Published in final edited form as:

J Neurol Sci. 2009 October 15; 285(1-2): 178–184. doi:10.1016/j.jns.2009.06.031.

Maintenance of White Matter Integrity in a Rat Model of Radiation-Induced Cognitive Impairment

Lei Shi^{b,d}, M. Constance Linville^d, Elizabeth Iversen^d, Doris P. Molina^d, Jessie Yester^d, Kenneth T. Wheeler^{a,e}, Michael E. Robbins^{a,c}, and Judy K. Brunso-Bechtold^{a,b,d}

^aBrain Tumor Center of Excellence, Wake Forest University School of Medicine, Winston-Salem, NC 27157

^bProgram in Neuroscience, Wake Forest University School of Medicine, Winston-Salem, NC 27157

^cDepartment of Radiation Oncology, Wake Forest University School of Medicine, Winston-Salem, NC 27157

^dDepartment of Neurobiology and Anatomy, Wake Forest University School of Medicine, Winston-Salem, NC 27157

^eDepartment of Radiology, Wake Forest University School of Medicine, Winston-Salem, NC 27157

Abstract

Radiation therapy is used widely to treat primary and metastatic brain tumors, but also can lead to delayed neurological complications. Since maintenance of myelin integrity is important for cognitive function, the present study used a rat model that demonstrates spatial learning and memory impairment 12 months following fractionated whole-brain irradiation (WBI) at middle age to investigate WBI-induced myelin changes. In this model, 12-month Fischer 344 x Brown Norway rats received 9 fractions of 5 Gy delivered over 4.5 weeks (WBI rats); Sham-IR rats received anesthesia only. Twelve months later, the brains were collected and measures of white matter integrity were quantified. Qualitative observation did not reveal white matter necrosis one year post-WBI. In addition, the size of major forebrain commissures, the number of oligodendrocytes, the size and number of myelinated axons, and the thickness of myelin sheaths did not differ between the two groups. In summary, both the gross morphology and the structural integrity of myelin were preserved one year following fractionated WBI in a rodent model of radiation-induced cognitive impairment. Imaging studies with advanced techniques including diffusion tensor imaging may be required to elucidate the neurobiological changes associated with the cognitive impairment in this model.

Keywords

fractionated WBI; forebrain; myelin; oligodendrocytes; axons

© 2009 Elsevier B.V. All rights reserved.

Corresponding author: Judy K. Brunso-Bechtold, Ph.D. Department of Neurobiology and Anatomy Wake Forest University School of Medicine Medical Center Boulevard Winston-Salem, NC 27157-1010 Phone: 336-716-4386 FAX: 336-716-4584 jbrunso@wfubmc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Radiation therapy is a cornerstone of modern cancer management and approximately half of all newly diagnosed cancer patients will receive radiotherapy at some point during the treatment of their disease [1]. Whole brain irradiation (WBI) following tumor resection or radiosurgery can effectively reduce tumor recurrence and is used prophylactically to kill metastatic cells that would otherwise seed the brain [2]. Along with the therapeutic benefits, however, WBI can lead to delayed, dose-dependent neurological complications including progressive cognitive impairment [3,4]. Importantly, the physical and social burden of these neurological side effects can be more debilitating and detrimental than the primary disease [3,5]. With the marked improvements in long-term cancer survival, radiation-induced neurotoxicity is an issue of increasing concern [6].

The pathophysiological changes characterizing late-delayed WBI-induced neurotoxicity are dynamic and complex, and involve both the brain vasculature [7,8] and parenchyma [9]. These late-delayed effects manifest more than 6 months following WBI and are progressive and irreversible [10]. Earlier studies of long-term WBI survivors [11] have revealed white matter changes such as periventricular white matter abnormalities, ventricular dilation, leukoencephalopathy, and diffuse demyelination [9,12,13]. It is important to note that published clinical data are derived from brain tumor patients. Not only are the brains exposed to the tumor microenvironment, but many patients also receive multimodality therapy, including surgery, radiosurgery, and/or chemotherapy. Use of such multimodality therapy provides significant biological challenges to normal brain tissue and can lead to severe neurotoxicity [2,3,14], confounding the interpretation of the effects of WBI [15]. Rodent models provide valuable tools to directly and thoroughly examine the late-delayed effects of WBI on brain white matter in the absence of the complicating effects of brain tumors or other treatment modalities. Indeed, recent studies have reported radiation-induced changes including apoptosis of oligodendrocytes and decreased expression of myelin-associated proteins in rodent spinal cord [16] as well as white matter necrosis in rat optic nerve neuropathy [17].

A recent study reported spatial learning and memory impairment 12 months following 45 Gy of WBI delivered as 9 fractions of 5 Gy whole-brain irradiation over 4.5 weeks to middle-aged Fisher 344 x Brown Norway (F344xBN) rats [18]. These cognitive impairments were associated with changes in glutamate receptors in the hippocampus, but not with a decrease in hippocampal neuron number [19]. This rat model of fractionated WBI at middle age is of clinical importance because the incidence of cancer with a propensity to metastasize to the brain such as lung cancer, breast cancer, and malignant melanoma, increases significantly by middle age [20]. The present study investigated potential late-delayed changes in brain white matter in this rat model of fractionated WBI.

Maintenance of white matter integrity is critically important for cognitive performance [21, 22] and a positive correlation has been established between cognitive performance and white matter integrity [23]. Moreover, in the aged population, a region-specific correlation between measures of cognitive performance and white matter integrity has been reported [24]. Accordingly, the present study quantified measures of white matter integrity 12 months following 45 Gy of fractionated WBI at middle age. By using the same experimental design that resulted in WBI-induced cognitive impairment [18], the rats in the previous study served as a behavioral index for the parallel cohorts of rats studied here. Using morphometric analysis of light and electron microscopic material, we quantified the size of major forebrain commissures, the number of oligodendrocytes, the size and number of myelinated axons, and the thickness of myelin sheaths and found no WBI-induced change on any of the measures. These findings suggest that the cognitive impairment present in this model one year after

fractionated WBI at middle age occurs in the absence of structural changes on these measures of myelin integrity.

2. Materials and methods

2.1. Animals and irradiation procedure

Male F1 Fischer 344 x Brown Norway(F344xBN) rats (Harlan Industries, Indianapolis, IN) were divided randomly into sham-irradiated (Sham-IR; n=15) and WBI (n=19) groups at 12 months of age. WBI rats were lightly anesthetized and irradiated twice per week for 4.5 weeks in a 444-TBq self-shielded ^{137}Cs irradiator using lead and Cerrobend shielding devices to collimate the beam so that the whole brain was irradiated [18,19]. A total dose of 45 Gy was delivered to the brain as 9 fractions of 5 Gy. The biologically effective dose (BED, 23) calculated for this regimen was 120 Gy3, assuming an α/β ratio of 3 for late radiation-induced damage [25] and the single dose equivalence was 17.6 Gy [48]. To ensure that each rat received the same midline brain dose, the dose was delivered to alternate sides of the head on alternate days. Sham-IR rats were anesthetized, but not irradiated. All rats were euthanized by an overdose of sodium pentobarbital at 24 months of age. All subsequent experimental procedures were carried out by investigators blinded to irradiation status. Following irradiation, Sham-IR and WBI rats were randomly allocated for evaluation by either light microscopy (LM) or electron microscopy (EM). The animal protocol for this study conforms to NIH guidelines and was reviewed and approved by the Animal Care and Use Committee of Wake Forest University Health Sciences to ensure the ethics of the research and the welfare of the animals. Rats were housed singly in a climate-controlled environment with a 12-hour light/dark cycle and provided food and water *ad libitum*. Steps have been taken to eliminate pain and suffering of animals.

2.2. Tissue preparation for LM

For LM analysis (n=7 Sham-IR; n=11 WBI), rats were perfused transcardially with saline followed by 4% paraformaldehyde in phosphate buffer. The brains were dissected from the cranial vault, postfixed overnight, cryoprotected in a graded sucrose, and blocked stereotaxically at Bregma 5.5 and Bregma -15 mm [26]. The brains were frozen in Tissue-Tek OCT (Optimal Cutting Temperature Compound; Sakura Finetek) on dry ice, and stored at -80°C . Tissue was cryostat-sectioned coronally (40 μm) and the sections were stored in antifreeze at -20°C until staining.

Heidenhain staining—Sections stained with the Heidenhain myelin stain were used to, i] assess white matter integrity, ii] determine neocortex and forebrain volumes, and iii] measure the decussation area of the major brain commissures at midline. Briefly, every 24th section through the cerebral cortex was washed in phosphate buffered saline (PBS), immersed in iron alum, washed in water, stained with hematoxylin, washed in water, and placed overnight in PBS [27]. Sections then were mounted onto slides, air-dried, dehydrated, cleared, and cover-slipped. Every-other section through the anterior commissure (AC) also was stained and processed as described.

Nissl staining—Nissl is a basophilic stain that provides clear visualization of both neurons and glia. Oligodendrocytes, the glia comprising the myelin sheaths, were quantified in Nissl-stained sections in both corpus callosum (CC) and AC. Previous studies have indicated that oligodendrocytes can be identified morphologically by their size, shape, and cytoplasmic density, as well as by their orientation parallel to axon bundles in white matter [28,29]. Oligodendrocytes identified using these criteria were quantified in order to count the entire population of myelinating glia. The available antibodies to oligodendrocytes only label subsets of oligodendrocytes. Consequently, the number of oligodendrocytes immunolabeled by the available antibodies, either individually or in combination, may not reflect the total

oligodendrocyte population. Briefly, every 12th section through the rostral-caudal extent of the CC and every-other section through AC were washed in PBS, mounted on slides, air-dried, washed in PBS and in water, stained in cresyl violet, washed in water, dehydrated, and coverslipped.

2.3. LM morphometric analysis

2.3.1. Volumetric determinations—Neocortex and forebrain volumes were measured in Heidenhain-stained sections using the Cavalieri method [31] and StereoInvestigator software. For these measurements, 16 sections through the rostral-caudal extent of the forebrain (Bregma 5.5 to −9.5 mm) were digitized (Duoscan T2500 scanner, AGFA). A grid (100 × 100 μm) was superimposed over the section (illustrated schematically for the right hemisphere in Figure 1A). The areas of the neocortex (cortex dorsal to the rhinal fissure, shown as the stippled area of the left hemisphere in Figure 1A) and the non-neocortex forebrain were calculated by the software from the grid size and the number of intersections lying over the tissue. The neocortex and total forebrain (neocortex + non-neocortex forebrain) areas were multiplied by the section thickness (40 μm) and separation between measured sections (960 μm) to determine the volumes.

2.3.2. Decussation areas of commissures—The decussation areas at midline of 3 major forebrain commissures were determined in Heidenhain-stained sections: CC (Figure 2A–D, Bregma 1.0 to −5.6 mm), AC (Figure 2A and B, Bregma −0.2 to −0.7 mm), and dorsal hippocampal commissure (DHC, Figure 2C and D, Bregma −1.8 mm to −5.2 mm). All sections were of equal thickness. In every 24th section through the CC and DHC and in every other section through the AC, the height of each commissure was measured at midline, multiplied by the section thickness (40 μm) and separation, and summed across the rostrocaudal extent of the commissure to derive the midline decussation area for that commissure. This method permitted derivation of commissure areas at midline from coronal sections.

2.3.3. Quantification of oligodendrocyte number—Oligodendrocytes in the CC and AC were quantified stereologically in Nissl-stained sections using the optical disector method [19,30] and StereoInvestigator software (MicroBrightField; Burlington, VT). Oligodendrocytes identified morphologically [28] by size, shape, cytoplasmic density, and alignment in relation to decussating fibers (Figure 3C, D, E, and F) were counted within 100 μm of the midline throughout the rostrocaudal extent of the CC and AC. Within each brain region, oligodendrocytes were quantified in counting boxes (counting frame = 25 × 25 μm, disector height = 10 μm) that were distributed in a systematically random manner by the software (sampling grid size: 187.55 × 66.13 μm² for CC and 88.8 × 66.9 μm² for AC). Oligodendrocytes to be counted were visualized by focusing through the disector height, and the N_v of cells per mm³ was calculated using the following equation: $N_v = \sum Q / (\sum a \cdot h)$, where Q is the number of oligodendrocytes per counting box, a is the area of the counting frame, and h is the height of the optical disector. The average number of oligodendrocytes per mm³ was derived for each section. Animal means for CC and AC were calculated by averaging the N_v from all the sections from each animal.

2.4. Tissue preparation for EM

Brain tissue from another cohort of rats (n=8 Sham-IR, n=8 WBI) was embedded in plastic for EM analysis of myelinated axons in AC and for additional LM analysis. These rats were anesthetized and perfused transcardially with 1.3 M cacodylate buffer (pH 7.4) followed by fixative (2% paraformaldehyde/2% glutaraldehyde). Brains were removed and vibratome-sectioned in the sagittal plane. Blocks containing AC were osmicated, dehydrated, embedded in araldite plastic, and sectioned at 1 μm (semithin sections) and 700 Å (thin sections). Semithin sections were mounted on slides, stained with toluidine blue, and coverslipped. Thin sections

were mounted on formvar-coated slot grids, stained with lead citrate and uranyl acetate, and viewed on a Zeiss 10-CA electron microscope.

2.5. Quantitative analysis of axon number and myelin thickness in the AC

In sagittal sections, the AC can be divided into a larger, darkly-stained anterior limb and a smaller, lightly-stained posterior limb [32] (Figure 5A and B). In semithin sections, the areas of both limbs and the total number of myelinated axons in each limb were quantified using StereoInvestigator software (counting box: $5 \times 5 \times 1 \mu\text{m}^3$; sampling grids, $100 \times 100 \mu\text{m}^2$, anterior limb; $75 \times 75 \mu\text{m}^2$, posterior limb). In thin sections, the myelin thickness and cross-sectional area of myelinated axons were quantified in the anterior limb. For this analysis, digitized electron micrographs (8000X) from randomly distributed anterior limb locations were visualized and myelin thickness was measured at 3 points around the axon perimeter (Figure 5C and D) using Scion Image software (NIH). The cross-sectional areas of the same myelinated axons were measured (Scion Image software). Mean myelin thickness and axon area were calculated for each animal from 750 axons per brain.

2.6. Statistical analysis

All data were analyzed using SigmaStat software (Systat Software Inc., Chicago, IL). Parameters were compared between Sham-IR and WBI groups with Student's *t* tests. All data are presented as the mean $\pm$ SEM with $p < 0.05$ considered as significant.

3. Results

3.1. Brain volume

Volume measurements (Figure 1B) revealed that the forebrain volume in WBI rats ($11.72 \pm 0.16 \times 10^2 \text{ mm}^3$) was significantly smaller ($p = 0.027$) than in Sham-IR rats ($12.42 \pm 0.24 \times 10^2 \text{ mm}^3$), but that the volume of neocortex did not differ between groups ($4.69 \pm 0.03 \times 10^2 \text{ mm}^3$ WBI vs $4.94 \pm 0.03 \times 10^2 \text{ mm}^3$ Sham-IR, $p = 0.092$).

3.2. Integrity and size of the major myelinated axon bundles

Qualitative comparison of myelin integrity in Heidenhain-stained sections did not reveal gross evidence of radiation-induced demyelination or white matter necrosis (Figure 2A and B). The integrity of the white matter is evident not only in the appearance of tracts such as CC and AC, but also in the distribution of myelinated axons to superficial layers of cerebral cortex.

Morphometric reconstruction of the CC, DHC, and AC in Heidenhain sections provides a measure of the decussation areas of the commissures at midline and a useful means to compare those myelinated fiber bundles between groups (Figure 2E). Although the area of the CC at midline is smaller in WBI than in Sham-IR rats, the difference fails to reach statistical significance ($3.42 \pm 0.13 \text{ mm}^2$ WBI vs $3.76 \pm 0.12 \text{ mm}^2$ Sham-IR, $p = 0.07$). Comparisons of the DHC and AC midline decussation areas indicate no WBI-induced difference (DHC: $0.58 \pm 0.03 \text{ mm}^2$ WBI vs $0.54 \pm 0.07 \text{ mm}^2$ Sham-IR, $p = 0.56$; AC: $0.36 \pm 0.01 \text{ mm}^2$ WBI vs $0.34 \pm 0.01 \text{ mm}^2$ Sham-IR, $p = 0.15$).

3.3. Quantification of AC and CC oligodendrocytes

As in Heidenhain-stained sections, the overall appearance of neural tissue in Nissl-stained sections was quite similar in Sham-IR and WBI rats (Figure 3A and B). Oligodendrocytes could be readily identified in rows oriented parallel to decussating axons in the CC (Figure 3C and D) and AC (Figure 3E and F) and were quantified throughout the anterior-posterior and dorsal-ventral extents of these commissures. However, since the lateral borders of the AC and CC cannot be defined unequivocally, quantification was limited to a $100 \mu\text{m}$ width on either

side of midline. No significant differences in the number of oligodendrocytes were found between the Sham-IR and WBI groups (CC: 3.3 ± 0.4 per mm^3 WBI vs $3.0 \pm 0.2 \times 10^5$ per mm^3 Sham IR, $p=0.12$; AC: $4.3 \pm 0.9 \times 10^5$ per mm^3 WBI vs $4.1 \pm 0.8 \times 10^5$ per mm^3 Sham IR, $p=0.65$) (Figure 4).

3. 4. Axon number in the AC

In order to provide a more thorough evaluation of the late-delayed effects of WBI on myelinated axons in the brain, $1\mu\text{m}$ sagittal sections of the AC were analyzed with light microscopy. Low-power photomicrographs of the AC from Sham-IR and WBI rats are shown in Figure 5A and B. As in the Heidenhain sections, myelinated axons in WBI rats appear quite similar to those in the Sham-IR group. Comparisons of the area of the darkly-stained anterior limb and lightly-stained posterior limb of the AC revealed no differences between the groups (anterior limb: $0.216 \pm 0.010 \text{ mm}^2$ WBI vs $0.215 \pm 0.007 \text{ mm}^2$ Sham-IR, $p=0.91$; posterior limb: $0.096 \pm 0.003 \text{ mm}^2$ WBI vs $0.093 \pm 0.003 \text{ mm}^2$ Sham-IR, $p=0.567$). The total area of the AC at midline ($0.313 \pm 0.012 \text{ mm}^2$ for WBI and $0.309 \pm 0.009 \text{ mm}^2$ for Sham-IR, $p=0.806$) was approximately the same as the decussation area of the AC at midline that was derived from the Heidenhain sections, validating the accuracy of these measures.

In these same sections, quantification of the total number of myelinated axons in the anterior limb, posterior limb, and entire AC revealed no radiation-induced difference (anterior limb: $1.05 \pm 0.08 \times 10^5$ WBI vs $1.05 \pm 0.07 \times 10^5$ Sham-IR, $p=0.96$; posterior limb: $0.55 \pm 0.03 \times 10^5$ WBI vs $0.56 \pm 0.03 \times 10^5$ Sham-IR, $p=0.79$; entire AC: $1.59 \pm 0.10 \times 10^5$ WBI vs $1.61 \pm 0.08 \times 10^5$ Sham-IR, $p=0.96$).

3. 5. Myelin thickness and cross-sectional area of myelinated axons in the AC

A detailed morphometric analysis of the myelin thickness and cross-sectional area of individual myelinated axons in the anterior limb of the AC was performed on thin sections. In order to analyze comparable axon populations, only myelinated axons cut perpendicular to the long axis of the axon as judged by axon shape were selected for analysis. Electron micrographs of these AC axons demonstrate the similarity in appearance of the myelin sheaths between the Sham-IR and WBI groups (Figure 5C and D, respectively). Moreover, the myelin sheath thickness does not differ ($p=0.58$) between the WBI ($0.0958 \pm 0.002 \mu\text{m}$) and Sham-IR ($0.090 \pm 0.010 \mu\text{m}$) groups and is consistent with published AC myelin sheath thickness in rodents [33,34]. A histogram of the frequency distribution of myelin thickness demonstrates the overall similarity in distribution patterns between groups (Figure 5E). Likewise, neither the cross-sectional areas of myelinated AC axons ($0.20 \pm 0.01 \mu\text{m}^2$ WBI vs $0.25 \pm 0.03 \mu\text{m}^2$ Sham-IR, $p=0.14$), nor their distribution (Figure 5F) differ between the two groups.

4. Discussion

The present findings indicate that myelin integrity is maintained in 24-month old F344xBN rats one year following 45 Gy of fractionated WBI, the same rodent model in which WBI leads to spatial learning and memory impairments as well as glutamate receptor abnormalities [18, 19]. Specifically, quantitative morphometric analyses of the white matter in the brains of WBI rats in this model did not reveal changes in the i] size of major forebrain commissures, ii] number of oligodendrocytes in AC or CC, iii] thickness of axon myelin sheaths in AC, or iv] number and size of myelinated axons in AC.

It has been well-established that WBI is associated with late-delayed cognitive impairments in the clinic [35,36] as well as in animal models [37,38,39,40]. In particular, the fractionated WBI regimen used in the present study has been shown to lead to impairment in the MWM test 12 months following WBI at middle age [18]. The animals in that study acted as the behavioral

index for the rats in the present study since both have the same experimental design including animal age, WBI dosage, and post-WBI survival period. Moreover, young (12 – 14 weeks) F344xBN rats receiving the same regimen of fractionated WBI that was used here, demonstrated impairments on the Novel Object Recognition (NOR) test, a memory task that involves the hippocampus as well as non-hippocampal regions [40,41]. Thus, following this regimen of fractionated WBI, both hippocampal- and non-hippocampal-dependent cognitive impairments occur in a chronic and progressive pattern that is similar to the development of brain injuries in patients following brain irradiation [10,42].

Despite the cognitive impairment evident in this model, qualitative examination revealed no gross evidence of white matter necrosis or frank demyelination. The lack of gross white matter change following fractionated WBI in this model is consistent with observations in recent studies suggesting that this WBI regimen did not result in gross histological changes indicative of demyelination, hemorrhage, or neuronal damage [18,40,43]. Similarly, in clinical studies, mild-to-moderate cognitive impairment has been reported to occur in the absence of neuroimaging abnormalities [44,45,46]. In an earlier rodent study in which 20 Gy of WBI was delivered as 5 fractions over 5 days (single dose equivalence, 11 Gy), the authors also found no evidence of myelin damage 1 week, 1 month, or 6 months after treatment [47]. Although frank demyelination and white matter necrosis have been reported in the rodent spinal cord [16] and optic nerve [17] following single doses of 22 Gy and 30 Gy, respectively, those doses were higher than both the 11 Gy single dose equivalence in the previously mentioned study and that in the present study (17.6 Gy). In addition, both the optic nerve and spinal cord are actually separate from the brain, and received collimated radiation to those structures rather than WBI. However, single doses of WBI greater than 20 Gy have been reported to result in a late-delayed dose-related necrosis of major white matter tracts including the corpus callosum [49], supporting the notion that the dose of fractionated WBI used in the present study is not sufficiently high to lead to gross white matter changes despite the fact that it leads to cognitive impairment.

Consistent with the cognitive impairment we have reported in this model, the WBI rats in the present study had a lower forebrain volume than the Sham-IR rats. This observation corroborates earlier reports suggesting that brain volume is a predictor of cognitive impairment [50,51]. Since we have previously reported that neuron number did not decrease in the hippocampus following WBI [19], the present study focused on potential myelin changes in the white matter of the brain. Myelin integrity in the brain is a major determinant of cognitive performance [23,24,52,53]. Moreover, earlier studies indicated white matter degeneration may have a greater contribution to cognitive decline in aging than does gray matter deterioration [24]. Since multiple regions of the brain may be involved in radiation-induced cognitive impairment [24,40], the present study analyzed myelin integrity in three major white matter pathways in the forebrain, the CC, AC, and DHC. By quantifying a broad range of morphometric measures related to myelinated axons, we provide a panel of criteria for objective assessment of the presence and extent of radiation-induced change to the structure of white matter.

Quantitative analysis of the structural features of white matter at both the light and electron microscopic level reveals maintenance of the basic elements of myelinated axons in the brains of WBI rats. Despite the decline in the overall volume of forebrain, the size of CC, AC, and DHC at midline did not differ between groups and no change was detected in oligodendrocyte number in either CC or AC one year following WBI at middle age. The stability of these measures is consistent with the absence of WBI-induced volume change seen here in neocortex and reported previously in hippocampus [19]. Taken together, these observations raise the possibility that the small difference in forebrain volume in fact may not be biologically meaningful, and instead may be due to the extremely small standard error of the data.

Nevertheless, it should be kept in mind that other neurobiological changes such as alterations in the extracellular matrix or in axodendritic synaptic fields could contribute to alterations in brain volume and merit future investigation.

Importantly, oligodendrocytes are the myelinating glia in the brain and the glial hypothesis of radiation-induced white matter damage suggests that progressive myelin pathology is due to a gradual radiation-induced loss of oligodendrocytes or their precursors [54]. Indeed, Kurita and colleagues found rapid apoptotic depletion of the oligodendrocyte population in 24 hours post-WBI [55]. The absence of late-delayed WBI-induced loss of oligodendrocytes in the present study does not rule out the possibility of a transient loss of oligodendrocytes that is compensated for by newly generated oligodendrocytes during the 12 months post-WBI period. This possibility would be consistent with the results of an earlier study showing an immediate radiation-induced depletion of oligodendrocyte progenitor cells within cortex, corpus callosum and hippocampus followed by a progressive repopulation by oligodendrocyte progenitor cells from non-irradiated cortical regions [56]. Because both the thickness of myelin sheath and the cross-sectional area of myelinated axons determine the speed at which impulses propagate along the myelinated fibers [34,57], we evaluated these measures at the ultrastructural level. Neither of these measures, nor the number of myelinated axons, differed in the AC following WBI, providing compelling evidence that the cognitive impairment in this model can occur in the absence of structural change to myelin integrity.

In summary, the 45 Gy dose of fractionated WBI at middle age that has been shown to lead to cognitive impairment does not result in either gross morphological changes to myelin integrity or subtle cellular changes indicative of demyelination. Nevertheless, the presence of cognitive impairment in this model suggests that WBI must elicit neurobiological changes that lead to altered neural processing. For example, it may be that WBI induces subtle alterations in myelin that are not detectable with the morphometric and histological techniques in the present study or changes to the extracellular matrix. In addition, recent studies using sensitive, non-invasive imaging tools have revealed subtle changes in metabolites in the brain of cognitively-impaired young adult WBI rats using the technique of magnetic resonance spectroscopy [58] as well as subtle changes associated with white matter damage using diffusion tensor imaging [54] in young WBI rats. Studies using these advanced techniques will be needed to further investigate neurobiological changes in this model of cognitive impairment following fractionated WBI at middle age.

Acknowledgments

This work was supported by NIH grants CA119990 and CA112593 as well as the Tab Williams Jr. Family Neuroscience Research Endowment

References

- [1]. Bentzen SM. Preventing or reducing late side effects of radiation therapy: radiobiology meets molecular pathology. *Nat Rev Cancer* 2006;6:702–713. [PubMed: 16929324]
- [2]. Armstrong CL, Gyato K, Awadalla AW, Lustig R, Tochner ZA. A critical review of the clinical effects of therapeutic irradiation damage to the brain: the roots of controversy. *Neuropsychol Rev* 2004;14:65–86. [PubMed: 15260139]
- [3]. Keime-Guibert F, Napolitano M, Delattre JY. Neurological complications of radiotherapy and chemotherapy. *J Neurol* 1998;245:695–708. [PubMed: 9808237]
- [4]. Shaw EG, Rosdhal R, D'Agostino RB Jr, Lovato J, Naughton MJ, Robbins ME, et al. Phase II study of donepezil in irradiated brain tumor patients: effect on cognitive function, mood, and quality of life. *J Clin Oncol* 2006;24:1415–1420. [PubMed: 16549835]

- [5]. Yabroff KR, Lawrence WF, Clauser S, Davis WW, Brown ML. Burden of illness in cancer survivors: findings from a population-based national sample. *J Natl Cancer Inst* 2004;96:1322–1330. [PubMed: 15339970]
- [6]. Mulhern RK, Merchant TE, Gajjar A, Reddick WE, Kun LE. Late neurocognitive sequelae in survivors of brain tumours in childhood. *Lancet Oncol* 2004;5:399–408. [PubMed: 15231246]
- [7]. Munter MW, Karger CP, Reith W, Schneider HM, Peschke P, Debus J. Delayed vascular injury after single high-dose irradiation in the rat brain: histologic immunohistochemical, and angiographic studies. *Radiology* 1999;212:475–482. [PubMed: 10429706]
- [8]. Lyubimova N, Hopewell JW. Experimental evidence to support the hypothesis that damage to vascular endothelium plays the primary role in the development of late radiation-induced CNS injury. *Br J Radiol* 2004;77:488–492. [PubMed: 15151969]
- [9]. Wassenberg MW, Bromberg JE, Witkamp TD, Terhaard CH, Taphoorn MJ. White matter lesions and encephalopathy in patients treated for primary central nervous system lymphoma. *J Neurooncol* 2001;52:73–80. [PubMed: 11451205]
- [10]. Tofilon PJ, Fike JR. The radioresponse of the central nervous system: a dynamic process. *Radiat Res* 2000;153:357–370. [PubMed: 10798963]
- [11]. Fujii O, Tsujino K, Soejima T, Yoden E, Ichimiya Y, Sugimura K. White matter changes on magnetic resonance imaging following whole-brain radiotherapy for brain metastases. *Radiat Med* 2006;24:345–350. [PubMed: 16958412]
- [12]. Reinhold HS, Calvo W, Hopewell JW, van der Berg AP. Development of blood vessel-related radiation damage in the fimbria of the central nervous system. *Int J Radiat Oncol Biol Phys* 1990;18:37–42. [PubMed: 2298633]
- [13]. Panagiotakos G, Alshamy G, Chan B, Abrams R, Greenberg E, Saxena A, et al. Long-term impact of radiation on the stem cell and oligodendrocyte precursors in the brain. *PLoS ONE* 2007;2:e588. [PubMed: 17622341]
- [14]. Fouladi M, Chintagumpala M, Laningham FH, Ashley D, Kellie SJ, Langston JW, et al. White matter lesions detected by magnetic resonance imaging after radiotherapy and high-dose chemotherapy in children with medulloblastoma or primitive neuroectodermal tumor. *J Clin Oncol* 2004;22:4551–4560. [PubMed: 15542806]
- [15]. Kitahara S, Nakasu S, Murata K, Sho K, Ito R. Evaluation of treatment-induced cerebral white matter injury by using diffusion-tensor MR imaging: initial experience. *AJNR Am J Neuroradiol* 2005;26:2200–2206. [PubMed: 16219822]
- [16]. Atkinson S, Li YQ, Wong CS. Changes in oligodendrocytes and myelin gene expression after radiation in the rodent spinal cord. *Int J Radiat Oncol Biol Phys* 2003;57:1093–1100. [PubMed: 14575841]
- [17]. Kim JH, Brown SL, Kolozsvary A, Jenrow KA, Ryu S, Rosenblum ML, et al. Modification of radiation injury by ramipril, inhibitor of angiotensin-converting enzyme, on optic neuropathy in the rat. *Radiat Res* 2004;161:137–142. [PubMed: 14731077]
- [18]. Shi L, Adams MM, Long A, Carter CC, Bennett C, Sonntag WE, et al. Spatial learning and memory deficits after whole-brain irradiation are associated with changes in NMDA receptor subunits in the hippocampus. *Radiat Res* 2006;166:892–899. [PubMed: 17149974]
- [19]. Shi L, Molina DP, Robbins ME, Wheeler KT, Brunso-Bechtold JK. Hippocampal neuron number is unchanged 1 year after fractionated whole-brain irradiation at middle age. *Int J Radiat Oncol Biol Phys* 2008;71:526–532. [PubMed: 18474312]
- [20]. Jemal A, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, et al. Cancer statistics, 2004. *CA Cancer J Clin* 2004;54:8–29. [PubMed: 14974761]
- [21]. Turken A, Whitfield-Gabrieli S, Bammer R, Baldo JV, Dronkers NF, Gabrieli JD. Cognitive processing speed and the structure of white matter pathways: convergent evidence from normal variation and lesion studies. *Neuroimage* 2008;42:1032–1044. [PubMed: 18602840]
- [22]. Chiang MC, Barysheva M, Shattuck DW, Lee AD, Madsen SK, Avedissian C, et al. Genetics of brain fiber architecture and intellectual performance. *J Neurosci* 2009;29:2212–2224. [PubMed: 19228974]
- [23]. Madden DJ, Spaniol J, Costello MC, Bucur B, White LE, Cabeza R, et al. Cerebral White Matter Integrity Mediates Adult Age Differences in Cognitive Performance. *J Cogn Neurosci*. 2008

- [24]. Ziegler DA, Piguet O, Salat DH, Prince K, Connally E, Corkin S. Cognition in healthy aging is related to regional white matter integrity, but not cortical thickness. *Neurobiol Aging*. 2008
- [25]. Van der Kogel, AJ. The nervous system: Radiobiology and experimental pathology. In: Scherer, E.; Streffer, C.; Trott, KR., editors. *Radiopathology of Organs and Tissues*. 1991. p. 191-212.
- [26]. Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. Vol. 1st Edition. Academic Press; San Diego, CA: 1986.
- [27]. Hutchins B, Weber JT. A rapid myelin stain for frozen sections: modification of the Heidenhain procedure. *J Neurosci Methods* 1983;7:289–294. [PubMed: 6188930]
- [28]. Hamidi M, Drevets WC, Price JL. Glial reduction in amygdala in major depressive disorder is due to oligodendrocytes. *Biol Psychiatry* 2004;55:563–569. [PubMed: 15013824]
- [29]. Hof PR, Haroutunian V, Friedrich VL Jr, Byne W, Buitron C, Perl DP, et al. Loss and altered spatial distribution of oligodendrocytes in the superior frontal gyrus in schizophrenia. *Biol Psychiatry* 2003;53:1075–1085. [PubMed: 12814859]
- [30]. Shi L, Linville MC, Tucker EW, Sonntag WE, Brunso-Bechtold JK. Differential effects of aging and insulin-like growth factor-1 on synapses in CA1 of rat hippocampus. *Cereb Cortex* 2005;15:571–577. [PubMed: 15319312]
- [31]. Mouton, PR. Principles and practices of unbiased stereology. 2002.
- [32]. Cheng CM, Joncas G, Reinhardt RR, Farrer R, Quarles R, Janssen J, et al. Biochemical and morphometric analyses show that myelination in the insulin-like growth factor 1 null brain is proportionate to its neuronal composition. *J Neurosci* 1998;18:5673–5681. [PubMed: 9671658]
- [33]. Ye P, Carson J, D’Ercole AJ. Insulin-like growth factor-I influences the initiation of myelination: studies of the anterior commissure of transgenic mice. *Neurosci Lett* 1995;201:235–238. [PubMed: 8786848]
- [34]. Ye P, Carson J, D’Ercole AJ. In vivo actions of insulin-like growth factor-I (IGF-I) on brain myelination: studies of IGF-I and IGF binding protein-1 (IGFBP-1) transgenic mice. *J Neurosci* 1995;15:7344–7356. [PubMed: 7472488]
- [35]. Crossen JR, Garwood D, Glatstein E, Neuwelt EA. Neurobehavioral sequelae of cranial irradiation in adults: a review of radiation-induced encephalopathy. *J Clin Oncol* 1994;12:627–642. [PubMed: 8120563]
- [36]. Surma-aho O, Niemela M, Vilkkilä J, Kouri M, Brander A, Salonen O, et al. Adverse long-term effects of brain radiotherapy in adult low-grade glioma patients. *Neurology* 2001;56:1285–1290. [PubMed: 11376174]
- [37]. Rola R, Raber J, Rizk A, Otsuka S, VandenBerg SR, Morhardt DR, et al. Radiation-induced impairment of hippocampal neurogenesis is associated with cognitive deficits in young mice. *Exp Neurol* 2004;188:316–330. [PubMed: 15246832]
- [38]. Yoneoka Y, Satoh M, Akiyama K, Sano K, Fujii Y, Tanaka R. An experimental study of radiation-induced cognitive dysfunction in an adult rat model. *Br J Radiol* 1999;72:1196–1201. [PubMed: 10703477]
- [39]. Madsen TM, Kristjansen PE, Bolwig TG, Wortwein G. Arrested neuronal proliferation and impaired hippocampal function following fractionated brain irradiation in the adult rat. *Neuroscience* 2003;119:635–642. [PubMed: 12809684]
- [40]. Robbins ME, Payne V, Tommasi E, Diz DI, Hsu FC, Brown WR, et al. The AT1 receptor antagonist, L-158,809, prevents or ameliorates fractionated whole-brain irradiation-induced cognitive impairment. *Int J Radiat Oncol Biol Phys* 2009;73:499–505. [PubMed: 19084353]
- [41]. Bussey TJ, Duck J, Muir JL, Aggleton JP. Distinct patterns of behavioural impairments resulting from fornix transection or neurotoxic lesions of the perirhinal and postrhinal cortices in the rat. *Behav Brain Res* 2000;111:187–202. [PubMed: 10840144]
- [42]. Gregor A, Cull A, Traynor E, Stewart M, Lander F, Love S. Neuropsychometric evaluation of long-term survivors of adult brain tumours: relationship with tumour and treatment parameters. *Radiother Oncol* 1996;41:55–59. [PubMed: 8961368]
- [43]. Brown WR, Thore CR, Moody DM, Robbins ME, Wheeler KT. Vascular damage after fractionated whole-brain irradiation in rats. *Radiat Res* 2005;164:662–668. [PubMed: 16238444]
- [44]. Dropcho EJ. Central nervous system injury by therapeutic irradiation. *Neurol Clin* 1991;9:969–988. [PubMed: 1758435]

- [45]. Sepe-Monti M, De Carolis A, Bomboi G, Castri P, Giubilei F. MRI evidence of bilateral hippocampal sclerosis in amnesic mild cognitive impairment. *Eur J Neurol* 2006;13:1031–1032. [PubMed: 16930374]
- [46]. van Harten B, Oosterman J, Muslimovic D, van Loon BJ, Scheltens P, Weinstein HC. Cognitive impairment and MRI correlates in the elderly patients with type 2 diabetes mellitus. *Age Ageing* 2007;36:164–170. [PubMed: 17350976]
- [47]. Mildenberger M, Beach TG, McGeer EG, Ludgate CM. An animal model of prophylactic cranial irradiation: histologic effects at acute, early and delayed stages. *Int J Radiat Oncol Biol Phys* 1990;18:1051–1060. [PubMed: 2347714]
- [48]. Fowler JF. Brief summary of radiobiological principles in fractionated radiotherapy. *Semin Radiat Oncol* 1992;2:16–21.
- [49]. Calvo W, Hopewell JW, Reinhold HS, Yeung TK. Time- and dose-related changes in the white matter of the rat brain after single doses of X rays. *Br J Radiol* 1988;61:1043–1052. [PubMed: 3208008]
- [50]. Pol, HE Hulshoff; Schnack, HG.; Mandl, RC.; Brans, RG.; van Haren, NE.; Baare, WF., et al. Gray and white matter density changes in monozygotic and same-sex dizygotic twins discordant for schizophrenia using voxel-based morphometry. *Neuroimage* 2006;31:482–488. [PubMed: 16497519]
- [51]. Moffat SD, Kennedy KM, Rodrigue KM, Raz N. Extrahippocampal contributions to age differences in human spatial navigation. *Cereb Cortex* 2007;17:1274–1282. [PubMed: 16857855]
- [52]. Charlton RA, Landau S, Schiavone F, Barrick TR, Clark CA, Markus HS, et al. A structural equation modeling investigation of age-related variance in executive function and DTI measured white matter damage. *Neurobiol Aging* 2008;29:1547–1555. [PubMed: 17451845]
- [53]. Makris N, Papadimitriou GM, van der KA, Kennedy DN, Hodge SM, Dale AM, et al. Frontal connections and cognitive changes in normal aging rhesus monkeys: a DTI study. *Neurobiol Aging* 2007;28:1556–1567. [PubMed: 16962214]
- [54]. Wang S, Wu EX, Qiu D, Leung LH, Lau HF, Khong PL. Longitudinal diffusion tensor magnetic resonance imaging study of radiation-induced white matter damage in a rat model. *Cancer Res* 2009;69:1190–1198. [PubMed: 19155304]
- [55]. Kurita H, Kawahara N, Asai A, Ueki K, Shin M, Kirino T. Radiation-induced apoptosis of oligodendrocytes in the adult rat brain. *Neurol Res* 2001;23:869–874. [PubMed: 11760880]
- [56]. Irvine KA, Blakemore WF. A different regional response by mouse oligodendrocyte progenitor cells (OPCs) to high-dose X-irradiation has consequences for repopulating OPC-depleted normal tissue. *Eur J Neurosci* 2007;25:417–424. [PubMed: 17284182]
- [57]. Peters A, Moss MB, Sethares C. Effects of aging on myelinated nerve fibers in monkey primary visual cortex. *J Comp Neurol* 2000;419:364–376. [PubMed: 10723011]
- [58]. Atwood T, Payne VS, Zhao W, Brown WR, Wheeler KT, Zhu JM, et al. Quantitative magnetic resonance spectroscopy reveals a potential relationship between radiation-induced changes in rat brain metabolites and cognitive impairment. *Radiat Res* 2007;168:574–581. [PubMed: 17973545]

Figure 1. Measurement of brain volume

A. To measure the size of neocortex (cortex dorsal to rhinal fissure, arrow, and indicated by stippled area on left) and non-neocortex forebrain (all other forebrain tissue), grids of pre-determined size (shown schematically on right) were overlaid on Heidenhain-stained sections using StereoInvestigator software. Each grid intersection over brain tissue was identified as neocortex or non-neocortex forebrain and the volume of each region was derived based on the total number of intersections, grid size, section thickness, and section separation. **B.** Forebrain volume (neocortex + non-neocortex forebrain), but not neocortex volume, was significantly smaller in whole-brain irradiated (WBI) compared to sham-irradiated (Sham-IR) rats ($p=0.017$ and $p=0.092$, respectively). Bar = 2.5 mm.

Figure 2. Measurement of commissure area at midline

White matter integrity and distribution appear similar in sections from Sham-IR (A) and WBI (B) rats processed with the Heidenhain stain for myelinated axons (Bregma -0.3). The areas of three major brain commissures at midline were measured: corpus callosum (CC), anterior commissure (AC), and the dorsal hippocampal commissure (DHC). Panel C (Bregma -4.5 mm) illustrates the CC and DHC at higher magnification. The same image is shown in D with different lighting to demonstrate the distinction between CC and DHC. Comparison of commissure areas at midline (E) revealed a trend for CC to be smaller in WBI than Sham-IR rats, but the difference failed to reach significance ($p=0.07$); neither AC nor DHC differed between groups ($p=0.15$ and $p=0.56$, respectively). Bar = 2.5mm (A & B); 0.8 mm (C & D).

Figure 3. Oligodendrocytes in corpus callosum and anterior commissure

The overall appearance of the brain tissue is similar in Nissl-stained sections from Sham-IR (A) and WBI rats (B). Oligodendrocytes in the CC (C and D) and AC (E and F) were identified at higher magnification by size, shape, cytoplasmic density, and alignment parallel to decussating fibers (insets illustrate magnification used for quantification). Bar = 2.5 mm (A & B); 250 μ m (C-F).

Figure 4. Oligodendrocytes quantification in corpus callosum and anterior commissure
Oligodendrocyte density did not differ between Sham-IR and WBI rats in either CC or AC ($p=0.12$ and $p=0.65$, respectively).

Figure 5. Anterior commissure at midline and measurement of myelin thickness and axon area in the anterior limb of anterior commissure

Light microscopic images of semithin, sagittal sections of the AC at midline from Sham-IR and WBI rats are shown in **A** and **B**, respectively. Electron microscopic images reveal that the ultrastructural appearance of myelinated AC axons was similar between Sham-IR and WBI rats (**C** and **D**, respectively). Asterisks indicate examples of myelinated axons that were analyzed. Neither the myelin thickness (measured at 3 positions indicated by white lines) nor the cross-sectional area of individual axons within myelin sheath differed between the two groups (see text). The distributions of myelin thickness (**E**) and of axon area (**F**) were similar in the Sham-IR and WBI groups. Ant = anterior limb; Post = posterior limb. Bar = 160 μm (**A** & **B**); Bar = 1 μm (**C** & **D**).