RADIATION-INDUCED BRAIN INJURY: NOT JUST A MICROGLIA AND HIPPOCAMPUS STORY ANYMORE

By

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DEDICATION

This work is dedicated to my mentor, Mike. He was a great mentor, boss, and friend. I wish I could still turn to him for guidance. I hope I can always learn from and model his example.
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>central nervous system</td>
<td>CNS</td>
</tr>
<tr>
<td>stereotactic radiosurgery</td>
<td>SRS</td>
</tr>
<tr>
<td>fractionated whole brain irradiation</td>
<td>fWBI</td>
</tr>
<tr>
<td>gray</td>
<td>Gy</td>
</tr>
<tr>
<td>quality of life</td>
<td>QOL</td>
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<td>interleukin-1β</td>
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<td>tumor necrosis factor alpha</td>
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<td>cyclooxygenase-2</td>
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<td>PPARδ</td>
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<td>ICAM-1</td>
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<tr>
<td>glial fibrillary acidic protein</td>
<td>GFAP</td>
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<td>SVZ</td>
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dentate gyrus  
granule cell layer  
neuronal stem cells  
whole brain irradiation  
renin-angiotensin system  
long term potentiation  
activity-regulated cytoskeleton-associated protein  
N-methyl-D-aspartic acid  
gamma-aminobutyric acid  
Alzheimer’s disease  
metabotropic glutamate receptor  
glutamate receptor  
*Corru Ammonis*  
angiotensin  
angiotensin converting enzyme inhibitors  
angiotensin II type 1 receptor antagonists  
angiotensinogen  
angiotensin converting enzyme  
neprilysin  
prolyl endopeptidase  
prolyl carboxypeptidase  
lipopolysaccharide  
prostaglandin E synthase  

DG  
GCL  
NSCs  
WBI  
RAS  
LTP  
Arc  
NMDA  
GABA  
AD  
mGluR  
GluR  
CA  
Ang  
ACEI  
ARB  
AGT  
ACE  
NEP  
PEP  
PCP  
LPS  
PGES
<table>
<thead>
<tr>
<th>Term</th>
<th>Abbreviation</th>
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<tr>
<td>dual specificity phosphatase 1</td>
<td>DUSP1</td>
</tr>
<tr>
<td>sodium vanadate</td>
<td>Na$_3$VO$_4$</td>
</tr>
<tr>
<td>dimethyl sulfate</td>
<td>Me$_2$SO$_4$</td>
</tr>
<tr>
<td>phenylmethylsulfonyl fluoride</td>
<td>PMSF</td>
</tr>
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<td>electromobility shift assay</td>
<td>EMSA</td>
</tr>
<tr>
<td>protein kinase C gamma</td>
<td>PKC$\gamma$</td>
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<tr>
<td>peroxisomal proliferator activated-receptor $\alpha$</td>
<td>PPAR$\alpha$</td>
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ABSTRACT

Elizabeth Diana Moore

RADIATION-INDUCED BRAIN INJURY: NOT JUST A MICROGLIA AND HIPPOCAMPUS STORY ANYMORE

Dissertation under the direction of Mike E. Robbins, Ph.D., Professor and Section Head of Radiation Biology, Department of Radiation Oncology (2009-2012), and Linda J. Metheny-Barlow, Ph.D., Assistant Professor and Interim Section Head of Radiation Biology, Department of Radiation Oncology (2013)

As the population of long-term cancer survivors increases, quality of life (QOL) is becoming an important aspect of cancer therapy. The majority of brain tumor patients surviving 6 months after fractionated whole brain irradiation (fWBI) will develop progressive, irreversible cognitive impairment that has a significant effect on their QOL. Although the mechanism(s) behind the development of radiation-induced brain injury, including cognitive impairment, are unknown, hippocampal microglia-mediated neuroinflammation and altered neuronal function are hypothesized to play a role. However, cognitive studies with renin-angiotensin system (RAS) blockers which increase Ang-(1-7), indicate that microglia and the hippocampus may not be the complete story. Thus, we hypothesized that radiation-induced brain injury may be prevented by i] targeting neuroinflammation in brain cells other than microglia, and by ii] altering neuronal function in other brain regions (e.g. cortex).
To determine if radiation-induced neuroinflammation occurs in brain cells other than microglia, we measured the inflammatory response in irradiated rat primary astrocytes. We demonstrated that radiation induces an astrocytic MAP kinase-mediated inflammatory response characterized by increases in i) IL-1β and IL-6 expression, ii) GFAP and Cox-2 protein, and iii) activation of transcription factors, AP-1 and NF-κB. Pretreatment with Ang-(1-7) inhibited this radiation-induced MAP kinase activation and inflammation. In addition, Ang-(1-7) treatment increased DUSP1, thus, Ang-(1-7) may prevent radiation-induced inflammation in astrocytes through increases in phosphatases. Taken together, these studies identify the astrocyte as a potential player and target for radiation-induced brain injury, including cognitive impairment.

To determine if brain regions other than the hippocampus are involved in radiation-induced brain injury, including cognitive impairment, we studied Homer1a expression that has been shown to be differentially regulated in the hippocampus and cortex in neurological diseases. fWBI differentially induces early changes in Homer1a expression and metabotropic glutamate 1 receptor (mGluR1) signaling in the rat hippocampus and cortex. These changes in Homer1a expression and mGluR1 signaling were prevented with both RAS blockers, L-158,809 and ramipril, that prevent radiation-induced cognitive impairment in rats. These studies indicate that the cortex is likely important for the development of radiation-induced brain injury, including cognitive impairment, and Homer1a regulation may be a potential target for preventing it.
CHAPTER I

General Introduction

1.1 Primary and Metastatic Brain Tumors: Treatment and Management

Over 1.6 million new cancer cases will be diagnosed in 2013; ~70,000 of these new cancer cases are primary brain tumors that are 1/3 malignant and 2/3 non-malignant (1). Additionally, ~30% of the new cancer patients will develop brain metastases (1). Thus, the number of patients diagnosed with a brain tumor each year is >250,000 and continues to increase with improvements in neuroimaging, better systemic treatment of primary tumors, and an aging population (2). Brain metastases are the most common intracranial tumor and a major source of cancer-related morbidity and mortality (3), arising predominantly from lung, breast, kidney, or skin cancer (1, 4).

About 120 types of brain and central nervous system (CNS) tumors were identified to date, and some have multiple subtypes (5). Each tumor type/subtype is genetically distinct, making the search for treatments or a cure extremely difficult. Brain tumors are most commonly treated by surgery, followed by radiation therapy, chemotherapy, or both (2). In the past two decades, little progress has been made in finding new treatments with the exception of adding temozolomide, a radiation sensitizer, during radiation therapy which increases overall survival by several months (6). The median survival of brain tumor patients (Table 1) also varies according to tumor type, location, number, and size (5).
Table 1: Median Survival for Brain Tumor Patients

<table>
<thead>
<tr>
<th>Primary Brain Cancer Type</th>
<th>Median Survival</th>
<th>Tumor Origin of Brain Metastases</th>
<th>Median Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glioblastoma</td>
<td>14 months</td>
<td>Breast Cancer</td>
<td>11 months</td>
</tr>
<tr>
<td>Grade III Astrocytoma</td>
<td>3 years</td>
<td>Lung Cancer</td>
<td>9 months</td>
</tr>
<tr>
<td>Grade III Oligoastrocytoma</td>
<td>4 years</td>
<td>Melanoma</td>
<td>7 months</td>
</tr>
<tr>
<td>Grade III Oligodendrogloma</td>
<td>5 years</td>
<td>Renal Cell Carcinoma</td>
<td>11 months</td>
</tr>
<tr>
<td>Grade II Astrocytoma</td>
<td>5 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade II Oligoastrocytoma</td>
<td>7.5 years</td>
<td></td>
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</tr>
<tr>
<td>Grade II Oligodendrogloma</td>
<td>10 years</td>
<td></td>
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</table>

Conventional treatment options for brain tumors include surgical resection, stereotactic radiosurgery (SRS) and fractionated large field partial or whole brain irradiation (fWBI) with or without chemotherapy (7). Radiotherapy and surgery are localized tumor treatments; thus, there is little systemic toxicity as seen with chemotherapy. Treatments for brain metastases vary according to the size, number of metastases, and location of the metastases within the brain as well as the origin of the primary tumor (7). Some tumor types are more radiosensitive than others (Table 1); small cell lung cancer is radiosensitive, while melanoma and renal cell carcinoma are more radioresistant (2, 8). Patients with accessible metastases and minimal extracranial disease often undergo surgery to reduce the tumor burden and intracranial pressure that lead to the patient’s death (7). SRS is typically reserved for patients who have 1-3 brain metastases that are either small or surgically inaccessible (7). The majority of metastatic brain tumor patients have >3 brain metastases which are often large, dispersed, and surgically unresectable. These patients receive fWBI for the treatment of their multiple brain metastases; thus, over 70% of brain tumor patients receive fWBI that increases their survival and decreases tumor associated neurological symptoms (9, 10). The commonly used fWBI regimen for primary brain tumor patients delivers a total dose of 60 Gy in 2
Gy fractions over 6 weeks; for metastatic brain tumor patients, a total dose of 30 Gy is delivered in 3 Gy fractions over 2 weeks (7).

Some patients receive a combination of surgery, SRS, and fWBI. Combination therapy of fWBI and SRS is associated with better local tumor control, increased tumor free survival, and decreased neurocognitive problems (7, 9, 11, 12). Although there are neurocognitive risks associated with cranial irradiation, the tumor itself can also cause neurocognitive deficits. Nevertheless, failing to administer fWBI greatly reduces survival to 1-3 months (10). Thus, fWBI remains the primary treatment modality for primary and metastatic brain tumors (7).

1.2 Radiation-Induced Brain Injury

Over 250,000 patients per year receive fWBI for the treatment of primary and metastatic brain cancer (1, 13). The effectiveness of this treatment modality is limited by the radiation dose that can be safely delivered to the normal tissue adjacent to the tumor (2). Radiation damages the normal tissue causing acute, early delayed, and late radiation-induced brain injury, depending on the onset time of the injury (14). Acute injuries occur days to weeks following irradiation and consist of headache, nausea/vomiting, edema and somnolence; these effects either resolve by themselves or with corticosteroid treatment (14). Early delayed radiation injuries start to appear at 1 to 6 months following radiotherapy and are characterized by a transient demyelination, somnolence, attention deficits and short term memory loss (14). Both of these types of injuries are manageable with current radiation therapy techniques and fractionation schedules. Consequently, the
acute and early delayed injuries from radiation do not present as a serious problem in the clinic (14).

However, ~90% of brain tumor patients who survive >6 months after fWBI are at risk for developing late radiation-induced brain injury (10, 12). This brain injury primarily consists of a progressive, irreversible cognitive impairment manifesting as a decrease in short-term memory, attention, concentration, and/or language proficiency (2, 9, 10, 15). All of these symptoms lead to quality of life (QOL) issues (14). QOL is a growing concern for cancer survivors as new systemic therapies continue to increase their life expectancy. QOL is now the second most important outcome after survival for brain tumor patients in clinical trials (2, 10, 16). Susan Sontag, a 16-year brain cancer survivor who received surgery, chemotherapy, and radiotherapy for a left temporal anaplastic astrocytoma described her mood as follows:

“Everything I do is slow. I walk, talk and think slowly… I still have no short-term memory… Much of the time I can’t even remember the names of relatives and close friends… I am always confused… Because I look normal and often sound normal, people assume I am normal. But I’m not… I’m more emotional. I cry a lot. And I get depressed a lot knowing that I will never have my competence back.” (Sontag Foundation Distinguished Scientists Awards ceremony speech at the Society for Neuro-Oncology Meeting, Toronto, Canada, November 20, 2004.)
Although the exact mechanism(s) behind radiation-induced brain injury is unknown, radiation decreases neural stem cells \((17, 18)\) and increase microglial activation \((19, 20)\), suggesting that impaired neurogenesis and neuroinflammation may play a role.

Currently, there are no successful long-term treatments or effective preventive strategies for radiation-induced cognitive impairment. Classically, it was thought that this type of injury developed from radiation-induced vascular and/or glial damage \((14, 21, 22)\). Nevertheless, gross vascular abnormalities and demyelination are often not observed in patients exhibiting severe cognitive impairment \((23)\). Recent studies suggested that additional factors may contribute to the development of radiation-induced cognitive impairment. Currently, there are four main hypotheses for the development of radiation-induced brain injury (Figure 1), i] vascular and glial damage, ii] decrease hippocampal neurogenesis, iii] neuroinflammation, and iv] altered neural function that produces changes in synaptic plasticity \((23)\). Although vascular and glial damage may play a role in the development of radiation-induced cognitive impairment, recent studies focused on the role of impaired neurogenesis, neuroinflammation, and changes in synaptic plasticity \((17, 19, 24-26)\).
Figure 1: Potential mechanisms underlying radiation-induced cognitive impairment.

Radiation-induced cognitive impairment likely involves dynamic interactions between multiple cell types in the brain. Brain irradiation causes changes in the vasculature, glial cell populations, hippocampal neurogenesis, neuroinflammation and neuronal function. All of these pathways are associated with the development of radiation-induced brain injury, including cognitive impairment (adapted from Schloesser et al., 2013) (23).

1.3 Radiation Induces Decreases in Neurogenesis

Impaired neurogenesis is associated with radiation-induced brain injury, including progressive cognitive impairment (27, 28). Neurogenesis is a complex process involving stem cell proliferation, migration, differentiation, and integration of newly formed neurons into the brain (29, 30). These processes depend on the specific neurogenic microenvironment (31). In the adult brain, neurogenesis occurs in two areas, the
subventricular zone (SVZ) of the forebrain and the dentate gyrus (DG) of the hippocampus (30, 32). The DG consists of 3 layers, an outer layer, granule cell layer (GCL) and inner layer/hilus (33, 34), with neurogenesis occurring at the interface of the GCL and hilus (29, 30). Neuronal stem cells (NSCs) in the DG are capable of both self-renewal, as well as generating new neurons and glial cells (29, 32, 35).

The hippocampus plays a major role in learning, consolidation, and retrieval of information (34). Thus, the majority of radiation-induced brain injury studies have focused on the hippocampus. Radiation elicits a broad range of insult to the brain, altering the microenvironment, inhibiting neurogenesis (17, 36) and triggering an inflammatory response (37). Irradiating the rodent brain results in a dose-dependent decrease in NSCs, decreased proliferation of the surviving NSCs, and decreased differentiation of NSCs into neurons (17, 36, 38). In young male rats, a single 10 Gy dose of whole brain irradiation (WBI) reduces new hippocampal neuron production to only 3% as compared to sham-irradiated controls (36). Reductions in hippocampal neurogenesis were correlated with radiation-induced cognitive impairment (27, 28) and radiation-induced increases in neuroinflammation (36, 39). For example, the anti-inflammatory agent, indomethacin, inhibits both radiation-induced decreases in neurogenesis and increases in neuroinflammation (39). However, other preclinical studies indicate that preservation of neurogenesis is not required to prevent radiation-induced cognitive impairment (20, 40, 41). Treating rats with a renin-angiotensin system (RAS) inhibitor (40, 41) or a proliferator-activated receptor δ (PPARδ) agonist (42, 43) does not prevent radiation-induced decreases in hippocampal neurogenesis; however, they do prevent radiation-induced cognitive impairment. Thus, decreased neurogenesis is not the
only mechanism responsible for producing radiation-induced brain injury, including cognitive impairment.

1.4 Radiation Induces Increases in Neuroinflammation

1.4.1 In Vivo Studies

Although the specific pathological mechanism(s) involved in the onset and progression of radiation-induced brain injury remains ill-defined, experimental evidence suggests that chronic inflammation/oxidative stress plays a major role (18, 37, 44, 45). Radiation activates microglia, the resident immune cells of the brain, triggering their secretion of proinflammatory cytokines and chemokines as well as generating free radicals and fatty acid metabolites, all of which are harmful to neurons and associated with neurodegeneration and impaired neuronal function (19, 45). Radiation induces both an acute and delayed inflammatory response in the brain in vivo (26, 46-48). Within 24 hours after a single dose of 15-35 Gy to the mouse brain, tumor necrosis factor alpha (TNFα), intracellular adhesion molecule 1 (ICAM-1), glial fibrillary acidic protein (GFAP) and monocyte chemotactic protein 1 (MCP-1) expression increase; expression of these inflammatory cytokines returns to basal levels by 3 days post-irradiation (48). However, several months to a year after fWBI or WBI, an increased number of microglia are found expressing ED1/CD68, a marker of microglial activation (44, 46, 49). Recently, cells expressing the major histocompatibility complex II (MHC II) and dendritic cell markers were observed in the brain starting 1 month after irradiation (48). Additionally, T lymphocytes were observed in the brain at 6 months after a single dose of 35 Gy in mice
(48). This suggests that the inflammatory response of the brain may include immune cells of both intracranial and extracranial origin.

1.4.1 *In Vitro Studies*

In murine BV-2 microglial cells, i) the transcription factors, activating protein 1 (AP-1) and nuclear factor kappa B (NF-κB), are activated by 30 min after irradiation, ii) MCP-1 and cyclooxygenase-2 (Cox-2) proteins are increased by 7 h after irradiation, and iii) expression of the inflammatory mediators, interleukin-1β (IL-1β) and TNFα, are increased by 24 h after a single dose of 10 Gy (26, 47, 50, 51). These effects may be mediated through radiation-induced activation/phosphorylation of the mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) proteins (40, 47, 52, 53). Radiation also increases total protein kinase-C alpha (PKCα) and phosphorylation of MEK and ERK in microglia (26, 47). Inhibition of this pathway with pharmacological agents or siRNA targeting ERK1 or ERK2, inhibits radiation-induced inflammation in microglia cell cultures (26, 47, 50). Furthermore, treatment with a PPARδ agonists prevent radiation-induced mitogen activated protein (MAP) kinase activation both *in vitro* (26) and *in vivo* (42), suggesting that the MAP kinase pathway plays a pivotal role in radiation-induced brain injury.

Although radiation induces neuroinflammation in co-cultures of microglia and astrocytes, radiation-induced neuroinflammation is thought to depend primarily on the radiation response of the microglia cells. Thus, the role of astrocytes in radiation-induced neuroinflammation is understudied. There is only one published study on the radiation response of astrocytes in monoculture, and this paper showed increases in Cox-2 (51). To
rectify this situation, a comprehensive study of the radiation response of primary astrocytes was undertaken. Chapter 2 identifies new players in radiation-induced inflammation and MAP kinase signaling in primary astrocytes and identifies a potential approach for inhibiting this signaling.

1.5 Radiation Induces Alterations in Neuronal Function

In addition to the hippocampus, there are other domains in the brain that are important for cognition and likely play an important role in the development of radiation-induced cognitive impairment. Prior studies suggested that conformal treatment plans may not lead to the same degree of cognitive impairment as WBI treatment plans, resulting in the hypothesis that there are specific brain regions that are more important than others in producing radiation-induced cognitive impairment (15, 54). Peiffer et. al. employed dose-volume histogram analyses of two prospective clinical trials to demonstrate that it is not the dose to the whole brain, but rather the dose to specific brain regions, such as the temporal lobes and hippocampus, that predicts subsequent radiation-induced cognitive impairment (2, 9). These authors proposed a neuroanatomical target theory for radiation-induced cognitive impairment. This theory suggests that it is the total dose delivered to specific brain regions during an fWBI regimen that may be the primary cause of cognitive impairment after radiotherapy, not the total dose to the normal tissue surrounding the tumor, unless the tumor is in one of these critical regions.

There is a growing interest in radiation-induced changes in neuronal function, particularly those involved in synaptic plasticity. Irradiating the rodent brain elicits changes in, i] hippocampal long term potentiation (LTP) (55), ii] expression of the
immediate-early gene activity-regulated cytoskeleton-associated protein (Arc) at the synapse (24), iii] N-methyl-D-aspartic acid (NMDA) receptor subunits (56), and iv] glutamatergic metabolism (57). Recently, Wu et. al. noted that irradiating isolated rat brain slices with single doses of 2-10 Gy led to decreases in tyrosine phosphorylation and removal of excitatory NMDA receptors from the cell surface while simultaneously increasing surface expression of the inhibitory gamma-aminobutyric acid (GABA) receptors at 30 min after irradiation (58). These changes corresponded with altered synaptic responses and inhibition of LTP (58). Although radiation-induced changes in synaptic plasticity were observed, there is no clear mechanism or target identified that could be used to prevent these changes in synaptic plasticity.

1.6 Homer1a and Cognition

Rodent models of aging suggest that subtle changes in neuronal function and synaptic plasticity, rather than a significant loss of neurons or synapses, are the critical underlying factors for age-related cognitive decline (59, 60). Indeed, one of the most successful clinical trials for modulating radiation-induced cognitive impairment involved the acetyl cholinesterase inhibitor, donepezil, a drug predominantly used to treat mild to moderate Alzheimer’s dementia (10, 61). Donepezil administered for 24 weeks, at 6 months after fWBI improved moderate dementia for ~2/3 of the irradiated brain tumor patients (10). Alzheimer’s disease (AD) and radiation-induced brain injury also share pathological similarities, including changes in myelination, NMDA receptors, and glutamate/glutamine levels (61-64). This suggests that the molecular mechanism(s)
responsible for the development of cognitive deficits in AD may also be involved in the
development of radiation-induced cognitive impairment.

Transient activation of the ERK signaling pathway results in the transcription of inflammatory cytokines in both preclinical models of AD (65) and radiation-induced brain injury (42). Activation of ERK also results in the transcription of early response genes involved in synaptic plasticity, including a protein only found in the nervous system, Homer1a (66). Homer1a mRNA levels were elevated in AD and likely to contribute to the development of AD cognitive deficits (67). Homer1a belongs to a family of scaffolding proteins that localize in the synapse and regulate intracellular calcium homeostasis (68, 69), gene transcription, signal transduction, and receptor trafficking (70, 71). The longer forms of Homer1 are constitutively expressed and have two functional domains (Figure 2), i) the EVH1 domain which binds to the NMDA receptors, Shank, metabotropic glutamate receptors 1 and 5 (mGluR1/5), and ryanodine receptors, and ii) a coiled coil structure which binds to other Homer forms (71). The Homer1a form lacks the coiled coil domain and disrupts both the scaffolding and signaling capabilities of the long forms of Homer proteins by competitively binding to the NMDA-Shank-mGluR1/5-ryanodine complex (70, 72). Thus, Homer1a binds solely to mGluRs and inhibits their binding to the synapse, thereby modifying glutamate signaling (71).
Figure 2: Model of the Homer postsynaptic protein complex and Homer1a. Long Homer forms bind to each other through the carboxy-terminal domains and to target proteins (i.e. mGluR1/5, NMDA receptors, Shank, and ryanodine receptors) via the EVH1 domain forming a cluster at the postsynaptic density area. Homer1a lacks the carboxy-terminal domain and competes with long Homer forms for binding to target proteins (Adapted from Shiraishi-Yamaguchi and Furuichi, 2007) (71).

Homer1a regulates both activity-induced post- and pre-synaptic remodeling (73) and dendritic axonal targeting of mGluR5 (74) and is transcriptionally induced in neurons after LTP (33, 75, 76) or seizures (77, 78). Hippocampal or striatal overexpression of Homer1a in rodents impairs hippocampal-dependent memory and motor performance in behavioral tasks (66). Homer1a expression is increased in AD (67), epileptic seizures (79), and Huntington’s disease (70). Homer1 knockout mice exhibit a schizophrenic-like phenotype with behavior (motivational and emotional), cognitive, sensorimotor processing, and glutamatergic abnormalities (80, 81). Decreased Homer1a expression in the whole brain (largely cortical expression) is associated with a loss of cognitive and motor function in mice (82). However, in a rat model of aging, increased expression of Homer1a and decreased mGluR5 signaling in the hippocampus were also associated with cognitive deficits (83). These data demonstrate that both increased and decreased
Homer1a expression has the ability to alter cognition, depending on the brain region involved.

fWBI alters LTP, impairs neuronal function (58, 84) and increases NR1 and NR2A, but not the NR2B subunits of the NMDA receptor in the CA1 region of the hippocampus of young adult male rats (56), suggesting that radiation alters glutamatergic signaling. Overexpression of Homer1a in the hippocampus also abolishes maintenance of CA3-CA1 long-term potentiation (LTP) (85), synaptic plasticity (86, 87), and impairs working memory (85). Memory/learning paradigms suggest that trafficking of GluR from intracellular compartments to the plasma membrane is an important “early” event during synaptic plasticity (88). Thus, it is likely that if radiation alters Homer1a expression in vivo, it will also alter glutamatergic signaling. However whether radiation modifies Homer1a expression in the brain has not yet been explored. To more fully understand the role of Homer1a in radiation-induced brain injury, Chapter 3 identifies and characterizes radiation-induced brain region specific changes in Homer1a as well as the upstream and downstream proteins involved in Homer1a signaling.

1.7 The Renin-Angiotensin System

One of the most successful approaches for preventing radiation-induced late injury in preclinical models is with blockers of the RAS (25, 89). Both angiotensin converting enzyme inhibitors (ACEIs) and angiotensin II type 1 receptor antagonists (ARBs) are known to prevent radiation-induced late injury in the kidney, lung, and brain (90).
The classic blood-borne RAS regulates both blood pressure and electrolyte balance, and consists of the precursor protein angiotensinogen (AGT), renin and ACE. In brief, AGT is generated in the liver and cleaved to Ang I by renin, an aspartyl protease synthesized in the kidney (90). Ang I is converted to Ang II by ACE, an exopeptidase produced in the lungs; Ang II is the main effector peptide of the RAS. Although Ang II can be converted to Ang III or Ang IV by the aminopeptidases A and N, the octapeptide hormone predominantly binds to the Ang II type 1 G-protein-coupled receptor. Through this receptor Ang II induces vasoconstriction and regulates blood pressure and fluid homeostasis (Figure 3).

**Figure 3: Diagram of the renin-angiotensin system**

Recent studies identified Ang-(1-7) as an important regulator of the RAS. Ang-(1-7) is generated from Ang I via neprilysin (NEP) or a prolyl endopeptidase (PEP) or from
Ang II by ACE2, a PEP, or a prolyl carboxypeptidase (PCP) (90). Ang-(1-7) binds the AT$_{(1-7)}$ receptor (Mas receptor), a G-protein coupled receptor. Ang-(1-7) is a vasodilator and opposes the effects of Ang II. The heptapeptide hormone has anti-inflammatory effects, anti-tumor effects, and the ability to inhibit proliferation and angiogenesis (91-94). Although RAS blockers attenuate the function of Ang II, the main mechanism for preventing radiation-induced late injury may be through increasing the anti-inflammatory peptide Ang-(1-7) (89). Given the potential role of inflammation and the effectiveness of RAS inhibition in preventing radiation-induced late injury, Ang-(1-7) may be a potential therapeutic intervention to prevent it. Chapter 2 explores this possibility in primary astrocytes.

1.8 The Renin-Angiotensin System in Radiation-Induced Brain Injury

The brain possesses its own intrinsic RAS, and preclinical studies suggest that blockade of Ang II, a pro-inflammatory peptide of the RAS, could be a potential therapeutic intervention for late radiation-induced brain injury (89). Indeed, blockade of the RAS with an ACEI or ARB decreases expression of inflammatory proteins (89), prevents AP-1 and NF-κB activation (25), and reduces oxidative stress (20, 90) in the rodent brain. Administration of the ARB, L-158,809 (41), or the ACEI, ramipril, before, during and after fWBI prevented cognitive impairment in the young adult male rat measured at ≥6 months post-irradiation (40).

RAS blockers could prevent radiation-induced cognitive impairment not only by attenuating the proinflammatory function of Ang II, but also by increasing Ang-(1-7) (89). Treatment with RAS inhibitors can increase the systemic levels of the anti-
inflammatory, anti-proliferative, and anti-fibrotic Ang-(1-7) (41). Ang-(1-7) modulates MAP kinase inflammatory pathways which are activated by radiation (91, 95). Ang-(1-7) decreases lipopolysaccharide (LPS)-stimulated inflammation in macrophages within the brain (96) and directly inhibits Ang II stimulated inflammation in endothelial cells (97) and leukocytes (98). Ang-(1-7) attenuates mitogen-stimulated increases in Cox-2 (93) and prostaglandin E synthase (PGES) (93, 99) as well as increases the dual specificity phosphatase 1 (DUSP1) (94), to reduce MAP kinase activation (100-102).

Although studies showed that RAS blockers have anti-inflammatory properties and increase the anti-inflammatory peptide, Ang-(1-7), the role of RAS blockers and/or Ang-(1-7) in radiation-induced brain injury was not been fully elucidated. To address this, the experiments in Chapter 2 were designed to determine if Ang-(1-7) can inhibit radiation-induced inflammation in primary astrocytes. In Chapter 3, Homer1a is identified as a key player in glutamatergic pathways that are altered by fWBI and restored by treatment with RAS blockers previously shown to protect against cognitive impairment. Taken together these studies identify, i) novel players in the radiation response of the brain, and ii) multiple functions for the RAS in radiation-induced brain injury.
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CHAPTER II

Angiotensin-(1-7) prevents radiation-induced inflammation in rat primary astrocytes through regulation of MAP kinase signaling

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2.1 Abstract

About 500,000 new cancer patients will develop brain metastases in 2013. The primary treatment modality for these patients is partial or whole brain irradiation which leads to a progressive, irreversible cognitive impairment. Although the exact mechanisms behind this radiation-induced brain injury are unknown, neuroinflammation in glial populations is hypothesized to play a role. Blockers of the renin-angiotensin system (RAS) prevent radiation-induced cognitive impairment and modulate radiation-induced neuroinflammation. Recent studies suggest that RAS blockers may reduce inflammation by increasing endogenous concentrations of the anti-inflammatory heptapeptide angiotensin-(1-7) [Ang-(1-7)]. Ang-(1-7) binds to the AT₁ receptor and inhibits MAP kinase activity to prevent inflammation. This study describes the inflammatory response to radiation in astrocytes characterized by radiation-induced increases in i) IL-1β and IL-6 gene expression; ii) Cox-2 and GFAP immunoreactivity; iii) activation of AP-1 and NF-κB transcription factors; and iv) PKCα, MEK and ERK (MAP kinase) activation. Treatment with U-0126, a MEK inhibitor demonstrates that this radiation-induced inflammation in astrocytes is mediated through the MAP kinase pathway. Ang-(1-7) inhibits radiation-induced inflammation, increases in PKCα and MAP kinase pathway activation (phosphorylation of MEK and ERK). Additionally Ang-(1-7) treatment leads to an increase in dual specificity phosphatase 1 (DUSP1). Furthermore, treatment with sodium vanadate (Na₃VO₄), a phosphatase inhibitor, blocks Ang-(1-7) inhibition of radiation-induced inflammation and MAP kinase activation, suggesting Ang-(1-7) regulates phosphatase activity to inhibit radiation-induced inflammation. These data
suggest that RAS blockers inhibit radiation-induced inflammation and prevent radiation-induced cognitive impairment not only by reducing Ang II by also through the up-regulation of Ang-(1-7).

**Keywords**

Angiotensin-(1-7); Radiation-induced Inflammation; Rat Primary Astrocytes; MAP Kinase signaling; Renin-angiotensin system
2.2 Introduction

Over 1.6 million new cancer cases will be diagnosed in 2013 (1) and approximately 500,000 of these individuals will develop brain metastases (2, 3). The number of patients diagnosed with brain metastases continues to increase with enhanced neuroimaging, improved systemic treatment of primary tumors, and an aging population (3, 4). The majority of metastatic brain tumor patients will receive some form of radiation therapy as part of their treatment (3-5), putting them at risk for developing late radiation-induced brain injury, including a progressive, irreversible decline in cognitive function which is associated with increased mortality and morbidity (3, 6, 7). Currently, there are no proven successful long-term treatments or effective preventive strategies for radiation-induced cognitive impairment (3, 7).

Although the specific pathological mechanism(s) involved in the onset and progression of radiation-induced brain injury remains ill-defined, experimental evidence suggests that chronic inflammation/oxidative stress plays a major role (8-11). Radiation activates co-cultures of microglia and astrocytes in vitro (12), increasing expression of pro-inflammatory mediators, including cyclooxygenase-2 (Cox-2) (13), interleukin (IL)-1β (14), IL-6 (15, 16), and tumor necrosis factor-α (TNF-α) (17). Additionally, radiation-induced increases in Cox-2 have been reported in monocultures of astrocytes (13). These effects may be partially mediated through radiation-induced activation/phosphorylation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein erk kinase (MEK) (18-21). To date there is a paucity of studies characterizing the radiation response of astrocytes. The results of this study demonstrate an important radiation-induced inflammatory signaling pathway in astrocytes.
Preclinical studies suggest that blockade of angiotensin (Ang) II, a pro-inflammatory peptide of the renin-angiotensin system (RAS), may serve as a potential therapeutic intervention for late radiation-induced organ injury (22). Attenuation of Ang II signaling via RAS inhibition prevents radiation-induced late effects in the kidney, lung and brain (23). Indeed, blockade of the RAS with an angiotensin converting enzyme inhibitor (ACEI) or angiotensin receptor blocker (ARB) reduces expression of inflammatory proteins (22), prevents AP-1 and NF-κB activation (24), and reduces oxidative stress (23) in the rodent brain. Previous studies in our lab demonstrate that administration of the ARB, L-158,809 (26), or the ACEI, ramipril, before, during and after fractionated whole brain irradiation (fWBI) prevents cognitive impairment in the young adult male rat assessed 6 months post-irradiation (21).

RAS blockers not only attenuate Ang II signaling, but also increase the generation of the anti-proliferative, anti-fibrotic, anti-inflammatory peptide, Ang-(1-7) (26, 27). Ang-(1-7), whose sequence is Asp-Arg-Val-Tyr-Ile-His-Pro, binds to the G protein-coupled AT(1-7) receptor and modulates MAP kinase inflammatory pathways which are activated by radiation (27, 28). Ang-(1-7) decreases lipopolysaccharide (LPS)-stimulated inflammation in macrophages within the brain (29) and directly inhibits Ang II stimulated inflammation in endothelial cells (30) and leukocytes (31). Ang-(1-7) attenuates mitogen-stimulated increases in Cox-2 (32) and prostaglandin E synthase (PGES) (33) and up-regulates the dual specificity phosphatase 1 (DUSP1) (34), to reduce MAP kinase activation (35-37). Thus, Ang-(1-7) treatment may prevent radiation-induced MAP kinase activation and inflammation to ameliorate radiation-induced cognitive impairment. In this study, primary cultures of rat astrocytes were incubated with Ang-(1-7) to
determine whether the heptapeptide hormone could effectively block the inflammatory response induced by radiation.
2.3 Materials and Methods

Materials

Ang-(1-7) (100 nM) and [D-Ala7]-Ang-(1-7) (1 µM) were dissolved in PBS (Bachem). The phosphatase inhibitor sodium vanadate (Na$_3$VO$_4$) (1 µM) (Sigma-Aldrich) was dissolved in water and the MEK inhibitor U-0126 (1 µM) (EMD Millipore), was dissolved in dimethyl sulfate Me$_2$SO$_4$. The following antibodies were used for this study: Goat anti-Cox-2 (1:1000) (Santa Cruz Biotechnologies), rabbit anti-p-MEK1/2 (1:1000) (Santa Cruz Biotechnologies), mouse anti-p-ERK1/2 (1:1000) (Santa Cruz Biotechnologies), rabbit anti-PKCα (1:20,000) (Cell Signaling) rabbit anti-DUSP-1 (1:1000) (EMD Millipore), Total MEK (1:1000) (Santa Cruz Biotechnologies), Total ERK (1:1000) (Santa Cruz Biotechnologies) and mouse anti-β-actin (1:10,000) (Sigma–Aldrich).

Astrocyte Isolation, Irradiation and Treatments

Astrocyte isolation and cell culture

Astrocytes were isolated from mixed glial cultures as described previously (38). In brief, brains taken from 1- to 3-day-old rat pups were minced, filtered, and plated in DMEM/F12 media (Invitrogen). At 100% confluency (1 week post plating), the mixed glial cultures were shaken at 200 rpm overnight at 37°C on a rotary shaker to separate the microglia and oligodendrocytes from the adherent astrocytes. The media, microglia and oligodendrocytes were removed; the astrocytes were trypsinized and subcultured in DMEM/F12 supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 IU/mL penicillin, 100 mg/mL streptomycin (Sigma-Aldrich), 100 mg/mL L-glutamine (Sigma-
Aldrich) and 15mM HEPES (Sigma-Aldrich). Astrocytes were maintained at 37°C in 5% CO2 and 95% air. Cultures used for experiments were determined to contain ≥99% astrocytes as assessed by, using double immunostaining for glial fibrillary acidic protein (GFAP) to identify astrocytes, and Iba1 (Wako) for microglia.

Irradiation

The culture medium was replaced with low serum media (0.5%) 24 h prior to irradiation to synchronize the cells. Cells were irradiated with a single dose of 10 Gy using a $^{137}$Cs irradiator at a dose rate of 3.57 Gy/min. Irradiations were conducted at room temperature and control cells received sham-irradiation; culture dishes were returned to the incubator and maintained at 37°C in 5% CO2 and 95% air following irradiation. The sampling times for inflammatory mediators, cytokines, and MAP kinase activation were based on historical laboratory data.

Treatment of Cultured Cells

Cultured astrocytes were incubated with 100 nM of Ang-(1-7), 1 μM of the AT$_{(1-7)}$ receptor antagonist D-Ala-Ang-(1-7), 1 μM of the MEK inhibitor U-0126 or 1 μM of the phosphatase inhibitor Na$_3$VO$_4$. For treatments with a single agent, astrocytes received Ang-(1-7), U-0126 or Na$_3$VO$_4$ for one hour prior to irradiation. When astrocytes were treated with a combination of the Ang-(1-7) and D-Ala-Ang-(1-7) or Na$_3$VO$_4$, cells were first treated with the inhibitor (ie, D-Ala-Ang-(1-7) or Na$_3$VO$_4$) for 15 min and then Ang-(1-7) was added 1 h prior to radiation.
**Immunoblot Hybridization**

Total cellular protein was harvested using M-PER lysis buffer (Pierce Biotechnology) supplemented with 1 mg/mL aprotinin (Sigma–Aldrich), 1 mM leupeptin (Sigma–Aldrich), 10 mg/mL phenylmethylsulfonyl fluoride (PMSF), 1 mM Na$_3$VO$_4$ (Sigma–Aldrich), and 150 mM NaCl. Lysates were centrifuged at 12,500 rpm for 30 min, and the supernatant was collected. Protein concentrations were measured using the Bradford assay method (Bio-Rad) at absorbance 595 nm. Five to 50 μg of protein were separated by SDS–PAGE. Protein was transferred to a polyvinylidene difluoride membrane at 35 V overnight, blocked in 5% milk in TBST (0.02 M Tris, 0.015 M NaCl, 0.05% Tween 20, pH 7.5), and incubated overnight with primary antibody. Membranes were washed, incubated with the appropriate horseradish peroxidase-conjugated secondary antibody, developed using the ECL detection system (GE Healthcare), and processed using a Kodak processing system. Films were scanned and densitometry was conducted to quantify the signal intensity using Adobe Photoshop Elements 6.0 to express protein levels as fold changes, with β-actin used as a loading control. No changes were observed in total levels of MEK or ERK in response to radiation or Ang-(1-7) (Supplementary Figure 1). Thus, the changes in p-MEK and p-ERK were normalized to the loading control, β-actin.

**RNA isolation and Quantitative Real Time Polymerase Chain Reaction with Taq-Man**

RNA was harvested using Trizol reagent (Invitrogen) according to the manufacturer's protocol. DNA contamination was removed by chloroform extraction (Ambion). Real-time PCR amplifications were conducted in a 20-μl reaction volume.
containing 2 μl cDNA, 10μl Taq-Man Master Mix (Applied Biosystems), 0.1 μM genespecific probe, upstream and downstream primers (Applied Biosystems), and 7 μl nuclease-free water. Real-time PCR was carried out in an ABI Prism 7000 at 50 °C for 2 min, 95 °C for 2 min, and 45 cycles of 95 °C for 15 min, 55 °C for 30 s, and 72 °C for 30 s. The fold changes in IL-1β (Applied Biosystems), and IL-6 (Applied Biosystems) gene expression were calculated using the comparative Ct (cross threshold) method.

Electromobility-shift assay (EMSA)

Cells were lysed on ice with Buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol (DTT); lysates were homogenized using a Dounce homogenizer, which was followed by centrifugation at 12,000 rpm for 10 min. The nuclear pellets were lysed with Buffer C (5 mM Hepes, pH 7.9, 1.5 mM MgCl2, 25% v/v glycerol, 400 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 mg/mL aprotinin, 2 mg/mL leupeptin, and 1 mM Na3VO4) followed by centrifugation at 12,000 rpm for 10 min to extract nuclear proteins. Protein concentrations were measured using the Bradford assay (Bio-Rad) at absorbance 595 nm. The EMSA procedure was performed using the Promega Gel-Shift Core Assay following the manufacturer's protocol. In brief, 10 μg of nuclear protein was incubated with 2 μl Binding Buffer (Promega) for 10 min. Consensus binding sequences of NF-κB (5’-AGTTGAGGGGACTTTCCCAGGC-3’ and 3’-TCAACTCCCCTGAAAGGGTGCCG-5’) and AP-1 (5’-CGCTTGATGAGTCAGCCGGAA-3’ and 3’-GCGAACTACTCAGTCGGCCTT-5’) were labeled with 10 μCi of γ-32P (GE Healthcare) and T4 polynucleotide kinase (Promega). The consensus sequences were
then incubated with the nuclear protein for 20 min and proteins were separated by electrophoresis on a 4% nondenaturing polyacrylamide gel. Band intensity on X-ray film was determined by densitometry and expressed as fold changes.

Statistical analysis

All data are expressed as the mean +/- SEM. Each experiment was repeated a minimum of three independent times. All analyses were carried out using GraphPad software (GraphPad Prism 5.0). T-tests were used for analysis of experiments comparing only sham to irradiated samples 2-Way ANOVAs were used to determine if there was a radiation or treatment effect. Bonferroni post-tests were used for all pair-wise comparisons. All results were considered statistically significant at the p<0.05 level.
2.4 Results

2.4.1 Radiation induces inflammation and activation of MAP kinase signaling in primary rat astrocytes.

A 10 Gy single dose of Cs\(^{137}\) radiation was administered to primary astrocytes to determine if radiation induced inflammation in monocultures of astrocytes. Radiation rapidly resulted in a 2-fold increase in IL-1\(\beta\) mRNA at 1 h post-irradiation (Figure 1A) and a delayed 2-fold increase in cytokine IL-6 at 7 h post-irradiation as compared to sham controls (Figure 1A). Enhanced immunoreactivity of inflammatory proteins, Cox-2 and GFAP, was also observed at 7 h post-irradiation as compared to sham controls (Figure 1B). In addition to increasing inflammatory markers, radiation caused an immediate activation of MAP kinase signaling at 30 min post-irradiation, characterized by a 2-fold increase in the total levels of PKC\(\alpha\), a 1.5-fold increase in phosphorylation of MEK and a 2-fold increase in phosphorylation of ERK compared to sham irradiated controls (Figure 1C). Furthermore, downstream MAP kinase pro-inflammatory transcription factors AP-1 and NF-\(\kappa\)B were activated 30 min post-irradiation as measured by EMSA and compared to sham controls (Figure 1D). Taken together, these data suggest that radiation causes an inflammatory response, potentially mediated through MAP kinase activation in primary rat astrocytes.

2.4.2 Radiation-induced inflammation in primary astrocytes occurs through activation of the MAP kinase pathway.

The MEK inhibitor, U-0126 was used to block MAP kinase signaling to determine if MAP kinase activation is a key mediator of radiation-induced inflammation
in primary astrocytes. Pretreatment with U-0126 1 h prior to irradiation prevented radiation-induced increases in phosphorylation of ERK and reduced basal levels of phosphorylated ERK as compared to sham controls (Figure 2A). Interestingly, MEK inhibition also prevented radiation-induced increases in upstream kinase PKCα, blocking any potential feedback loop in the MAP kinase cascade (Figure 2B). Radiation-induced inflammation was also abolished as a result of MEK inhibition. Radiation-induced increases in IL-1β mRNA 1 h post-irradiation as well as IL-6 and Cox-2 at 7 h post-irradiation were blocked with U-0126 treatment as compared to sham controls (Figures 2C, 2D, and 2E). Of note, U-0126 treatment in sham-irradiated astrocytes did not significantly alter inflammatory cytokine or mediator levels. These findings indicate that radiation-induced inflammation is likely mediated through MAP kinase activation in primary astrocytes.

2.4.3 Ang-(1-7) inhibits radiation-induced inflammation and MAP kinase activation through the AT(1-7) receptor.

Primary rat astrocytes were pretreated with 100 nM Ang-(1-7) for 1 h followed by a 10 Gy single dose of radiation. Inflammatory markers and MAP kinase activation were evaluated to determine whether Ang-(1-7) attenuates radiation-induced inflammation. A greater than 2-fold increase in IL-1β mRNA compared to the control cell concentration was observed 1 h post-irradiation (Figure 3A). A 1 h pre-incubation of the astrocytes with Ang-(1-7) prevented the radiation-induced increase in IL-1β gene expression. Irradiation of primary rat astrocytes also up-regulated IL-6 mRNA at 7 h post-irradiation, and Ang-(1-7) treatment for 1 h before radiation prevented the increase (Figure 3B). The
attenuation of radiation-induced increases in both IL-1β and IL-6 gene expression by Ang-(1-7) in cultured rat astrocytes was effectively blocked by the AT(1-7) receptor antagonist D-Ala-Ang-(1-7) (Figures 3A and 3B), indicating a receptor-mediated mechanism. The enhanced immunoreactivity of inflammatory proteins Cox-2 and GFAP observed 7 h post-irradiation as compared to control cells was also blocked by pretreatment with Ang-(1-7). Similarly, the D-Ala-Ang-(1-7) effectively prevented the Ang-(1-7) mediated inhibition of radiation-induced increases in Cox-2 or GFAP (Figures 3C and 3D). Sham irradiated primary astrocytes treated with Ang-(1-7), D-Ala-Ang-(1-7), or a combination of the two treatments showed no changes in the regulation of these inflammatory cytokines or proteins.

Pretreatment with Ang-(1-7) 1 h prior to irradiation also prevented radiation-induced increases in MAP kinase signaling. Western blot hybridization analyses showed that radiation increased the total levels of PKCα in primary cultures of rat astrocytes by ~2-fold at 30 min after irradiation (Figure 3E). The effect was attenuated by incubation of the cells with 100 nM Ang-(1-7) 1 h prior to irradiation. Radiation also increased phosphorylation of MEK and ERK in the astrocytes (Figures 3F and 3G), an effect that was blocked by incubation with Ang-(1-7) 1 h prior to irradiation of the cells. The AT(1-7) receptor antagonist, D-Ala-Ang-(1-7), blocked Ang-(1-7) prevention of radiation-induced increases in PKCα and p-ERK. However, treatment with D-Ala-Ang-(1-7) alone caused an increase in p-MEK in sham-irradiated cells, which precludes the ability to confirm that the Ang-(1-7) decrease in p-MEK is mediated through the Mas receptor. The increase in the downstream transcription factors, AP-1 and NF-κB, 30 min post-irradiation was prevented by Ang-(1-7) treatment. The effect was inhibited by addition of D-Ala-Ang-(1-
7), suggesting that Ang-(1-7) inhibits the activation of the MAP kinase pathway after irradiation through the AT$_{1,-7}$ receptor-mediated mechanism (Supplemental Figure 2, Panels A and B).

2.4.4 Ang-(1-7) upregulates dual specificity phosphatase-1 (DUSP1) to inhibit radiation-induced activation of MAP kinase signaling and inflammation in primary astrocytes.

Na$_3$VO$_4$, an inhibitor of phosphatase activity, was used to determine if activation of phosphatase activity is a mechanism for Ang-(1-7) inhibition of radiation-induced MAP kinase signaling and inflammation in primary astrocytes. Pretreatment with phosphatase inhibitor, Na$_3$VO$_4$, restored radiation-induced phosphorylation of MEK and ERK (Figure 4A and 4B). Furthermore, Cox-2 enhanced immunoreactivity after irradiation was not prevented by Ang-(1-7) in the presence of Na$_3$VO$_4$ (Figure 4C). These data indicate that Ang-(1-7) modulation of MAP kinase activation and inflammation by radiation may occur through the upregulation of a phosphatase.

Ang-(1-7) modulates MAP kinase signaling via up-regulation of DUSP1 models of breast cancer fibrosis (4) and cardiac remodeling (5), suggesting that the heptapeptide hormone may regulate DUSP1 in MAP kinase-mediated inflammation. Pretreatment with Ang-(1-7) increased immunoreactivity of DUSP1 in primary astrocytes and the combination of pretreatment with Ang-(1-7) and radiation increased total levels of DUSP1 by 3 fold (Figure 4D). Radiation alone had no significant effect on DUSP1 concentrations compared sham-irradiated cells. Interestingly, pretreatment with Na$_3$VO$_4$ prevented these Ang-(1-7) mediated effects on DUSP1, suggesting that changes in the total levels of DUSP1 are partially regulated by phosphatase activity. Thus, these data
suggest that Ang-(1-7) may mediate radiation-induced MAP kinase signaling and inflammation through up-regulation of DUSP1.
2.5 Discussion

Preclinical studies have identified radiation-induced neuroinflammation as a potential mechanism for the development of radiation-induced brain injury, including progressive cognitive impairment (3, 24). While the brain microenvironment consists of different cell types including microglia, oligodendrocytes, neurons, endothelial cells, and astrocytes, in vivo and in vitro studies have primarily focused on the radiation-induced neuroinflammation in microglia. Radiation initiates inflammation through MAP kinase signaling in immortalized murine microglia BV-2 cells (39, 40) and activates microglia (CD68/ED1 marker) at 6 mo following fWBI in rodent models (21, 25). Radiation studies of mixed glial cultures (microglia and astrocytes) suggest that microglia are the primary mediators of radiation-induced neuroinflammation and are necessary to activate astrocytes (12, 13). However, previous studies with RAS blockers demonstrate that preventing activation of microglia may not be sufficient to protect against radiation-induced cognitive decline. Treatment with the ARB, L-158,809, or the ACEI, ramipril, inhibits radiation-induced cognitive impairment, but only ramipril prevents microglial activation in the hippocampus (21, 25, 26). Thus, it is likely that other brain cells play a role in the pathogenesis of radiation-induced brain injury and are affected by the RAS blockers.

This is the first study to demonstrate a radiation-induced MAP kinase inflammatory signaling mechanism in astrocytes. Astrocytes constitute approximately 50% of the total glial cell population within the brain and represent a heterogeneous class of cells which perform diverse functions including modulation of synaptic transmission and secretion of neurotropic factors (43). In response to injury, astrocytes become
reactive, proliferate and increase expression of GFAP (43, 44). Irradiation of the rat and mouse brain causes gliosis and increases expression of GFAP (17). Radiation triggers astrocytes to express Cox-2 and ICAM-1 that are likely to contribute to breakdown of the blood-brain barrier and subsequent infiltration of leukocytes (13). In the study reported here, radiation activates MAP kinase signaling to induce increases in inflammatory cytokines IL-6 and IL-1β and inflammatory mediators Cox-2, GFAP, AP-1 and NF-κB in primary astrocytes. Furthermore, treatment with Ang-(1-7), an anti-inflammatory peptide of the RAS, prior to irradiation inhibits radiation-induced inflammation in astrocytes, likely through MAP kinase inhibition. Ang-(1-7) prevents radiation-induced increases in PKCα, p-MEK and p-ERK. Ang-(1-7) also increases DUSP1, a phosphatase of ERK. These data suggest that astrocytes play an inflammatory role in radiation-induced brain injury and RAS blockers are likely to function as potential therapeutics for prevention of radiation-induced brain injury, via MAP kinase inhibition.

RAS blockers may prevent radiation-induced cognitive impairment by not only attenuating the function of Ang II, but also by increasing Ang-(1-7) (22). Treatment with RAS blockers increases systemic levels of anti-inflammatory, anti-proliferative, and anti-fibrotic Ang-(1-7) (26). Ang-(1-7) has been shown to modulate several phosphatases in different disease models. Ang-(1-7) inhibits proliferation of cardiac fibroblasts in a rodent model of myocardial infarction (42, 45) and reduces fibrosis in orthotopic models of breast cancer through a reduction in MAP kinase activation and an increase in DUSP1 (34). LPS-stimulated inflammation in macrophages is also inhibited by treatment with Ang-(1-7) (29). In the study reported here, Ang-(1-7) inhibits radiation-induced ERK phosphorylation and Ang-(1-7) treatment alone and in combination with radiation,
significantly increases total levels of DUSP1, suggesting that reduction in MAP kinase signaling through an increase in DUSP1 may serve as a potential mechanism for the prevention of radiation-induced inflammation in primary astrocytes. This study also identifies p-MEK as a target for Ang-(1-7) modulation of MAP kinase signaling. Ang-(1-7) inhibits radiation-induced increases in p-MEK in primary astrocytes. However, this study does not identify the specific mechanism for how Ang-(1-7) decreases p-MEK because Ang-(1-7) regulates multiple phosphatases (36, 46, 47). Consistent with this, the phosphatase inhibitor Na<sub>3</sub>VO<sub>4</sub> inhibited Ang-(1-7) from preventing radiation-induced increases in p-MEK and p-ERK. Together these data suggest that Ang-(1-7) increases phosphatases to inhibit radiation-induced inflammation and MAP kinase signaling in primary astrocytes.

Based on the findings described above, Figure 5 outlines a proposed model for radiation-induced inflammation in primary astrocytes and modulation of this radiation response by Ang-(1-7). Radiation activates MAP kinase signaling by increasing total levels of PKCα and phosphorylating MEK and ERK. MAP kinase activation induces increases in activation of transcription factors AP-1 and NF-κB. Activation of these transcription factors increases expression of inflammatory cytokines IL-1β and IL-6 and inflammatory mediator Cox-2, generating an inflammatory phenotype and increased expression of GFAP in astrocytes. Ang-(1-7) inhibits this radiation response in astrocytes by reducing MAP kinase activation. Ang-(1-7) binds to the AT<sub>1</sub>(1-7) receptor and increases DUSP1, potentially attenuating radiation-induced ERK phosphorylation. This effect is amplified by Ang-(1-7) also directly decreasing total levels of PKCα and inhibiting phosphorylation of MEK. The MEK inhibitor U-0126 also prevented radiation-induced
increases in PKCα, suggesting that downstream mediators of MAP kinases may contribute to radiation-induced changes in PKCα and p-MEK. Inhibition of MAP kinase signaling prevented radiation-induced increases in transcription factors AP-1 and NF-κB and induction of inflammatory factors IL-1β, IL-6, and Cox-2. Phosphatase inhibition by Na3VO4 prevented Ang-(1-7) from inhibiting radiation-induced MAP kinase activation, suggesting that Ang-(1-7) alters phosphatase activity as a mechanism for preventing radiation-induced inflammation in primary astrocytes. Taken together, this study indicates that Ang-(1-7) signaling can prevent radiation-induced MAP kinase activation and inflammation in primary astrocytes and is a likely therapeutic for preventing radiation-induced brain injury, including a progressive cognitive impairment.

Radiation-induced brain injury in vivo is a multicellular process that consists of a chronic and persistent inflammatory response, which is associated with decreased neurogenesis and impaired neuronal function (3, 48). It is unlikely that RAS blocker inhibition of radiation-induced cognitive impairment is solely due to the reduction in astrocyte inflammation by Ang-(1-7). The AT(1,7) receptor is ubiquitously expressed throughout the brain (49) and it is likely that Ang-(1-7) blocks radiation-induced inflammation in multiple cell types. This study indicates that Ang-(1-7) inhibits radiation-induced inflammation by MAP kinase inhibition in astrocytes. Of note, MAP kinase signaling is a proposed mechanism for radiation-induced inflammation in other brain cell types including microglia (12, 13, 39, 40) and treatment with PPAR agonists can decreases p-ERK in irradiated rat brain and inhibit radiation-induced cognitive impairment (50). Thus Ang-(1-7) may also inhibit radiation-induced inflammation in
multiple brain cell types in vitro and in vivo, and its efficacy should be tested in other cells types and in an animal model.

There is currently no clear standard of care for preventing radiation-induced brain injury, including a progressive cognitive impairment (3). Several current clinical trials of potential pharmacological mediators of cognitive impairment are being developed based on preclinical data suggesting that anti-inflammatory agents can prevent MAP kinase activation and radiation-induced brain injury (21, 26, 40, 51-53). With the caveat that acute results in tissue culture may not directly extrapolate to the late effects in brain, our data suggests that RAS blockers may prevent radiation-induced brain injury via Ang-(1-7) inhibition of MAP kinase mediated astrocyte inflammation. Furthermore, Ang-(1-7) possesses anti-tumorigenic properties in preclinical (34, 54, 55) and clinical cancer studies (56, 57). Thus RAS blockers and/or Ang-(1-7) therapies may serve a dual purpose, targeting tumor proliferation and protecting normal tissue from radiation-induced inflammation. Consequently, these studies indicate the importance of MAP kinase signaling in radiation-induced brain injury and suggest that targeting this pathway may, in part, ameliorate radiation-induced cognitive impairment.
Acknowledgements

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Figure 1: Radiation induces inflammation and activation of MAP kinase signaling in primary rat astrocytes. Primary rat astrocytes were subjected to sham irradiation or a single dose of 10 Gy of $^{137}$Cs γ ray irradiation and analyzed as indicated. **Panel A**: IL-1β and IL-6 mRNAs levels were determined by Taq-man real time PCR on mRNAs isolated 1h or 7h post-irradiation, respectively, and normalized to 18S mRNA. **Panel B**: Cox-2 and GFAP protein were analyzed by Western blot hybridization of lysates isolated 7h post-irradiation; β-actin was used as a loading control. **Panel C**: Lysates isolated 30min post-irradiation were analyzed by Western blot for total PKCα, p-MEK, and p-ERK; β-actin was used as a loading control. **Panel D**: Nuclear lysates isolated 30min post-irradiation were subjected to EMSA using AP-1 and NF-κB consensus oligonucleotides. Data is expressed as fold changes of Mean ± S.E.M from three independent experiments. *p<0.05, **p<0.01, ***p<0.001; n=3
Figure 2: The MEK inhibitor, U-0126, prevents radiation-induced MAP kinase activation and inflammation. Primary rat astrocytes were pretreated with 1 µM U-0126 or vehicle control for 1 h, then irradiated with a single dose of 10 Gy of 137Cs γ rays or sham irradiated and analyzed as indicated. **Panel A**: p-ERK, **Panel B**: PKCa, and **Panel C**: Cox-2 were analyzed by Western blot hybridization at 30min (A, B) or 7h (C) post-irradiation; β-actin was used as loading control as compared to sham and untreated controls. **Panel D**: IL-1β (1h) mRNA and **Panel E**: IL-6 (7h) mRNA levels were analyzed using Taq-man Real-time PCR and normalized to 18S mRNA. Data was expressed as fold changes of Mean ± S.E.M from three independent samples of cells. *p<0.05, **p<0.01, ***p<0.001; ##, p≤ 0.01 U-0126 response; n=3
Figure 3: Ang-(1-7) inhibits radiation-induced MAP kinase activation and inflammation through the AT(1-7) receptor. Primary rat astrocytes were pretreated with 100 nM Ang-(1-7) for 1 h, 1 μM D-Ala-Ang-(1-7) for 30 min or the combination of 1 μM D-Ala-Ang-(1-7) for 30 min and then 100 nM of Ang-(1-7) for 1 h prior to a single dose of 10 Gy of 137Cs γ irradiation and analyzed as indicated. Panel A: IL-1β and IL-6 mRNA levels were measured with Taq-man real time PCR at 1h or 7h post-irradiation, respectively, and normalized to 18S mRNA. Panel B: Cox-2 and GFAP protein and Panel C: PKCα, p-MEK, and p-ERK protein were analyzed by Western blot hybridization at 7h (B) or 30min (C) post-irradiation; β-actin used as a loading control. Data was expressed as fold changes of Mean ± S.E.M from three independent samples of cells. *p<0.05 radiation response, **p<0.01 radiation response, ***p<0.005 radiation response; ##, p≤ 0.01 Ang-(1-7) response; ###, p≤ 0.001 Ang-(1-7) response; ††, p≤ 0.01 D-Ala-Ang-(1-7); †††, p≤ 0.001 D-Ala-Ang-(1-7) response; n=3
Figure 4: Sodium vanadate (Na$_3$VO$_4$), a phosphatase inhibitor, prevents Ang-(1-7) inhibition of radiation-induced increases in MAP kinase activation and Cox-2. Primary rat astrocytes were pretreated with 1 μM Na$_3$VO$_4$ for 1 h, 100 nM of Ang-(1-7) for 1 h or the combination of 1 μM Na$_3$VO$_4$ and 100 nM of Ang-(1-7) for 1 h prior to a single dose of 10 Gy of $^{137}$Cs γ irradiation and analyzed as indicated. Panel A: p-MEK, Panel B: p-ERK, Panel C: Cox-2 and Panel D: DUSP1 protein were analyzed 30 min (A, B, D) or 7h (C) post-irradiation by Western blot hybridization and compared to untreated controls, β-actin was used as a loading control. Data was expressed as fold changes of Mean ± S.E.M from three independent samples of cells. **p<0.01 radiation response, ***p<0.005 radiation response; #, p≤ 0.01 Ang-(1-7) response; ###, p≤ 0.001 Ang-(1-7) response; †††, p≤ 0.001 Na$_3$VO$_4$ response; n=3
Figure 5: Proposed model outlining the role of Ang-(1-7) in the modulation of the radiation-induced inflammatory response in primary astrocytes. Irradiation of primary astrocytes leads to an increase in MAP kinase signaling and activation of the transcription factors AP-1 and NF-κB to enhance the expression of IL-1β, IL-6 and Cox-2. Ang-(1-7) inhibits radiation-induced MAP kinase activation by binding to the AT(1-7) receptor and inhibiting radiation-induced increases in PKCα, and MAP kinase activation. Ang-(1-7) may inhibit radiation-induced inflammation by increasing phosphatase activity, likely through up-regulation of DUSP1 and other phosphatases.
Supplemental Figure 1: Radiation or Ang-(1-7) treatment does not alter total levels of MEK or ERK. Primary rat astrocytes were subjected to sham irradiation, a single dose of 10 Gy of 137Cs γ irradiation or treated with 100 nM Ang-(1-7) for 1 h and analyzed by Western blot as indicated. Panel A: Total MEK was measured 5 min to 2 h post-irradiation; Panel B: Total MEK 1 h after treatment with Ang-(1-7); Panel C: Total ERK was measured 5 min to 2 h post-irradiation; Panel D: Total ERK 1 h after treatment with Ang-(1-7). β-actin was used as a loading control. Data is expressed as fold changes of Mean ± S.E.M from three independent samples of cells.
Supplemental Figure 2: Ang-(1-7) inhibits radiation-induced MAP kinase activation of transcription factors AP-1 and NF-κB through the AT<sub>(1-7)</sub> receptor. Primary rat astrocytes were pretreated with Ang-(1-7), D-Ala-Ang-(1-7) or the combination of D-Ala-Ang-(1-7) and Ang-(1-7) for 1h prior to a single dose of 10 Gy of 137Cs γ irradiation. Nuclear lysates isolated 30min post-irradiation were subjected to EMSA using AP-1 (Panel A) and NF-κB (Panel B) consensus oligonucleotides. Data was expressed as fold changes of Mean ± S.E.M from three independent samples of cells. *p<0.05 radiation response, **p<0.01 radiation response, ***p<0.005 radiation response; ##, p≤ 0.01 Ang-(1-7) response; ###, p≤ 0.001 Ang-(1-7) response; n=3.
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CHAPTER III

Differential Expression of Homer1a in the Hippocampus and Cortex Likely Plays a Role in Radiation-Induced Brain Injury

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3.1 Abstract

Fractionated partial or whole brain irradiation (fWBI) is the primary treatment for metastatic brain tumors. Despite reducing tumor burden and increasing lifespan, progressive, irreversible cognitive impairment occurs in >50% of the patients who survive >6 months after fWBI. The exact mechanism(s) responsible for this radiation-induced brain injury are unknown; however, preclinical studies suggest that radiation modulates the extracellular receptor kinase (ERK) signaling pathway which is associated with cognitive impairment in many neurological diseases. In the study reported here, we demonstrated that the ERK transcriptionally-regulated early response gene, Homer1a, was up-regulated in the hippocampus and down-regulated in the cortex of young adult male Fischer 344 X Brown Norway rats at 48 h after 40 Gy of fWBI. Two months after fWBI, these changes in Homer1a expression correlated with a down-regulation of the hippocampal metabotropic glutamate receptor 1 (mGluR1) and protein kinase Cγ (PKCγ), as well as an up-regulation of cortical mGluR1 and PKCγ. Two drugs that prevent radiation-induced cognitive impairment in rats, the angiotensin type-1 receptor blocker, L-158,809, and the angiotensin converting enzyme inhibitor, ramipril, reversed the fWBI-induced Homer1a expression at 48 h in the hippocampus and cortex and restored mGluR1 and PKCγ to the levels in sham-irradiated controls at 2 months after fWBI. These data indicate that Homer1a is, i) a brain region specific regulator of fWBI–induced brain injury, including cognitive impairment and ii) potentially a druggable target for preventing it.

Keywords: Homer1a expression; fWBI; hippocampus; cortex; Renin-angiotensin system
3.2 Introduction

Over 250,000 patients per year receive fractionated partial or whole brain irradiation (fWBI) for the treatment of primary and metastatic brain cancer (1, 2). The effectiveness of this treatment modality is limited by the radiation dose that can be safely delivered to the normal tissue adjacent to the tumor (3). The majority of patients that receive fWBI are at risk for developing late radiation-induced brain injury which primarily consists of a progressive, irreversible cognitive impairment manifesting as a decrease in short-term memory, attention, concentration, and/or language proficiency (3, 4). Although the exact mechanism(s) behind radiation-induced brain injury are unknown, radiation has been reported to increase microglia activation (5, 6) and decrease neurogenesis (7, 8), suggesting that neuroinflammation and impaired neurogenesis play a role.

Currently, there are no long term treatments for the prevention of radiation-induced brain injury. However, preclinical studies have led to the development of several ongoing clinical trials. In rodent models of radiation-induced brain injury, the peroxisomal proliferator activating receptor alpha (PPARα) agonist, fenofibrate, and the anti-inflammatory drug, indomethacin, prevent radiation-induced decreases in hippocampal neurogenesis (9, 10) and radiation-induced cognitive impairment [Dana Greene-Schloesser, PhD, personal communication]. Additionally, partial restoration of neuronal populations by implantation of neural stem cells or embryonic stem cells has been reported to prevent radiation-induced cognitive impairment in nude rats (11, 12). As a result, radiotherapists are currently attempting to prevent radiation-induced brain injury by shielding the hippocampus (13), one of two sites of neurogenesis in the brain (14).
However, hippocampal shielding has not always proven to be effective at preventing cognitive impairment, suggesting that other brain regions contribute to the development of radiation-induced brain injury (3, 4).

Our lab has focused on the role of neuroinflammation in radiation-induced brain injury. In vitro studies have identified that radiation generates reactive oxygen species (15) and activates the MAP kinase mediated inflammatory response in brain cells (16, 17). Blocking radiation-induced MAP kinase signaling with either PPARα or PPARδ agonists (16, 18) or the renin-angiotensin system (RAS) heptapeptide, angiotensin-(1-7) [EDM, personal communication], inhibits the induction of inflammatory cytokines (e.g. IL-6, Cox-2, MCP-1) in cultured microglia or astrocytes. Furthermore, blockade of the RAS with the angiotensin type-1 receptor blocker (ARB), L-158,809 (19), or the angiotensin converting enzyme inhibitor (ACEI), ramipril (20), prevents fWBI-induced cognitive impairment, but does not protect fWBI-induced decreases in hippocampal neurogenesis in young adult male rats. Thus, the mechanism(s) for developing fWBI-induced brain injury, including cognitive impairment, and how to prevent it have not been fully elucidated.

Brain region specific radiation responses may partially account for the difficulty in elucidating the mechanism(s) for the development of fWBI-induced brain injury and generating a successful strategy to prevent it. For example, recent studies by Peiffer et al. have shown that fWBI-induced damage to brain regions other than the hippocampus may be able to predict which irradiated brain tumor patients will develop cognitive impairment (4). It has also been reported that radiation induces brain region specific changes in white matter (21), acetylcholinesterase (22), and cerebral metabolism (23).
Moreover, brain regions vary in their cell density, number, and type (24). Consequently, these biochemical, cellular, and structural variations may be responsible for the brain region specific radiation response that is observed in the clinic (4).

One of the most successful clinical trials for modulating radiation-induced cognitive impairment employed the acetylcholinesterase inhibitor, donepezil, a drug predominantly used to treat mild to moderate Alzheimer’s dementia (25, 26). Administration of donepezil for 24 weeks, beginning 6 months after fWBI improved moderate dementia in 2/3 of the irradiated brain tumor patients (25). Radiation-induced brain injury and Alzheimer’s disease (AD) also share pathological similarities, including changes in myelination, NMDA receptors, and glutamate/glutamine levels (22, 26-28). This suggests that the molecular mechanism(s) responsible for the development of cognitive deficits in AD may also be involved in the development of radiation-induced cognitive impairment.

Activation of the ERK signaling pathway has been shown to result in the transcription of inflammatory cytokines in both AD (29) and a preclinical model of radiation-induced brain injury (30). Also, activation of ERK has been shown to result in the transcription of early response genes involved in synaptic plasticity, including a protein, Homer1a (31). Homer1a mRNA levels have been shown to be elevated in AD and may contribute to the development of AD cognitive deficits (32). Homer1a is a truncated form of the long Homer1 protein which contains both an EVH1 and carboxy-terminal domain. The Homer1 EVH1 domain binds to metabotropic glutamate family 1 receptors while the carboxy-terminal domain binds other proteins within the postsynaptic complex at the cell membrane (33). Homer1a lacks the carboxy-terminal domain. Thus,
Homer1a binds solely to family 1 metabotropic glutamate receptors (mGluR) and inhibits their binding to the synapse. (34). Overexpression of Homer1a in the hippocampus is known to abolish maintenance of CA3-CA1 long-term potentiation (LTP) (35), synaptic plasticity (36, 37), and impair working memory (35). Radiation also alters LTP and impairs working memory (38). However, whether radiation modifies Homer1a expression in the brain has not yet been explored. In the present study, we tested the hypotheses that, i) radiation alters Homer1a expression similar to what is observed in AD, and ii) Homer1a is potentially a druggable target for preventing radiation-induced brain injury, including cognitive impairment.
3.3 Materials and Methods

Materials

The ARB, L-158,809, (Merck Pharmaceuticals, Whitehouse Station, NJ) was dissolved in water at a concentration of 20 mg/L and administered to rats in their drinking water. The ACEI, ramipril, (Pfizer, Inc, New York, NY), was also dissolved in water at a concentration of 15 mg/L and administered to rats in their drinking water. The following antibody concentrations were used in this study: 1:1000 rabbit anti-mGluR1 (Millipore, Billerica, MA), 1:5000 rabbit anti-p-GluR1 (Ser831; PhosphoSolutions, Aurora, CO), 1:4000 rabbit anti-p-GluR2 (Ser880; PhosphoSolutions), 1:2000 rabbit anti-GluR2/3 (Millipore), 1:1000 rabbit anti-PKCγ (Abcam, Cambridge, MA), 1:1000 mouse anti-p-ERK1/2 (Santa Cruz Biotechnologies, Dallas, TX), 1:1000 ERK1/2 (Cell Signaling, Beverly, MA), and 1:10,000 mouse anti-β-actin (Sigma–Aldrich, St. Louis, MO).

Animals

Forty-eight young adult (10–12 weeks old) male Fischer 344 x Brown Norway rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and housed in pairs on a 12:12 light:dark schedule with access to food and water ad libitum. All animal handling and experiments were performed in strict accordance with the Declaration of Helsinki and the NIH Guide for Care and Use of Laboratory Animals as approved by the Wake Forest University School of Medicine Institutional Animal Care and Use Committee.
Experimental Design

After an acclimation period of 2 weeks, rats were randomized to 6 treatment groups (n=8): Group 1: sham-irradiation, Group 2: L-158,809 alone, Group 3: ramipril alone, Group 4: fWBI alone, Group 5: fWBI + L-158,809, and Group 6: fWBI + ramipril. Rats in Groups 2, 3, 5 and 6 were administered the drug 3 days before initiating fWBI, and then continuously for the duration of the experiment. Fresh drinking water with/without drug was supplied every other day.

Irradiation Procedure

A total dose of 40 Gy was delivered in 8 fractions of 5 Gy/fraction, twice per week for 4 weeks as described previously (39). Briefly, all irradiations were performed in a 267 TBq (7,214 Ci) self-shielded $^{137}$Cs irradiator using lead and Cerrobend shielding devices to collimate the beam so that the whole brain, including the brain stem, was irradiated. The average dose rate to the midline of the brain was $\sim$4 Gy/min; the eyes and body received $\sim$15% and $\sim$3% of the brain dose, respectively. To ensure that the rats received the same midline brain dose, each lightly anesthetized (Ketamine [75 mg/kg]/xylazine [7 mg/kg]) rat had the twice-weekly dose delivered to alternate sides of the head on alternate days. Sham-irradiated rats were anesthetized at the same time as the fWBI rats.

Tissue Collection

Four rats from each treatment group were euthanized 48 h (24 rats) or 2 months (24 rats) after the completion of fWBI. The brains were removed rapidly and grossly
dissected into cortex and hippocampus. The sections were then flash frozen in liquid nitrogen and stored at -80°C.

*Immunoblot Hybridization*

Total cellular protein was harvested from the frozen hippocampal and cortical tissues using M-PER lysis buffer (Pierce Biotechnology, Rockford, IL) supplemented with 1 mg/mL aprotinin (Sigma–Aldrich), 1 mM leupeptin (Sigma–Aldrich), 10 mg/mL phenylmethylsulfonyl fluoride (PMSF), 1 mM Na3VO4 (Sigma–Aldrich), and 150 mM NaCl. After homogenization, the tissue lysates were centrifuged at 12,500 rpm for 30 min, and the supernatant collected. Protein concentrations were measured using the Bradford assay (Bio-Rad, Hercules, CA) at an absorbance 595 nm. Aliquots (25-30 μg) of protein were loaded onto a 10% polyacrylamide gel, and the protein separated by SDS–PAGE electrophoresis. The separated proteins were transferred to polyvinylidene difluoride membranes (Life Sciences) at 35 V overnight, blocked in 5% milk in TBST (0.02 M Tris, 0.015 M NaCl, 0.05% Tween 20, pH 7.5), and incubated overnight with the desired primary antibody. The membranes were then washed, incubated with the appropriate horseradish peroxidase-conjugated secondary antibody, and developed using the ECL reagent (GE Healthcare, Piscataway, NJ), and a Kodak film processor (Rochester, NY). Films were scanned and densitometry performed to quantify the protein using Adobe Photoshop Elements 6.0. All protein levels were expressed as fold changes, with beta actin used as the loading control.
RNA isolation and Quantitative Real Time Polymerase Chain Reaction with Taq-Man

RNA was harvested from the frozen hippocampal and cortical tissue using Trizol reagent (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. DNA contamination was removed by chloroform extraction (Ambion, Austin, TX). Real-time PCR amplifications were conducted in a 20 μl reaction volume containing 2 μl cDNA, 10 μl Taq-Man Master Mix (Applied Biosystems), 0.1 μM Homer1a specific probe, upstream primer, and downstream primer (Applied Biosystems), and 7 μl nuclease-free water. Real-time PCR was carried out in an ABI Prism 7000 at 50 °C for 2 min, 95 °C for 2 min, and 45 cycles of 95 °C for 15 min, 55 °C for 30 s, and 72 °C for 30 s. The fold changes in Homer1a gene expression were calculated using the comparative Ct method (40).

Statistical analysis

All data are expressed as the mean and +/- SEM. All analyses were carried out using GraphPad 5.0 (GraphPad Prism Software). Analyses of the experiments comparing irradiated data to sham-irradiated data were performed using a Student’s t-test for equal sample sizes. Analyses to determine if there was a radiation or drug effect were performed using 2-Way ANOVAs; group comparisons were measured using Bonferroni post-hoc tests. All results were considered statistically significant at the p<0.05 level.
3.4 Results

3.4.1 fWBI alters Homer1a expression in the hippocampus and cortex of young adult male rats.

To determine if fWBI alters Homer1a expression in the brain similar to what is observed in AD, we measured the Homer1a expression in both the hippocampus and cortex at 48 h and 2 months after fWBI. Homer1a expression increased ~2 fold in the hippocampus and decreased ~50% in the cortex at 48 h after fWBI compared to sham-irradiated controls (Fig. 1A). At 2 months after fWBI Homer1a expression was decreased ~70% in the hippocampus and ~50% in the cortex compared to sham-irradiated controls (Fig. 1B). Homer1a is known to be regulated by ERK activation (41) and single doses of irradiation have been shown to activate ERK in vitro (16, 17) and in vivo (30). Consequently, we measured p-ERK in the hippocampus and cortex to determine if fWBI modulation of Homer1a may be associated with the activation of ERK at 48 h and 2 months. fWBI-induced an ~2-fold increase in p-ERK in the hippocampus and an ~50% decrease in p-ERK in the cortex at both 48 h (Fig. 1C) and 2 months (Fig. 1D) after fWBI compared to sham-irradiated controls. Taken together, these data suggest that fWBI alters Homer1a expression in a brain region specific manner (or gray vs. white matter manner), consistent with the brain region specific radiation responses observed in preclinical (22, 23) and clinical studies (4, 21).

3.4.2 mGluR1 and its downstream effector, PKCγ, are decreased in the hippocampus at 2 months after fWBI
Homer1a binds to mGluR1 and inhibits mGluR1 binding to the synapse (42). Homer1a overexpression is associated with decreased LTP and working memory (35). Finally, hippocampal decreases in mGluR1 and its downstream effectors have been reported to be associated with a decline in cognition (43). Consequently, we measured the total levels of hippocampal mGluR1 and its downstream effector, PKCγ, a kinase that has been shown to be activated by Ca\(^{2+}\) release from mGluR1 complexes at 2 months after fWBI (42). Total hippocampal mGluR1 levels decreased ~80% at 2 months after fWBI compared to sham-irradiated controls (Fig. 2A). Furthermore, changes in mGluR1 activation have been shown to alter phosphorylation of AMPA GluR1 at Ser831 (44). However, at 2 months after fWBI, we did not detect a change in the levels of p-GluR1 (Fig. 2B) or changes in other AMPA receptors, total AMPA GluR2/3 (Fig. 2C) and AMPA p-GluR2 (Fig. 2D), suggesting that fWBI only alters the total levels of mGluR1. Also, fWBI induced an ~60% decrease in the total levels of the downstream effector, PKCγ (Fig. 2E), suggesting that a decrease in mGluR1 levels has the potential to produce a decrease in mGluR1 downstream signaling after fWBI. These data suggest that fWBI produces an early increase in hippocampal Homer1a expression (Fig. 1A, B) which correlates with a decrease in hippocampal mGluR1 (Fig. 2A) and its downstream effectors at 2 months after fWBI (Fig. 2E), similar to what is found in other neurodegenerative conditions such as epileptic seizures (45, 46), Huntington’s disease (47) and AD (32, 48).

3.4.3 mGluR1 and its downstream effector, PKCγ, are increased in the cortex at 2 months after fWBI
Unlike the hippocampus, the total levels of cortical mGluR1 increased ~2-fold (Fig. 3A), and its downstream effector, PKCγ, increased ~3 fold (Fig. 3E) compared to sham-irradiated controls at 2 months after fWBI. Similar to the hippocampus, fWBI did not alter the total levels of cortical p-GluR1 (Fig. 3B), GluR2/3 (Fig. 3C) or p-GluR2 (Fig. 3D). These data suggest that fWBI produces a sustained decrease in cortical Homer1a expression (Fig. 1A, B) which correlates with an increase in cortical mGluR1 (Fig. 3A) and its downstream effectors at 2 months after fWBI (Fig. 3E), similar to that reported in mouse models of aging (49).

### 3.4.4 L-158,809 and ramipril prevent the changes in hippocampal Homer1a expression and its downstream signaling after fWBI

Blocking the RAS with the ARB, L-158,809 or the ACEI, ramipril, has been shown to prevent fWBI-induced hippocampal-independent cognitive impairment (19, 20). In the present study, both L-158,809 and ramipril prevented the fWBI-induced increase in hippocampal Homer1a expression at 48 h after fWBI (Fig. 4A), but only L-158,809 had the same effect at 2 months (Suppl. Fig. 1). These RAS blockers restored the hippocampal mGluR1 levels to the sham-irradiated control levels at 2 months (Fig. 4B). Similarly, both drugs prevented the fWBI-induced changes in p-ERK (Fig. 4C) upstream of Homer1a, and PKCγ (Fig. 4D) downstream of mGluR1. L-158,809 and ramipril did not affect the levels of p-GluR1 (Suppl. Fig. 2A), total GluR2/3 (Suppl. Fig. 2B) or p-GluR2 (Suppl. Fig. 2C). These data suggest that L-158,809 and ramipril are able to prevent the early fWBI-induced increase in hippocampal Homer1a expression and restore
the mGluR1 signaling which is likely important for hippocampal-dependent cognitive function.

3.4.5 L-158,809 and ramipril prevent the changes in cortical Homer1a expression and its downstream signaling after fWBI

Similar to the hippocampus results, both L-158,809 and ramipril prevented the decrease in cortical Homer1a expression at 48 h after fWBI (Fig. 5A), but only L-158,809 had the same effect at 2 months (Suppl. Fig. 3). These RAS blockers restored the cortical mGluR1 levels to the sham-irradiated control levels at 2 months after fWBI (Fig. 5B). Similarly, both drugs prevented the fWBI-induced changes in the upstream and downstream proteins, p-ERK (Fig. 5C) and PKCγ (Fig. 5D), respectively. L-158,809 and ramipril did not affect the levels of p-GluR1 (Suppl. Fig. 4A), total GluR2/3 (Suppl. Fig. 4B) or p-GluR2 (Suppl. Fig. 4C). These data suggest that L-158,809 and ramipril are able to prevent the early fWBI-induced decrease in cortical Homer1a expression and restore mGluR1 signaling which is likely important for cortical-dependent cognitive function.
3.5 Discussion

The majority of brain tumor patients surviving >6 months after radiation therapy will develop late radiation-induced brain injury predominantly manifesting as a progressive, irreversible cognitive impairment. In the study reported here, we demonstrated that the ERK transcriptionally-regulated early response gene, Homer1a, is differentially regulated in the hippocampus and cortex of young adult male rats at 48 h after a total 40 Gy dose of fWBI (Fig. 1A). Furthermore, treatment with L-158,809 or ramipril, drugs known to prevent fWBI-induced cognitive impairment in rats (19, 20), prevented fWBI-induced changes in Homer1a expression in both the hippocampus and the cortex at 48 h after fWBI (Figs. 4A, 5A), but only L-158,809 had the same effect at 2 months (Suppl. Figs. 1, 3). Homer1a belongs to a family of scaffolding proteins that localize in the synapse and regulate intracellular calcium homeostasis (50, 51), gene transcription, signal transduction, and receptor trafficking (34, 41). The longer forms of Homer1 are constitutively expressed and have two functional domains, i) the EVH1 domain which binds to the Shank, mGluR1/5, and ryanodine receptors, and ii) a coiled coil structure which binds to other Homer forms (34). The Homer1a form lacks the coiled coil domain and disrupts both the scaffolding and signaling capabilities of the long forms of Homer proteins by competitively binding to the Shank-mGluR1/5-ryanodine complex (41, 42).

Homer1a is transcriptionally induced in neurons after seizures (45, 52) and in the hippocampus during LTP (53-55). Homer1a regulates activity-induced post and presynaptic remodeling (56) and dendritic axonal targeting of mGluR5 (57). Overexpression of Homer1a in the rodent hippocampus or striatum impairs hippocampal-dependent
memory and motor performance in behavioral tasks (31). Homer1a expression is increased in AD (32) and animal models of epileptic seizures (58) and Huntington’s disease (41). Homer1 knockout mice exhibit a schizophrenic-like phenotype with behavioral (motivational and emotional), cognitive, sensorimotor processing, and glutamatergic abnormalities (59, 60). In a rat model of aging, increased expression of Homer1a and decreased mGluR5 signaling in the hippocampus were associated with cognitive deficits (61). Conversely, decreased Homer1a expression in the whole brain (largely cortical expression) was also associated with a loss of cognitive and motor function in mice (49). These data demonstrate that, depending on the brain region involved, both increased and decreased Homer1a expression can lead to cognitive impairment. Our data showing that Homer1a is differentially expressed in the hippocampus and cortex after fWBI (Fig. 1A) is consistent with this concept.

The reason for the differential regulation of Homer1a expression in the hippocampus and cortex is not clear. We speculate that the brain microenvironment is likely to contribute to this differential signaling. Brain regions vary in their cellular distribution (24), and this may contribute to their response to different stimuli (62, 63). Moreover, it has been shown that astrocytes, microglia and neurons isolated from different brain regions respond differently to injury (64-66). Therefore, it is not unique that fWBI induces specific brain region-mediated responses in Homer1a expression.

The study reported here indicates that fWBI induces changes in p-ERK, Homer1a, mGluR1, and PKCγ (Figs. 1-3). In the hippocampus, fWBI increased phosphorylation of ERK and transcription of Homer1a at 48 h after fWBI (Fig. 1A, C). This increase in early hippocampal Homer1a expression correlated with a decrease in mGluR1 (Fig. 2A) and its
downstream effector, PKCγ (Fig. 2E) at 2 months after fWBI. In contrast to the hippocampus, fWBI decreased p-ERK and Homer1a expression at 48 h in the cortex (Fig. 1A, D). This was concomitant with a large increase in mGluR1 (Fig. 3A) and PKCγ (Fig. 3B) at 2 months after fWBI. fWBI did not alter p-GluR1, total GluR2/3 or p-GluR2 at 2 months after fWBI (Suppl. Figs. 2, 4). Although Homer1a has also been shown to regulate phosphorylation of GluR1 (44) and GluR2 (50), our data suggests that Homer1a is regulating mGluR1 signaling after fWBI. At 2 months after fWBI, only L-158,809 prevented the fWBI-induced changes in Homer1a expression in both the hippocampus and cortex (Suppl. Figs. 1, 3). In addition, ERK activation does not match Homer1a regulation in the hippocampus at 2 months after fWBI, likely due to ERK’s involvement in many other processes (55, 67, 68). Therefore, we suspect that the role of Homer1a in fWBI-induced cognitive impairment appears to be limited to an “early” event after irradiation.

Memory/learning paradigms suggest that trafficking of mGluR1 from an intracellular compartment to the plasma membrane is an early event during neural plasticity (43). Thus, the changes in mGluR1 (Figs. 2A, 3A) at 2 months after fWBI may result in alterations in hippocampal and/or cortical neural plasticity. Of note, our rat model of fWBI-induced brain injury does not exhibit hippocampal-independent cognitive impairment until 6 months after fWBI, similar to what is observed clinically (3). Given that these hippocampal and cortical fWBI-induced early changes in Homer1a expression and delayed changes in mGluR1 signaling were prevented by L-158,809 and ramipril, drugs that also prevent fWBI-induced hippocampal-independent cognitive impairment
(19, 20), we speculate that this signaling mechanism is important for radiation-induced cognitive impairment.

It is important to note that all of the data in this study describing the relationship between Homer1a expression and mGluR1 signaling is correlative rather than causative. Unfortunately, our analysis was limited to Homer1a expression because a suitable antibody is not available for measuring Homer1a protein. Future experiments that measure Homer1a expression in the hippocampus and cortex over time periods up to 6 months after fWBI will be required to elucidate the role of Homer1a and mGluR1 signaling in fWBI-induced cognitive impairment. Finally, fWBI studies with Homer1a knockout mice (50) could further our understanding of the role of Homer1a and mGluR1 signaling in fWBI-induced cognitive impairment.

The ARB, L-158,809, and the ACEI, ramipril, both prevent fWBI-induced cognitive impairment in rats (19, 20) and inhibit the early fWBI-induced differential expression of Homer1a in the hippocampus (Fig. 4A) and cortex (Fig. 5A). Although ramipril ameliorates radiation-induced neuroinflammation, our results suggest that ramipril may have other targets for preventing fWBI-induced cognitive impairment (20). The data reported here indicate that components of the p-ERK-Homer1a-mGluR1-PKCγ signaling pathway may be druggable candidates for preventing fWBI-induced cognitive impairment. ERK is the first member of the pathway and has been shown to be activated both in vitro (16, 17) and in vivo (30) by ionizing radiation. Moreover, ERK inhibition has been associated with decreases in radiation-induced neuroinflammation (16, 17, 30). In the study reported here, both L-158,809 and ramipril inhibited fWBI-induced changes in p-ERK. However, ERK is not an attractive druggable target to prevent of fWBI-
induced brain injury because ERK is involved in numerous biochemical and cellular processes in several organs (68). Thus, inhibition of ERK is likely to have negative effects on one or more organ systems in the body (69). To our knowledge, there are no reports of Homer1a having an important function, other than in cardiac myocytes (70), outside the nervous system (34). Consequently, this makes Homer1a an attractive druggable target for the prevention of fWBI-induced brain injury, including cognitive impairment.

In summary, Homer1a, a protein only expressed in the nervous system, was up-regulated in the hippocampus and down-regulated in the cortex of young adult male rats after fWBI, similar to what is observed in other neurodegenerative conditions. These early changes in Homer1a expression resulted in altered mGluR1 and PKCγ levels in both the hippocampus and cortex at 2 months after fWBI. Furthermore, treatment with L-158,809 or ramipril, which prevent fWBI-induced cognitive impairment in rats, restored early Homer1a expression as well as mGluR1 and its downstream effector levels to those in sham-irradiated controls. Taken together, these data suggest that early Homer1a expression, i) plays an important role in the development of fWBI-induced brain injury, including cognitive impairment, and ii) is potentially a druggable target for preventing it.
Acknowledgements:

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Figure 1: fWBI alters Homer1a expression in the hippocampus and cortex of young adult male rats. Hippocampal and cortical Homer1a mRNA levels were determined by Taq-man real time PCR on mRNAs isolated 48 h (A) or 2 months (B) after fWBI, and the data normalized to 18S mRNA levels. Hippocampal and cortical p-ERK proteins were analyzed by Western blot hybridization of lysates isolated 48 h (C) and 2 months (D) after fWBI; β-actin was the loading control. Data are expressed as the Mean ± S.E.M. from 4 rats. *p<0.05, ***p<0.001
Figure 2: mGluR1 and its downstream effector, PKCγ, are decreased in the hippocampus at 2 months after fWBI. Total mGluR1 (A), p-GluR1 (B), total GluR2/3 (C), p-GluR2 (D), and total PKCγ (E) protein levels were analyzed by Western blot hybridization at 2 months after fWBI; β-actin was the loading control. Data are expressed as the Mean ± S.E.M. from 4 rats. ***p<0.001
Figure 3: mGluR1 and its downstream effector, PKCγ, are increased in the cortex at 2 months after fWBI. Total mGluR1 (A), p-GluR1 (B), total GluR2/3 (C), p-GluR2 (D), and total PKCγ (E) protein levels were analyzed by Western blot hybridization at 2 months after fWBI; β-actin was the loading control. Data are expressed as the Mean ± S.E.M. from 4 rats. ***p<0.001
Figure 4: L-158,809 and ramipril prevent the changes in hippocampal Homer1a expression and its downstream signaling after fWBI. Homer1a mRNA levels were determined by Taq-man real time PCR on mRNAs isolated 48 h after fWBI, and the data normalized to 18S mRNA levels (A). Total mGluR1 at 2 months after fWBI (B), p-ERK at 48 h after fWBI (C), and total PKCγ at 2 months after fWBI (D) protein levels were analyzed by Western blot hybridization; β-actin was the loading control. Data are expressed as the Mean ± S.E.M. from 4 rats. *p<0.05, **p<0.01
Figure 5: L-158,809 and ramipril prevent the changes in cortical Homer1a expression and its downstream signaling after fWBI. Homer1a mRNA levels were determined by Taq-man real time PCR on mRNAs isolated 48 h after fWBI, and the data normalized to 18S mRNA levels (A). Total mGluR1 at 2 months after fWBI (B), p-ERK at 48 h after fWBI (C), and total PKCγ at 2 months after fWBI (D) protein levels were analyzed by Western blot hybridization; β-actin was the loading control. Data are expressed as the Mean ± S.E.M. from 4 rats. *p<0.05, ***p<0.001.
Supplemental Figure 1: Only L-158,809 prevents the changes in hippocampal Homer1a expression at 2 months after fWBI. Homer1a mRNA levels were determined by Taq-man real time PCR on mRNAs isolated 2 months after fWBI, and the data normalized to 18S mRNA levels. Data are expressed as the Mean ± S.E.M. from 3-4 rats.
Supplemental Figure 2: L-158,809, and ramipril, did not alter the hippocampal p-GluR1, GluR2/3, or p-GluR2 levels at 2 months after fWBI. Hippocampal p-GluR1 (A), total GluR2/3 (B), and p-GluR2 (C) protein levels were analyzed by Western blot hybridization at 2 months after fWBI; β-actin was the loading control. Data are expressed as the Mean ± S.E.M. from 4 rats.
Supplemental Figure 3: Only L-158,809 prevents the changes in cortical Homer1a expression at 2 months after fWBI. Homer1a mRNA levels were determined by Taqman real time PCR on mRNAs isolated 2 months after fWBI, and the data normalized to 18S mRNA levels. Data are expressed as the Mean ± S.E.M. from 3-4 rats.
Supplemental Figure 4: L-158,809, and ramipril, did not alter the cortical p-GluR1, GluR2/3, or p-GluR2 levels at 2 months after fWBI. Cortical p-GluR1 (A), total GluR2/3 (B), and p-GluR2 (C) protein levels were analyzed by Western blot hybridization at 2 months after fWBI; β-actin was the loading control. Data are expressed as the Mean ± S.E.M. from 4 rats.
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CHAPTER IV

General Discussion and Future Directions

4.1 Clinical Problem

In 2013, approximately 1.6 million new cancer cases will be diagnosed in the United States; about 550,000 of these cases will have or develop some form of a brain tumor, either primary or metastatic (1). About 120 types of brain and CNS tumors were identified to date with the most common intracranial tumor being a brain metastases arising from lung, breast, kidney, or skin cancer (1, 2). Brain tumors are most commonly treated by surgery, radiation therapy, chemotherapy, or a combination of these modalities (3). The majority of brain tumor patients receive fWBI delivered as a total dose of 30 Gy in 3 Gy fractions over 6 weeks as part of their treatment (4). The others usually receive some form of SRS where 18-22 Gy are delivered to the tumor volume in a single treatment (3, 5). Although fWBI may result in irreversible late radiation-induced brain injury, including cognitive impairment, failing to administer fWBI greatly reduces the survival of most brain tumor patients to 1-3 months (6). Thus, despite potential QOL issues, fWBI remains the preferred treatment modality for primary and metastatic brain tumors (7).

Currently, the only two published studies to date of a clinical trial for modulating radiation-induced cognitive impairment involved the acetyl cholinesterase inhibitor, donepezil (6, 8) and the NMDA receptor blocker, memantine, and neither prevented radiation-induced cognitive impairment (9). Preclinical studies demonstrated that RAS
blockers can prevent the development of radiation-induced brain injury, including cognitive impairment (10-12); however their mechanism(s) of action is not fully known. Although both the ARB, L-158,809 (10, 13), and the ACEI, ramipril (11), prevent radiation-induced cognitive impairment, neither prevent the decrease in neurogenesis and only ramipril inhibits hippocampal radiation-induced microglial activation. This led to the development of two hypotheses, i] the mechanism by which RAS blockers prevent/ameliorate radiation-induced cognitive impairment is not solely by inhibiting microglia activation, and ii] targeting the hippocampus alone is not sufficient for prevention of radiation-induced brain injury, including cognitive impairment.

4.2 The Role of Astrocytes in Radiation-Induced Brain Injury

Late radiation-induced brain injury is a dynamic, complex process. It involves an initial acute inflammatory response classically characterized as activation of microglia (14-16). Radiation activates microglia, the resident immune cells of the brain, triggering secretion of proinflammatory cytokines and chemokines as well as generating free radicals and fatty acid metabolites, all of which are harmful to neurons and associated with neurodegeneration and impaired neuronal function (17, 18). Our in vitro studies indicate that radiation-induced inflammation not only occurs in microglia (15, 19), but also in astrocytes (Chapter 2, Fig. 1). Astrocytes account for the majority of the glial cell population in the brain and outnumber neurons 9:1 (20). Their main function is to support both neuronal transmission and synaptic plasticity (20). Astrocytes also work in collaboration with endothelial cells to generate and maintain the BBB (20).
exposed to an insult (e.g. brain trauma), these cells become hypertrophic, express high levels of GFAP (18, 20, 21), and are referred to as reactive astrocytes. Reactive astrocytes express the inflammatory mediators, IL-6, Cox-2, and MCP-1, which exacerbate the severity of CNS disorders (20-23). Thus, it is likely that a radiation-induced inflammatory response in astrocytes contributes to radiation-induced brain injury, including cognitive impairment (18).

Radiation-induced inflammation in brain cells other than microglia may also partially explain data observed in animal studies where agents that prevent radiation-induced cognitive impairment do not always inhibit radiation-induced microglial activation (10, 13). There is limited evidence that radiation also induces an inflammatory response in endothelial cells (24, 25). Studies in immortalized rat brain microvascular endothelial cells (RBMEC) indicate that radiation increases, i] reactive oxygen species, ii] NF-κB activation, and iii] ICAM-1 expression (24). Because the RAS peptide, Ang-(1-7), i] exhibits antiangiogenic effects in endothelial cells (26) and ii] inhibits the radiation-induced inflammatory response of primary astrocytes (Chapter 2, Fig. 1), it is reasonable to speculate that Ang-(1-7) may also inhibit the acute inflammatory response in endothelial cells. Inhibition of this endothelial inflammatory response could potentially strengthen the BBB and prevent the infiltration of activated extracranial immune cells into the brain that may contribute to radiation-induced brain injury, including cognitive impairment. Future studies should examine this possibility of protecting the BBB.

Radiation-induced inflammation in astrocytes may also explain oligodendrocyte cell death observed after high single doses (> 10 Gy) (27, 28). Oligodendrocytes are glial cells which produce the myelin sheath on neuronal axons (27). High single dose WBI
induces apoptosis of oligodendrocyte progenitor cells, greatly reducing the population of new oligodendrocytes (27-30). This is consistent with studies demonstrating that inflammatory cytokines released from astrocytes contribute to oligodendrocyte cell death (31). However, under clinically relevant fWBI regimens, oligodendrocyte number and gross axonal structure are unaltered 6-12 months after irradiation in rats (30, 32).

It has been proposed that subtle changes in white matter integrity are likely responsible for the brain injury that leads to cognitive impairment (30, 32, 33). Radiation-induced structural alterations in myelination can be measured using diffusion tensor imaging (DTI) (34). Changes in DTI, such as decreases in fractional anisotropy (FA) and increases in the apparent diffusion coefficient (ADC), correlate with decreases in cognition (34). Although fWBI does not induce gross structural changes or DTI changes in FA in heavily myelinated white matter, fWBI induces FA changes in the lightly myelinated white matter of the superficial parietal cortex in rats (32). These data suggest that the important radiation targets may be the less myelinated or unmyelinated cortical axons, the extracellular matrix, or the synaptic fields rather than the heavily myelinated tracts. Thus, radiation-induced astrocyte inflammation could compromise oligodendrocyte function and promote white matter integrity changes that might contribute to the development of radiation-induced cognitive impairment.

Measuring the levels of proinflammatory mediators secreted by reactive astrocytes over a prolonged post-irradiation period in vivo is a challenge. In general, radiation-induced increases in inflammatory mediators were measured only in lysates from whole brain or specific brain regions, not in specific cell types (14, 35-37). In addition, these studies only measured inflammation at acute time points after irradiation.
Future studies should be designed to determine if, i) radiation induces an inflammatory response in astrocytes in vivo, and ii) drugs such as the RAS blockers can prevent this inflammation. In brief, brain cells could be double-labeled with GFAP, an astrocyte marker, and an inflammatory marker (e.g. Cox-2) that is elevated in astrocytes in vitro (Chapter 2, Fig. 1). If these studies demonstrate that astrocytes can be targeted to prevent a radiation-induced inflammatory response in the brain, it may be possible to develop CNS specific agents for the prevention of radiation-induced brain injury, including cognitive impairment.

A caveat to our work in Chapter 2 is that our in vitro results may not directly translate to the in vivo situation because Ang (1-7) may not have the same effect in the complex brain microenvironment. Furthermore, acute studies on the radiation-induced inflammatory response have not been definitively linked to late radiation-induced brain injury, including cognitive impairment (4). Although the studies in Chapter 2 demonstrate an acute inflammatory response in astrocytes and identify a potential approach to preventing this response, it is difficult to directly extrapolate these in vitro results to the clinic. Thus, in vivo studies should be undertaken to determine if the RAS blockers can prevent radiation-induced inflammation in brain cell populations other than microglia. This is especially important for preventing radiation-induced brain injury, including cognitive impairment, because microglia are “brain macrophages” and macrophages are present throughout the body. These systemic macrophages perform a number of important immune functions (39). Thus, targeting the microglia/macrophages to prevent radiation-induced brain injury could have negative systemic effects. If one could inhibit radiation-induced neuroinflammation by inhibiting the response of a CNS
specific cell, such as astrocytes, then interventions could be targeted to the brain with little to no systemic toxicity.

4.3 Ang-(1-7): Anti-Tumor Agent and Normal Tissue Protector

Ang-(1-7) has anti-tumor effects in sarcoma (40), breast (41), lung (42, 43), and prostate cancer (44, 45). Ang-(1-7) inhibits tumor cell proliferation via inhibition of MAP kinase signaling (41, 45, 46), similar to its mechanism of action for inhibiting radiation-induced inflammation in cultures of primary astrocytes (Chapter 2, Fig. 3). Thus, interventions that increase Ang-(1-7) during radiation therapy may aid in reducing the tumor burden as well as protecting against radiation-induced brain injury, including cognitive impairment.

The studies in Chapter 2 used Ang-(1-7) to inhibit radiation-induced inflammation as a potential mechanism for RAS blocker mediation of radiation-induced cognitive impairment. Unfortunately, the ability of Ang-(1-7) to cross the BBB, or its ability to prevent radiation-induced cognitive impairment, is unknown. Our attempt to perform an in vivo study in which Ang-(1-7) was directly administered to the brain via intracerebroventricular cannulas failed because of technical difficulties. The lack of evidence demonstrating the ability of Ang-(1-7) to cross the BBB or inhibit radiation-induced cognitive impairment limits its ability to move into the clinic as a potential therapeutic for radiation-induced brain injury, including cognitive impairment. However, the RAS blockers that, i] cross the BBB (10, 47), ii] prevent radiation-induced cognitive impairment in rodent models (10, 11), and iii] increase the concentration of the anti-inflammatory, anti-tumor, Ang-(1-7) peptide in brain cells (12) make them prime
candidates for future clinical trials to prevent/ameliorate radiation-induced brain injury, including cognitive impairment. A clinical trial with ramipril is currently under development at Wake Forest and expected to begin accrual in early 2014.

Standard radiation therapy today uses temozolomide, a radiation sensitizer, in combination with fWBI for primary brain tumors (4, 48). Temozolomide increases radiation damage in the tumor by methylating the DNA and saturating DNA methyl transferases (48-50). Because the MAP kinase pathway regulates the transcription of methyl transferases, such as O6-methyl-guanine methyl transferase which removes methyl groups from the DNA (51, 52), inhibition of the MAP kinase pathway by Ang-(1-7) should produce a more effective tumor cell kill when combined with the current clinical regimen. Future in vitro and in vivo studies should be designed to test the tumor cell kill effectiveness of a combined modality treatments using temozolomide, Ang-(1-7), and radiation.

4.4 The Role of Altered Neuronal Function in Radiation-Induced Brain Injury

The evidence supporting changes in neuronal function as a contributing factor to the development of radiation-induced brain injury, including progressive cognitive impairment, is limited. Early radiation studies assumed that neurons were radioresistant because they did not divide (53). With the discovery of adult neurogenesis in the brain, neurons are now known to respond negatively to radiation. Most studies focused on radiation-induced neuronal changes in the hippocampus, one of the two sites of neurogenesis in the adult brain (54, 55). Radiation induces changes in hippocampal
cellular activity (56, 57), synaptic efficiency/spike generation (57, 58), and neuronal gene expression (59, 60). Single or fractionated WBI of the rodent brain induces changes in, i] expression of activity-regulated cytoskeleton-associated protein (Arc) (60), ii] N-methyl-D-aspartic acid (NMDA) receptor subunits (33, 61), iii] hippocampal long-term potentiation (LTP) (62, 63), and iv] glutamatergic signaling (61, 64), all of which are important for synaptic plasticity and cognition.

However, there are several contradictory studies on radiation-induced changes in neuronal function. For example, two studies by the same group used magnetic resonance spectroscopy (MRS) in an attempt to identify biomarkers for radiation-induced brain injury (10, 65). The initial study detected radiation-induced increases in brain metabolites, including increases in glutamate/glutamine at 12 month after fWBI that appeared to correlate with cognitive impairment (65). A subsequent study by this group could not detect changes in these brain metabolites at 6 months when cognitive impairment was already detectable (10, 66). A possible explanation for these results is that the MRS measurements in these studies used different voxel sizes that included several brain regions (10, 65). Using much smaller voxels (2.5 mm$^3$) that covered single brain regions in the rat, it has been shown that radiation induces changes in DTI and FA only in the superficial parietal cortex, but not in the hippocampus (67). Recently, it has been demonstrated that the radiation dose delivered to specific brain regions may predict cognitive outcome, rather than the dose to the whole brain (68). Thus, it is likely that future studies of radiation-induced changes in brain metabolites need to be performed using relatively small voxels in the brain regions most likely involved in cognition.
To date, radiation-induced changes in neuronal function in specific brain regions were poorly characterized. Although several rodent studies reported radiation-induced changes at the molecular level in the hippocampus, these studies neglected to assess other areas of the brain (60, 69). Irradiating the rodent brain decreases hippocampal NMDA subunits (33, 61), neurogenesis (14, 70), and LTP (63, 71) as well as increases neuroinflammation. This increased neuroinflammation and decreased neurogenesis in the hippocampus has often been accepted as being responsible for the results of radiation studies describing both hippocampal-dependent and hippocampal-independent cognitive impairment (10, 11). However, treatments with various PPAR agonists and RAS inhibitors prevent radiation-induced brain injury, including cognitive impairment, but they do not always prevent the radiation-induced changes in hippocampal neuroinflammation and neurogenesis (Table 1) (10, 11, 13, 36, 72, 73).

Table 1. Preclinical studies of interventions targeting radiation-induced brain injury

<table>
<thead>
<tr>
<th>Drug</th>
<th>Model</th>
<th>Prevents Radiation-Induced Decreases in Neurogenesis</th>
<th>Prevents Radiation-Induced Increases in Neuroinflammation</th>
<th>Prevents Radiation-Induced Cognitive Impairment</th>
<th>Prevents Radiation-Induced Cognitive Impairment</th>
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<tr>
<td>L-158,809 (ARB)</td>
<td>Rat</td>
<td>No</td>
<td>No</td>
<td>ND</td>
<td>Yes</td>
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<tr>
<td>Ramipril (ACEI)</td>
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<td>Yes</td>
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<tr>
<td>Fefofibrate (PPARα agonist)</td>
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<td>No</td>
<td>No</td>
<td>No Radiation Alone Effect</td>
<td>Yes</td>
</tr>
<tr>
<td>Fefofibrate (PPARα agonist)</td>
<td>Mouse</td>
<td>Yes</td>
<td>Yes</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Pioglitazone (PPARγ agonist)</td>
<td>Rat</td>
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</tbody>
</table>

N/D = Not Done

For example, both the ARB, L-158,089, and the PPARα agonist, fenofibrate, fail to prevent either the decrease in hippocampal neurogenesis or the increase in hippocampal neuroinflammation, but they prevent hippocampal-independent radiation-
induced cognitive impairment (Table 1). Thus, genes that are differently expressed in brain regions after irradiation need to be identified to better understand the development of radiation induced cognitive impairment, and how to prevent it.

4.5 Homer1a: A Potential Modulator of Radiation-Induced Brain Injury

The early response gene, Homer1a, is a potential candidate that may be differentially expressed in brain regions after irradiation. Homer1a has been shown to be involved in synaptic plasticity (74). It is differentially expressed in specific brain regions in patients that have Alzheimer’s disease (75) and preclinical models of epileptic seizures (76), Huntington’s disease (77), and age-impaired cognition (78, 79). Homer1a belongs to a family of scaffolding proteins that localize in the synapse and regulate intracellular calcium homeostasis, gene transcription, signal transduction, and receptor trafficking (80-83). Its main function is to bind solely to GluRs and inhibit their binding to the synapse, thereby modifying glutamate signaling (Chapter 1, Fig. 2) (81). Decreased Homer1a expression in whole brain lysates (largely cortical expression) is associated with a loss of cognitive and motor function in mice (78). Conversely, increased hippocampal Homer1a expression is associated with cognitive deficits in a rat model of aging (79). Given Homer1a’s function and its altered expression in neurodegenerative conditions, it is likely to play a brain region specific role in radiation-induced brain injury, including cognitive impairment. However, to date no studies identified a radiation effect on Homer1a expression and/or its downstream signaling.
Brain region specific radiation-induced changes in Homer1a were identified in Chapter 3 (Figs. 1-3) as a potential mechanism for modulating glutamate signaling. Although the exact role that glutamate signaling plays in radiation-induced cognitive impairment is undefined, a recently published clinical study demonstrated that the NMDA receptor antagonist, memantine, can slow the cognitive impairment in brain cancer patients treated with fWBI (9). Interestingly, hippocampal overexpression of Homer1a greatly alters AMPA and NMDA signaling in the rodent brain (84, 85). Thus, radiation-induced changes in glutamate signaling may not only result in cognitive impairment, but targeting the glutamate signaling pathway may be a way to prevent it. To determine if radiation-induced changes in glutamate signaling are associated with changes in Homer1a expression, both in vitro and in vivo studies should be performed. In vitro studies should involve measuring changes in the glutamate receptor protein after irradiation of cultured neurons where Homer1a expression can be altered via siRNA knockdown or lentiviral overexpression. If knocking down Homer1a expression decreases glutamate receptor protein in vitro, then these studies should be extended in vivo using the Homer1a knockout mouse.

The Worley lab at Johns Hopkins generated Homer1 and Homer1a knockout mice for Alzheimer’s disease related studies (83, 86, 87). Although Homer1 knockout mice exhibit schizophrenic-like characteristics (86, 87), it is not yet known if Homer1a knockout mice have cognitive defects. However, since the Homer1a knockout mouse was generated on a C57Bl6 background (83), and this strain exhibits radiation-induced brain injury, including cognitive impairment (36), pre- and post-irradiation cognitive studies are likely to be informative. Because brain region specific increases and decreases
in Homer1a expression are associated with cognitive impairment (78, 79), it is difficult to predict if knocking out Homer1a would “protect” or “exacerbate” radiation-induced cognitive impairment. However, it is reasonable to hypothesize that a complete loss of Homer1a is likely to exacerbate radiation-induced cognitive impairment. Other future studies might involve a cell type specific, conditional knockout Homer1a mouse. The Worley lab (personal communication) is currently developing these animals, and they should be available for potential radiation studies starting in early 2014.

In addition to cognitive testing experiments, or if cognitive data cannot be obtained from the knockout mice, future studies on the role of Homer1a in radiation-induced brain injury could be explored using LTP as an end-point. Homer1a overexpression in the hippocampus impairs working memory and abolishes maintenance of CA3-CA1 LTP (88). Thus, it is likely that radiation-induced increases in hippocampal Homer1a expression could impact CA3-CA1 LTP. Studies that collect recordings from brain slices of irradiated rodents should also determine if changes in Homer1a expression affect cortical and/or hippocampal LTP.

The importance of evaluating the exact role that Homer1a plays in radiation-induced brain injury, including cognitive impairment, stems from our ability to inhibit the radiation-induced Homer1a expression changes with the ARB, L-158,809, or the ACEI, ramipril. Both of these drugs inhibit hippocampal-independent radiation-induced cognitive impairment in preclinical models (10, 11) and inhibited radiation-induced changes in Homer1a expression in both the hippocampus and cortex (Chapter 3, Figs. 4, 5). Furthermore, radiation-induced changes in mGluR1 and its downstream target, PKCγ, were inhibited with these RAS blockers (Chapter 3, Figs. 4, 5). This indicates that
Homer1a may be a druggable target for preventing radiation-induced brain injury, including cognitive impairment.

4.6 Not Just a Microglia and Hippocampus Story Anymore

In summary, the data in Chapter 2 indicate that radiation induces a MAP kinase mediated inflammatory response in astrocytes. Previously, it was thought that astrocytes could generate a radiation-induced inflammatory response only in the presence of microglia that were activated by radiation. This new information expands our model of radiation-induced cognitive impairment to now include the astrocyte as a contributor to radiation-induced neuroinflammation (Figure 1). Furthermore, our data identifies the astrocyte as a new CNS-specific cellular target for preventing radiation-induced brain injury. Consequently, radiation-induced brain injury is not just a microglia story anymore.
Figure 1: Revised model of radiation-induced cognitive impairment.

Figure 1: Revised mechanisms underlying radiation-induced cognitive impairment. Astrocytes are now included as contributors to neuroinflammation and Homer1a is now included as a contributor to altered neuronal function. Updates are highlighted in red print. (adapted from Schloesser et al., 2013) (89).

In Chapter 3, our post-fWBI data indicate that Homer1a expression is, i) increased in the hippocampus resulting in decreased mGluR1 signaling, and ii) decreased in the cortex resulting in increased mGluR1 signaling. Furthermore, the ARB, L-158,809, and the ACEI, ramipril, reverse these radiation-induced changes in Homer1a expression and its signaling in both the cortex and hippocampus. Moreover, both L-158,809 and ramipril prevent hippocampal-independent cognitive impairment, but neither restores both hippocampal neurogenesis and prevents hippocampal neuroinflammation. This new
information expands our model of radiation-induced cognitive impairment to now include Homer1a as a contributor to altered neuronal function in both the cortex and hippocampus (Figure 1). Consequently, radiation-induced brain injury, including cognitive impairment, is not just a hippocampus story anymore.
References


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76. Wong ML, Smith MA, Licinio J, Doi SQ, Weiss SR, Post RM, et al., Differential effects of kindled and electrically induced seizures on a glutamate receptor (GluR1) gene expression. Epilepsy research 1993; 14, 221-7.


# Elizabeth D. Moore

## EDUCATION

<table>
<thead>
<tr>
<th>Period</th>
<th>Institution</th>
<th>Program</th>
<th>Concentration</th>
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<tr>
<td>08-2008 - 10-2013</td>
<td><strong>Wake Forest University</strong>, School of Medicine, Winston-Salem, NC</td>
<td><em>Doctor of Philosophy in Cancer Biology</em></td>
<td>Radiation Oncology</td>
<td>The use of therapeutics (blockers of the renin-angiotensin system) to prevent damage to the normal brain (cognitive deficits) caused by radiation therapy administered to treat brain tumors.</td>
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<tr>
<td>08-2011 - 10-2013</td>
<td><strong>Wake Forest University</strong>, Schools of Business, Winston-Salem, NC</td>
<td><em>Masters in Business Administration</em></td>
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<tr>
<td>08-2003 - 05-2008</td>
<td><strong>University of North Carolina</strong>, School of Allied Health Sciences, Chapel Hill, NC</td>
<td><em>Bachelor of Science in Clinical Laboratory Science</em></td>
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## PROFESSIONAL EXPERIENCE

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<tr>
<td>05-2013 – Current</td>
<td>Sr. Analyst, Business Development</td>
<td>Merz Pharmaceuticals LLC, Greensboro, NC</td>
<td>L ead a new team project identifying novel technologies developed at the University level in medical and aesthetic dermatology</td>
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<td>F acilitated working relationships and contracts with US Universities for research and development purposes</td>
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<td>S elected potential technologies to be added to Merz’s MedDerm portfolio (to be completed in August 2013)</td>
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<td>G enerated Wolters Kluwer reports for project development and planning</td>
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<td>A ttended project management meetings and participated in analyzing new contracts and acquisitions</td>
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<tr>
<td>07-2011 – Current</td>
<td>Chair and former Vice-Chair, Scholars-in-Training Committee</td>
<td>Radiation Research Society</td>
<td>I dentified and recruited over 35 different influential radiation scientists to speak at a workshops for scholars in training (SITs) in the radiation sciences (i.e. medical students, graduate students, residents and postdoctoral trainees) at the national Radiation Research Society (RRS) meeting in New Orleans, LA (Sept 2013), RRS meeting in San Juan, Puerto Rico (September 2012) and Intercontinental Congress of Radiation Research meeting in Warsaw, Poland (August 2011)</td>
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<td>C reated networking events for SITs including conference socials and mentors luncheons as well as facilitated social networking via online job sharing on the RRS SIT website, and Facebook</td>
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<td>A ccrued sources of financial support to fund the SIT workshops, mentor’s luncheons and travel awards for Scholars In Training (SIT) ~ $100,000/yr (in 2012 and 2013)</td>
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<td>G enerated monthly updates of new opportunities within radiation sciences and recognized SIT achievements</td>
</tr>
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11-2012 – 02-2013  Case Writer, Wake Forest University Biotechnology Case Competition, Winston-Salem, NC

- Formulated and designed a business school case with Boston Scientific involving undertaking of a women’s health business unit into China (currently at $500 million in revenue, looking to grow to $1 billion in the next few years)
- Case was used for competition where 10 national student teams proposed how (i.e. Joint venture, greenfield), where (i.e. hospital size, location) and with what medical devices Boston Scientific should enter into China
- Evaluated proposals and presentations from each competing team – winning team won $10,000
- First student author (non-faculty author) to write a case for the WFU Biotechnology Case Competition
- Invited by both Wake Forest University and Boston Scientific to write next year’s case or to lead the Wake Forest Competition team

09-2012 – 02-2013  Consultant and Co-Author, Feasibility Study for Heavy Ion Metal Accelerator, Houston, TX

- Proposed the creation of the first clinically accessible Heavy Ion Metal Accelerator to be built in the US ~ estimated initial investment is over $60 million
- Analyzed the current US market for radiation therapy including current technology available in the US [location and type (gamma knife, protons)] therapy usage rate (radiation type and cancer treated), research currently being done with heavy ion radiation (clinical and preclinical; nationally and internationally)
- Developed business model, including risk assessment (internal and external), for use of the accelerator
- Established a working model for financial support (clinical- and research-based use) of the accelerator
- Presented proposal to a senior board member of the hospital – proposal was later taken to the entire hospital board and well received
- Requested to generate a similar feasibility study for the implementation of a Heavy Ion Metal Accelerator for another facility
- Onsite consultation with Dr. Atsushi Kitagawa, Head of the Promotion of Carbon Therapy at the Heavy Ion Metal Accelerator in Chiba, Japan in May 2013 (first clinical Heavy Ion Metal Accelerator worldwide)

SCIENTIFIC MANUSCRIPTS
Moore EL, Kooshki M, Metheny-Barlow L, Gallagher PE, Robbins ME. Angiotensin-(1-7) prevents radiation-induced inflammation in rat primary astrocytes through regulation of MAP kinase/DUSP1 signaling. *Free Radical Biology and Medicine.* (submitted June 2013)

Moore EL, Kooshki M, Metheny-Barlow L, Robbins ME. Fractionated Whole brain irradiation modulates homer1a expression and glutamate 1 receptors in a brain region specific manner. *Radiation Research.* (submitted July 2013)

**AWARDS AND HONORS**

Radiation Research Scholar in Training Travel Award 2013, $800
American Association for Cancer Research Scholar in Training Travel Award, 2013, $1,500
Wake Forest University Scholarship for PhD/MBA Program, 2012-2013, $17,500
Radiation Research Scholar in Training Travel Award 2012, $885
Wake Forest University Scholarship for PhD/MBA Program, 2011-2012, $17,500
Radiation Research Scholar in Training Travel Award 2011, $700
Graduate School Stipend 2008-Current, $25,000 annually

**OTHER SKILLS**

*Competitive Swimmer:* Recent accomplishments - 5th place, 1 mile open water championship, 09-2012; 3rd place, 1 mile open water 2012; 1st place Butterfly, 2nd place Freestyle, Frank Clark Swim Meet 2011

*Senior Swim Instructor/Coach:* Currently the lead swim instructor at the Jerry Long Family YMCA (since 2009); teach all levels ranging from toddler to adult; train new instructors in how to teach lessons (private lessons for individual swim practice and group sessions for general procedures)