distinguish between the two contexts, but the TMZ animals could not. The areas where they showed most inhibition was in their delay to distinguish the difference between the shock and safe contexts, which they were not able to do in any statistically meaningful way until the 5th day; two full days later than their healthy control counterparts. Throughout the entire test, TMZ animals consistently had higher rates of freezing than controls. This finding could be caused by one of a few different things. One, the TMZ animals could be over-generalizing their surroundings leading to unclear and indistinguishable conclusions about what this elevated level of freezing means. Two, they could be demonstrating high anxiety as a response to the test; or three, this finding could be showing a cognitive or memory impairment. To draw more concrete conclusions about these results,tests of anxious behaviour in rodents were utilized.

Anxiety is reported by roughly 50% of the patients that undergo chemotherapy, regardless of their feelings surrounding their diagnosis (Dey et al., 2020; Lin et al., 2011). Both the EPM and the shock-probe are measures of anxiety used in rodent studies, and both of these tests are known to both be DG dependent (Degroot & Treit, 2002). In the hippocampus, defensive behaviours are regulated by the ventral pole, and spatial learning by the dorsal hippocampus (Bertoglio et al.,2006; Degroot & Treit, 2002). In the EPM, the control animals spent statistically significantly more time in the open arms compared to the TMZ animals, however, the TMZ animals made more entries into the closed arms than the controls. This data appears to almost be conflicting, and leaves the reason for this finding requiring further exploration. One thought is that this could be caused by a more hyper-active animal, with TMZ-

treated animals running back and forth between the open and closed arms. However, the entries to the open arms were statistically similar between treatment groups, indicating that this was not the case. Therefore, the number of stretch-attending postures were explored to determine if this explained the difference between the groups. Stretch-attending behaviours look like lowering of the back and elongating the animal's body while moving forward slowly or not moving at all, as a form of guarded risk-assessment as a result of exploratory anxiety (Holly et al., 2016). By examining the data from the animal's stretch-attending postures, it was evident that the TMZ animals were performing this behaviour at a significantly higher rate than controls. What this data suggests then is that TMZ animals were engaging in this guarded behaviour likely as a result of higher levels of anxiety than the controls. When considering how the TMZ and control animals did not differ significantly in the number of open arm entries, these findings show that the differences were not a result of a motor problem resulting from the chemotherapy and that the TMZ treatment results in anxiogenic behaviour in Long-Evans rats. Specifically, the TMZ animals show a mild anxiety phenotype, stretching their neck out into the open arms and leaving without entering. Furthermore, research conducted in both humans (Bakhiet, Ali & Bakhiet, 2021; Mitchell et al., 2013) and animals (Dey et al., 2020; Kitamura et al., 2013) have shown that chemotherapy is related to higher rates of anxiety than controls, indicating that my findings here are consistent with current relevant literature.

The second test of anxious behaviour that was conducted for this study was the shockprobe test. This test is a measure of anxious behaviour in rats, as well as coping style when faced with an anxiogenic threat (Fucich, & Morilak, 2018). The immobility of animals responding to the perceived threat of the probe is considered a maladaptive approach to coping and indicative of higher levels of stress, while probe-burying is indicative of lower levels of stress and an active, adaptive coping style (Fucich & Morilak, 2018). To the best of this author's

knowledge, there hasn't been another chemotherapy study conducted with the use of the shockprobe test to assess coping strategies in animals to compare these findings to. However, a study by Fucich & Morilak (2018), found that rodents with chronic stress and psychiatric illnesses showed a maladaptive approach to the shock-probe test, with greater immobility and reduced probeburying behaviours (Fucich & Morilak, 2018). These findings were consistent with the findings from this study, with TMZ treated animals presenting a robust reduction of burying, and more time spent immobile than controls. Further, these findings are interesting because they suggest that maladaptive coping style might be correlated to chemotherapy treatment, something that there seems to be no current studies on.

4.3 TMZ is efficient in suppressing AHN in animals given a longer treatment cycle

The last hypothesis of my study tested the neurobiological effects of TMZ following the longer treatment cycle and behavioural testing. For this, three types of staining measures were used. The first was doublecortin (DCX), which is a cytoskeleton-associated protein that is expressed during adult neurogenesis (Klempin et al., 2011). TMZ suppressed the production of DCX+ cells in this study by 30%, and like the findings of the dose-seeking pilot, this suppression was found to be specifically in the ventral dentate. This suggests that the neurogenesis of the ventral dentate are selectively impaired during the treatment of TMZ; a finding that has also been noted in prior literature (Egeland et al., 2017). However, the results of my study were less robust than others. Egeland et al. (2017) found a 60% reduction of DCX+ cells following a 25 mg/kg TMZ treatment, with 49% less cells in the ventral DG, but no significant decrease in the dorsal DG; and Garthe et al. (2009) reported a reduction of proliferating neurons by more than 80% in mice, also with a 25 mh/kg dose.

The difference between my findings and others could be the result of key differences in experimental designs. Garthe et al. (2009) ran a similar dose-seeking study but utilized C57BL/6 mice instead of Long-Evans rats. This could mean that TMZ has a more robust effect on mice instead of rats, or that there are gene-specific impacts in one of these species that alters the impact of treatment. Further differences also can be seen in the injection schedule. For my study, injections were given two times daily at a rate of 25 mg/kg per injection for a total of 50 mg/kg, but the methods of Garthe et al. (2009) indicated that they only injected their animals once daily with the 50 mg/kg dose instead. Hypothetically, this could change the impact of the drug enough to yield different results. In the Egeland et al. (2017) study, animals were given only a 25 mg/kg dose for three consecutive days each week for a total of six weeks, which differed from the 50mg/kg dose the animals in my study were given three times a week for five weeks. They then allowed the animals to recover for six weeks and conducted three weeks of behavioural testing, which in its entirety was a fundamentally different design from mine and may be the cause of these differences. However, it is interesting that the 25 mg/kg dose in this study yielded such robust results.

Synaptophysin was the second stain used in this study, which stains for synaptophysin-positive synaptic boutons that are sensitive to cognitive deficits, and stress (Calhoun et al., 1998; Thome et al., 2001), and allows us to examine the entire hippocampus for structural changes. What was found was that the synaptic boutons of the TMZ animals had an impact on the dendritic CA3, CA1 and a slight effect on the DG subfields of the hippocampus proper. These findings showed that there was a reduction in synaptophysin and immunoreactivity, implying that TMZ was able to disrupt these connections. Given the pyramidal structure of the neurons in the CA3 of the hippocampus, it stands to reason that a disruption of the connections between the CA3 cells that project axon terminals onto the CA1

would result in structural changes and functioning of the hippocampus, and coincidentally also behavioural and mood alterations. The decrease that was seen in the Stratum Oriens, a relatively cell-free layer of the hippocampus located below the stratum lucidum that is occupied by mossy fiber axons, suggests that this was really a decrease in mossy fiber inputs (Cappaert et al., 2015).

Long-term potentiation (LTP) plays a major role in synaptic plasticity and is believed to be pertinent for the formation of new memories (Kumar, 2011). The Stratum Radiatum is located in the CA1 region and is the area well-known to be potentiated for LTP (Tao-Cheng, 2014). LTP occurs through an excitation of synapses in the hippocampus, producing varied sequences of patterns in different areas (Tao-Cheng, 2014). Therefore, it is expected that a reduction of synapses in the Stratum Radiatum would impact this firing process and result in an impairment of memory. The findings of this study indicate that TMZ treatment suppresses synapses in the CA1, and as a result, of LTP and the formation of new memories. This suggests that TMZ treatment impacted the ability to form new memories.

The last stain that was used in this study was Egr1, a marker for encoding an immediate early gene (IEG) protein (Saha et al., 2021). Immediate early genes are important for coordinating molecular signals engaged in learning, memory and plasticity (Minatohara et al., 2016). This study showed a reduction in both the IEG expression in the DG cell layer and CA1 subfield. The reduction of IEG expression in the DG cell layer indicates that the TMZ impaired memory encoding in the TMZ-treated animals, and the reduction in the CA1 subfield suggests an impairment in memory retrieval (Bartsch et al., 2011; Piatti et al., 2013). This further suggests that the TMZ animals experienced a memory impairment.

My findings on the contextual fear discrimination test, as well as the results from the synaptophysin and Egr1 staining demonstrated that TMZ creates a suppression of AHN that

results in a learning impairment associated with decreased functionality for discrimination. This supports my original hypothesis that the hippocampus is being impacted by the chemotherapy and results in the cognitive impairments that survivors report. In conjunction with these results, the conclusions from my study were also able to show that TMZ impacted mental health and increased anxiety behaviours; in particular, passive anxiety behaviours. This is consistent with current research in human chemotherapy survivors who also complain of increased anxiety (Bakhiet et al., 2021; Lee et al., 2017; Mitchell et al., 2013; Oswald et al., 2022). The research on this in humans is currently mixed however, with many researchers attributing anxiety in chemotherapy to the result of the cancer diagnosis and impending treatment, instead of a cognitive impairment as a result of chemotherapeutic side-effects (Lee et al., 2017; Oswald et al., 2022). It seems that in human studies, the idea that increased anxiety could be considered as an independent side effect that occurs with the drug has not yet been fully considered. Therefore, I believe it to be interesting and important to the current research that this study was able to demonstrate a link between chemotherapy and anxiolytic behaviour as a result of the chemotherapy itself.

4.4 General Discussion

Ultimately, this study has provided evidence that TMZ selectively suppresses AHN in the ventral pole of the DG, accounting for the selective behavioural inhibition that was witnessed during testing. I feel confident in saying that TMZ impairs the structure of the hippocampus at the biomechanical level which results in cognitive impairments such as impaired memory, learning, and an increase in anxious behaviours. Specifically, I feel confident stating that TMZ impairs memory encoding and retrieval which can lead to the ‘fog’ symptoms that have been

expressed by survivors. I as well have shown evidence of TMZ cancer treatment as a contributor to maladaptive approaches to anxiety.

While this study was able to show important findings to add to the current research on CICI, I also acknowledge that there are a few notable limitations. The first being the small sample size for each group. While a larger sample would be more ideal, there have been meaningful other studies conducted that have concluded important and note-worthy findings without a larger sample size; and according to some researchers, may even yield more truthful results by allowing all aspects of the study to be properly controlled (Indrayan & Mishra, 2021). Furthermore, studies in both human and rodent that have also conducted similar research to my study with larger groups have still found the same conclusions (Cheng et al., 2017; Lee et al., 2017; Nokia et al., 2012). Therefore, despite this being an arguable limitation, I firmly believe that it does not change the impact of the results.

Second, there is the possibility that TMZ has indirect effects on other neurobiological processes that may have been overlooked. The second area of the brain that continually makes neurons into adulthood is the Subventricular zone (SVZ) of the olfactory bulb (Lledo, Alonso & Grubb, 2006). My study did not examine the SVZ section of the brain since I did not find any reports of impairments associated with the olfactory zone in any of the prior research I reviewed. However, some research has shown that the SVZ is also subject to suppression of neurogenesis from external factors like TBI and alcohol use, which I believe indicates that there is a possibility then that chemotherapy could have an impact on the progenitor cells of this area as well (Acosta et al., 2013; Hansson et al., 2010). If chemotherapy is cytotoxic to the neural progenitor cells of the hippocampus, then they may be cytotoxic to the cells of the SVZ, and result in further impairments or confounds that are going unacknowledged. Despite how this may lead to findings that are being overlooked, the olfactory bulb and the hippocampus are found in entirely different

areas of the brain and involved with different processes, so I feel confidently that even if these is an impact of the SVZ, there would be no impact of this on the findings of this study.

The amygdala is a structure attached to the hippocampus that is involved in emotional processing and attention (Gallagher & Chiba, 1996), and some of the research conducted with chemotherapy survivors has also indicated an issue with attention (Anderson et al., 2019; Dias-Carvalho et al., 2021; Schagen et al., 1999). While the hippocampus also plays a part in attention processing, there is the possibility that the amygdala may also be impacted in some way. The finding of anxiogenic behaviour on both the EPM as well as the shock-probe test, as well as the impaired discrimination on the contextual discrimination test, could indicate an extended impairment of the amygdala, which is known to be integral to the process of fear circuitry (Ressler, 2010). It would be beneficial to explore the amygdala and potential lesions in future studies. My attempts at exploring current research on any relationship between the amygdala and chemotherapy yielded no results to discuss further.

Third, it is my belief that the findings of the contextual discrimination test and the posthumous staining indicate that TMZ suppresses the pattern separation process that results in contextual discrimination learning impairments. Pattern separation is a process that takes place in the hippocampus by activating different sequences of cells to distinguish overlapping information. This is a process that is performed in the DG whereby EC inputs are converted into place-like fields (Rolls, 2013). Further down the perforant path, the CA3 completes the stored patterns using sparse coding representations (Rolls, 2013). Neurogenesis is also known to be a contributor to this process, playing an important role in encoding and retrieval of these memories (Johnston et al., 2016). Previous research has also indicated that the contextual discrimination test is a test engaging pattern separation, whereby an impairment in pattern separation is found when neurogenesis is impacted (McHugh et al., 2007; Sahay et al., 2011; Tronel et al., 2011).

The findings of my study successfully showed a suppression of the perforating hippocampal neurons, as well as an impairment on the contextual discrimination test, a suppression of the synaptic boulons in the CA3, and a reduction of immunoreactivity in the same area. This evidence has me concluding that the memory impairment suffered by the TMZ animals during the contextual discrimination test is a result of a suppression of AHN resulting in an impairment of the pattern separation process.

Finally, the last limitation of my findings that I acknowledge was not remaining consistent with the use of BrdU staining from our pilot study to our main study. BrdU is a marker of DNA synthesis, repair, and cell proliferation (Taupin, 2007); while DCX is thought to be a better marker of plasticity and a cytoskeleton-associated protein that is expressed during adult neurogenesis (Klempin et al., 2011); so while I can use both to view expression of the AHN, it would have been beneficial to compare the shorter timeline of chemotherapy to the longer timeline using one stain to support the original findings as consistently as possible. However, similar studies have used a variety of staining measures: Ki-67, BrdU, DCX, NeuN, etc and have all concluded a marked suppression in AHN related to chemotherapy (Briones & Woods, 2011; Christie et al., 2012; Hinduja et al., 2015; Raafat et al., 2023; Rendeiro et al., 2016), so the change in stain is not something that changes the findings of our results in any meaningful way.

With the findings of this study being incorporated into the body of scientific literature, I could see the importance for future studies to expand further beyond what has been concluded here. For this study, I examined the effects of TMZ on the rat brain in a short-term time frame. However, several studies on CICI have concluded that impairments in human chemotherapy survivors can persist for several years afterwards (Ahles et al., 2002; Jansen et al., 2011). Future studies could be conducted with a longer time frame between the end of treatment and behavioural testing to see if the longevity of these cognitive experiences change with the

progression of time. While this will look different in a rat model due to a shorter lifespan and age milestones, it would still benefit the research to see if these findings are persistent with the increased passage of time, and to explore what both the behavioural expression and the neurobiological effects look like over time. Further, I believe it would be important to the scientific literature to recreate this study in the future and examine some of the aspects more broadly. In essence, the findings of asymmetrical neuronal suppression between the ventral and dorsal DG should be examined more deeply to establish why this occurs and when it can be expected to occur. For our study, we were limited in the way we differentiated between the dorsal and ventral poles, since this was not originally part of our hypothesis, so we took an average of the cells for this reason.

Further, the use of TMZ for this study came from the findings of previous research that indicated that TMZ is able to cross the BBB; which is one of the reasons why it is so predominantly used for glioblastoma (Agarwala & Kirkwood, 2000; Nokia et al., 2012). However, in much of the research discussing CICI, the subjects used are female breast cancer survivors due to the higher survival rate associated with breast cancer. As other researchers have pointed out, many chemotherapeutics are known to cross the BBB (Nokia et al., 2012), however not all of them do (Angeli et al., 2020). Most of the research in human for CICI come from breast cancer patients since breast cancer has such a low mortality rate (Bender et al., 2006; Cheng et al., 2017; Christie et al., 2012; Eide & Feng, 2020; Schagen et al., 1999; Whittaker et al., 2022). Further investigating the cognitive effects of chemotherapy with drugs that are being used in human research that is commonly reporting CICI would be an added benefit to the current body of CICI research. In addition to this, repeating this study with female rats would also be of benefit to see if differences occur due to sex differences. With so much of the CICI research

being reported in women, repeating this study with female rodents would provide the opportunity to explore sex differences and how they may be impacting the research findings.

Overall, my hope is that this data will add to the literature of chemotherapy side-effects for human cancer survivors, giving better insight into the mechanisms at work in CICI. My findings showed that there is in fact an impairment in the hippocampus caused by chemotherapeutics, and that this impairment is selective to the SGZ of the ventral DG, as well as to the dendritic connections made throughout the rest of the hippocampus proper, impacting areas of the CA1, CA3 and DG respectively; leading to memory impairments and increased anxiety behaviours. This research is beneficial to both medical practitioners and future researchers, and it is my hope that these findings do two things: one, I hope that these findings helps medical practitioners to consider proper informed consent on chemotherapy and its expected outcomes to patients prior to accepting treatment, and two, that it helps future research be conducted to search for a solution to these impairments by understanding what mechanisms are being impacted and what cognitive effects they are having.

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