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Effects of Proton Radiation and Pomegranates on Hippocampus and Behavior

Melissa S. Dulcich
Loma Linda University

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The Effects of Proton Radiation and Pomegranates on Hippocampus and Behavior

by

Melissa S. Dulcich

A Dissertation submitted in partial satisfaction of The requirements for the degree of Doctor of Philosophy in Experimental Psychology

June 2013
Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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<td>5-bromo-2’-deoxyuridine</td>
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<td>DCX</td>
<td>Doublecortin</td>
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<td>DG</td>
<td>Dentate Gyrus</td>
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<td>NASA</td>
<td>National Aeronautics and Space Administration</td>
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<td>LEO</td>
<td>Lower Earth Orbit</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>SPE</td>
<td>Solar Particle Event</td>
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<td>GCR</td>
<td>Galactic Cosmic Radiation</td>
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<tr>
<td>LET</td>
<td>Linear Energy Transfer</td>
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<tr>
<td>MeV/n</td>
<td>Megaelectron Volts per Nucleon</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
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<tr>
<td>SI</td>
<td>Standard Internationale</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>SGZ</td>
<td>Subgranular Zone</td>
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<td>GCL</td>
<td>Granular Cell Layer</td>
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<td>AD</td>
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<td>HZE</td>
<td>High-Ionizing High-Energy Particles</td>
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<td>EVA</td>
<td>Extra-vehicular Activity</td>
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<td>TST</td>
<td>Tail Suspension Test</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
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<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
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<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>ROI</td>
<td>Region of Interest</td>
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<tr>
<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
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<tr>
<td>SPSS</td>
<td>Statistical Product and Service Solutions</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>PJ</td>
<td>Pomegranate Juice</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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ABSTRACT OF THE DISSERTATION

The Effects of Proton Radiation and Pomegranates on Hippocampus and Behavior

by

Melissa S. Dulcich

Doctor of Philosophy, Graduate Program in Experimental Psychology
Loma Linda University, June 2013
Dr. Richard E. Hartman, Chairperson

Exposure to ionizing radiation may have deleterious effects on physical and mental health, with an increased risk of proton radiation for astronauts traveling outside Earth’s atmosphere into lower earth orbit. In animal models, radiation has been shown to suppress neurogenesis in the subgranular zone of the hippocampus, a key area for learning and memory. Furthermore, some evidence suggests that compounds found in fruits and vegetables (e.g. polyphenols) may offer some protection against the cellular effects of radiation. Few studies have looked at the effects of proton radiation on the central nervous system, even though proton radiation is the most prevalent ionizing radiation in space. This study determined whether a polyphenol-rich diet could offer enough protection from the effects of radiation to maintain complex cognitive and fine motor skills such as those required by astronauts. Ninety-six C57BL/6 mice (48 receiving pomegranate juice and 48 receiving sugar water control in their drinking bottles) underwent a battery of behavioral tests to assess baseline cognitive and motor functions and were then either irradiated with proton radiation (2 Gy at 150 MeV/n at 1-2 Gy/min) or sham irradiated. 2 Gy is the approximate dose of radiation to which an astronaut on a long-term mission to Mars may be exposed. Post-radiation behaviors were assessed 1-2
days after irradiation and again two months later to determine radiation-related behavioral changes. Radiation affected depression- and avoidance behaviors. Pomegranate juice was found to be protective against radiation-induced depression, but had gender-specific affects for avoidance behavior. In addition, pomegranate juice seemed to have a greater effect on males than females, inducing behavior similar to that of females in tests of motor coordination and activity levels. Furthermore, the birth of new glial cells and neurons in the hippocampus was quantified using immunohistochemistry for BrdU+/DCX+ cells. Radiation suppressed cell proliferation (BrdU+) and neurogenesis in the dentate gyrus (BrdU+/DCX+) of the hippocampus. Pomegranate juice increased neurogenesis (DCX+) in the hippocampus of non-irradiated mice. These data highlight the importance of understanding the complex gender-specific interactions between diet, behavior, and neuropathology, and suggest that pomegranate juice may protect against some radiation-induced deficits.
CHAPTER ONE
INTRODUCTION

Since its launch in the 1960s, the space program has been vital in its contribution to knowledge in several fields. With its dedication to exploration, technology development and scientific research, the National Aeronautics and Space Administration (NASA) programs have increased our knowledge in areas such as astronomy, crystal growth, space physics, aeronautics, and astrobiology, to name a few. In addition, these programs have allowed us to understand our planet more completely, while simultaneously exploring the possibility of expanding human habitation out into space.

NASA’s Space Transportation System program, the manned launch vehicle program that began in 1981, was scheduled for mandatory retirement in 2011 (President Bush offers new vision for NASA, 2004). It was to be replaced with the Constellation program, an elaborate design focusing on exploration outside the Earth environment. However, in 2010, President Obama announced plans for NASA to initiate the National Space Policy of the Obama administration in its stead (The White House, 2010). In this scaled back version of the Constellation program, it is projected that a manned mission to Mars will take place by 2035, preceded by an asteroid mission by 2025. Missions to date have always yielded the concern of radiation exposure effects on astronauts; yet, expanding exploration outside of lower earth orbit (LEO) into deep space missions can leave astronauts vulnerable to the risks of increased radiation due to the length of time these missions will require. The outcome on the physical and mental health of astronauts due to increased radiation of these longer missions, as well as possible therapeutic interventions, needs to be explored.
Ionizing Radiation

Ionizing radiation is a major concern for astronauts (Durante & Cucinotta, 2008; White & Averner, 2001). Ionizing radiation consists of high-energy charged particles (protons, electrons, and atomic nuclei) and high energy electromagnetic radiation (gamma- and X-rays) that can easily pass through barriers such as human tissue (Buckley Jr., 2006). In addition, neutrons, which have no charge, can also produce ionization as they collide with protons, which in turn impact electrons leading to the formation of ions. Ionization occurs when high-energy photons or particles interact with electrons and nuclei in atoms. Energy is transferred from the radiation to the atom, ejecting electrons from their orbit around the nucleus, thus resulting in a charged particle (Schobel et al., 2010). Ionizing radiation can cause damage in living tissue when the ejected electrons collide with and excite organic molecules. These excited molecules can become ionized, thus creating free radicals, a major player in oxidative stress-induced cell damage (Joubert & Foray, 2007). In addition, radiation can also cause cellular damage when it directly ionizes molecules such as DNA, thereby damaging key components of cell stability (Foray, Arlett, & Malaise, 1997; Joubert et al., 2008). In comparison, non-ionizing radiation, such as microwaves, radio waves, and visible light, cannot remove electrons from their orbit and therefore yields less biological damage in comparison to ionizing radiation.

 Ionizing radiation comes from three sources: the Earth (magnetic core, medical procedures, nuclear devices), the sun (solar wind, solar particle events or SPEs) and the Milky Way Galaxy (galactic cosmic radiation or GCR) (Radiation & Air, 2007). Living organisms on Earth are largely protected from excessive ionizing radiation due to the
Earth’s mass and atmosphere. In addition, since ionizing radiation consists of charged particles, its pathway can be altered when it encounters Earth’s magnetic field, pulling ions away from the bulk of the Earth toward the polar regions, thus further protecting the inhabitants of Earth from extensive amounts of radiation-induced damage. Although this magnetic field is largely protective, particle radiation can become trapped and held by Earth’s magnetic core within this field, creating a space around the Earth called the radiation belts or Van Allen belts. The Van Allen belts consist of two zones, largely composed of protons and electrons, with the outer zone largely made up of protons and the inner zone of electrons (Maalouf, Durante, & Foray, 2011). Astronauts in lower Earth orbit (LEO) are often at risk from this trapped radiation (Nachtwey & Yang, 1991). Also, astronauts on interplanetary missions must pass through these belts en route to Mars or to asteroids and are at increased risk of radiation exposure in this space (Nachtwey & Yang, 1991). Furthermore, once astronauts on deep space missions do pass through the magnetic field, they will be exposed to even greater levels of space radiation emitting from the sun (solar wind, SPEs) and the galaxy (GCRs) (Hellweg & Baumstark-Khan, 2007; Simonsen, Wilson, Kim, & Cucinotta, 2000).

All ionizing radiation from space consists mainly of protons and other charged particles. Solar wind is a consistent source of radiation and contains many chemical elements, although it primarily consists of protons and electrons. SPEs (solar flares and coronal mass projections) pose an even greater risk for astronauts (Hellweg & Baumstark-Khan, 2007; Simonsen, et al., 2000). A solar flare, consists of 92% protons, 6% helium nuclei and less than 2% heavier nuclei (Maalouf, et al., 2011) and emits a massive flow of ionizing radiation. Directly following a SPE, there is an electromagnetic
energy burst that can reach the Earth in approximately 8 minutes. This precedes a flux of high energy charged particles that travels at much higher speeds in comparison to solar wind, reaching the Earth in tens of minutes (Phillips, 2005). This type of event can leave an astronaut outside the magnetosphere vulnerable to dangerous levels of radiation (Shea & Smart, 1994). For example, the Apollo missions had to leave the magnetosphere on their way to the moon. Between Apollo 16 and Apollo 17 (August 1972), a SPE occurred that would have been lethal to astronauts had it occurred while they were exposed on the surface of the moon (Townsend, Shinn, & Wilson, 1991). Due to the length of space flight necessary for deep space missions, the likelihood of an astronaut encountering at least one SPE is extremely high. For example, a manned trip to Mars would require at least a total of 8-12 months of travel round trip to Mars and back as well as a stay of 12-18 months on Mars (Mewaldt et al., 2005). It is almost certain astronauts will encounter one SPE during this time, and encountering two SPEs is very likely.

Galactic cosmic rays (GCRs), the major source of radiation danger for astronauts, consist of high energy, high mass particles flowing into our solar system from our Milky Way Galaxy and beyond (Hellweg & Baumstark-Khan, 2007). GCRs are primarily subatomic particles such as protons, electrons and atomic nuclei stripped of all electrons due to their speed of travel. Specifically, GCRs are composed of 87% protons, 12% helium nuclei, 2% electrons and 1% heavier nuclei (Hellweg & Baumstark-Khan, 2007). The amount and type of ionizing radiation present can change with the 11-year solar cycle. For example, during solar minimum (low solar activity), GCRs increase while SPEs decrease; this relationship reverses during solar maximum (high solar activity) (Badhwar & O'Neil, 1994; Benton & Benton, 2001). In fact, at solar minimum, the
radiation level obtained from GCRs is similar to the current annual limits for astronauts on LEO missions (Mewaldt et al., 2005). GCRs are difficult to shield against, and are therefore a major source of concern for manned flights to Mars.

*Proton Radiation*

Early research concerned with the effects of ionizing radiation on astronauts used gamma- and X-radiation. Recent research is focusing on heavier ions, such as iron, due to the extensive damage these ions can cause in living tissue. However, proton radiation, which constitutes the largest percentage of space radiation for both GCRs and SPEs, can also be detrimental to the health of the astronaut (Hsiao & Stewart, 2008), and should therefore be a continued focus of study.

Proton radiation is a high energy charged particle radiation with low linear energy transfer (LET) properties, with a peak energy range of approximately 150 MeV/n. Charged particle radiations deposit the total energy to a limited range of cells, unlike photon radiations (i.e. gamma and X-rays) which demonstrate exponential absorption at low levels, thus dispersing and minimizing tissue damage. If photon radiation increases, each cell receives more radiation, but if charged particle radiation increases, the number of cells receiving radiation increases. In addition, as protons collide with other matter, they produce secondary particles (other protons, neutrons, and alpha particles) that can extend the ionization trajectory of the original particles. Secondary electrons (δ-rays) are also produced, and their ejection from the ionization path can also affect surrounding tissue. Finally, as charged particles pass through matter, they ionize and lose energy along the path trajectory. As proton radiation traverses materials, the density of ionization
increases prior to the end of its movement, reaching a maximum shortly before the energy reaches zero. This is known as the Bragg Peak. This is unlike photon radiation, which mainly loses energy exponentially. The nature of charged particle radiation leads to a greater biological impact compared to that of photon radiation (Ghosh, Narang, Sarma, & Krishna, 2011; Hada & Sutherland, 2006). Proton radiation is considered to be a form of low-LET radiation, along with gamma- and X-radiation, thus depositing less energy along its path through tissue; it was previously thought that this implied levels of cellular damage similar to those seen in photon radiation. However, research suggests that proton radiation can elicit similar damage to that seen in high-LET radiations (such as iron) (Hada & Sutherland, 2006), indicating that the trajectory of proton particle radiation, compared to photon radiation, may lead to differences in biological effects.

**Biological Effects of Ionizing Radiation**

On Earth, we are exposed to radiation daily; however, for most humans, this background radiation equals approximately 0.5 milli-Gray per year (mGy/yr) and is not enough to cause significant health issues (Todd, 2003). The Gray is a Standard Internationale (SI) unit that measures absorption of ionizing radiation. Specifically it measures one joule of ionizing radiation per kilogram of mass (Measures, Laboratory, & Standards, 1970), and can be applied to any matter (humans, rocks, etc). As the NASA program heads in the direction of long-term space missions, the biological effects of this absorbed radiation from possible constant exposure of solar winds, SPEs, GCRs, and radiation belts, are a focus of much concern. Overall, an astronaut on a three-year mission will be exposed to at least 1 Gy and possibly up to 2 Gy radiation (Parsons & Townsend,
2000; Simonsen, et al., 2000; Stephens, Townsend, & Hoff, 2005). Furthermore, the negative effects of radiation exposure can continue long after astronauts return to Earth. Increased exposure can lead to accumulation of radiation-induced tissue damage and inflammation that can result in loss of cells and possible physical changes in the organism both during and after prolonged radiation exposure (Hellweg & Baumstark-Khan, 2007). Astronauts must maintain good physical and mental health in order to perform complex cognitive and motor skills on the job; therefore it is vital that the biological effects from radiation exposure are well understood, so that healthy functioning levels can be maintained during and after these long-term missions.

Effects of Ionizing Radiation on Cellular Tissue

The biological effects of ionizing radiation can occur short term or long term and include radiation sickness, immune damage, cardiovascular problems, muscle and bone loss, cataracts and cancer (Buckley Jr., 2006). Additionally, a growing body of evidence is demonstrating the deleterious effects ionizing radiation can have on the central nervous system (CNS). Specifically, cells in the hippocampus area, a region that helps process learning and memory, could be hit and damaged with GCRs (Curtis et al., 1998). In fact, research to date indicates that the effects of ionizing radiation on the CNS yields effects similar to those seen in senescence or Alzheimer’s disease, a neurodegenerative disease of age (Lowe, Bhattacharya, Marchetti, & Wyrobek, 2009; Vlkolinsky et al., 2010). The full extent of damage to the CNS from ionizing radiation is difficult to predict; however, this remains an important area to research as cell loss in the CNS can be permanent since there are few actively dividing cells in this system.
Radiation-induced biological damage occurs when ionizing radiation transfers energy to tissue molecules causing them to become ionized and excited. Additionally, these ionized molecules may produce a secondary flux of ionizing particles. This process can result in oxidative stress-induced cell damage (Acharya et al., 2010). Oxidative stress occurs when there is an imbalance between the cells oxidation and antioxidant processes (Halliwell, 1985). When radiation energy hits human tissue, it produces an overabundance of oxidation, including reactive oxygen species (ROS; i.e., free radicals, peroxides) via modulation of genes involved in oxidative stress (Baluchamy et al., 2010a), thus leading to an imbalance of these processes. The brain is especially vulnerable to this process, due to its high oxygen content and low antioxidant properties. In healthy tissue, ROS production aids in the aging process and normal cellular functions; however, in excess, it can trigger damage to DNA, proteins and lipids (Mitchell, Russo, Kuppusamy, & Krishna, 2000; Wu et al., 1999). Most molecular damage resulting from low exposures of radiation can be repaired (Jaroudi et al., 2009). Even damage to the more complex DNA strands can be ameliorated or tolerated if the damage is not too extensive. Yet increased exposure to ionizing radiation can lead to single and double-strand DNA breaks, affecting vital gene expressions (Hsiao & Stewart, 2008; Tariq et al., 2011). In fact, DNA fragmentation has been found to occur with several different types of ion radiation: proton, carbon, helium, iron and gamma (Alloni et al., 2010). If cellular processes cannot repair this damage, the result is cell injury, carcinogenesis, exacerbation of the aging process, and apoptosis (programmed cell death) (Jaroudi, et al., 2009; Spitz, Azzam, Li, & Gius, 2004).
Furthermore, ionizing radiation has been shown to induce a modulation of genes involved in complex signaling pathways resulting in an upregulation of microglial inflammation and pro-inflammatory cytokines within the central nervous system (Baluchamy et al., 2010b; Cameron, Tanapat, & Gould, 1998). Pro-inflammatory cytokines are protein molecules involved in immune response that send signals to other cells and induce inflammation in response to injury (Cheung et al., 2002; Dinarello, 2000), subsequently increasing ROS expression (Little, 2000). This radiation-induced inflammation and pro-inflammatory cytokine increase has been found to induce stress hormones that can block neurogenesis (the production of new neurons) in certain brain regions such as the hippocampus (Cameron, et al., 1998; Rola et al., 2008). New neurons are produced in the dentate gyrus of the hippocampus throughout life in the mammalian brain. Active neural precursor/stem cells in the subgranular zone (SGZ) produce new granule cells for the dentate gyrus; these cells migrate to the granular cell layer (GCL) and molecular layer and “plug in” (Eriksson et al., 1998; van Praag et al., 2002). As we age, this process can slow down (Bernal & Peterson, 2011; Lazarov, Mattson, Peterson, Pimplikar, & van Praag, 2010); in addition, diseases of age, such as Alzheimer’s disease (AD), can also inhibit this process (Gang et al., 2011; Rodriguez & Verkhratsky, 2011).

Several animal studies demonstrate that radiation insult can induce apoptosis and block neurogenesis in the hippocampus, thereby accelerating this aging process (Yang et al., 2010). For example, adult mice exposed to 5 Gy cranial gamma irradiation demonstrated neural apoptosis and decreased neurogenesis in the dentate gyrus of the hippocampus (J. S. Kim et al., 2010). Also, high doses of X-radiation, a high-energy electromagnetic radiation similar to gamma radiation, induced apoptosis and inhibited
neurogenesis in the subgranular cells of the dentate gyrus in adult C57BL/129S wildtype mice (Li, Aubert, & Wong, 2010; Tada, Parent, Lowenstein, & Fike, 2000); this inhibition of neurogenesis was also seen one month post-6Gy X-radiation (Manda, Ueno, & Anzai, 2009). Furthermore, this X-ray dose-dependent inhibition of neurogenesis was associated with significant inflammation in C57BL mice receiving whole brain irradiation (Mizumatsu et al., 2003). These deficits are also seen in younger animals. Juvenile rats irradiated with 1, 2, or 3 Gy X-irradiation also demonstrated significant decrease of brain weight and multiple apoptotic cells in the sub-ventricular zone of the dentate gyrus of the hippocampus six hours after irradiation. The sub-ventricular zone is another site of neural stem cells and is known for neurogenesis (Lim & Alvarez-Buylla, 1999). This suggests that a buildup of irradiation may cause irreversible damage on neural stem cells resulting in brain growth retardation and dysfunction (Amano et al., 2002). Further evidence demonstrates that prevention of inflammation can reverse these deficits. One study demonstrated that indomethacin, which blocks inflammation, was able to increase neurogenesis after cranial irradiation (Monje, Toda, & Palmer, 2003).

Little research has been done on the biological effects of proton radiation in the hippocampus, yet some evidence indicates similar effects are seen here. Exposure to proton radiation was shown to enhance caspase-3 production and apoptotic DNA fragmentation in the brain of mice (Baluchamy, et al., 2010b); caspase 3 is known to play a central role in cell apoptosis. Also, proton radiation may lead to continued altered functioning in the CNS. One study demonstrated that, although low doses of proton radiation led to transient decrease in erythropoiesis, a process involving the red bone
marrow in which red blood cells are produced, changes in neuronal tissues were seen many weeks after irradiation (Chang et al., 2010).

Thus, oxidative stress, leading to DNA fragmentation and inflammation, seems to be a key factor in the inhibition of neurogenesis and possible apoptosis, and finding ways to reduce the impact of these factors may protect against these effects.

**Effects of Ionizing Radiation on Cognition and Behavior**

Hippocampal neural deficits are often associated with poor behavioral performance on tests that measure learning and memory (Manda, et al., 2009). As previously mentioned, radiation-induced deficits emulate Alzheimer’s disease, the most common type of dementia, and, in a rat model for dementia, after animals were injected with beta-amyloid peptides (plaques causing neural deficits), neuronal apoptosis within the hippocampus increased significantly. Furthermore, the amount of apoptosis correlated with learning and memory deficits in the treated group as compared with control and sham groups (Guo, Deng, Wang, Huang, & Guan, 2011).

In addition, ionizing radiation-induced apoptosis and reduced neurogenesis in the hippocampus has also been found to result in significant learning, memory, and motor deficits in animals. In one study, 2x5 Gy gamma radiation doses, delivered at differing time points for a total of 10Gy, impeded neurogenesis in the dentate gyrus of the hippocampus and caused significant deficits in an inhibitory avoidance memory task in rats; these results were not seen in the sham-treated animals (Jahanshahi, Khoshbin Khoshnazar, Azami, & Heidari, 2011). Furthermore, this fractioned dose was more effective at inhibiting neurogenesis compared with a single dose of 10Gy (Jahanshahi, et
al., 2011), indicating that extensive exposure to ionizing radiation over time can induce more severe neurological effects than a one-time exposure. Additionally, young mice pups demonstrated learning deficits associated with inhibition of neurogenesis in the granule layer of the hippocampus when irradiated with a 6 Gy radiation dose (Barlind, Karlsson, Bjork-Eriksson, Isgaard, & Blomgren, 2010). Mice irradiated with 5 Gy X-rays, demonstrated decreased spatial memory retention that was correlated with inhibited hippocampal neurogenesis (Rola, Raber, et al., 2004).

These effects can be seen long term as well. Juvenile mice, exposed to 0.35 and 0.5 Gy X-radiation in utero, demonstrated significant impairment in a post natal spatial discrimination learning task, and higher radiation dosage was associated with increased deficits (Sienkiewicz, Haylock, & Saunders, 1994). High energy charged particles (HZE), such as the heavier ions also lead to significant spatial learning and memory deficits, as measured by the radial arm maze, 9 months post-radiation (Shukitt-Hale, Casadesus, Cantuti-Castelvetri, Rabin, & Joseph, 2003). Also, motor deficits similar to those seen in Parkinson’s disease have been found in rodents exposed to high-energy particles (Joseph, Erat, & Rabin, 1998).

Finally, long-term missions can lead to deleterious psychological effects that may be exacerbated by conditions of extensive radiation exposure. Although there is a careful screening process to select mentally healthy individuals that can withstand the rigors of this high stress job, several factors stemming from the high amounts of stress of an astronaut’s job can take their toll mentally and physically and can contribute to psychological problems such as clinical depression and anxiety (Newberg, 1994). Chronic isolation, low light levels, too little or too much work, separation from family,
lack of sleep and fatigue, dissatisfaction with the mission, and relationship conflict can all lead to depression in astronauts during a mission (Buckley Jr., 2006; Tarzi, Kennedy, Stone, & Evans, 2001). In addition, anxiety can result from the apprehension that accompanies the hazards of deep space travel. Furthermore, a condition known as asthenia may occur. Asthenia (aka neurasthenia) is not classified as a distinct disorder in the United States. In the Diagnostic and Statistical Manual IV-TR (APA, 2000), it is considered an undifferentiated somatoform disorder. Asthenia has been diagnosed, however, within the Russian space program. Under that system, symptoms of asthenia include, fatigue, irritability, sleep disturbances, conflicts, other symptoms similar to clinical depression, and cognitive disturbances in learning and memory, leading to performance errors (N. Kanas et al., 2001). Altogether these psychosocial factors can inhibit the success of space missions. There is a strong connection between the immune system, the brain and psychosocial factors such as depression & anxiety (Conti & Fulcheri, 2010). Individuals with anxiety or mood disorders have a much poorer prognosis for most diseases, disorders, and injuries (Maes, Kubera, Obuchowiczwa, Goehler, & Brzeszcz, 2011). In addition, depression has been associated with increased oxidative stress and decreased antioxidant enzyme activity, leading to subsequent DNA damage (Maes et al., 2011). Furthermore, depression has been shown to possibly occur in response to neuroinflammation, increased oxidative stress, decreased neurogenesis and increased apoptosis (Maes, Kubera, et al., 2011). Stress can also lead to an increase in inflammatory immune response, and aging has been shown to exacerbate this association (Sparkman & Johnson, 2008). Oxidative stress has also been shown to induce stress related “anxiety-like” behaviors and downregulate antioxidant enzymes while promoting
neuroinflammatory factors (Salim et al., 2011). This suggests that stress-related anxiety and depression may result from radiation-induced oxidative stress and subsequent apoptosis and decreased neurogenesis. In fact, one study demonstrated that X-irradiation simultaneously led to deficient neurogenesis and increased anxiety-like and depression-like behavior in mice (Snyder, Soumier, Brewer, Pickel, & Cameron, 2011). Therefore, therapeutic interventions that reduce oxidative stress and/or neuroinflammation may ameliorate depressive- and stress related “anxiety-like” behaviors. Very little research has been done in this area, yet understanding this link between psychosocial factors and neurophysiological responses is clinically relevant for possible future interventions for long-term mission astronauts, since the effects of stress-related anxiety and depression can lead to mission failure.

**Protective Effects of Antioxidants in Radiation-induced Deficits**

It has been estimated that 2/3 of the damage caused by radiation is mediated by free radicals (Hall, 1994). Antioxidants inhibit the oxidation of other molecules and can either chemically repair damaged molecules or interfere with damaging radicals before they can insult tissue (Weiss & Landauer, 2000). In addition, they can indirectly attenuate inflammation and apoptosis (Rasheed, Akhtar, & Haqqi, 2010). There are several different types of antioxidants, including drugs (i.e., Edaravone, amifostine) and the naturally occurring compounds, such as vitamins and those found in fruits and vegetables. Some types seem to be more effective than others in warding off the negative effects of radiation-induced damage.
Antioxidant Drugs

In one study, Edaravone, a neuroprotective agent and a novel free-radical scavenger, was found to protect human neural stem cells from radiation-induced apoptosis in the subgranular zone of the dentate gyrus post radiation. Furthermore, Edaravone ameliorated spatial memory deficits in the Morris water maze (Motonura, Ogura, Natsume, Yokoyama, & Wakabayashi, 2010). However, other drugs have not been as effective. Amifostine, a cytoprotectant used to counteract the side effects of chemotherapy radiation exposure during treatment of cancers, was not found to be radioprotective in radiation-induced brain injury and cognitive deficits in rats (Gorker et al., 2011); in addition, there are several adverse effects found with amifostine treatment, including nausea, diarrhea, vomiting, and hypotension. Tempol, a nitrooxide antioxidant, has been found to protect against cellular apoptosis and neural inflammation in the brains of animals (Wilcox, 2010). However, Tempol and other nitrooxide antioxidants can be neurologically toxic. High levels of these antioxidant agents were found to increase excitatory potentials and epileptiform activity in the hippocampus of guinea pigs (Hahn, Lepinski, DeLuca, Mitchell, & Pellmar, 1995). In addition, to date, Tempol has only been used in humans as a topical drug to protect against radiation-induced alopecia (a loss of hair from the head or body), and more research would need to be done to assess its radioprotective abilities and safety for astronauts. Due to the expense of manufacture and the possible adverse effects of antioxidant agents, research has focused on the naturally occurring antioxidants as radioprotectants.
Naturally Occurring Antioxidants

Vitamin E, vitamin C, and cysteine are naturally occurring antioxidants that also have been shown to ameliorate the toxic effects of radiation. Cysteine, a compound found in red peppers, garlic, and broccoli, was among the first antioxidants to demonstrate radioprotective abilities in animals (Weiss & Landauer, 2000); however, at high levels, this antioxidant can be toxic to brain cells (Roberts, Koch, Detrick, Warters, & Lubec, 1995). In animal studies, vitamins C and E (alpha-tocopherols), have both been found to protect against radiation-induced DNA damage (Pence & Yang, 2000). Also, fetuses administered vitamin E in their drinking water and then X-irradiated, showed an increase in DNA concentration and a decrease in lipid peroxide formation compared to rat fetuses treated only with water, thus demonstrating the radioprotective effects of vitamin E on neural development (Tanaka, Iwasaki, Inomata, Nasu, & Nishimura, 1986). Pretreatment with Vitamin E also demonstrated its radioprotective effects against DNA damage in mouse leukocytes (Konopacka, Widel, & Rzeszowska-Wolny, 1998). In humans, dietary vitamin E & C, has been associated with a decreased risk of radiation-induced cancer (Kennedy & Todd, 2003). The benefits of these vitamins in protecting against ionizing radiation-induced CNS insult in human subjects needs to be explored. Furthermore, vitamin antioxidants have limited radioprotective capabilities. In cases of high dose radiation, the effectiveness of these compounds decreases (Weiss & Landauer, 2000). Studies analyzing the effectiveness of vitamin supplements demonstrated no increased protection beyond dietary recommendations (Choi, Benzie, Collins, Hannigan, & Strain, 2004), and the use of these alone may not prove beneficial to astronauts on long-term missions in space.
**Fruit Polyphenols**

Fruits contain powerful antioxidants that can impart extensive protective properties against oxidative stress-induced damage. In fact antioxidant polyphenols found in fruits have been shown to be neuroprotective and to slow down the aging process (Joseph, Denisova, Bielinski, Fisher, & Shukitt-Hale, 2000; Joseph, Shukitt-Hale, & Lau, 2007; Lau, Shukitt-Hale, & Joseph, 2005). These have also been shown to ameliorate symptoms of several age-related diseases including those seen in AD (Lau, et al., 2005). Therefore, these dietary antioxidants may help alleviate and protect against radiation-induced neurotoxicity and subsequent behavioral deficits that might occur due to deep space missions.

The antioxidant polyphenols found in fruits have especially demonstrated a protective effect against oxidative stress-induced CNS damage. Antioxidants found in blueberries and strawberries have been shown to protect against spatial learning deficits (Shukitt-Hale, Carey, Jenkins, Rabin, & Joseph, 2007) and operant responding (Rabin, Shukitt-Hale, Joseph, & Todd, 2005) in rodents exposed to HZE radiation, suggesting these antioxidants may be protective against radiation-induced hippocampal damage. HZE particle radiation is a type of high-LET radiation and can often lead to more extensive tissue damage compared to low-LET radiations. Therefore if antioxidants can be neuroprotective in heavy ion radiation, this implies that it can protect organisms from low-LET radiation-induced insults.
Pomegranate Polyphenols

Pomegranates have long been used as a traditional remedy for various ailments. Pomegranates have a high polyphenol count and have been shown to have antioxidant, anti-inflammatory, and anti-apoptotic properties (Aviram et al., 2000; Aviram et al., 2004; Braga, Leite, et al., 2005; Braga, Shupp, et al., 2005; de Nigris et al., 2005; Kaplan et al., 2001; Rosenblat, Volkova, Coleman, & Aviram, 2006; Rozenberg, Howell, & Aviram, 2006; Seeram et al., 2005). In fact, one study showed a 2-3 times greater antioxidant capacity of pomegranates compared to other antioxidant sources, such as red wine or green tea (Gil, Tomas-Barberan, Hess-Pierce, Holcroft, & Kader, 2000).

Pomegranate polyphenols include flavonoids (i.e., quercetin) and tannins (i.e., punicalagins and ellagitannins). Ellagitannins are derived from ellagic acid and carbohydrate. Flavonoids and tannins have specifically been shown to decrease oxidative stress by deactivating ROS (Rosenblat, et al., 2006) and scavenging free radicals (Gil, et al., 2000; Rosenblat, et al., 2006).

As mentioned previously, there seems to be an enhanced aging effect occurring in the brains of radiation-exposed individuals similar to that seen in individuals with Alzheimer’s disease. Antioxidants found in pomegranates have been found to be neuroprotective in the hippocampus of mice with Alzheimer’s plaques (Hartman et al., 2006). In this study, Hartman used a 1:20 diluted concentration of pomegranate juice and found this dosage to be significant to ameliorate spatial learning deficits in the Morris water maze and decrease amyloid plaque load in the hippocampus. Also, rat pups subjected to traumatic brain injury had less tissue loss in the hippocampus when exposed neonatally to pomegranate juice (Loren, Seeram, Schulman, & Holtzman, 2005) as
compared to controls, demonstrating the neuroprotective effects of pomegranate polyphenols.

Ellagic acid has also been shown to scavenge free radicals, ROS, and reactive nitrogen species (Priyadarsini, Khopde, Kumar, & Mohan, 2002). Additionally, ellagic acid inhibits copper-mediated dopamine-induced oxidation of DNA and subsequent DNA damage (W. A. Spencer, Jeyabalan, Kichambre, & Gupta, 2011); this suggests pomegranate polyphenols may also inhibit radiation-induced DNA oxidation and damage. Although these isolated pomegranate polyphenols have antioxidant properties when acting individually, research suggests that pomegranate polyphenols combined synergistically act to prevent oxidative stress-induced damage. Indeed, one study analyzing the anti-apoptotic properties of pomegranate juice found the whole juice to be more effective than any one isolated component (Seeram, et al., 2005). Altogether, this evidence suggests that the polyphenols found in pomegranates could be an effective therapy against radiation-induced neurogenesis inhibition and apoptosis. Furthermore, ethanol extract from pomegranate seeds was found to decrease depressive-like behaviors similar to imipramine (an antidepressant drug) treatment, induce anxiolytic effects similar to benzodiazepines (a commonly prescribed anxiolytic agent), and display anti-nociceptive effects almost equivalent to morphine in both old and young Swiss albino mice; these results were attributed the extract’s antioxidant powers within the CNS (Kumar, Maheshwari, & Singh, 2008). This suggests that deficits of any depressive- or stress related “anxiety-like” behavior (i.e., avoidance behavior in the face of a potential threat) due to radiation-induced oxidative stress might be ameliorated by antioxidants found in pomegranates. No studies to date have looked at the radioprotective effects of
pomegranate antioxidants; however, the evidence indicates that the antioxidants found in this fruit may be beneficial in ameliorating the negative effects of ionizing radiation.

**Gender and Ionizing Radiation Effects on Astronaut Health and Behavior**

Women have been part of the space program since its inception. The first woman flew in space in 1963, and the first American woman followed in 1983. There have been a total of 55 female astronauts and 14 female astronaut candidates. Women comprise ~11% of the astronauts who have flown in space. There are both benefits and disadvantages to mixed gender crews; women typically have less upper body strength and decreased arm reach, but greater flexibility compared to men, making women approach extra-vehicular activity (EVA) and other jobs from a unique angle. Psychosocial studies often highlight the benefits of mixed-gender crews, although sexual tensions in space can be a concern. Men tend to be more physically aggressive and competitive than the average woman (Archer, 2004; Bettencourt & Miller, 1996; Bettencourt, Talley, Benjamin, & Valentine, 2006; Niederle & Vesterlund, 2008), specifically toward other men (Geary, 1998). The presence of women on a crew mission has been attributed to improved sociability of the group via moderation of the men’s behavior (Rosnet, Jurion, Cazes, & Bachelard, 2004), and the diffusion of tensions and hostility seen among male-only crew (Kanas & Manzey, 2003). This may be important on long-term missions, since the length of the mission may increase the chances of hostile behavior and lack of group cooperation, which can lead to the downfall of a mission.
Specific health issues for astronauts in space travel highlight gender differences in bone loss, orthostatic response, and disease risk, indicating differential effects of the space environment on men and women. Post-menopausal bone loss may be exacerbated in space, where bone loss is a concern for any astronaut. Menopause typically occurs around the age of 50; however, it is still considered natural when it occurs at age 40. Since the average age of past women astronauts is 39 years, this is a concern. Estrogen replacement is one answer; however increasing estrogen levels can lead to a higher risk for breast cancer (Clemons & Goss, 2001). Selective estrogen-receptor modulators (SERMs), such as raloxifene, allow for estrogen-like affects on bone mass preservation, but act as antagonists in breast and uterine tissues (Barrett-Connor et al., 2004; Sporn, Dowsett, Mershon, & Bryant, 2004), thereby offering a better treatment for women astronauts. Women are also more likely to have decreased orthostatic tolerance and increased presyncopal experiences in comparison with men (Custaud et al., 2002; Harm et al., 2001). Presyncope is a condition of lightheadedness, slight nausea and a feeling of faintness that is usually a result of loss of blood pressure when rising from a seated or supine position. Gender differences in cardiovascular processes may be the cause of this tolerance difference (Gotshall, 2000; Gotshall, Tsai, & Frey, 1991). Not every woman experiences this problem, however, and both men and women prone to this intolerance are weeded out through the screening process. Other gender differences are seen as well. Men are more likely to develop kidney stones both before and after space flight (Harm, et al., 2001), and post-menopausal women receiving estrogen therapy after space travel may be at a lower risk for cataract development (Dynlacht et al., 2008), a common problem seen in astronauts post-space travel. Overall, gender-mediated health effects have not
been an overwhelming concern as these health differences may be overcome with appropriate forethought and preventative measures. Yet, this highlights the different biological effects that can occur between the sexes when exposed to ionizing radiation and other factors in the space environment.

Little to no research, however, has looked at the biological effects of ionizing radiation on the CNS of women, even though other evidence indicates there may be gender-mediated differences. Women are more likely to suffer age-related cognitive impairments associated with the hippocampus, and they have a faster declination of visual-spatial skills compared to men (Small, La Rue, Komo, Kaplan, & Mandelkern, 1995). Also, women are 3 to 4 times more susceptible to developing Alzheimer’s disease, a neurodegenerative disease (Gao, Hendrie, Hall, & Hui, 1998).

Animal studies demonstrate gender differences as well. Female mice are more likely than male mice to demonstrate age-related cognitive deficits (Benice, Rizk, Kohama, Pfankuch, & Raber, 2006) related to hippocampus function. Also, female mice irradiated with heavy ion radiation (iron) demonstrated inhibition of hippocampal neurogenesis (Rola, Otsuka, et al., 2004), which could affect performance. In fact, hippocampus-dependent fear conditioning was impeded in female mice, but not male mice exposed to iron irradiation (Villasana, Rosenberg, & Raber, 2010). This suggests possible gender differences in how radiation can affect the CNS, and this should be studied further in order to ascertain the biological and behavioral effects of, as well as putative therapies for, ionizing radiation in future female astronauts.
Use of C57Bl/6 Mice for this Study

To analyze the effects of proton radiation in a controlled experiment, animal research must be conducted. The animals must be affordable, available for purchase and should have hippocampal brain structure and neuronal activity comparable to humans. C57Bl/6 mice fit all requirements. The hippocampus is similar across a range of mammalian species from rodents to humans, and mice have similar hippocampal structure and activity compared to humans (West, 1990). Neuronal impairments within hippocampal regions of the brains of mice and humans both demonstrate similar physiological and behavioral differences (Dillon, Qu, Marcus, & Dodart, 2008; Rempel-Clower, Zola, Squire, & Amaral, 1996), and many previous studies use rodents to better understand hippocampus-related behavioral deficits and their correlated neuropathological problems (D’Hooge & De Deyn, 2001). C57BL/6 mice are the most commonly used wildtype mice that are commercially available and are budget friendly.

Estrous Cycle, Behavior and Neurogenesis of C57Bl/6 female mice

Research demonstrates that, although gender-mediated differences are present in many disorders (Qureshi & Mehler, 2010), animal studies using mice do not often utilize both male and female mice for gender-mediated difference analysis. Furthermore, unless studying a female-specific disorder, most studies choose to use male mice. These research patterns are often due to the uncertainty of how estrous cycling-induced hormone changes might affect physiology and behavior.

The mouse estrous cycle lasts ~4-6 days and consists of four phases: (a) the proestrus phase, where follicular growth occurs; (b) the estrus phase, or the “in heat”
phase when estradiol is high; (c) the metestrus phase, marked by lowered estradiol levels and small amounts of progesterone; and (d) the diestrus phase, marked by increasing progesterone levels and re-absorption of the endometrium.

It has been thought that species-specific behavioral changes may be affected by the phases of the estrous cycle. For the purpose of the present study, research reporting on how estrous cycle phases affect behavior and hippocampal neurogenesis in the C57Bl/6 mouse is presented.

One study showed no difference between the 4 phases of the cycle on open field, rotarod, tail-flick, and hotplate tests, but mice had significantly longer immobility on the tail suspension (TST) during the metestrus phase as compared to the proestrus and diestrus phases (Meziane, Ouagazzal, Aubert, Wietrzych, & Krezel, 2007). Other research showed that the estrous cycle had no effect on acoustic startle response amplitude, habituation, prepulse inhibition or prepulse facilitation (Plappert, Rodenbucher, & Pilz, 2005). Estrous females did demonstrate lowered spatial memory performance as measured in a 1-day water maze test, compared to proestrus and metestrus mice; however when data were averaged for 3 spatial trials, there was no significant differences in performance across the estrous phases (Frick & Berger-Sweeney, 2001). Probe tests measured 30 minutes post spatial trials were significantly different among groups, with estrous females performing worse than proestrus or metestrus females; however, 24 hours post-spatial trials, there was no significant difference in performance among the estrous groups (Frick & Berger-Sweeney, 2001). Although another study found activation of certain hippocampal receptors and markers in mice during proestrus (high-estradiol phase), there was no performance difference on an
object placement test of spatial memory (J. L. Spencer, Waters, Milner, & McEwen, 2008). In addition, no sex differences were evident between 5-month-aged female and male mice in the cued and spatial water maze (Frick, Burlingame, Arters, & Berger-Sweeney, 2000).

Furthermore, in adult C57Bl/6 mice, no overall gender differences in hippocampal neurogenesis are apparent, and the estrous cycle does not influence the production of new SGZ cells in female mice. This suggests that adult neurogenesis is not influenced by estrous cycle-dependent hormone changes (Lagace, Fischer, & Eisch, 2007).

Aims of the Current Study

The present study will analyze possible behavioral and neuropathological changes in male and female C57Bl/6 mice as a result of the effects of a radiation dose equivalent to that which might be seen on long-term manned missions to Mars. Additionally, the putative protective effects of the polyphenol antioxidants found in pomegranate juice against radiation-induced deficits will be analyzed.

Aim 1

Explore effects of gender on behavioral and neuropathological effects of radiation.

Hypothesis 1: Female mice will demonstrate greater deficits on hippocampal-dependent behavioral tests and tests of affect (water maze, zero maze, tail suspension) compared to male mice.

Hypothesis 2: Female mice will demonstrate greater neurogenesis inhibition in the SGZ and GCL of the dentate gyrus compared to male mice.
Aim 2
Explore effects of diet on behavioral and neuropathological effects of radiation.

*Hypothesis 1*: Radiation will lead to deficits in hippocampal-dependent behavioral tests and tests of affect (water maze, zero maze, tail suspension), and pomegranate juice will protect against these deficits.

*Hypothesis 2*: Radiation will lead to neurogenesis inhibition in the hippocampus of mice exposed to radiation, and pomegranate juice will ameliorate these deficits.

Aim 3
Explore interactions between effects of gender and diet on behavioral and neuropathological effects of radiation, as well as behavioral/biomarker correlations.

*Hypothesis 1*: Pomegranate juice will ameliorate enhanced behavioral deficits in female mice, and they will be expected to perform similarly to male mice.

*Hypothesis 2*: Pomegranate juice will ameliorate increased neurogenesis inhibition in female mice, so that they will not differ from their male counterparts.

*Hypothesis 3*: Enhanced neurogenesis inhibition within the hippocampus will be correlated with deficits in hippocampal dependent behavioral tests and tests of affect.
CHAPTER TWO

METHODS

Experimental Design

Male and female C57Bl/6 mice (4 months old) were separated into 4 groups (pomegranate/no-pomegranate, radiation/no-radiation), yeilding a total of 8 groups (see Table 1). Forty-eight animals were started on a pomegranate juice diet immediately and continued on this diet until time of sacrifice. The pomegranate juice was administered in the animal’s drinking water at a 1:20 ratio. Forty-eight animals did not receive pomegranate preparation, but received a sugar placebo in their drinking water.

Table 1

Projected animal group breakdown

<table>
<thead>
<tr>
<th>Pomegranate Juice</th>
<th>Placebo</th>
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<tbody>
<tr>
<td>Radiation</td>
<td></td>
</tr>
<tr>
<td>Male: n=12</td>
<td>Male: n=12</td>
</tr>
<tr>
<td>Female: n = 12</td>
<td>Female: n = 12</td>
</tr>
<tr>
<td>No Radiation</td>
<td></td>
</tr>
<tr>
<td>Male: n=12</td>
<td>Male: n=12</td>
</tr>
<tr>
<td>Female: n = 12</td>
<td>Female: n = 12</td>
</tr>
</tbody>
</table>

Animals were allowed to acclimate for 1 week. After acclimation, animals were tested on a battery of behavioral tests for 2 weeks. These tests assessed a host of cognitive, motor, and learning abilities (i.e. water maze, openfield test, zero maze, etc). After the first round of behavioral testing, animals were subjected to radiation or sham treatment. Half of the mice were placed into well-ventilated acrylic boxes, where they
were then irradiated with a whole body radiation beam of proton radiation (2 Gy at 150 MeV/n for 1-2 Gy/min) for ~5-10 minutes. The other half were placed in the boxes for the equivalent amount of time as the radiation group, but no radiation was administered.

Approximately 20-48 hours post-radiation, mice were tested for motor skills/coordinaiton and activity levels. Eight weeks post-irradiation, animals underwent behavioral tests similar to the first battery of baseline tests. After this second round of behavioral testing, the mice were deeply anesthetized with Euthasol and perfused via the left ventricle with saline until euthanization. Once euthanization was confirmed, the animals’ brains were removed and placed in paraformaldehyde for 24 hours, then washed in saline, frozen with dry ice and stored at -20 degrees Celsius until sectioning. See Figure 1 for timeline of experimental design.

![Experimental Design Timeline](image)

*Figure 1. Timeline of the experimental design.*
**Animals**

Ninety-six C57Bl/6 mice (48 males; 48 females), 4 months in age, were obtained from Charles River laboratory and housed approximately 3-6 per cage, depending on gender (each cage was gender specific). All mice were maintained on a 12-hour light/dark schedule and fed standard rodent chow *ad libitum*. All animals were treated in accordance with the requirements of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, as well as the specifications stipulated by the Loma Linda University Institutional Animal Care and Use Committee. One animal was “gained” and 7 animals were “lost” during the experimental process. Two male mice died: one shortly after arriving at Loma Linda University most likely from the stress of transportation, and one after being confined to the radiation cube (no radiation). Five female mice were removed from the study due to pregnancy. Also, during the course of the study, it was discovered that Charles River laboratory had accidentally included an extra female mouse during one shipment. (See Table 2 for final group numbers).

Table 2

*Final animal group breakdowns for behavioral tests*

<table>
<thead>
<tr>
<th></th>
<th>Pomegranate Juice</th>
<th>Placebo</th>
</tr>
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<td><strong>Radiation</strong></td>
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<td>Male: n=11</td>
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<tr>
<td></td>
<td>Female: n = 10</td>
<td>Female: n = 12</td>
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</table>
Pomegranate Preparation

Pomegranate juice (PomWonderful) was diluted 1:20 into filtered water. The mice drank ~ 6-8 ml/day. Full-strength pomegranate juice consists of 84% water, 14% carbohydrates, 0.48% ash, 0.4% citric acid, 0.1% protein, 0.02% fat and 1% other, including polyphenols (phenolic acids and flavonoids). Phenolic acids include 115 ppm ellagic acid and 5 ppm gallic acid. Flavonoids include 1880 ppm hydrolysable tannins (e.g., gallotannins, ellagittannins, punicalagin) and 369 ppm anthocyanins and their glycosides (e.g., cyanidin, delphinidin, pelargonidin). PomWonderful pomegranate juice was purchased from local grocery stores and kept refrigerated at 4 ° Celsius throughout the experiment. The solution was prepared as needed (as often as the animals’ water needed changing), and was administered by diluting the solution into the animal’s drinking water. Animals had ad libitum access to the water. Animals not receiving pomegranate juice received an equivalent sugar water placebo emulating the sugar content of the pomegranate juice (85% sucrose, 7.5% D-(+)- glucose, 7.5% D-fructose).

Behavioral Testing

At 1 week and 12 weeks after arrival, animals were tested on a 2-week long battery of behavioral tests designed to assess a wide variety of behavioral domains. In addition, at approximately 20-24 hours post-radiation, mice were tested on motor skills (rotarod) and activity levels (openfield test) and at approximately 45-48 hours post-radiation, mice were tested on an additional motor skills/coordination test (balance beam test) to assess any acute post-radiation behavioral changes. (See Table 3 for 8-week post-radiation testing schedule).
Table 3

Two-week schedule of behavioral tests for baseline and 8-weeks post-radiation

<table>
<thead>
<tr>
<th>Week</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
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<tbody>
<tr>
<td>1</td>
<td>Open Field</td>
<td>Zero maze</td>
<td>Rotarod</td>
<td>Open Field</td>
<td>Zero maze</td>
<td>Rotarod</td>
</tr>
<tr>
<td>2</td>
<td>Cued Water Maze</td>
<td>Spatial 1 Water</td>
<td>Probe 1; Spatial 2</td>
<td>Probe 2; Spatial 3</td>
<td>Probe 3</td>
<td>Tail Suspension Test</td>
</tr>
</tbody>
</table>

Learning and Memory

The water maze is the standard test of learning and memory abilities in rodents (Hartman, et al., 2006; Morris, 1984). This test of spatial navigation requires the animal to learn the location of a hidden (submerged) platform in a pool of water using visual cues from around the room. The water maze consists of a metal pool (110 cm diameter) filled with water made opaque with white tempera paint. The pool contains a round platform (11 cm diameter) that the animal can step on to escape the water. For each trial, an animal was released nose against the wall into the pool at one of four release points and allowed to swim to the platform. The animals were given 10 trials (60 s maximum) per day. When an animal did not find the platform in the allotted 60 s, the experimenter manually guided the mouse to the platform. An overhead camera recorded the animals’ swim paths, allowing for quantification of distance, latency, proximity to target, and swimming speed by a computerized tracking system (Noldus Ethovision). Each animal received 10 trials on four consecutive days, making a total of 40 trials per animal. Trials were performed in blocks of 5 each day (2 trials per block), with approximately 30 min
intervals between blocks. As performance improves, escape latency and swim path length generally decrease.

**Day 1: Cued Water Maze**

This is a control task for assessing sensorimotor and/or motivational deficits that could affect performance during the Spatial phase. For this phase, the surface of the escape platform is visible (1 cm above the surface of the water), and a pole is placed on top of the platform to make its location even more obvious. The platform’s location varied across the 5 blocks that day. The animals were released into the pool opposite the location of the platform and were allowed to remain on the platform for 5 s.

**Days 2-4: Spatial Water Maze**

For this task, the surface of the escape platform is submerged 1 cm below the surface of the opaque water, so the mouse must find the platform based on its relationship to the spatial cues rather than direct visualization. The location of the platform changed each day for 3 days. After finding the platform, the animals were allowed to remain on it for 5 s. Twenty-four hours after each Spatial day, the animals were given a “probe” trial in which the platform was removed from the water maze, and the animal was allowed to search the pool for 60 s. The amount of time spent searching the probe quadrant was measured, as well as the total number of times that the animal crosses over the former location of the platform. An hour later, the platform was placed back into the pool in its new location, and the next set of 10 trials was administered.
General Avoidance Levels

The elevated zero maze consists of a plastic ring, 100 cm outer diameter, 10 cm wide, with 35 cm walls enclosing two opposing quadrants and elevated off the floor. The room lights are dimmed and halogen lights directly illuminate the open spaces of the maze. Animals were initially placed in the center of one of the open spaces, and their activity was monitored for the duration of five minutes. The percent amount of time spent within an enclosed space was calculated. Each animal received two trials, with the second trial being conducted 48 hours after the first.

General Activity Levels / Movement Patterns

In the open field test, animals were placed in a 49 cm x 36 cm opaque open-topped plastic bin and allowed to explore for the duration of thirty minutes while the movements of each animal were recorded by an overhead camera and analyzed by a computerized tracking system (Noldus Ethovision). Various parameters were analyzed, including the distance the animal moved and the percent time spent moving. Each animal, with the exception of the acute post-radiation trial, received two trials, with the second trial being conducted 48 hours after the first. For the acute post-radiation trial, each animal received one trial only.

Learned Helplessness

In the tail suspension test, mice were suspended by the tail with adhesive tape that was attached approximately 1 cm from the tip of the tail. The other end of the tape was wrapped around a hook embedded in the center of the ceiling of a wooden box measuring
19cm x 21cm and 40 cm in height. When suspended, the animal's rostral end was approximately 20cm from the floor of the device. The box is enclosed on all sides except one (for viewing), and the room lighting and sound were kept to a minimum in order to diminish visual and acoustical disturbances. In addition, the nature of the box kept each animal visually isolated. This device was modeled after the Automatic Tail-Suspension test designed by Panlab, Harvard Apparatus. While the mouse tried to escape its position, two assistants blinded to treatment group individually rated the mouse on immobility and agitation for the duration of 6 minutes. The time that the animal remained immobile during the final 4 minutes of a 6-minute duration was recorded (Kwon et al., 2010). Each animal received one 6-minute trial. Immobility was operationally defined as a complete lack of voluntary movement on the part of the mouse (Steru, Chermat, Thierry, & Simon, 1985). The animal was counted as immobile even if it was still swinging back and forth from a previous struggle, but was now completely still in its voluntary movements. The animal was also rated immobile if it was curled up, appearing to rest while holding its front paws to its back paws but was not currently struggling or moving.

**Sensorimotor / Coordination**

The accelerating rotarod (Columbus Instruments) is a test of sensorimotor coordination and balance. It consists of a 3 cm diameter rotating horizontal cylinder. The mouse was placed onto the cylinder and had to continuously walk forward to avoid falling. Latency to fall off was the dependent variable. Performance over days of testing was a measure of motor learning. The mice were tested every other day for 2 days of trials (with the exception of the acute post-radiation trial where the animals received only
1 day of testing). Three blocks of 2 consecutive trials were administered per day: 2 stationary (learning) trials (at 5 RPM steady), 2 trials that started at 5 RPM and accelerated by 3 RPM every 5 s, and 2 trials that started at 5 RPM and increased by 3 RPM every 3 seconds. Each steady trial lasted up to 60 s, and each accelerating trial lasted up to 120 s. There was approximately 45 minutes to an hour between trials.

**Motor Skills**

The balance beam test, used only during the acute post-radiation behavioral testing, is designed to assess motor skills, balance and coordination. The beam was 62 cm tall, 0.5 cm wide, and 60 cm in length with every 5 cm along the beam marked for purposes of distance measurement. Animals were placed in the center of the beam and their movement was monitored for the duration of 60 s. Distance the animal traveled along the beam, the amount of times the animal reversed its direction on the beam, and the time the animal spent on the beam (out of 60 s) were recorded. Each animal was given 2 trials, approximately 45 min apart.

**Irradiation**

After behavioral testing, animals were transferred to the Loma Linda University Protons Treatment Facility, where half of the mice were placed into acrylic boxes (3 x 3 x 6 cm) ventilated with holes on the sides. The mice were not anesthetized, but movement was limited to comfortable breathing during proton irradiation. Mice were irradiated with a single, whole body radiation beam of proton radiation (2 Gy at 1-2 Gy/min) delivered at 150 MeV/n. Treatment did not exceed 10 minutes. Several studies looking at the
irradiation of individuals suffering from diseases such as cancer often use head-only techniques; however, astronauts will be exposed fully to radiation. As such, whole body radiation procedures were chosen in order to better emulate the true nature of radiation-exposure in space. Indeed, studies looking at astronauts’ risk of ionizing radiation exposure in space use whole-body methods (Shukitt-Hale, et al., 2007). Sham-irradiated controls were placed into the same boxes for the same length of time as the animals receiving the radiation but were not irradiated.

**BrdU Injections**

5-bromo-2′-deoxiuridine (BrdU) is a reagent commonly used as a marker for cell proliferation. BrdU is structurally similar to thymidine and is incorporated into DNA during the synthesis-phase of the cell cycle in place of thymidine; therefore it can serve as a marker for new cell division. Mice received an intraperitoneal injection of BrdU (Sigma, St. Louis, MO), at 50 mg/kg body weight (20 mg/ml stock, prepared in warm PBS with 0.007 N NaOH). Once solution was prepared (pH 7.0) and allowed to cool to room temperature, it was divided into aliquot parts of 4 mL and stored at -20°C. Each time the mice were given a new injection, the aliquot of BrdU was removed from the freezer, allowed to thaw (~30-60m), then warmed to 50-60°C in order to get rid of the white precipitates that had formed in the solution. The solution was then allowed to cool to room temperature and injected directly after. Injections were given twice a day (morning and afternoon) every day for three days prior to sacrifice in order to label newborn cells in the hippocampus. This protocol was adapted from recommendations for mice (Wojtowicz & Kee, 2006).
Animal Euthanasia and Tissue Preparation

The fourth day after the first BrdU injection, mice were deeply anesthetized with an intraperitoneal injection of Euthasol at doses appropriate for body weight and perfused transcardially with 4% paraformaldehyde (PFA) in a phosphate buffer saline (PBS) solution. After euthanization, the brain was removed from the skull and immediately placed in 4% PFA and stored at 4°C for 24 hours. Following 3x10min rinses with PBS, brains were immersed and fixed in 30% sucrose solution in PBS for 48 hours, then air-dried, frozen on dry ice, and stored at -20°C. Brains were sectioned coronally in 50 μm sections from the rostral to caudal end on a cryostat (Leica CM 1950) and tissue sections were stored free-floating at -20°C in cryoprotectant made of a PBS solution with 1% polyvinylpyrrolidone (PVP) and 50% ethylene glycol.

Histology & Immunohistochemistry

A subset of tissue was tested for cell proliferation and neurogenesis. See Table 4 for final group sizes for histological and immunohistochemical procedures.

Table 4

*Group sizes for histological and immunohistochemical procedures*

<table>
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<tr>
<th></th>
<th>Pomegranate Juice</th>
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</tr>
</thead>
<tbody>
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<td>Male: n=7</td>
</tr>
<tr>
<td></td>
<td>Female: n = 7</td>
<td>Female: n = 6</td>
</tr>
<tr>
<td><strong>No Radiation</strong></td>
<td>Male: n=5</td>
<td>Male: n=6</td>
</tr>
<tr>
<td></td>
<td>Female: n = 6</td>
<td>Female: n = 5</td>
</tr>
</tbody>
</table>
BrdU and DCX

To break the double-strand structure of the DNA so that BrdU label detection was possible, sections were treated with 2M hydrochloric acid for 60 min at 37°C and then neutralized with 0.1 M boric acid in TBS (pH 8.5) for 20 min at room temperature. Sections were then blocked with a solution of 1% BSA in PBS. To specifically detect newly dividing neurons, tissue underwent double immunofluorescence labeling with two primary antibodies: rat monoclonal antibody against BrdU (1:200; Abcam, Cambridge, MA), and rabbit polyclonal antibody against doublecortin (DCX) (1:300; Abcam, Cambridge, MA) in 0.25%BSA, 0.25% Triton X-100 in PBS for 72 hours at 4°C. Sections were then washed in PBS and incubated with appropriate biotinylated secondary antibodies (Alexa Fluor, Jackson ImmunoResearch Laboratories, West Grove, PA) for 90 min at room temperature.

Counts were taken for BrdU⁺, DCX⁺, and BrdU⁺/DCX⁺ cells. Images were obtained by ImagePro. Tissue sections were magnified at 40x for the SGZ and the GCL of the dentate gyrus (see Figure 2). Images were then uploaded to ImageJ and positively labeled cells were counted within parameters around the region of interest (ROI) created by an observer blinded to experimental conditions for the following regions: the SGZ and the GCL of the dentate gyrus. For dentate gyrus analysis, standard counting sections were chosen from brains sampled at approximately 1.94 mm behind the Bregma. Measurements of tissue area (µm²) were obtained for each ROI, and cell density was calculated by dividing regional cell count with its respective area.
Figure 2. A coronal section of the hippocampus showing the dentate gyrus (dashed outline), the granular cell layer (solid outline), the hilar region, and the molecular layer (above the granular cell layer). The subgranular zone is a small region spanning the upper portion of the hilus and the lower portion of the granular cell layer.

**DAPI**

DAPI, or 4’, 6-diamidino-2-phenylindole, binds to the A-T (adenine-thymine) pairing in double-stranded DNA and is used to stain the nucleus of numerous cell types which allows for the counting of individual cells. In the current study, each tissue section was removed from cryoprotectant, washed 5 x 10min in PBS and mounted on Gold+ double-frosted slides (Fisher Scientific, Pittsburgh, PA), with the mounting medium Vectashield with DAPI (Vector, Vector Laboratories, Burlingame, CA). Two out of 24 serial sections were analyzed for both right and left hippocampus. Images were acquired using ImagePro and magnified 10x. Quantification proceeded the same as for BrdU/DCX.

**Statistical Analyses**

All statistical analyses were carried out using SPSS. A mixed design ANOVA with 3 between-subjects variables (gender, pomegranate, radiation) and 2 repeated measures (time point: Baseline vs. Post-Radiation, & test day) was conducted on all
behavioral data. To control for sphericity and compound symmetry due to repeated measures in the test-day (10 trials), the Huynh-Feldt correction to the degrees of freedom was used to determine the $p$-value. For the neurological data between-subjects univariate ANOVA’s were conducted with 3 between subjects groups with two levels each (Gender: Male/Female, Radiation: Radiation/Sham, Diet: Pomegranate/Placebo). In analyses where no statistical differences were found between groups (i.e. gender, radiation, or pomegranate diet), groups were collapsed to enhance statistical power. In cases where normality was violated, an inverse transformation was conducted and run on the univariate ANOVA. In cases where transformations did not correct the normality violation, a log linear analysis or chi-square test was run to achieve probability and odds-ratios on main effects and interactions. Pearson product-moment coefficient was used to determine correlations between behavioral and biological variables that did not violate normality. When a variable violated normality, Kendall’s tau was used. An alpha level of 0.05 was used for all tests of statistical significance.
CHAPTER THREE

RESULTS

Hippocampal Area

Although tissue samples were all taken from approximately the same Bregma level, variations of hippocampus size may have still occurred between animals, therefore, a measure of the area for each ROI was obtained to determine if cell counts were valid. No significant differences of area were found between animals for any group in any ROI, indicating that cell counts were valid (see Figure 3 for a representation). However, as DAPI had to be analyzed using separate tissue, areas for ROIs for DAPI stained tissue were also measured. There was a significant difference between the areas of the SGZ for radiation groups, with mice in the radiation group having significantly larger SGZ areas than sham mice, $F(1, 43) = 7.53, p = .009$ (Figure 4). Therefore, in this paper, adjusted cell counts will be reported for all biological results to stay consistent. However, the $p$-values of raw cell counts are reported along with the $p$-values of adjusted cell counts in a table at the end of this section.
**Figure 3.** There were no significant differences in area of tissue among any groups.

**Figure 4.** Radiation group tissue larger than sham group in the SGZ of the dentate gyrus, *p*=.009.
Hippocampal Cell Counts

**DAPI**

The only significant difference among basic cell counts was a gender difference. Females had more cells in the SGZ of the dentate gyrus than males, $F(1, 39) = 6.61$, $p = .014$, $\omega^2 = .09$, power = .09 (Figure 5).

![DAPI - DG](image)

*Figure 5. Females had more cells than males in the dentate gyrus, * $p = .014$.*

**Hippocampal Plasticity: BrdU**

BrdU is a marker for new cell growth, but alone it does not specify type of cell. However, BrdU counts may give an indication of general plasticity of the hippocampus within its respective regions. Radiation suppressed cell growth in the SGZ, $F(1, 38) = 11.20$, $p = .002$, $\omega^2 = .23$, power = .33; in addition, a pomegranate juice diet was found to
increase cell growth in the SGZ of the dentate gyrus for non-irradiated mice, $F(1, 38) = 6.60, p = .014, \omega^2 = .08$, power $= .08$ (Figure 6a,b & 7a-d).

Figure 6a &b. Radiation suppressed new cell growth, and pomegranate diet increased cell growth in non-irradiated animals, *$p < .02$. 
Figure 7a-d. BrdU (cell proliferation). Radiation suppressed cell growth in the SGZ (b & d), and pomegranate diet allowed for more cell growth for non-irradiated animals (a) in the SGZ of the DG, but not control diet (c). Scale bar = 50 µm.
Furthermore, there was a trend for non-irradiated males to have greater cell growth in the GCL compared to their female counterparts, but radiation suppressed this. Radiation did not affect females, $F(1, 38) = 3.81, p = .058, \omega^2 = .05$, power =.06 (Figure 8 & 9a-d).

Figure 8. Non-irradiated males had more cell growth in the GCL of the hippocampus than females, but irradiation suppressed this, *$p=.058$. 

Figure 9a-d. BrdU (cell proliferation) in the GCL. Non-irradiated males (a) had more cell proliferation in the GCL of the hippocampus compared to other groups (b-d). Scale bar = 50 µm.
Also, pomegranate juice affected males and females differently. Pomegranate-fed males showed significantly more cell growth in the SGZ compared to pomegranate-fed females and control diet males, and, for the female mice, a control diet led to more cell growth than a pomegranate diet, $F(1, 38) = 16.36, p < .001, \omega^2 = .23$, power = .33 (Figure 10 & 11a-d).

![BrdU - SGZ](image)

*Figure 10.* Males fed a pomegranate diet had increased cell growth over control fed males, while control fed females had greater cell growth than pomegranate fed females, *$p < .01$. 
Figure 11a-d. BrdU (cell proliferation) in SGZ. Pomegranate-fed males (a) had greater cell proliferation in the SGZ of the DG compared to other groups (b-d). Scale bar = 50 µm.

**Neurogenesis: DCX**

Doublecortin alone is often used as a marker for neurogenesis. Radiation significantly suppressed DCX-marked neurogenesis in the SGZ, $F(1, 38) = 17.22, p < .01$; $\omega^2 = .22$, power = .31, and the GCL, $F(1, 36) = 15.50, p < .001$, $\omega^2 = .18$, power = .21 of
mice (Figure 12). Pomegranate juice, however, had a radiation-dependent affect. Non-irradiated animals on a pomegranate juice diet had greater neurogenesis as measured by DCX in the SGZ compared to control-fed animals and irradiated animals, $F(1, 38) = 6.01$, $p = .019$, $\omega^2 = .11$, power = .11, (Figure 13a). Although, not significant, this same trend was seen in the GCL of the hippocampus, $F(1, 36) = 3.65$, $p = .064$, $\omega^2 = .04$, power = .06 (Figure 13b). For representative images, see Figure 14 (a-d).

![Graph showing neurogenesis in SGZ and GCL](image)

*Figure 12.* Radiation suppressed neurogenesis in both the SGZ and the GCL of the dentate gyrus, *p* < .01.
Figure 13a & b. Radiation suppressed neurogenesis (a & b); pomegranate juice increased neurogenesis in the SGZ of non-irradiated mice, but not radiated mice (a), *p<.02. This same trend was also seen in the GCL, p=.064.
Figure 14a-d. Doublecortin (DCX) marked neurogenesis. Radiation suppressed the number of DCX positive cells in the SGZ of the dentate gyrus (B & D). Pomegranate juice (A) but not control diet (C) increased neurogenesis in the SGZ of non-irradiated mice, but not radiated mice (B). Scale bar = 50 µm.

Neurogenesis: BrdU/DCX

There was a significant association between radiation group (radiated or sham) and whether or not BrdU⁺/DCX⁺ cells were found in the SGZ of the hippocampus, $\chi^2(1) = 10.47$, $p=.001$ (Figure 15a & b) and the GCL of the hippocampus, $\chi^2(1) = 8.63$, $p=.003$ (Figure 16a & b). This seems to represent the fact that based on the odds ratio non-
irradiated animals were 8.09 times more likely to express BrdU+/DCX+ cells in the SGZ and 5.43 times more likely to express these cells in the GCL. This suggests that radiation suppressed neurogenesis in these regions of the dentate gyrus (Figures 17a-d).

*Figure 15a & b.* Mice were 8.09 times as likely to express BrdU+/DCX+ cells in the SGZ of the hippocampus if they were not exposed to radiation, *p* < .01.
Figure 16a & b. Mice were 5.43 times more likely to express BrdU+/DCX+ cells in the GCL of the hippocampus if they were not exposed to radiation, $p = .003$. 
Figure 17a-d. Co-localization of BrdU (cell proliferation) and DCX (neurogenesis). Sham animals displayed co-localization in the SGZ (a) and GCL (c), while no BrdU+/DCX+ cells were found after radiation for either SGZ (b) or GCL (d). Scale bar = 50 µm.
Table 5

*Summary of immunohistological findings*

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<th>Antibody</th>
<th>Region</th>
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Acute Post-Radiation Behavior

General Activity

There were no activity differences among animals in the open field test at approximately 20 hours following proton irradiation (Figure 18a & b).

Figure 18a & b. No significant differences among the groups. Neither radiation nor pomegranate diet affected males or females.
**Motor Skill: Rotarod**

When rotarod performance was assessed within approximately 24 hours post-radiation, the radiation had no effect (Figure 19a), but females performed better than males, $F(1, 79) = 6.02, p=0.016, \omega^2 = 0.05$, power = .08 (Figure 19b), and in general, animals on a pomegranate juice diet performed better than control-fed animals, $F(1, 79), = 4.60, p = 0.035, \omega^2 = 0.04$, power = .07 (Figure 19c).

![Rotarod - Fast Accelerating](A)

*Figure 19a.* Radiation had no significant effect on rotarod performance.
Figure 19b. Females performed better than males on the fast-accelerating rotarod 1 day post-radiation, *p<0.02.

Figure 19c. Animals on a pomegranate diet performed better than animals on a control diet on the fast-accelerating rotarod 1 day post-radiation, *p<0.04.
Motor Skill: Balance Beam

At approximately 48 hours post-radiation, motor skills/coordination was tested with the balance beam. Radiation and pomegranate diet had a different affects on males and females. For both outcome measures (distance traveled and reversals made), females were more active than males. For both outcome measures, radiation increased activity level for control-fed males, but pomegranate juice ameliorated this effect. Neither radiation nor diet affected females for either measure. Distance traveled: $F(1, 81) = 4.05, p=0.048, \omega^2=.03$, power = .06 (Figure 20a-d); reversals: $F(1, 81) = 6.52, p=0.013, \omega^2=.05$, power = .08 (Figure 21a-d). Note: graphs C and D show the same data from graphs A and B presented somewhat differently.
Figure 20a & b. Females traveled a greater distance than males. Radiation increased activity in males, but pomegranate juice ameliorated this affect, *p*<.05.
Figure 20c & d. Females traveled a greater distance than males. Radiation increased activity in males, but pomegranate juice ameliorated this affect, *p<.05.
Figure 21a & b. Females reversed more than males. Radiation increased reversal in males, but pomegranate juice ameliorated this affect, *p<.02.
Figure 21c & d. Females reversed more than males. Radiation increased reversal in males, but pomegranate juice ameliorated this affect, *p<.02

Long-Term Behavior

Test Effects

For all tests there was a test effect at the post-radiation time point (data not shown). Animals demonstrated that they were more familiar with the testing process during the final battery of behavioral tests (8 weeks after baseline tests). For the water
maze (all tests: cued, spatial, and probe), mice were better able to find the platform or spent more time in the platform area (probe) at the post-radiation time point compared with baseline performance, suggesting a test effect: cued, $F(1, 82) = 326.48, p < 0.01$; spatial, $F(1, 82) = 20.77, p<0.01$; probe, $F(1, 82) = 5.07, p = 0.027$. For the tail-suspension test, mice demonstrated greater depression-like behavior during the post-radiation trial (compared to baseline) regardless of radiation group $F(1, 82) = 31.95, p<0.01$, suggesting a greater familiarity with the test the second time around. For the zero maze, animals spent more time in the dark during the post-radiation time point compared with baseline, $F(1, 82) = 44.57, p < 0.001$. Rotarod tests demonstrated a similar pattern; all animals performed better during the post-radiation time point (compared to baseline) for all tests (steady: $F(1, 82) = 80.23, p<0.001$; slow accelerating: $F(1, 82) = 61.93, p<0.001$; fast accelerating: $F(1, 82) = 22.61, p<0.001$). Finally, in the open field test, animals generally had less activity (spent less time exploring the novel environment) during the post-radiation time point, $F(9,738) = 11.86, p <0.001$, again suggesting a familiarity with the test. Despite these test effects, there were several effects due to differences among the independent variables: gender, radiation, and treatment for the various cognitive and behavioral tests.

**Learning/Memory**

No differences were seen between the radiation and diet groups at baseline. For the cued test, male mice found the platform more quickly than female mice during baseline testing; however, this effect disappeared at the post-radiation testing point, $F(1, 82) = 4.95, p=0.029, \omega^2=.07, \text{power} = .10$ (Figure 22). Radiation had no effect on any water maze test (cued, spatial, or probe: Figure 23a & b). For animals that were not
exposed to radiation, males on a pomegranate diet and females in general learned to locate the position of the hidden platform more quickly than control-fed males, $F(6.08, 249.32) = 2.56, p = .019, \omega^2 = .02$, power = .06 (Figure 24). There were no other gender effects seen for spatial or probe tests.

Figure 22. Male mice found the cued platform more quickly than females during baseline testing; however, at the post-radiation time point there were no gender differences, $p<0.03$. 
Figure 23a & b. There were no significant differences due to radiation for any group.
Figure 24. For non-irradiated animals, male mice on pomegranate juice diet learned the placement of the hidden platform more quickly (similar to females in general) compared with control-fed male mice, $p=0.019$.

**Depression-like Behavior**

The most striking interaction between radiation and pomegranate treatment on behavior was observed in the tail suspension test, which provides an assessment of depression-like behaviors. Compared to baseline (in which no differences were detected among any of the groups), radiation induced depression-like behaviors (i.e. the mice gave up sooner) in control-fed mice, but the pomegranate-fed mice were protected from this effect ($F(1, 82)=4.92$, $p<.03$, $ω^2=.03$, power = .06; see Figure 25a & b). Note: graph B shows the same data from graph A somewhat differently. There was no difference in the performance of males versus females on this test.
Figure 25a & b. Radiation increased depression-like behaviors in control-fed mice, but pomegranate juice protected against this effect, * $p<.03$.

**Avoidance Behavior**

Radiation and pomegranate treatment had gender-specific effects in the elevated zero maze test (Figure 26a-d), which provides an assessment of avoidance behaviors in the face of a potential threat ($F(1, 82)=9.19, p<.004, \omega^2=.08, \text{ power} = .12$). Note: graphs 26 C and D show the same data from graphs 26 A and B somewhat differently. Overall,
pomegranate-treated mice spent more time in the dark quadrants than control-treated mice ($F(1, 82)=7.12, p<0.01, \omega^2=.06, \text{power} = .09$). Normal (non-irradiated control fed) females spent more time hiding in the dark quadrants than normal males ($F(1, 82)=7.67, p<0.008, \omega^2=.06, \text{power} = .09$), suggesting heightened avoidance behaviors in females as compared to males. Pomegranate juice led to more time spent in the dark (greater avoidance behavior) for irradiated females, compared to control-fed irradiated females, suggesting that radiation induced more exploration of the open quadrants in females (indicating lowered avoidance and/or increased exploratory behavior), and that this effect was blocked by the pomegranate treatment. However, both radiation and pomegranate treatment induced behavior in males similar to that seen in females in this test (i.e. spending more time hiding in the dark). The effect of pomegranate on these behaviors in males was attenuated by radiation exposure. There were no significant differences when comparing baseline with post-radiation groups.
Figure 26a &b. Control females spent more time in the dark than control males (suggesting heightened avoidance). Radiation may have induced more exploration of the open quadrants in females (suggesting reduced avoidance and/or abnormal exploratory behavior), but this effect was blocked by pomegranate. Interestingly, radiation did not affect males' performance, but pomegranate consumption in non-irradiated males induced behavior similar to those of females in this test, * $p<0.004$. 
Figure 26c& d. Control females spent more time in the dark than control males (suggesting heightened avoidance). Radiation may have induced more exploration of the open quadrants in females (suggesting reduced avoidance and/or abnormal exploratory behavior), but this effect was blocked by pomegranate. Interestingly, radiation did not affect males' performance, but pomegranate consumption in non-irradiated males induced behavior similar to those of females in this test, * $p<0.004$. 
Motor Skills

Although, the irradiated and sham mice did not differ on post-radiation rotarod performance, which provides an assessment of sensorimotor coordination and balance, the mice that were ultimately assigned to the radiation group had pre-existing performance deficits on the fast accelerating rotarod (Figure 27a). Sham mice had a greater difference from baseline compared to irradiated mice (Figure 27b), $F(1, 82) = 5.22, p=0.025, \omega^2=.11, \text{ power } = .18$, suggesting a possible radiation effect given equal baseline performance. Indeed, while sham mice significantly improved from baseline to post-radiation time point, $t(44) = -4.52, p = .003$, radiation mice did not ($p = .09$).
Figure 27a & b. There was a significant difference in how the animals assigned to each group (radiation vs. sham) performed at baseline compared to post-radiation time point (9a). In looking at differences from baseline, sham animals demonstrated a greater difference from baseline performance compared to irradiated animals, *p*<0.03(9b).

Males generally performed more poorly (fell off more quickly) than females on all rotarod tasks both before and after radiation except in the steady rotarod test measured at baseline (Figure 28a & b). Baseline Tests: (a) Slow: $F(1, 87) = 5.69, p = 0.02, \omega^2=0.05,$
power = .08; (b) Fast: $F(1, 87) = 4.72, p=0.033, \omega^2=.04, \text{ power } = .06$. Post-Radiation Tests: (a) Steady: $F(1, 82) = 4.60, p=0.035, \omega^2=.04, \text{ power } = .06$; (b) Slow: $F(1, 82) = 5.28, p=0.024, \omega^2=.05, \text{ power } = .08$; (c) Fast: $F(1, 82)=4.15, p<.05, \omega^2=.03, \text{ power } = .06$.

However, pomegranate treatment improved the performance of males to that of females on the fast accelerating rotarod, post-radiation (Figure 29), although it did not further improve the performance of females ($F (1,82) = 4.20, p<.05, \omega^2=.03, \text{ power } = .06$). Diet did not affect rotarod performance for either the slow or steady rotarod tests.
Figure 28a & b. Although males and females performed similarly during the first learning trials of the steady rotarod (a), for all other tests females performed better than males (a & b), *p<0.05.
Figure 29. Males fell off the rotating beam faster than females during the fast accelerating rotarod, but pomegranate treatment improved their performance to that of females, *p<0.05.

Activity Levels

Radiation had no effect on the open field test, which provides an assessment of overall activity levels and patterns. Females traveled more than males during baseline testing but not during post-treatment testing and had a significantly greater difference from baseline compared to males (Figure 30), $F(1, 9629.44) = 9.31, p = 0.003, \omega^2 = .09$, power = .13, suggesting females explored the environment significantly more than males at the initial testing point. However, 8 weeks later, this overall gender difference disappeared, and diet mediated activity for males but not females. Specifically, for males, pomegranate juice prevented the lower activity seen in control-fed males, leading to more activity similar to that of female animals on this test, $F(8.75, 708.94)=2.23, p<0.03, \omega^2=.05$, power = .05 (Figure 31a & b).
Figure 30. Females traveled more during baseline and had a greater difference from baseline compared to males. *$p=0.003$. 

*Openfield*
Figure 31a & b. Radiation had no effect on the open field test. Pomegranate treatment prevented the lower activity for male mice (a), but not female mice (b), *p<0.03.
Table 6

Summary of significant behavioral findings and their associated p-values

<table>
<thead>
<tr>
<th>Test</th>
<th>Summary of Significant Behavioral Findings</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water maze</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cued</td>
<td>Males better at finding cued platform at baseline only</td>
<td>&lt;.03</td>
</tr>
<tr>
<td>Spatial</td>
<td>PJ led males to perform more similar to that of female mice (control-fed males took longer to learn the spatial task).</td>
<td>&lt;.02</td>
</tr>
<tr>
<td>Probe</td>
<td></td>
<td>n.s.</td>
</tr>
<tr>
<td>Tail Suspension</td>
<td>Radiation increased immobility, but PJ restored it</td>
<td>&lt;.03</td>
</tr>
<tr>
<td>Zero maze</td>
<td>PJ made sham males perform similar to that of female mice (&gt; time in dark)</td>
<td>&lt;.004</td>
</tr>
<tr>
<td></td>
<td>Radiation induced greater exploration in females</td>
<td>&quot;&quot;</td>
</tr>
<tr>
<td>Open field</td>
<td>Females more active than males at baseline</td>
<td>&lt;.01</td>
</tr>
<tr>
<td></td>
<td>PJ made males perform similar to female mice in activity</td>
<td>&lt;.03</td>
</tr>
<tr>
<td>Rotarod</td>
<td>Sham group significantly improved from baseline, radiation group did not.</td>
<td>&lt;.03</td>
</tr>
<tr>
<td></td>
<td>Females better than males*</td>
<td>&lt;.05</td>
</tr>
<tr>
<td></td>
<td>PJ led males to perform similar to female mice</td>
<td>&lt;.05</td>
</tr>
<tr>
<td></td>
<td>PJ improved performance**</td>
<td>&lt;.04</td>
</tr>
<tr>
<td>Balance Beam</td>
<td>Females more active than males**</td>
<td>&lt;.05</td>
</tr>
<tr>
<td></td>
<td>Radiation increased male activity, PJ ameliorated this**</td>
<td>&quot;&quot;</td>
</tr>
</tbody>
</table>

*Result was found for both long-term and acute post-radiation data. **Data found only for acute post-radiation tests.

Behavioral Correlations

Pomegranate-fed males had improved activity and decreased avoidance behaviors across tests. Specifically, pomegranate-fed males that demonstrated better motor skills/coordination also had increased activity in the open field test of locomotor activity, $r = .45$, $p = .028$ (Figure 32). Furthermore, pomegranate-fed males that had increased
activity in the open field displayed less avoidance behavior on the zero maze, $r = -0.61$, $p = 0.002$ (Figure 33). Also, in general males (but not females) that found the hidden platform more efficiently in the spatial water maze also had motor skills on the rotarod test, $r = -0.46$, $p = 0.001$ (Figure 34).

![Graph](image)

**Figure 32.** Pomegranate-fed males that performed better in the rotarod test of motor skills had greater activity in the open field test of locomotor activity, $p = 0.028$. 

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Figure 33. Pomegranate-fed males that had increased locomotor activity on the open field test also spent less time in the dark on the zero maze, \( p = .002 \).

Figure 34. Males, but not females, that performed better on the spatial water maze (lower score), also performed better on the rotarod test of motor skills/coordination, \( p = .001 \).
Furthermore, irradiated females that demonstrated greater levels of avoidance behavior on the zero maze test also had poorer performance on the rotarod (fell of the quickly), $r = -.45$, $p = .035$ (Figure 35). Also, non-irradiated females on a control diet that displayed greater depression-like behavior on the tail suspension test showed greater avoidance behavior in the zero maze, $r = .61$, $p = .036$, (Figure 36).

*Figure 35.* Irradiated females that performed more poorly on the rotarod showed greater avoidance behaviors, $p = .035$. 
Figure 36. Non-irradiated females on a control diet that showed greater depression-like behavior also showed more avoidance behavior, $p = .036$.

Surprisingly, some behavioral tests were correlated with tests in an unexpected way. In general, mice that had better performance on the rotarod test of motor activity had increased depression-like behaviors, $r = .21$, $p = .046$ (Figure 37). In addition, non-irradiated mice on a control diet that showed increased avoidance behavior on the zero maze (more time spent in the dark), also had better performance on the rotarod test of motor skills, $r = .49$, $p = .018$ (Figure 38).
Figure 37. In general, mice that had decreased mobility on the tail suspension test had increased motor skill performance on the rotarod, $p = .046$.

Figure 38. Non-irradiated control-fed mice that had better motor skills on the rotarod test spent more time in the dark on the zero maze, showing greater avoidance behavior, $p = .018$. 
Table 7

Summary of significant correlations among behavioral tests with their respective Pearson’s r coefficient

<table>
<thead>
<tr>
<th>Correlated Tests</th>
<th>Results</th>
<th>Pearson's r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotarod/Open field</td>
<td>PJ-fed males that had better motor skills were more active</td>
<td>0.45</td>
</tr>
<tr>
<td>Open field/Zero maze</td>
<td>PJ-fed males that were more active spent more time in the dark</td>
<td>-0.61</td>
</tr>
<tr>
<td>Water maze/Rotarod</td>
<td>Males that had better motor skills did better on water maze</td>
<td>-0.46</td>
</tr>
<tr>
<td>Rotarod/Zero maze</td>
<td>Irradiated females with poorer motor skill had more avoidance behavior</td>
<td>-0.45</td>
</tr>
<tr>
<td>Tail Suspension/Zero maze</td>
<td>Non-irradiated females on control diet had both increased depression-like &amp; avoidance behavior</td>
<td>0.61</td>
</tr>
<tr>
<td>Rotarod/Tail Suspension</td>
<td>In general mice that had better motor skills had greater depression-like behavior</td>
<td>0.046</td>
</tr>
<tr>
<td>Rotarod/Zero maze</td>
<td>Non-irradiated control fed mice that had better motor skills spent more time in the dark</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Behavioral and Biological Correlations

To explore whether decreased neurogenesis and/or cell proliferation was associated with poorer performance in behavioral tests, correlations were measured. Kendall’s tau was used for BrdU/DCX variable due to severe normality violation and Pearson’s r was measured for normal data.

Decreased cell proliferation in the SGZ (as measured by BrdU) was associated with less activity in the open field test of locomotor activity, $r = .46, p = .023$ (Figure 39).
Increased motor skills/coordination on the rotarod was associated with increased cell proliferation in the GCL of the dentate gyrus, but only for female mice, $r = .57, p = .005$ (Figure 40). Also, decreased neurogenesis (DCX) was associated with less avoidance behaviors in female mice (less time spent in the dark), $r = .45, p = .027$ (Figure 41).

**Figure 39.** Less activity was associated with fewer new cells in the SGZ of the dentate gyrus, $p = .005$. 

![Graph showing the relationship between distance traveled and BrdU+ cells in the SGZ](image-url)
Figure 40. Greater motor skills was associated with increased cell proliferation in the GCL of the dentate gyrus for female mice, $p = .023$.

Figure 41. Females that had decreased neurogenesis in the DG also spent less time in the dark on the zero maze, suggesting lowered avoidance behavior, $p = .027$.

Furthermore, decreased neurogenesis (as measured by DCX) in the total dentate gyrus (significant for SGZ & GCL) was associated with decreased activity in the open
field test of activity, $r = .44$, $p = .002$ (Figure 42). Decreased neurogenesis was also associated with less time spent searching for a previously hidden platform in the correct quadrant during the water maze probe test, $r = .34$, $p = .021$ (Figure 43). Poorer performance on the spatial water maze was found to be associated with decreased neurogenesis (DCX) in the GCL ($r = -.44$, $p = .033$; Figure 44) only for control-fed mice and in the SGZ and GCL for irradiated mice only, $r = -.59$, $p = .002$, and $r = -.56$, $p = .004$, respectively (Figure 45a & b).

![Graph](image)

**Figure 42.** Decreased neurogenesis in the dentate gyrus was associated less activity in the open field, $p = .002$. 
Figure 43. Decreased neurogenesis was associated with less time spent searching in the platform quadrant during the probe water maze test, $p = .021$.

Figure 44. Poorer performance in the spatial water maze test was associated with decreased neurogenesis in control-fed mice, $p = .033$. 
Figure 4a & b. Irradiated mice demonstrated an association between decreased neurogenesis in the SGZ (a) and the GCL (b) and poorer performance on a test for learning and memory, $p < .01$. 
Table 8

Summary of significant associations between decreased cell proliferation and neurogenesis and behavioral tests

<table>
<thead>
<tr>
<th>Variables</th>
<th>Results</th>
<th>Pearson's r</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BrdU</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGZ/Open field</td>
<td>Decreased cell proliferation associated with decreased activity</td>
<td>0.41</td>
</tr>
<tr>
<td>GCL/Rotarod</td>
<td>Increased cell growth was associated with increased motor coordination</td>
<td>0.46</td>
</tr>
<tr>
<td><strong>DCX</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total DG/Zero maze</td>
<td>Decreased cell growth was associated with decreased avoidance behaviors in females</td>
<td>0.45</td>
</tr>
<tr>
<td>Total DG/Open field</td>
<td>Decreased neurogenesis associated with decreased activity</td>
<td>0.44</td>
</tr>
<tr>
<td>GCL/Probe</td>
<td>Decreased neurogenesis associated with decreased performance</td>
<td>0.34</td>
</tr>
<tr>
<td>GCL/Water maze; SGZ/Water maze</td>
<td>Decreased neurogenesis was associated with decreased performance in the spatial water maze for control fed(a) and irradiated mice only(b1 &amp; b2)</td>
<td>-.44 (a) -.59 (b1) -.56 (b2)</td>
</tr>
</tbody>
</table>
CHAPTER FOUR

DISCUSSION

Effects of Radiation, Diet, and Gender on the Hippocampus

The purpose of this study was to explore the effects of proton radiation on behavior and neurogenesis in the hippocampus, as well as the putative protective effects of pomegranate juice on radiation-induced deficits in both male and female mice. We expected radiation to have a negative effect on the hippocampus. Indeed, we found that radiation decreased neurogenesis in the dentate gyrus of the hippocampus, specifically in the SGZ and GCL. The SGZ is the site of cellular proliferation in the hippocampus. Progenitor cells develop, mature and migrate through the GCL. Our results indicate that radiation interferes with this process. This is consistent with previous research findings. C57BL/6 mice irradiated with a long exposure (1-4 hours) of a low dose (0.01Gy/min) of proton radiation for one hour demonstrated an alteration in genes related to apoptosis and cell regeneration in the hippocampus for up to 8-weeks post-radiation (Chang, et al., 2010). Heavy ion radiation has also proven to be deleterious to neurogenesis, decreasing the percentage of immature neurons in the SGZ of the hippocampus significantly as radiation dose increases (Rola, Otsuka, et al., 2004). In addition, C57BL/6J mice receiving photon irradiation (8 Gy) to the whole brain at a young age demonstrated decreased neurogenesis in the GCL of the hippocampus as measured by DCX-positive cells, and this effect continued throughout adulthood. In fact, it was reported that irradiation almost completely suppressed neurogenesis in these areas, (Roughton, Kalm, & Blomgren, 2012). This is similar to what was demonstrated in the current study. Very few DCX-positive cells were evident in irradiated animals.
Not only did radiation decrease neurogenesis, but it also affected the development of new cells as measured by BrdU. BrdU does not specify cell type, but does incorporate itself into the DNA of any newly developing cell. Thus it is often used as an indicator of cellular proliferation. We did find that BrdU was decreased in the SGZ of irradiated mice. Again, this is consistent with the literature (Zou et al., 2012). 56Fe radiation led to significantly fewer BrdU-positive proliferating cells in the SGZ of the dentate gyrus in C57Bl/6 mice and these cells decreased at a dose-dependent rate; as dose increased, cell expression decreased dramatically. Furthermore, these effects can be seen up to 3 months post-irradiation and have been associated with behavioral deficits in learning and memory (Rola, Otsuka, et al., 2004; Rola, Raber, et al., 2004). Altogether these findings not only suggest that radiation, including proton radiation, has a deleterious effect on neurogenesis and cell proliferation in the hippocampus, but these affects can lead to long-term changes in the brains of these mice.

Furthermore, research suggests that these affects may be attenuated by antioxidant mechanisms (T. T. Huang, Zou, & Corniola, 2012; Zou, et al., 2012). Although pomegranate juice was not found to specifically mitigate radiation-induced neurogenesis suppression and cell growth deficiency within the dentate gyrus, it did lead to greater neurogenesis in the SGZ of the dentate gyrus of non-irradiated animals, suggesting that antioxidants either promote neurogenesis or protect against its suppression or both. Research indicates that antioxidants, such as those in green tea (epigallocatechin-3-gallate or EGCG) and grapes (resveratrol), upregulate mRNA and protein markers that promote growth of neural progenitor cells in the hippocampus of rodents (Wang et al.,
2012), as well as reduce injury-induced neuroinflammation and microglia response (Gatson et al., 2013) that can be damaging to cell growth.

We were expecting that pomegranate juice would ameliorate radiation-induced injury; however, this was not the case. It is possible this was due to treatment length and radiation dose, as well as bioavailability of pomegranate polyphenols. Given the low bioavailability of pomegranate polyphenols in crossing the blood brain barrier (J. P. Spencer, Vauzour, & Rendeiro, 2009), 3 months on pomegranate juice may not have been long enough to see a strong effect of polyphenol antioxidants. In addition, although 2 Gy is a relatively low dose, it is a relatively large dose in the context of exposure for astronauts. Therefore, it may be that our dose was too strong and our treatment timeframe too short to see any neuroprotective effects.

Although we did not find a difference in the expression of neurogenesis between male and female mice, non-irradiated males expressed greater cellular proliferation in the GCL of the hippocampus than non-irradiated females. This is in contrast with current studies where non-irradiated female C57BL/6 mice expressed more BrdU-positive markers for cell proliferation than males (Roughton, et al., 2012). This seemingly contradictory finding may be explained by the type of behavioral tests used in these respective studies. Roughton et al. (2012) tested male and female mice on open field (locomotor activity) and IntelliCage (operant conditioning). This current study used several other test measures including the Morris water maze (spatial learning and memory). In general, neurogenesis increases after learning; however, there seems to be a gender-specific hippocampal response to the spatial water maze task. Male rodents demonstrate greater BrdU-labeled cell proliferation in the GCL than females days after
spatial water maze training (Chow, Epp, Lieblich, Barha, & Galea, 2012). Therefore, the greater cellular proliferation of the GCL found in males over females in this study may have been the result of spatial water maze training days prior to perfusion.

In addition, pomegranate juice increased cellular proliferation in the SGZ of male mice over control diet males and pomegranate diet females, suggesting that an antioxidant-rich diet may be more advantageous for males in promoting cell growth. These effects seen in males, but not females, might be explained by the presence of phytoestrogens in pomegranates (Auerbach et al., 2012; Kaufman & Wiesman, 2007). Research indicates that estrogen, known as the female sex hormone, has a similar structure to that of antioxidants and may provide a neuroprotective benefit (Behl & Manthey, 2000). In fact, pomegranate seeds have been reported to be the richest plant source of estrogens (Mori-Okamoto, Otawara-Hamamoto, Yamato, & Yoshimura, 2004). Therefore, it may be that a diet rich in antioxidant polyphenols is more beneficial to males who have less estrogen than females.

**Effects of Radiation, Diet, and Gender on Cognition and Behavior**

It was also the purpose of this study to explore the effects of radiation on behavior and whether pomegranate juice provides some measure of protection from these radiation-induced changes. The long-term behavioral data from this study have been recently published (Dulcich, 2013). Our findings demonstrated that mice exposed to proton radiation displayed more depression-like behaviors than non-irradiated mice, but that pomegranate juice blocked this deficit. Tests that provide the latency at which an animal stops struggling to escape an inescapable situation are often used to measure
depression-like behaviors in animal models (Steru, et al., 1985). Other studies have also reported the amelioration of depression-like behavior in animals receiving pomegranate treatment. Pomegranate extract successfully reduced depression-like behaviors in mice demonstrating ovariectomy-induced depression-like symptoms (Mori-Okamoto, et al., 2004). In addition, pomegranate seed extract was found to decrease depression-like behaviors similar to imipramine (an antidepressant drug) treatment in young and old mice (Kumar, et al., 2008).

This putative protective effect of antioxidant administration against depression-like behavior has also been demonstrated with other antioxidants. For example, male Kunming mice subjected to chronic unpredictable mild stress showed increased depression-like behaviors on the tail suspension test, but 25 mg/kg and 50 mg/kg of tea polyphenols ameliorated this stress-induced effect (Y. Liu et al., 2013). Similarly, female Swiss mice also exposed to chronic unpredictable mild stress also demonstrated an increase in depression-like behavior on the tail suspension test, but ascorbic acid had an antidepressant-like effect similar to that seen with fluoxetine, an SSRI antidepressant. Additionally, stress-induced depression-like behavior was correlated with an increase in oxidative stress in the hippocampus of these mice, and ascorbic acid reduced these markers (Moretti et al., 2012). Together with the findings of the current study, this research suggests a link between depression and oxidative stress. In fact, studies show depression is associated with increased oxidative stress and decreased antioxidant enzyme activity, leading to subsequent DNA damage (Maes, Leonard, et al., 2011). Furthermore, depression may occur in response to neuroinflammation, increased oxidative stress, decreased neurogenesis and/or increased apoptosis (Maes, Kubera, et al.,
2011). Specifically, radiation-induced suppression of hippocampal neurogenesis in rodents has led to depression-like behavior (Snyder, et al., 2011), thus suggesting that the incidence of depression may increase with radiation exposure and its associated oxidative stress induction. In fact, research indicates not only that stress decreases SGZ cell proliferation, but that this is linked to behavioral outcomes that may be specific to “controllable” versus “uncontrollable” stress. Male rats exposed to a foot shock in an inescapable condition, demonstrated significantly decreased neurogenesis in the SGZ compared to male rats exposed to the same amount of stress/shock, but with control to escape (Shors et al., 2007). In the current study, radiation did indeed decrease neurogenesis in the dentate gyrus of the hippocampus; however, this decrease was not found to be correlated with depression-like behaviors. However, according to Zhao et al. (2008), a link between decreased hippocampal neurogenesis and depression-like behaviors on a test such as the tail suspension test is not necessarily a matter of a simple correlation. Antidepressants have repeatedly been shown to increase neurogenesis; however, the behavioral effects of antidepressants on SGZ neurogenesis seem to depend on several factors, including species, the genetic background of the animal, and expression of serotonin and noradrenalin in that animal. Direct correlations between depression-like behavior and neurogenesis are not always obvious (Zhao, Deng, & Gage, 2008). In addition, it has been suggested that the link between antidepressants, neurogenesis, and behavior may be in the regulation of synaptic signaling/function in newborn (and adult) neurons, since antidepressants have been shown to regulate proteins involved in synaptic transmission and dendritic spine formation in the hippocampus (Hajszan, MacLusky, & Leranth, 2005; Zhao, et al., 2008) Therefore, the mechanisms
underlying the antioxidant and anti-inflammatory properties of pomegranate juice and its putative protective qualities against depression may be more complicated than could be measured in this experiment.

We hypothesized that female mice would demonstrate greater-learned helplessness in contrast with male mice; however, males and females performed similarly on the test of depression-like behavior. Studies on gender-specific depression-like behaviors on the tail suspension test are inconsistent. Liu and Gershenfeld (2001) tested eleven different strains of male and female mice on the tail suspension test. On baseline tests, four strains (including C57BL/6J mice) were found to have significant gender differences with females displaying greater immobility (thus greater depression-like behavior) compared to males (X. Liu & Gershenfeld, 2001). However, other research reported no gross differences between male and females on the tail suspension test among several mouse strains (including C57BL/6 mice), although C57Bl/6 mice did show some decreased immobility after unpredictable chronic mild stress. In addition, specific effects of gender differences found in this study, showed increased depression-like behaviors were more likely to be seen in male mice, rather than female mice (Mineur, Belzung, & Crusio, 2006). This may be due to housing issues. To minimize housing costs, mice are group-housed. In a cage of females, there is generally no fighting for hierarchy, but this is not necessarily true for a cage of males. Thus, males are exposed to potentially greater stress levels than females overall simply due to housing arrangements. As it has been previously stated, stress leads to increased levels of depression-like behavior on this test. This additional stress may mask any gender differences that might otherwise be evident (Mineur, et al., 2006). However, the studies that have reported gender differences in
depression-like behavior also group-housed males. Therefore, other factors may need to be considered. In the present study, diet mediated radiation-induced depression behaviors, and although diet was not found to significantly mediate gender-specific responses in the tail suspension test, there may have been enough variability in gender-specific diet responses to mask an overall gender difference.

This study also demonstrated gender specific differences in avoidance behaviors (in the face of a potential threat) and some interesting interactions of radiation and pomegranate juice. Control females demonstrated greater avoidance behaviors compared to control males on the zero maze test, spending more time in the dark enclosed quadrants. Other studies have also shown that C57BL/6 female mice spend more time in the dark enclosed arms of the elevated plus maze compared to males (An et al., 2011; Hartman, Kamper, Goyal, Stewart, & Longo, 2012). The elevated plus maze is also a test of avoidance behavior for mice and is analogous to the elevated zero maze. Interestingly, radiation induced more exploration of the open quadrants (suggesting abnormally low avoidance levels and/or increased risky behavior) in the female mice of our study. Pomegranate treatment blocked this effect of radiation on female behavior. Pomegranate treatment also increased hiding in the enclosed quadrants for non-irradiated male mice, inducing behaviors similar to that seen in female mice (i.e. greater avoidance behavior), and radiation reduced this effect in males.

One study reported that exercise-induced elevated levels of hippocampal neurogenesis were correlated with increased avoidance behavior on the O-maze (analogous to the zero maze), the open field, and dark/light box, but that radiation attenuated this effect, leading to reduced avoidance behaviors in mice, thus suggesting a
direct connection between hippocampal neurogenesis and stress-related anxiety (Fuss et al., 2010). In fact, there was an association between increased neurogenesis in females and increased time spent in the dark on the zero maze in this study. In addition, radiation decreased neurogenesis in the dentate gyrus for all mice in this study. It is possible that the antioxidant and anti-inflammatory effects of pomegranate may have ameliorated deleterious effects of radiation on hippocampal neurogenesis in our female mice. Specifically, we speculate that radiation reduced natural levels of avoidance behavior in the face of a potential threat, and that this is linked to reduced neurogenesis in the hippocampus, and that pomegranate juice restored natural avoidance behaviors in our female mice. Indeed, dietary polyphenols have actually been shown to stimulate neurogenesis in the hippocampus (S. J. Kim et al., 2008; Valente et al., 2009).

Our findings also demonstrate that radiation may have had an effect on motor coordination and balance performance as measured by the accelerating rotarod. Although we did not find a significant difference between the groups after radiation, the baseline groups that had not yet undergone irradiation were significantly different in their performance. The animals that ended up being assigned to the “radiation” group demonstrated superior motor coordination and balance to that of the animals ultimately assigned to the “sham” group. Further tests indicate that while the “sham” group improved significantly from baseline, the group that was exposed to radiation did not; thus suggesting radiation did affect these mice. Research reporting radiation effects on motor balance and coordination (as measured by the rotarod) are somewhat mixed. Some studies indicate that radiation does affect motor performance on the rotarod. Mice irradiated at 10 Gy (gamma) performed significantly worse on the accelerating rotarod
than sham mice tested 2-weeks post-radiation (Dayger, Villasana, Pfankuch, Davis, & Raber, 2011). However, when C57Bl/6J mice (either sedentary or exercise group), were exposed to a lower dose of radiation repeatedly across time (3 bouts of 5 Gy gamma radiation), they did not demonstrate any significant difference from controls on an accelerating rotarod performance 3.5 months post-radiation regardless of treatment group (Clark et al., 2008). In fact, this occurred despite the fact that radiation significantly reduced neurogenesis in the hippocampus of these mice, suggesting that hippocampal neurogenesis does not necessarily affect motor performance. Other studies also report conflicting findings. C57BL/6 mice exposed to 3 or 4 Gy proton radiation were significantly impaired on steady but high speed (18 RPM) rotarod performance within 24 hours (3 Gy only) and 1, 2, 4, 8 & 12 weeks (3 & 4 Gy) following radiation; however, a second experiment (starting at 2 weeks and continuing through week 12, did not show this same effect (Pecaut et al., 2002). In the current study, 2 Gy proton radiation did not affect mice within 24-hours post-radiation; however, these results should be interpreted with caution given the difference in baseline. Further tests revealed that both sham and radiation groups improved significantly from baseline, suggesting no radiation effect. This difference from literature may also be explained in the radiation dosage difference. Pecaut et al. (2002) reported a radiation-induced motor deficit within 24-hours post-radiation for one experiment, but the dose was 3 Gy. Radiation delivered at 2 Gy, as in the current study, may not have been at a high enough dose to elicit acute post-radiation deficits.

Interestingly, at 48 hours post-radiation, radiation increased motor activity of control-fed male mice on the balance beam test, but pomegranate juice ameliorated this.
No effect of radiation or diet was seen in females. It is unclear why our results indicate that radiation would increase “motor skills” for males while an antioxidant would decrease “motor skills.” There is very little research on the effects of ionizing radiation exposure and balance beam activity. Studies have shown that brain injury and disease can decrease motor coordination and balance on this test (more foot slips) and that treatment with antioxidant properties can protect against these deficits (Germano, Imperatore, d'Avella, Costa, & Tomasello, 1998; Kalonia, Kumar, & Kumar, 2011). These outcomes contradict what we found. However, these studies recorded number of foot slips and latency to first beam crossing, whereas we focused on total distance traveled and successful reversal. Therefore, it may be that our measures were targeting activity level on the beam rather than coordination per se, and that radiation led to increased agitation in males in the balance beam environment, which pomegranate juice decreased in males. However, this needs to be explored further to see if this bears out.

Our findings also demonstrated that pomegranate juice treatment led to gender-specific behavioral changes even in the absence of radiation-induced deficits. Measures of sensorimotor coordination and balance showed that females performed significantly better than males on the rotarod across most tasks, in agreement with the current body of literature. Female C57BL/6J mice that either exercised or were sedentary, and then either assigned to a sham radiation group or exposed to 3 sessions of 5Gy gamma radiation performed significantly better on the rotarod across all groups (Clark, et al., 2008). Also, hypertensive female (BPL/2) and normal blood pressure female mice (BPN/3) performed better on the rotarod compared to their male counterparts (Hartman, et al., 2012).
Furthermore, in the current study, this sensorimotor/coordination gender difference was mediated by pomegranate juice. Specifically, pomegranate treatment improved the rotarod performance of males to that of females on the fast accelerating task (the most difficult rotarod test), but not the steady or slow tasks. This suggests that pomegranate polyphenols may have a protective effect for difficult motor skills tasks for males. Several studies point to the protective effects that anti-inflammatory and antioxidant-rich polyphenols have on motor functioning. Extra virgin olive oil, rich in polyphenols and anti-inflammatory healthy fats, showed a protective effect against age-related motor deficits as measured by the rotarod and reduced inflammation in the cerebellum for C57BL/6J mice (Pitozzi et al., 2012). Also, male Fischer rats fed a diet of 50% Concord grape juice had improved functioning in age-related motor deficits compared to controls (Shukitt-Hale, Carey, Simon, Mark, & Joseph, 2006). In fact, there is evidence that several types of antioxidant-rich fruits, including strawberries, blueberries, and cranberries play a protective role against age-related motor deficits (Willis, Shukitt-Hale, & Joseph, 2009). Oxidative stress increases in the aged brain and it has been shown to be ameliorated by antioxidants. These studies, however, were conducted on male rodents and may have missed a gender-specific interaction of antioxidants on motor coordination and balance. In one study, male and female C57BL/6 x CBA mice (bred to express Charcot Marie tooth disease) were tested on the rotarod after two months of treatment with ascorbic acid. Initially, females performed better than males, but after treatment, although both males and females showed significant improvement, males improved significantly more than females (Passage et al., 2004), suggesting a greater effect on males.
Proton radiation was not found to have an effect on locomotor activity in our mice either within the day following radiation nor 8 weeks post-radiation; however, this is consistent with published proton radiation literature. C57BL/6 mice exposed to 0, 3, or 4 Gy proton radiation showed no effects of radiation on general ambulation in the open field test at any time tested (24 hrs, 1, 2, 4, 8, or 12 weeks) post-radiation (Pecaut, et al., 2002). Even though radiation had no effect on the open field test, we saw a similar pattern of pomegranate juice on males as reported for the rotarod test. Pomegranate treatment led to greater total distance traveled for male mice, inducing behavior similar to that of female mice, suggesting a dietary effect on exploratory behaviors. Again, this seems to point to the hormonal differences between males and females. It has been reported that female rodents tend to display greater locomotion/exploratory behavior in this test, and this corroborates our finding that females were more active than males during baseline open field tests. Female Long-Evans rats were found to be more active than male rats on a hole board/open field paradigm in a study examining gender differences in stress-related behavior and neuroendocrine function (Duchesne, Dufresne, & Sullivan, 2009). In addition, one study explored activity levels in males and females of 15 different mouse strains and showed that female C57BL/6 mice were more active compared to their male counterparts (O'Leary, Gunn, & Brown, 2013). Interestingly, this activity difference may be linked to an estrogen receptor gene. ERKO male mice are deficient in estrogen receptors, and this deficiency can lead to decreased masculine behaviors (such as aggression and ejaculation) making them behave more similarly to female mice. Ogawa et al. (1997) showed that ERKO males not only demonstrated decreased/shortened aggression behaviors, but also displayed more activity, behaving
similarly to the female mice in the open field compared to wildtype males (Ogawa, Lubahn, Korach, & Pfaff, 1997).

It may be that the antioxidant, anti-inflammatory and anti-apoptotic properties of pomegranate juice had a beneficial effect on males, increasing cell proliferation and thus activity. Research has linked decreased cell proliferation in the hippocampus with decreased activity in the open field test (Hayashi, Takashima, Murayama, & Inokuchi, 2008). In the current study, both increased cell proliferation and increased neurogenesis were associated with greater activity in the open field test. Studies have shown that a diet including natural antioxidants is linked to increased activity in tests of the open field and may ameliorate stress- or injury-induced decrements. Adult male Swiss mice treated for 30 days with anthocyanins, a phytochemical with antioxidant properties found in berries, showed increased activity in the open field test compared to controls. Furthermore, increased levels of anthocyanin dosage resulted in increased activity (Barros et al., 2006). Also, male C57BL/6 mice receiving a treatment that induced dopaminergic neurotoxicity demonstrated decreased activity in the open field test, but 3 weeks of curcumin treatment increased activation over that of non-treatment groups (Ojha, Rastogi, Devi, Agrawal, & Dubey, 2012). Additionally, chronic unpredictable mild stress lead to decreased activity in the open field in male Kunming mice, but treatment with tea polyphenols ameliorated this affect (Y. Liu, et al., 2013).

Furthermore, male mice on a pomegranate diet performed similarly to females and learned the position of the hidden platform in the spatial water maze more quickly compared to male mice on a control diet. Interestingly, most of the gender-specific effects of pomegranate treatment functioned to make the performance of males more
similar to the performance of females. Again, these effects seen in males, but not females, might be explained by the presence of phytoestrogens in pomegranates (Auerbach, et al., 2012; Kaufman & Wiesman, 2007).

We did not detect a significant effect of radiation on spatial learning and memory 2 months post-radiation as measured by the water maze, but this is consistent with the recent literature on spatial learning and proton irradiation. One study measured male Sprague-Dawley rats irradiated with a proton beam at 0, 1.5, 3, & 4 Gy and did not see any behavioral differences in spatial learning and memory at any dose 6-7 weeks post-radiation (Shukitt-Hale, Szprengiel, Pluhar, Rabin, & Joseph, 2004). However, unpublished data from our laboratory indicate that a long-term effect may develop. Wildtype mice exposed to 0.5 Gy whole body radiation did not demonstrate any spatial learning differences compared to controls at 3 months post-radiation, but irradiated mice demonstrated spatial learning deficits at 6 months post-radiation (compared to controls). This is in contrast to heavy atomic nuclei and high energy level particle (HZE) radiation. Even low doses of HZE, such as 56-Fe, have been shown to cause spatial learning deficits in the water maze, (Shukitt-Hale, et al., 2007), sometimes as early as 30 days post-radiation (Manda, Ueno, & Anzai, 2008). Furthermore, these deficits have been ameliorated by compounds found in fruits such as strawberries and blueberries (Shukitt-Hale, et al., 2007), and alpha lipoic acid, which can have antioxidant effects (Manda, et al., 2008), suggesting that HZE particle radiation may cause more extensive hippocampal damage via oxidative stress than proton radiation, and that antioxidants may be protective against radiation-induced hippocampal damage. Even though there was no direct effect of radiation on spatial learning, we did see an association between neurogenesis and water
maze performance. Mice that showed less neurogenesis also had poorer performance on the probe test. These mice spent less time in the target quadrant searching for the platform. In addition, we saw an association between decreased neurogenesis and poorer performance on the spatial water maze, but only for irradiated mice on a control diet. So, although there was no cause/effect relationship of irradiation on this test, there seems to be some relationship with decreased neurogenesis and performance.

Although it has been demonstrated that male mice outperform female mice in the water maze (Arters, Hohmann, Mills, Olaghere, & Berger-Sweeney, 1998; Jonasson, 2005), this difference was not found in the current study. The one exception was in the cued test where males outperformed females only in baseline tests; this gender difference disappeared after irradiation, suggesting that females learned how to perform the cued test and improved performance across time. The literature is inconsistent in demonstrating sex differences in the water maze. As mentioned previously, there is typically a male advantage, but other factors may yield different results. For example, a specific test task order may yield gender-specific advantage. When a Spatial task was performed prior to the Cued task, there were no gender differences in performance; however, when the task order was reversed (Cued first followed by Spatial), males outperformed females (Berger-Sweeney, Arnold, Gabeau, & Mills, 1995). In addition, one study showed that 5 and 25-month old male and female C57BL/6NIA mice performed similarly on the water maze, but that 17-month old females had longer path length than 17-month old males (Frick, et al., 2000). Furthermore, one study showed that ICR female mice had shorter escape latencies and spent more time in the target quadrant compared to males (Ge, Qi, Qiao, Wang, & Zhou, 2013). A meta-analysis of the current
body of literature has shown that these inconsistencies arise from several factors: different species used, different testing protocols between experiments, variation in dependent variable (i.e., latency vs. path length), age of the rodent, and pre-training occurrence (Jonasson, 2005).

Turning to human research, we find another possible reason for these differences. Historically, human studies have reported that in general, human males have greater spatial navigation abilities compared to human females (Linn & Petersen, 1985; Maccoby, 1974); however, this literature has been criticized for using only basic measures of mental rotation and spatial visualization in determining spatial abilities. More recent studies, using updated operational definitions of spatial learning (navigation of more complex environments), have demonstrated results indicating a sexual dimorphism exists (Saucier, 2002). There seems to be gender-specific brain activation during the water maze task. One study (Sneider, Sava, Rogowska, & Yurgelun-Todd, 2011), showing no human sex differences on virtual water maze task performance, reported that different brain regions were recruited for males and females (as measured by fMRI) performing the hidden platform spatial task. Although both sexes recruited bilateral hippocampus activation, females recruited more right hippocampus and posterior parahippocampal activity and males more left hippocampus and anterior parahippocampal activity. It has been suggested that the hippocampus processes spatial maps of locations in an environment (Burgess, Maguire, & O'Keefe, 2002), while the parahippocampal lobes processes navigational relevance to targeted object and specializes in encoding and recognizing topographical information (Janzen & van Turennout, 2004). Specifically, the anterior parahippocampal region processes the
characteristics of an item, and the posterior parahippocampal region processes the “object in environment context” information (Eichenbaum, Yonelinas, & Ranganath, 2007). These noted gender-specific region activations suggest sex-dependent cognitive strategies during spatial processing. In addition, during the learning phase of this task, females, but not males, showed increased activation in the parahippocampus region as well as in the cingulate cortex, an area also known for processing learning and memory, suggesting gender-specific encoding strategies of spatial information.

Overall, there is a decided advantage for males in much of the literature, but until there are better controls on sexual dimorphism, age, species, testing protocols, etc, gender-specific difference reports (or the lack thereof) will be difficult to fully interpret.

**Possible Mechanisms of Pomegranate Juice on Cellular and Behavioral Changes**

This study was not able to determine by what mechanisms pomegranate juice may be inducing neurogenesis, cell proliferation, and/or changes in cognition and behavior. However, research is beginning to shed light on the possible mechanisms that may underlie the neuroprotective abilities of pomegranate juice. Pomegranates may protect the brain (and therefore behavior) from the effects of radiation by a number of potential mechanisms. Radiation particles can strike DNA, causing damage and apoptosis. Particles can also strike water molecules, generating reactive oxidative species that cause inflammation, vascular damage, and suppressed neurogenesis. Pomegranate’s antiapoptotic, antioxidant, anti-inflammatory, and provascular (via nitric oxide synthase) properties may protect against these effects (See Figure 46).
Figure 46. Putative protective mechanisms of pomegranates.

Oxidative stress occurs due to both endogenous (oxidation via cellular actions) and exogenous factors (sun, pollution, toxins, etc.). When the balance of oxidation and antioxidant enzymes is lost, ROS (i.e. free radicals) are upregulated. Free radicals contain unpaired electrons, which are highly unstable. Free radicals scavenge the body looking to donate or take an extra electron, thereby damaging the body. Antioxidants either provide the second electron to make up a pair, thereby stabilizing the molecule, or break down the free radical, neutralizing it, and making it harmless (Cheeseman & Slater, 1993). In essence, antioxidants may scavenge free radicals, thereby diminishing damage to cells
and allowing the growth of new cells. Indeed, an antioxidant, found in green tea, (-)-
epigallocatechin-3-gallate (called EGCG) has been shown to promote neurogenesis in the
hippocampus (Yoo et al., 2010), and can diminish oxidative-stress induced damage in
other neurodegenerative diseases (Ehrnhoefer et al., 2006). Specifically, pomegranate
extract has been shown to scavenge free radicals and decrease oxidative stress in several
animal studies (Jurenka, 2008).

However, recent studies indicate that the flavonoids found in antioxidant-rich
fruits such as pomegranates are metabolized extensively, and that relatively low levels of
fruit-derived flavonoids pass through the blood brain barrier and directly scavenge free
radicals by donating an electron (J. P. Spencer, et al., 2009). Rather, it seems that these
antioxidants and their metabolites may indirectly affect the process of neurogenesis via
mechanisms of influencing neural cellular signaling pathways that affect neuronal
survival and differentiation (Williams, Spencer, & Rice-Evans, 2004). For example,
antioxidants may promote neurogenesis by increasing brain derived neurotrophic factor
(BDNF) (Stangl & Thuret, 2009). BDNF is a protein found in the nervous system, and
specifically in the hippocampus of the CNS. It is involved the survival of existing
neurons as well as the development and differentiation of new neurons (E. J. Huang &
Reichardt, 2001). In addition, antioxidants may also promote neurogenesis by increasing
blood flow to the brain. Several studies indicate that antioxidants increase vasculature
and reduce blood pressure (J. P. Spencer, et al., 2009), and increase cerebral blood flow
(Dinges, 2006; Fisher, Sorond, & Hollenberg, 2006; Francis, Head, Morris, &
Macdonald, 2006) while simultaneously upregulating nitric oxide (Heiss et al., 2006). In
turn, this may indirectly affect neurogenesis by increasing the vasculature of the
“neurogenesis niche” in the subgranular zone of the hippocampus. The “neurogenesis niche” is the term coined for describing the unique environment that allows for neurogenesis in an area of the body noted for its inability to produce new neurons (the brain). Within this niche, neural progenitor cells are clustered around blood vessels (Ohab, Fleming, Blesch, & Carmichael, 2006), and angiogenesis may occur simultaneously with the promotion of neurogenesis (Palmer, Willhoite, & Gage, 2000). Angiogenesis is the process by which new blood vessels are formed from pre-existing vessels. Nitric oxide is involved in the promotion of angiogenesis (Shaul, 2002) and thus its upregulation can also indirectly affect neurogenesis, and flavonoids have been shown to upregulate nitric oxide (Heiss, et al., 2006).

Another factor that may be responsible for the neuroprotective effects of pomegranate juice is its anti-inflammatory properties. The inflammatory response is an early, non-specific immune reaction to tissue damage or pathogen invasion. Inflammation of the central nervous system (CNS) is characterized by increased glial activation, pro-inflammatory cytokine concentration, blood-brain-barrier permeability, and leukocyte invasion. When injury of the CNS occurs, microglia are activated to the injury site. They change into an amoeboid shape by altering gene expression, and this alteration of gene expression activates mediators that are important in the normal function of microglia. However, in extended injury (chronic neuroinflammation), these mediators can be detrimental to the neural environment (Streit, 2006). Some of the factors microglia can release include pro-inflammatory cytokines and proteases. Long term, these can lead to neurodegeneration and degradation of the extracellular membrane (Griffiths, Neal, & Gasque, 2007). One major contributor that is believed to propagate this
neuroinflammatory process is interleukin (IL)-1 beta, a pro-inflammatory cytokine that is up-regulated in several diseases including Alzheimer's disease (AD), Parkinson's disease, multiple sclerosis, and other neurodegenerative disorders (McGeer, McGeer, & Yasojima, 2000; Yirmiya & Goshen, 2011).

Therefore, treatments for inflammation focus on blocking microglia activation, which is dependent upon mitogen-activated protein kinase (MAPK), and on inhibiting cytokine synthesis. Pomegranate extract has been shown to be beneficial in treating inflammation; it was shown to attenuate microglia activation and reduce concentrations of TNF-alpha, a pro-inflammatory gene, in the brains of rodents (Rojanathammannee, Puig, & Combs, 2013). Pomegranate fruit extract (POMx) has also been shown to inhibit pro-inflammatory cytokines, such as IL-1, (IL)-6 and IL-8, and downregulate MAPK (Rasheed et al., 2009; Rasheed, et al., 2010).

**Future Directions**

Determining the mechanisms behind the effects of radiation and/or pomegranate juice on cellular proliferation in the brain and behavior is beyond the scope of the current study. Future projects should focus on determining levels of oxidative stress, inflammation, and/or apoptosis to narrow down possible mechanisms for neurogenesis promotion or deficiency. Also, counting neurons in the hippocampus would determine whether radiation-suppressed neurogenesis ultimately affects the total number of mature neurons in this region. In addition, an exploration of cell proliferation in striatal &/or cerebellar regions should be conducted to better understand the effects of radiation and/or pomegranate diet on motor skills. Finally, although differences in performance on spatial
learning and memory tasks were not detected in this study, it is possible that the irradiated mice used compensatory non-spatial search strategies to solve these tasks (Garthe, Behr, & Kempermann, 2009; Pop et al., 2013). Thus swim/search strategy analysis may provide a more sensitive measure of subtle differences than the overall finding of the platform. Analyses of spatial swim/search strategies for this project are currently underway in our lab.

**Conclusions**

Our findings suggest that radiation decreased neurogenesis and cell proliferation in the dentate gyrus of the hippocampus, and that this may have led to changes in behavior. In addition, pomegranate supplementation may provide protection against radiation-induced changes in affective behaviors, and some of these effects may be gender specific. Furthermore, pomegranates may provide gender specific benefits to sensorimotor coordination. Our findings thus highlight the importance of understanding gender differences in the effects of radiation as well as pomegranate treatment. Women have been part of the space program since its inception and comprise ~11% of the astronauts who have flown in space. Previous research has demonstrated that radiation affects males and females somewhat differently; specific health issues for space travel highlight gender differences in bone loss, orthostatic response, and disease risk, indicating differential effects of the space environment on men and women (Custaud, et al., 2002; Dynlacht, et al., 2008; Harm, et al., 2001). Altogether, pomegranate consumption seems to induce beneficial effects, and the possible underlying mechanisms should be explored to better understand their putative benefits.
REFERENCES


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APPENDIX A

IMAGES OF TISSUE FOR EACH GROUP FOR BRDU/DCX
APPENDIX A (CON)

IMAGES OF TISSUE FOR EACH GROUP FOR BRDU/DCX
APPENDIX B

IMAGES OF TISSUE FOR EACH GROUP FOR DCX
APPENDIX B (CON)

IMAGES OF TISSUE FOR EACH GROUP FOR DCX
APPENDIX C

IMAGES OF TISSUE FOR EACH GROUP FOR BRDU
APPENDIX C (CON)

IMAGES OF TISSUE FOR EACH GROUP FOR BRDU

Male
Control Diet
Sham

Male
Pomegranate
Radiation

Female
Control Diet
Sham

Female
Pomegranate
Radiation