EFFECTS OF AT₁R ANTAGONIST L-158,809 ON NORMAL BRAIN TISSUE INJURY FOLLOWING WHOLE-BRAIN IRRADIATION

BY

KELLY R. CONNER

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Approved By:

David R. Riddle, Ph.D., Advisor

Examinining Committee:

Patricia E. Gallagher, Ph.D., Chairwoman

Michael E. Robbins, Ph.D.

Judy K. Brunso-Bechtold, Ph.D.

Ronald W. Oppenheim, Ph.D.
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Ang   angiotensin
Ang (1-7) angiotensin (1-7)
ACE   angiotensin converting enzyme
ACE2 angiotensin converting enzyme 2
ACEi angiotensin converting enzyme inhibitor
AT1R angiotensin II type 1 receptor
AT1RA angiotensin II type 1 receptor antagonist
AT2R angiotensin II type 2 receptor
AGT angiotensinogen
BBB blood brain barrier
BP blood pressure
BrdU 5-Bromo-2’-deoxyuridine
BN Brown Norway
CA Cornu Ammonis
CBF cerebral blood flow
CNS central nervous system
DG dentate gyrus
DCX doublecortin
ECM extra cellular matrix
fi fimbria
F344 Fischer 344
GPCR G-protein coupled receptor
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GCL</td>
<td>granule cell layer</td>
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<tr>
<td>Gy</td>
<td>Gray</td>
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<td>h</td>
<td>hour</td>
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<tr>
<td>IF</td>
<td>immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>IL-1β</td>
<td>interleukin one beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin six</td>
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<tr>
<td>Iba1</td>
<td>ionizing Ca²⁺ binding adaptor protein one</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>LTP</td>
<td>long term potentiation</td>
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<tr>
<td>MHC II</td>
<td>major histocompatibility complex two</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MWM</td>
<td>Morris Water Maze</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
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<tr>
<td>NPC</td>
<td>neural precursor cell</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PrC</td>
<td>perirhinal cortex</td>
</tr>
<tr>
<td>PCL</td>
<td>pyramidal cell layer</td>
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<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
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<td>RECA</td>
<td>rat endothelial cell antigen</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>ROI</td>
<td>region of interest</td>
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<tr>
<td>RAS</td>
<td>renin angiotensin system</td>
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<tr>
<td>SR</td>
<td>stratum radiatum</td>
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<tr>
<td>SO</td>
<td>stratum oriens</td>
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<tr>
<td>SGZ</td>
<td>subgranular zone</td>
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<td>SVZ</td>
<td>subventricular zone</td>
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<td>TIMPs</td>
<td>tissue inhibitors of metalloproteinases</td>
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<td>TGF-β</td>
<td>transforming growth factor <em>beta</em></td>
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<td>TNF-α</td>
<td>tumor necrosis factor <em>alpha</em></td>
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<td>VEP</td>
<td>visually evoked potential</td>
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<td>whole brain irradiation</td>
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Primary and metastatic brain cancers are commonly treated with partial or whole-brain irradiation (WBI). Twenty to fifty percent of patients that receive WBI develop progressive cognitive dysfunction caused by injury to irradiated normal brain tissue. The cellular and molecular mechanisms underlying radiation-induced normal brain tissue injury have not been fully elucidated. Neuroinflammation, in the form of microglial activation, and decreased hippocampal neurogenesis frequently occur in animal models before and concurrent with radiation related cognitive deficits. Pharmacological interventions that reduce neuroinflammation and/or promote neurogenesis may ameliorate WBI-induced cognitive deficits. Blockade of the renin-angiotensin system (RAS) is an attractive target since: (1) RAS blockade ameliorates radiation-induced injury to normal lung, kidney, and optic nerve tissue, (2) angiotensin II (Ang II) can act as a pro-inflammatory cytokine via its Ang II type 1 receptor (AT₁R), (3) the brain has its own intrinsic RAS, and (4) AT₁R blockade, by oral administration of the AT₁R antagonist L-158,809, ameliorated impairment in the novel object recognition task after fractionated WBI.

The work in this dissertation tested the hypothesis that blocking the intrinsic brain RAS with L-158,809 before, during, and after WBI would prevent radiation-induced neurobiological changes. The first study investigated whether L-158,809 treatment
altered WBI-induced changes in hippocampal neuroinflammation and neurogenesis following a single dose of 10 Gy WBI. We found that L-158,809 treatment increased Ang II type 2 receptor expression in irradiated rats but had minimal, if any, effect on other analyzed markers of neuroinflammation and neurogenesis. Previous studies indicate that tissue responses to a single dose of radiation differ from responses to a fractionated dose; therefore, the second study evaluated whether L-158,809 treatment mediates its cognitive benefits via modulation of microglial population dynamics and/or the promotion of hippocampal proliferation and neurogenesis following a 40 Gy fractionated WBI dose. Our findings suggest the cognitive benefits of L-158,809 treatment before, during, and after fractionated WBI are not mediated through changes in the investigated indicators of cellular dynamics. Although cell population sizes in irradiated rats were not greatly altered by L-158,809 treatment, microglial function and the contribution of immature neurons to cognition may differ between these conditions.
CHAPTER I

INTRODUCTION

Approximately 220,000 patients are diagnosed with primary or metastatic central nervous system (CNS) tumors each year (1,2). Radiotherapy, usually in the form of large field or whole-brain irradiation (WBI), is a front-line treatment for primary and metastatic brain tumors. WBI is also used as a prophylactic measure to prevent highly metastatic cancers from spreading to the brain (1,3-6). WBI has proven efficacy and, as radiation dose is increased, survival and cancer regression are improved. The dose that can safely be administered, however, is limited by concomitant damage to irradiated normal tissues, which can produce functional deficits that manifest several months to years after radiotherapy. Unfortunately, 20–50% of long term cancer survivors, defined as those surviving five years and more post-diagnosis, that receive WBI suffer progressive cognitive deficits that are thought to develop from damage to normal tissue (7-9). Neuropsychological effects of WBI include impairments in processing speed, attention, spatial information processing, working memory, executive function, and other forms of learning and memory. Learning and memory processes associated with hippocampal function are typically affected (10-12). Radiation-induced cognitive deficits can occur in the absence of gross histological changes to normal brain tissue or cancer recurrence (13-16). The cellular and molecular mechanisms underlying these cognitive deficits have not been fully explained nor are there currently any approved preventative measures or successful treatments for the cognitive dysfunction resulting from radiation-induced brain injury (17).
Blockade of the renin-angiotensin system (RAS) effectively prevents radiation damage to normal lung, kidney, and optic nerve tissue and has been demonstrated to prevent WBI-induced cognitive dysfunction (18–21). The experiments conducted for this dissertation add to current knowledge of in vivo cellular responses to WBI both with and without concomitant RAS blockade using the angiotensin II (Ang II) type 1 receptor (AT1R) antagonist L-158,809.

Section I: WBI - background, neurogenesis, and inflammation

WBI

Gamma radiation causes cell death either directly through irreparable DNA strand breaks or indirectly through cell damage from free radicals and other inflammatory mediators. The majority of radiation-induced cell death of non-cancerous brain cells is the result of these indirect effects (22). Non-proliferating, differentiated normal tissue is more resistant than highly proliferative cancer cells to direct DNA damage from radiation, but indirect radiation effects on normal cell function are thought to cause the cognitive deficits that become apparent months to years after radiotherapy (22).

The standard fractionation regimen for treatment of CNS tumors consists of 2 Gy daily fractions six times per week for six weeks for a total dose of 66–68 Gy. Total dose per fraction and overall treatment time have been associated with increased risk of normal tissue necrosis in the treatment of tumors (23,24). Current prophylactic radiotherapy fractionation regimens usually consist of a total of 35 gray (Gy, the radiation unit used clinically) delivered in 2.5 Gy fractions over 14 treatment days, but differences
in dose, timing, and fractionation do not significantly alter median patient survival time (6,25). Such prophylactic fractionation schemes have reduced the occurrence of brain necrosis, which is rarely seen below 60 Gy, and is within the range of clinically effective total doses, 20–80 Gy. The use of individual fractions in all regimens between 2–3 Gy reduces the risk of neurotoxicity that can manifest long after the completion of radiotherapy (6,9,14,23,26). Cognitive dysfunction, however, is still a significant risk even with modern fractionated WBI (27).

Three phases of WBI-induced normal tissue injury

WBI-induced normal tissue injury is classified into three phases (acute, early delayed, and late delayed) based on when it manifests in relation to radiotherapy. Patients may or may not exhibit all three phases. Also, the risk of progressive cognitive deficits associated with late delayed effects cannot be predicted by the presence or absence of acute and/or early delayed effects.

**Acute:** Acute effects occur during or within a few weeks of treatment and include transient worsening of neurological deficits, drowsiness, headache, and/or nausea. Acute effects are attributed to cerebral edema, are commonly and effectively treated with corticosteroids, and usually spontaneously reverse.

**Early Delayed:** Early delayed effects, also referred to as subacute or consequential, usually develop one to six months after radiotherapy and are symptomatically similar to acute effects. They are the result of transient, diffuse demyelination and/or temporary changes in blood-brain barrier (BBB) permeability. Like acute effects, they spontaneously reverse.
Late Delayed: Late delayed effects can manifest from six months to years after WBI. Unlike acute and early delayed effects, late delayed effects are usually permanent and progressive. Late delayed histological changes can include vascular abnormalities and white matter necrosis. The progressive cognitive deficits associated with late delayed effects negatively impact quality of life. As cancer treatments are improved, more cancer patients survive to present with late delayed cognitive effects, thus, increasing the need for effective preventative interventions and/or treatments to improve cognition function (14,16,17,25,28,29,30).

Classical vs. current theories of radiation injury

Traditionally, radiation-induced injury was thought to result directly from the loss of proliferating cells because of irreparable DNA damage. The long delay before the onset of dysfunction was attributed to a long replication time for the proliferating cells, specifically oligodendrocyte and vascular progenitors, in the tissue(s) of interest. Recently, a paradigm shift has occurred with respect to the mechanisms of radiation-induced brain injury. Radiation damage involves complex interactions between several cell types, including endothelial cells, astrocytes, oligodendrocytes, microglia, and neurons (14,17,31). Numerous animal studies have revealed that these dynamic interactions produce microenvironmental changes that may contribute to WBI-induced cognitive deficits, including decreased proliferation and neurogenesis in the subgranular zone (SGZ) of the hippocampus and chronic neuroinflammation (15,32-38).
Neurogenesis

Adult neurogenesis occurs in two specialized regions of the brain, the subventricular zone (SVZ), where neurogenesis serves as a source of new neurons to the olfactory bulb, and the SGZ of the hippocampus (40,41). These regions have certain features permissive to adult neurogenesis. For instance, hippocampal astrocytes, but not spinal chord astrocytes, can induce proliferating cells to differentiate into neurons in vitro; neural precursor cells (NPCs) are in close proximity to astrocytes in vivo (42). Also, NPCs are found in tight clusters within 5–6 µm of microvessels. This close association between NPCs and the microvasculature, referred to as the neurogenic niche, is critical for maintaining neurogenesis (43-45).

Neurogenesis consists of a multi-step process in which NPCs undergo expansion and differentiation. In the hippocampus, surviving daughter cells directed toward a neuronal fate begin to express markers associated with immature neurons, e.g. doublecortin (DCX), and migrate from the SGZ into the granule cell layer (GCL). They send axons into the mossy fiber tract to CA3 and dendrites to the molecular layer of the dentate gyrus (DG) and are selected for survival by successfully incorporating into the existing network (32,42). Neurogenesis is thought to contribute to hippocampal-dependent learning and memory by increasing the number of connections and the processing capacity of the network. New neurons have greater synaptic plasticity and a lower threshold for long-term potentiation (LTP), a cellular model of long term memory, than fully differentiated mature neurons, characteristics conducive to increasing network flexibility (46).
Many conditions can affect neurogenesis. Physical exercise increases NPC proliferation, whereas learning and memory tasks promote the survival and integration of immature neurons into the existing network. Environmental enrichment and certain insults, e.g. ischemia or seizures, also increase neurogenesis (33,43,46,47). Circumstances that reduce neurogenesis include stress and glucocorticoids, lipopolysaccharide (LPS) administration, and radiation (43,45,48-50).

WBI, neurogenesis, and cognition

The WBI-induced reduction in neurogenesis correlates with decreased performance in hippocampal-dependent cognitive tasks. A number of animal studies have been conducted to determine the effects of a single dose of WBI on learning and memory tasks and the cellular mechanisms behind these deficits. Rats receiving either a 20 or 25 Gy single dose of WBI were impaired on both the T-maze (35 weeks) and Morris Water Maze (MWM, 46 weeks) even in the absence of white matter necrosis (51). Two studies from Joseph Fike’s group demonstrated cognitive deficits in young adult mice three months after a single dose of five or 10 Gy WBI that were associated with reduced proliferation and neurogenesis (35,36). Snyder et al. (2005) reported that a single 10 Gy dose in adult rats produced deficits in the MWM six and eight weeks after irradiation and these deficits were also accompanied by a significant reduction in the neurogenesis (52).

Although several studies, including a number from our group at Wake Forest University School of Medicine, have demonstrated detrimental effects of more clinically relevant, fractionated WBI on cognition in animal models (13,21,53-55), only one has concurrently measured indicators of proliferation (BrdU labeling) and neurogenesis
(BrdU/NeuN labeling). In young, male Wistar rats, 24 Gy given as eight 3 Gy fractions reduced proliferation, but did not impair performance on the novel object recognition (NOR) task one or three weeks after WBI. Irradiated rats did show transient impairment in the hippocampal-dependent T-maze: they were impaired one and three weeks after WBI, but were not impaired at seven weeks (56). Considering the link between neurogenesis and cognition, further investigation is needed to determine if the prolonged suppression of hippocampal proliferation and neurogenesis demonstrated in single dose studies is maintained and whether it contributes to cognitive dysfunction after fractionated doses similar to those used in the clinic.

**WBI Effects on Proliferation and Neurogenesis**

Proliferation and the production of newborn neurons are significantly reduced by WBI in both the SGZ of the hippocampus and the SVZ of the lateral ventricles (57-62). Acute reductions in proliferating cells, hours to days, are attributed to the apoptosis of cells undergoing mitosis at the time of irradiation. In rodents, WBI-induced apoptosis peaks between 6–12 h and is complete by 24–48 h following doses as low as 0.4 Gy (15,32,39,63,64).

Interestingly, some studies using different proliferation markers have reported a spike in proliferation back to or beyond control levels one week after a low to moderate (2 Gy to 23.7 Gy) single dose of WBI, which is hypothesized to represent the system attempting to return to equilibrium (39,58,65,66). This attempt at recovery is not sustained and proliferation is continually suppressed for at least 3 months in rats (15,34-36,39). One study in mice, however, reported similar levels of proliferation, as indicated
by Ki67 labeling, between irradiated animals and sham-irradiated controls at both one week and one month after a 4 Gy dose. They concluded proliferation recovered completely after WBI (66).

WBI not only reduces proliferation, but it also affects cell fate. Neurogenesis is particularly sensitive to irradiation while gliogenesis is preserved (17,34,36,52). The sensitivity of neurogenesis to radiation is attributed to microenvironmental changes. Monje et al. (2002) reported that non-irradiated NPCs transplanted into an irradiated hippocampus differentiated into glial cells, but irradiated NPCs can differentiate into neurons in vitro suggesting changes in the irradiated parenchyma alters the fate of newborn cells. This study also illustrated a disruption in the neurogenic niche following a single 10 Gy dose of WBI. The disruption in the association between proliferating cells and the microvasculature raises the question of whether radiation damage to the vasculature causes the radiation-induced decrease in neurogenesis. A more recent study from Fike’s group demonstrated that it is radiation to the parenchyma and not the microvasculature that results in the greatest reduction in neurogenesis (67). Fike’s group has also reported that cognitive training and environmental enrichment, two conditions that increase neurogenesis in normal animals, also increase neurogenesis in irradiated animals; however, neurogenesis is not returned to sham-irradiated levels by these interventions (33,36).

Inflammation reduces neurogenesis, and WBI-induced increases in activated microglia occurs concomitant with reductions in neurogenesis (15,17,34,36,68). Monje et al. (2003) reported that treatment with indomethacin, a non-steroidal anti-inflammatory drug (NSAID), reduced the number of activated microglia and increased the number of
cells adopting a neuronal phenotype two months after 10 Gy WBI. These findings suggest that reducing WBI-induced inflammation may rescue neurogenesis after WBI.

**Inflammation**

There are two facets of the tissue response to any injury: inflammation and recovery. Inflammation can be further divided into overlapping acute and chronic phases. Acute inflammation includes the stereotypical inflammatory response common to all injuries in which immune cells are attracted to the site of injury, release pro-inflammatory mediators, and phagocytosis debris or pathogens. Chronic inflammation occurs when the acute response is not brought to a close either due to repeated injury or a self-perpetuating response (69), and has been associated with the WBI-induced late delayed effects in humans (70,71). Increased inflammation, demarcated by an increase in activated microglia, cytokine production, and ROS production, has also been extensively reported in animal models of WBI, see (32) for review.

The recovery stage of the general injury response can have two outcomes: repair or replacement. In repair, damaged cells are replaced with the same cell type, which results in a greater level of functional recovery. Replacement involves just that, replacement with some other cell type, usually resulting in scar formation and fibrosis; function is usually impaired (69). Replacement typically leads to problems after whole body irradiation when fibrosis of the lung and kidney result in severe functional deficits, reviewed by (72).
**Microglia**

The brain is considered to be immuno-privileged because the BBB prevents immune cells in the blood, comprising the adaptive arm of the immune system, from freely infiltrating the brain. Under normal conditions, the brain’s response to insults is limited to innate immune responses. Microglia, discovered in 1932, are the innate immune cells of the brain and constitute up to 20% of glial cells, approximately 10% of cells in the brain (73-75). The original view of microglia was as phagocytes that simply removed pathogens and/or cell debris after an injury and as a source of inflammatory mediators. Recently, however, this paradigm has shifted. Now, microglia are recognized to play a role in several pathologies, such as Alzheimer’s Disease (76,77,78) and normal biological processes ranging from development to aging (73,79-81).

Microglia have distinct morphologies associated with their level of activation and function. The default morphology for microglia in a healthy, young brain consists of cells with small somas and abundantly ramified process. These cells are referred to as “resting” microglia. They are homogeneously distributed with cell to cell distances of about 50–60 µm (82). Their highly motile processes are constantly surveying the surrounding tissue in which they contact and interact with nearby neurons, astrocytes, and blood vessels (82). Therefore, “surveying” microglia may be a more accurate term than “resting” (83). Resting microglia do not phagocytose but do constitutively express certain markers, e.g. CD11b (complement 3 receptor)(84) and ionized calcium-binding adaptor protein-1 (Iba1) (85). Unlike neurons that are fully differentiated and do not divide, the resting microglial population is constantly, albeit slowly, turned over mostly through proliferation of resident microglia. Infiltrating blood born monocytes can transmigrated
across the intact BBB and become microglia, but their contribution to the microglia population is minimal in the absence of injury (86,87).

Microglia become activated in response to injury and/or infection, and are a primary source of inflammatory mediators in the brain, e.g. cytokines, ROS, and reactive nitrogen species (88,89). Three stages of activation have been described for microglia \textit{in vitro}: 1) neurotrophic only, 2) mixed, in which both neurotoxic and neurotrophic molecules are produced and secreted, and 3) neurotoxic only. The neurotrophic only stage corresponds to the default stage in normal tissue and the mixed stage occurs in response to insult, e.g. LPS. The neurotoxic stage, however, has not been demonstrated \textit{in vivo} (90).

The morphology of activated microglial is characterized by shortened processes and enlarged somas. Upon activation, microglia up-regulate expression of activation markers, such as CD68, a lysosomal glycoprotein associated with their role as phagocytes, and major histocompatibility complex II (MHC II) involved in antigen presentation. A hallmark of the microglial response to injury is a massive expansion of the microglial population that peaks within days of the injury and slowly returns to normal through apoptosis over the ensuing weeks and months (91-94).

Innate immunity is not as well controlled as adaptive immunity; therefore, the substances secreted by activated microglia can inadvertently have deleterious effects on surrounding neurons (48). These harmful effects have been correlated with decreased neurogenesis and alterations in the ability of neurons to process information (95) indicating that they may contribute to WBI-induced cognitive deficits.
Inflammation and microglial responses to injury, however, are not always detrimental but can be beneficial under certain circumstances. Microglia can secrete neurotrophic factors that promote neuronal survival and regeneration in several injury models including ischemia, excitotoxicity, and nerve lesions (77,90). To further complicate issues, cytokines classically held to be pro-inflammatory; such as TNF-α and IL-6, can be neuroprotective depending on the microenvironmental conditions (89,96). Also, many cytokines signal through multiple receptors, often with opposing actions (for example, TNFR1 and TNFR2 for TNF-α or AT1R and AT2R for Ang II, see below). The final outcome of inflammation may, therefore, depend on the balance between pro- and anti-inflammatory responses (17,89,93,97). Consequently, instead of preventing or stopping inflammation all together, driving the WBI-induced microglial response towards a neurotrophic/neuroprotective phenotype may be the best course of action.

*Inflammation and WBI*

As stated above, radiation causes an inflammatory response in normal tissue that is associated with decreased neurogenesis. Cytokines, such as TNF-α, IL-1β, IL-6, TGF-β, are also part of the WBI-induced inflammatory response, and may play an important role in normal tissue injury. In fact, pretreatment with TNF-α and IL-1 protects mice from a lethal dose of whole body irradiation, while IL-6 pretreatment increases radiosensitivity (22). Most studies of radiation-induced cytokine expression, however, were very acute and showed transient peaks in expression within hours of irradiation followed by a return to normal levels within days (70,98-102). Unfortunately, most of these studies used semi-quantitative measures to assess gene expression. It remains to be
determined if alleviating WBI-induced cytokine production and microglial activation will ameliorate radiotherapy associated cognitive dysfunction.

Section II: RAS

RAS: an overview

The systemic RAS is a hormonal system involved in numerous processes. It mediates fluid tolerance through regulating drinking and thirst, is implicated in blood pressure (BP) maintenance, and regulates sexual behavior by controlling the secretion of sex hormones from the pituitary gland (103,104). Ang II, an eight amino acid peptide, is the most extensively studied biologically active peptide of the RAS. Ang II is the primary mediator of the aforementioned actions of the systemic RAS (105).

The systemic RAS consists of a cascade beginning with the pro-protein angiotensinogen (AGT). AGT is produced by the liver and released into the circulation where it is cleaved by renin, released from the kidneys, to the 10 amino acid peptide Ang I. Both AGT and Ang I are inactive, but are the source of several biologically active peptides produced by subsequent cleavage (Figure 1). Ang I is cleaved to Ang II via angiotensin-converting enzyme (ACE) bound to vascular endothelial cell membranes (106-108). Several serine proteases can convert Ang I to Ang II independently from ACE activity, a route for Ang II production that is unaffected by ACE inhibitors (ACEi) commonly used to treat high BP (109). In addition to binding to its two receptors (see below), Ang II can be metabolized by numerous peptidases to shorter active peptides (e.g. Ang III, Ang IV, and Ang (1 – 7)) or inactive peptides (103,110,111).
Figure 1. Systemic RAS Cascade. Pro-proteins and peptides are listed within ovals. Cleavage enzymes are listed beside arrows. Receptors and as yet unidentified receptors are in pentagons.
AGT → Ang I → Ang II → AT1R → AT2R → Ang III → peptidases → AT3RI/AT1R

AGT → Ang I → Ang II → ACE2 → Ang (1-7) → Mas

ACE & Serine proteases
Ang II binds to two high affinity receptors, the AT₁ and angiotensin II type 2 (AT₂) receptors. Both Ang II receptors are seven-transmembrane G-protein coupled receptors (GPCR). The AT₁R mediates most of the classical effects of Ang II. AT₁R activation leads to vasoconstriction, the release of pro-inflammatory cytokines, and proliferation (110,112-114). It is the most prominently expressed Ang II receptor in adult mammals with high expression levels in a several tissues including certain areas of the brain, such as the circumventricular organs. In contrast, the AT₂R is highly expressed in the fetus with expression in most regions declining after birth, but high expression is maintained in the adult in select tissues and certain areas of the brain, e.g. the amygdala (103,104,110,111,115,116); the AT₂R is up-regulated after injury (117,118). The AT₂R mediates several actions in opposition to those regulated by the AT₁R including vasodilatation, neuronal differentiation and regeneration, cerebroprotection under hypoxic conditions, and, in certain circumstances, apoptosis (104,111,116,119-122).

Besides the systemic RAS, there exist several independently regulated tissue specific RAS in the kidney, lung, heart, peripheral blood vessels, adrenal glands, and brain (107,123). Recent studies indicate that, in addition to its regulation of BP and water balance, the tissue specific RAS play a significant role in radiation-induced injury to normal tissue (18).

**RAS blockade and radiation-induced tissue injury**

**RAS blockade and radiation nephropathy and lung fibrosis and pneumopathy**

In 1986, Robbins and Hopewell demonstrated that treatment with an ACEi decreased the early effects of radiation on the kidneys (124). ACE, however, cleaves
more than just Ang I and Ang II can be created by non-ACE pathways (109); therefore, it is possible that the beneficial effects of ACEi treatment were not mediated by RAS blockade. Several studies by Moulder’s group have addressed this issue. In the kidney, the AT₁R antagonist (AT₁RA) L-158,809 was superior to the ACEi captopril in mitigating radiation injury to the kidney. AT₁R antagonism and ACE inhibition, however, produce similar results if treatment is not started until after the kidney is dysfunctional. ACE inhibition and AT₁R antagonism may, therefore, employ different mechanisms during prophylaxis and treatment of tissue dysfunction (125).

The lungs also contain a tissue specific RAS. Ward et al. (1988 and 1992) showed that ACE inhibition prevents lung fibrosis following irradiation, reviewed by (31). Moulder’s group later showed that the AT₁RA L-158,809, similar to results in the kidney, was superior to ACEi treatment in preventing radiation-induced lung fibrosis and pneumopathy (20). RAS blockade following irradiation, therefore, is beneficial to damaged normal tissue in at least two different organs suggesting that other organs possessing a tissue specific RAS may be amenable to RAS blockade as well.

RAS blockade can be discontinued for the treatment of radiation nephropathy 26 weeks after a single 10 Gy dose of total body irradiation to mice, but must be continuous in order to prevent the development of pneumopathy and lung fibrosis (126). The differential effects of RAS blockade in the kidney and lung suggest that the normal tissue damage from radiation can differ between organs and/or tissue specific RAS may play different roles in radiation-induced injury in individual organs. In contrast to the demonstrated benefits of manipulating the RAS for preventing radiation-induced injury to
the kidneys and lungs, the role of the RAS in radiation-induced brain injury is only beginning to be explored.

The brain/CNS RAS

A CNS specific RAS was discovered independently by two groups in 1988, Mendelsohn et al. and Unger et al. (127). In the brain, AGT is produced primarily in astrocytes while renin is localized to both neurons and glia. Brain ACE is on most cell membranes, but can be released as a soluble enzyme as well. Ang II is found in synaptic vesicles; both of its receptors can be found on neurons in several brain regions including the hippocampus (Figure 2). Ang II can modulate pre- and post-synaptic activity in several neurotransmitter systems, regulate cerebral blood flow (CBF), is involved in BBB maintenance, and can influence cognition, including hippocampal LTP (104,123,128-134).

Neither neurons nor astrocytes contain a complete RAS system; therefore, intercellular interactions are necessary for functional outcomes (129,135,136). Microglia, however, do possess a complete RAS, which is up-regulated upon microglial activation, providing another link between the brain RAS and inflammation (137). Also, several neurological diseases and stroke activate the brain RAS, and RAS blockade has beneficial effects in these instances (121,138-149). AT$_1$R antagonism, and subsequent AT$_2$R up-regulation and activation, is also associated with anti-depressive and anxiolytic effects and improved outcome after stroke (104,142,150-152).
Figure 2: Brain RAS cascade. Pro-proteins and peptides are listed within ovals with locations in parentheses. Cleavage enzymes listed beside arrows. Receptors and possible receptors are in pentagons.
AGT (astrocytes)

Renin (glia & neurons)

Ang I

ACE (glia & neurons, especially synaptosomes and synaptic vessels)

Ang II (synaptic vessels)

ACE2 (glia & neurons?)

peptidases

Ang (1-7)

Mas (neurons)

AT4R/IRAP? (neurons)

Ang IV

AT3R?/AT1R (astrocytes & neurons)

AT1R (astrocytes & neurons)

AT2R (neurons & astrocytes)
Considering the role of Ang II in inflammation, neuronal population dynamics, and cognition, RAS blockade is an attractive target for the treatment of radiation-induced brain injury. Kim and colleagues (2004) conducted the first study of the effects of blocking the CNS RAS in the treatment of radiation injury. In a well-characterized rat model of optic neuropathy, using a single 30 Gy dose to the exposed optic nerve, the ACEi ramipril ameliorated radiation effects on mean peak latencies of visually evoked potentials (VEP) in 75% of treated animals six months after irradiation. Treated animals also exhibited insignificant morphological changes in their optic nerves, e.g. slight bulging of the optic nerve at the chiasm (19).

There remain, however, a few important issues to be addressed before treatment strategies targeting the brain RAS are taken from the bench to the neuro-oncology clinic. The first of which is whether or not treatment with a RAS blocker will provide radioprotection to the tumor. AT1R expression is up-regulated in a number of cancers. RAS blockade with an ACEi or AT1RA inhibits tumor growth, decreases tumor angiogenesis, and decreases expression of several pro-inflammatory cytokines in a colon cancer rat model, cultured prostate cancer cells, and cultured rat C6 glioma cells (153-156). Treatment with an AT1RA leads to more circulating Ang II, which in turn leads to higher concentrations of smaller, yet biologically active peptides like Ang (1-7), an inhibitor of cultured human lung cancer cell proliferation (157). In the brain, however, the higher concentration of systemically circulating Ang II is unlikely to effect local Ang II concentrations since it cannot cross the BBB. AT1RAs that do cross the BBB may, however, increase local Ang II levels, which could increase the local production of active
Ang II metabolites. The evidence suggests that RAS blockade in cancer patients should not hinder the efficacy of radiation therapy and may, in fact, promote tumor removal.

Second, the timing of RAS blockade initiation in relation to radiotherapy must be determined. The renal nephropathy studies indicate that ACEi and AT₁RA treatment could be postponed up to three weeks after completion of radiotherapy without reducing the benefits of treatment (126,158). Ramipril treatment started two weeks after optic nerve radiosurgery significantly improved VEPs and reduced morphological changes (19,159). Since RAS blockers are anti-tumorogenic it may, however, be best to begin RAS blockade before WBI.

The final issue relates to how long RAS blockade must be continued after irradiation. Again, Moulder’s group (1998 and 2002) found that treatment in rats is only necessary for weeks three through 10 following whole body irradiation to effectively prevent nephropathy. In the lung, however, discontinuing treatment resulted in an almost immediate increase in fibrosis, signifying that it may be necessary to continue ACEi treatment for the life of the animal or patient (126,160,158). A recent study by our group at Wake Forest University, showed that L-158,809 treatment beginning three days before the start of a 40 Gy fractionated regimen of WBI and continued for five weeks following the last 5 Gy fraction ameliorated WBI-induced cognitive deficits in the novel object recognition task (21). This finding suggests that blocking the brain RAS may only be necessary during a critical period, similar to that in the irradiated kidney. The cellular and molecular mechanisms by which L-158,809 mediates its cognitive benefits, however, are unknown.
Section III: Summary

The brain RAS is an increasingly attractive target for the treatment of radiation-induced brain injury for a number of reasons. (1) RAS blockers are well tolerated by patients. (2) RAS blockers alleviate radiation damage in other organs, e.g. the lung, kidney, and optic nerve. (3) The brain RAS influences cognitive function and RAS blockade prevents or ameliorates cognitive dysfunction after WBI (21,134,161). (4) Inflammation creates an environment permissive to cancer development and growth, is a component of the normal tissue response following WBI (154), and the AT$_1$R promotes inflammation through the production of pro-inflammatory cytokines and ROS (137,162). (5) WBI-induced inflammation is correlated with decreased hippocampal neurogenesis, which is associated with hippocampal-dependent cognitive dysfunction. The balance between AT$_1$R and AT$_2$R activation could alter proliferation and neurogenesis in the hippocampus. Beneficial neuropsychological effects of L-158,809 treatment may, therefore, be mediated by reducing inflammation and/or rescuing neurogenesis.

The experiments conducted for this dissertation tested the hypothesis that blockade of the brain RAS with the AT$_1$RA L-158,809 before, during, and after WBI would decrease the radiation-induced microglial response and rescue neurogenesis. We blocked the brain RAS with an AT$_1$RA instead of an ACEi since: (1) Ang II can be formed through ACE-independent pathways (109), (2) AT$_1$RAs are as effective as ACEis in preventing radiation damage to the kidney and lung (72), and (3) ACE can cleave non-RAS related biologically active peptides, e.g. bradykinin, lutenizing hormone-releasing hormone, opioid peptides, etc. (109). The AT$_1$RA L-158,809 was chosen over other
available AT$_1$RAs because it: (1) has approximately 20 times higher affinity for the AT$_1$R than losartan, the classical AT$_1$RA (163), (2) attenuates radiation-induced damage in the kidney and lung (31,126,160,164), and (3) is known to alleviate WBI-induced cognitive deficits following a 40 Gy fractionated dose of WBI (21).

A two-pronged approach was adopted so that the effects of L-158,809 treatment could be examined after both a single dose and a fractionated dose of WBI. Chapter II outlines experiments using a single 10 Gy dose in young, male Fischer 344 (F344) rats because multiple fractions could obscure more acute tissue responses to radiation injury if neurobiological changes critical to the development of cognitive dysfunctions began during the course of fractionated WBI. Also, the majority of the current literature on normal tissue damage following WBI used single doses of WBI. Fractionated WBI, however, is used for clinical WBI. Therefore, findings from single dose studies concerning the cellular and molecular mechanisms underlying normal tissue damage, and any promising intervention, should be substantiated in a more clinically relevant model before progressing to clinical testing. Chapter III discusses experiments using behaviorally characterized F344 x Brown Norway (BN) rats to determine the effects of L-158,809 treatment on normal tissue responses following a 40 Gy fractionated dose of WBI. The experiments in this dissertation contribute to the current knowledge of in vivo cellular responses to both single dose and fractionated WBI, and examine possible mechanisms of action of an appealing intervention, L-158,809 treatment, for the prevention of normal tissue damage that culminates in cognitive dysfunction following WBI.
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CHAPTER II

EFFECTS OF THE AT₁ RECEPTOR ANTAGONIST L-158,809 ON RADIATION-
INDUCED NEUROINFLAMMATION AND NEUROGENESIS

Kelly R. Conner, M. Elizabeth Forbes, Won H. Lee, Yong W. Lee,
and David R. Riddle

The following manuscript is in preparation for publishing in Journal of Neuroscience Research and is reprinted with permission from authors. Stylistic variations are due to the requirements of the journal. M.E. Forbes assisted with animal care and euthanasia. W.H. Lee performed the qRT-PCR. K. Conner collected all other data, performed data analyses, and prepared the manuscript. Dr. D. Riddle acted in an advisory and editorial capacity.
ABSTRACT

Whole-brain irradiation (WBI), a frontline therapy for primary brain tumors and metastases, produces progressive cognitive deficits in 20–50% of patients that survive for six months or more. The cellular and molecular mechanisms underlying these deficits remain poorly understood, but radiation clearly increases neuroinflammation and reduces cell proliferation and neurogenesis, changes that have been associated with the cognitive dysfunction that manifests after WBI. Among other effects, blockers of the renin-angiotensin system (RAS) have anti-inflammatory properties and have been demonstrated to reduce radiation injury in the lung, kidney, and optic nerve. The present study investigated whether treatment with the angiotensin II type 1 receptor antagonist (AT₁RA) L-158,809 before, during, and after a single 10 Gy dose of WBI to young male F344 rats reduced neuroinflammation and/or rescued neurogenesis. Expression of cytokines, angiotensin II receptors (AT₁R and AT₂R) and angiotensin-converting enzyme 2 (ACE2) were evaluated by real-time PCR 24 hours and one and 12 weeks after WBI. At the latter time points total microglial number and the numbers and percentages of proliferating and activated microglia were analyzed in the granule cell layer and hilus of the hippocampus, and microglial proliferation, non-microglial proliferation, and neurogenesis were quantified in the hippocampal subgranular zone. Our results suggest that L-158,809 treatment increases Ang II receptor expression (one week) and TNF-α expression (12 weeks) but has minimal effects on WBI-induced changes in microglial population dynamics and neurogenesis in the hippocampus.
INTRODUCTION

Each year over 220,000 patients are diagnosed with primary brain and CNS cancers or brain metastases (Eichler and Loeffler, 2007; Jemal et al., 2008). These tumors commonly are treated with large-field or whole-brain irradiation (WBI) (Choy and Milas, 2003; Silasi et al., 2004), which is very effective in killing cancer cells. Unfortunately, 20–50% of patients that undergo WBI present months to years later with radiotherapy-associated progressive cognitive deficits that decrease their quality of life (Imperato et al., 1990; Crossen et al., 1994; Johannesen et al., 2003). The cellular and molecular mechanisms underlying these cognitive deficits are not fully understood and currently there are neither prophylactic measures nor effective therapies.

Chronic neuroinflammation occurs following WBI and may contribute to WBI-induced cognitive dysfunction (Tofilon and Fike, 2000; Rola et al., 2004). Activated microglia (indicated, for example, by increased expression of the late endosomal protein CD68) can alter neuronal function by producing cytokines and/or trophic factors and by modulating synaptic plasticity (Kempermann and Neumann, 2003). Neuroinflammation also alters the neuronal microenvironment and decreases neurogenesis in regions of the adult brain (Monje et al., 2003; Ehninger and Kempermann, 2008; Rosi et al., 2008) and is correlated with WBI-induced hippocampal-dependent cognitive dysfunction (Abayomi, 1996; Armstrong et al., 2004; Shaw and Robbins, 2006). Pharmacological interventions that modulate inflammation and/or protect or promote neurogenesis may, therefore, ameliorate WBI-induced cognitive dysfunction.

An attractive therapeutic target for WBI-induced brain injury is pharmacological blockade of the renin-angiotensin system (RAS). Although the systemic RAS is
classically viewed as a hormonal system involved in blood pressure and fluid balance, elements of the RAS have other effects and (Gard, 2002; Wright et al., 2002), several organs, including the brain, have an intrinsic RAS that functions independently from the systemic RAS (Carey and Siragy, 2003; Gendron et al., 2003). Angiotensin II (Ang II) is the best characterized biologically active RAS peptide and, via the angiotensin II type 1 (AT₁R) and type 2 (AT₂R) receptors, contributes to inflammatory responses and neuronal function in the brain (Culman et al., 2001; Culman et al., 2002; de Cavanagh et al., 2004). Therefore, RAS blockade may ameliorate WBI-induced neuroinflammation and/or restore neurogenesis.

The focus of the current study was modulation of radiation-induced neurobiological changes by RAS inhibition. In previous studies treatment with angiotensin-converting enzyme inhibitors (ACEi) or AT₁R antagonist (AT₁RA) ameliorated radiation damage to the lung and kidney (Molteni et al., 2000; Moulder et al., 2003; Moulder and Cohen, 2007) and the ACEi ramipril ameliorated radiation damage in the optic nerve (Kim et al., 2004). An AT₁RA was selected for this experiment since ACE can cleave non-RAS related biologically active peptides, such as bradykinin and opioid peptides (Igic and Behnia, 2003), making it difficult to interpret effects of ACE inhibition. L-158,809 was chosen over other AT₁RAs because it: i) has 20 times higher affinity for the AT₁R than losartan, the classical AT₁RA (Chang et al., 1992), ii) attenuates radiation-induced damage in the kidney and lung (Moulder et al., 1998a; Moulder et al., 1998b; Molteni et al., 2000; Cohen et al., 2007), and iii) ameliorates radiation-induced cognitive dysfunction after fractionated WBI (Robbins et al., 2009).
This study assessed whether blocking the brain RAS with the AT₁RA L-158,809 ameliorates the increase in neuroinflammation and decrease in neurogenesis that follows a 10 Gy dose of WBI. Hippocampal cytokine and Ang II receptor expression and the number of microglia and the percentages of proliferating or activated microglia in the dentate gyrus (DG) granule cell layer (GCL)/hilus were assessed as indicators of neuroinflammation. In addition, effects of WBI and L-158,809 treatment on the densities of proliferating cells and immature neurons in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus were quantified.

**MATERIALS AND METHODS**

**Animals**

Sixty male Fischer 344 rats were purchased from Harlan Sprague Dawley, Inc., (Indianapolis, IN). Rats were housed individually on a 12:12 h light: dark cycle with *ad libitum* access to food and water and were acclimated for three weeks prior to irradiation at twelve weeks of age. The animal facility at Wake Forest University School of Medicine is accredited by the American Association for Accreditation of Laboratory Animal Care and complies with Public Health Service-National Institutes of Health and institutional policies and standards for laboratory animal care. All procedures were approved by the Institutional Animal Care and Use Committee.

**Treatment and Radiation Procedures**

L-158,809 treatment (20 mg/L in the drinking water, Merck & Co., Inc., Rahway, NJ) began 5 days before WBI and continued throughout the course of the study. Fresh
drinking water with or without L-158,809 was provided every other day; animals were weighed weekly and the volume of water consumed was recorded and used to monitor drug dosage over the course of the experiment (averaging 2 mg/kg/day). Animals were randomly assigned to one of four conditions: 1) sham-irradiated (Sham), 2) WBI, 3) sham-irradiated plus L-158,809 (Sham + AT₁RA), and 4) WBI plus L-158,809 (WBI + AT₁RA). Rats were anesthetized using a ketaset/xylazine mixture (80/4 mg/kg body weight; Butler Animal Health Supply, Dublin, OH) and were irradiated in a ¹³⁷ Cs irradiator with collimating devices for delivery to the whole brain and lead shielding to protect the eyes and body. Sham rats were anesthetized but not irradiated. Irradiated rats received 10 Gy at an average dose of 3.85 Gy/min. To ensure equal midline radiation exposure, half the dose (5 Gy) was delivered to each side of the head. We tested effects of a single 10 Gy dose of WBI, rather than a fractionated dose, since most data concerning WBI-induced neurobiological changes is from single dose studies and since, in the weeks following fractionated irradiation, it is difficult to differentiate short term effects of a recent fraction from longer term effects of earlier fractions.

Rats from each of the four conditions were divided into three survival groups (n = 4–8/group) sacrificed at 24 hours, one week, and 12 weeks post-irradiation/anesthesia. These endpoints were chosen based on a previous study of WBI in young rodents that demonstrated an early decrease in proliferation within the SGZ (24 hours) followed by a transient increase (one week) in proliferation and then a sustained decrease in proliferation and neurogenesis (12 weeks) (Tada et al., 2000). An increase in the number of activated microglia in the DG GCL/hilus (12 weeks) has also been reported after WBI (Tada et al., 2000; Monje et al., 2003; Mizumatsu et al., 2003).
Tissue Processing

Animals were deeply anesthetized with sodium pentobarbital (150 mg/kg body weight, Ovation Pharmaceuticals, Inc., Deerfield, IL) and decapitated. Brains were rapidly extracted and hemisected at the midline. The hippocampus was dissected from the right hemisphere, quickly frozen in liquid nitrogen and stored at -80°C until processed for qRT-PCR. The left hemisphere was fixed by immersion in 0.1M phosphate-buffered 4% paraformaldehyde (pH 7.4) for 48 h before being cryoprotected in sucrose and embedded in Tissue Freezing Medium (TFM, Triangle Biomedical Sciences, Inc., Durham, NC). Serial coronal sections through the entire hippocampal formation (bregma –1.8 to –6.9) (Paxinos and Watson, 1998) cut at 40µm (one week) or 60µm (12 week) on a cryostat were collected in an antifreeze solution (1:1:2 ethylene glycol, glycerol, and 0.1 M phosphate-buffer, pH 7.4) and stored at -20°C until processed for immunohistochemistry (IHC) or immunofluorescence (IF).

Real-time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Quantitative RT-PCR using fluorogenic 5’nuclease assay technology with TaqMan® probes and primers (Applied Biosystems, Foster City, CA) was conducted for gene expression analyses. Briefly, total RNA was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s protocol. One microgram of total RNA was reverse transcribed at 25 °C for 15 min, 42 °C for 45 min, and 99 °C for 5 min in 20 µl of 10 mM Tris-HCl, pH 9.0, containing 5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 1 unit/µl of recombinant RNasin, 15 units/µg of AMV reverse transcriptase, and 0.5 µg of random hexamers. Amplification of individual genes was performed on an Applied Biosystems 7300 Real-Time PCR System using TaqMan®
Universal PCR Master Mix and a standard thermal cycler protocol (50 °C for 2 min before the first cycle, 95 °C for 15 sec and 60 °C for 1 min, repeated 45 times). TaqMan® Gene Expression Assays (amplification efficiencies of 100%) for rat tumor necrosis factor alpha (TNF-α, Assay ID: Rn99999017_m1, amplicon length: 108 nucleotides), interleukin 1 beta (IL-1β, Assay ID: Rn999990099_m1, amplicon length: 100), interleukin 6 (IL-6, Assay ID: Rn99999011_m1, amplicon length: 90), tumor growth factor beta (TGF-β1, Assay ID: Rn00821748_g1, amplicon length: 71 nucleotides), AT_1aR (Assay ID: Rn01435427_m1, amplicon length: 99), AT_2R (Assay ID: Rn00560677_s1, amplicon length: 71), and GAPDH (TaqMan® Rodent GAPDH Control reagent, catalogue number 4308313), as an endogenous control, were used. The threshold cycle (C_T), which indicates the fractional cycle number at which the amount of amplified target gene reaches a fixed threshold, was determined from each well using the Applied Biosystems Sequence Detection Software v1.2.3 and relative quantification was calculated by the comparative C_T method as described previously.(Livak and Schmittgen, 2001; Deng et al., 2003; Lee et al., 2004)

**Immunohistochemistry and Immunofluorescence**

Systematically random 1-in-12 series of sections through the entire anterior-to-posterior extent of the DG for each animal were selected for immunolabeling. Sections from equal numbers of rats representing the four treatment groups were processed and analyzed in cohorts. Material from each sacrifice group (24 hours, one week and 12 weeks after WBI) was processed and evaluated independently.
The antifreeze solution was washed from sections using 0.1M tris-buffered saline, pH 7.5 (TBS); then endogenous peroxidase activity was reduced by incubation for 30 minutes in 0.6% hydrogen peroxide (H₂O₂) in TBS. Sections were incubated for one h at room temperature in TBS containing 5% normal serum and 0.3% Triton X-100 prior to incubation overnight with primary antibody in the same solution at 4°C. Primary antibodies were rabbit monoclonal anti-Ki67 (clone SP6; a marker for proliferating cells (Kee et al., 2002), AbCam, Cambridge, MA, 1:200), rabbit polyclonal anti-ionized calcium-binding adaptor molecule-1 (Iba1; labels all macrophages/microglial, (Ito et al., 1998), Wako, Richmond, VA, 0.083 µg/ml), and mouse monoclonal anti-CD68 (clone ED1, a lysosomal label of phagocytic macrophages/microglia (Damoiseaux et al., 1994), AbD Serotec, Raleigh, NC, 2.5 µg/ml). For sections immunolabeled with Ki67, antigen retrieval was performed by incubation in 10 mM sodium citrate buffer, pH 6, at 90°C for 10 minutes prior to the H₂O₂ incubation (described above). Primary antibodies were detected with biotinylated secondary antibodies (1:300) and visualized using peroxidase-conjugated avidin-biotin complex (ABC Elite kit) with Vector SG (Ki67), diaminobenzidine (Iba1), or nickel enhanced diaminobenzidine (ED1) as peroxidase substrates (Vector Laboratories, Inc., Burlingame, CA). Ki67 and Iba1 were labeled in the same series of sections; labeling and visualization of Ki67 was performed as described above and then the sections were treated with 1% H₂O₂ in TBS for 30 minutes (room temperature) prior to labeling of Iba1. All sections were counterstained for 20 minutes with 100µM of the nucleic acid stain Sytox Green (Invitrogen-Molecular Probes, Carlsbad, CA) to visualize cell layers in the hippocampal formation and facilitate contour drawing for stereological and density measurements (described below). Sections were
mounted from TBS onto superfrost Plus slides (Fisher Scientific, Hampton, NH), blotted of residual buffer, air dried 30 minutes, dehydrated using a graded series of ethanol, cleared in xylene, and coverslipped using Cytoseal 60 permanent mounting medium (VWR International, Inc., West Chester, PA).

Labeling of neuroblasts and immature neurons in the dorso-medial DG was evaluated using goat polyclonal anti-doublecortin antibody (DCX (Brown et al., 2003), Santa Cruz, Santa Cruz, CA, 1 µg/ml). Sections were washed with TBS, incubated 1 h in TBS containing 5% normal donkey serum and 0.3% Triton X-100 and then incubated overnight at 4°C with addition of primary antibody. DCX was visualized using Cy-5 conjugated, highly cross-adsorbed donkey anti-goat secondary antibody (Jackson ImmunoResearch, West Grove, PA, 7.5 µg/ml). Sections were stained with Sytox Green as described above; mounted from TBS onto superfrost Plus slides and blotted of residual buffer and coverslipped using Biomedia Gel Mount (Fisher Scientific, Hampton, NH). Slides were sealed using Cytoseal 60 to prevent dehydration and stored in the dark at 4°C to prevent photobleaching.

**Qualitative Analysis**

All analyses were performed on cohorts sampling equally across experimental groups. The experimenter was blinded to experimental condition using coded slides. Stereological estimates of the total number of microglia (Iba1+ cells) and numbers of proliferating (Ki67+/Iba1+) and activated (ED1+) microglia in the DG GCL/hilus were obtained using the StereoInvestigator system (MBF Bioscience, Williston, VT) on an Olympus BX51 microscope. A 10x objective was used to draw contours defining the
hippocampal regions of interest (ROI) and counting of IHC-labelled cells was performed with a planapochromatic 60x oil-immersion objective (NA 1.42). The DG GCL/hilus ROI was defined as the area encompassing the GCL and including the hilus. In more anterior sections in which the dorsal and ventral blades of the DG GCL did not meet a line connecting the two blades was drawn to create a closed contour. Stereological parameters were as follows: 100 µm X 100 µm counting frame, 225 µm X 225 µm sampling grid size, 3 µm guard zone, and 10 µm disector height (one week) or 15 µm disector height (12 weeks). Average coefficient of error was 0.047 (Schmitz and Hof, 2000).

The low density and non-uniform distribution of all other IHC processed markers did not lend themselves to analysis using StereoInvestigator; therefore, they were exhaustively counted within the DG GCL/hilus (Iba1+/Ki67+ cells and ED1+ cells) or the SGZ (Iba1+/Ki67+ cells Iba1+/Ki67+ cells). These analyses were carried out, as previously described (Lichtenwalner et al., 2001; Schindler et al., 2008), using a modification of the optical disector (Gundersen et al., 1999) on the NeuroLucida system (MBF Bioscience, Williston, VT). Counts within the DG GCL/hilus are expressed as the estimated total number of positive cells within the entire left hemisphere DG GCL/hilus. Percentages of proliferating and activated microglia were calculated from their estimated total numbers compared to the total number of all microglia. The SGZ was defined as the region extending 25 µm on either side of the border between the GCL and hilus. Counts of proliferating cells within the SGZ were expressed as densities (cells per millimeter of SGZ examined).
DCX\(^+\) cells typically occurred in clusters and were difficult to analyze in thick sections using widefield microscopy. DCX\(^+\) cells, therefore, were counted using a Leica TCS SP2 confocal microscope with a 63x oil-immersion objective (NA 1.4, Leica Microsystems, Brannockburn, IL) moving field-by-field along the extent of the SGZ. Z-stacks (1 \(\mu\)m steps) through the depth of the section in approximately 25 fields per section were required to perform density measurements. Preliminary investigations established that the relative densities of DCX\(^+\) cells (comparing individuals or groups) were not different whether a single section or a series of several sections was analyzed for each animal; one section per animal provided a reliable estimate of the density of DCX-labeled cells in the SGZ. Thus, an equivalent coronal section from each animal was used to estimate the production of newborn neurons in the dorso-medial DG. The density was expressed as the number of DCX\(^+\) cells per millimeter of SGZ. For both Ki67 and DCX immunolabeled cells within the SGZ, all cells were counted except those in the top focal plane of each section to avoid overestimation.

Statistical Analysis

Data are given as the mean ± standard error of the mean (SEM). All data were analyzed using Sigmastat 3.0 (SYSTAT Software, San Jose, CA). Effects of irradiation status (Sham vs. WBI), drug status (water vs. L-158,809), and interactions were evaluated using two-way ANOVAs and Holm-Sidak post hoc test with a significance threshold of \(p \leq 0.05\). Large irradiation effects on several variables resulted in non-normal distributions and/or unequal variance in the data, violating the assumptions of the ANOVA. Since this raised the probability of type 1 errors, in those cases we first used
post hoc Student t-tests to test for effects of drug treatment within sham-irradiated or irradiated groups, adjusting the $p$ value for multiple comparisons. If the data were not normally distributed even within the irradiated or sham groups, a post hoc Mann-Whitney Rank Sum test was performed. Only significant $p$ values for interactions between irradiation and L-158,809 treatment and post hoc t-tests and Mann-Whitney Rank Sum test are reported.

RESULTS

Effects of WBI and AT$_1$R Antagonism on Cytokines and Elements of the Brain RAS

Many neural injury models, including radiation, show increases in the expression of pro-inflammatory cytokines (Chiang et al., 1997; Kim et al., 2002; Gaber et al., 2003). The pro-inflammatory actions of Ang II are produced via activation of the AT$_1$R (for review see (Dagenais and Jamali, 2005)); therefore, we tested whether treatment with the AT$_1$RA L-158,809 would affect expression levels of cytokines known to be influenced by Ang II. We also evaluated expression of the two Ang II receptors and ACE2 in order to further understand the effects of AT$_1$RA and WBI on the local brain RAS. Quantitative RT-PCR was used to evaluate mRNA expression at 24 hours, one week and 12 weeks after a single 10 Gy dose of WBI (Table I).

Cytokine expression. By 24 hours after WBI TNF-α expression was reduced 25-30%; the extent of the decrease was not affected by drug treatment. TNF-α levels in the irradiated rats returned to (WBI + L-158,809) or slightly exceeded (WBI only) the level in sham rats at one and twelve weeks post-irradiation. In addition, at twelve weeks a small but statistically significant increase in TNF-α expression was evident in drug-
Table 1. Gene expression following WBI and AT1RA. Values represent mean fold induction (range) normalized to values for GAPDH, endogenous control, and relative to Sham+H2O. *Represents values significantly different from Sham+H2O ($p < 0.05$), **from Sham+AT1RA ($p < 0.05$), or † from WBI+H2O ($p < 0.05$), †† interaction ($p = 0.03$), (n = 3-5).
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treated rats without WBI. IL-6 expression was decreased by WBI approximately 60% at one week post-irradiation, regardless of drug treatment, but was not affected by WBI or drug treatment at 24 hours or twelve weeks. Expression levels of IL-1 and TGF-β were not affected by WBI or drug treatment at any time point.

*Ang II receptors and ACE2.* Levels of mRNA for the AT₁R and the AT₂R were only minimally affected by WBI and treatment with L-158,809. At one week post-irradiation small drug-induced increases in AT₁R and AT₂R expression were apparent in sham and irradiated animals, respectively. ACE2 expression was not affected by WBI or drug treatment at any time point.

**Effects of WBI and AT₁R Antagonism on the Microglial Response in the DG GCL/hilus**

Iba1, ionized calcium-binding adaptor molecule 1, is located in the cytosol of all microglia and infiltrating monocytes regardless of activation state (Ito et al., 1998) (for convenience Iba1⁺ cells will be referred to here as microglia). Microglia were homogeneously distributed in the hippocampus, as shown in Fig. 1 (A and B) and no gross histological damage was evident in sections from irradiated animals. Iba1-labeled cells typically had a ramified morphology indicative of “resting” microglia (right inset Fig. 1B); this was true even for those expressing the proliferation marker Ki67 (Ki67⁺/Iba1⁺ cells, left inset Fig. 1B).

The ED1 antibody labels CD68, a lysosomal/endosomal associated membrane glycoprotein involved in phagocytosis and widely used as a marker of activated microglia and macrophages (Damoiseaux et al., 1994). As for Iba1 labeling, we did not attempt to
Fig. 1. Markers of microglia, proliferating cells, and immature neurons in male F344 rats. 

**A** and **B** illustrate Ki67 labeling of proliferating cells (gray) and Iba1 labeling of microglia (brown) in sham (**A**) and irradiated (**B**) animals at 1 week after WBI; typical examples of labeled cells, including Ki67⁺/Iba1⁺ proliferating microglia, are shown at higher magnification in insets. **C** and **D** demonstrate ED1 labeling in sham (**C**) and irradiated (**D**) rats at 12 weeks after WBI. Labeled cells are shown at higher magnification in insets. In **E** and **F** an immature neuron is evident in the SGZ of a sham animal at the 1 week time point, demonstrated by DCX labeling (red) alone (**E**) and in a merged image (**F**) showing the distribution of all nuclei stained with Sytox green (green). Scale bars = 50μm, 25μm (inset).
distinguish between activated microglia and infiltrating macrophages and ED1+ cells are referred to as activated microglia for convenience. Representative ED1 labeling can be seen in Fig. 1 (C and D). The lysosomal ED1 labeling often appeared as individual or small groups of puncta near the nucleus of labeled.

We hypothesized that AT1R antagonism mediated beneficial effects on WBI-induced normal brain tissue injury by reducing neuroinflammation; therefore, we compared the number of all microglia (all Iba1+) and the number of proliferating (Ki67+/Iba1+) or activated (ED1+) microglia in the DG GCL/hilus in animals from each experimental condition. WBI reduced the total number of microglia (all Iba1+ cells) by approximately 25% within the DG GCL/hilus one week after WBI. At 12 weeks after WBI, however, there was no difference in total microglial number between experimental conditions (Fig. 2A). WBI, regardless of drug treatment, greatly increased both proliferating (Fig. 2B) and activated (Fig. 2C) microglia at one week and 12 weeks, although the WBI-induced increase was much smaller at 12 weeks than at one week.

To better understand the dynamics of the microglial population we further analyzed the percentage of proliferating (Fig. 3A) and activated (Fig. 3B) microglia in the DG GCL/hilus. One week after WBI, the percentage of proliferating microglia was increased approximately 98% in irradiated animals compared to their sham controls; proliferating microglia appeared to be decreased (~19%) in irradiated animals treated with L-158,809 compared to those without drug, but the reduction was not statistically significant. Microglia proliferation remained elevated (~75%) in irradiated rats compared to sham controls at 12 weeks post-irradiation (regardless of drug treatment), albeit to a smaller extent than at one week.
Fig. 2. Quantitative analysis of microglial numbers in the GCL/hilus. WBI significantly reduced the number of Iba1+ microglia in the GCL/hilus one week after WBI but not at 12 weeks (A). WBI increased the number of proliferating microglia (C, Ki67+/Iba1+ cells) and activated microglia (D, ED1+ cells) at both one week and 12 weeks post-irradiation *p ≤ 0.001 versus sham control; n = 5 animals/experimental group.
Fig. 3. Quantitative analysis of the proportion of proliferating or activated microglia in the GCL/hilus. WBI increased the percentage of proliferating microglia (A) and activated microglia (B) at both one week and 12 weeks post-irradiation. Activated microglia were too rare in sham animals at one week to allow stereological quantification. \( p^* \leq 0.05 \quad **< 0.001 \) versus sham control; \( n = 5 \) animals/experimental group.
At one week after WBI too few ED1 labeled cells were apparent in sham-irradiated animals to permit stereological analysis, so the percentage of activated microglia was calculated only for irradiated animals. L-158,809 treatment had no effect on the approximately 45-48% of microglia in irradiated animals that were CD68⁺. Activated microglia were analyzed in all four experimental conditions at 12 weeks after WBI, at which point the number was sufficient in sham rats to permit quantitation. Microglial activation remained elevated in irradiated animals (~ 60% greater than shams), although the magnitude to the irradiation response was noticeably lower than at one week after WBI. At 12 weeks as at one week, L-158,809 treatment had no influence on the percentage of activated microglia.

Effects of WBI and AT₁R Antagonism on the Proliferation and Neurogenesis in the SGZ

Proliferating cells typically appear in clusters in the SGZ, as illustrated in Fig. 1 (A and B). The density of all Ki67⁺ cells within the SGZ was calculated as a measure of total proliferation. The densities of Ki67⁺/Iba1⁻ cells (non-microglial proliferating cells) and Ki67⁺/Iba1⁺ cells (proliferating microglia) were analyzed in order to determine the contribution of proliferating microglia to the WBI-induced proliferative response (Fig. 4).

One week after WBI, total proliferation was statistically equivalent across all experimental conditions (Sham: 771.2 ± 56.72, WBI: 682.72 ± 53.77, Sham + AT₁RA: 636.65 ± 31.18; WBI + AT₁RA: 648.72 ± 56.56 Ki67⁺ cells/mm). When the proliferating population was divided into proliferating non-microglia and proliferating microglia, however, effects of WBI and L-158,809 treatment were apparent. WBI reduced the
Fig. 4. Effects of WBI and AT₁R antagonism on proliferation in the SGZ. At one week after treatment WBI decreased the density of proliferating non-microglial cells but increased the density of proliferating microglia, such that there was no net difference in the total proliferating population across the experimental groups. L-158,809 treatment also decreased proliferating non-microglia in the sham animals at one week. Twelve weeks after treatment, the overall density of proliferating cells was lower in irradiated rats; the density of proliferating, non-microglial cells was decreased while the density of proliferating microglia remained elevated, albeit to a smaller extent than at one week. L-158,809 treatment decreased proliferating microglia in irradiated rats at 12 weeks. *p < 0.05 vs. sham controls, #p < 0.05 vs. sham without L-158,809 treatment, ##p < 0.05 vs. irradiated rats without L-158,809 treatment; n = 5 animals/experimental group; far left bar = Sham, second bar = Sham + AT₁RA, third bar = WBI, far right bar = WBI + AT₁RA.
density of proliferating non-microglia in both irradiated groups compared to their sham controls (~ 31%). L-158,809 treatment also significantly reduced proliferating non-microglia (~ 18%) but only in sham animals. WBI significantly increased the density of proliferating microglia one week after WBI (~ 98% greater than sham animals).

Twelve weeks following WBI, radiation decreased the total population of Ki67+ cells by approximately 45%. A WBI-associated reduction in non-microglia proliferation was apparent at 12 weeks in both irradiated groups compared to their sham controls (~ 48%), whereas an increase in the density of proliferating microglia remained apparent in irradiated rats (~ 82% greater in WBI compared to sham animals). At this time point, L-158,809 treatment further decreased proliferating microglia in irradiated rats (~ 39% lower than irradiated rats without drug treatment).

The density of immature neurons (DCX+ cells) in the SGZ was analyzed since neuroinflammation can detrimentally affect neurogenesis (Fig. 1, E and F). At one and 12 weeks after WBI, irradiation resulted in a robust reduction in the density of immature neurons (~ 95% at one week, irradiated animals compared to sham animals; ~ 56% at 12 weeks, Fig. 5). L-158,809 treated, irradiated rats had approximately 52% fewer DCX+ cells/mm than water-treated, irradiated rats one week after WBI, but this drug-induced difference was not apparent at the 12 week time point.

**DISCUSSION**

RAS blockade has anti-inflammatory effects, ameliorates radiation-induced inflammation in the lung and kidney (Cohen et al., 2002; Molteni et al., 2007), and can modulate proliferation and neurogenesis (Hahn et al., 1994; Moriyama et al., 1995; Datta
Fig. 5 Effects of WBI and AT₁R antagonism on neurogenesis. WBI reduced the density of DCX⁺ cells at both time points; L-158,809 treatment further reduced the density of immature neurons in irradiated rats but only at one week. *p < 0.001 vs. sham controls (by Mann-Whitney at one week and Student’s t-test at twelve weeks), #p <0.05 vs. irradiated rats without L-158,809 treatment (by Student’s t-test at one week); n = 5 animals/experimental group.
et al., 1999; Gendron et al., 2003; Suzuki et al., 2003; Ruiz-Ortega et al., 2007). Therefore, we hypothesized that L-158,809 treatment would alleviate WBI-induced inflammation and/or rescue neurogenesis, two neurobiological changes implicated in WBI-induced cognitive dysfunction (Monje et al., 2003; Mizumatsu et al., 2003; Raber et al., 2004; Rola et al., 2004; Rosi et al., 2008).

Cytokine Expression following WBI and AT₁R Antagonism

Previous experiments demonstrated that WBI increases cytokine expression several fold within two to four hours and that the expression of most cytokines returns to normal levels by 24 hours post-irradiation (Hong et al., 1995; Chiang et al., 1997; Kyrkanides et al., 1999). The pattern of expression after WBI is similar to that seen in response to other nervous system injuries (e.g., sciatic nerve lesions, middle cerebral artery occlusion, and lipopolysaccharide (LPS) administration (Buttini et al., 1996; Streit et al., 1998; Gregersen et al., 2000; Silasi et al., 2004; Tanaka et al., 2006). Given evidence that at least TNF-α undergoes a second elevation in the months after a single dose of WBI (25 Gy, (Chiang et al., 1997)), we tested whether TNF-α or the other cytokines showed sustained elevations in the weeks after a 10 Gy dose. Overall, WBI- and AT₁RA-induced changes in cytokine expression were modest and the biological significance of the small (less than one fold) changes remains to be established. Nevertheless, the present data demonstrate that TNF-α expression is moderately suppressed at one day after 10 Gy WBI (presumably following a robust increase several hours earlier) and then increases in subsequent weeks, as described previously for a 25 Gy dose. The elevation was significant only in rats that were not treated with L-158,809,
suggesting some ability of the AT<sub>1</sub>RA to reduce the radiation-induced increase in the cytokine. Increased expression of TNF-\(\alpha\) at one week post-irradiation was accompanied by decreased expression of IL-6, perhaps due to a feedback interaction between the two cytokines that have been demonstrated previously (Feghali and Wright, 1997). Although these analyses of samples representing the entire hippocampus revealed only modest modulation of cytokine levels by WBI and RAS inhibition, it should be recognized that greater regulation may occur at the levels of individual cells or cell types.

**Ang II Receptor Expression following WBI and AT<sub>1</sub>R Antagonism**

AT<sub>1</sub>R antagonism raises plasma levels of Ang II (Culman et al., 2002; Robbins et al., 2009) and increases the availability of Ang II to bind to AT<sub>2</sub>Rs. Since WBI also may alter the activity of the brain RAS it was important in the present study to assess Ang II receptor levels to determine whether changes, particularly in AT<sub>2</sub>R expression may contribute to WBI-induced changes and improved cognitive function in irradiated rats treated with the drug (Robbins et al., 2009). L-158,809 treatment transiently increased AT<sub>1a</sub>R expression in sham-irradiated rats, consistent with up-regulation in the face of reduced ligand-binding (Wolf and Ritz, 2005). AT<sub>2</sub>R expression was altered only with the combination of WBI and L-158,809 treatment. AT<sub>2</sub>R expression is up-regulated following a number of neural injuries and serves a neuroprotective and neuroregenerative function by promoting neuronal survival and migration and modulating neuronal excitability (Makino et al., 1996; Unger, 1999; Gendron et al., 2003; Kagiyama et al., 2003). Increased availability of Ang II to the AT<sub>2</sub>R when AT<sub>1</sub>R is blocked also may have contributed to the increased expression of the former since Ang II binding, somewhat
paradoxically, up-regulates AT₂R expression (Shibata et al., 1997). Overall, however, the biological significance of the small changes in expression of AT₁R and AT₂R mRNA is unclear, particularly since Western blot analysis of the AT₁R did not reveal changes at the protein level (antibodies suitable for analysis of the AT₂R were not available).

**Microglial Responses to WBI and AT₁R Antagonism**

WBI clearly alters microglial population dynamics in the hippocampus. One week post-WBI, many microglia were proliferating and/or activated in irradiated rats, even though the total number of microglia in the GCL/hilus was significantly reduced (see also (Schindler et al., 2008)). Increased microglial proliferation concurrent with a decrease in total population size indicates that there is substantial turnover in the microglial population in the weeks following WBI (Ladeby et al., 2005). WBI-induced microglial proliferation and activation remained elevated at 12 weeks but the response was not as robust as at the one week time point, suggesting a gradual normalization of the microglial population, at least with respect to markers typically assessed in studies of radiation-induced neural changes.

Although L-158,809 treatment did not affect proliferation or activated microglia in the DG GCL/hilus in irradiated animals, the contributions of microglia, especially proliferating and/or activated microglia, to the surrounding microenvironment may not necessarily be equivalent between irradiated animals with and without L-158,809 treatment. Microglia can release trophic factors that can increase neuronal survival after injury (Nakajima and Kohsaka, 2004; Streit et al., 2005). Therefore, microglia in L-158,809 treated, irradiated rats may not be pro-inflammatory, but instead may be a source
of anti-inflammatory signals and/or trophic factors for surrounding neurons, contributing to an attempt at repair. Additional studies will be required to further characterize the WBI-induced microglial response with and without L-158,809 treatment.

**Proliferation and Neurogenesis in Response to WBI and AT₁R Antagonism**

WBI-induced suppression of SGZ proliferation and neurogenesis is well documented (Sakagawa et al., 2000; Monje et al., 2002; Madsen et al., 2003; Raber et al., 2004; Rola et al., 2004) and has been correlated with deficits in hippocampal-dependent cognitive tasks (see (Monje and Palmer, 2003) for review). Since Ang II receptors can influence cell proliferation and neuronal differentiation and migration (Culman et al., 2002; Gendron et al., 2003), the effects of AT₁R antagonism were investigated to determine if this pharmacological intervention would restore the WBI-induced deficits in SGZ proliferation and neurogenesis. As in previous studies (Tada et al., 2000; Schindler et al., 2008), WBI had no effect on the total number of proliferating cells in the SGZ at one week after WBI, but analysis of the phenotype of those cells revealed increased proliferation of microglia and decreased proliferation of non-microglial cells. The increased proliferation of microglia at one week post-WBI was similar to the microglial expansion seen in other injury models (Streit et al., 1999; Ladeby et al., 2005). Microglial proliferation was greatly reduced at 12 weeks after WBI and non-microglial proliferation remained suppressed, resulting in an overall decrease in proliferation similar to that reported previously (Tada et al., 2000). L-158,809 treatment had only small effects on proliferation in the SGZ. The AT₁RA significantly reduced non-proliferating microglia in sham-irradiated rats and reduced proliferating microglia in irradiated rats 12 weeks after
WBI, consistent with a reduction in proliferative and pro-inflammatory actions of the AT1RA.

In addition to effects on proliferating cells, WBI greatly reduced the density of newborn (DCX+) neurons. In contrast to some previous reports, in the present study neurogenesis in irradiated rats appeared to gradually increase with time, demonstrating some capacity for recovery. L-158,809 treatment exacerbated the WBI-induced decrease in DCX labeling at one week post-irradiation. Although effects on the size of the DCX+ population could arise secondary to effects on the proliferation of progenitors, the demonstrated ability of the AT2R to promote cell differentiation (Unger, 1999; Rosenstiel et al., 2002; Kaschina and Unger, 2003) raises the intriguing possibility that increased AT2R activity and decreased AT1R activity in the presence of AT1R antagonists accelerated the development and integration of newborn neurons in the DG. Such effects could contribute to the amelioration of WBI-induced cognitive deficits by L-158,809 (Robbins et al., 2009), as might effects of Ang II receptors on synaptic transmission (Wright and Harding, 1994; Armstrong et al., 1996; Blume et al., 1999; Culman et al., 2002; Pan, 2004).

**Drug efficacy**

The limited ability of L-158,809 treatment to ameliorate these measures of WBI-induced neurobiological changes, despite previous demonstrations of anti-inflammatory effects (Moulder et al., 1998b; Molteni et al., 2000), requires consideration of dosing and the drug’s access to the brain. Several observations indicate that L-158,809, as delivered in the present study (20 mg/L in the drinking water): i) crosses the blood-brain barrier
(BBB), ii) affects cognition through neural, rather than peripheral effects, and iii) might alter neuroinflammation. Although no direct measures of BBB permeability to L-158,809 are available, the compound is more lipophilic and, therefore, more likely to cross the BBB than losartan, a more widely studied drug of the same class (Gohlke et al., 2001; Thone-Reineke et al., 2004). L-158,809 at this dose ameliorates radiation-induced cognitive deficits (Robbins et al., 2009) without affecting blood pressure (Moulder et al., 1998b; Gilliam-Davis et al., 2007) suggesting L-158,809 directly affects the CNS. Moreover, in the present study the drug altered non-microglial SGZ proliferation in sham-irradiated rats and neurogenesis in irradiated rats, suggesting central actions. L-158,809 reduces radiation-induced inflammation in the kidney and lung (Moulder et al., 1998a; Moulder et al., 1998b; Molteni et al., 2000; Cohen et al., 2007), consistent with anti-inflammatory actions. Thus, it appears that L-158,809 acts directly within the brain and can influence inflammatory responses in at least some tissues. Whether the drug modulates inflammatory mechanisms and microglial responses not assessed in these analyses or does not act through inflammatory pathways in the brain at all remains to be established.

**Conclusion**

L-158,809 treatment ameliorates cognitive deficits 28 and 54 weeks after WBI in young adult rats (Robbins et al., 2009) but has only limited ability to modulate inflammatory cytokines and microglia or to rescue neurogenesis, the most extensively studied WBI-induced changes in the hippocampus. Given that RAS inhibitors have significant promise for reducing radiation-induced cognitive dysfunction in cancer
patients, additional studies are required to elucidate mechanisms of normal tissue injury that may be modulated by this class of drugs.

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CHAPTER III

EFFECTS OF THE AT₁ RECEPTOR ANTAGONIST L-158,809 ON
MICROGLIAL ACTIVATION, POPULATION SIZE, AND ON NEUROGENESIS
AFTER FRACTIONATED WHOLE-BRAIN IRRADIATION

Kelly R. Conner, Valerie Payne, M. Elizabeth Forbes, Mike E. Robbins, and
David R. Riddle

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ABSTRACT

Cognitive dysfunction develops in approximately 50% of patients who receive whole-brain irradiation (WBI). The mechanisms underlying these deficits are unknown. A recent study demonstrated that treatment with the angiotensin II type 1 receptor antagonist (AT\textsubscript{1}RA) L-158,809 before, during, and after fractionated WBI prevents or ameliorates radiation-induced cognitive deficits in adult rats. Given that i) AT\textsubscript{1}RAs may function as anti-inflammatory drugs, ii) that inflammation contributes to radiation injury, and iii) that radiation-induced inflammation contributes to deficits in hippocampal neurogenesis, we tested whether the cognitive benefits of L-158,809 treatment were associated with amelioration of the sustained neuroinflammation and changes in neurogenesis that are induced by WBI. In rats examined 28 and 54 weeks after fractionated WBI, the number of microglia in the hippocampus and the radiation-induced increase in activated microglia in the hippocampus and perirhinal cortex were not affected by L-158,809 treatment. Similarly, treatment with L-158,809 did not ameliorate the WBI-induced decrease in proliferating cells and immature neurons in the hippocampus. These findings suggest that L-158,809 does not prevent or ameliorate WBI-induced cognitive deficits by modulation of chronic inflammatory mechanisms, but rather may reduce radiation-induced changes that occur earlier in the post-irradiation period and only gradually lead to cognitive dysfunction.
INTRODUCTION

Approximately 220,000 patients are diagnosed each year with primary or metastatic brain cancer (1-3). Partial or whole-brain irradiation (WBI) is an effective treatment for primary and/or metastatic brain tumors and also is used prophylactically to prevent metastases to the brain, the second most common site of metastatic cancer (4). WBI has proven efficacy in eliminating neoplasms; however, approximately 50% of long-term cancer survivors who receive WBI develop progressive cognitive dysfunction attributed to normal tissue damage (5-7). The cellular and molecular bases of WBI-induced cognitive dysfunction have yet to be fully elucidated.

Neuroinflammation is a significant component of the brain’s response to radiation (8,9). Neuroinflammation following WBI, evidenced by an increase in the number of activated (e.g. CD68 expressing) microglia, is accompanied by decreased hippocampal proliferation and neurogenesis, which have been associated with hippocampal-dependent cognitive deficits that commonly develop after WBI (10,11). Interventions that modulate inflammation and/or promote neurogenesis may, therefore, provide protection against the normal tissue damage that is thought to lead to radiation-induced cognitive deficits.

Pharmacological blockade of the renin-angiotensin system (RAS) is an attractive therapeutic target against WBI-induced brain injury. Although the systemic RAS has classically been viewed as a hormonal system regulating blood pressure and fluid balance (12,13), several organ specific systems exist, including one in the brain, and function independently from the systemic RAS (14,15). Angiotensin II (Ang II) is the best characterized of the biologically active RAS peptides and signals through angiotensin II type 1 (AT₁) and angiotensin II type 2 (AT₂) receptors (AT₁R, AT₂R). Ang II is involved
in inflammatory responses and neuronal function in the brain (16-18). RAS blockade, via angiotensin-converting enzyme inhibitors (ACEis) or AT$_1$R antagonists (AT$_1$RAs), ameliorates radiation-induced injury in the lung, kidney, and optic nerve (19-21). Therefore, blockade of the brain RAS may ameliorate WBI-induced neuroinflammation and/or restore neurogenesis in the brain. AT$_1$RAs are as effective as ACEis in preventing radiation damage to the lung and kidney (22), so in this study, we blocked the brain RAS with an AT$_1$RA instead of an ACEi because ACE can cleave biologically active peptides that are not related to the RAS, e.g. bradykinin and opioid peptides (23), which complicates interpretation of experimental effects.

The development of a robust and tractable animal model of radiation-induced cognitive dysfunction greatly facilitates the development and testing of novel therapies such as RAS inhibition. We have demonstrated that young adult male rats exposed to a 40 Gy fractionated WBI regimen develop deficits in multiple cognitive domains. Deficits in the hippocampal-independent novel object recognition task (NOR) and in the hippocampal-dependent radial arm maze and Morris water maze developed with similar time courses (24-26), suggesting radiation damage occurs throughout the brain, not just in regions of ongoing neurogenesis, e.g. the dentate gyrus (DG) of the hippocampus. Moreover, treatment with the AT$_1$RA L-158,809 prevented or ameliorated WBI-induced cognitive deficits in the NOR task, indicating RAS blockade may be effective in preventing delayed radiation-induced cognitive dysfunction.

In the present study we tested the hypothesis that L-158,809 prevents or ameliorates WBI-induced cognitive dysfunction through chronic modulation of neuroinflammation and/or by protecting neurogenesis in the hippocampus. Brains were
obtained from rats that were behaviorally characterized at 28 and 54 weeks after completion of fractionated WBI and exhibited radiation-induced deficits in the NOR task that were ameliorated by treatment with L-158,809 (26). Quantification of total microglial number and/or of microglial activation in the granule cell layer and hilus (GCL/hilus) of the DG and in the CA3 region of the dorsal hippocampus served as indicators of neuroinflammation. Given the accumulating evidence that sustained deficits result from radiation-induced inflammation and contribute to cognitive deficits (11,27,28), the densities of proliferating cells and immature neurons were evaluated in the subgranular zone (SGZ) of the hippocampus, a region of the brain where neurogenesis continues throughout adulthood (29). In addition, we also assessed the microglial activation in the perirhinal cortex (PrC) since i) the NOR task is perirhinal dependent and ii) radiation-induced inflammatory processes likely act widely in the brain to produce deficits.

METHODS AND MATERIALS

Animals and Irradiation Procedures

Adult (10-12 week old) male Fischer 344 x Brown Norway (F344xBN) rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and housed in pairs on a 12:12 h light-dark schedule with food and water available ad libitum. The animal facility at Wake Forest University Health Sciences (Winston-Salem, NC) is accredited by the American Association for Accreditation of Laboratory Animal Care and complies with all Public Health Service-National Institutes of Health and institutional policies and
standards for laboratory animal care. All protocols described herein were approved by the
Institutional Animal Care and Use Committee.

After a two week acclimation, rats were randomized to four experimental groups: 1) sham-irradiated (Sham), 2) WBI, 3) sham-irradiated plus the AT$_1$RA, L-158,809 (Sham + AT$_1$RA, Merck & Co., Inc., Rahway, NJ), and 4) WBI plus L-158,809 (WBI + AT$_1$RA). Fractionated WBI (40 Gy in eight fractions of 5 Gy, twice/week for 4 weeks at 4.41 Gy/min) was performed in a self-shielded $^{137}$Cs irradiator with collimating devices for delivery to the whole brain and lead shielding to protect the body and eyes as previously described in detail (26,30). All rats, including shams, were anesthetized using a ketamine/xylazine mixture (75/5 mg/kg body weight, i.p.) before each irradiation session. The twice-weekly WBI dose was administered to alternate sides of the head on alternate days to make sure each animal received the same midline dose over the course of the treatment. L-158,809 treatment (20 mg/L in the drinking water) began three days before the start of fractionated WBI and continued until subjects were euthanized either 28 weeks or 54 weeks after the final WBI fraction. Fresh drinking water with or without L-158,809 was provided every other day; animals were weighed weekly and the volume consumed was recorded and used to monitor drug dosage over the course of the experiment (averaging 2 mg/kg/day).

Tissue Processing

Rats were deeply anesthetized with sodium pentobarbital (150 mg/kg body weight) and decapitated 28 or 54 weeks after the completion of fractionated or sham WBI. Each brain was extracted rapidly and hemisected at the midline; the right
hemisphere was flash frozen in liquid nitrogen and stored for other analyses while the left hemisphere was immersion fixed in phosphate-buffered 4% paraformaldehyde (pH 7.4) for 24 h at 4°C. The fixed hemispheres were cryoprotected in sucrose, embedded in Tissue Freezing Medium (TFM, Triangle Biomedical Sciences, Inc., Durham, NC) and stored at -80°C until sectioned. Serial coronal sections through the entire hippocampal formation (bregma –1.8 to –6.8) (31) were cut at 60 µm on a cryostat, collected in antifreeze solution (1:1:2 ethylene glycol, glycerol, and 0.1 M sodium phosphate-buffer, pH 7.4), and stored at -20°C until processed for immunohistochemistry (IHC) or immunofluorescence (IF).

Immunohistochemistry and Immunofluorescence

Systematically random series were selected for immunolabeling. For analysis of the hippocampal regions1-in-12 (GCL/hilus) and 1-in-6 (dorsal CA3) series of sections representing the entire anterior-to-posterior extent of the hippocampus were labeled and analyzed. Given the difficulty of defining absolutely the borders of the PrC we did not attempt to quantitate absolutely the number of activated microglia in the region but rather assessed the density in a well-defined region of PrC (see below) in three sections per animal from the series used for analysis of GCL/hilus. Sections from equal numbers of rats representing the four treatment groups were processed and analyzed in cohorts. Material from each group, 28 weeks or 54 weeks following WBI (n = 6 rats/condition), was processed and evaluated independently.

The antifreeze solution was washed from the sections using 0.1 M tris-buffered saline, pH 7.5 (TBS) and then the endogenous peroxidase activity was reduced by
incubating sections for 30 minutes in 1% hydrogen peroxide (H₂O₂) in TBS. Sections were incubated for 1 h at room temperature in TBS containing 5% normal serum and 0.3% Triton X-100 prior to incubation overnight with primary antibody in the same solution at 4°C. The primary antibodies used were: rabbit monoclonal anti-Ki67 (clone SP6; a marker for proliferating cells (32), AbCam, Cambridge, MA, 1:200), rabbit polyclonal anti-ionized calcium-binding adaptor molecule1 (Iba1; labels all macrophages/microglia, (33), Wako, Richmond, VA, 0.083 µg/ml), mouse monoclonal anti-CD68 (clone ED1; a lysosomal label for phagocytic macrophages/microglia (34), AbD Serotec, Raleigh, NC, 2.5 µg/ml). For sections immunolabeled with Ki67, antigen retrieval via incubation in 10 mM sodium citrate, pH 6, at 90°C for 10 minutes was performed prior to the H₂O₂ incubation (described above). Primary antibodies were detected with biotinylated secondary antibodies (1:300), amplified using a peroxidase-conjugated avidin-biotin complex (Vectastain ABC Elite kit) and visualized using Vector SG (Ki67), diaminobenzidine (Iba1), or nickel enhanced diaminobenzidine (CD68/ED1) peroxidase substrates (Vector Laboratories, Inc., Burlingame, CA). Ki67 and Iba1 were labeled in the same series of sections; labeling and visualization of Ki67 was performed as described, then sections were treated with 1% H₂O₂ in TBS for 30 minutes (room temperature) prior to labeling of Iba1. All sections were counterstained for 20 minutes with 100 µM of the nucleic acid stain Sytox Green (Invitrogen-Molecular Probes, Carlsbad, CA) to permit identification of the cell layers in the hippocampal formation and facilitate contour drawing for stereological analyses (described below). Sections were mounted from TBS onto superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), blotted of residual buffer, air dried for 30 minutes, dehydrated using a graded series of ethanol,
cleared in xylene and coverslipped using Cytoseal 60 permanent mounting medium (VWR International, LLC, West Chester, PA).

Labeling of neuroblasts and immature neurons in the dorso-medial SGZ was evaluated using goat polyclonal anti-doublecortin antibody (DCX (35), Santa Cruz, Santa Cruz, CA, 1 µg/ml). Sections were washed with TBS, underwent antigen retrieval via incubation in 10 mM sodium citrate, pH 6 at 90°C for 15 minutes, incubated for 1 h in TBS containing 5% normal donkey serum and 0.3% Triton X-100 and then incubated overnight at 4°C with addition of primary antibody. DCX was visualized using Cy-5 conjugated, highly cross-adsorbed donkey anti-goat secondary antibody (Jackson ImmunoResearch, West Grove, PA, 7.5 µg/ml). Sections were counterstained with Sytox Green as described above and then mounted from TBS onto superfrost Plus slides, blotted of residual buffer and coverslipped using Biomega Gel Mount (Fisher Scientific, Pittsburg, PA). Slides were sealed using Cytoseal 60 to prevent dehydration and were stored in the dark at 4°C, to reduce photobleaching.

Quantitative Analyses

All analyses were performed blinded to the experimental condition using coded slides and cohorts sampling equally across the four experimental groups. Identical analyses were performed for material from both experimental time points, 28 and 54 weeks after fractionated WBI. Total numbers of microglia (Iba1+) and of activated, phagocytic microglia (ED1+) were estimated stereologically in the GCL/hilus and dorsal CA3 using the optical fractionator technique (36,37) using the Stereo Investigator system (MBF Bioscience, Williston, VT) on an Olympus BX51 microscope (Olympus America,
Contours defining hippocampal regions of interest (ROI) were drawn using a 10x objective and then IHC-labeled cells within each optical disector were counted using a planapochromatic 60x oil-immersion objective (NA 1.42). The percentage of total microglia (Iba1+) that were activated (ED1+) was computed from analyses performed on adjacent sections from each animal and compared across experimental conditions. The GCL/hilus ROI was defined as the area containing the GCL and hilus/polymorph layer of the DG (Fig. 1). In more anterior sections in which the dorsal and ventral GCL were not fused into a closed contour a line connecting the suprapyramidal and infrapyramidal blades of the GCL was drawn to create a closed contour. The dorsal CA3 region of interest included all strata of CA3. A closed contour was constructed at the border of CA3 and the DG by connecting the two blades of the DG. The pyramidal layer of CA2 was included within the ROI to determine the dorsal border between CA3/CA2 and CA1. Analyses of CA3 were restricted to the dorsal CA3 (bregma –2.8 through −4.0) (38) given its established role in cognitive functions demonstrated to be sensitive to WBI (39,40). For all optical fractionator analyses, a 12 µm disector height, 2 µm guard zones, and 100 µm x 100 µm counting frame were used. Table 1 shows the sampling grid size and coefficient of error (CE) (41) for each hippocampal subregion analyzed at each time point investigated.

The hippocampus has been the focus of much recent analysis of radiation-induced normal tissue injury in the brain but radiation-induced deficits are not limited to hippocampal-dependent tasks. The animals in this study were behaviorally characterized using a PrC-dependent version of the NOR task (26); therefore, the density of activated microglia in PrC also was assessed. Since the PrC lacks readily identifiable anatomical
**Fig. 1.** ROI contours for analyses in GCL/hilus, dorsal CA3, and PrC. SGZ within DG indicated by dashed line (adapted from Paxinos and Watson, 1998 with permission (31)).
### TABLE 1

**Stereological parameters**

<table>
<thead>
<tr>
<th>Time point</th>
<th>Region</th>
<th>Object</th>
<th>Sampling grid size (µm²)</th>
<th>Mean CE Schmitz-Hof⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>28 weeks³</td>
<td>GCL/hilus</td>
<td>Total microglia (Iba1⁺)</td>
<td>275 x 275</td>
<td>0.055</td>
</tr>
<tr>
<td>Dorsal CA3</td>
<td></td>
<td>Activated microglia (ED1⁺)</td>
<td>175 x 175</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total microglia (Iba1⁺)</td>
<td>250 x 250</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>Dorsal CA3</td>
<td>Activated microglia (ED1⁺)</td>
<td>225 x 225</td>
<td>0.075</td>
</tr>
<tr>
<td>54 weeks³</td>
<td>GCL/hilus</td>
<td>Total microglia (Iba1⁺)</td>
<td>275 x 275</td>
<td>0.054</td>
</tr>
<tr>
<td>Dorsal CA3</td>
<td></td>
<td>Activated microglia (ED1⁺)</td>
<td>225 x 225</td>
<td>0.053</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total microglia (Iba1⁺)</td>
<td>300 x 300</td>
<td>0.058</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Activated microglia (ED1⁺)</td>
<td>250 x 250</td>
<td>0.061</td>
</tr>
</tbody>
</table>

³n = 6 per each experimental group  
⁶Coefficient of error (CE) for individual estimates.
borders it is not well suited to stereological analysis. Density measures were performed using the NeuroLucida system (MBF Bioscience, Williston, VT) and a modification of the optical disector (37) as described previously (42,43). Three sections (located between bregma –3.5 and –5.5) were analyzed for each animal. The ROI was defined as a 400 µm wide area centered on the rhinal fissure and extending from the pial surface to the white matter (Fig. 1).

The SGZ is one of two regions in the brain where continual neurogenesis can occur throughout life. It is located along the border between the cell body dense GCL and the cell body sparse hilus. The density of proliferating cells (Ki67+ cells) within the SGZ (defined as the region extending 25 µm either side of the boundary between the hilus and the GCL) was quantified using a modification of the optical disector as described previously (42,43) and was expressed as the density of Ki67+ cells per millimeter of SGZ examined. Counts were performed using the NeuroLucida system (MBF Bioscience, Williston, VT) and a 60x oil-immersion objective. Sections were labeled sequentially for Ki67 and Iba1 to permit analyzing specifically the proliferation of microglia, but qualitative examination revealed that at 28 and 54 weeks post-irradiation proliferating microglia constituted a very minor (< 1.5%) component of the proliferating cells in the SGZ and of the microglial population in the GCL/hilus or CA3. Therefore, Ki67+/Iba1+ cells were not quantified separately.

Immature neurons (DCX+ cells) were quantified as a measure of the production of new neurons. DCX+ cells typically occurred in clusters and could not be analyzed accurately in thick sections using widefield microscopy. DCX+ cells were counted using a Leica TCS SP2 confocal microscope with a 63x oil-immersion objective (NA1.4, Leica
Microsystems, Brannockburn, IL) moving field-by-field along the extent of the SGZ. Counting the labeled cells in a single hemi-section required imaging z-stacks (1 µm steps) through the depth of the section in approximately 25 fields. Preliminary analyses established that the relative densities of labeled cells (comparing individuals or groups) was equivalent whether one section or a series of several sections was analyzed for each animal, indicating that a single section provided a reliable estimate of the density of DCX-labeled cells in the SGZ. Thus, a single, equivalent coronal section from each animal was used to estimate the production of new neurons in the dorso-medial DG, expressed as the density of DCX+ cells per millimeter of SGZ. For both Ki67 and DCX immunolabeled cells within the SGZ all cells were counted except those in the top focal plane of each section to avoid overestimation.

Statistical Analysis

Data are given as the mean ± standard error of the mean (SEM). All data were analyzed with Sigmastat (SYSTAT Software, San Jose, CA). Two-way analysis of variance (ANOVA) was used to test for main effects of irradiation status (sham vs. WBI) and drug status (water vs. L-158,809) and for interactions. Since all handling of animals and processing of tissue was performed separately for the two time points ANOVAs were also run separately for each time point. For significant main effects, pairwise multiple comparisons were performed using the Holm-Sidak test. A p value of ≤ 0.05 was considered the threshold for significance.
RESULTS

Previous studies demonstrated i) that fractionated WBI of young adult male F344xBN rats leads to a chronic, progressive decrease in cognitive function, demonstrated by deficits in performance in a NOR task, and ii) that treatment with the AT1RA L-158,809 prevents or ameliorates these radiation-induced cognitive changes (26). Given the evidence that the brain RAS may act, in part, through inflammatory mechanisms, we used tissue from behaviorally characterized animals to test whether the cognitive benefits of AT1RA treatment in irradiated rats are associated with amelioration of WBI-induced changes in markers of WBI-induced neuroinflammation or cell turnover, neurobiological changes that are thought to contribute to WBI-induced cognitive dysfunction.

Effects of WBI and AT1R Antagonism on Total Microglial Number and on Number and Percentage of Activated Microglia

Iba1, ionized calcium-binding adaptor molecule1, is located in the cytosol of all microglia and infiltrating monocytes regardless of activation state (33). Iba1+ cells are referred to here as microglia for convenience but with the recognition that Iba1 labeling does not distinguish between microglia and macrophages that might be recruited from the periphery under inflammatory conditions. Photomicrographs in Fig. 2 (panels A and B) show the homogeneous distribution of microglia in the hippocampus. Qualitative examination revealed no noticeable difference between irradiated and sham rats in the morphology of Iba1+ cells; most had the ramified morphology described for “resting” microglia (44-46). Anti-ED1 antibody recognizes the rat homologue of human CD68, a
Fig. 2. Markers of microglia, proliferating cells, activated microglia, and immature neurons in the hippocampus of F344 x Brown Norway rats. A and B illustrate Ki67 labeling of proliferating cells (gray) and Iba1 labeling of microglia (brown) in sham (A) and irradiated (B) animals; typical examples of labeled cells are shown at higher magnification in insets. C and D demonstrate ED1 labeling in the GCL/hilus (C) and CA3 (D, indicated structures within CA3 are pyramidal cell layer (PCL), stratum oriens (SO), stratum radiatum (SR), and fimbria (fi)). Labeled cells are shown at higher magnification in insets. In E and F an immature neuron in the SGZ, demonstrated by DCX labeling (red) alone (E) and in a merged image (F) showing the distribution of all nuclei stained with Sytox green (green). Scale bars = 50 μm, 25 μm (inset).
lysosomal/endosomal associated membrane glycoprotein involved in phagocytosis, expressed only in activated microglia and macrophages (34). Representative photomicrographs of ED1 labeling are shown in Fig. 2 (panels C and D). Consistent with the expected lysosomal location of CD68, ED1 labeling typically appeared as individual or small groups of puncta near the nucleus of labeled cells.

The total number of microglia (Iba1+ cells), the number of activated microglia (ED1+ cells), and the proportion of microglia that were activated (% ED1+ cells) were analyzed in order to assess changes in microglial population following WBI. We hypothesized that L-158,809 might affect cognition by modulating the size of the microglial population and/or reducing microglial activation.

In the GCL/hilus (Fig. 3 panel A), neither WBI nor AT1RA treatment significantly affected total microglial number at 28 weeks after WBI. At 54 weeks after WBI, however, the total number of microglia was reduced by 17% in irradiated animals (compared to shams) in the absence of AT1RA treatment. This reduction was not seen in drug treated animals. Radiation increased the number of activated microglia in the GCL/hilus at 28 weeks (F (1, 20) = 5.625, p < 0.05 for the main effect of radiation; no significant increase by post hoc tests within the water- or drug-treated group) but not at 54 weeks post-irradiation (Fig. 4 panel A). The percentage of activated microglia was increased significantly (24%) in WBI vs. Sham animals at 28 weeks in rats without drug treatment but not in rats receiving L-158,809 (Fig. 4 panel C). Fifty-four weeks after WBI, the percentage of activated microglia did not differ significantly among the experimental groups.
Fig. 3. Quantitative analysis of total microglial number in the GCL/hilus and dorsal CA3. WBI significantly decreased the total microglial population in the GCL/hilus (A) but not in CA3 (B) 54 weeks after WBI. *$p < 0.05$ vs. sham controls; white bars = Sham, dark gray bars = WBI, light gray bars = Sham + L-158,809, black bars = WBI + L-158,809.
Fig. 4. Quantitative analysis of activation state in the GCL/hilus, dorsal CA3, and PrC. Irradiation increased the number of activated microglia in the GCL/hilus (A, see text) but not in CA3 (B) at 28 weeks. At 28 weeks post-irradiation WBI increased the percentage of activated microglia in the GCL/hilus in water treated animals (C) and in CA3 in L-158,809-treated rats (D). WBI also significantly increased the density of ED1 microglia in the PrC of rats both with and without drug treatment (28 week, E). \*p < 0.05 vs. sham controls; experimental groups as indicated in Fig. 3.
Neither WBI nor L-158,809 treatment significantly altered the total microglial number (Iba1+ cells, Fig. 3 panel B) or number of activated microglia (ED1+ cells, Fig. 4 panel B) in the dorsal CA3 at either time point, but in the L-158,809 treated animals WBI significantly increased the percentage of activated microglia (19%, Fig. 4 panel D). Neither WBI nor AT1R antagonism affected the proportion of activated microglia in the dorsal CA3 region at 54 weeks following WBI.

WBI significantly increased the density of activated microglia in the PrC at 28 (115%, \( p \leq 0.001 \)) but not at 54 weeks post-irradiation (Fig. 4 panel E). There was no effect of L-158,809 treatment at either time point.

The experimental design of this study did not permit direct, statistical comparisons between the two survival periods but qualitative assessment indicated that microglial activation was consistently greater in the 54 week group than in the 28 week group. In those instances in which radiation-induced increases in activation were apparent at 28 but not at 54 weeks, it appeared that the number and proportion of activated microglia in sham animals were higher at the later than at the earlier time point, rather than the levels decreasing in irradiated animals at the later time point decreasing to that in shams. In the hippocampus the number and density of ED1+ cells increased over time in all groups and reached a common level.

*Effects of WBI and AT1R Antagonism on Proliferation and Production of Immature Neurons in the SGZ*

Proliferating cells were observed, typically in clusters, in the SGZ (Fig. 2 panels A and B). The density of proliferating Ki67+ cells in the SGZ was calculated for rats from
all four experimental conditions at 28 and 54 weeks after completing WBI (Fig. 5). In agreement with previous studies examining proliferation after WBI, we saw a significant radiation-induced reduction in the density of proliferating cells in the SGZ at both time points. WBI resulted in a 76% reduction in the density of proliferating cells at 28 weeks and a 54% decrease at 54 weeks after irradiation. Treatment with L-158,809 had no effect at either time point.

Figure 2 (panels E and F) includes representative sections immunolabeled for DCX and counterstained with Sytox green. The photomicrographs show cytosolic DCX immunoreactivity illustrating the location of the cell bodies of immature neurons in the SGZ. Apical dendrites can also be seen extending through the GCL to the molecular layer of the DG where entorhinal afferents provide input to the hippocampus via the perforant pathway (47).

Immature neurons (DCX⁺ cells) were quantified (Fig. 6) within the SGZ as an indicator of neurogenesis. Since not all newborn neurons are incorporated into functional networks (29), the density of DCX⁺ cells is an indirect estimate of functional neurogenesis. By 28 weeks post-irradiation WBI reduced the density of DCX⁺ cells by 69% (Sham vs. WBI) and 60% (Sham + AT₁RA vs. WBI + AT₁RA). L-158,809 treatment alone reduced the density of immature neurons by 35% (Sham vs. Sham + AT₁RA). Neither WBI nor AT₁RA treatment resulted in significant changes in the density of immature neurons at 54 weeks post-irradiation; there was a trend toward lower density in irradiated animals without drug treatment but not in those receiving L-158,809 (Fig. 6).
**Fig. 5.** Effects of fractionated WBI and AT$_1$R antagonism on SGZ proliferation. WBI decreased the density of proliferating cells in the SGZ at both 28 and 54 weeks post-irradiation. L-158,809 had no effect on proliferation at either time point. The experimental design did not permit statistical comparisons between the two survival periods but the density of proliferating cells in sham rats in the 54 weeks group was less than half that in the 28 weeks group, presumably due to an aging-related decline. $p^* < 0.001$ **0.01 vs. sham controls; experimental groups indicated as in Fig. 3.
Fig. 6. Effects of fractionated WBI and AT$_{1}$R antagonism on neurogenesis. Significant WBI- and AT$_{1}$RA-induced reductions in the density of immature neurons were evident only at 28 weeks following fractionated WBI. The density of immature neurons was equivalent across all groups at 54 weeks. The effects of WBI and drug treatment were not additive; AT$_{1}$R blockade in irradiated rats did not reduce the density of DCX$^{+}$ cells below that seen in irradiated rats without drug treatment. *$p < 0.001$ vs. sham controls; #$p < 0.01$ vs sham without L-158,809 treatment; experimental groups indicated as in Fig. 3.
DISCUSSION

Treatment with the AT₁RA L-158,809 before, during, and after fractionated WBI preserves cognitive function in our rodent model of radiation-induced brain injury (26). The animals in the current study had radiation-induced deficits and AT₁RA-dependent benefits in the non-hippocampal dependent NOR task. The irradiation regimen used here also induces deficits in hippocampal-dependent tasks (e.g. the partially-baited radial arm maze and Morris water maze; (24,25)) and we expect ongoing studies will demonstrate that L-158,809 treatment ameliorates those deficits as well. Based on the evidence that inflammatory mechanisms underlie radiation-induced brain injury (11,27,28,48) and previous demonstrations of anti-inflammatory effects of AT₁RA (49,50), we hypothesized that the beneficial effects of L-158,809 on cognition following WBI involve chronic modulation of microglial activation, possibly also resulting in protection of proliferation and neurogenesis in the hippocampus. The present results indicate that the original hypothesis must be revised.

Microglial Response Following WBI and AT₁R Antagonism

Given that: i) increased inflammation, as indicated by changes in the microglial population, occurs after WBI (8,11,27,28,51), ii) AT₁R activation activates microglia in several injury models and in vitro (18,52-54), and iii) AT₁R blockade reduces inflammation and ameliorates injury following ischemia (55,56) it was reasonable to expect AT₁R antagonism would attenuate WBI-induced microglial activation. This study, however, revealed no robust effects of L-158,809 treatment after WBI on the total microglial number or the number or percentage of microglia exhibiting an activated
phenotype (ED1+) in the GCL/hilus or CA3 region of the hippocampus, or on the density of ED1+ cells in the PrC. It is unlikely that this was due to insufficient dose, delivery, or anti-inflammatory efficacy of the drug. Several observations support the conclusions that L-158,809 as delivered in the present study (20 mg/L in the drinking water) i) crosses the blood-brain barrier (BBB), ii) affects cognition through neural, rather than peripheral effects, and iii) might modulate inflammatory pathways in the brain. Although no direct assessment of its transport across the BBB is available, L-158,809 is more lipophilic, and therefore more likely to cross the BBB, than losartan, a more widely studied drug of the same class (57,58). With respect to CNS effects of the drug, L-158,809 at this dose ameliorates radiation-induced cognitive deficits (26) without affecting blood pressure (59,60), consistent with a CNS-mediated action. The demonstration in the present study that the drug altered neurogenesis in sham-irradiated rats at the 28 weeks time also suggests central actions. With respect to possible anti-inflammatory actions, L-158,809 clearly is effective at reducing radiation-induced inflammation in the kidney and lung (59,61-63). Thus, it appears that L-158,809 does act within the brain and can modulate inflammation. Since no changes in inflammatory markers were seen in these animals at the later time points at which preservation of cognition is apparent, the beneficial effect of AT1R antagonism following WBI may involve anti-inflammatory mechanisms acting in the first weeks following WBI. This possibility is consistent with the observation that L-158,809 treatment for only five weeks following WBI partially ameliorated the cognitive deficit from WBI in the NOR task measured at 26 weeks (26).

This study provided a critical, but limited, initial assessment of microglial changes following WBI and RAS inhibition. ED1 labeling of CD68 is well established as
a marker of microglial activation following WBI and other pro-inflammatory challenges. Robust WBI-related increases in CD68 expression occur in rodents examined at a few days up to three months after a single dose of WBI (11,28,42,48). In the current study fractionated WBI resulted in significant increases in the number and proportion of ED1\(^+\) cells at 28 weeks but not at 54 weeks after WBI. The magnitude of the increase in activated microglia in irradiated compared to sham animals at 28 weeks was smaller than that seen at the more acute time points investigated in previous single dose studies and was not statistically significant in all regions under all conditions. The smaller relative increase at 28 weeks after WBI (compared to shorter survival periods in previous studies) and the absence of a significant irradiation effect at 54 weeks were not due to decreased activation in irradiated animals with longer survival periods, but rather to a robust aging-related increase in microglial activation (see also (42)). Thus, microglial activation, as evidence by CD68 expression, is sustained chronically following fractionated WBI, but substantial aging-related changes eventually produce comparable levels of activation in non-irradiated animals.

In the present study, there was not compelling evidence that L-158,809 treatment altered the number of microglia or their expression of CD68 several months after WBI. At 54 weeks post-irradiation the number of microglia in the GCL/hilus was decreased relative to sham controls in irradiated rats without AT\(_1\)R blockade but not in those receiving L-158,809. Similarly, the percentage of ED1\(^+\) microglia in the GCL/hilus was increased at 28 weeks in rats without drug treatment but not in those receiving the antagonist. These radiation-induced changes in rats without drug treatment but not in those receiving the AT\(_1\)RA suggest the drug may have some protective effect, but this is
contradicted by the observation that in CA3 WBI increased the proportion of microglia that were ED1\(^+\) in L-158,809 -treated rats but not in those without the drug. Overall, it appears that RAS inhibition has only modest, if any, effects on radiation-induced changes in these measures of microglial function.

Although CD68 expression was not robustly regulated by RAS inhibition, radiation and the brain RAS may affect other microglial responses that were not evident with our analyses. For instance, microglia, even in an activated state, can release trophic factors that increase neuronal survival after injury (64,65). Therefore, microglia in irradiated animals treated with L-158,809 may produce anti-inflammatory signals and/or trophic factors that either increase survival of or modulate information processing in nearby neurons. Studies to further characterize microglial phenotypes in irradiated brains both with and without L-158,809 treatment are ongoing.

**SGZ Proliferation and Neurogenesis following WBI and AT\(_1\)R Antagonism**

Numerous studies of radiation-induced brain injury have established that WBI results in a sustained decrease in neurogenesis due to both death of progenitor cells and ongoing changes in the microenvironment that are regulated, at least in part, by inflammatory signals (10,11,27,48,66). Thus, hippocampal neurogenesis was an important component of the present study. Even in the absence of demonstrable effects on neuroinflammation, assessing proliferation and neurogenesis was critical since hippocampal neurogenesis is regulated by other factors that might be influenced by AT\(_1\)RAs. For example, AT\(_1\)R antagonism increases AT\(_2\)R activation, which promotes
neuronal differentiation, maturation, and migration (as opposed to AT1R activation, which can stimulate proliferation (15,17)).

In this study, L-158,809 treatment did not ameliorate the WBI-induced decreases in SGZ proliferation or in DCX density. L-158,809 treatment did, however, significantly reduce DCX density in non-irradiated rats (28 weeks group). The latter suggests AT1RAs can influence neurogenesis but by suppressing, not enhancing, production of new neurons or by speeding up maturation so that new neurons spend less time in the DCX expressing stage of neurogenesis (29). The density of DCX⁺ cells in irradiated animals was similar to sham levels at 54 weeks post-WBI, even though total proliferation remained suppressed in irradiated animals, which indicates that the number of proliferating cells adopting a neuronal fate increases in the months following WBI. Although DCX⁺ cells did not differ in number among experimental groups at 54 weeks, the number of newborn neurons that contribute to function could differ between irradiated animals with and without L-158,809 treatment. Additional cell fate studies will be required to determine whether more newborn neurons survive and are integrated in L-158,809 treated animals, a possible mechanism by which AT1R antagonism could influence cognitive dysfunction.

*Other possible targets of AT1R regulation*

AT1R antagonism could be beneficial to cognition through several other mechanisms, including direct effects at the synaptic level. First, Ang II is localized to synaptic vesicles and can modulate both pre- and post-synaptic transmission; several laboratories have demonstrated AT1-mediated inhibition of long-term potentiation, a cellular model of learning and memory (17,67-70). Second, the RAS also regulates
extracellular matrix (ECM) turnover and alters cell-cell and cell-ECM interactions that affect synaptic formation and strength. For example, AT$_1$RAs suppress AT$_1$-induced expression of TGF-β, a fibrogenic cytokine involved in extracellular matrix deposition (49). Such suppression could increase synaptic plasticity. Third, AT$_1$R antagonism may improve cognitive function following WBI through effects on the vasculature, since WBI causes vascular changes that produce ischemic/hypoxic conditions (71). Beneficial cognitive effects of AT$_1$RAs could be mediated by increasing cerebral blood flow and alleviating local, WBI-induced hypoxic conditions (17).

Conclusions

In summary, L-158,809 treatment before, during, and after fractionated WBI prevents or ameliorates the progressive cognitive deficits that arise long after the completion of radiotherapy. The findings of this study suggest that these cognitive benefits are not mediated through chronic modulation of microglia or regulation of neurogenesis, mechanisms that have been the focus of many studies of radiation-induced brain injury, but rather through other modulation of changes in the first few weeks following irradiation. Given the clinical promise of AT$_1$RAs, studies of radiation-induced cognitive dysfunction and its modulation by RAS inhibitors must continue to explore different mechanisms that may contribute to the drugs’ beneficial effects.

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CHAPTER IV

DISCUSSION

WBI remains a standard of care in the neuro-oncology clinic. Although an effective treatment for both primary and metastatic tumors, WBI can have high quality of life costs in the form of progressive cognitive deficits (1-3). The cellular and molecular interactions that occur in response to WBI and culminate in cognitive dysfunction have not been fully elucidated (4,5). It is imperative that these interactions be clarified as they represent possible therapeutic targets within the dysfunctioning system. Testing of attractive therapeutic agents, even those that fail to work as hypothesized, improve our understanding as to which tissue responses are causative and which neurological changes only correlate with cognitive dysfunction.

Late delayed effects become measurable several months to years following radiotherapy (6-8). The cellular and molecular components of the WBI response that eventually result in clinically detectable cognitive dysfunction are likely initiated much earlier. The experiments described in this dissertation contribute to the knowledge base of normal tissue damage hypothesized to cause WBI-associated cognitive deficits and investigated the mechanisms of action of an attractive therapeutic agent.

RAS blockade, via the AT_1 RA L-158,809, was investigated as a possible therapeutic intervention to ameliorate neuroinflammation and/or rescue neurogenesis after WBI. Experiments were conducted using both a single dose and a fractionated dose of WBI. The use of a single 10 Gy dose permitted us to analyze acute changes that occur before the onset of measurable cognitive deficits without confounds associated with
multiple radiation fractions. For instance, determining when an acute neurobiological change occurred in relation to individual fractions is difficult. It is important, however, to use animal models that mimic clinical conditions to increase the likelihood of successfully translating therapies from the bench to the bedside. We, therefore, investigated neuroinflammation and neurogenesis following a 40 Gy fractionated dose of WBI at time points when L-158,809 treatment alleviated WBI-induced cognitive dysfunction.

Section I: Neuroinflammation

Neuroinflammation, usually reported as increased expression of the lysosomal membrane associated glycoprotein CD68, is a well documented WBI-induced response (9-11), and increased microglial activation correlates with WBI-induced cognitive deficits (10). The brain RAS may influence the effects of WBI-induced inflammation through regulation of cytokine production and microglial proliferation and/or activation. Thus, it was of interest to determine whether L-158,809 treatment prevented radiation-induced neuroinflammation.

Cytokine Expression after a single dose 10 Gy WBI

The experiments in Chapter II investigated the hypothesis that oral administration of the AT1RA L-158,809 before, during, and after a single 10 Gy dose of WBI would ameliorate WBI-induced neuroinflammation. Quantitative RT-PCR was used to determine mRNA expression levels of cytokines known to be involved in the WBI response (12-16) and to be influenced by RAS signaling, usually via the AT1R (17-23).
WBI did not induce the large changes in expression reported in most previous studies, but this may be due to methodological differences: time points after irradiation, species and strain differences, whole brain versus hippocampal homogenates, and radiation dose. Changes in cytokine mRNA expression peaks early, around 4–8 h, and is usually at or near control levels by 24 h (12) suggesting that we missed the peak in expression. This is further supported by findings from our collaborators. Their data indicates that our single dose model causes peak cytokine expression at 4–8 h with levels returning to or near control levels by 24 h after WBI (24). Our results, however, are similar to findings by Kalm et al. (2009) in the developing rat brain (postnatal day 9) following a single 8 Gy WBI dose and microarray gene analysis, a method with quantitative abilities comparable to qRT-PCR. They found that several cytokines, including TNF-α, but not IL-6 or IL-1β, were significantly upregulated at 6 h post-WBI but had returned to control levels by 24 h and remained at baseline for at least 8 weeks post-WBI (25).

Although we detected only a few small, but statistically significant, changes in cytokine expression there were some interesting findings, for example, the relationship between TNF-α and IL-6 responses at 24 h and one week. TNF-α expression returned to or slightly exceeded sham levels at one week, up from a WBI-induced decrease at 24 h, and was accompanied by a WBI-induced decrease of IL-6 expression. Considering TNF-α and IL-6 can influence the expression of each other (26), it is possible these results illustrate an ongoing cycle. Chiang et al. (1997) reported an acute increase in TNF-α expression at 4 h, followed by spikes in expression at 2–3 months and again at 6 months following a single 25 Gy dose of WBI in mice. We detected a similar radiation-induced increase in TNF-α expression only in animals not treated with L-158,809 at our 12 week
time point, coinciding with Chiang’s 2–3 month time point. The lack of an irradiation effect in L-158,809 treated irradiated animals and a drug effect in sham animals at the 12 week time point suggest that L-158,809 does have some influence on cytokine expression following WBI. Also, the biological relevance of these small changes in mRNA expression remains to be established.

McBride’s group (2004), of which Chiang and Hong’s studies were a part, has hypothesized that WBI sets in motion a chronic inflammatory response in which waves of inflammatory mediators are expressed. These cycles may signify failed attempts to end inflammation, may not have the same functional outcome, and could represent periods during which the system is more amenable to interventions. The evidence of a cyclical interaction between cytokines at our more acute time points is in line with this hypothesis. The reelevation of mRNA expression and evidence of an irradiation and drug interaction on TNF-α at our 12 week time point further supports the possibility of an ongoing cyclical response in cytokine expression. Additional studies are needed, however, to corroborate if a cyclical response is reoccurring in our model and to determine the biological effects of individual cycles.

Expression of the two Ang II receptors was also analyzed given that Ang II decreases AT₁R expression and, somewhat paradoxically, increases AT₂R expression (27,28). AT₁R antagonism increased AT₁a R mRNA expression, but only in sham-irradiated animals and only at the one week time point. Lack of any sustained increase in expression may indicate a compensatory action of the brain RAS in response to chronic inhibition. The transient increase in mRNA expression was not reflected at the protein level. The antibody used in the Western blots, however, recognized both the AT₁a and
AT₁b receptors which may contribute to the disparity between gene and protein expression levels (Appendix Figure 1).

Interestingly, L-158,809 treatment increased AT₂R mRNA expression only in irradiated animals at one week after WBI. The lack of a sustained increase at 12 weeks post-WBI may reflect cyclical regulation of the receptor similar to that seen with cytokines. Alternatively, normal levels of AT₂R expression may be sufficient to mediate effects when expression occurs under pathological conditions (29). AT₂R expression is up-regulated after a number of CNS injuries in which it is linked to neuronal survival and regeneration and decreased proliferation. In ischemia, AT₂R activation protects against N-methyl-D-aspartic acid (NMDA)-induced neurotoxicity and increases CBF through vasodilation (30,31). Similar histopathological changes are seen after both WBI and global ischemia (32). Combined with results from our collaborators suggesting an association between capillary loss and cognitive dysfunction following 40 Gy fractionated WBI (33), increased AT₂R expression could be beneficial by increasing neuronal survival through by preventing excitotoxicity, or increasing CBF and ameliorating WBI-induced hypoxia.

Finally, angiotensin-converting enzyme 2 (ACE2) expression was assessed since AT₁R antagonism should increase levels of Ang II available to be cleaved to Ang (1-7), which like AT₂R activation has actions in opposition to AT₁R-mediated actions of Ang II (34). No effect of either irradiation or drug was detected at any time point.

Microglial population dynamics after WBI

Microglia are the innate immune cells of the brain and a source of both inflammatory mediators and several neurotrophic substances (35-40). Changes in the
microglial population, in the form of increased proliferation and/or activation, typically accompany insults to the brain, including LPS injection, ischemia, nerve lesion, traumatic brain injury, stress, and radiation (41-48). Population expansion, via massive proliferation, is an acute response in other injury models and returns to control levels within weeks to months after an acute injury (49-51). Reports of microglial responses to WBI, however, have been limited to changes in the density of activated microglia only up to two months after irradiation, including a case study of a patient who received a radiation boost to the brain two months prior to death (10,11,52,53). Ang II is involved in microglial activation and cytokine production, thereby influencing the microglial population (31,54). Therefore, we investigated the effects of WBI and L-158,809 treatment on multiple aspects of the microglial population after either a single 10 Gy dose or fractionated 40 Gy dose of WBI.

The acute microglial proliferation seen after other injuries is indeed part of the DG GCL/hilus response to WBI. Similar to other injuries, microglial proliferation was a major component of the acute radiation response. Although microglial proliferation remained elevated 12 weeks after WBI it was greatly reduced compared to the one week response (Figures 2B and 3A in Chapter II). Surprisingly, increased microglial proliferation did not increase the total, Iba1+, microglial population. In fact, WBI significantly reduced the total microglial population during the height of the proliferative response. This reduction in total number of Iba1+ microglia at one week following WBI is in agreement with that reported in the hippocampus and SVZ of postnatal day 9 rats one week after a single 8 Gy dose of WBI (55). The proliferative response was also accompanied by an increase in activated microglia. Considering apoptosis plays a role in
controlling the microglial population after excitotoxicity (56), the reduction in total number of microglia one week after WBI suggests that microglia are induced to proliferate, express activation markers, provide some function, and are then removed by apoptosis. The overall microglial population returns to sham levels even though the number and the percentages of proliferating and activated microglia remain elevated compared to controls. Given that microglial proliferation was greatly reduced at 12 weeks post-WBI and the acute nature of the proliferative response in other neural injury models, we were not surprised to discover that microglial proliferation no longer significantly contributed to the radiation response at the much later time points investigated after fractionated WBI.

Microglial proliferation reduces infarct size after transient ischemia, partly through secretion of cytokines such as TNF-α (5,57). Although it has not been directly tested the acute microglial proliferation following other types of injury, e.g. nerve lesion and radiation, may be pivotal in controlling damage from these injuries. If microglial proliferation is beneficial after an insult, preventing all components of the inflammatory processes from the onset of injury onward could prove counterproductive. In such circumstances, preservation of the microglial proliferative response under L-158,809 treatment may make RAS blockade a better treatment option compared to other anti-inflammatory therapies, such as NSAIDs that have limited efficacy in preventing radiation-induced normal tissue injury (58), if administered prior to WBI or immediately after WBI.

Microglial activation is also greatest at more acute time points following WBI. Approximately 50% of all microglia express the activation marker ED1 one week after a
single 10 Gy dose of WBI while sham animals had almost none. By 12 weeks, activated microglia constituted only about 13% of microglia in irradiated animals and had risen, presumably due to an aging effect (36,59), to just over 5% in sham-irradiated animals (Figures 2C and 3B, Chapter II). Considering the precipitous drop in the percentage of activated microglia in irradiated animals over time, accompanied with the aging associated increase in sham-irradiated animals, we hypothesized that any remaining radiation affects on the percentage of activated microglia will eventually be masked by aging related increases in microglial activation. Results from Chapter III support this hypothesis. By the time cognitive deficits are manifested following fractionated WBI, microglial number, the number of activated microglia, and the percentage of activated microglia are only slightly affected by WBI (Figure 2 in Chapter III). When significant differences occurred or trends were apparent, they were in the directions seen at the earlier time points after a single dose of WBI; i.e. WBI occasionally lowered the number of total microglia, but increased microglia activation.

The NOR protocol used to behaviorally characterize these animal is PrC-dependent; therefore, the density of activated microglia was analyzed in the PrC to ensure WBI and L-158,809 treatment had similar effects on neuroinflammation in a brain region other than just the hippocampus (Figure 2E in Chapter III). WBI increased the density of activated microglial in the PrC at 28 weeks, but the effects of WBI were no longer evident at 54 weeks. The lack of a radiation effect on the density of activated microglia 54 weeks after WBI appears to be due to an increase in microglial activation in sham-irradiated animals as opposed to a reduction in irradiated animals. As mentioned before, aging related increased microglial activation could be masking any remaining WBI
effects on microglial activation. We think the discrepancy between the hippocampal response and the PrC response at 28 weeks is because of differences in the methods used to quantify activated microglia, stereological estimates of total numbers and the percentage of total microglia calculated from those stereological counts in the hippocampus versus density measurements from a region of interest within the PrC. The PrC lacks well defined anatomic borders that negate the use of stereology requiring another method to quantify microglial activation within the PrC. Although L-158,809 treatment improved performance on the NOR task in irradiated rats it had no effect on the density of activated microglia. This finding suggests that microglial activation is either not involved in PrC-dependent cognitive function or AT1R blockade alters microglial responses through mechanisms other than ED1 expression, for example, influencing microglial phenotype and/or interactions with other surrounding cell types.

Our findings are in agreement with those from Fike’s group suggesting that changes in the microglial population, at least in the hippocampus, occur during the latent period between irradiation and the manifestation of cognitive deficits (5). If increased microglial activation has a causal link, not just a correlative link, to WBI-induced cognitive dysfunction, at least in PrC-independent tasks, then interventions aimed at altering the microglial response should be employed either as prophylactic or mitigation therapy as opposed to treatment therapy once cognitive dysfunction is evident. Figure 1 provides a graphical representation of microglial population dynamics following WBI.

Although L-158,809 treatment did not ameliorate WBI-induced changes in microglial numbers. Microglia in water-treated, irradiated rats may play different roles in the hippocampus and/or the PrC compared to microglia in L-158,809 treated, irradiated
Figure 1. Microglial population dynamics following WBI. Microglial proliferation, small dashed line, is an acute response and returns to sham levels quickly. Microglial activation, solid line, is greatest at acute time points and continues longer than proliferation followed by an age related increase months to years after WBI. Sham animals, solid red line, also experience an age related increase in microglial activation.
rats. For instance, activation of the AT₂R, or at least the prevention of AT₁R activation, could be driving activated microglia toward a neuroprotective/neuroregenerative phenotype. ED1 labeling in our model usually consisted of one or two labeled lysosomes near the nucleus of labeled cells suggesting these microglial may not be fully functioning phagocytes like those seen after other injury models such as LPS injection.

Previous studies indicate that microglia can retain their ramified, resting morphology in which they do not contribute to a pro-inflammatory environment, but up-regulate activation markers. This state appears to represent a primed condition as upon further challenge, such as LPS injection, these microglia express higher levels of pro-inflammatory cytokines than microglia in naïve animals (60). WBI priming of microglia could explain why not all patients that receive WBI suffer cognitive deficits and/or the long latency between radiotherapy and the clinical manifestation of cognitive deficits. The 20–50% of patients that experience cognitive dysfunction following WBI may have suffered an unreported, or unrealized, secondary injury that prompted an exaggerated response from radiation-primed microglia, which was highly detrimental to neuronal function.

WBI effects on microglia could also be compounded by aging effects over time. Patients classified as “successful agers” may correspond to the patients that do not develop WBI-induced cognitive dysfunction. Aging itself is accompanied by increased microglial activation (46); therefore, WBI-primed microglia may become increasingly activated, toward a pro-inflammatory/neurotoxic phenotype, as the effects of aging accumulate. The patient population receiving WBI is predominantly middle aged, and older individuals are more susceptible to WBI-induced cognitive dysfunction than
younger adults (61,62). In the patient population aging may prime microglia making them more susceptible to further activation in response to a challenge such as WBI. In support of this theory, a recent study from our laboratory revealed activation of microglia is, in fact, exacerbated in older animals following WBI (63).

**Future Directions: Neuroinflammation**

*Cytokines and Ang II receptors*

The qRT-PCR data in Chapter II was generated using RNA from the whole hippocampus while analyses of microglial population size were restricted to specific hippocampal subregions. We used whole hippocampal homogenates to ensure RNA integrity since RNA degrades quickly and sub-dissection of the hippocampus is time consuming. The lack of large fold differences in mRNA expression could represent a dilution artifact if hippocampal subregions have different expression levels. It is more likely, however, that the several fold peak in expression is an acute phenomenon and large changes in cytokine expression do not contribute to the feedforward mechanism thought to underlie the chronic WBI-induced inflammatory response since studies reporting large expression peaks at earlier times used RNA from the whole brain, or at least whole hemispheres (12,13,16). Microarray gene analysis from developing rat brains following a single 8 Gy dose of WBI also suggests the cytokine response is acute since any cytokines found to be upregulated at 6 h post-WBI had returned to control levels by 24 h and remained at baseline at least 8 weeks after WBI (25).
Further characterization of microglial phenotypes, pro-inflammatory versus neuroprotective, following WBI and L-158,809 treatment is necessary to determine whether RAS blockade modulates other components of the WBI-induced inflammatory response; it has little effect on microglial number. *In situ* hybridization experiments would be superior to qRT-PCR from hippocampal subregions since *in situ* hybridization facilitates the determination of cytokine and Ang II receptor cellular sources. *In situ* hybridization is also preferred over flow cytometry. The tissue processing required for flow cytometry disrupts the spatial relationship between cell types, which could provide insight into functional interactions, and may artificially activate microglia.

The identification of cellular sources and the maintenance of spatial relationships by *in situ* hybridization are valuable because the timing and the conditions in which cytokine and Ang II receptor expression occurs can determine the biological outcome of their expression. For example, astrocytic IL-6 shifts NPCs away from a neuronal fate toward a glial fate (64) but neuronal IL-6 induces neuroprotective microglial proliferation after nerve transection (65). Concerning the AT2R, over expression in the absence of Ang II binding induces apoptosis in neuronal cultures (27) but promotes neuronal differentiation, neurite outgrowth, and neuronal migration *in vivo* (66). In the vasculature, activation of the AT2R may prevent microvascular remodeling or increase CBF following WBI. Also, AT2R is up-regulated in monocytes as they mature into functioning macrophages and may serve a similar function in the activation of microglia (31). In order to determine the cellular mechanisms underlying WBI-induced injury and L-158,809 treatment-induced benefits we must have a better understanding of the environment in which cytokines and Ang II receptors are expressed.
Microglial population dynamics

Although we expanded microglial population analyses to dorsal CA3 in Chapter III, our results indicated no difference in the microglial response to WBI between the DG or CA3 hippocampal subregions. It may be of interest, however, to extend analyses into CA1 in future studies. Data from our laboratory indicates that WBI has similar effects on the density of both the total microglial population and activated microglia one and 10 weeks after a single 10 Gy WBI dose in CA1 as in the DG and CA3 (67). Whether L-158,809 treatment has any effect in CA1 remains to be ascertained.

CA1 is inherently different than the DG or CA3. Functional outcomes of microglial responses to RAS blockade, therefore, could vary between regions. Qualitative examination of sections labeled for major histocompatibility complex II (MHC II, another marker of microglial activation) revealed a startling lack of expression in CA1 compared to both the DG and CA3, which had clusters of robustly labeled cells (data not shown) regardless of irradiation status, whereas other microglial markers, e.g. ED1 and Iba1, have a more consistent distribution throughout the hippocampus and cortex. Although these apparent differences in microglial marker expression suggest inflammation in CA1 is different than in the other hippocampal subregions, it also implies the existence of partially overlapping microglia populations that could have different functions (68,69). A more in depth characterization of microglial subpopulations based on the different markers (e.g. Iba1 versus CD11b/OX-42 and activation markers such as ED1 compared to MHC II) and their functions would behoove the neuroinflammation field as a whole. As suggested above, in situ hybridization studies would be a useful avenue for such investigations.
Our collaborators also reported WBI-induced increases in NMDA receptor subunits only in CA1 one year after fractionated WBI (70). The NMDA glutamate receptor is involved in synaptic plasticity and ischemic excitotoxicity (71), whereas microglial activation, through glutamate clearance (72), and the up-regulation of neuronal AT2 receptors are neuroprotective during hypoxia. RAS signaling influences the outcome of NMDA receptor activation. Following ischemia, AT2R activation prevents NMDA receptor mediated down-regulation of anti-apoptotic Bcl-2 and increases CBF by augmenting NMDA-induced nitric oxide (NO) (30,73). If WBI produces hypoxic conditions, as suggested from evidence of vessel loss and occlusion (74,75), then microglial and AT2R activation may prevent WBI-induced ischemic excitotoxicity in L-158,809 treated animals.

Section II: SGZ proliferation and neurogenesis

Suppression of proliferation and neurogenesis in the SGZ following WBI is well documented, including one small study in humans. Reductions in SGZ proliferation and neurogenesis are associated with the hippocampal-dependent cognitive deficits that develop after WBI (76,78,77,10,79,53). AT1R antagonism increases AT2R activation, which has been implicated in neuronal differentiation and migration in several injury models (80,81,82,66). The effects of RAS blockade via the AT1RA L-158,809, therefore, were investigated to determine if this pharmacological intervention would restore the WBI-induced deficits in SGZ proliferation and neurogenesis.
Proliferation

Previous reports suggest that proliferation in the SGZ, as indicated by BrdU incorporation (83) or Ki67 (84), is unaffected by radiation one week after a single 4-10 Gy dose of WBI. Reactive astrogliosis does not significantly contribute to SGZ proliferation following low doses of WBI suggesting gliogenesis is not responsible for the recovery in proliferation seen at one week from the immediate obliteration seen hours to days after WBI. Clusters of proliferating cells within the neurogenic niche can ultimately produce a number of cell types, including neurons, astrocytes, oligodendrocytes, and endothelial cells (85). The SGZ also contains resident microglia, which are the primary source of new microglia in the healthy adult brain (39,86), and our previous findings revealed an acute proliferative microglial expansion in the DG GCL/hilus. Therefore, we wished to determine if proliferating microglia comprised a significant portion of the proliferating population in the SGZ. We used Ki67 as our proliferation marker because it is expressed by cells in all stages of mitosis. BrdU is only incorporated into DNA during S-phase and is diluted with subsequent divisions (87). Also, Ki67 does not require injecting the animals, which may cause stress and stress-induced inflammation.

In agreement with previous reports, total proliferation was similar across conditions one week after a single 10 Gy dose of WBI, but proliferating microglia compose a significant proportion of proliferating cells in the irradiated SGZ at this time. The increase in proliferating microglia is accompanied by a corresponding decrease in non-microglial proliferation. The seemingly equivalent change in number, however, is coincidental. It cannot be due to newborn cells being directed from a non-microglial fate
into a microglial fate as these subpopulations are derived from different progenitor populations (39). L-158,809 treatment reduced non-microglial proliferation in sham-irradiated animals at the one week time point, which we attribute to the anti-proliferative effects of AT1R antagonism (82).

Unlike Ben Abdallah’s (2007) findings that proliferation recovers by one week and remains at control levels thereafter, our 12 week post-WBI results corroborate the long term suppression of SGZ proliferation more commonly reported following a single dose of WBI (9,10,77,79,83). Microglia proliferation, as in the DG GCL/hilus, was greatly decreased in the SGZ 12 weeks after a 10 Gy WBI dose. L-158,809 treatment in irradiated animals further reduced microglia proliferation suggesting L-158,809 treatment may bring the microglial proliferative response to an end more quickly, at least within a region of the brain permissive to adult neurogenesis.

The reduction in microglial proliferation in irradiated animals 12 weeks after WBI is not accompanied by an equivalent increase in non-microglial proliferation. These findings suggest that although microglial proliferation, and the secretion of cytokines and/or trophic factors associated with it, may play a role in regulating non-microglial proliferation, and ultimately cell fate; it is not the only mechanism mediating the WBI-induced suppression of non-microglial proliferation.

SGZ proliferation is also significantly reduced by fractionated WBI, at least up to 42 days after WBI completion (78). The study in Chapter III provides the first evidence that a clinically relevant, fractionated dose of WBI results in a chronic reduction of SGZ proliferation. Again, microglial proliferation was no longer a significant component of the proliferating population; therefore, our data reflect a WBI-induced suppression of
non-microglial proliferation. Of note, non-microglial proliferation, regardless of the irradiation status of the animal, was lower at the later time point in both the single dose and fractionated dose studies and most likely reflects an age effect on proliferation (88). Since L-158,809 treatment had only small effects on proliferation at shorter time points after a single dose of WBI, it was not surprising L-158,809 had no effect on the density of proliferating cells at the time points investigated following fractionated WBI. It remains to be seen, however, whether L-158,809 treatment alters cell fate and/or survival of daughter cells born after single or fractionated doses of WBI.

Neurogenesis

The majority of the current literature is based on the effects of single WBI doses on measures of neurogenesis up to two months in rats, or up to nine months in gerbils, post-WBI (9,10,77,79). The only fractionated radiation (24 Gy dose) study including an analysis of neurogenesis investigated neurogenesis only up to 42 days after the final fraction (78). Although the above mentioned studies usually assessed proliferation at acute time points, days to weeks after WBI, none measured neurogenesis before one month after WBI. The impairment in neurogenesis following radiotherapy, both with and without chemotherapy, is also seen in human beings and is a long term consequence, evident up to at least 23 years after radiation (53).

In agreement with earlier studies, we found reduced neurogenesis after either a single dose (both one week and 12 weeks) or fractionated dose (only 28 weeks) of WBI. The production of new neurons, although still significantly reduced compared to sham-irradiated controls, has begun to recover by 12 weeks following the acute annihilation of
newborn neurons at one week after 10 Gy WBI. Similar to effects on proliferation, neurogenesis in sham-irradiated animals was reduced over time indicative of the known aging effects on neurogenesis (89). We hypothesize that as neurogenesis decreases with age in sham-irradiated animals and neurogenesis continues to recover in irradiated animals densities will cease to differ among conditions. Evidence from the fractionated study in Chapter III supports this hypothesis. The density of DCX$^+$ cells was no longer different between conditions 54 weeks after fractionated WBI, when these animals are middle aged (approximately 16 months old).

An interesting observation from irradiated animals was that although the production of immature neurons appears to recover with time there is a continued reduction in non-microglial proliferation, see Figure 2 for a graphical representation of the proliferative and neurogenic response to WBI over time. Therefore, as the microenvironment becomes less conducive to microglial proliferation and more permissive for neurogenesis it remains restrictive to non-microglial proliferation. This raises the question as to which cell type(s) constitutes the discrepancy between increasing neurogenesis and decreasing non-microglial proliferation. The other glial cells, astrocytes and oligodendrocytes, are unlikely suspects considering gliogenesis is relatively preserved, if not slightly elevated, following radiation doses and fractionation regimens similar to those utilized here (9,10,83,90,91). Loss of endothelial progenitors, a component of the neurogenic niche (85), could account for the continued down-regulation of total SGZ proliferation following WBI and/or the disruption of the relationship between clusters of proliferating cells and the microvasculature reported by Monje et al. (2002). A persistent loss of endothelial progenitors may also explain the
Figure 2. SGZ proliferation and neurogenesis following WBI. Microglial proliferation, small dashed line, is a component of the one week spike in total proliferation. Non-microglial proliferation, long dashed line, is constitutively suppressed by WBI below control levels, red long dashed line. Neurogenesis, solid line, eventually recovers to control levels, red solid line, followed by further suppression over time due to aging effects.
WBI-induced decrease in vessel density and vessel length proceeding impaired performance on the partially baited radial arm maze in our fractionated model. (33,75).

L-158,809 treatment had intriguing effects on the density of DCX$^+$ immature neurons. Originally, we hypothesized AT$_1$R blockade would increase AT$_2$R activation, thereby promoting neurogenesis, but when drug effects were detected L-158,809 treatment was associated with decreased DCX density. DCX, expressed during the stage of neurogenesis when recently born neurons are selected for survival or elimination, is an indirect marker of neurogenesis given that not all immature neurons will integrate into the network and survive(92). Since AT$_2$R activation is involved in neuronal migration and maturation, including modulating neuronal firing rates that play a role in selection for survival and integration (66,93), it is possible that L-158,809 induced reductions in DCX density reflect faster maturation instead of an actual reduction in neurogenesis. Even if L-158,809 treatment is speeding up neurogenesis so that cells spend less time in the DCX expressing state, it does not necessarily mean more neurons are ultimately surviving and contributing to cognition; they could be selected for elimination more quickly.

**Future Directions: Proliferation and Neurogenesis**

Double labeling with the proliferation marker Ki67 and the microglial marker Iba1 allowed us to differentiate between proliferating microglia and proliferating non-microglial cells. Multi-labeling with Ki67 and other cell specific markers would permit further delineation of the proliferating non-microglial population. Expanding analyses just to include an endothelial specific marker, e.g. rat endothelial cell antigen (RECA)
(85), would be a reasonable first step given that gliogenesis is relatively unchanged by doses of WBI similar to ours. Cell fate studies using BrdU would also shed light on the cell types comprising the non-microglial population and determining if L-158,809 induced decreases in immature neuron density are due to fewer neurons being produced or faster maturation and integration.

Considering the role of the RAS in angiogenesis, future studies should also address the ability of L-158,809 treatment to ameliorate WBI-induced alterations in the microvasculature. Such studies would further complement proliferation experiments in which endothelial progenitors are examined. If these endothelial and microvascular studies reveal L-158,809 effects, then studies to determine whether L-158,809 also restores the close relationship between proliferating clusters and the microvasculature would be warranted.

Section III: Drug efficacy and alternative hypotheses

Oral L-158,809 treatment and the brain RAS

L-158,809 treatment did not ameliorate WBI-induced changes in neuroinflammation and neurogenesis as hypothesized. This introduces concerns of whether the drug gained access to the brain and whether the dose was sufficient to produce the hypothesized changes. Although we have no direct measure of L-158,809 crossing the BBB (for example, detectable levels in the cerebral spinal fluid; this was not done because it required too many animals in order to extract and pool enough CSF to be of use) it is reasonable to assume that it does. Losartan, the original AT1RA, does have a
limited ability to cross the BBB and block some of the AT₁R within the BBB (94). Telmisartan, another AT₁RA, is more lipophilic than losartan and crosses the BBB more efficiently because of its increased lipophilicity (95), suggesting that the higher lipophilic characteristics of L-158,809 compared to losartan should increase its ability to cross the BBB. Once L-158,809, or any other AT₁RA, crosses the BBB, it may increase BBB permeability to the drug since AT₁ receptors on astrocytes are involved in BBB maintenance (96). Therefore, it is reasonable to conclude that L-158,809 is capable of crossing the BBB.

There is also evidence that our dose and timing of administration should have been sufficient to alter WBI-induced brain injury if L-158,809 mechanisms of action involved modulation of the analyzed endpoints. L-158,809, at the dose and mode of administration used here (20 mg/L in the drinking water): (1) effectively reduces radiation-induced inflammation in the kidney and lung (4,97-100), (2) ameliorates radiation-induced cognitive deficits (101), (3) decreases neurogenesis (one week after a 10 Gy dose of WBI in irradiated rats and 28 weeks in sham-irradiated rats), and (4) significantly reduced proliferating microglia in the SGZ 12 weeks after a single 10 Gy WBI dose. Beneficial cognitive effects of L-158,809 treatment are unlikely to be due to peripheral mechanisms such as superior BP control since our dose does not affect blood pressure in normotensive animals (4,102). Therefore, beneficial cognitive effects are most likely mediated through the alteration of neural mechanisms.

Although L-158,809 treatment ameliorated WBI-induced cognitive deficits in the NOR task, it remains to be determined whether L-158,809 treatment has similar efficacy in alleviating deficits in hippocampal-dependent cognitive tasks; tasks more likely to be
directly affected by neurobiological changes in the hippocampus. Deficits in the hippocampal-dependent partially baited radial arm maze and MWM has also been demonstrated in the fractionated WBI rat model developed by our group at Wake Forest University (33,70) at time points similar to those when deficits in the NOR task were manifested. This model is an excellent candidate for future studies addressing L-158,809 treatment effects on hippocampal-dependent tasks.

Alternative hypotheses

RAS blockade and the synapse

RAS blockade could produce beneficial cognitive effects following WBI without direct effects on inflammation. In rats, increased density and widening of the pre-synaptic pole and a reduction in pre-synaptic vesicles are seen 90 days after 40 Gy fractionated WBI, at least in the cortex (74). Ang II is found in synaptic vesicles and has both pre- and post-synaptic effects on neurotransmission in several neurotransmitter systems; therefore, Ang II may directly influence information processing (82,103,104). Also, AT$_2$R activation can alter expression of ion channels involved in action potentials leading to enhanced firing rates (66). Ang II synaptic effects associated with the glutamate system may influence neuronal maturation and integration into the hippocampal network given that NMDA receptor activation is important in neurogenesis (93) and AT$_2$R activation can modulate NMDA receptor signaling (105,106).

The RAS is directly involved in LTP. Ang II, via AT$_1$R activation, inhibits LTP in DG granule cells, CA1 pyramidal cells, and the amygdala (107-109). Blockade of the AT$_1$R with L-158,809 may, therefore, enhance LTP. In order to test this, and determine if
synaptic transmission is altered following WBI, future studies including electrophysiology experiments using brain slices would be valuable. Such studies would also allow direct blockade of the AT2R, which cannot be accomplished with oral administration because currently available AT2R antagonists do not readily cross the BBB. It is important to determine the role of AT2R activation in this model considering that many beneficial effects of AT1R antagonism are currently attributed to increased AT2R activation. Considering evidence that AT2R blockade is beneficial in the irradiated kidney (110) the favorable effects of L-158,809 treatment may not be AT2R mediated. Acute and delayed radiation-induced gene expression is, however, under genetic control (50). Radiation responses in individual organs may, therefore, differ as well as their responses to different pharmacological interventions (for example, differences in response to RAS blockade in the irradiated lung compared to the irradiated kidney (97)) further supporting the need to directly test the contribution of AT2R activation in the irradiated brain.

The RAS can also modulated ECM production (22). Ang II, via AT1R activation, induces TGF-β expression (21). The L-158,809-induced suppression of TGF-β expression (Chapter II) may increase synaptic plasticity by decreasing ECM deposition. Other biologically active RAS peptides can also influence both LTP and regulate ECM composition.

Other active peptides of the RAS

Ang II is not the only biologically active RAS peptide. Ang III has actions similar to Ang II, probably because it binds to AT1R with high affinity. Blockade with an
AT₁RA should inhibit many of the actions mediated through Ang III activity. Ang (1-7) and Ang IV are also active, have receptors in the hippocampus, and can influence aspects of learning and memory (111).

Ang (1-7) binds to its own GPCR, Mas. Similar to AT₂R activation, Mas activation usually leads to outcomes opposing AT₁R-mediated functions, i.e. Mas activation is vasodilatory and antiproliferative (112). If WBI is creating a hypoxic microenvironment, Ang (1-7) may contribute to the amelioration of tissue damage by increasing CBF. Also, Ang (1-7) enhances LTP in CA1 through Mas receptor activation (113). AT₁R antagonism could increase Ang (1-7) production from the metabolism of free Ang II via ACE2, which is not affected by either ACEis or AT₁RAs, thereby increasing activation of Mas receptors and enhancing LTP.

Ang IV has its own high affinity receptor, the AT₄ receptor. The AT₄ receptor is not a GPCR like other Ang receptors. Evidence suggests it is a trimeric receptor including insulin-responsive aminopeptidase (IRAP) as an alpha subunit. Activation of the AT₄ receptor can increase CBF, influence vascular remodeling and angiogenesis, increase proliferation of neuronal cultures, inhibit neurite outgrowth, and increase kidney function. Ang IV also acts as an endogenous ACEi to block cleavage of Ang I to Ang II (6,107,114-117). Ang IV enhances LTP, exploratory behavior, all aspects of spatial learning (such as acquisition, retrieval, and storage), and the learning of both passive and conditioned avoidance tasks (6,116-123). In addition, Ang IV could influence learning and memory by modulating the effects of acetylcholine (ACh) release and/or the remodeling of the ECM (117,124-127). Ang IV has been linked to the expression and activation of matrix metalloproteinases (MMPs) as well as their inhibitors, tissue
inhibitors of metalloproteinases (TIMPs) (115,121,127). Activated MMPs rearrange the ECM by cleaving ECM proteins leading to the restructuring of cell-cell and cell-ECM interactions that either strengthens, as in LTP, or weakens synapses.

Although there is some evidence to suggest that LVV-hemorphin-7, another naturally occurring peptide in the brain, could be the endogenous AT$_4$R ligand instead of Ang IV (123,128) it remains controversial. Therefore, Ang IV should not, as yet, be removed from consideration as a mediator of the beneficial effects of L-158,809 treatment.

Agonists and antagonists exist for both Ang (1-7) and Ang IV. Electrophysiology studies in tissue slices from irradiated and L-158,809 treated animals, using Ang (1-7) and Ang IV agonists and antagonists, would further clarify the role of the brain RAS in normal brain tissue responses to WBI. Figure 3 represents possible mechanisms by which AT$_1$R antagonism could mediate beneficial outcomes following WBI and/or other neural injuries.

Section IV: Conclusions

The work presented here builds on current knowledge concerning the brain’s response to WBI. Our studies are the first to show that, similar to several other neural injury models, microglial proliferation is a major component of the acute response to WBI but is not a part of later responses. We also corroborated previously reported WBI effects on proliferation and neurogenesis; however, our results suggest that neurogenesis recovers over time. Therefore, neurogenesis may not be as heavily involved in WBI-
Figure 3. Mechanisms of AT$_1$R antagonism. L-158,809 treatment could produce several neurobiological effects either by preventing actions mediated by the AT$_1$R, freeing Ang II to bind to AT$_2$R, and/or allowing the metabolism of unbound Ang II into smaller, biologically active angiotensin peptides, e.g. Ang IV and Ang (1-7), which activate their own receptors. Depending on which angiotensin peptides are present and which receptors are activated, biological effects of AT$_1$R blockade could range from changes in neuronal differentiation, neuronal excitability, neuronal proliferation, non-neuronal proliferation, alterations in LTP, or vasodilation.
induced cognitive deficits in adults as previously suggested. The recovery in neurogenesis occurs, however, in parallel with continued suppression of SGZ proliferation. The identity of the lost progenitors is currently unknown. As in previous studies, we found microglial activation to be involved in acute (one week) and shorter term (12 weeks) responses to a single dose of WBI. The level of microglial activation is reduced over time, and, according to the results of our fractionated study, is no longer a major contributing element of the tissue response to WBI by the time cognitive deficits develop.

L-158,809 did affect proliferation and neurogenesis, possibly through its enhancement of AT\textsubscript{2}R expression. Our findings suggest that L-158,809 treatment has little effect on overall microglial population size. It remains to be seen whether L-158,809 influences the inflammatory response in other ways, for example, by influencing microglial phenotype. Although RAS blockade did not ameliorate the WBI-induced reduction in neurogenesis or prevent changes in the elements of the WBI-induced microglial response studied here, RAS blockade should not be abandoned as a promising intervention to prevent WBI-induced normal tissue damage. L-158,809 treatment improved cognition following a fractionated dose of WBI (101) and several mechanisms of action that could mediate this cognitive improvement remain to be evaluated.
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APPENDIX

Figure 1. Mean relative AT$_1$R levels in F334 rat hippocampus and entorhinal cortex following WBI. Average relative ODu calculated from densitometry of Western blot gels of whole hippocampal (top) or entorhinal cortex (bottom) homogenates 24hr or 1 week after a single 10 Gy dose of WBI. n = 4. No statistically significant differences in AT$_1$R protein expression were detected.
Post-irradiation AT$_1$R levels in F344 Rat Hippocampus

A

Post-irradiation AT$_1$R levels in F344 Rat Entorhinal Cortex

B

Mean Relative ODu ± SEM

0.0 0.5 1.0 1.5

24 hr 1 week

Sham
Sham+L-158,809
WBI
WBI+L158,809

0.0 0.5 1.0 1.5

24 hr 1 week

Sham
Sham+L-158,809
WBI
WBI+L158,809

204
Figure 2. Effects of WBI and AT$_1$R antagonism on systemic ACE activity and plasma levels of Ang II. Plasma from animals sacrificed 12 weeks after a single 10 Gy dose of WBI was analyzed using radioimmunoassay (RIA) to measure systemic ACE activity and levels of Ang II. Neither WBI nor AT$_1$R antagonism altered ACE activity. AT$_1$R antagonism did significantly increase circulating levels of Ang II regardless of irradiation status. *$p = 0.001$ versus sham control; n = 4.
SCHOLASTIC VITAE

KELLY R. CONNER

BORN: November 26, 1980, Greensboro, North Carolina

UNDERGRADUATE STUDY: Appalachian State University
Boone, North Carolina
Bachelor of Science (B.S.), Biology,
Chemistry (minor), 2003

GRADUATE STUDY: Wake Forest University Health Sciences
Winston-Salem, North Carolina
Ph.D. Neuroscience, complete 2009

SCHOLASTIC AND PROFESSIONAL EXPERIENCE:

Pre-doctoral fellowship, Wake Forest University Health Sciences

HONORS AND AWARDS:

NIH Predoctoral Fellowship, 2003 – 2004
Individual Predoctoral Fellowship:
Ruth L. Kirschstein National Research Service Award, 2007 – 2009

PROFESSIONAL SOCIETIES:

Association of Southeastern Biologists, 2002 – 2003
Western North Carolina Chapter of the Society for Neuroscience, 2005 – present
Society for Neuroscience, 2006 – present
Radiation Research Society, 2007 – present

MEETINGS ATTENDED:

Society for Neuroscience 34rd Annual Meeting, New Orleans, LA, 2003
Society for Neuroscience 35th Annual Meeting, San Diego, CA, 2004
Society for Neuroscience 37th Annual Meeting, Atlanta, GA, 2006

PUBLICATIONS:
Journal articles:


Abstracts:

