

**EFFECTS OF CANCER THERAPIES ON THE BRAIN – FROM MOLECULAR  
MECHANISMS TO BEHAVIORAL OUTCOMES**

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## **ABSTRACT**

Based on the most recent estimates by the Canadian Cancer Society, two in five Canadians will develop cancer in their lifetimes. There is mounting evidence that anti-cancer radiation and chemotherapy cause central nervous system side-effects, including declines in cognitive function, memory, and attention. The mechanisms of these effects are not well understood.

Here, we investigated the molecular and cellular effects of two main anti-cancer treatment modalities—radiation therapy and chemotherapy—on the brain using established experimental rodent models. Using a rat model, we showed that radiation therapy-like exposures cause molecular and cellular changes in the brain and impacts animal behavior. Using a mouse model, we also determined that the cytotoxic chemotherapy agents cyclophosphamide and mitomycin C induce oxidative DNA damage and impact molecular and epigenetic processes in the brain.

Our results may be used to develop new strategies and interventions to prevent and mitigate radiation and chemotherapy effects on the brain.

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## LIST OF ABBREVIATIONS

5-aza – 5-aza-2'-deoxycytidine  
5mC – 5-methyl-cytosine  
5hmC – 5-hydroxymethylcytosine  
8-Oxo-2'-deoxyguanosine  
AID - orbital frontal cortex  
ANOVA - analysis of variance  
APE1 - apurinic/aprimidinic endonuclease  
BCL2 - B-cell lymphoma 2  
BDNF - Brain-derived neurotrophic factor  
BER – base excision repair  
CNS – central nervous system  
CA1 - hippocampus  
CdxA - caudal type homeobox A  
CFI-SAGES – Canada Foundation for Innovation – Southern Alberta Group for Epigenetics Studies  
Cg3 – medial frontal cortex region  
cGy – centigray  
CPP - cyclophosphamide  
DNMT – DNA methyltransferase  
DSB – double strand breaks  
ECL – enhanced chemiluminescence  
 $\gamma$ H2AX – phosphorylated histone H2AX  
MeCP2 – methyl CpG binding protein 2  
miRNA/miR – microRNA  
mRNA – messenger RNA  
PAGE – polyacrylamide gel electrophoresis  
PCR – polymerase chain reaction  
PI3K – phosphatidylinositol 3-kinases  
RIN – RNA integrity number  
RT-PCR – reverse transcription PCR  
SEM – standard error of the mean  
EAAT2 - excitatory amino acid transporter 2  
EGF - epidermal growth factor  
ELK1 - ETS domain-containing protein  
EPM - Elevated-Plus Maze  
FDR - false discovery rates  
FGF-2 - fibroblast growth factor-2  
GATA-1 - globin transcription factor 1  
GnRH - Gonadotropin-releasing hormone  
Gy – Gray  
HIF-1 - hypoxia-inducible transcription factor  
IR - ionising radiation  
Jak-STAT signaling  
JNK - c-Jun N-terminal kinases

MAPK - Mitogen-activated protein kinases  
MDS - multidimensional scaling  
MMC - mitomycin C  
NOR - Novel Object Recognition  
OGG1 - 8-Oxoguanine glycosylase  
PFC - prefrontal cortex  
RT - radiation therapy  
SRY - sex-determining region Y  
TBX18 - T-box transcription factor  
TET - Ten-eleven translocation methylcytosine dioxygenase

## **CHAPTER 1: INTRODUCTION**

## **CANCER AND CANCER TREATMENTS**

### **CANCER INCIDENCES**

The Canadian Cancer Society predicts that 2 out of 5 Canadians will develop cancer in their lifetime, and 1 out of 4 will go on to die from it. In Canada, cancer is the leading cause of death, exceeding heart disease. Cancer incidences have been rising, and it has been theorized that this can be due to the following factors: exposure to known and/or unknown carcinogens, population growth, aging, etc. As such, around 191,000 new cases of cancer and 76,000 cancer-related deaths have been predicted to occur in Canada in 2014. Meanwhile, the American Cancer Society predicts that in 2015, there will be 1,658,370 new diagnosed cancer cases in America. Based on the statistics, cancer is the second most common cause of death in the US, and it is surpassed only by heart disease (<http://www.cdc.gov/nchs/fastats/leading-causes-of-death.htm>). Due to the recent advancements in cancer research and treatment, 63% of diagnosed cancer patients will survive at least 5 years after their diagnosis ([www.cancer.ca](http://www.cancer.ca)).

The high incidences of cancer pose a significant burden on patients and society. In the United States, the annual direct and indirect costs of cancer, from medical costs to the associated costs of productivity loss, were estimated by the National Institutes of Health to be at around \$226 billion (Contreras and Kennedy, 2013). Therefore, high cancer rates constitute a tremendous burden in terms of human suffering. A lot of the above is due to side effects of cancer treatments themselves, and there are a growing number of people now living with the long-term side effects of cancer treatments. A lot still has to be done to ensure that cancer survivors have the best possible quality of life and suffer minimum side effects.

## **CANCER TREATMENTS/THERAPIES**

The elevated cancer rates have resulted in increased awareness, leading to the outpouring of research seeking new ways to improve cancer prevention, the effective early detection and precise diagnostics, and, most important of all, the effective treatment options. For example, modern and efficient technologies for cancer screening have led to the detection and diagnosis of cancer at early stages of the disease and have increased the levels of detection of non-invasive tumors or early-stage localized invasive cancers. These advances have led to high cure and survival rates and almost a normal life expectancy among early-stage cancer patients (Etzioni et al., 2003)

Even if cancers are detected in the higher or terminal stages, a wide array of treatment options exists to combat and manage the disease. Numerous advances in the development of new treatment modalities - from new technologies and more precise measurements for radiation delivery to new chemotherapeutic drugs and regimens - have led to the better management, reduced recurrences, and increased survival rates in several types of cancer.

The main treatment options are commonly based on the type and stage of each individual patient's tumor as well as on the assessment of the individualized risks of possible treatment complications and side effects. The treatment plan is founded on the physician's suggestions and the physician's and patient's choices made from a range of therapeutic options. Surgery is commonly used for an initial removal of primary tumor(s). Systemic therapy is a form of treatment that influences the entire body. It is applied through the bloodstream and includes hormonal therapy, anti-inflammatory therapy, and chemotherapy. Radiation therapy relies on the use of radiation for the

targeted destruction of tumors. In general, the vast majority of cancer patients undergo surgery for the removal of the tumor that is followed by radiation therapy and/or chemotherapeutic treatments

## **RADIATION AND RADIATION EFFECTS**

### **RADIATION THERAPY AND IONIZING RADIATION**

Radiation therapy (RT) employs ionizing radiation to eradicate cancer cells and tumors (ACS, 2014a). Several different types of ionizing radiation are used for therapeutic purposes – charged particles, X-rays and gamma rays. Ionizing radiation can be externally applied to the body using radiation therapy equipment (external-beam radiation therapy) or can be directed via placing capsules with radioactive material inside the body (brachytherapy) (ACS, 2014b, ACS, 2014a). Moreover, radioactive compounds, such as radioactive iodine used to combat thyroid cancers, can be administered systemically via the bloodstream. More than half of all cancer patients receive some type of radiation therapy during their treatment course. The majority of them receive external beam-focused radiation therapy. All patients undergo ionizing radiation-based diagnostics(ACS, 2014b, ACS, 2014a).

### **DIRECT RADIATION EFFECTS**

Ionizing radiation is often referred to as a double-edged sword. Indeed, on the one hand, it is one of the indispensable mainstream diagnostic and treatment modalities; on the other hand, it is a potent DNA-damaging agent (Koturbash et al., 2011a, Frankenberg-Schwager, 1990). Radiation exposure was proven to cause various DNA lesions, such as single- and double-strand breaks, base and nucleotide damages, and DNA and protein crosslinks. Recent data suggest that ionizing radiation induces around



850 pyrimidine lesions, 450 purine lesions, 1000 single-strand breaks and 20-40 double-strand breaks per cell per Gy of gamma-irradiation through linear energy transfer (Lomax et al., 2013).

High doses of ionizing radiation ensure that cells suffer massive DNA damage that is beyond repair. Cells with overwhelming levels of DNA damage undergo apoptosis and thus are eliminated from the body. The faulty repair of radiation-induced DNA damage can lead to adverse genetic changes such as mutations and chromosomal aberrations (Little, 2006). The accumulation of radiation-induced DNA damage and mutations paralleled by radiation-induced changes in key processes of cellular regulation often underlie the development of cancer as well as other pathologies (Little, 2000, Barcellos-Hoff, 2005, Barcellos-Hoff et al., 2005, Sowa et al., 2006).

Besides causing DNA damage, radiation exposure also disrupts a variety of processes in the exposed cells. It can trigger changes in gene expression and cell cycle control, disrupt mitochondrial processes, differentiation and apoptotic cell death (Amundson et al., 2003, Deckbar et al., 2011, Lomax et al., 2013), and it also leads to global genome instability (Sowa et al., 2006). Furthermore, radiation effects are also epigenetically mediated, as reviewed in (Szumiel, 2015, Dincer and Sezgin, 2014, Merrifield and Kovalchuk, 2013, Aypar et al., 2011, Kovalchuk and Baulch, 2008).

## **RADIATION EFFECTS ON THE BRAIN**

Recent studies have proven that the brain is indeed sensitive to irradiation, and radiation therapy impacts a wide array of brain functions causing cognitive decline, memory deficits and fatigue, as well as brain tumours in exposed individuals (Koturbash et al., 2011a). The extent and severity of radiation effects on the brain depends upon

radiation dose. Exposure to high-dose IR results in profound functional and morphological changes in brain tissues and leads to debilitating cognitive decline (Mizumatsu et al., 2003a, Andres-Mach et al., 2008). Low doses can also induce a wide array of cognitive impairments and deficits even without any significant morphological alterations (Andres-Mach et al., 2008).

While the effects of high doses of radiation on the brain have been studied and are reasonably well understood, the effects and mechanisms of the brain's response to low doses of radiation remain obscure (Yin et al., 2003). Recent studies have proven that radiation effects are also age-, brain region- and sex-specific (Koturbash et al., 2011a, Hudson et al., 2011, Silasi et al., 2004). Amongst various brain regions, the prefrontal cortex and the hippocampus are most sensitive to irradiation (Andres-Mach et al., 2008, Mizumatsu et al., 2003b, Madsen et al., 2003, Fike et al., 2007, Rola et al., 2004a, Rola et al., 2004b, Kornev et al., 2005).

The hippocampus is one of the two active sites of neurogenesis in the mammalian brain (Gage, 2000, Christian et al., 2014). The proliferation of neuronal precursors in the subgranular zone of the dentate gyrus generates cells that further migrate to the granule cell layer and differentiate into mature neuronal and glial phenotypes (Palmer et al., 1997). The prefrontal cortex (PFC) is a key regulatory region that collects inputs from all other cortical regions and plans and directs an array of motor, cognitive and social behaviours (Kolb et al., 2012).

The hippocampus is very sensitive to radiation exposure. Radiation exposure results in a loss of cells in the CA1 ammonic field (Chaillan et al., 1997), and it impacts gene and protein expression in the PFC (Hudson et al., 2011, Kornev et al., 2005, Silasi

et al., 2004). Irradiation is a well-known cause of apoptosis and neurogenesis inhibition in the dentate subgranular zone (Mizumatsu et al., 2003a); this blockage persists for a prolonged period of time. A recent mouse model-based study reported a profound and persistent reduction of neuronal proliferation as well as numbers of new neurons in the stem cell niche of the hippocampal dentate gyrus at 72 hours and one month after exposure to 20 Gy of cranial irradiation (Rao et al., 2011). Furthermore, behavioural characterization demonstrated sizeable and persistent hippocampus-dependent learning deficits after irradiation (Rao et al., 2011).

The PFC is also very sensitive to different environmental stresses, including ionizing radiation. Overall, it is currently well accepted that radiation damages normal brain tissues through a variety of weakly understood mechanisms, thus resulting in profound cognitive impairment and a significant life-long disability.

#### **INDIRECT BYSTANDER EFFECTS**

For several decades, it has been accepted that the biological effects of radiation exposure are attributable to the direct DNA-damaging effects of irradiation in exposed tissues. Nonetheless, this concept has been challenged by numerous experiments that prove that cells that were not directly exposed to radiation demonstrated responses that were characteristic of directly irradiated cells (Mothersill and Seymour, 2005, Mothersill and Seymour, 2004). The enigmatic and intriguing radiation-induced ‘bystander effects’ were discovered by Nagasawa and Little in the early 90s, who uncovered that unirradiated cells co-cultured with irradiated cells demonstrated elevated rates of sister chromatid exchanges (Nagasawa and Little, 1992).

From then on the radiation-induced ‘bystander’ effects were observed in both

naïve cells that were in contact with directly irradiated cells and in naïve cells that received some irradiation “distress” signals from the directly exposed cells via the growth medium (Morgan and Sowa, 2005, Morgan, 2012, Morgan and Sowa, 2007, Mothersill and Seymour, 2004, Morgan, 2003b, Morgan, 2003a). Bystander effects have been observed in a large number of studies that have been performed using cell culture models (Hu et al., 2006, Maguire et al., 2005, Maguire et al., 2007, Zhou et al., 2000, Kovalchuk and Baulch, 2008), tissue explants (Belyakov et al., 2002, Belyakov et al., 2006, Kovalchuk and Baulch, 2008), spheroids (Persaud et al., 2005) or three-dimensional artificial human tissue models (Sedelnikova et al., 2007, Belyakov et al., 2005, Kovalchuk and Baulch, 2008), and could be induced by a co-culture of naïve cells and tissues with exposed ones or by transferring a bystander-conditioned medium to naïve cells (Morgan and Sowa, 2005, Morgan and Sowa, 2007, Kovalchuk and Baulch, 2008). Nowadays, bystander effects are commonly accepted as an ubiquitous outcome of exposure to ionizing radiation (Mothersill and Seymour, 2004).

Moreover, bystander effects also occur in the whole organism context when radiation exposure causes the release of soluble factors into the circulating blood that are capable of inducing chromosome damage in the cultured cells and tissue explants. Such factors were reported in the plasma of radiation therapy patients and individuals who were accidentally exposed to ionizing radiation (Hollowell and Littlefield, 1968, Marozik et al., 2007, Pant and Kamada, 1977), also reviewed in (Kovalchuk and Baulch, 2008, Morgan and Sowa, 2007, Mothersill et al., 2004).

Bystander effects have been shown to be important within organs when one organ part was exposed or within organisms when one paired organ was irradiated

(Khan et al., 1998). For example, irradiation of the lung base caused molecular and cellular changes in the shielded lung apex in a rat model. When one lung was irradiated, a significant increase of DNA damage was found in the unexposed shielded bystander lung (Khan et al., 1998, Khan et al., 2003). Similar outcomes were noted during partial liver irradiation (Brooks, 2004). Bystander effects also occurred within the entire organism (Koturbash et al., 2006, Koturbash et al., 2007, Koturbash et al., 2008b, Koturbash et al., 2008c, Tamminga et al., 2008) when one part of the animal's body or animal's head was exposed to radiation and another part was protected by a lead shield (Koturbash et al., 2007, Koturbash et al., 2006, Tamminga et al., 2008, Koturbash et al., 2008b).

On a molecular level, bystander effects manifest as increases in DNA damage and mutations, changes in gene expression, altered levels of cellular proliferation and apoptosis, as reviewed in (Morgan and Sowa, 2005, Morgan and Sowa, 2007, Mothersill and Seymour, 2003, Mothersill and Seymour, 2004). Similar to direct radiation exposure, radiation-induced bystander effects affect all key elements of cellular epigenetic controls, as reviewed in (Merrifield and Kovalchuk, 2013, Ilnytskyy and Kovalchuk, 2011, Kovalchuk and Baulch, 2008, Dickey et al., 2011, Mothersill and Seymour, 2012). In several rodent model-based experiment cranial exposure caused molecular bystander effects in animals' shielded spleens, livers and gonads. IR-induced bystander effects persist for a long time following irradiation (Tamminga et al., 2008, Koturbash et al., 2008c, Koturbash et al., 2008b, Koturbash et al., 2006, Koturbash et al., 2007, Koturbash et al., 2008a, Koturbash, 2008).

## **BYSTANDER EFFECTS IN THE BRAIN**

While cranial exposure was documented to cause bystander effects in somatic organs, very little is known about the existence of bystander effects on a shielded brain upon irradiation of distal somatic organs. A handful of studies show bystander effects in the brain. A report by Mancuso and colleagues showed the occurrence of radiation-induced bystander responses in the neonatal murine cerebellum after X-ray exposure of the remainder of the body using radiosensitive Patched-1 (Ptch1) heterozygous mice (Mancuso et al., 2008). The same group showed induction of bystander effects in the brain using the connexin43 mutant mouse (Mancuso et al., 2011). Still, a lot has to be learned about the existence, magnitude, mechanisms and consequences of radiation-induced bystander effects in the brain, and their contributions to the side effects of radiation therapy.

## **THE EFFECTS OF CHEMOTHERAPY**

### **CHEMOTHERAPY: AN OVERVIEW**

Chemotherapy uses single agents, or combinations of various toxic agents, to eradicate cancer cells. In the vast majority of cases, it is carried out systemically, administered intravenously through the bloodstream, and thus targets not only cancer cells, but all of the cells in the body. Cytotoxic chemotherapy, which is often referred to as non-targeted chemotherapy, uses drugs that enter the bloodstream and kill cancer cells by hindering their ability to divide and grow, and by inducing apoptosis. Essentially, cytotoxic chemotherapy targets all of the dividing cells in the body (DeVita et al., 2005).

Depending on the individualized treatment goals involved, systemic

chemotherapy may be administered in three ways: as primary (neoadjuvant) therapy, adjuvant therapy, or combination chemotherapy. Neoadjuvant chemotherapy aims to reduce tumour size prior to surgical or radiation treatments. In an adjuvant setting, chemotherapy is applied to eradicate any residual cancer cells or tumours after other treatments have been carried out, such as radiation or surgery. Combination chemotherapy is administered in direct combination with other treatments, or is applied by itself, involving a set of chemotherapy agents. Depending on the cancer's type, stage, and grade, as well as on the other treatments used, chemotherapy can cure cancer, control cancer, or be used for cancer palliation to decrease tumour burden, ease pain, and alleviate tumour pressure on the organs (DeVita et al., 2005, Rizzo, 2002). The most common cytotoxic chemotherapy agents are described in the subsections below (Rizzo, 2002, ACS, 2015).

### **Alkylating Agents**

Alkylating agents attach an alkyl group ( $C_nH_{2n+1}$ ) to DNA, usually to the seventh nitrogen atom of the guanine base of DNA, and thus prevent DNA replication. Alkylating agents include (i) nitrogen mustards (cyclophosphamide, mustargen, melphalan, ifosfamide), (ii) nitrosoureas (carmustine, lomustine), and (iii) alkyl sulfonates (busulfan). Monoalkylating agents can react only with one N-7 of guanine, while dialkylating agents react with two different N-7 guanine residues. Some of them act immediately, while others require conversion into active substances (such as cyclophosphamide)(ACS, 2015).

Cyclophosphamide (CPP) is an antineoplastic agent that is used in combination chemotherapy regimens for solid tumours. It undergoes activation in the liver, and

attaches the alkyl group to the guanine base of DNA, at the number seven nitrogen atom of the imidazole ring. This interferes with DNA replication by forming intra- and inter-strand DNA crosslinks (Soffietti et al., 2014). Mitomycin C (MMC), which is a product isolated from the *Streptomyces* species, is an antineoplastic antibiotic that, like CPP, works as an alkylating agent to inhibit DNA synthesis (Soffietti et al., 2014, ACS, 2015).

### **Anthracyclines**

Anthracycline anti-tumour agents were originally derived from *Streptomyces* in the 1960s. They are red aromatic polyketides that occur in variety of forms, due to their structural differences and the different sugar residues attached to them. Adriamycin, or doxorubicin, is one of the most widely-used chemotherapeutic agents with a broad activity spectrum. It is commonly prescribed in combination with other agents, and is one of the most effective drugs for the treatment of various solid tumours, such as breast, lung, ovarian, and other cancers. The mechanism by which anthracyclines inhibit cancer is still not completely clear, but one of them involves DNA intercalation and reactive oxygen species generation (ACS, 2015).

### **Vinca alkaloids and other spindle inhibitors**

The vinca alkaloids are a subset of chemotherapy agents derived from the Madagascar periwinkle plant, which include vinblastine and vincristine. They are the second most-used class of chemotherapy agents. Mechanistically, vinca alkaloids bind tubulin and inhibit the formation of spindle microtubules (ACS, 2015).

### **Antimetabolites**

Antimetabolite agents include folic acid, pyrimidine, and purine analogues, and



were among the first effective chemotherapeutic agents to be discovered. They have low molecular weights and structures that are very similar to the bases of nucleic acids, and they interfere with DNA synthesis by inhibiting enzymes that partake in DNA and RNA synthesis. Amongst them is 5-fluorouracil (5-FU), which is used to treat breast, head and neck, adrenal, pancreatic, gastric, colon, rectal, esophageal, liver, bladder, prostate, and other cancers (ACS, 2015).

### **Kinase Inhibitors**

Tyrosine kinase inhibitors (TKIs) are chemotherapy agents that inhibit protein tyrosine kinases. TKIs were developed based on mechanistic knowledge about the cell cycle regulation and molecular signaling pathways that are commonly affected in cancers. They were the first type of molecular targeted chemotherapy drugs (Arora and Scholar, 2005).

### **Topoisomerase Inhibitors**

Topoisomerase inhibitors are agents that block both topoisomerase type-I and type-II enzymes, and thus interfere with cell division. Topoisomerase I inhibitors include camptothecin, while topoisomerase II inhibitors include doxorubicin, etoposides, and mitoxantrone. They are used to eradicate rapidly dividing and growing cancers (ACS, 2015).

Recent research advances have led to the development of novel treatments that can be tailored to each individual patient's tumours. These include the use of monoclonal antibodies, small molecule inhibitors, and immunotoxins (ACS, 2015).

### **SIDE EFFECTS OF CHEMOTHERAPY**

The vast majority of cytotoxic chemotherapy agents target rapidly dividing cells,

including both cancer cells and normal cells that are growing and dividing. As such, these agents can have numerous toxic side effects. Rapidly dividing cells occur in the skin and hair follicles, gastrointestinal tract, and bone marrow, which means that chemotherapy causes side effects such as hair loss, skin changes, gastro-intestinal syndromes, and dysfunction of the bone marrow, among many others (Rizzo, 2002, DeVita et al., 2005).

### **NEUROTOXICITY OF CHEMOTHERAPY**

The brain is the key coordinating organ that is responsible for every function of our bodies. Cancer treatment side effects also manifest in central nervous system (CNS) toxicity (Soffietti et al., 2014). Recent research shows that chemotherapy agents are in fact more toxic to healthy brain cells than to the cancer cells that they were designed to treat (Han et al., 2008). Chemotherapeutic drugs cause side effects in the cognitive domains of memory, attention, processing speed, and executive function, and these chemotherapy-induced cognitive dysfunctions can persist even after treatment (Seigers and Fardell, 2011, Seigers et al., 2015, Seigers et al., 2010a, Seigers et al., 2008, Seigers et al., 2009, Seigers et al., 2013, Seigers et al., 2010b, Christie et al., 2012). This condition is known as “chemo brain” (Mitchell and Turton, 2011). The duration of chemo brain symptoms ranges from short to long (Ahles et al., 2002, Ahles et al., 2005, Ahles et al., 1998), with around 35% of patients reporting side effects for months to years after the cessation of their treatments. Furthermore, reports by the International Cognitive Workshop suggest that cancer-treatment-related cognitive side effects can persist for as long as five to 10 years after treatment completion (Mitchell and Turton, 2011, Vardy et al., 2008).

Numerous studies have reported the occurrence of chemotherapy-related changes in cognitive functions (Ahles et al., 2012, Ahles and Saykin, 2002, Wefel et al., 2015, Wefel et al., 2014, Wefel and Schagen, 2012). Cognitive dysfunction refers to the subjective experience of having deficits in one's cognitive function. In breast cancer alone, more than 60 studies have investigated and found various degrees of association between chemotherapy and cognitive impairments (Wefel and Schagen, 2012).

Nevertheless, it is not clear which cognitive domains are most affected and most vulnerable to chemotherapy treatment. This is due to the multifactorial nature of the neuropsychological tests used in various clinical studies (O'Farrell et al., 2013). In a longitudinal study by O'Farrell et al., researchers found four cognitive factors that were affected in cases of chemo brain: processing speed, working memory, visual memory, and verbal memory. These test findings fit well with patients' self-reports of experiencing losses in cognitive function (O'Farrell et al., 2013). At the same time, other studies have found that self-reported cognitive function impairment is weakly correlated with testing performance on neurocognitive tasks (Castellon and Ganz, 2009). However, this dichotomy may suggest that tests of neurocognitive tasks may be not wholly accurate in assessing how well patients perform in their everyday lives. Subjective reports of impairment from patients, while providing grounds that issues occur in post-chemotherapy treatment, are based on assignments of cognitive tests that assess a particular cognitive domain. These do not yield any information on the molecular and cellular changes that go on in the brain and serve as a foundation for cognitive deficits.

#### **MECHANISMS OF CHEMO BRAIN**

The underlying mechanisms of chemotherapy-related cognitive dysfunction are

not well understood (Kaiser et al., 2014). Recently, increasing amounts of data have shown that chemotherapy, contrary to previous belief, does impose toxic effects on the cellular populations of the central nervous system (CNS) (Kaiser et al., 2014).

It has been found that chemotherapy caused oxidative stress, inhibited neuronal proliferation and differentiation, activated microglia, induced apoptosis, and altered levels of histone modification and chromatin remodeling lead to the aberrant expression of neurotrophin and neurogenic proteins in the brains of experimental animals (Seigers and Fardell, 2011, Seigers et al., 2015, Seigers et al., 2010a, Seigers et al., 2008, Seigers et al., 2009, Seigers et al., 2013, Seigers et al., 2010b). These molecular changes are associated with altered neurogenesis and deficits in learning and memory processes (Mustafa et al., 2008, Briones and Woods, 2011, Christie et al., 2012). While the molecular and cellular mechanisms of chemo brain are still not well understood, the frequency and timing of chemo brain occurrence and persistence suggest that the origins of chemo brain may be epigenetic and associated with aberrant global gene expression patterns (Wang et al., 2015).

## **EPIGENETICS**

Epigenetic changes are defined as "meiotically heritable and mitotically stable alterations in gene expression" that "include DNA methylation, histone modification and RNA-associated silencing" (Jaenisch and Bird, 2003, Kovalchuk, 2013, Jirtle, 2013). Cytosine DNA methylation was the first epigenetic alteration identified and is one of the most widely studied and well-known epigenetic mechanisms. DNA methylation refers to the addition of a methyl group, obtained from the methyl donor S-adenosyl-L-methionine to the fifth carbon atom in the cytosine pyridine ring. This reaction is

catalyzed by DNA methyltransferases (DNMT1, DNMT3a and DNMT3b) (Goll and Bestor, 2005, Rountree et al., 2001, Weber et al., 2007, Wilson et al., 2006, Weber et al., 2005, Robertson, 2001) and results in the formation of 5-methylcytosine. In mammalian somatic cells this stable post-synthetic epigenetic mark is found at cytosine residues of CpG dinucleotides methylated to 60-80% (Weber and Schubeler, 2007). DNMT1 is the key enzyme involved in the maintenance of DNA methylation patterns after DNA replication (Liang et al., 2002, Kovalchuk, 2013). DNMT3a and DNMT3b are de novo methyltransferases that target unmethylated and hemimethylated sites and establish new methylation patterns (Weber and Schubeler, 2007, Okano et al., 1999, Goll and Bestor, 2005). The association of DNA methylation with transcriptional repression is facilitated by methyl CpG-binding domain (MBD) proteins, which preferentially interact with methylated DNA (Klose and Bird, 2006a, Robertson and Wolffe, 2000, Robertson, 2002, Hendrich and Tweedie, 2003, Jaenisch and Bird, 2003, Kovalchuk, 2013).

DNA methylation is a primary epigenetic regulator of gene expression. DNA hypermethylation is the gain of methylation at specific sites that are unmethylated under normal conditions whereas DNA hypomethylation is the loss of methylation at sites that, under normal conditions, are methylated. As such, hypermethylated gene promoters lead to an “off” state of gene expression while those less methylated are deemed as “on” (Jaenisch and Bird, 2003). DNA methylation is crucial for normal development, cell proliferation, and maintenance of genome stability in an organism, and for responses to the environment (Klose and Bird, 2006b, Shames et al., 2007, Scarano et al., 2005, Robertson and Wolffe, 2000, Jirtle, 2013). DNA methylation is closely connected with the other components of chromatin structure, primarily - histone modifications. In this

instance, however, there are in excess of 50 post-translational modifications that may occur at key amino acid residues within histones of the nucleosome. Histone modifications encompass acetylation, methylation, and phosphorylation. Ubiquitination and sumoylation are also important in transcriptional regulation and genome stability (Weidman et al., 2007, Jenuwein and Allis, 2001). In addition, epigenetic control of gene expression can be facilitated by means of small regulatory RNAs. Exposures to direct and bystander irradiation as well as chemotherapy significantly alter DNA methylation and other constituents of epigenetic control (Kovalchuk and Baulch, 2008).

Recent studies have identified a second type of cytosine modification in mammalian DNA, 5-hydroxymethylcytosine (5-hmC); it is a new type of epigenetic modification. It was shown that 5-hmC constitutes an oxidative derivative of 5-methylcytosine (5-mC) (Globisch et al., 2010). It is a key participant and intermediate in the process of DNA demethylation. In fact, the process of DNA demethylation was rather unclear until the discovery of DNA hydroxymethylation. TET (Ten-Eleven-Translocation) proteins oxidize 5-mC to 5-hmC, which can then be further modified, leading to the demethylation of DNA (Guo et al., 2011, Wu and Zhang, 2011).

Within the genome, 5-hmC occurs predominantly within the gene regions, including exons and untranslated regions, while in the introns and intergenic regions, the levels of 5-hmC are depleted, reviewed in (Sherwani and Khan, 2015). High levels of genomic 5-hmC were shown in embryonic stem cells and in the murine brain (Wen and Tang, 2014). Furthermore, the presence of 5-hmC was identified in various mammalian cells and tissues, suggesting its role in the maintenance of DNA methylation and regulation of gene expression (Globisch et al., 2010).

Epigenetics is of special interest to neuroscientists because it can provide a molecular basis for the complex mechanisms underlying cognition and behavior. In recent years, more and more research has been providing evidence that neurological processes are indeed regulated by epigenetic components that govern gene expression: DNA methylation, histone modification and chromatin remodeling, and non-coding RNAs (Kovalchuk and Kovalchuk, 2012). Neurological processes orchestrated by epigenetics include: neuron development and function, neuronal plasticity, and memory formation just to name a few. During brain development, DNA methylation is believed to be important in regulating the proliferation of neural stem cells and their differentiation into neurons and glial cells (Mattson, 2003).

DNA hydroxymethylation is also important for the brain development and function, as its levels change during neurodevelopment (Chen et al., 2014). Research by Szulwach and colleagues proved that 5-hmC-mediated epigenetic changes are critical for neurodevelopment and play a key part in various neurological diseases (Szulwach et al., 2011). Later, numerous studies confirmed the importance of DNA hydroxymethylation in Alzheimer's disease, Huntington's disease, Parkinson's disease, malignant gliomas and autism, just to name a few (Sherwani and Khan, 2015, Kovalchuk and Kovalchuk, 2012). A handful of studies show that exposure to environmental agents such as phenobarbital, ascorbic acid, diethylstilbestrol and hydroquinone cause changes in the levels of cellular 5-hmC and TET proteins, reviewed in (Dao et al., 2014). Nevertheless, nothing is known of the effects of any other agents on the levels of hydroxymethylation.

## **THE KEY BRAIN REGULATORY REGIONS – THE PREFRONTAL CORTEX AND HIPPOCAMPUS**

### **THE PREFRONTAL CORTEX**

The prefrontal cortex is at the foremost section of the frontal lobes. It is involved in “executive functions,” such as working memory, decision-making, planning, judgment, and social behavior. It is also responsible for abstract thinking and for regulating behavior. The prefrontal cortex undergoes prolonged development, and is extensively interconnected with other cortical, subcortical, and brain stem sites (Kolb et al., 2012).

The dorsal lateral prefrontal cortex is interconnected with the brain regions involved in attention, cognition, and action, while the ventral prefrontal cortex is connected to regions involved with emotion. The prefrontal cortex (PFC) is also believed to store short-term memory, and is important for top-down processing, which occurs when behaviors are guided by internal states or intentions. It guides the control of cognitive actions, and representations in the PFC can function as templates, by providing top-down signals to other regions of the brain that direct the flow of activity along the pathways necessary to accomplish a task (Miller and Cohen, 2001).

### **THE HIPPOCAMPUS**

The hippocampus is part of the limbic system, and is located within the medial temporal lobe. It is involved in several cognitive processes, including spatial navigation and memory processing. The hippocampus is also involved in the storage of long-term memory, and plays a major role in declarative memory, which concerns things that can be recalled with purpose, such as facts or events.



The hippocampus is comprised of the dentate gyrus and the cornu ammonis, each of which can be broken down into different strata. Within the dentate gyrus, the polymorphic layer is the topmost. It connects many interneurons, as well as the axons of dentate granule cells passing through on their way to the CA3 (one of the strata of the cornu ammonis). The dentate gyrus' stratum granulosum contains the cell bodies of the dentate granule cells, while its inner layers, the stratum moleculare, contain synapses (Kolb and Whishaw, 2014).

Neurogenesis within the hippocampus occurs in the subgranular zone of the dentate gyrus. This layer contains several types of cells, of which the most prominent are the neural stem cells. These exist in various developmental stages. This layer also contains astrocytes, as well as endothelial cells and blood vessels that come together to create an environment that supports the neuroblasts and regulates their proliferation, migration, and differentiation. The hippocampus is sensitive to reductions in the global oxygen level of the body. Even non-fatal hypoxia (periods of oxygen deprivation) can result in damage to the hippocampus (Kolb and Whishaw, 2014).

## **NEUROGENESIS**

Neurogenesis is the process by which functional neurons are generated from precursors. While previously thought to occur in mammals only during the embryonic and perinatal stages, anatomical evidence of the presence of newly-generated dentate granule cells has been found in postnatal rats' hippocampi (Altman and Das, 1965). In the early 1960s, it was believed that the nervous system was not capable of regeneration and the first evidence of adult neurogenesis came in 1969, with the identification of the rostral migratory stream by Joseph Altman. Neurogenesis was then shown to occur in

rats and birds, and it was later demonstrated that adult neurogenesis takes place in humans (Eriksson, 1998) and in non-human primates.

It is currently accepted that adult neurogenesis occurs in the subgranular zone of the hippocampal dentate gyrus, and in the subventricular zone that makes up the lining of the lateral ventricles in the forebrain. Neuroblasts produced in the subventricular zone migrate to the olfactory bulb via the rostral migratory stream to become interneurons. Meanwhile, new dentate granule cells are produced in the subgranular zone, in the dentate gyrus. New neurons increase memory capacity, reduce overlap between different memories, and add temporal information to memories. The learning process itself has been linked to neuronal survival.

New neurons arise from the division of neural precursor cells, which become either neurons or astrocytes/oligodendroglia. Neuroblasts travel to the olfactory bulb through tubes created by astrocytes in the rostral migratory pathway. Astrocytes encourage both precursor cell proliferation and the maturation of precursor cells into neurons. The precursor cells grown on these glia do so at a faster rate than those grown on fibroblasts, and are also more likely to become neurons (Kolb and Whishaw, 2014).

An important brain chemical in neurogenesis is brain-derived neurotrophic factor (BDNF), which is needed during the proliferation of hippocampal precursor cells to trigger their maturation into neurons. Other growth factors, such as fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) also serve to stimulate neuronal proliferation (Baldauf and Reymann, 2005).

There are a number of factors influencing neurogenesis. Physical activity and environmental enrichment have been shown to affect both how many cells are born and

how many survive in rodents' dentate gyri (Kolb and Whishaw, 2014). Meanwhile, physical stress factors, such as radiation exposure and exposure to toxic chemotherapy agents, can block neurogenesis.

## **THEORY AND HYPOTHESES**

### **RATIONALE**

It is of utmost importance to ensure that cancer patients achieve the best possible quality of life and suffer minimum side effects from their treatments. The most common anticancer treatments include radiation therapy and chemotherapy, although current literature provides evidence that both radiation therapy and chemotherapy exposures exert negative effects on the brain. Recent studies have proven that the brain is very sensitive to radiation exposure, and direct brain irradiation impacts a wide array of brain functions causing cognitive declines, memory deficits and fatigue, in addition to brain tumours in some exposed individuals. Furthermore, cranial exposure also causes molecular bystander effects in the shielded somatic organs and gonads of animals (Tamminga et al., 2008, Koturbash et al., 2006, Koturbash et al., 2007), albeit very little is known about the existence of bystander effects in the shielded brain upon the irradiation of distal somatic organs. Chemotherapeutic agents cause an array of side effects, including memory loss and cognitive dysfunction, which can persist long after the completion of cytotoxic treatment (Seigers et al., 2008) and cause the condition commonly known as chemo brain (Han et al., 2008) (Mustafa et al., 2008, Briones and Woods, 2011, Christie et al., 2012).

Nevertheless, the molecular mechanisms and precise cellular and behavioural repercussions of radiation and chemotherapy are poorly understood and need to be investigated. The frequency and timing of brain irradiation effects and chemo brain occurrence strongly suggest that these deleterious effects on the brain may be epigenetic and associated with aberrant global gene expression patterns.

## **THEORY**

Based on the aforementioned, here we proposed a new theory of radiation brain and chemo brain whereby the mechanisms that underlie the neurotoxic side effects of radiation therapy and chemotherapy on the brain are epigenetically regulated and associated with altered gene expression. Our analysis focused on the hippocampus and prefrontal cortex (PFC), and was based on their pivotal roles in memory, learning and executive functions.

**Hypothesis 1** - Head and liver irradiation cause direct and bystander changes in the animal brain *in vivo*, and molecular changes are correlated with neuroanatomical changes and behavioural outcomes.

**Hypothesis 2** - Chemotherapy agents cause persistent epigenetic and gene expression changes in the animal brain *in vivo*.

### **THE FOLLOWING OBJECTIVES WERE FORMULATED TO TEST OUR HYPOTHESES**

1. To analyse the molecular gene expression changes caused by the direct and bystander radiation therapy, such as low-dose irradiation on the brain, and to correlate those with the neuroanatomical characteristics and behavioural outcomes (Chapters 2 and 3).

2. To analyse the molecular epigenetic and gene expression changes in the brain upon exposure to cytotoxic chemotherapy agents (Chapter 4).

## **KEY FINDINGS**

In Chapter 1, we provide a review of the most current literature and summarise the current knowledge regarding cancer therapies, focusing on radiation therapy and chemotherapy, the effects of radiation therapy and diagnostics on the brain,

chemotherapy effects on the brain and the role of epigenetic changes in the brain's responses to radiation and cytotoxic chemotherapy.

Chapter 2 summarises our study, which was the first to conduct a large-scale analysis of the molecular, neuroanatomical, and behavioural consequences of direct and bystander low-dose irradiation on the rodent brain. The key findings are that: (i) direct head exposure to radiation doses as low as 24.5 cGy induces persistent, albeit small, increases in DNA damage, as measured by the levels of  $\gamma$ H2AX and affects gene expression in the PFCs of exposed animals; (ii) bystander effects exist in the brain after liver irradiation and manifest as a small increase in DNA damage, as measured by the levels of  $\gamma$ H2AX and as altered gene and protein expression; (iii) both head and liver irradiation reduce dendritic space (and, thus, synapse numbers) in measures of spine density, dendritic complexity, and dendritic length; (iv) the neuroanatomical effects are brain region-specific and are more pronounced in females; and (v) both head and liver irradiation alters behaviour. Bystander effects described in Chapter 2 may be caused by some blood-derived factors; they may also be due to very small, scattered irradiation doses received by the brain.

Chapter 3 discusses the effects of scatter radiation on the brain. This is the first study showing that very low, clinically relevant doses of bystander scatter irradiation alter gene expression, induce changes in dendritic morphology, and lead to behavioural deficits in exposed animals. The key outcomes of this study are that: (i) bystander scatter irradiation affects the brain; (ii) bystander scatter irradiation with a clinically-relevant dose as low as 0.115 cGy causes changes in gene expression in the PFC tissues of females, but not males; (ii) bystander scatter irradiation reduces spine density, dendritic

complexity and dendritic length; (iii) the bystander scatter-induced neuroanatomical changes are brain region-specific and are much more pronounced in females; and (iv) bystander scatter irradiation causes behavioural deficits in female animals, but not in male animals.

These constitute seminal findings because, for quite some time, the brain was considered a radiation-resistant organ, and only very high doses were thought to exert harmful effects on it.

Chapter 4 summarises a study of molecular effects on chemo brain – a severe post-chemotherapy side effect – using a murine model. Here, we analysed epigenetic and gene expression changes in the hippocampus and PFC tissues of mice 24 hours and three weeks after treatment with cytotoxic chemotherapy agents mitomycin C (MMC) and cyclophosphamide (CPP). These agents have been reported to cause chemo brain, but the mechanisms of their effects have not been elucidated. The key findings of this study are that: (i) MMC and CPP treatments lead to drug-, sex- and brain region-specific and persistent changes in global gene expression profiles; (ii) chemotherapy agents cause changes in the global levels of DNA methylation and DNA hydroxymethylation, and lead to increased levels of oxidative DNA damage; (iii) and, changes caused by MMC exposure persist for up to three, weeks and were the most pronounced in the PFC tissues of female animals.

In sum, the experiments described herein present some key evidence that the mammalian brain is negatively affected by direct, bystander and scatter radiation exposures and by cytotoxic chemotherapy. The effects are sex- and brain region-specific and persistent. In addition, our data suggest that the female prefrontal cortex is

especially sensitive to radiation and chemotherapy treatments, much more so than the male one, and that it is one of the most stress-sensitive regions of the mammalian brain.

Finally, Chapter 5 provides a short discussion of the most important findings and the outlook for the future research developments, and propose some possible behavioural strategies to mitigate the deleterious neuroanatomical and behavioural consequences of radiation exposure.



## **CHAPTER 2: LIVER IRRADIATION CAUSES DISTAL BYSTANDER EFFECTS IN THE RAT BRAIN AND AFFECTS ANIMAL BEHAVIOUR**

Chapter 2 has been submitted in its entirety:

Kovalchuk A., Mychasiuk R., Muhammad A., Hossain S., Ilnytskyy S., Ghose A., Kirkby C., Ghasroddashti E., Kovalchuk O., Kolb B. Liver irradiation causes distal bystander effects in the rat brain and affects animal behaviour. *Scientific Reports* (in review)

## **ABSTRACT**

Radiation therapy not only produces effects on targeted organs, but can also influence shielded bystander organs, such as the brain in targeted liver irradiation. The brain is sensitive to radiation exposure, and irradiation causes significant neuro-cognitive deficits, including attention deficits in attention, concentration, memory, and executive and visuospatial functions. The mechanisms of their occurrence are not understood, although they may be related to the bystander effects.

We pioneered the analysis of the induction, mechanisms, and behavioural repercussions of bystander effects in the brain upon liver irradiation in a well-established rat model. Here, we show for the first time that bystander effects occur in the prefrontal cortex and hippocampus regions upon liver irradiation, where they manifest as altered gene expression and somewhat increased levels of  $\gamma$ H2AX. We also report that bystander effects in the brain are associated with neuroanatomical and behavioural changes, and are more pronounced in females than in males.

## INTRODUCTION

While ionizing radiation (IR) is a key mainstream diagnostic and treatment modality, it is also a potent DNA-damaging agent capable of inducing a variety of diseases, including cancer. Today, radiation therapy (RT) is one of the main sources of IR exposure. Recent studies have proven that the brain is very sensitive to irradiation, and RT impacts a wide array of brain functions, causing cognitive decline, memory deficits, fatigue, and brain tumours in exposed individuals, reviewed in (Greene-Schloesser and Robbins, 2012, Marazziti et al., 2012) . The extent and severity of IR's effects on the brain depend upon its dosage (Greene-Schloesser and Robbins, 2012). While the effects of high doses of IR have been studied and are reasonably well understood, the effects and mechanisms of the brain's response to low doses of IR need to be analyzed in more detail (Yin et al., 2003, Greene-Schloesser and Robbins, 2012, Kempf et al., 2015).

Brain irradiation effects are specific to age, brain region, and sex (Koturbash et al., 2011a, Silasi et al., 2004, Kempf et al., 2014b). Of the various brain regions, the prefrontal cortex and the hippocampus are particularly sensitive to IR (Monje and Palmer, 2003, Fike et al., 2007, Rola et al., 2004b, Lonart et al., 2012, Parihar et al., 2015). The hippocampus is one of two active sites of neurogenesis in the mammalian brain (Gage, 2000). The proliferation of neuronal precursors in the subgranular zone of the dentate gyrus generates cells that migrate further to the granule cell layer and differentiate into mature neuronal and glial phenotypes (Palmer et al., 1997).

The prefrontal cortex (PFC) is a main regulatory region that collects input from all other cortical regions; it plans and directs an array of motor, cognitive, and social behaviours. The PFC receives inputs from the ventral tegmental area and connects with

virtually all regions of the forebrain. It is susceptible to harmful factors like stress, which can lead to abnormal functioning (Kolb et al., 2012).

Irradiation is a well-known cause of apoptosis, and it blocks neurogenesis in the dentate subgranular zone (Mizumatsu et al., 2003a). Such blockages persist for prolonged periods of time. IR exposure results in a loss of cells in the hippocampal CA1 subfield, reduces spine density and dendritic length in the dentate gyrus. The PFC is also very sensitive to different environmental stresses, including IR, which impacts gene and protein expression in the PFC (Kornev et al., 2005, Silasi et al., 2004, Parihar and Limoli, 2013, Parihar et al., 2015).

In addition, while it has been broadly accepted for several decades that the biological effects of radiation exposure are attributable to the direct damaging effects of irradiation in exposed tissues, this paradigm has been challenged by numerous experiments proving that even those cells that are not directly traversed by IR exhibit responses that are very typical of directly irradiated cells (Mothersill and Seymour, 2004). Such IR-induced ‘bystander’ effects have been seen in both naïve cells that were in contact with directly irradiated cells and naïve cells that received certain irradiation ‘distress’ signals from the directly exposed cells (Morgan and Sowa, 2005).

Cranial exposure causes a wide array of molecular bystander effects in animals’ shielded spleens, livers, and gonads (Tamminga et al., 2008, Koturbash et al., 2008b, Koturbash et al., 2007). IR-induced bystander effects persist for a long time following irradiation. While cranial exposure has been shown to cause bystander effects in somatic organs, very little is known about the potential bystander effects caused by the irradiation of distal somatic organs on a shielded brain.

Here, we present the first evidence that bystander effects occur in the brain as a result of liver irradiation; these effects manifest as altered gene and protein expression and DNA damage. They are associated with neuroanatomical and behavioural changes, and are more pronounced in females than in males.

## **RESULTS**

### **Liver irradiation model to study bystander effects in the brain**

We studied bystander effects in the brain by exposing the liver of an experimental animal to IR while protecting the rest of the body with a medical-grade lead shield (Fig. 2.1). We compared this result to the effects of head irradiation. Detailed dose analyses revealed that directly irradiated brains received doses of 24.5 centigrays (cGy). When the liver was irradiated, the brain received a small scatter dose of 0.125 cGy (Kirkby et al., 2013). Both doses belong to the low-dose radiation range.

### **Persistence of DNA damage in exposed and bystander PFC tissues *in vivo***

The induction of DNA damage constitutes a well-established bystander effect endpoint (Sedelnikova et al., 2007). To analyze the levels of DNA damage, we assayed for the presence of H2AX phosphorylation in the hippocampus and PFC tissues of control, liver-exposed, and head-exposed animals. H2AX is a member of the H2A histone family that becomes phosphorylated at S139 ( $\gamma$ H2AX) as one of the earliest cellular responses to double-strand breaks in DNA (Sedelnikova et al., 2003). Two weeks after exposure,  $\gamma$ H2AX was virtually undetectable by western blot in the PFCs of unirradiated control rats, but was found, albeit in small amounts, in the PFCs of head-irradiated female and male rats (Fig. 2.1). Furthermore, small amounts of  $\gamma$ H2AX were detected in the PFCs of female rats that had been subjected to liver irradiation, which

could be indicative of increased levels of bystander DNA damage in these animals. No  $\gamma$ H2AX was detected in the PFCs of liver-irradiated male rats. No changes in the  $\gamma$ H2AX levels were seen in the hippocampi of head- or liver-exposed male and female rats (data not shown).

### **Gene expression in the brain tissues of control and exposed rats**

Bystander effects often manifest as aberrant gene expression profiles. Therefore, we next analysed the global transcriptomes of the PFC and hippocampal tissues in male and female rats following liver irradiation using Illumina-based massively parallel sequencing. Transcriptome profiling revealed profound sex- and region-specific differences in gene expression. In males, liver irradiation affected one particular predicted gene in the hippocampus and PFC: ENSRNOG00000043197.

In females, twenty-two genes were differentially expressed in bystander PFC tissues following liver irradiation (Table 2.1). To further substantiate our results, we conducted western immunoblotting for the protein products of two differentially expressed genes: Tbx18 and Eaat2. Compared with the case of control rats, both genes were down-regulated in the bystander PFC tissues of the liver-exposed female rats. The levels of both the TBX18 and EAAT2 proteins were also lower in the PFC tissues of the liver-exposed female rats than in those of the control rats (Table 2.1, Fig. 2.3).

### **Neuroanatomical changes induced by head and liver irradiation**

Because we had witnessed slightly increased levels of  $\gamma$ H2AX and altered gene expression, we next analysed the neuroanatomical characteristics of the brains of head- and liver-irradiated animals. Our overall finding was that radiation applied to the liver or the head produced extensive changes in dendritic organisation in all regions measured,

with the effects being most extensive in the PFC. The effects were greater in the head irradiation group than in the liver irradiation group. Many of the effects were sexually dimorphic, as the females tended to be more affected than the males. In most measures, the males (including the control males) had higher values than the females. We considered each region in turn (see Figure 2.4 for examples of the neurons from Cg3, AID, and CA1; Figure 2.5 for spine data; and Figure 2.6 for dendrite data). Note that only the basilar fields were drawn for AID and CA1, whereas both the apical and basilar fields were drawn for Cg3 and Par1.

### ***Medial Prefrontal Cortex (Cg3)***

The greatest effects of irradiation were seen in Cg3 in both sexes. Two-way analysis of variance (ANOVA) was run for all analyses, with ‘Treatment’ and ‘Sex’ as factors (F).

*Apical field spine density.* Spine density was reduced in both irradiation groups. ANOVA revealed significant effects for treatment ( $F[2,68]=12.5$ ,  $p<.001$ ), and sex ( $F[1,68]=3.83$ ,  $p=.05$ ), but not their interaction ( $F[2,68]=0.66$ ,  $p=.52$ ). The sex difference reflected greater spine density in males than in females.

*Apical field branching.* Apical branching was reduced in females, but increased in the male head irradiation group. ANOVA revealed significant effects for treatment ( $F[2,68]=6.3$ ,  $p=.003$ ), sex ( $F[1,68]=187.9$ ,  $p<.001$ ), and their interaction ( $F[2,68]=12.25$ ,  $p<.001$ ). The sex difference reflected greater branching in males than in females, and the interaction reflected opposite effects of head irradiation in the two sexes.

*Apical field dendritic length.* Irradiation reduced dendritic length following both types of irradiation. ANOVA revealed significant effects for treatment ( $F[2,68]=8.9$ ,  $p<.001$ ), sex ( $F[1,68]=56.3$ ,  $p<.001$ ), and their interaction ( $F[2,68]=3.6$ ,  $p=.03$ ). Males

had longer dendrites than females, and the effect of radiation was larger in females than in males.

*Basilar field spine density.* Spine density was reduced in both irradiation groups. ANOVA revealed significant effects for treatment ( $F[2,68]=11.3$ ,  $p<.001$ ) and sex ( $F[1,68]=5.3$ ,  $p=.02$ ), but not for their interaction ( $F[2,68]=1.4$ ,  $p=.88$ ). The sex difference again reflected greater spine density in males than in females.

*Basilar field branching.* The results for the basilar field branching were similar to those for the apical branching, with sexually dimorphic irradiation effects. ANOVA revealed no significant effect for treatment ( $F[2,68]=11.15$ ,  $p=.32$ ), but showed significant effects for sex ( $F[1,68]=22.12$ ,  $p<.001$ ) and the interaction ( $F[2,68]=5.0$ ,  $p=.009$ ). The sex difference resulted from the existence of more complex neurons in the males, and the interaction reflected an increase in branching in the male head irradiation group (compared to a decrease in both female irradiation groups).

*Basilar field dendritic length.* The effects on basilar length were complex: the head irradiation reduced length, but the liver irradiation did not. ANOVA revealed significant effects for treatment ( $F[2,68]=3.02$ ,  $p=.05$ ) and sex ( $F[1,68]=3.93$ ,  $p=.05$ ), but not for their interaction ( $F[2,68]=0.12$ ,  $p=.25$ ).

#### ***Orbital Prefrontal Cortex (AID)***

*Basilar field spine density.* Spine density was reduced overall from irradiation, but was higher in males. ANOVA revealed significant effects for treatment ( $F[2,68]=4.7$ ,  $p<.013$ ) and sex ( $F[1,68]=51.8$ ,  $p<.001$ ), but not their interaction ( $F[2,68]=0.6$ ,  $p=.56$ ).

*Basilar field branching.* Although ANOVA revealed no effects for treatment ( $F[2,68]=1.39$ ,  $p=.25$ ), there were significant effects for sex ( $F[1,68]=20.9$ ,  $p<.001$ ) and



the interaction ( $F[2,68]=6.68$ ,  $p=.002$ ). The interaction resulted from a significant drop in branching in the females, but not in the males. Once again, males had more complex cells than females.

*Basilar field dendritic length.* As in the branching results, ANOVA revealed no effects of treatment ( $F[2,68]=1.8$ ,  $p=.17$ ); there were, however, significant effects for sex ( $F[1,68]=9.8$ ,  $p=.003$ ) and the interaction ( $F[2,68]=4.4$ ,  $p=.02$ ). Moreover, as in the branching, the interaction resulted from a significant drop in length in the females, but not in the males.

### ***Hippocampus (CA1)***

The effects of irradiation in the hippocampus were surprisingly small, relative to those found in the prefrontal regions, and were seen only in males.

*Basilar field spine density.* Irradiation significantly reduced spine density in males, but not in females. In addition, as in other regions, males had higher spine densities than females. ANOVA revealed significant effects for treatment ( $F[2,68]=4.1$ ,  $p=.02$ ), sex ( $F[1,68]=2.7$ ,  $p=.07$ ), and their interaction ( $F[2,68]=9.3$ ,  $p<.001$ ).

*Basilar field branching.* There were no significant effects of irradiation on branching. ANOVA found no effects for treatment ( $F[2,68]=0.58$ ,  $p=.56$ ), sex ( $F[1,68]=2.2$ ,  $p=.14$ ), or their interaction ( $F[2,68]=1.4$ ,  $p=.88$ ).

*Basilar field dendritic length.* There was a significant effect of head irradiation, but not liver irradiation, on dendritic length. ANOVA found an effect for treatment ( $F[2,68]=3.02$ ,  $p=.05$ ), but not for sex ( $F[1,68]=0.00$ ,  $p=1.00$ ) nor the interaction ( $F[2,68]=.15$ ,  $p=.6$ ). Although there was a trend of shorter dendrites in both irradiation groups, it was only significant in the head groups.

### ***Parietal Cortex (Par1)***

*Apical field spine density.* There was a significant effect (reduced spine density) of irradiation in females, but not in males. ANOVA showed no significant effect for treatment ( $F[2,68]=2.24$ ,  $p=.12$ ), but did show effects for sex ( $F[1,68]=8.7$ ,  $p=.004$ ) and the interaction ( $F[2,68]=3.68$ ,  $p=.03$ ). The interaction reflected the decrease in females, but not in males.

*Apical field branching.* There was no effect of irradiation in either sex, although males had more branching than females. ANOVA found no significant effects for treatment ( $F[2,68]=1.45$ ,  $p=.24$ ) or the interaction ( $F[2,68]=2.05$ ,  $p=.137$ ), but did show a significant effect for sex ( $F[1,68]=4.5$ ,  $p=.037$ ).

*Apical field dendritic length.* There were no significant effects of either sex or irradiation on apical dendritic length. ANOVA showed no significant effects for treatment ( $F[2,68]=0.96$ ,  $p=.39$ ), sex ( $F[1,68]=.21$ ,  $p=.65$ ), or the interaction ( $F[2,68]=0.75$ ,  $p=.45$ ).

*Basilar field spine density.* As with the apical spines, there was a significant reduction from irradiation in females, but not in males. ANOVA showed significant effects for treatment ( $F[2,68]=6.29$ ,  $p=.003$ ), sex ( $F[1,68]=25.0$ ,  $p<.001$ ), and their interaction ( $F[2,68]=6.75$ ,  $p=.002$ ). The interaction reflected the decrease in females, but not in males.

*Basilar field branching.* There was an unexpected effect of an increase in branching in the head irradiation groups. ANOVA (Treatment x Sex) found an effect for treatment ( $F[2,68]=3.01$ ,  $p=.05$ ), but not for sex ( $F[1,68]=0.19$ ,  $p=.66$ ) or the interaction ( $F[2,68]=.342$ ,  $p=.71$ ).

*Basilar field dendritic length.* There were no significant effects of either sex or irradiation on basilar dendritic length. ANOVA showed no significant effects for treatment ( $F[2,68]=1.02$ ,  $p=.37$ ), sex ( $F[1,68]=1.36$ ,  $p=.25$ ), or their interaction ( $F[2,68]=0.37$ ,  $p=.69$ ).

### **Behavioural changes induced by head and liver irradiation**

Overall, both head and liver radiation affected animal behaviour in both the activity test and the Elevated-Plus Maze (EPM), but not in the novel object recognition test (Fig. 7), which is a test of memory. We considered each separately.

*Activity.* Both head and liver irradiation significantly reduced activity, with the reduction being about 20 percent in males and 15 percent in females (see Fig. 7). A two-way ANOVA found a significant effect for treatment ( $F[2,25]=3.90$ ,  $p=.03$ ) and sex ( $F[1,40]=10.78$ ,  $p<.01$ ), but not for their interaction ( $F[2,35]=.08$ ,  $p=.92$ ).

*Elevated-Plus Maze.* Head irradiation significantly reduced the time spent by both male and female animals in the open arms of the maze (described further in the Behavioural Analysis section below), but liver irradiation only reduced activity in males (Fig. 5). A two-way ANOVA found a significant effect for treatment ( $F[2,35]=6.07$ ,  $p<.01$ ), but not for sex ( $F[1,40]=2.35$ ,  $p=.13$ ). It also found a nonsignificant trend toward an interaction ( $F[2,35]=2.69$ ,  $p=.08$ ). The interaction trend reflected the absence of a treatment effect in the female liver radiation group.

*Novel Object Recognition.* There were no significant effects of radiation on novel object recognition (Fig. 2.7). A two-way ANOVA found no significant effects for treatment ( $F[2,25]=1.62$ ,  $p=.21$ ), sex ( $F[1,40]=1.95$ ,  $p<.17$ ), or their interaction ( $F[2,35]=2.37$ ,  $p=.29$ ). However, inspection of Figure 2.7 suggests a trend toward a

reduced amount of time spent with the novel object in the female radiation groups. Given this observation, we performed a posthoc t-test comparing the female head irradiation and non-irradiation groups and found a significant difference ( $p < .05$ ).

## **DISCUSSION**

This study is the first to conduct a large-scale analysis of the molecular, neuroanatomical, and behavioural consequences of direct and bystander low-dose irradiation on the rodent brain. The key findings of this study are that: 1) direct head exposure to 24.5 cGy causes persistent albeit small increase in DNA damage as measured by the levels of  $\gamma$ H2AX and affects gene expression in the PFCs of exposed animals; 2) bystander effects of liver exposure to a dose as low as 0.125 cGy in the shielded bystander brain manifest as an increase in the levels of  $\gamma$ H2AX and as altered gene and protein expression; 3) both head and liver irradiation reduce dendritic space (and, thus, synapse number) in measures of spine density, dendritic complexity, and dendritic length; 4) the neuroanatomical effects are brain-region-specific, and are more pronounced in females; and 5) both head and liver irradiation alters behaviour.

The direct low-dose IR and the bystander-induced DNA damage in the brain represent an interesting observation. We analysed the DNA damage by studying the levels of phosphorylated histone H2AX ( $\gamma$ H2AX).  $\gamma$ H2AX is a well-accepted marker of DNA strand breaks (Sedelnikova et al., 2003), and bystander cells were reported to exhibit an accumulation of  $\gamma$ H2AX (Sokolov et al., 2007).

Our study is the first to show the presence of  $\gamma$ H2AX in the shielded bystander brains. Two weeks after exposure, found the presence of  $\gamma$ H2AX in the PFCs of female rats subjected to head and liver irradiation, which suggests increased albeit small levels

of direct and bystander DNA damage in these animals. This may be due to inefficient DNA repair, or to the fact that small amounts of damage may be simply overlooked by DNA repair systems. In the future, it will be important to analyse and confirm the precise nature of the DNA damage caused by direct and bystander irradiation in the PFC.

Although low-dose radiation effects, as well as bystander effects, have been shown to cause changes in gene expression in affected cells (Kalanxhi and Dahle, 2012), nothing was previously known about the effects of liver irradiation on gene expression in distal bystander brain tissues. In this study, we analysed the global transcriptomes of PFC and hippocampal tissues in male and female rats following liver irradiation and uncovered interesting sex- and brain-region-specific changes in gene expression. In males, liver irradiation affected one predicted gene in the hippocampus and PFC: ENSRNOG00000043197. This locus is a predicted target of several transcription factors, such as CdxA, GATA-1, SRY, HFH-2, and p300. Interestingly, SRY is the sex determination factor that is expressed only in males, which may explain why this gene is up-regulated in a sex-specific manner. Further analysis is needed to explore this gene, its function, and its regulation in detail.

For the first time, we noted significant and sex-specific alterations in gene expression profiles, in which twenty-two genes were affected in female PFCs (Table 2.1). Differentially expressed genes included those involved in the function of the blood-brain barrier, neuroinflammation, and apoptosis. Amongst these there was the *Eaat2* gene. Its altered expression were also confirmed to exist on the protein level. EAAT2 belongs to excitatory amino-acid transporters, which are also known as glutamate transporters, a family of neurotransmitter transporters that affect brain function and development.

EAAT2 is also one of the major glutamate transporters expressed in astroglial cells that governs approximately 90 percent of total glutamate uptake. Loss of EAAT2 function has been associated with the development of neurodegenerative diseases, such as Alzheimer's and Huntington's diseases, amyotrophic lateral sclerosis, and malignant glioma (Su et al., 2003, Kim et al., 2011). The down-regulation of EAAT2 therefore may have negative consequences on PFC function. The roles of EAAT2 in direct and bystander radiation responses need to be further explored.

Our study found that liver irradiation also negatively affects the expression of collagens in the bystander PFCs of female animals. Collagens protect against neuronal apoptosis and are engaged in the blood-brain barrier function (Cheng et al., 2011, Baeten and Akassoglou, 2011). Previous studies have reported that exposure to 10 or 40 Gy of  $\gamma$ -rays leads to reduction in collagen levels and dysfunction of the blood-brain barrier (Lee et al., 2012). Additionally, we noted decreased levels of Slit-Robo Rho GTPase-activating protein 3 and Pecanex-like protein 1. Pecanex exerts a neurogenic role in *Drosophila* (Gilbert et al., 1992). The Slit-Robo pathway has been shown to play a role in axonal regeneration after nerve injury (Madura et al., 2004). Moreover, disruption of some of the Slit-Robo Rho GTPase-activating proteins has been linked to the development of infantile epileptic encephalopathy (Saito et al., 2012). The roles of these proteins in low-dose radiation and bystander effects also need to be further examined.

Most importantly, our study showed extensive bystander effects in brain morphology, which manifested as decreased spine density, dendritic length, and dendritic complexity in the two PFC regions measured, the parietal cortex, and the hippocampus. These effects were generally more profound in females, and the largest effects were

found in the PFC regions, with surprisingly small effects seen in the hippocampus. While previous studies have shown conclusively that irradiation interferes with neurogenesis, leading to cognitive impairment (Parihar and Limoli, 2013, Parihar et al., 2015), still fairly little is known about the effects of irradiation on mature neurons. We chose to examine dendritic measures in several brain regions that did not have adult neurogenesis. The sizes of dendritic fields and the densities of the spines provide indirect measures of synaptic organisation and number. Reductions in these measures are correlated with several brain disorders, including dementia, Down syndrome, and fragile-X syndrome, and changes (e.g., increases or decreases in the synaptic space) have been associated with learning (Markham and Greenough, 2004). Parihar and Limoli (Parihar and Limoli, 2013) showed in a study on male mice that higher levels of cranial irradiation than those used in the current study reduced spine density and dendritic complexity in the dentate gyrus of the hippocampus. Their observed effects were much larger than those observed in this study, which used much lower irradiation doses and looked at CA1. Chakraborti et al. (Chakraborti et al., 2012) also studied spine densities in male mice, and found decreased spine densities in both the dentate gyrus and CA1 of the hippocampus. Most recent study by Pahirar et al. established that low, space-relevant doses of charged particles reduced dendritic complexity and spine density in PFC of male mice (Parihar et al., 2015).

Thus, the novel neuroanatomical findings here are that: 1) both direct head and indirect (bystander-to-liver) irradiation in low doses reduces synaptic space in both the hippocampus and the neocortex, and 2) these changes are sexually dimorphic and areal-specific. Indeed, the effects in both prefrontal regions measured were far more extensive

than those seen in the hippocampus.

The current study also showed that neuroanatomical and brain molecular changes were associated with behavioural change, as well. The decrease in activity associated with both head and bystander irradiation was unexpected, given that lesions to the PFC or the hippocampus tend to increase activity (Kolb, 1974). The irradiated animals did not have lesions; instead, they experienced a reorganisation of circuits, which presumably accounts for the difference. The irradiation also increased anxiety, a result commonly associated with stressful experiences. Curiously, the bystander effect on anxiety was only observed in males, possibly reflecting the male-specific changes in spine density in both AID and CA1. Finally, the novel object recognition test, which is a test of memory, showed head irradiation to have an effect on females, but not on males. This sexually dimorphic effect may be associated with the decrease in dendritic branching and length found in female, but not male, Cg3 neurons. Lesions of the medial PFC are associated with deficits in temporal order memory, such as the memory used for current tasks (Hannesson et al., 2004) .

Bystander effects in the brain were previously shown in two studies by Mancuso and colleagues (Mancuso et al., 2011, Mancuso et al., 2008), who analysed changes in the cerebellums of two mutant mouse strains following irradiation of the animal bodies. In our study, we also observed bystander effects in the cerebellum, which manifested as altered levels of protein expression (data not shown). The changes in the cerebellum, however, were less pronounced than those in the PFC and the hippocampus. Moreover, our study engaged in a detailed analysis of the scatter dose received by the brain during liver exposure. The dose (0.125 cGy) was very low, nevertheless it could cause some of



the observed effects. Low doses were previously shown to exert strong mutagenic potential on exposed cells and tissues. Aside from the small scatter dose, a blood-borne bystander signal may also exist. Such a signal may originate in exposed blood cells and spread via the blood. Indeed, numerous blood cells are located in the exposure field during the exposure of the liver (which received 30 cGy). Blood cells are sensitive to irradiation and can undergo apoptosis or necrosis. They then release a variety of soluble factors that are small enough to cross the blood-brain barrier and damage it. The molecular identity of the bystander factors needs to be defined, and the roles played by these factors, and by small scatter doses in bystander effects, should be further investigated.

## **MATERIALS AND METHODS**

### **Animal Model and Tissue Sampling**

Forty four male and forty four female three-month-old Long Evans rats (Charles River) were used in this study. Sixteen male and sixteen female animals were used for molecular profiling, and twenty eight male and twenty eight female animals were used for neuroanatomical analysis and behavioural testing.

The animals were housed in a pathogen-free controlled facility with a 12 h light/dark cycle and given food and water ad libitum. The animals were randomly allocated to the following groups: head-exposed, liver-exposed and sham-treated control, as described before (Kirkby et al., 2013). For irradiation, the animals were anaesthetised through intra-peritoneal injections of ketamine/xylazine (50/5 mg/kg). The anaesthesia was well tolerated, and no side effects were observed. Head- and liver-exposed animals received X-ray irradiation delivered to the surface of the respective area of their body; a medical-grade lead shield protected the rest of the body. Specifically, for each target organ, a lead apron (0.05 cm Pb-equivalent) was used for shielding. A 1.7 cm by 3.5 cm oval was cut into the apron in order to define a primary field, and the apron was then placed on the rats. The doses delivered to the respective organs were determined as follows: for head exposure, a dose to the brain constituted 24.5 cGy. In the liver-exposed/brain bystander scenario, the centre-to-centre distances between organs were roughly 6.0 cm brain-to-liver. During liver exposure, the brain was shielded, but it still received a dose of 0.125 cGy due to radiation scattering (Kirkby et al., 2013). The handling and care of the animals were conducted in accordance with the

recommendations of the Canadian Council for Animal Care and Use. The University of Lethbridge Animal Welfare Committee approved all procedures.

### **Molecular profiling**

For molecular analysis, the animals were euthanized 14 days after irradiation. Upon sacrifice, the brain areas (hippocampus and prefrontal cortex) were sampled and snap-frozen.

### ***Gene Expression Analysis***

The hippocampus and prefrontal cortex tissues of three animals per group were used for the analysis of the gene expression profiles. RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA); purified using an RNAeasy kit (Qiagen), according to the manufacturer's instructions; and quantified using Nanodrop2000c (ThermoScientific). Next, RNA concentration and integrity were determined using 2100 BioAnalyzer (Agilent). Sequencing libraries will be prepared using Illumina's TruSeq RNA library preparation kits. Gene expressions were determined using the Illumina deep sequencing platform at the University of Lethbridge CFI-SAGES Facility.

Statistical comparisons between the control and exposed groups within each tissue type were performed using the DESeq Bioconductor package (version 1.8.3) and the baySeq Bioconductor package (version 1.10.0). The clustering of the samples was assessed using multidimensional scaling (MDS) plots built using the plotMDS function of the edgeR Bioconductor package. MA plots showing the relationship between the average level of expression and the log2 fold change were built for each of the comparisons. Features with false discovery rates (FDR) < 0.1 (10% false positive rate) were considered differentially expressed between conditions.

### ***Western Immunoblotting***

Western immunoblotting was conducted as described previously (Silasi et al., 2004). Membranes were stained overnight using primary antibodies against  $\gamma$ H2AX (1:500, Cell Signaling, Danvers, MA), TBX18 and EAAT2 (1:1000, Abcam, Toronto, ON), and actin (1:2000, Abcam, Toronto, ON). Primary antibody binding was detected using horseradish peroxidase-conjugated secondary antibodies and the Enhanced Chemiluminescence Plus System (Amersham Biosciences, Baie d'Urfe, Quebec). Chemiluminescence was detected using a FluorChem HD2 camera with FluorChem software (Cell Biosciences). The membranes were stained with Coomassie blue (BioRad, Hercules, CA) to confirm equal protein loading. Signals were quantified using the NIH Image J64 software and normalized relative to actin or Coomassie staining.

### **Histological Processing and Neuroanatomical Analysis**

For neuroanatomical analysis rats were given an overdose of sodium pentobarbital solution i.p. and perfused with 0.9% saline solution intracardially 14 days after exposure. Their brains were removed from their skulls, weighed and preserved in Golgi-Cox solution for 14 days, followed by transfer to 30% sucrose solution. The brains were sliced at a thickness of 200  $\mu$ m on a vibratome and fixed on gelatinized slides. The slides mounted with brain sections will be processed for Golgi-Cox staining, following the protocol described (Gibb and Kolb, 1998).

Pyramidal cells were drawn from layer 3 of Cg3 and AID (medial and orbital prefrontal regions, respectively) and from the CA1 region of the hippocampus, according to Zilles' cortical atlas (Zilles, 1985). Individual neurons were traced using a camera lucida mounted on a microscope. For dendritic branching and length, a total of 10 cells

(5/hemisphere) were traced at 250X for each brain region. The averages of the cells from each hemisphere comprised the data points used for statistical analysis. Spine density was measured at 1000x and calculated by counting the number of spines on a length of distal dendrite that was least 50 microns in length. The exact length of the dendrite segment was calculated, and the density was expressed per 10  $\mu\text{m}$ . Five segments were drawn per hemisphere from different neurons, and a mean value was calculated to use as the unit of measurement (Muhammad and Kolb, 2011).

Branch order, which is an estimate of dendritic complexity, was used to measure the number of dendritic bifurcations. Dendritic length was calculated using a Sholl analysis, which is an estimation of dendritic length that counts the number of dendritic branches that intersect concentric circles spaced 25  $\mu\text{m}$  apart. Length is estimated by multiplying the number of dendritic intersections by 25.

### **Behavioural analysis**

Testing occurred two weeks after exposure.

#### ***Activity Box***

The activity levels of the rats were measured two weeks after irradiation (Raza et al., 2015). The rats were placed into an Accusan activity-monitoring box. The system consisted of an electronically fitted Plexiglas box measuring 41 x 41 x 30.5 cm that recorded the movements of each rat. The rats were left in the boxes for 10 min., and their exploratory behaviours were recorded in five 2-min intervals. The data were recorded using the VersaMax TM computer software. The five intervals were summed, and the results are reported as the average of the total activity/distance travelled.

### ***Elevated Plus Maze***

Approximately three weeks after irradiation, the rats were tested in the Elevated Plus Maze (EPM) as previously described (Raza et al., 2015). The EPM was constructed from black Plexiglas, with a base measuring 94 cm high, two open arms measuring 10 cm wide and 40 cm long and two closed arms measuring 10 cm wide, 40 cm long, and 40 cm high. The maze was located in an empty room, and filming occurred with the lights on. The camera for filming was placed at the end of one of the open arms in a slightly elevated position. Filming occurred for five minutes. Rats were placed with their front paws in the center of the square maze facing a closed arm. Their performances were scored by a researcher blinded to experimental conditions, who measured the time spent in the open arms and the time spent in the closed arms. Reduced time spent in the open arms was taken as a measure of anxiety.

### ***Novel Object Recognition (NOR)***

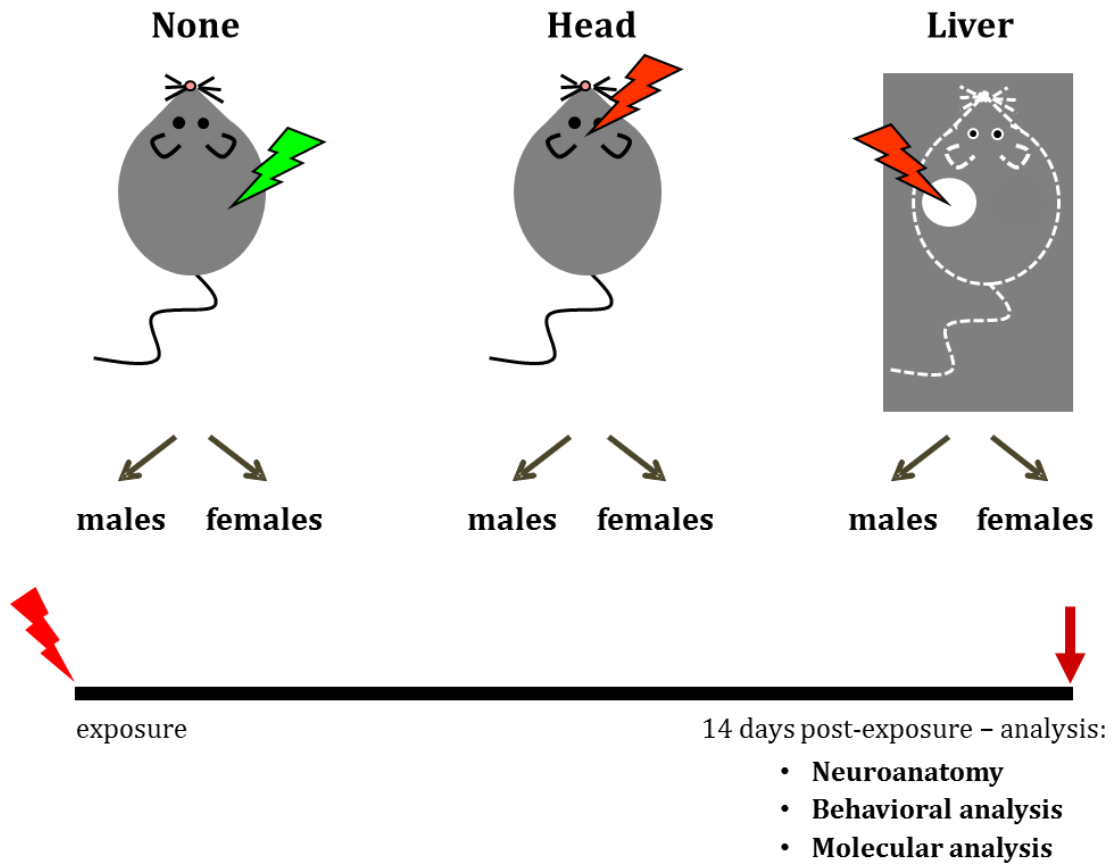
Testing was performed as described (Richards et al., 2012) and occurred two weeks after exposure. In brief, an NOR for temporal order memory was run in three separate trials, each starting one hour apart, on filming day. The rats were placed in a 48 cm × 48 cm × 52 cm white plastic container for five minutes three days prior to filming to habituate them to the testing conditions. On filming day, the initial trial involved placing two identical objects in the base of the tub. The animals were then left to explore the objects for four minutes. The second trial began one hour later and involved placing different identical objects in the tub with the animals for four minutes. The third trial consisted of placing the rats in the plastic container with one object from the first trial and one object from the second trial for four minutes. The time spent with each of the

objects was calculated in the third trial. A rat was considered to be in contact with an object if its nose was within 2 cm of the object.

### **Statistical analysis**

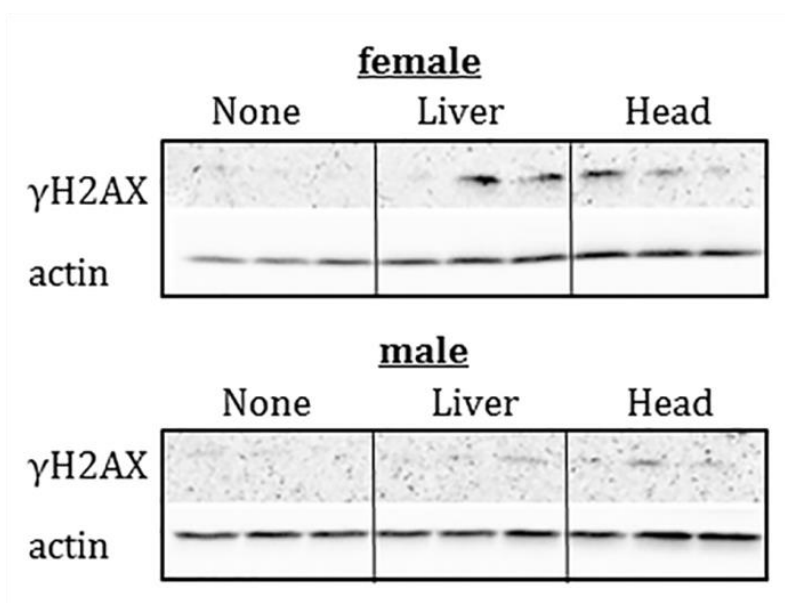
All statistical analyses were carried out using SPSS 16.0 (Richards et al., 2012). Each rat was used as a unit of analysis. Two-way ANOVAs using treatment (control, head-exposure, liver exposure) and sex (M/F) as factors were run to compare the behavioral outcomes in control and exposed rats.

## FIGURES

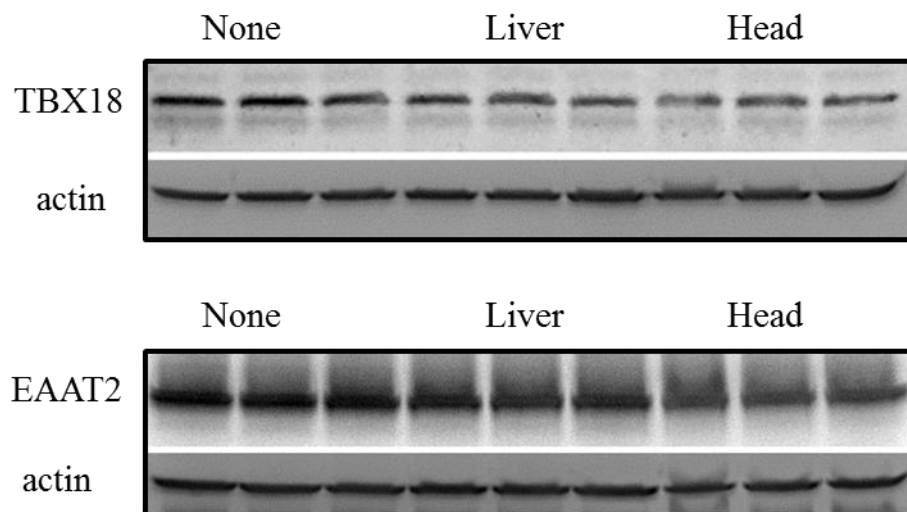


**Figure 2.1:** Experimental scheme.

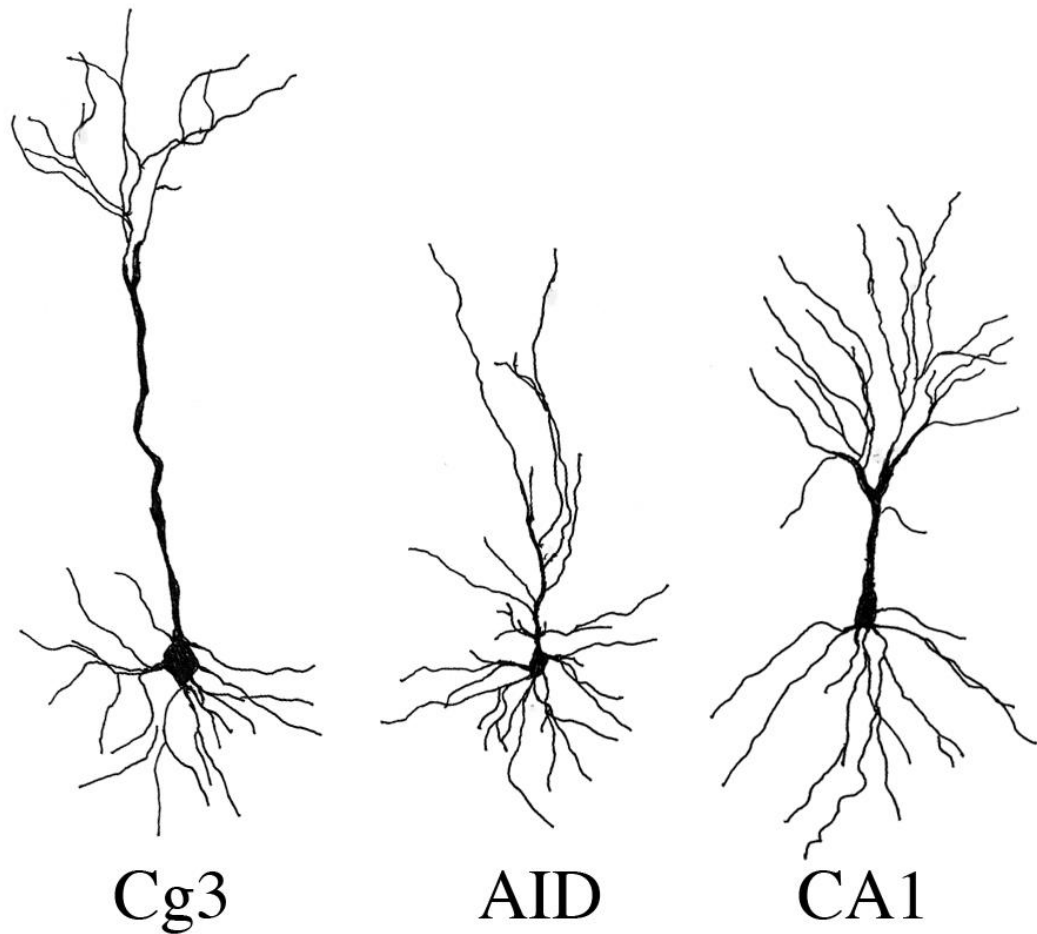




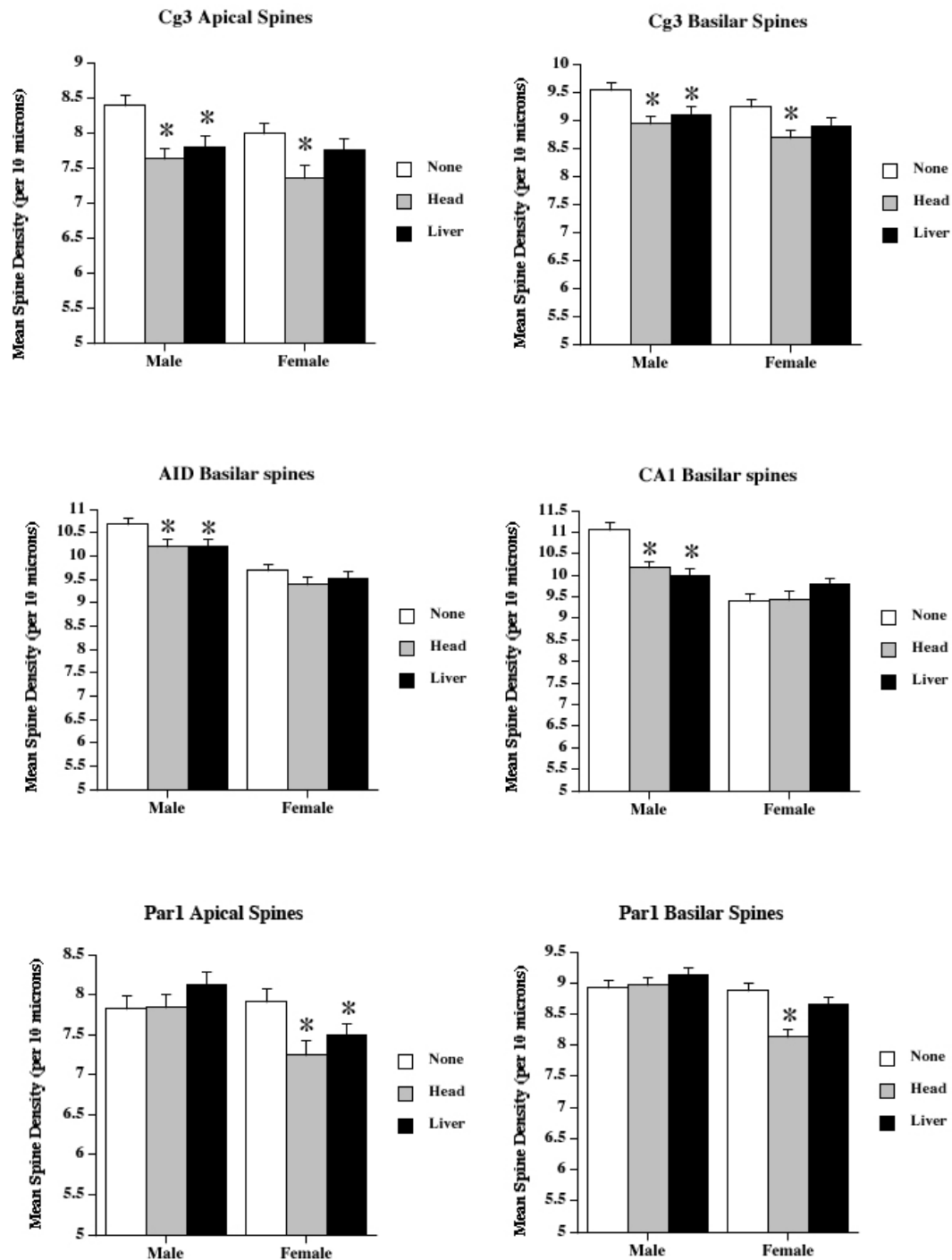
**Figure 2.2:** Levels of phosphorylated H2AX ( $\gamma$ H2AX ) in PFC tissues of head- and liver-irradiated female and male animals. Lysates from PFC tissues were immunoblotted using antibodies against  $\gamma$ H2AX.



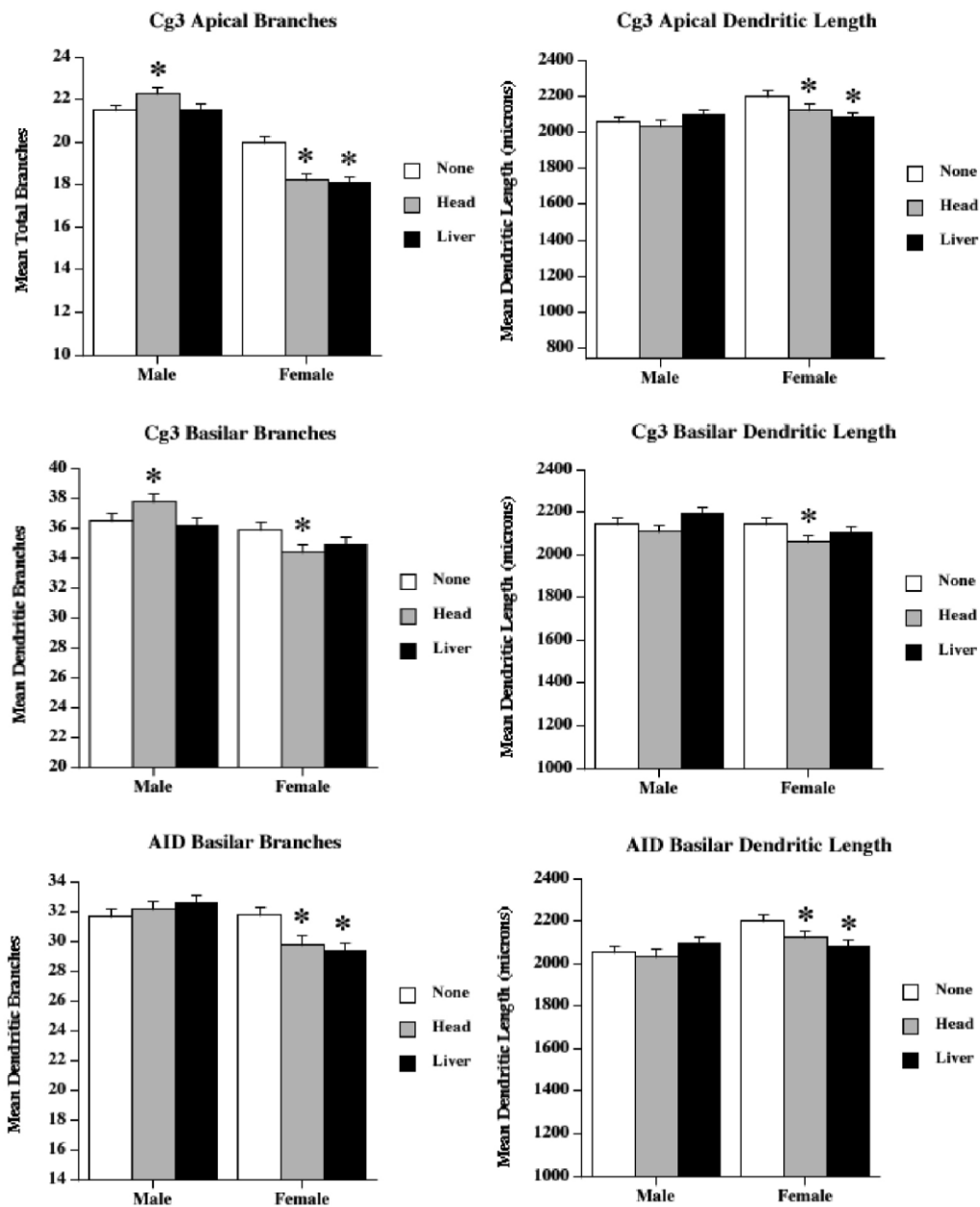
**Figure 2.3:** Levels of TBX18 and EAA2 in PFC tissues of head- and liver-irradiated female animals. Lysates from PFC tissues were immunoblotted using antibodies against TBX18 and EAA2.



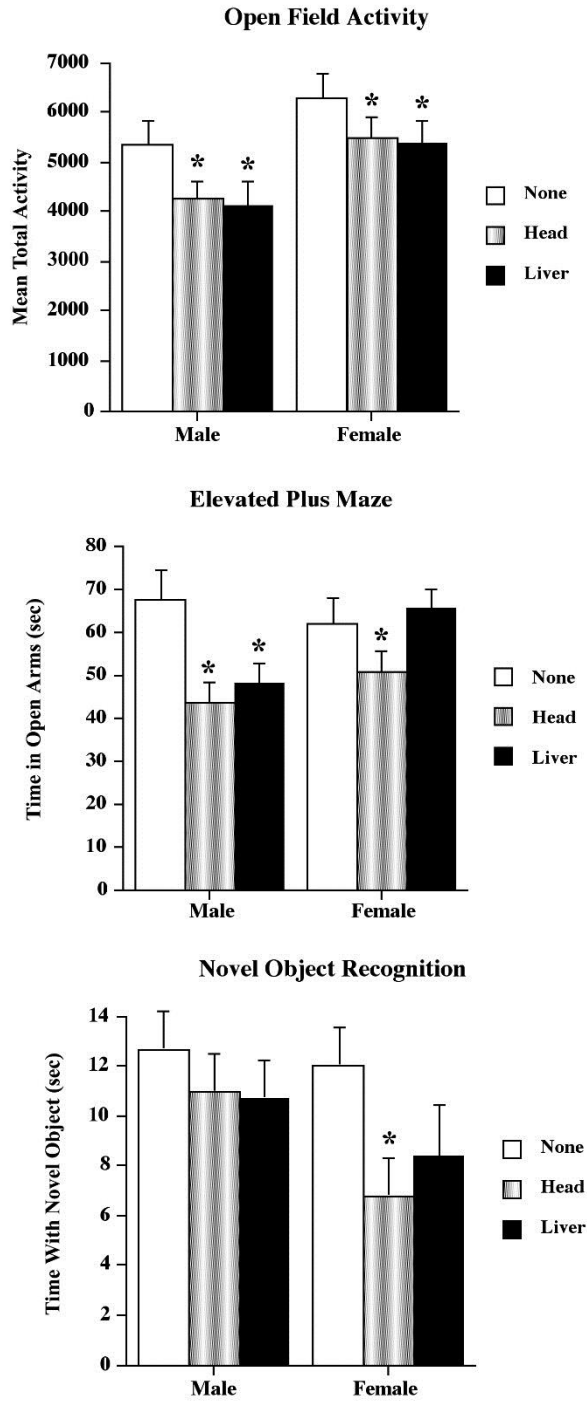
**Figure 2.4:** Representative samples of camera lucida drawings of pyramidal neurons used for spine density and dendritic analysis in medial prefrontal cortex (Cg3), orbital frontal cortex (AID), and hippocampus (CA1) of male and female rats exposed head or liver irradiation.



**Figure 2.5:** Low dose radiation exposure affects spine density. The density of dendritic spines (spines/10 $\mu$ M) in medial prefrontal cortex (Cg3), orbital frontal cortex (AID), parietal cortex (Par1), and hippocampus (CA1) of male and female rats upon head or liver irradiation. \*Significantly different from the control unexposed animals.



**Figure 2.6:** Low dose radiation exposure causes changes in dendritic branching and length. Apical and basilar branching and dendritic length in medial prefrontal cortex (Cg3) and orbital frontal cortex (AID) of male and female rats upon head or liver irradiation. \*Significantly different from the control unexposed animals  $p < .05$  or better.



**Figure 2.7:** Low dose head and liver irradiation exposure affect animal behavior. Graphical representation of the behavioral data for the Open Field Activity, Elevated Plus Maze and Novel Object Recognition tests. \*Significantly different from the control unexposed animals  $p < .05$  or better.

**Table 2.1:** List of genes differentially expressed in the PFC of female rats upon liver irradiation. Levels of corresponding proteins were determined by western immunoblotting and were significant ( $p < 0.05$ )

<b>Gene</b>	<b>Gene expression (Log2Fold)</b>	<b>Protein level (Fold)</b>
Collagen alpha-2(I)	-1.21	
T-box transcription factor TBX18	-1.30	-1.09
Fibronectin Anastellin	-0.82	
Collagen alpha-1(I) chain	-1.00	
Excitatory amino acid transporter 2	-0.61	-1.24
Inactive carboxypeptidase-like protein X2	-1.13	
Insulin receptor substrate 2	-0.75	
ENSRNOG00000024374 Uncharacterized protein	-0.55	
Adenylate cyclase type 1	-0.65	
Putative RNA-binding protein 3	0.52	
Biregional cell adhesion molecule-related/down-regulated by oncogenes (Cdon) binding protein	-0.57	
Collagen alpha-1(III) chain	-1.20	
Cadherin-1E-Cad/CTF1E-Cad/CTF2E-Cad/CTF3	-1.55	
Retinal dehydrogenase 2	-0.81	
Insulin-like growth factor-binding protein 2	-0.77	
Pecanex-like protein 1	-0.40	
ENSRNOG00000001249 Uncharacterized protein	-1.03	
SLIT-ROBO Rho GTPase-activating protein 3	-0.40	
Fibulin-1	-0.72	
Tubulin polymerization-promoting protein	-0.47	
Collectin-12	-0.91	
ENSRNOG00000019462 Uncharacterized protein	-0.39	

**CHAPTER 3: PROFOUND AND SEXUALLY DIMORPHIC EFFECTS OF  
CLINICALLY-RELEVANT LOW DOSE SCATTER IRRADIATION ON THE  
BRAIN AND BEHAVIOR**



## **ABSTRACT**

Irradiated cells can signal damage and distress to both close and distant naïve bystander neighbors. While studies have shown that bystander effects occur in the shielded brain of animals upon body irradiation, their mechanism remains unexplored. Bystander effects may be caused by some blood-derived factors; they may also be due to very small scatter doses received by the brain. In order to establish the roles of low doses of scatter irradiation in the brain bystander effects, we developed a new model for bystander scatter irradiation analysis whereby one rat was irradiated while a recipient rat was placed adjacent to it and received a true scatter-only dose.

Here, we provide the first experimental evidence that very low, clinically relevant doses of bystander scatter irradiation alter gene expression, induce changes in dendritic morphology, and lead to behavioral deficits in exposed animals.

We showed that exposure to scatter irradiation dose as low as 0.115 cGy caused changes in gene expression and reduced spine density, dendritic complexity, and dendritic length in the prefrontal cortex tissues of females, but not males. In the hippocampus, scatter irradiation altered neuroanatomical organization in males but not in females. Moreover, low dose bystander scatter irradiation caused behavioral deficits in the exposed animals.

## INTRODUCTION

Ionizing radiation is a well-established DNA damaging agent that can exert a wide array of effects in exposed cells (Morgan and Sowa, 2005). However, it is also clear that radiation effects occur beyond the exposed tissues and cells that signal damage and distress to both the close and distant unexposed naïve neighbors, thus giving rise to the “bystander effect” (Morgan and Sowa, 2005, Morgan, 2012, Morgan and Sowa, 2007, Mothersill and Seymour, 2004, Morgan, 2003b, Morgan, 2003a).

Nagasawa and Little in the 1990s discovered radiation-induced bystander effects. Since then, a wide array of studies have reported the existence of bystander effects in cell culture models (Hu et al., 2006, Maguire et al., 2005, Maguire et al., 2007, Zhou et al., 2000, Kovalchuk and Baulch, 2008), tissue explants (Belyakov et al., 2002, Belyakov et al., 2006, Kovalchuk and Baulch, 2008), and artificial human tissue models (Sedelnikova et al., 2007, Belyakov et al., 2005, Kovalchuk and Baulch, 2008).

Bystander effects were confirmed to manifest in the whole-organism context. It has been shown that radiation exposure results in a release of soluble DNA damaging “clastogenic” factors into the circulating blood. When applied to the recipient cell culture, these factors induce chromosome damage (Hollowell and Littlefield, 1968, Marozik et al., 2007, Pant and Kamada, 1977); also reviewed in (Kovalchuk and Baulch, 2008, Morgan and Sowa, 2007, Mothersill et al., 2004). Likewise, bystander effects have been shown to be important within the exposed organs when one part of an organ is irradiated (Khan et al., 1998). Bystander effects also occur in distant shielded bystander organs and tissues (Koturbash et al., 2006, Koturbash et al., 2007, Koturbash et al., 2008b, Koturbash et al., 2008c, Tamminga et al., 2008). Amongst those, bystander

effects were seen in somatic organs and in gonads upon cranial irradiation. Yet, very little was known about the existence of bystander effects in the brain upon exposure of somatic organs. Mancuso and colleagues (Mancuso et al., 2011, Mancuso et al., 2008) reported the presence of bystander effects in the cerebellums of mutant mouse strains following irradiation of the animals' bodies. In our most recent study, we showed that liver irradiation causes molecular and neuroanatomical changes in shielded bystander brains that affect animal behavior (Chapter 2).

In our initial study, liver irradiation-induced bystander changes manifested in the hippocampus and the prefrontal cortex (PFC) tissues of rats. The hippocampus, one of the two active sites of adult neurogenesis, is responsible for memory consolidation (Gage, 2000). The prefrontal cortex (PFC) coordinates a wide array of motor, cognitive, and social behaviors. It receives inputs from all other cortical regions as well as the ventral tegmental area and connects with almost all regions of the forebrain (Kolb et al., 2012). Nevertheless, the mechanisms of bystander effects remain obscure.

As such, bystander effects in the brain may be caused by some enigmatic clastogenic factors previously described by others; or they may also be due to very small scatter doses received by the brain during liver irradiation. Such scatter doses for the bystander brain of liver-irradiated rats constituted around 0.115 cGy and belonged to the under-investigated area of low doses; as of now very little is known about the effects of low, clinically, and occupationally relevant doses of radiation on the brain.

In order to establish whether or not low bystander scatter irradiation doses exert any influence on the brain, we developed a new model for bystander scatter irradiation analysis. In this model, one rat was irradiated, and a recipient bystander rat was placed

adjacent to it while receiving a true bystander scatter exposure. Here we report that bystander scatter low-dose irradiation causes changes in gene expression, alters dendritic morphology, and induces behavioral deficits in exposed female, but not male, rats.

## **RESULTS**

### **Lack of scatter irradiation-induced DNA damage in a rat model**

We studied true scatter/bystander effects in the brain by exposing the liver of an anaesthetized target experimental animal to radiation while protecting the rest of its body and the body of the adjacent bystander animal with a medical-grade lead shield (Fig. 3.1). Even low doses of ionizing radiation cause DNA damage. Bystander effects also manifest themselves as increased levels of DNA damage. Therefore, we first checked whether or not bystander scatter radiation caused DNA damage in the hippocampus and PFC tissues of male and female rats. To study the scatter irradiation-induced DNA damage, we assayed for the levels of phosphorylated histone H2AX ( $\gamma$ H2AX). To our surprise, we did not detect any H2AX phosphorylation in either the hippocampus or the PFC tissues of the scatter-exposed animals in two independent technical iterations of the experiment (data not shown). This may indicate that no DNA damage was induced by low-dose bystander scatter irradiation or that any damage was effectively repaired within two weeks of the exposure, which was when the analysis was conducted.

### **Scatter irradiation-induced gene expression analysis**

Low-dose radiation effects were previously shown to cause aberrant gene expression in various cells and tissues. Therefore, we proceeded to analyze the global gene expression profiles of the hippocampus and PFC tissues of control and bystander scatter-exposed male and female rats. An initial transcriptome analysis revealed that 741

genes were statistically significantly up-regulated and 1,135 genes were significantly down-regulated in the PFC tissues of the scatter-exposed female rats compared to the controls (adjusted P-value < 0.05 and absolute log 2 Fold Change > 0.58, which corresponded to a 1.5 fold difference in expression between the groups) (Fig. 3.2). Differentially expressed genes were distributed all across the genome with no obvious hot-spots at any of the chromosomal locations. Upon application of even more restricted criteria (adjusted P-value < 0.05 and absolute log 2 Fold Change > 1), 1,045 genes were found to be significantly differentially expressed in the PFC tissues of the scatter-exposed females compared to the controls, with 101 genes significantly up-regulated and 944 significantly down-regulated in the scatter-exposed animals when compared with the controls.

In contrast to the massive transcriptome response observed in the females, only 11 genes were significantly differentially expressed in the PFC of the bystander scatter-exposed males compared to the controls ( $P < 0.05$ ) (Fig. 3.2). All of them were up-regulated, and two genes were commonly up-regulated in the PCF tissues of both the male and female animals. These were the glutathione S-transferase A3 and the beta globin minor genes. In relation to the hippocampus, only two genes were up-regulated in the males, and no significant changes were noted in the hippocampal tissues of the bystander scatter-exposed females (Fig. 3.2).

To gain further insight into the functional significance of the observed gene expression changes, we conducted an in-depth KEGG pathway analysis. This analysis revealed a significant up-regulation of the pathways involved in oxidative phosphorylation, DNA replication, proteasome, ribosome, RNA transport, nucleotide

excision repair, and other pathways in the prefrontal cortex of the bystander scatter-exposed female animals compared to the controls.

Compared to the controls, the down-regulated pathways in the PFC of the bystander scatter-exposed animals included those involved in calcium signaling, neuroactive ligand–receptor interaction, phosphatidylinositol signaling system, GnRH signaling pathway, Gap junction, Fc epsilon RI signaling, Jak–STAT signaling, and Fc gamma R–mediated phagocytosis pathways, to name a few. Compared to the controls, axon guidance, MAPK signaling, and neurotrophin signaling pathways were also down-regulated in the PFC of the scatter-exposed females (Fig. 3.3).

Expression of several differentially regulated genes belonging to the MAPK and neurotrophin signaling pathways was confirmed on the protein level. In concordance with the gene expression results, the levels of BDNF were up-regulated whereas the levels of JNK and BCL2 were down-regulated in the PFC tissues of the scatter-exposed female rats. The trend toward down-regulation observed in the ELK1 protein was not statistically significant (Fig. 3.4).

### **Scatter radiation-induced neuroanatomical changes**

Having observed the profound transcriptome changes in the PFC tissues of the scatter-exposed animals, we then investigated whether or not aberrant gene expression was associated with neuroanatomical changes in the PFC (both the medial PFC (Cg3) and orbital PFC (AID) regions), parietal cortex, and hippocampus. Overall, we found that scatter irradiation caused noticeable and sexually dimorphic changes in dendritic organization, affecting the length, branching, and density of the spines in the PFC and hippocampus. The females were more affected than the males. We analyzed each region

separately. The spine density data are presented in Figure 3.5 the branching data - in Figure 3.6, and dendritic length – in Figure 3.7. Only the basilar fields were drawn for AID and CA1, whereas both the apical and basilar fields were drawn for Cg3 and Par1 regions of the prefrontal cortex.

### ***Medial Prefrontal Cortex (Cg3)***

The greatest effects of scatter irradiation were seen in Cg3 in both sexes. Two-way analysis of variance (ANOVA) was performed for all analyses with “Radiation” and “Sex” as factors.

*Apical field spine density.* The spine density was reduced in both scatter-irradiated males and females. The sex difference reflected greater spine density in males than in females. ANOVA revealed significant effects for radiation ( $F[1,57] = 10.1, p = .002$ ) and sex ( $F[1,57] = 6.12, p = .017$ ) but not for their interaction ( $F[1,57] = .032, p = .859$ ).

*Apical field branching.* Apical branching was reduced in scatter-irradiated females; however, no significant effect was seen in scatter-irradiated males. The sex difference reflected greater branching in males than in females. ANOVA revealed significant effects for radiation ( $F[1,57] = 16.5, p = .000$ ) and sex ( $F[1,57] = 67.9, p = .000$ ) as well as for their interaction ( $F[1,57] = 7.57, p = .008$ ).

*Apical field dendritic length.* Apical dendritic length was reduced in scatter-irradiated females; however, no significant effect was seen in scatter-irradiated males. The sex difference reflected greater branching in scatter-irradiated males than in scatter-irradiated females. ANOVA revealed significant effects for radiation ( $F[1,57] = 23.6, p < .0001$ ), sex ( $F[21,57] = 40.9, p < .0001$ ), and the interaction ( $F[1,57] = 9.46, p = .003$ ).

*Basilar field spine density.* The spine density was reduced in both scatter-irradiated males and females. The sex difference reflected greater spine density in males than in females. ANOVA revealed significant effects for radiation ( $F[1,57] = 11.1, p = .002$ ) and sex ( $F[1,57] = 5.38, p = .024$ ) but not for their interaction ( $F[1,57] = .002, p = .968$ ).

*Basilar field branching.* Basilar branching was reduced in scatter-irradiated females; however, no significant effect was seen in scatter-irradiated males. The sex difference reflected greater branching in scatter-irradiated males than in scatter-irradiated females. ANOVA revealed significant effects for radiation ( $F[1,57] = 4.69, p = .035$ ) and for sex ( $F[1,57] = 5.47, p = .023$ ) but not for their interaction ( $F[1,57] = .767, p = .385$ ).

*Basilar field dendritic length.* The effects on basilar length were similar in males and females: scatter irradiation reduced the length. There were no sex differences observed. ANOVA revealed significant effects for radiation ( $F[1,57] = 25.7, p = .000$ ) but not for sex ( $F[1,57] = .559, p = .458$ ) nor their interaction ( $F[1,57] = .703, p = .405$ ).

### ***Parietal Cortex (Par1)***

*Apical field spine density.* Scatter irradiated females had lower spine density compared to control. No sex difference was observed. ANOVA showed significant effects for radiation ( $F[1,57] = 4.00, p = .005$ ) but not for sex ( $F[1,57] = .008, p = .930$ ) nor their interaction ( $F[1,57] = .720, p = .400$ ).

*Apical field branching.* No effect of irradiation was observed in either sex to their respective controls. However, there was a sex difference in that more branching was observed in irradiated males than in irradiated females but not between control males and females. ANOVA showed no significant effects for radiation ( $F[1,57] = .070, p = .792$ ).



but did show a significant effect for sex ( $F[1,57] = 8.31, p = .006$ ); however, no significant effect was observed for their interaction ( $F[1,57] = 1.78, p = .188$ ).

*Apical field dendritic length.* Irradiation had no significant effect on the apical dendritic length in males but the apical dendritic length was significantly decreased in females. A sex difference was observed, with irradiated females showing shorter apical dendrites than irradiated males. ANOVA showed no significant effects for radiation ( $F[1,57] = 2.04, p = .159$ ), sex ( $F[1,57] = 2.08, p = .155$ ), nor their interaction ( $F[1,57] = 2.28, p = .137$ ).

*Basilar field spine density.* Irradiation did not affect basilar spine density. Thus, ANOVA showed no significant effects for radiation ( $F[1,57] = 1.75, p = .192$ ), sex ( $F[1,57] = 1.90, p = .174$ ) nor their interaction ( $F[1,57] = 1.17, p = .284$ ).

*Basilar field branching.* Irradiation did not affect basilar branching. ANOVA showed no significant effects of radiation ( $F[1,57] = 1.42, p = .239$ ), sex ( $F[1,57] = .003, p = .958$ ), their interaction ( $F[1,57] = .560, p = .458$ ).

*Basilar field dendritic length.* Irradiation did not affect basilar dendritic length. ANOVA showed no significant effects for radiation ( $F[1,57] = .035, p = .852$ ) sex ( $F[1,57] = .281, p = .598$ ), nor their interaction ( $F[1,57] = 1.77, p = .189$ ).

#### ***Orbital Prefrontal Cortex (AID)***

*Basilar field spine density.* The spine density was reduced in both scatter irradiated females and males. A sex difference was observed, with irradiated females showing lower spine density than males. ANOVA showed a significant effect of radiation ( $F[1,57] = 17.1, p < .0001$ ), and sex ( $F[1,57] = 58.8, p = .000$ ) but not their interaction ( $F[1,57] = 1.47, p = .230$ ).

*Basilar field branching.* No significant effect of irradiation was observed on branching. ANOVA showed no significant effects for radiation ( $F[1,57] = 1.88$ ,  $p = .176$ ); however, it showed significant effects for sex ( $F[1,57] = 6.55$ ,  $p = .013$ ) as well as for their interaction ( $F[1,57] = 7.63$ ,  $p = .008$ ). A sex difference resulted from a significant decrease in branching in females (both irradiated and controls) compared to males (who had more complex cells than females).

*Basilar field dendritic length.* The dendritic length was increased in irradiated males but not females. ANOVA showed significant effects for radiation ( $F[1,57] = 4.49$ ,  $p = .039$ ) but not for sex ( $F[1,57] = 2.18$ ,  $p = 0.145$ ); however, it showed a significant effect for their interaction ( $F[1,57] = 17.5$ ,  $p < .0001$ ). The interaction was complex reflecting shorter dendrites of irradiated females than irradiated males but control females had longer dendrites than control males.

### ***Hippocampus (CA1)***

The effects of irradiation in the hippocampus were surprisingly small relative to those found in the prefrontal regions, and they were only observed in males.

*Basilar field spine density.* Irradiation significantly reduced the spine density in males but not in females. A sex difference was observed, with females showing lower spine density than males. ANOVA showed significant effects for radiation ( $F[1,57] = 12.9$ ,  $p = .001$ ), sex ( $F[1,57] = 67.0$ ,  $p = .001$ ), as well as for the interaction ( $F[1,57] = 7.87$ ,  $p = .007$ ).

*Basilar field branching.* No significant effects of irradiation were observed on branching. ANOVA showed no significant effects for radiation ( $F[1,57] = .266$ ,  $p = .608$ ) but it did show a significant effect for sex ( $F[1,57] = 8.63$ ,  $p = .005$ ), but not for the

interaction ( $F[1,57] = .047$ ,  $p = .829$ ). A sex difference resulted from a significant decrease in branching in females (both irradiated and controls) compared to males (who had more complex cells than females).

*Basilar field dendritic length.* Irradiation significantly reduced the dendritic length in males but not in females. There was a sex difference whereby irradiated females had longer dendrites than irradiated males. ANOVA showed no significant effect for radiation ( $F[1,57] = 2.04$ ,  $p = .159$ ), but it did show a significant effect for sex ( $F[1,57] = 8.67$ ,  $p = 0.005$ ) and the interaction ( $F[1,57] = 5.36$ ,  $p = .024$ ).

### **Behavioral changes induced by scatter irradiation**

Overall, scatter irradiation significantly affected behaviour in the Elevated-Plus Maze (EPM) test but not in the activity nor the novel object recognition tests (Fig. 3.8).

*Activity.* There was no effect of irradiation on activity. There were no sex differences. Two-way ANOVA showed no significant effect for radiation ( $F[1,15] = .123$ ,  $p = .732$ ), sex ( $F[1,15] = .114$ ,  $p = .741$ ) nor their interaction ( $F[1,15] = 2.01$ ,  $p = .173$ ).

*Elevated-Plus Maze.* Scatter irradiation significantly increased the time spent by both male and female animals in the closed arms of the maze. Two-way ANOVA showed a significant effect for radiation ( $F[1,15] = 6.79$ ,  $p = .023$ ), but not for sex ( $F[1,15] = 4.60$ ,  $p = .053$ ), nor the interaction ( $F[1,15] = .026$ ,  $p = .874$ ).

*Novel Object Recognition.* There were no significant effects of scatter radiation on novel object recognition. There were no sex differences. Two-way ANOVA showed no significant effects for radiation ( $F[1,15] = 1.37$ ,  $p = .265$ ), sex ( $F[1,15] = .004$ ,  $p = .950$ ), nor the interaction ( $F[1,15] = .179$ ,  $p = .680$ ).

## DISCUSSION

Here, we developed a new model to study in vivo effects caused by low dose scatter irradiation. Using this model we were able to show that very low, clinically relevant, doses of bystander scatter irradiation altered gene expression in brain, induced changes in dendritic morphology, and led to behavioral changes in exposed animals.

The key findings of our study are that: (i) bystander scatter irradiation consisting of a clinically-relevant dose as low as 0.115 cGy caused massive changes in gene expression in the PFC tissues of females, but much smaller changes in males; (ii) overall bystander scatter irradiation reduced spine density, dendritic complexity, and dendritic length, the effects being brain-region-specific and more pronounced in females; and (iii) bystander scatter irradiation causes behavioral changes in exposed animals.

These constitute seminal findings since, for quite some time, the brain was considered a radiation-resistant organ and only very high doses were thought to exert harmful effects on it (USNRC, 2003). Therefore, previous key efforts have focused on understating the consequences of cranial radiotherapy, and numerous studies have been conducted to analyze the effects of high, therapeutically-relevant doses of radiation on the brain. High-dose cranial radiation therapy was proven to be neurotoxic and to cause severe cognitive impairments in patients (Raber, 2010). High-dose cranial radiation therapy was shown to cause oxidative stress, alterations in neurogenesis, and changes in synaptic and dendritic markers (Raber, 2010).

Studies have emerged showing that the brain is much more sensitive to irradiation than previously considered and that high, medium, and low doses of radiation can cause molecular and cellular changes in the brain and induce cognitive decline (Sweet et al.,

2014, Acharya et al., 2015). We were able to show that very low, computed tomography-like, doses can induce a wide array of molecular and cellular changes in the brain, as well as lead to cognitive deficits.

Among the various brain regions, the hippocampus, which is one of the key sites of adult neurogenesis, has been suggested as the most sensitive to radiation (Mizumatsu et al., 2003b). Ionizing radiation was shown to have a profound effect on cells in the dentate gyrus as it causes apoptosis and persistent reduction in proliferating SGZ precursor cells (Tada et al., 2000). Monje and colleagues (Monje et al., 2002) showed that exposure of the rat brain to 10Gy of X-rays almost completely abolished the production of new neurons, causing surviving precursor cells to adopt a glial phenotype. Exposure of young adult mice to 10Gy of Cs-137 irradiation led to significant and persistent decline in spine density in the dentate gyrus at both one week and one month after exposure. Irradiation also altered spine morphology and resulted in decreases in the proportion of mushroom spines (Chakraborti et al., 2012).

Exposure of mouse hippocampal neuronal HT22 cells to low and moderate doses (0.5 Gy, 1.0 Gy and 4.0 Gy) of gamma-irradiation led to profound proteome alterations (Kempf et al., 2014a). Furthermore, low (<2Gy) dose radiation was shown to affect the hippocampal microenvironment and modulate inflammatory responses (Acharya et al., 2015). A new study by Kempf and colleagues (2015) revealed that exposure to 0.1 or 0.5 Gy of gamma radiation affected the signaling pathways related to the mitochondrial and synaptic functions in the hippocampus and cortex tissues of exposed mice (Kempf et al., 2015).

While the majority of studies of brain radiation effects have focused on neurogenesis and the hippocampus, the effects of radiation on other brain regions such as the PFC are under-investigated (Silasi et al., 2004, Kempf et al., 2014a). For example, the PFC receives inputs from all other cortical regions and plans and guides various motor, cognitive, affective, and social behaviors. It is sensitive to a wide array of stimuli, including hormones, drugs, toxic chemicals, stress, and social experiences (Kolb et al., 2012). In our previous study, we showed that direct and bystander irradiation led to altered dendritic morphology and aberrant gene expression in the PFC tissues of exposed female rats (Chapter 2). Here, for the first time, we show that very low, clinically, and occupationally-relevant doses of radiation affect gene expression in the PFC in a sex-specific manner.

Exposure to just 1.15 mGy of scatter X-rays down-regulated several key pathways involved in neuronal organization, differentiation, and plasticity, including the axon guidance pathway. Within this pathway, we noted decreased levels of several genes, such as ephrin B2, chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1) CXCL12, semaforin genes, and most importantly, Cofilin-2 and Rac1 genes. Altered expression of Cofilin-2 and Rac1 upon irradiation was very recently reported by Kempf and colleagues (2014) (Kempf et al., 2014a), who exposed mouse hippocampal neuronal HT22 cells or adult mice to 0.5 Gy, 1.0 Gy, and 4.0 Gy and observed changes in the Rac1-Cofilin pathway (which regulates synaptic actin filament formation, the maintenance of a proper spine, synapse morphology, and is crucial for learning and memory (Kempf et al., 2014a)). Even though the doses used in our study were much lower than those used by Kempf and colleagues, the roles of Rac-Cofilin pathways in

low-dose radiation responses in the PFC need to be analyzed further, especially given the fact that radiation-induced changes were observed in two different rodent species – mice and rats.

Numerous affected genes have been previously shown to play a key role in brain function. Amongst the down-regulated genes, there was neuroligin 3, a neuronal cell surface protein that is thought to be involved in cell-cell interactions and the formation or maintenance of synaptic junctions. Scatter irradiation also decreased the levels of glutamate dehydrogenase 1, a key enzyme that partakes in the regulation of learning and memory by increasing the turnover of the neurotransmitter glutamate. The glutamate receptor-interacting protein 2 and the gamma-aminobutyric acid (GABA) A receptor, beta 2 gene were also down-regulated.

The transcriptome response to 0.115 cGy of the bystander scatter dose was much more profound than the response seen in the bystander liver irradiation model in which the head received 0.125cGy of the scatter dose (Chapter 2). The difference may be due to the presence of bystander signal in the irradiated blood of the animals. Moreover, some of the observed changes may be protective in nature, such as an increase in the levels of the brain-derived neurotrophic factor (BDNF), one of the key factors involved in neuronal survival (Gorski et al., 2003, Vigers et al., 2012).

We observed an alteration in the dendritic morphologies of the medial PFC and orbital cortex, the parietal cortex, and the hippocampus. In the medial PFC (Cg3), irradiation led to a reduced apical and basilar field spine density in males and females compared to non-irradiated animals. Compared to controls, apical field branching, apical dendritic lengths and basilar fields branching were reduced in scatter-irradiated females

but not irradiated males. These changes reflect an overall decrease in synaptic space, and presumably synapse number, related to the low dose scatter irradiation.

Scatter-induced changes were less pronounced in the parietal cortex than the medial PFC. In the parietal cortex scatter irradiation only reduced the apical field dendritic length in the exposed females but not the males. In the orbital cortex, scatter exposure caused a reduction of basilar field spine density and dendritic length in both males and females.

Taken together, the data show that scatter-irradiation-induced neuroanatomical changes were more pronounced in the prefrontal cortex of females than males. In addition, the medial PFC and orbital cortex were more susceptible to low-dose radiation effects than the parietal cortex. Our study shows the differential sensitivity of prefrontal cortical regions to irradiation and the profound sexual dimorphism of low-dose irradiation effects in the PFC.

We noted an important congruence between alterations in global gene expression levels and neuroanatomical changes seen in the PFC of scatter-exposed females. As well, the observed down-regulation of axon guidance, calcium signaling, neuroactive ligand–receptor interaction, phosphatidylinositol, MAPK and neurotrophin signaling pathways in female but not male animals might explain the reduction in dendritic length, dendritic branching and spine density detected in female but not male animals.

In contrast to the PFC, bystander scatter radiation in the hippocampus significantly reduced spine density and basilar field dendritic length in males but not females. Therefore, the male hippocampus might be more sensitive to irradiation than the female



hippocampus. Future studies should examine behavioral tasks such as learning of spatial navigation tasks as these are especially sensitive to hippocampal dysfunction.

The changes observed in the animals' behavior were intriguing. We noted that scatter irradiation showed a trend towards decreased levels of open-field activity in the experimental female rats. In males, the trend, although not statistically significant, was to increased open-field activity (Fig. 3.8). In the elevated plus maze, irradiated males and females both spent more time in the closed arms of the maze, indicating increased anxiety levels in the irradiated animals of both sexes compared to controls. Neither female nor male scatter-irradiated rats exhibited impaired performance in a novel object recognition test (Fig. 3.8). They spent the same amount of time with the novel object compared to control rats (Fig. 3.8).

In sum, this is the first study showing that low dose scatter irradiation influences the brain and behavior in a sex-specific way. Whereas our previous studies suggested that low-dose irradiation could impact behavior, no prior data existed on the behavioral consequences of small scatter dose-induced effects on the brain. The mechanisms of sex-specificity of the observed molecular, cellular and behavioural changes need to be further elucidated and may be associated with different gonadal hormone signalling in males and females.

Almost all medical radiation procedures result in radiation scattering, and scatter doses to the brain need to be analyzed. Moreover, cognitive outcomes of radiation therapy to somatic organs and tissues other than the brain have not been extensively studied in humans. Nevertheless, a substantial number of reports indicate bothersome cognitive impairments following radiation treatments. Are these due to the scatter? This

clinical question needs to be addressed in future studies. It is of paramount importance to further analyze sex differences in order to ensure that everyone is well-protected against the deleterious effects of scatter irradiation during radiation diagnostic and treatment procedures.

This is the first study of its kind providing behavioral evidence that very low doses of scatter irradiation can exert profound deleterious effects on the mammalian brain and impact the behavior of animals, and that the effects are more pronounced in females. These data may serve as a roadmap for future translational approaches aimed at understanding whether females are indeed more susceptible to the effects of scatter irradiation.

## **MATERIALS AND METHODS**

### **Animal Model and Tissue Sampling**

Twenty male and twenty female three-month-old Long-Evans rats (Charles River) were used in this study. Half of the animals were used for molecular and neuroanatomical profiling and the other half for behavioural testing.

The animals were housed in a pathogen-free controlled facility with a 12-hour light/dark cycle and were given food and water ad libitum. The animals were randomly allocated to the following groups: (i) scatter-exposed, and (ii) sham-treated control.

For irradiation, the animals were anaesthetised with an intra-peritoneal injection of ketamine/xylazine (50/5 mg/kg b.w.). The anaesthesia was well-tolerated and no side effects were observed. Irradiated animals (10 rats) received X-ray irradiation delivered to the surface of their bodies over the liver; a medical-grade lead shield protected the rest of the body and the entire body of the recipient bystander scatter rat that was placed

adjacent to the liver-irradiated rat. The data from the liver-exposed animals were compared to cranial exposure in another group of animals elsewhere (see Chapter 2). Here we report only on the scatter – exposed and control animals.

A lead apron (0.05 cm Pb-equivalent) was used for shielding. A 1.7 cm by 3.5 cm oval was cut into the shielding in order to define a primary field over the liver of the exposed rat and was placed on both the irradiated and the scatter bystander rats (Fig. 3.1). During exposure, the brains of recipient bystander animals were shielded, but they still received a dose of 0.115 cGy due to radiation scattering (Kirkby et al., 2013).

For molecular analysis, the animals were humanely sacrificed 14 days after irradiation. The handling and care of the animals were conducted in accordance with recommendations from the Canadian Council for Animal Care and Use. The University of Lethbridge Animal Welfare Committee approved all the procedures. After sacrifice, brain areas (hippocampus and prefrontal cortex) were sampled and snap-frozen for RNA, protein and DNA extraction.

## **Molecular analysis**

### ***Gene Expression Analysis***

The hippocampus and prefrontal cortex (PFC) tissues of three animals per group were used for the analysis of gene expression profiles. In brief, RNA was extracted from the hippocampus and the PFC tissues using TRIzol® Reagent (Invitrogen, Carlsbad, CA), further purified using an RNAeasy kit (Qiagen), and quantified using a Nanodrop2000c (ThermoScientific). Afterwards, RNA integrity and concentration were established using a 2100 BioAnalyzer (Agilent). Sequencing libraries were prepared using Illumina's TruSeq RNA library preparation kits, and global gene expression

profiles were determined using the Illumina deep-sequencing platform at the University of Lethbridge CFI-SAGES Facility. Statistical comparisons between the control and exposed groups within each tissue type were performed using the DESeq Bioconductor package (version 1.8.3) and the baySeq Bioconductor package (version 1.10.0). Clustering of the samples was assessed with multidimensional scaling (MDS) plots built using the plotMDS function from the edgeR Bioconductor package. MA plots showing the relationship between the average level of expression and the log2 fold change were created for each of the comparisons. Features with a false discovery rate (FDR) < 0.1 (10% false positive rate) were considered differentially expressed between conditions.

### ***Western Immunoblotting***

Western immunoblotting was conducted as described previously (Silasi et al., 2004). In brief, approximately 50 mg of hippocampus or PFC tissues were sonicated in ice-cold 1% SDS and immediately boiled. Protein concentrations were ascertained using the Bradford assay (BioRad, Hercules, CA). Equal amounts of protein (10-30 µg) were separated by SDS-PAGE into slab gels of 10-15% polyacrylamide and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Baie d'Urfé, Quebec). The membranes were incubated with primary antibodies against BDNF, JNK, ELK (1:1000, Cell Signaling), BCL2 (1:200, Abcam) and actin (1:2000, Abcam) overnight at 4° C. Primary antibody binding was detected using horseradish peroxidase-conjugated secondary antibodies and the Enhanced Chemiluminescence Plus System (Amersham Biosciences, Baie d'Urfé, Quebec). Chemiluminescence was detected using a FluorChem HD2 camera with FluorChem software (Cell Biosciences). The membranes were stained with Coomassie blue (BioRad, Hercules, CA) to confirm equal protein loading. Signals

were quantified using NIH Image J64 software and normalised relative to actin or Coomassie staining.

## **Neuroanatomy**

### ***Perfusion and Staining***

Two weeks post-exposure, the experimental animals were given an overdose of sodium pentobarbital solution intraperitoneally and perfused with 0.9% saline solution intracardially. Their brains were removed from their skulls then weighed and preserved in a Golgi-Cox solution for 14 days, followed by transfer to a 30% sucrose solution for at least three days. Next, the brains were sliced at a thickness of 200  $\mu\text{m}$  on a vibratome and fixed on gelatinised slides. The slides mounted with brain sections were processed for Golgi-Cox staining following the protocol as previously described (Gibb and Kolb, 1998).

### ***Anatomy***

As described previously, pyramidal cells were drawn from layer 3 of Cg3 and AID (medial and orbital prefrontal regions, respectively) and from the CA1 region of the hippocampus, according to Zilles' cortical atlas (Zilles, 1985). Individual neurons were traced using a camera lucida mounted on a microscope. For dendritic branching and length, a total of 10 cells (5/hemisphere) were traced at 250X for each brain region. The averages of the cells from each hemisphere comprised the data points used for statistical analysis. Spine density was measured at 1000x and calculated by counting the number of spines on a length of distal dendrite that was at least 50 microns in length. The exact length of the dendrite segment was calculated, and the density was expressed per 10  $\mu\text{m}$ .

Five segments were drawn per hemisphere from different neurons, and a mean value was calculated to use as the unit of measurement (Muhammad and Kolb, 2011).

As an estimate of dendritic complexity, we studied the branch order that was used to measure the number of dendritic bifurcations. Dendritic length was calculated using a Sholl analysis, which includes an estimation of dendritic length that counts the number of dendritic branches that intersect in concentric circles spaced 25  $\mu\text{m}$  apart. Length is estimated by multiplying the number of dendritic intersections by 25.

### **Behavioural analysis**

The sequence of behavioural testing was as follows: the novel object recognition test, the activity box test, followed by the elevated plus maze (EPM) test.

#### ***Novel object recognition (NOR)***

Testing was performed as described (Richards et al., 2012) and occurred two weeks after exposure. NOR for temporal order memory was run in three separate trials starting 1 hour apart on filming day. The rats were placed in a white plastic container 48 cm  $\times$  48 cm  $\times$  52 cm for 5 min three days prior to filming to habituate them to testing conditions. On filming day, the initial trial consisted of two identical objects in the base of the tub and leaving the animals to explore them for 4 min. The second trial began 1 hour later; different identical objects were placed in the tub with the animal for 4 min. The third trial involved the rat in the plastic container with one object from the first trial and one object from the second trial for 4 min. The time spent with each of the objects was calculated in the third trial. An animal was considered to be in contact with an object if its nose was within 2 cm of the object.

### ***Activity box***

Testing was performed as previously described (Richards et al., 2012). Activity was measured two weeks after irradiation. Rats were placed in an Accuscan® activity monitoring system consisting of electronically fitted Plexiglas® boxes measuring 41 cm × 41 cm × 30.5 cm that recorded the movements of each individual rat. Rats were placed into a box for 10 minutes, and their exploratory behaviour was recorded in five 2-minute intervals. Data were recorded using VersaMax™ computer software. The key measure analysed was overall activity/distance travelled.

### ***Elevated plus maze (EPM)***

Testing was performed as described (Raza et al., 2015) and occurred two weeks after exposure. In brief, the EPM was constructed from black Plexiglas®, with a base measuring 94 cm high. The two open arms measured 10 cm wide and 40 cm long. The two closed arms measured 10 cm wide and 40 cm long, and had walls measuring 40 cm high. The maze was housed in an empty room, and lights were on during filming. The camera for filming was placed at the end of an open arm slightly above the maze. Rats were placed with their front paws in the centre of the square maze facing a closed arm. Each rat was filmed for 5 min and was scored for the time spent in the closed arms and the time spent in the centre of the maze. An animal was considered to be in an arm when the first half of its body was inside the arm.

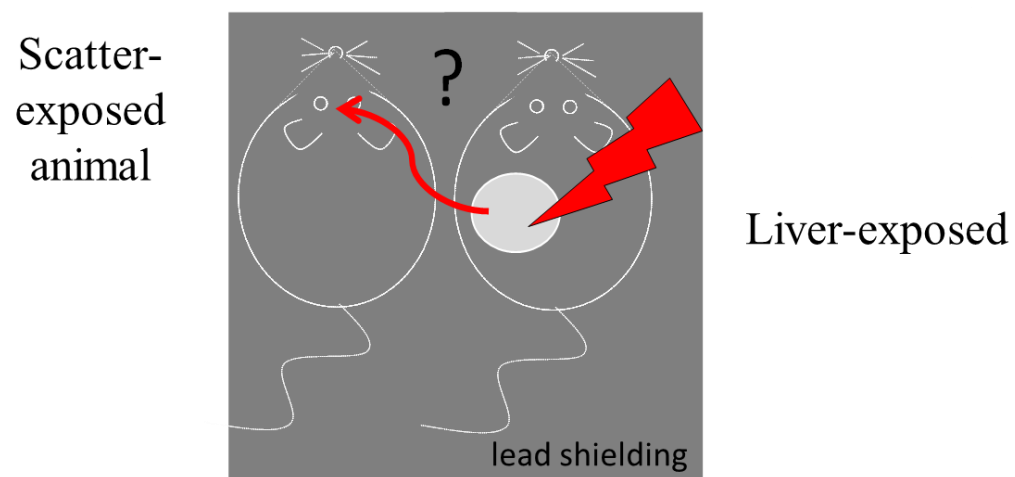
### **Statistical analysis**

All statistical analyses were carried out using SPSS 16.0 (Richards et al., 2012). Each rat was used as a unit of analysis. Two-way ANOVAs with treatment (control,

scatter exposure) and sex (M/F) as factors were run to compare the behavioural outcomes in both control and exposed rats.



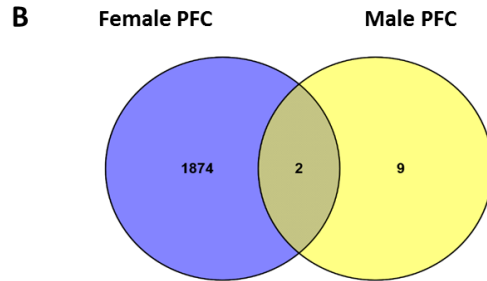
## FIGURES



**Figure 3.1:** Induction of bystander scatter effects in vivo

**A**

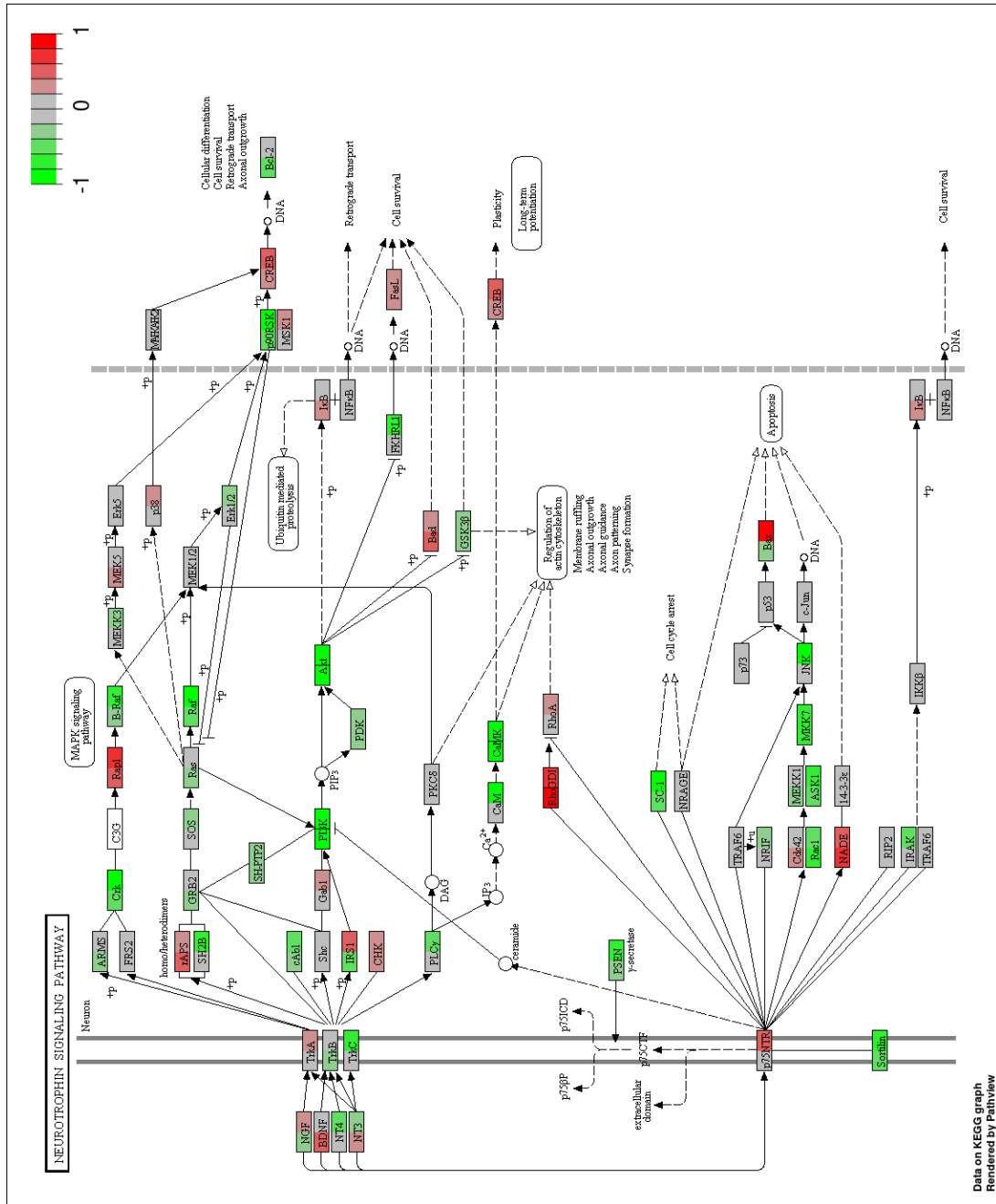
Tissue	Females			Males		
	Total genes	Up-regulated	Down-regulated	Total genes	Up-regulated	Down-regulated
Prefrontal cortex	1876	741	1135	11	11	0
Hippocampus	0	0	0	2	2	0
Cerebellum	0	0	0	0	0	0



**Figure 3.2:** Scatter radiation affects gene expression in the brain.

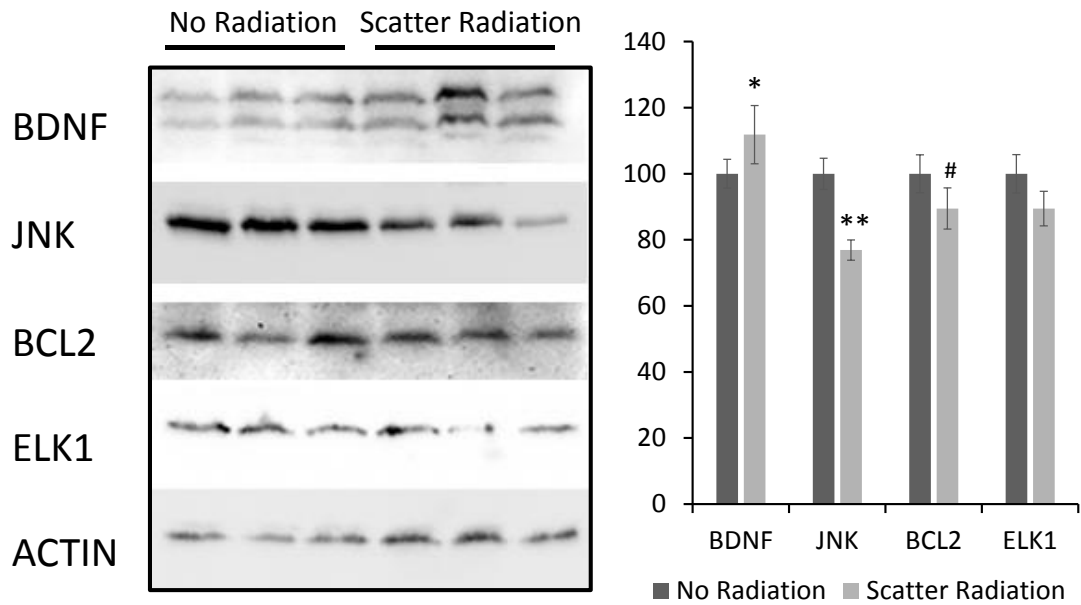
- A.** Global gene expression profiling in the prefrontal cortex, hippocampus and cerebellum tissues of scatter radiation-exposed male and female animals.
- B.** Venn diagrams depicting differences and similarities between scatter-induced gene expression changes in the prefrontal cortex (PFC) tissues of male and female rats.



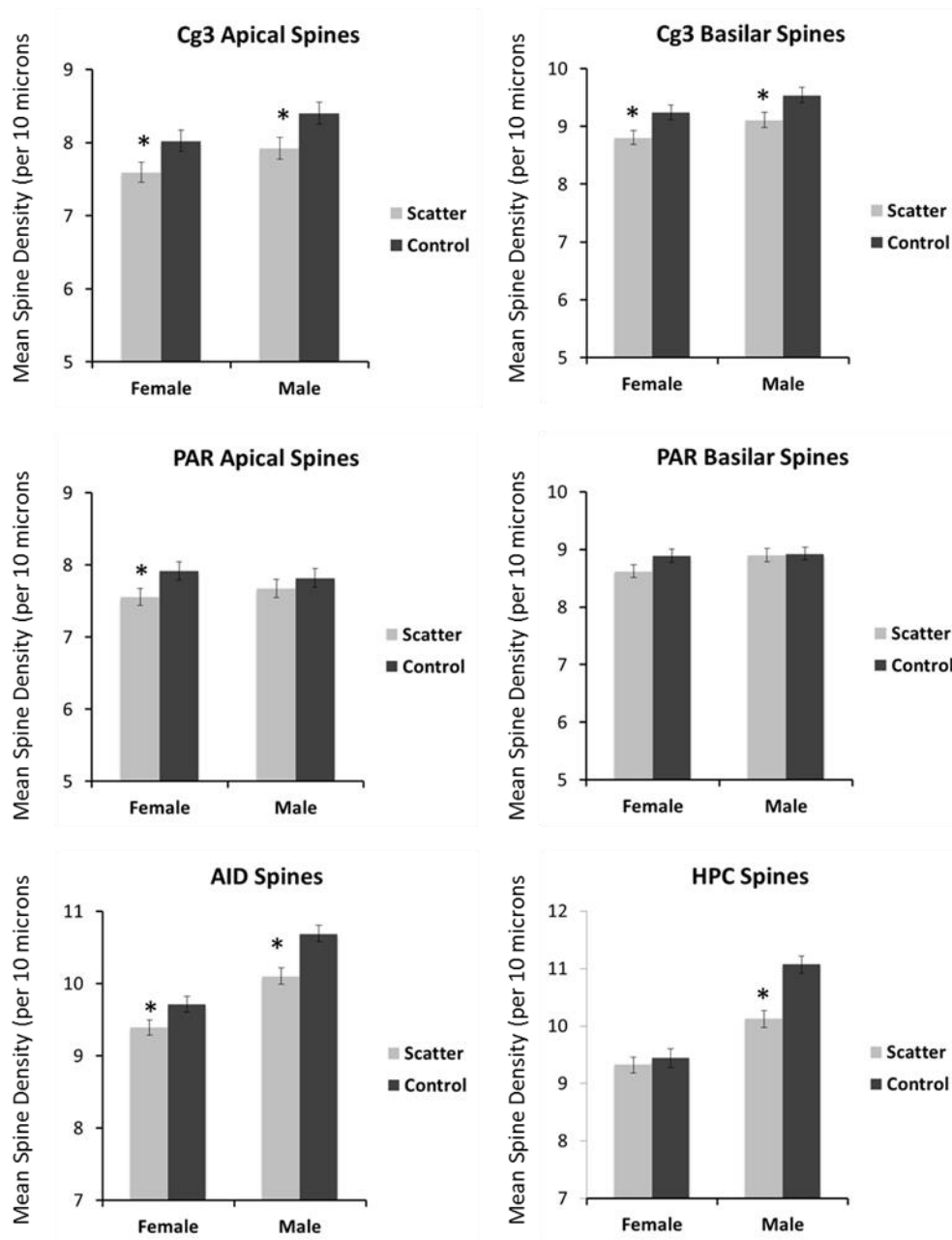


B

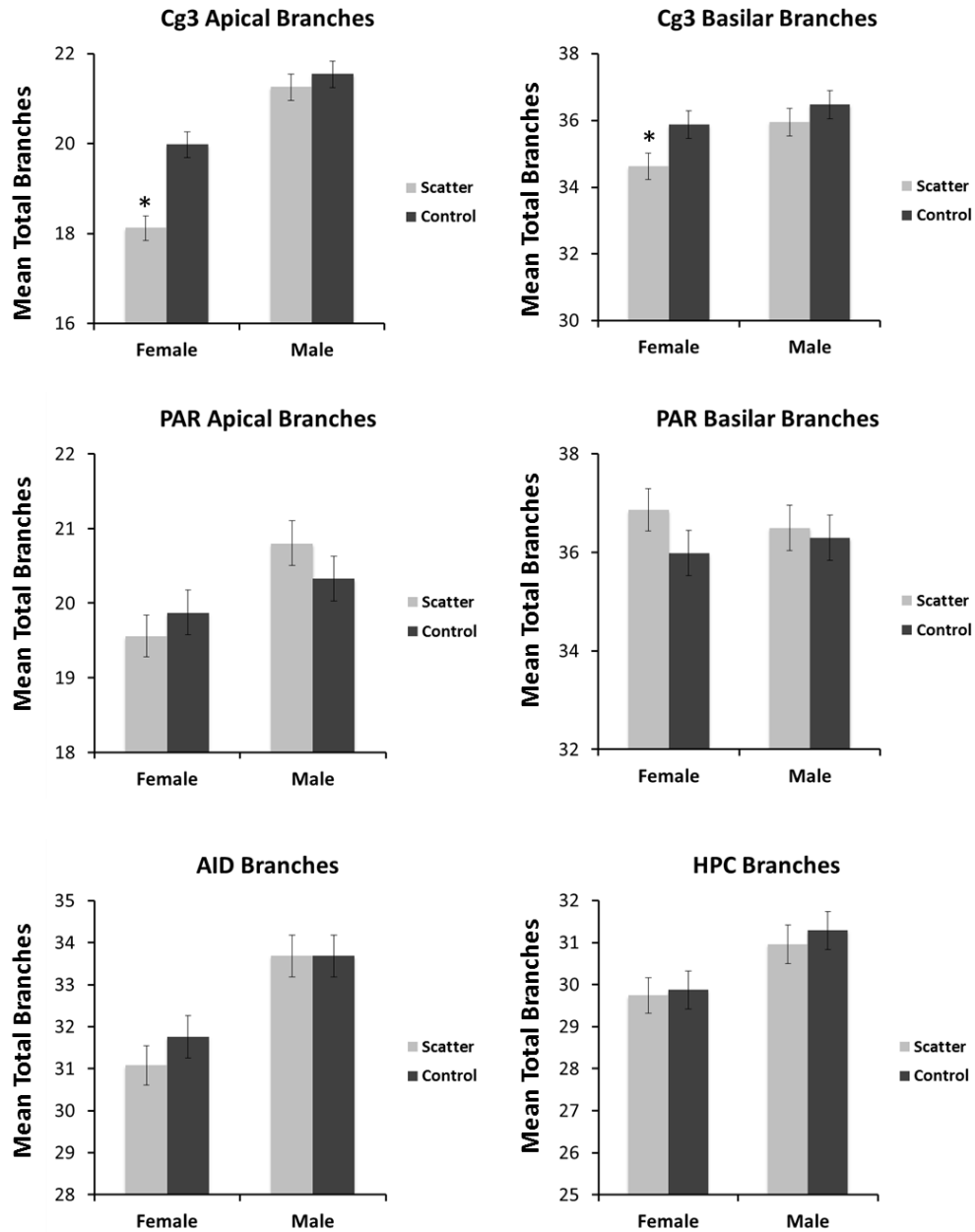
**Figure 3.3:** The KEGG axon guidance (A) and the KEGG neurotrophin signaling pathway (B) (Huang da et al., 2009b, Huang da et al., 2009a, Huang et al., 2007). Red denotes elevated expression as compared to control; green denotes decreased expression as compared to control.



**Figure 3.4:** Levels of BDNF, JNK, BCL2 and ELK1 in PFC tissues of scatter-irradiated female animals. Lysates from PFC tissues were immunoblotted using antibodies against BDNF, JNK, BCL2 and ELK1. Protein levels relative to those of control animals are shown as the means  $\pm$  SD; \*\*  $p<0.001$ ; \* $p<0.05$ ; # $p<0.10$ , Student's *t*-test.

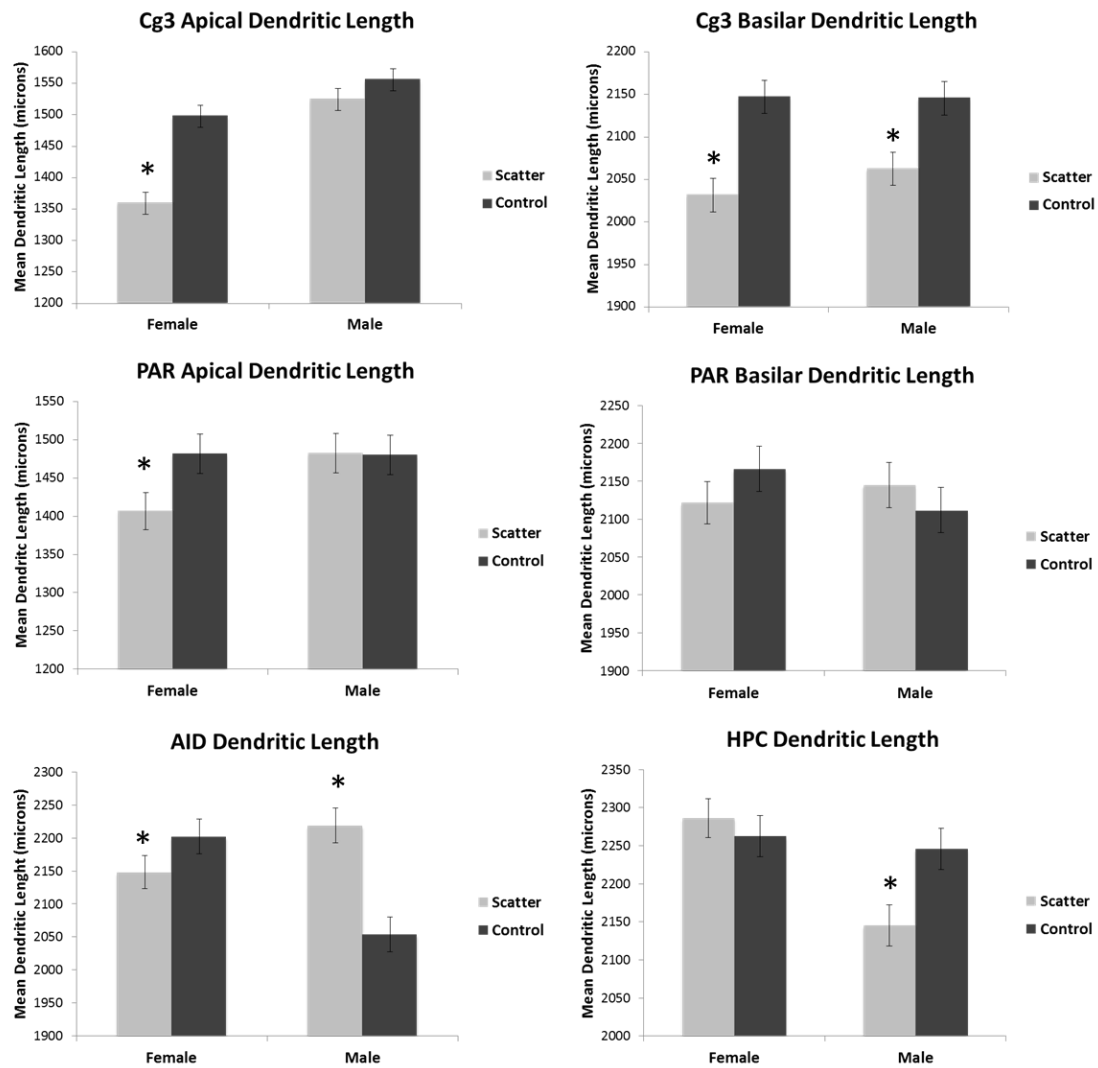


**Figure 3.5:** Scatter radiation exposure affects spine density. The density of dendritic spines (spines/10 $\mu$ M) in medial prefrontal cortex (Cg3), orbital frontal cortex (AID), parietal cortex (Par1), and hippocampus (CA1) of male and female rats upon low dose scatter irradiation. \*Significantly different from the control unexposed animals;  $p < 0.05$  or better.



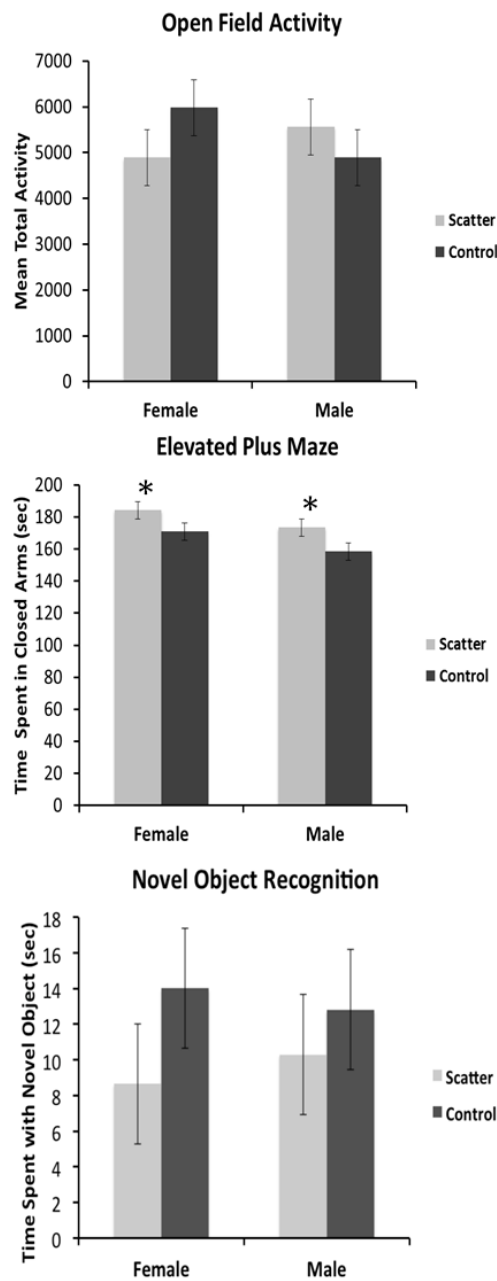
**Figure 3.6:** Scatter radiation exposure causes changes in dendritic branching. Apical and basilar branching in medial prefrontal cortex (Cg3), orbital frontal cortex (AID), parietal cortex (Par1), and hippocampus (CA1) of male and female rats upon scatter irradiation.

\*Significantly different from the control unexposed animals  $p < .05$  or better.



**Figure 3.7:** Scatter radiation exposure causes changes in dendritic length. Dendritic length in medial prefrontal cortex (Cg3), orbital frontal cortex (AID), parietal cortex (Par1), and hippocampus (CA1) of male and female rats upon scatter irradiation. \*Significantly different from the control unexposed animals  $p < 0.05$  or better.





**Figure 3.8:** Scatter irradiation exposure affects animal behavior. Graphical representation of the behavioral data for the Open Field Activity, Elevated Plus Maze and Novel Object Recognition tests. \*Significantly different from the control unexposed animals  $p < 0.05$  or better.

## **CHAPTER 4: CYTOTOXIC CHEMOTHERAPY AGENTS**

### **CYCLOPHOSPHAMIDE AND MITOMYCIN C CAUSE PERSISTENT GENE EXPRESSION CHANGES, OXIDATIVE DNA DAMAGE AND EPIGENETIC ALTERATIONS IN THE PREFRONTAL CORTEX AND HIPPOCAMPUS**

## **ABSTRACT**

The development of new chemotherapeutic agents and regimens for cancer therapy has led to increasing rates of survival in cancer patients. However, chemotherapy agents cause numerous side effects, including central nervous system toxicity. Recent research shows that chemotherapy agents are more toxic to healthy brain cells than to cancer cells they were designed to treat. They cause a wide array of side effects, including memory loss and cognitive dysfunction that can persist long after the completion of cytotoxic treatment. This condition is known as chemo brain. While the molecular and cellular mechanisms of chemo brain are not well investigated, the frequency and timing of its occurrence and its persistence suggests that chemo brain is epigenetic in nature and is associated with aberrant global gene expression patterns.

Here, we analyzed the effects of two commonly used cytotoxic chemotherapy drugs—cyclophosphamide (CPP) and mitomycin C (MMC)—on gene expression and epigenetic processes in the murine brain, focusing on the PFC and hippocampal regions.

We, for the first time, show that CPP and MMC treatment led to profound sex- and brain region-specific alterations in gene expression profiles. Gene expression changes were most prominent in the PFC tissues of female animals 3 weeks after MMC treatment, and the gene expression response was much more profound for MCC than CPP exposure.

MMC exposure also resulted in an accumulation of an oxidative DNA damage marker 8-oxo-2'-deoxyguanosine (8-oxodG) and a decrease in the 8-oxodG repair protein OGG1 in the PFC of female animals 3 weeks after treatment. MMC treatment let

to decreased global DNA methylation and increased DNA hydroxymethylation in the PFC tissues of female animals.

The majority of changes induced by chemotherapy in the PFC tissues of female mice resembled ones that occurred during the brain aging processes. Therefore, our study establishes initial mechanistic links between chemotherapy-induced chemo brain and brain aging, and provides an important roadmap for future analysis.

## INTRODUCTION

Based on a recent report by the American Cancer Society, 14.1 million new cancer cases were diagnosed worldwide in 2012, and 8 million of those occurred in developed countries. By 2030, newly diagnosed cancer cases are projected to reach 21.7 million worldwide (<http://www.cancer.org/research/acsresearchupdates/more/10-must-know-2015-global-cancer-facts>). The development of new chemotherapeutic agents and regimens for cancer therapy has led to increasing rates of survival in cancer patients; therefore, it is important to ensure that cancer survivors suffer minimal side effects and have a good quality of life.

Chemotherapy agents cause a wide array of side effects, including central nervous system (CNS) toxicity (Soffietti et al., 2014, Ahles et al., 2012), and recent research shows that chemotherapy agents are more toxic to healthy brain cells than to the cancer cells they were designed to treat (Han et al., 2008). Numerous studies have provided evidence of the occurrence of chemotherapy-cognitive dysfunction (Kaiser et al., 2014, Moore, 2014, Vardy et al., 2008). As reported by Wefel and Schagen (2012), with regard to breast cancer alone, more than 60 studies have investigated and found various degrees of association between chemotherapy and cognitive impairments (Wefel and Schagen, 2012).

Chemotherapy-induced CNS side effects impact the cognitive domains of attention, memory, processing speed, and executive function (Seigers and Fardell, 2011, Seigers et al., 2015, Seigers et al., 2010a, Seigers et al., 2008, Seigers et al., 2009, Seigers et al., 2013, Seigers et al., 2010b, Christie et al., 2012, Joly et al., 2011), causing a condition that has been termed *chemo brain* (Mitchell and Turton, 2011). The persistence of chemo brain manifestations ranges from short to long (Ahles et al., 2012, Ahles et al., 2002, Ahles et al., 2005), with

about 35% of patients reporting side effects for months to years after the cessation of their treatments. Furthermore, data from the International Cognitive Workshop (Venice, Italy, October 2006) indicate that chemo brain's cognitive side effects can persist for as long as five to ten years after the completion of treatment (Mitchell and Turton, 2011, Vardy et al., 2008). In order to prevent and mitigate chemo brain side effects, it is important to fully understand the mechanisms that are affected by chemotherapy agents in the brain.

The proposed mechanisms of chemo brain include increased oxidative stress, chronic inflammation, inhibition of neuronal proliferation, differentiation and disruption of hippocampal neurogenesis, induction of apoptosis, alterations in brain blood flow, changes in metabolism, disruption of the blood-brain barrier, and white matter dysfunction (Raffa, 2011, Lyons et al., 2011b, Briones and Woods, 2014, Briones et al., 2015, Christie et al., 2012, Han et al., 2008, Joshi et al., 2010, Seigers and Fardell, 2011).

Although the molecular mechanisms underlying chemo brain have been assessed in clinical studies, analyses are difficult to conduct because of large inter-patient variability regarding numerous factors, such as treatment protocols, disease status, and co-morbidities (Yang et al., 2012, Wefel et al., 2011, Myers, 2010). Therefore, much of the recent chemo brain research has employed cell lines as well as rodent models, as reviews show (Seigers and Fardell, 2011, Seigers et al., 2015). Several model-based studies have reported that chemotherapy exposure has caused oxidative stress, inhibited neuronal proliferation and differentiation, increased apoptosis, and altered levels of histone modification and chromatin remodeling, thus leading to aberrant expression of neurotrophin and neurogenic proteins in the brains of experimental animals. These molecular changes

were associated with altered neurogenesis and deficits in learning and memory processes (Mustafa et al., 2008, Briones and Woods, 2014, Christie et al., 2012).

The frequency and timing of chemo brain occurrence and persistence suggest that chemo brain may be epigenetic in nature and may be associated with aberrant gene expression profiles. Epigenetic changes are meiotically heritable and mitotically stable alterations that regulate gene expression and genome stability; they include DNA methylation and hydroxymethylation, histone modification, and non-coding RNA regulation (Wang et al., 2015).

DNA methylation is critical to neurogenesis (Jobe et al., 2012). Furthermore, chemotherapy drugs may alter epigenetic homeostasis (Csoka and Szyf, 2009) and the recently discovered DNA hydroxymethylation plays a pivotal role in brain development and is implicated in various neurological diseases (Szulwach et al., 2011). These findings provide a strong rationale for the epigenetic background of chemo brain (Wang et al., 2015). Epigenetic changes affect gene expression and are pliable and reversible. Therefore, analysis of epigenetic and gene expression patterns of chemo brain may open a new avenue for its prevention or reversal.

The vast majority of chemo brain studies have focused on the hippocampus, due to its involvement in several cognitive processes, including spatial navigation, memory processing, storage of long-term memory, and declarative memory. Yet, almost nothing is known about chemotherapy's effects on the prefrontal cortex (PFC), a key regulatory region that is involved in executive functions, such as working memory, decision-making, planning, judgment, social behavior, and abstract thinking.

Here, we set out to analyze the effects of two commonly used cytotoxic chemotherapy drugs — cyclophosphamide (CPP) and mitomycin C (MMC) — on gene expression and epigenetic processes in the murine brain, focusing on the PFC and hippocampal regions. We demonstrated that CPP and MMC treatment led to profound sex- and brain region-specific alterations in gene expression profiles, as well as caused oxidative DNA damage and changes in global levels of DNA methylation and hydroxymethylation.

## **RESULTS**

### **Analysis of differential gene expression in response to CPP and MMC**

Global gene expression profiling provides a mechanistic insight into a milieu of molecular processes and pathways associated with exposures to various genotoxic and non-genotoxic agents, as well as with a variety of disease conditions. Our aim was to use an Illumina Bead Array platform to conduct an in-depth gene expression analysis of the prefrontal cortex (PFC) and hippocampal tissues of male and female animals three weeks after exposure to cyclophosphamide (CPP) or mitomycin C (MMC).

The differential gene expression analysis revealed no notable changes in the prefrontal cortex after exposure to CPP, regardless of the sex of analyzed animals (Fig.4.1A). In contrast, changes in gene expression in response to MMC were evident three weeks after exposure. Thirty-six genes were upregulated and 166 genes were downregulated in females, while 2 and 16 genes were upregulated and downregulated, respectively, in males, following MMC exposure (the adjusted p-value <0.05 and fold change 1.5) (Fig4.1A).



The hippocampus, in contrast to the PFC, contained almost no genes that were differentially expressed three weeks after exposure: only a single gene was upregulated in male hippocampal tissues after MMC treatment ( $p < 0.05$ , fold change 1.5) (Fig.4.1B). Exposure to CPP led to the upregulation of a single gene in the males at three weeks post exposure, while ten genes were downregulated in the hippocampal tissues of female animals ( $p < 0.05$ , fold change 1.5) (Fig.4.1C). The MMC-induced changes were brain-region specific, and the MMC-induced changes in gene expression were seen only in the PFC and not in the hippocampus (Fig.4.1A and 4.1B).

We also established baseline sex differences in gene expression by comparing the profiles of the male and female PFC and hippocampal tissues of the control (saline-treated) animals. Three weeks after treatment, the PFC tissues of the control males differed from those of the females in the expression of 166 genes ( $p < 0.05$ , fold change 1.5), whereas their hippocampus tissues differed in only six genes ( $p < 0.05$ , fold change 1.5) (Fig.4.2). The comparison of gene expression in the hippocampus versus the frontal cortex revealed differential expression in 1800 and 1721 genes in females and males, respectively ( $p < 0.05$ , fold change 1.5).

### **Detailed analysis of gene enrichment pathways**

We sought further insight into the functional significance of the observed gene expression changes by conducting an in-depth study of signalling pathways. First, we annotated genes that were differentially expressed in response to MMC or CPP by performing a functional annotation Gene Ontology (GO) terms analysis using the DAVID bioinformatics platform.

Among the genes that were downregulated at three weeks post treatment in the PFC of females exposed to MMC, enrichment was seen in the positive regulation of the Notch signaling pathway (more than 50 fold) and in neural crest cell differentiation (more than 10 fold). In the males, the endoplasmic reticulum and endosome pathways were enriched. The findings for males and females showed no overlap. In the upregulated genes found at three weeks post treatment in the PFC tissues of females exposed to MMC, enrichment was observed in the GO terms of olfactory receptor activity (Table 4.1). Too few genes were changed in the males to warrant the same analysis that was conducted for the female groups.

We further visualized the genes and networks altered in response to MMC and CPP using WikiPathways and KEGG pathways. The PFC of females exposed to MMC at three weeks showed downregulated genes belonging to dopaminergic neurogenesis (Fig.4.3) and oxidative phosphorylation pathway (Fig.4.4).

### **Chemotherapy-induced oxidative damage**

A number of the observed chemotherapy-induced gene expression changes indicated an altered cellular redox status and oxidative stress (Kesler, 2014). An increase in oxidative stress has been proposed as one of mechanisms of chemo brain (Wang et al., 2015, Joshi et al., 2005). Based on these findings, we assessed the levels of 8-oxo-2'-deoxyguanosine (8-oxodG) in genomic DNA from PFC and hippocampal tissues of MMC and CPP exposed male and female animals. The 8-oxodG molecule, one of the predominant and best-studied molecular markers of oxidative DNA damage, is formed by the action of reactive oxygen species (Dizdaroglu and Jaruga, 2012, Dizdaroglu et al., 2002). Exposure to CPP caused a small but statistically significant ( $p=0.007$ ) increase in

8-oxodG levels in the PFC tissues of female, but not male, animals at 3 weeks post-treatment. Exposure to MMC led to a statistically significant increase in the 8-oxodG levels in the PFC tissues of male mice ( $p=0.016$ ), while the PFCs of MMC-exposed females showed a strong trend towards an increase in 8-oxodG levels, but the difference did not reach statistical significance ( $p=0.146$ ) (Fig.4.5). In the hippocampal tissues, CPP exposure had no effect on the levels of 8-oxodG in either male or female animals, while MMC exposure led to significantly elevated levels in females ( $p=0.002$ ) and an increasing, but not statistically significant trend in males ( $p=0.179$ ) (Fig.4.5). As such, MMC was a more potent inducer of oxidative DNA damage when compared to CCP.

We explored potential mechanisms for the persistence of 8-oxodG in the genomic DNA of the PFC tissues of the MMC-exposed animals by determining the expression of key base excision repair proteins involved in the repair of oxidative DNA damage. In addition, the expression of base excision repair proteins is another well-accepted marker of oxidative DNA damage (Shpyleva et al., 2014, Rusyn et al., 2004, Rusyn et al., 2005). Our analysis of the levels of 8-oxoguanine glycosylase (OGG 1) and apurinic/apyrimidinic endonuclease 1 (APE1) using western immunoblotting revealed a statistically significant reduction in the levels of OGG1 ( $p=0.045$  and  $p=0.044$ ) in the PFC tissues of the MMC- and CPP-exposed female animals, respectively (Fig.4.6). No statistically significant changes were observed in the levels of APE1 (Fig.4.6).

#### **Analysis of global DNA methylation in the PFC and hippocampal tissues of chemotherapy-exposed mice**

Several studies have suggested that aberrant DNA methylation may occur because of oxidative DNA damage (Shpyleva et al., 2014, O'Hagan et al., 2011, Olinski et al.,

2014). Aberrant DNA methylation is also associated with altered gene expression patterns (Liyanage et al., 2014). With this in mind, we analyzed the status of global DNA methylation in the hippocampal and PFC tissues of CPP- and MMC-exposed male and female mice. We determined the levels of 5-methyl-cytosine (5mC) and 5-hydroxymethyl-cytosine (5-hmC) in the genomic DNA of PFC and hippocampal tissues of chemotherapy-treated animals at 3 weeks post-exposure.

We found a statistically significant decrease in the level of 5-mC in the global DNA of PFC tissues of MMC-treated female mice ( $p=0.025$ ), whereas the level of 5-hmC was significantly increased ( $p=0.017$ ). MMC-treated male mice showed an increase in the 5hmC levels in the hippocampus ( $p=0.018$ ), but no changes were observed in the 5mC levels. CPP treatment had no effect on the levels of 5mC or 5-hmC in any of the studied tissues (Fig.4.7).

We further explored the mechanisms of the decrease in the level of 5mC and the increase in the level of 5hmC in the PFC tissues of MMC-exposed females by determining the levels of proteins that establish and maintain these epigenetic modifications (Hamidi et al., 2015). MMC exposure led to a significant down-regulation of the cellular levels of maintenance DNA methyltransferase DNMT1 ( $p=0.032$ ), and *de novo* DNA methyltransferase DNMT3a ( $p=0.017$ ) in the female PFC tissues at 3 weeks post-treatment (Fig.4.8). In parallel, the levels of the methyl-CpG binding protein MECP2 were increased ( $p=0.044$ ). The levels of Tet1 and Tet 2 were unaffected (data not shown).

## DISCUSSION

This study is the first in-depth analysis of gene expression profiles in the PFC and

hippocampus tissues of male and female mice three weeks after treatment with the chemotherapy agents MMC and CPP. The key findings of our study were that: (i) chemotherapy altered the gene expression profiles in the murine PFC and hippocampal tissues; (ii) gene expression changes were most prominent in the PFC tissues of female animals three weeks after MMC treatment; (iii) gene expression responses were much more profound for MMC than CPP exposure; (iv) MMC exposure resulted in accumulated 8-oxodG, decreased global DNA methylation, and increased DNA hydroxymethylation in the PFC tissues of female animals; and (v) the majority of the changes induced by MMC in the PFC tissues of female mice resembled those that occur during the aging processes. As such, the PFC of both male and female animals appears not to be sensitive to CPP treatment, although the reasons for this apparent resistance require further analysis.

Our data also suggest that the PFCs of females may be more vulnerable than those of males in the long term, as the significant changes observed in females at three weeks post-exposure to MMC were not apparent in males. We found that MMC exposure leads to profound alterations in global gene expression, affecting pathways responsible for oxidative stress and other effects. We also demonstrate that MMC exposure leads to the accumulation and persistence of 8-oxodG in the PFC tissues of female animals at three weeks after exposure. This accumulation was paralleled by decreases in levels of 5-methylcytosine and increases in levels of 5-hydroxymethylcytosine.

The elevated level of the oxidative stress biomarker 8-oxodG, and the decreased amount of key BER protein (OGG1) that repairs 8-oxodG, strongly suggest that MMC induces oxidative DNA damage and oxidative stress (Rusyn et al., 2005, Rusyn et al.,

2004, Shpyleva et al., 2014). The 8-oxodG biomarker is a prevalent and well-studied component of oxidative DNA lesions (Dizdaroglu and Jaruga, 2012, Dizdaroglu et al., 2002). Various studies have established the important role of altered cellular redox status in the pathophysiology of major neurological diseases and conditions, such as Alzheimer's disease, Parkinson's disease, stroke, autism, and many others (Liu et al., 2011, Lovell and Markesbery, 2007, Nakabeppu et al., 2007, Shpyleva et al., 2014). Oxidative stress is also viewed as one of the potential mechanisms of chemo brain (Wang et al., 2015, Joshi et al., 2005), but the associated processes and pathways linking oxidative stress and chemo brain have not yet been analyzed, beyond claims of the effect of chemotherapy treatment on the brain.

This study, for the first time, shows that there is a persistent decrease in the levels of OGG1 in the PFC of chemotherapy-exposed female animals. OGG1 is a glycosylase involved in the initial steps of recognition and removal of 8-oxodG, a cytotoxic and mutagenic lesion, through the highly conserved base excision repair pathway (Nishimura, 2002, Klungland and Bjelland, 2007). OGG1 is the first enzyme in BER, and thus the success of 8-oxodG removal heavily depends on proper OGG1 function. OGG1 is highly expressed in the brain, where it works to protect neurons against oxidative DNA damage and apoptosis, and to maintain proper neuronal connectivity (Liu et al., 2011). This gene is crucially important for brain development (Wong et al., 2008, Larsen et al., 2006). In addition, the loss or inhibition of OGG1 and subsequent accumulation of 8-oxodG in the genome are associated with cancer, neurodegenerative diseases, metabolic diseases, obesity, and autism (Lovell and Markesbery, 2007, Nakabeppu et al., 2007, Shpyleva et al., 2014, Kinnersley et al., 2014, Klungland and Bjelland, 2007, Nohmi et al., 2005,

Osorio et al., 2014, Sampath et al., 2012). Along with these and other pathological conditions, OGG1 loss has been implicated in brain aging (Cardozo-Pelaez et al., 2012, Liu et al., 2011, Swain and Rao, 2012).

Another key finding of the present study is the decrease in 5mC and concurrent increase in 5hmC in the PFC tissues of female animals three weeks after exposure to MMC. Ours is the first study to report changes in these epigenetic markers in the context of chemo brain. The observed decrease in 5mC, which is indicative of global DNA hypomethylation, may be attributed to the observed reduction in the levels of maintenance and de novo DNA methyltransferases DNMT1 and 3a. It may also reflect the presence of oxidative lesions themselves, which have been previously shown to decrease the expression and activity of DNMTs, leading to aberrant DNA methylation patterns.

The reduced levels of DNMT1 and 3a constitute another important finding. These proteins are important for the maintenance of DNA methylation and synaptic plasticity in the adult brain (Feng et al., 2010). Their reduced expression has been associated with blast-induced neurotrauma (Bailey et al., 2015), loss of synaptic functions in forebrain neurons (Feng et al., 2010), several neurodegenerative diseases, and brain aging. Inactivating mutations in DNMT1 are associated with hereditary sensory neuropathy and adult-onset dementia (Klein et al., 2011). The observed persistent increase in the levels of 5hmC is also important, because changes in DNA hydroxymethylation have been implicated in neurodegeneration and brain aging (Sherwani and Khan, 2015, van den Hove et al., 2012).

Recent studies have linked decreases in 5mC and increases in 5-hmC to newly discovered demethylation functions of Tet proteins and the Tet-mediated enzymatic oxidation of 5mC (Piccolo and Fisher, 2014, Liyanage et al., 2014). Nevertheless, in the present study, the cellular levels of Tet proteins were found to be unchanged following MMC exposure. Two recent studies have reported similar accumulations of 5hmC in brain tissues without changes in Tet levels (Shpyleva et al., 2014, Chen et al., 2012). Thus, the increase in 5hmC observed in the present study is perhaps not associated with its role in DNA demethylation. In any case, its precise roles, as well as its locus specificity, need further clarification.

Altered cellular levels of 5mC and 5 hmC, in turn, may affect gene expression as well as genome stability, which emphasizes the importance of analyzing the precise locus-specific distribution and plasticity of these changes in chemo brain, and in other neurological conditions. Increased oxidative stress, altered methylation, and aberrant gene expression were all observed in the PFC of MMC-exposed female animals three weeks post-treatment. This may indicate the possible interconnectivity of these molecular processes. Indeed, MMC can cause oxidative damage, which leads to altered DNA methylation and aberrant gene expression. The aberrant expression of oxidative pathways, as shown in our study, may also lead to the accumulation of oxidative damage, thereby creating a "damage loop" and causing persistence of adverse molecular changes in the PFC (Fig.4.9).

The cytotoxic chemotherapy-induced changes observed in the PFC constitute yet another seminal finding of this study. The vast majority of previous chemo brain studies have focused on the hippocampus, due to its role in neurogenesis, whereas the PFC has



been under-investigated, despite being a key regulatory region. Several clinical research articles have pointed out the important role of the PFC in chemo brain (Mu et al., 2015, Kesler et al., 2011, McDonald et al., 2013), while one mouse model-based analysis has reported PFC effects upon the application of the targeted cancer drug everolimus (Dubois et al., 2014). The current study opens up new avenues for the analysis of chemo brain and the role of PFC damage in its etiology and pathogenesis.

The molecular changes observed in this study of chemo brain have also been found to play key roles in neurodegeneration and aging (see Fig.4.9). Indeed, increased oxidative damage and altered levels of DNA methylation and hydroxymethylation are established molecular signs of aging (Irier and Jin, 2012, van den Hove et al., 2012, Swain and Rao, 2012, Romanucci and Della Salda, 2015). Furthermore, a recent clinical research article has suggested the existence of a link between aging and cancer treatments, and calls for basic and model-based research to clarify the link between these two key clinical conditions (Kesler, 2014). In light of this need, our study establishes the initial mechanistic links between chemotherapy-induced chemo brain and brain aging, and provides an important roadmap for future analysis.

## **MATERIALS AND METHODS**

### **Chemotherapy treatment**

Forty-five day old male and female BALB/c mice were randomly allocated to the following groups: (i) mitomycin C-treated; (ii) cyclophosphamide-treated; (iii) controls (10 ♂ and 10 ♀). Treated animals received either MMC (5 mg/kg) or CPP (200 mg/kg) as 2 consecutive injections every other day, and were euthanized 3 weeks after chemotherapy to examine the persistent effects on the brain.

## Gene Expression Analysis

The hippocampus and prefrontal cortex (PFC) tissues of four animals per group were used for the analysis of gene expression profiles. Differential expression analyses were performed by the Illumina® GenomeStudio software using an Illumina-custom model. In brief, RNA was extracted from the hippocampus and the PFC tissues using TRIzol® Reagent (Invitrogen, Carlsbad, CA), further purified using an RNAeasy kit (Qiagen), and quantified using Nanodrop2000c (ThermoScientific). Afterwards, RNA integrity and concentration were determined using 2100 BioAnalyzer (Agilent). Gene expression profiles were determined using Illumina MouseRef-8 v2.0 Expression BeadChip at the University of Lethbridge CFI-SAGES Facility. Differential expression analyses were performed by the Illumina® GenomeStudio software using an Illumina-custom model. To process the data, all expression values were made positive by adding an offset (minus minimum value plus one). Next, all expression values were transformed into Log2 values and normalized by the quantile method. To increase the statistical power of the outcome, the genes that had detection p-values in all the 8 compared samples (4 controls and 4 treatments) greater than or equal to 0.01 were removed. The adjusted p-values from moderated t-statistics (Smyth, 2004) were calculated using linear model and Bayes moderation. For the identification of differentially expressed genes, we have compared treatment samples (MMS and CPP) to appropriate sex (male or female), tissue type (prefrontal cortex or hippocampus) and controls (treated with saline). Differentially expressed genes were then extracted on the basis of the adjusted p-value 0.05 and fold change 1.5. Several comparisons within control groups were made. These

included comparison between male and female control samples, as well as between hippocampus and prefrontal cortex.

The functional annotations of differentially expressed genes were performed using DAVID, GO (Gene Ontology) Elite, and GO-TermFinder (Boyle et al., 2004). In DAVID, fold enrichment (FE) of a certain pathway was calculated according to the following formula:  $FE = n/N / x/X$ , where  $n$  – the number of genes involved in a given pathway in a given sample,  $N$  – the total number of genes changed in a given sample,  $x$  – the total number of genes in the genome belonging to a given pathway,  $X$  – the total number of genes in the genome. Pathways were visualized using WikiPathways and DAVID Bioinformatics Resources 6.7 KEGG Pathways (Huang et al., 2009b, Huang et al., 2009a, Huang et al., 2007).

### **Analysis of 8-oxo-7-hydrodeoxyguanosine, 5-methylcytosine, and 5-hydroxymethylcytosine in cerebellar DNA**

DNA was extracted from PFC and hippocampal tissues using the Qiagen DNeasy Kit. The levels of 8-oxodG, 5mC, and 5hmC in mouse PFC and hippocampal tissue DNA were measured by liquid chromatography combined with electrospray tandem mass spectrometry (LC-MS/MS) as described previously (Shpyleva et al., 2014, James et al., 2013) using four samples per each group.

### **Western Immunoblotting**

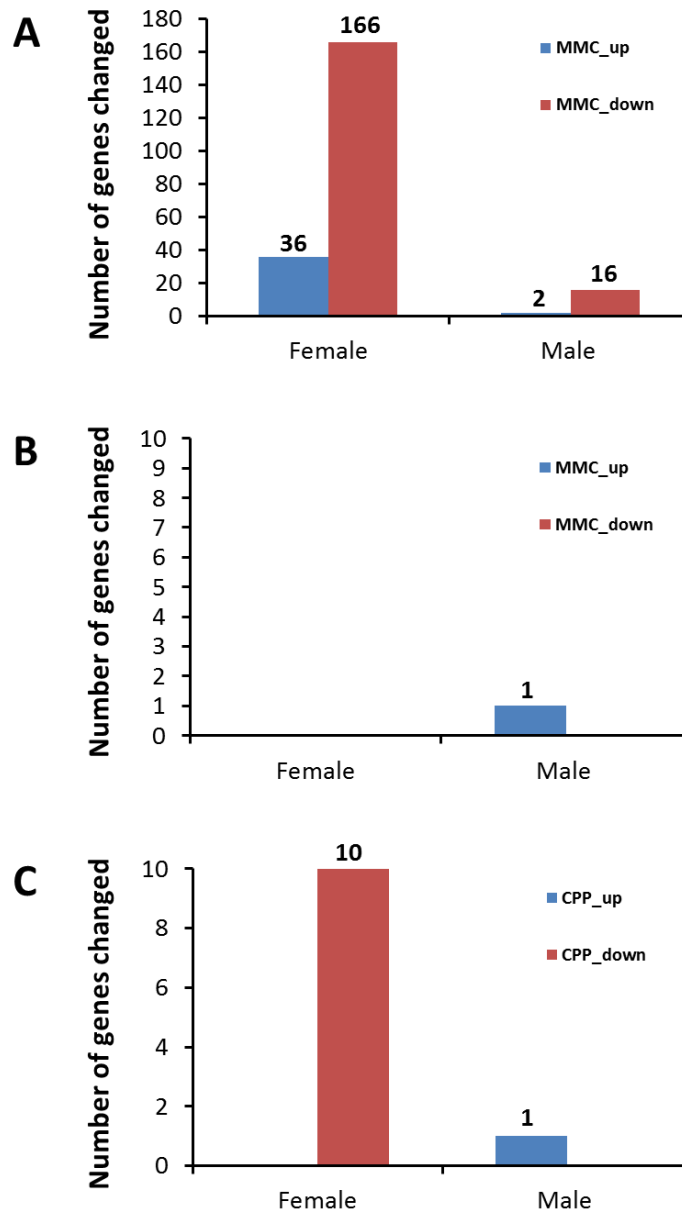
Western immunoblotting was conducted as described previously (Silasi et al., 2004). The membranes were incubated with primary antibodies against APE1, OGG1, DNMT1, DNMT3a, MeCP2 (1:1000, Abcam) and actin (1:2000, Abcam) overnight at 4°C. Primary antibody binding was detected using horseradish peroxidase-conjugated

secondary antibodies and the Enhanced Chemiluminescence Plus System (Amersham Biosciences, Baie d'Urfé, Quebec). Chemiluminescence was detected using a FluorChem HD2 camera with FluorChem software (Cell Biosciences). The membranes were stained with Coomassie blue (BioRad, Hercules, CA) to confirm equal protein loading. Signals were quantified using NIH Image J64 software and normalised relative to actin or Coomassie staining.

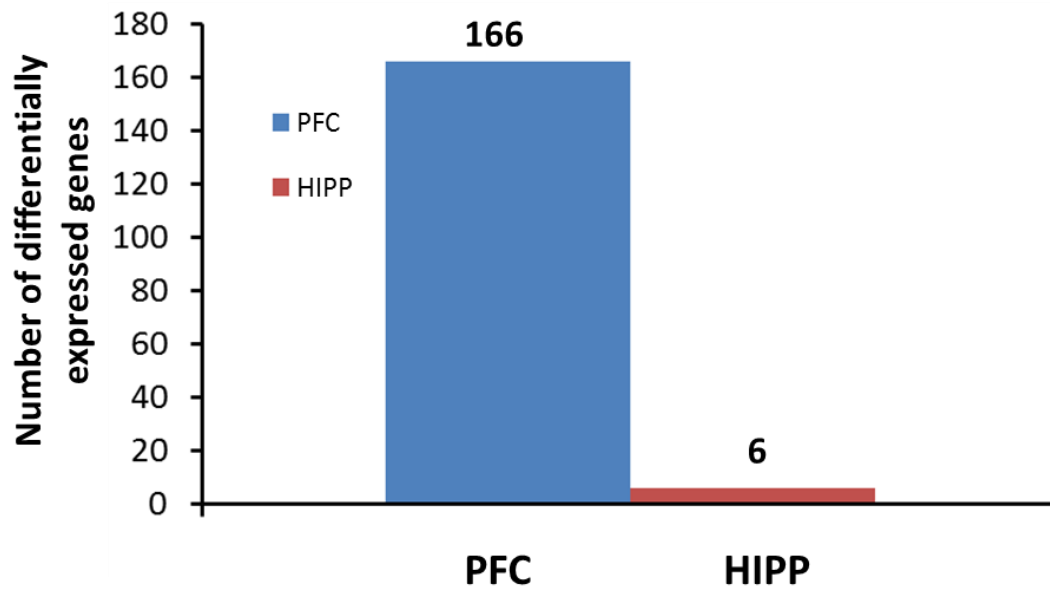
### **Statistical analysis**

All statistical analyses for DNA methylation, oxidative stress and protein levels were carried out using Microsoft Excel. Each mouse was used as a unit of analysis.

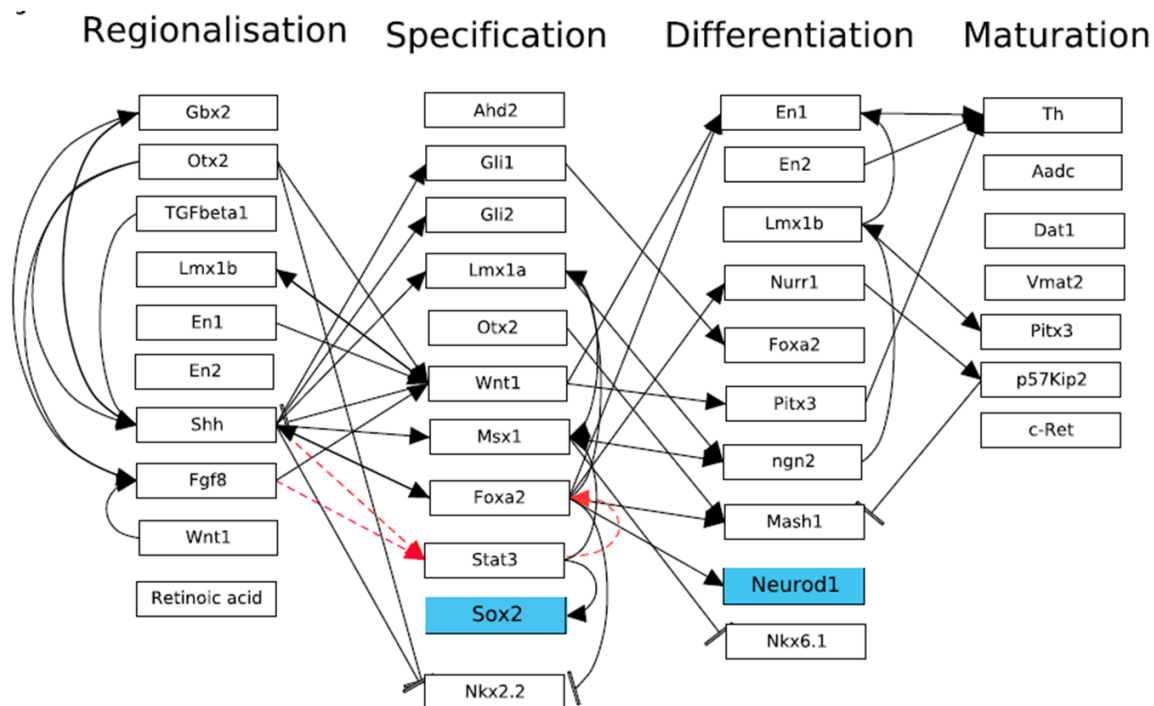
## FIGURES



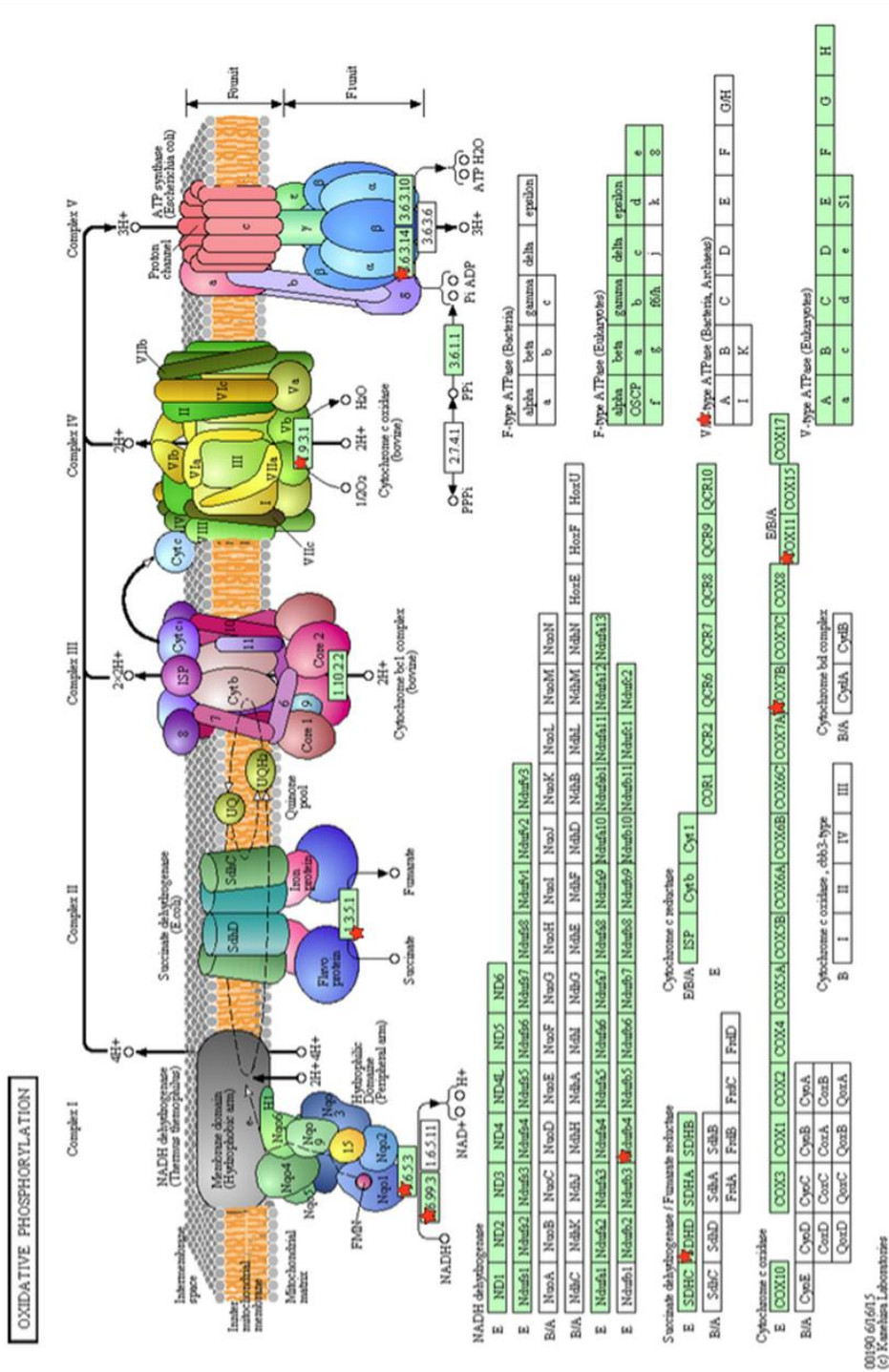
**Figure 4.1:** Number of up- and down-regulated genes in the prefrontal cortex and hippocampus of male and female animals exposed to MMC or CPP. A – number of differentially expressed genes in PFC of animals in response to MMC; B - number of differentially expressed genes in hippocampus of animals in response to MMC; C - number of differentially expressed genes in hippocampus of animals in response to CPP



**Figure 4.2:** Comparison of gene expression in the PFC and hippocampus tissues of males and females. Sex differences in gene expression in the PFC and hippocampus tissues. PFC – prefrontal cortex; HIPP – hippocampus.

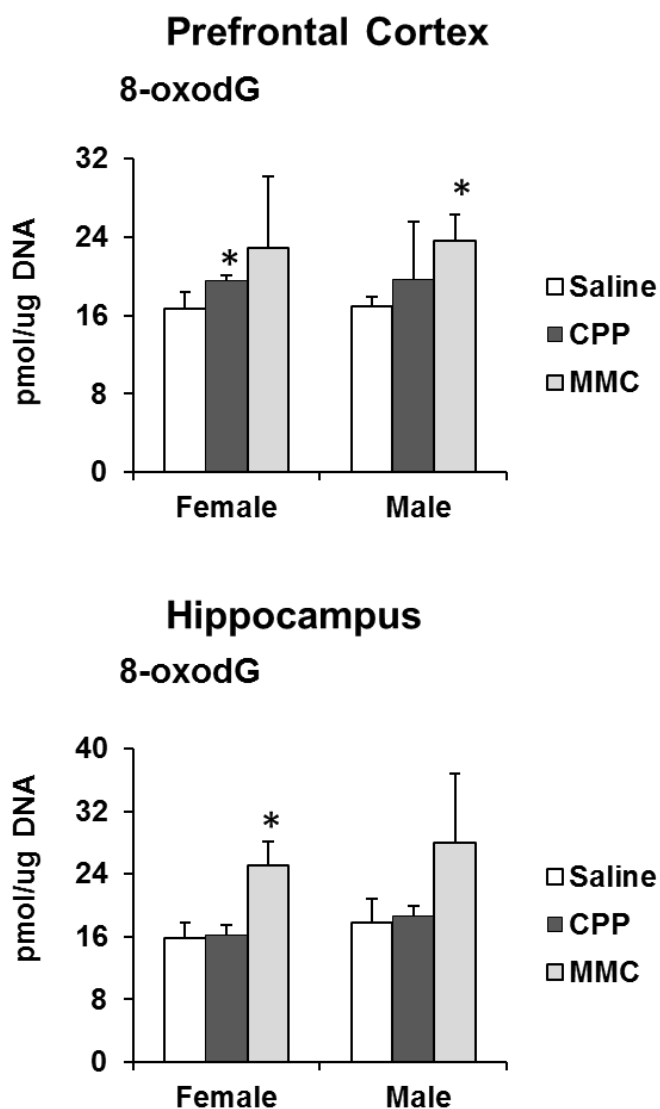


**Figure 4.3:** Visualization of genes downregulated in Dopaminergic Neurogenesis pathway in the PFC of females 3 weeks after MMC exposure (WikiPathways). Downregulated genes are shown in blue.

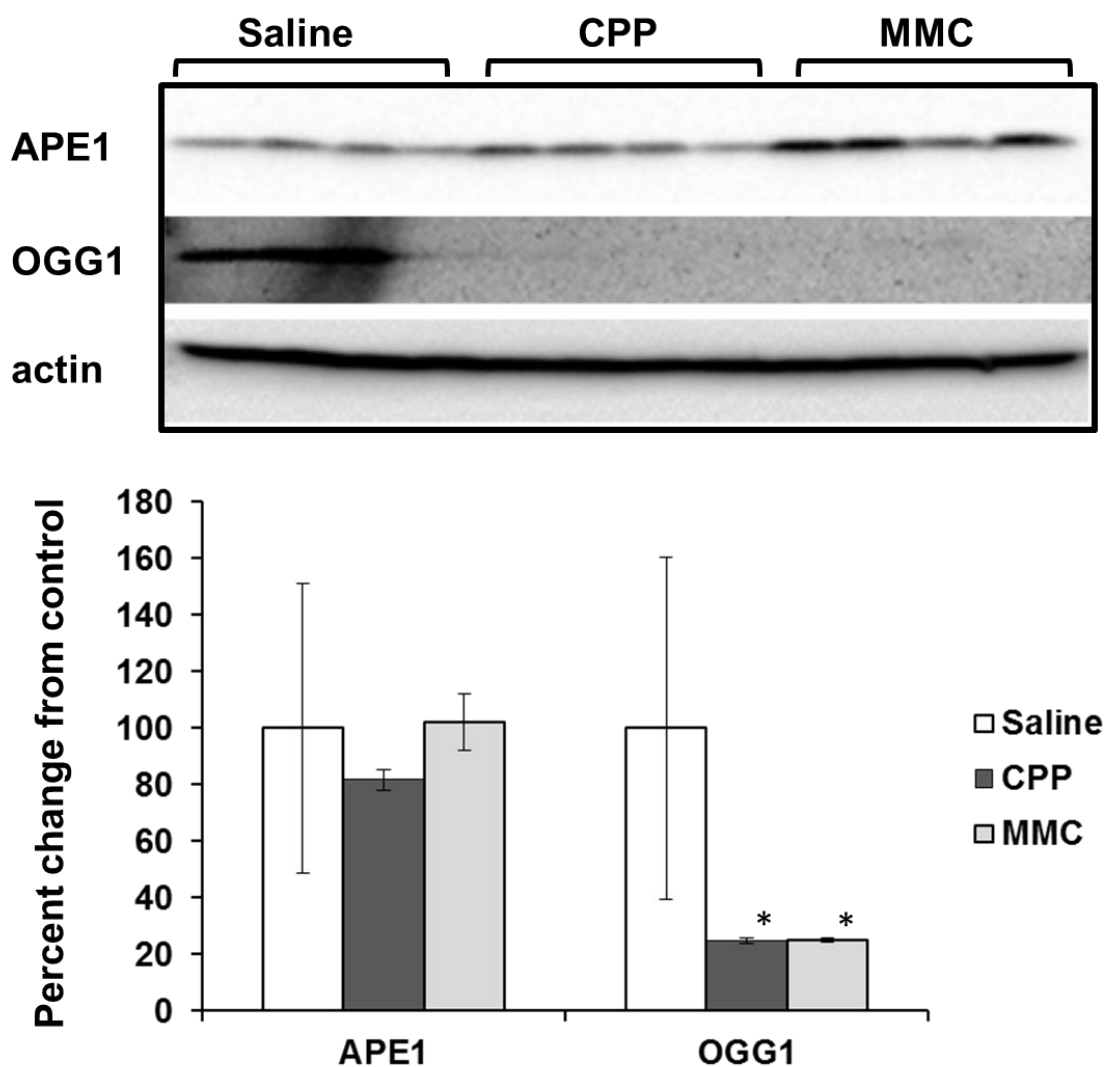


**Figure 4.4:** Visualization of genes downregulated in the KEGG oxidative phosphorylation pathways in the PFC of females 3 weeks after MMC exposure (DAVID Bioinformatics Resources 6.7)(Huang da et al., 2009b, Huang da et al., 2009a, Huang et al., 2007). Stars denote genes that were down-regulated in the PFC tissues of MMC-exposed female animals

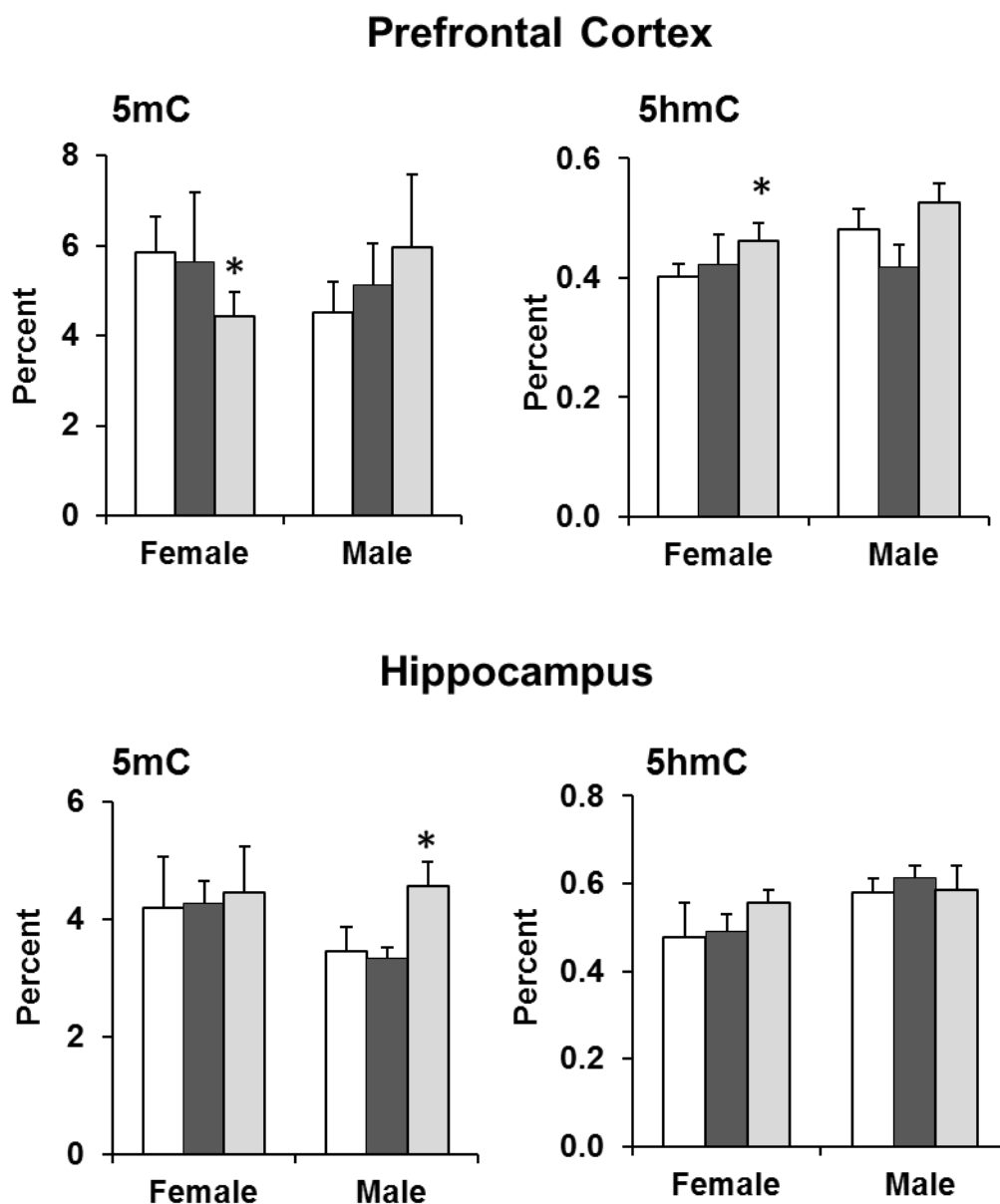




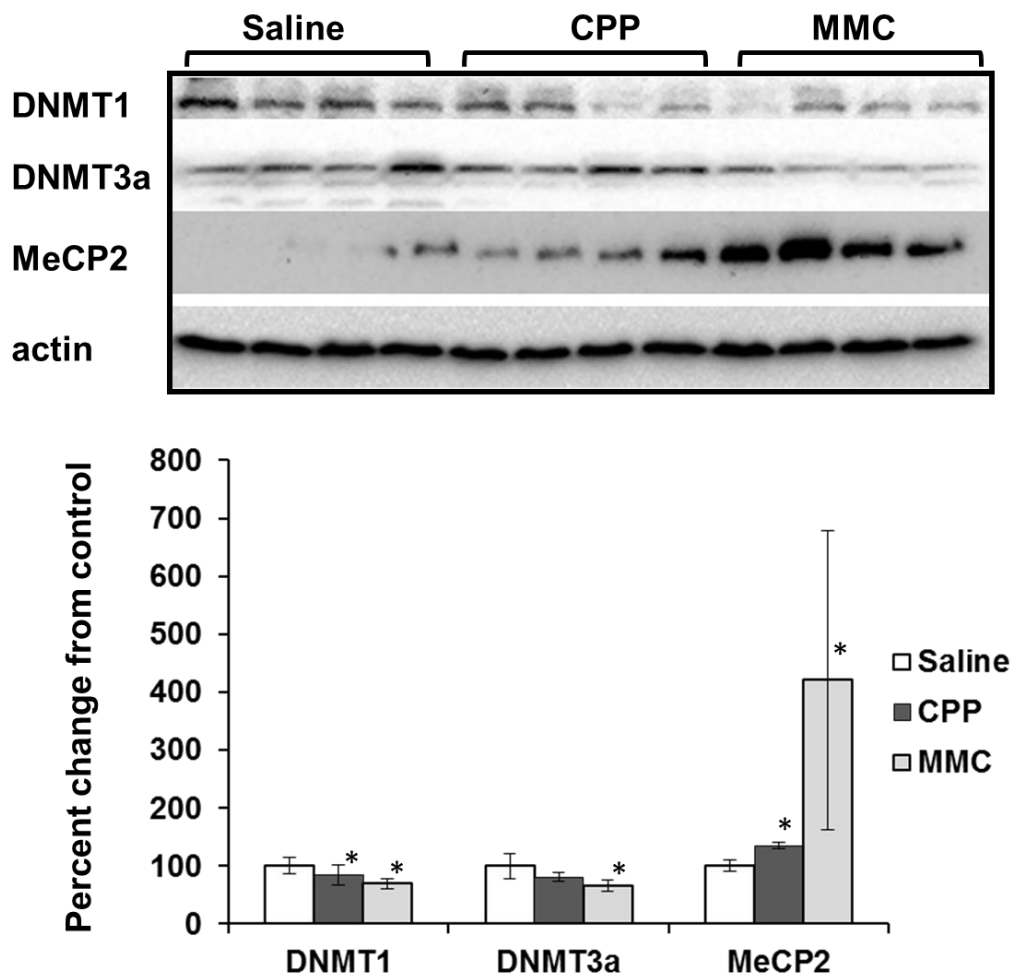
**Figure 4.5:** Oxidative DNA damage in the PFC and hippocampus tissues of chemotherapy-exposed animals. Levels of 8-oxo-7-hydrodeoxyguanosine (8-oxodG) in genomic DNA isolated from the PFC and hippocampus of male and female mice (mean  $\pm$  SD, n=4). ); \*p<0.05, Student's t-test.



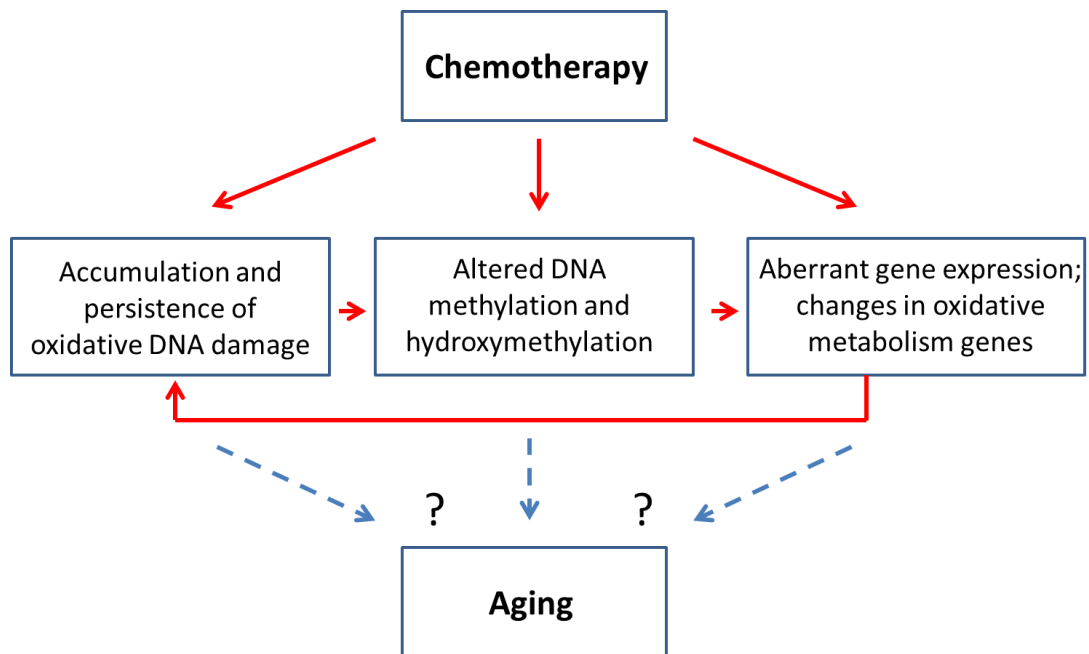
**Figure 4.6:** Levels of APE1 and OGG1 in the PFC tissues of chemotherapy-exposed female animals 3 weeks after treatment. Lysates from PFC tissues were immunoblotted using antibodies against APE1, OGG1 and actin. Protein levels relative to those of control animals are shown as the means  $\pm$  SD; \* $p$ <0.05, Student's t-test.



**Figure 4.7:** Levels of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) in the genomic DNA of the in the PFC and hippocampus tissues of chemotherapy-exposed animals. Mean  $\pm$  SD, n=4; \*p<0.05, Student's t-test.



**Figure 4.8:** Levels of DNMT1, DNMT3a and MeCP2 in the PFC tissues of chemotherapy-exposed female animals 3 weeks after treatment. Lysates from PFC tissues were immunoblotted using antibodies against DNMT1, DNMT3a, MeCP2 and actin. Protein levels relative to those of control animals are shown as the means  $\pm$  SD; \* $p$ <0.05, Student's t-test.



**Figure 4.9:** Chemotherapy-induced changes may be connected to the aging-related changes - model scheme.

## TABLES

**Table 4.1:** DAVID annotation analysis. “Count” - shows the number of genes belonging to a defined GO:term in a particular comparison group, “%” – shows the percentage these genes represent out of all genes changed in a particular comparison group, “P-value” – shows significance (only the GO:terms that had p-value lower than 0.05 are shown) and “fold enrichment” – shows fold enrichment (FE) of a certain pathway, calculated according to the following formula:  $FE = n/N / x/X$ , where n – the number of genes involved in a given pathway in a given sample, N – the total number of genes changed in a given sample, x – the total number of genes in the genome belonging to a given pathway, X – the total number of genes in the genome.

Term	Count	%	P-value	Fold Enrichment
<b>PFC_MMC_Female_3 weeks_Up</b>				
olfactory receptor activity	5	16.67	0.037	3.56
<b>PFC_MMC_Female_3 weeks_Down</b>				
positive regulation of Notch signaling pathway	2	1.45	0.036	54.86
neural crest cell differentiation	3	1.45	0.028	11.35
<b>PFC_MMC_Male_3 weeks_Down</b>				
endoplasmic reticulum part	3	21.43	0.006	20.63
endosome	3	21.43	0.009	17.40
<b>HIPP_CPP_Female_3 weeks_Down</b>				
zinc-finger	4	40	0.013	6.62
zinc ion binding	4	40	0.049	3.77
von Willebrand factor, type C	3	30	1.83E-04	130.26

## **CHAPTER 5: GENERAL DISCUSSION AND FUTURE DIRECTIONS**

There is mounting evidence that cancer treatments cause numerous deleterious effects, including central nervous system (CNS) toxicity (Mitchell and Turton, 2011, Wang et al., 2015, CCS, 2015, Steen et al., 2001). Chemotherapy-caused CNS side effects encompass changes in cognitive function, memory, and attention, to name a few (Wang et al., 2015, Soffietti et al., 2014). Even though chemotherapy treatment-induced side effects occur in 16–75% of all patients (Myers, 2009), the mechanisms of these effects are not well understood and need to be further explained. While exposure to ionizing radiation also causes cognitive dysfunction (Loganovsky et al., 2015), induces neuroinflammation and inhibits neurogenesis (Monje and Palmer, 2003, Andres-Mach et al., 2008, Greene-Schloesser and Robbins, 2012), the effects of radiation therapy-like and radiation diagnostics-like exposures (e.g., bystander effects and scatter radiation-induced effects) on the brain need to be analyzed.

In these studies, we investigated the molecular and cellular effects of two main anti-cancer treatment modalities—radiation therapy and chemotherapy—on the brain using established experimental rodent models. Using a rat model, we showed that radiation therapy-like exposures to direct, bystander, and small-dose scatter irradiation cause molecular and cellular changes in the brain and negatively impact the animal's behavior. Using a mouse model, we also determined that cytotoxic chemotherapy drugs CPP and MMC induce oxidative DNA damage and impact molecular and epigenetic processes in the brain.

Both treatments influenced the prefrontal cortex (PFC) more than the hippocampus. This is a novel finding because the majority of animal model-based studies focused on the effects in the hippocampus due to its established role in adult



neurogenesis and memory formation (Andres-Mach et al., 2008, Monje and Palmer, 2003, Rola et al., 2004b, Mustafa et al., 2008, Briones and Woods, 2011, Christie et al., 2012). The PFC, though, has been overlooked in animal models of radiation and chemotherapy treatment despite its key role in regulating crucial executive functions, such as planning, decision-making, behavioural inhibition, and working memory, among others (Faw, 2003, Kolb et al., 2012).

In our study, direct irradiation of the head, bystander irradiation of the liver, and scatter irradiation caused notable and persistent gene expression changes in the PFC tissues of female rats. Likewise, MMC treatment caused alterations of gene expression in the PFC tissues of female mice that persisted for three weeks post treatment. Changes in the hippocampus tissues were small to negligible.

Another finding of our analysis revealed that molecular epigenetic changes induced by chemotherapy and molecular, cellular, neuroanatomical, and behavioural changes induced by cranial, bystander, and scatter radiation treatments showed a sex-difference and were much more pronounced in female animals. The majority of earlier animal studies used male animals (Joshi et al., 2010, Lyons et al., 2011a, Lyons et al., 2011b, Parihar and Limoli, 2013, Christie et al., 2012, Acharya et al., 2015) and, thus, could not provide the complete picture of the brain's response to radiation and chemotherapy treatment. Overall, brain functions are well-documented as being 'sexed' and 'gendered' (Ngun et al., 2011), and numerous sex differences have been documented in autism spectrum disorder (Hu et al., 2015, Mottron et al., 2015), the development of substance use and abuse (Kuhn, 2015), regulation of neuro-inflammatory responses (Acaz-Fonseca et al., 2015), and the effects of adolescent stress

(Pyter et al., 2013), among others. Synaptic patterns and neuronal density are sexually dimorphic, and males and females display dissimilar patterns of transmitting, regulating, and processing biomolecules, including neurotransmitters, and different patterns of behavior in response to certain stimuli, reviewed in (Ngun et al., 2011). Therefore, it is absolutely imperative to use both male and female animals in any model study. An earlier study by Silasi et al. (Silasi et al., 2004) reported significant sex differences in brain responses to single doses and multiple, fractionated doses of direct, total body irradiation.

The mechanisms of sex differences in radiation and chemotherapy responses need to be studied in further detail. These mechanisms may be due to differences in hormonal status and an intricate interplay between gene expression regulation by sex hormones and radiation and chemotherapy (Silasi et al., 2004, Ngun et al., 2011). Furthermore, sex hormone-associated epigenetic changes and chromatin remodelling need to be studied. The outcome of our radiation and chemotherapy analyses may be used as a roadmap for the future examination of occurrences and mechanisms of brain responses to stressors such as chemotherapy and irradiation in males and females.

#### **FUTURE PERSPECTIVES**

In this study, we used the Illumina mRNA profiling technology to determine that radiation and chemotherapy exposures cause gene expression changes in the rodent brain, even though mRNAs constitute only a small portion of cellular RNA makeup. Genome sequencing, as well as recent advances in non-coding RNA biology, have shown that more than 98% of our genes encode RNA molecules that are never translated into proteins (Ponting and Belgard, 2010, Stein, 2004). These non-coding RNAs

(ncRNAs) are structurally and functionally diverse, and many of them partake in regulation of cellular proliferation, differentiation, apoptosis, stress responses, and control of genome stability (Gibb et al., 2011, Iorio and Croce, 2012, Koturbash et al., 2011b), (Mattick and Makunin, 2006). Among the large repertoire of cellular ncRNAs, microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs) are implicated as important players in the regulation of neuronal development and function, aging and neurodegeneration, and a variety of neurological diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, stroke, Huntington's disease and brain cancers, reviewed in (Lardenoije et al., 2015, Szafranski et al., 2015, Iyengar et al., 2014, Kovalchuk and Kovalchuk, 2012).

Previous studies suggest that miRNAs play regulatory roles in gene expression in the brain's responses to total body irradiation (Koturbash et al., 2011a, Shi et al., 2012). Yet, nothing is known on the effects of head, bystander, or scatter irradiation on the brain's miRNAs or piRNAs. Chemo brain has not been explored in terms of the small ncRNA domain. Future research dissecting the effects of radiation and chemotherapy on the non-coding RNAs in the brain is both interesting and important.

In this study, we determined that chemotherapy exposure causes changes in global genome DNA methylation and hydroxymethylation. These epigenetic phenomena are essential regulators of gene expression (Jaenisch and Bird, 2003, Lardenoije et al., 2015, Wen and Tang, 2014). Our data show the overall net changes in the amount of 5mC and 5hmC in the genome but lack details on the genomic distribution and locus specificity of the observed changes. It has been shown that alterations in DNA methylation occur in defined regions (Weber and Schubeler, 2007). Future studies ought

to be conducted to determine the distribution and plasticity of DNA methylation and hydroxymethylation in a quantitative fashion and to correlate the genome-wide and promoter-specific DNA methylation and hydroxymethylation patterns with the levels of gene expression (Weber et al., 2007, Wilson et al., 2006, Wen and Tang, 2014). This will help to analyze the regulation of gene expression by radiation and chemotherapy exposure. In addition, looking into the role of transcription factors in the regulation of gene expression responses to radiation and chemotherapy drugs would be likewise important.

This study focused on the effects of two cytotoxic chemotherapy agents on the brain — MMC and CPP. Central nervous system (CNS) side effects have been reported to occur upon exposure to ‘targeted’ chemotherapy drugs, such as proteasome inhibitors (bortezomib), topoisomerase inhibitors, bevacizumab, trastuzumab, and small molecule tyrosine kinase inhibitors (TKIs), to name a few (Soffietti et al., 2014). Amongst those, bevacizumab is a recombinant monoclonal antibody that blocks angiogenesis by inhibiting vascular endothelial growth factor A. Trastuzumab (i.e., Herceptin) is a monoclonal antibody that interacts with the HER2. Gefitinib is one of many oral small molecule TKIs that block the ErbB-1 receptor (Gupta and El-Rayes, 2008). The molecular targets of many of these agents are involved in cancer, but they may also be important for brain function. Little is known about the effects of targeted drugs on the brain or on the mechanisms of chemo brain induction by these new targeted chemotherapy agents. While new techniques are being developed to better tailor individual drugs to individual patients using new platforms, such as the OncoFinder algorithm (Borisov et al., 2014, Buzdin et al., 2014, Lezhnina et al., 2014), it will also be

important to conduct individualized predictions of any possible side effects, especially severe ones that involve the CNS. Recent modifications to the OncoFinder algorithm allow the personalized screening of nootropic drugs (Jellen et al., 2015). With thorough animal studies, OncoFinder may be further developed and enabled to predict possible targeted chemotherapy-induced brain side effects.

Here we have shown that radiation exposure causes molecular epigenetic, neuroanatomical, and behavioral changes in the exposed animals. Given that chemotherapy exposure led to molecular epigenetic changes, analyzing neuroanatomical and behavioral post-chemotherapy outcomes is an interesting area for future study. Moreover, our studies and the available data on chemo brain used healthy animal models that, while treated with chemotherapy drugs, lacked one important component—the presence of an actual tumor. To gain a full understanding of the molecular mechanisms and pathways affected in chemo brain, it is essential to study chemo brain in tumour-bearing animals. Such models have been developed using TumorGraft<sup>®</sup> technology, created by Champions Oncology (Hidalgo et al., 2011). With TumorGraft<sup>®</sup> technology, a piece of a patient's tumor is removed and engrafted into immune-deficient mice. The mice are then subjected to chemotherapy treatments in order to determine the best one for each individual tumor. It can be hypothesized that chemo brain will manifest itself in tumor-bearing mice and will be more pronounced in the treated animals than in the untreated ones, while the presence of a tumour itself will also affect molecular networks in the brain.

Like the effects of chemo brain, the effects of bystander and scatter-radiation should also be studied using tumor-bearing animals. This will allow an examination of

the roles of tumors and therapy on the brain. It will also yield important information on the relative extent of brain side effects caused by effective and ineffective treatments.

On another note, the phenomena of chemo brain and radiation brain have not been fully explored in the ageing domain. Chemotherapy and radiation therapy may cause changes leading to neuroinflammation and brain aging. The mechanism and role of cancer treatment-caused aging-related changes need to be analysed, as this will allow the development of strategies for prevention and mitigation of treatment-induced neurodegeneration and aging.

Even more crucial would be the study of chemo- and radiation treatment side effects in adolescents and children. For children in developed countries, cancer is the second most common cause of death after accidents. There are 10,000 children living with cancer in Canada today, and 1500 new cases are diagnosed each year. Among these, leukemia is the most common pediatric cancer, accounting for 30% of all malignancies diagnosed annually in children aged younger than 15 ([http://childhoodcancer.ca/education/facts\\_figures](http://childhoodcancer.ca/education/facts_figures)). In 1960, the survival rate of pediatric leukemia patients was very low, at about 10%. Nowadays, 80–85% of leukemia patients survive, but many of them suffer debilitating side effects, including severe manifestations of chemo brain, leading to huge losses in productive years of life. In the future, animal model studies can help shed light on the molecular mechanisms and behavioral repercussions of pediatric radiation and chemo brain effects.

One other poorly studied aspect of chemo brain and irradiation effects is the possibility of treatments that might reverse, or at least reduce, the behavioral effects of the cancer treatments. Such treatments could be based upon treatments devised for

rehabilitation after brain injuries in animal models such as complex housing, exercise, tactile stimulation, and psychomotor stimulants, among others. Because radiation exposure (direct, bystander, and scatter) affects dendritic space, reduces the brain's ability to produce new neurons, and alters behavior, mitigation efforts should focus on restoring these parameters.

In recent years, much effort has been focused on developing new strategies for the prevention and mitigation of deleterious radiation effects on healthy tissues and organs, including the brain. These include numerous studies of natural products and medicinal plants with radio-protective properties, reviewed in (Arora et al., 2005), but their use in brain radiation protection has not been fully explored. The concurrent use of natural products and anti-cancer therapies may result in unknown deleterious side effects due to possible interactions between the components and metabolites of natural products with allopathic medications. Moreover, the safety and efficacy of the vast majority of natural products and herbal remedies have not been properly evaluated in large-scale clinical trials.

Some recent efforts have focused on drug repurposing and used current agents with neuroprotective properties to ameliorate radiation effects. Amongst those, the minocycline antibiotic, which was shown to have neuroprotective properties in several experimental models of neurological diseases, was reported to improve the cognitive performance of rats after high dose whole brain irradiation (Zhang et al., 2014). Histone deacetylase inhibitor valproic acid and L-alpha-glycerylphosphorylcholine (GPC) were reported to have radioprotective effects in the rat brain (Zhou et al., 2015, Plangar et al., 2014). GPC treatment led to significant protection against cognitive decline and cellular

radiation damage (Plangar et al., 2014). Even though these studies suggest some progress in the search for radiation neuroprotectors, applications of pharmacological and natural product-based regimens after radiation exposure are still considered difficult and impractical.

However, environmental enrichment and exercise may provide a plausible avenue for exploration of brain irradiation protection. Studies have explored the positive effects of environmental enrichment and exercise on the brain. Exercise was reported to boost neurogenesis, increase synaptic plasticity in the hippocampus, and improve cognitive function, spatial memory, and learning in rodents (Arida et al., 2011). Exercise regimens augmented cognitive function in several brain disease models, including delaying age-related cognitive decline; preventing and mitigating cognitive deficits in models of Alzheimer's, Parkinson's and Huntington's diseases; assisting in recovery from brain injury; and protecting from deleterious effects of chronic stress (Pang and Hannan, 2013). In humans, exercise and physical activity improved cognitive function and was associated with a reduced risk of dementia and Alzheimer's disease (Verdelho et al., 2012, Scarmeas et al., 2009).

Several studies have shown that exercise can help prevent the loss of learning and memory after high-dose whole brain irradiation (Naylor et al., 2008, Wong-Goodrich et al., 2010). Additionally, radiation effects could be mitigated by environmental enrichment that included access to a running wheel (Fan et al., 2007). These data were further substantiated by Ji et al. who showed that the enrichment and exercise-induced protective effects of radiation-induced cognitive impairments were associated with increased levels of BDNF (Ji et al., 2014).



Environmental enrichment is a much broader concept than exercise alone. Environmental enrichment constitutes exposure of animals to positive physical and social stimulation that is superior to their routine housing and care conditions (Rosenzweig and Bennett, 1996). Physical enrichment includes structural modifications to animal housing, such as increased floor space and the introduction of structural features and objects that allow animals to climb, run, jump and explore new areas, interact, play, and exercise. These often include additional nesting materials, plastic toys, tunnels, and running wheels, climbing ladders, swings, etc. These objects are changed regularly to stimulate the exploration and curiosity of animals. Social enrichment refers to housing animals in groups that allow them to play and interact with each other. Ideally, both physical and social enrichments have to be combined (Johansson and Ohlsson, 1996), (Simpson and Kelly, 2011).

Several studies have shown the positive effects of environmental enrichment on animal's development and better recuperation in test models of various neurological conditions, including Alzheimer's disease, Huntington's disease, Rett syndrome, and stroke (Hirase and Shinohara, 2014, Pang and Hannan, 2013, Nithianantharajah and Hannan, 2006). Both physical and social environmental enrichment synergistically influence brain plasticity, increase neurogenesis, and enhance learning and memory (Bekinschtein et al., 2011). Moreover, parental environmental enrichment was reported to be crucial for animal development (Mychasiuk et al., 2012).

Since environmental enrichment was reported to have numerous positive, protective, and mitigating effects in models of neurological diseases and animals exposed to high doses of whole brain irradiation, one could predict that environmental

enrichments may be very effective to counteract deleterious neuroanatomical and behavioural effects of low dose head, bystander, and scatter irradiation. In a set of initial studies, we compared the effects of low dose head, bystander, and scatter irradiation on animals housed in both the environmental enrichment condos and standard housing.

We used the same environmental enrichment approach as previously described by Mychasiuk et al. (Mychasiuk et al., 2012). The environmental enrichment condo constituted a large steel cage (2 ft × 4 ft × 6 ft) with three levels that were connected by ramps and bars, which allowed animals to climb and explore and thus have more movement and exercise. The enrichment condo also contained diverse "toys" that were changed on a weekly basis to continuously encourage interest and exploration. Moreover, different food treats, such as peanut butter, fruit and Cheerios, were placed around the condo in order to promote more exploration, climbing and activity. Four animals were housed per condo. The standard housing condition consisted of regular shoebox cages (48 cm × 25 cm × 20 cm). The standard housing animals received the same food treats as offered to the enriched house animals (Mychasiuk et al., 2012).

We noted that the irradiated animals that were housed in enriched condos exhibited fewer radiation-induced behavioural deficits than those housed in standard conditions. Moreover, enriched condo conditions also ameliorated radiation-induced neuroanatomical changes. These studies must be continued and further substantiated and may serve as a foundation for the development of new methods to prevent low-dose radiation effects on the brain. Such methods may in turn be important because low dose brain radiation exposure occurs during radiation therapy and diagnostics and in occupational and environmental conditions.

Preclinical animal model data can serve as a foundation for the research and development of new chemo brain and radiation brain biomarkers. Our studies can be used as a roadmap for the development of tests that will predict sensitivity to radiation and chemo brain side effects. To find effective biomarkers, molecular changes observed in the brain must first be correlated with those observed in blood. Those markers (small RNAs or mRNAs) that will be correlated between blood and the brain in animal models may be further explored to determine their usefulness in human studies. Last, but not least, animal models may be used to develop future strategies and interventions to prevent and mitigate chemo brain and radiation-brain.

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