Behavioral Effects of Focal Irradiation in a Juvenile Murine Model

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Chemotherapy has been successfully used to reduce radiation dose and volume for most pediatric patients. However, because of the failure of chemotherapeutic agents to cross the blood-brain barrier and the lack of response of some brain tumors to these agents, radiation therapy is still used to treat many childhood cancers with CNS involvement. In this study, we investigated the radiation effects on cognition and dendritic structure in the hippocampus in juvenile male mice. Twenty-one-day-old male C57BL/6 mice were irradiated using the small animal radiation research platform (SARRP). Animals were exposed to either a 10 Gy single dose or 10 Gy $\times$ 2 fractionated doses of X-ray cranial radiation. Five weeks after irradiation, animals were tested for hippocampus-dependent cognitive performance in the Morris water maze. Significant impairment in spatial memory retention was observed in the probe trial after the first day of hidden-platform training (first probe trial) in animals that received either 10 Gy single-dose or 10 Gy $\times$ 2 fractionated doses. However, by day 5, mice that received a 10 Gy single dose showed spatial memory retention in the probe trials, whereas mice that received the 20 Gy fractionated doses remained impaired. During Y-maze testing, animals exposed to radiation were impaired; the irradiated mice were not able to distinguish among the three Y-maze arms and spent approximately the same amount of time in all three arms during the retention trial. Radiation significantly compromised the dendritic architecture and reduced spine density throughout the hippocampal trisynaptic network. © 2018 by Radiation Research Society

INTRODUCTION

Whole-brain radiation therapy continues to be the standard care for 20% of children with acute lymphoblastic

leukemia (ALL) and for children diagnosed with T-cell ALL or B-precursor ALL and overt central nervous system (CNS) involvement at diagnosis (1). Unfortunately, children receiving radiation therapy are at significant risk for cognitive problems (2) that reduce academic, social and vocational attainment (3). Radiation-induced effects are hypothesized to result from dynamic interactions between the multiple cell types within the brain, including astrocytes, endothelial cells, microglia, neurons and oligodendrocytes (4, 5). However, the precise mechanisms behind radiation-induced memory deficits remain elusive, and there is a critical need for efficacious interventions that target symptoms associated with neurocognitive late effects in pediatric cancer survivors (6).

Cognitive decline after cranial irradiation can manifest as decreases in working memory, cognitive control and flexibility, processing speed, visual searching and planning and attention (7). In the 1970s and 1980s, conformal radiation therapy (CRT) was commonly used in the treatment of ALL because of its effectiveness at killing cells. However, survivors were at high risk of long-term morbidity, including endocrine insufficiencies, second malignant neoplasms, metabolic complications and cardiovascular morbidity (8). In addition to killing neurons, CRT has been associated with destruction of microvasculature in the CNS, which results in white matter necrosis (9). Because white matter in the brain contributes to the formation of synapses and the passing of information between neurocircuitry, white matter necrosis could potentially lead to decreased synapses and subsequent decreased learning and memory capabilities.

Dendrites are the tree-like sets of neuronal fine processes that receive signals from other neurons, and dendritic spines are individual thorn-like structures that are the sites of individual synapses. The morphology of each of these structures influences many aspects of neuronal function, including action potential propagation and information processing. For example, the size of a dendritic spine head is correlated with the strength of the synapse (10), and abnormalities in spine number and morphology have been observed in a number of neurological disorders (11). It is hypothesized that changes in the morphology of dendrites

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and dendritic spines alter synaptic plasticity and neurotransmission, resulting in cognitive dysfunction (10, 12, 13).

Data obtained by us and others have identified the capability of ionizing radiation to compromise the structure of neurons in the hippocampus, an area critical for the acquisition (learning), consolidation and retrieval of declarative memories (14). Gamma-ray and proton doses spanning from 0.1–10.0 Gy have been found to cause significant, dose-responsive reductions in dendritic complexity and spine density in the hippocampus lasting at least one month postirradiation (15–17). However, the majority of these studies have focused on adult murine models. In the current study, mouse brains were irradiated on postnatal day 21, an age approximately equivalent to a juvenile child (18). The purpose of this study was to investigate how focal cranial irradiation affects cognitive performance and dendrite complexity in a juvenile murine model.

METHODS

Animals

Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME), 21 days old ($n = 30$), were used in this study. Mice were irradiated at 3 weeks old. Behavioral testing was performed when mice were 2 months old. Sacrifice was done after behavioral testing when mice were 3 months old. The mice were housed under a constant 12 h light/dark schedule and were cared for in compliance with the University of Arkansas for Medical Sciences (UAMS) Institutional Animal Care and Use Committee.

Small Animal Radiation Research Platform

Male mice were irradiated using the small animal radiation research platform (SARRP). Animals were anesthetized with 1% isoflurane inhalation for the duration of exposure at 0 Gy ($n = 10$), 10 Gy ($n = 10$) or 20 Gy ($n = 10$). Each mouse was placed prone on the horizontal bed in the SARRP. A cone beam computed tomographic image of each mouse was obtained to deliver image-guided radiation targeted to the brain at 60 kVp and 0.8 mA; reconstruction was accomplished with the use of 720 projections. From Merislice, image precision targeting of the brain was determined to avoid the eyes and frontal lobe. One group received one round of 5 Gy $\times$ 2 fractions of X-ray irradiation from 90° and –90° gantry angles with an isocenter tissue depth of 5 mm. A second group received a second round of 5 Gy $\times$ 2 fractions X-ray irradiation with the use of the same gantry and isocenter parameters 1 week later. The brain-targeted radiation doses were delivered with a 0.5-mm copper filter with a 5 $\times$ 5 mm collimator using 220 kVp and 13 mA. All sham-irradiated mice were anesthetized and placed in the SARRP for an equivalent amount time as the irradiated mice. Both the 10 Gy single-dose and 20 Gy fractionated-doses groups were anesthetized at the delivery time of the second fraction to control for effects of anesthesia on cognition.

Y-maze

The Y-maze assesses short-term spatial memory and exploratory activity in a novel environment (19). It consists of three acrylic glass arms (45 $\times$ 15 $\times$ 30 cm), each designated as either the starting arm, the novel arm or the familiar arm. The test consisted of two sets of trials performed 4 h apart. Before the experiment, the animals were assigned to two arms (start and familiar) to which they were exposed during the first phase of the test (training trial). A unique object was secured at

the end of each arm to differentiate start, familiar and novel arms. Allocation of arms (start, familiar and novel) was counterbalanced within each experimental group. During the training trial, mice were placed at the end of the start arm and allowed to freely move and explore the start and familiar arms for 5 min. The novel arm was blocked during the training trial. After 4 h, the second trial (testing trial) was performed, during which mice had free access to all three arms and were allowed to explore the Y-maze for 5 min. Each session was recorded on a CCD video camera located above the maze for automatic behavioral analysis with EthoVision software version 11 (Noldus Information Technology, Sterling, VA).

Morris Water Maze

Assessment of hippocampus-dependent cognitive performance was performed after the Y-maze test. A circular pool (140 cm diameter) was filled with opaque water (24°C), and mice were trained to locate a visible platform (200 lux luminescence). To determine if radiation affects the ability of mice to swim or learn the water maze task, mice were first trained to locate a clearly marked platform (visible platform, days 1 and 2) by means of visual cues strategically placed around the pool. During visible-platform training, the platform was moved to a different quadrant of the pool for each session. Mice were subsequently trained to locate the platform when it was hidden beneath the surface of the opaque water (days 3–5). Hidden-platform training (acquisition) requires mice to learn the location of the hidden-platform on the basis of extramaze spatial cues. In this training, the platform location was kept constant. For both visible- and hidden-platform paradigms, mice were placed into the water facing the edge of the pool in one of nine random locations. There were two daily sessions, spaced 2 h apart, each consisting of three trials (with 10-min intertrial intervals), with the start location changing for each. A trial ended when the mice located the platform. Mice that failed to locate the platform within 60 s were led to the platform by the examiner's placing a finger in front of the mouse's swim path, and the mice were made to stay on the platform for 10 s. Mice that found the platform were taken out of the pool after they were physically on the platform for a minimum of 3 s. Distance moved and probe trials were recorded with the EthoVision XT video tracking system (Noldus Information Technology) set at 6 samples/sec.

To measure spatial memory retention, probe trials (platform removed) were performed 1 h after the last trial on each day of hidden-platform training (i.e., three separate probe trials). For the probe trials, mice were placed into the water in the quadrant opposite the target quadrant (i.e., where the platform was previously located during hidden-platform training) and were allowed to search for the platform for 60 s. The time spent in the target quadrant was compared with the time spent in the three nontarget quadrants. We also used the average velocity and distance to the platform as a measure of performance for the visual- and hidden-platform sessions.

Golgi Staining and Tissue Preparation

Immediately after sacrifice, half-brains ($n = 4$) were subjected to Golgi-Cox staining. Samples were immersed in mercuric chloride solution for impregnation for 2 weeks. Next, samples were immersed in post-impregnation buffer for 2 days. Half-brains were then cut at 150 μ m in 1 $\times$ phosphate buffered saline (PBS) with a microtome. Samples were then placed in wells and washed with 0.01 M PBS buffer (pH 7.4) with Triton™ X-100 (0.3%) (PBS-T). Samples were stained with ammonium hydroxide solution and then immersed in a post-staining buffer (superGolgi Kit; Bioenno Tech, LLC, Santa Ana, CA). Sections were then washed in PBS-T and mounted on 1% gelatin-coated slides and allowed to dry. Sections were then dehydrated with ethanol solutions, cleaned in xylenes and coverslipped with Permount™ mounting medium (Thermo Fisher Scientific™ Inc., Rockford, IL).

Spine Density and Spine Morphology

Spine analyses were performed with blinding to the experimental conditions on coded Golgi-impregnated brain sections containing the dorsal hippocampus. Spines were examined on dendrites of dentate gyrus (DG) granule neurons and apical (stratum radiatum) and basal (stratum oriens) dendrites of CA1 and pyramidal neurons. The neurons that satisfied the following criteria were chosen for analysis in each of the experimental groups: 1. presence of untruncated dendrites; 2. consistent, dark Golgi staining along the entire extent of the dendrites; and 3. relative isolation from neighboring neurons to avoid interference with analysis (20). Three to five dendritic segments, each at least 20 nm in length (21), were analyzed per neuron, and 6–7 neurons were analyzed per brain. Neurons that met staining criteria were traced with the use of a 60× oil objective, a computerized stage and NeuroLucida software version 11 (MBF Bioscience, Williston, VT).

Leukocyte Collection

Animals were sedated using isoflurane (5%) and ~500 µl of blood was collected from the inferior vena cava. Needles were heparinized to prevent clotting. Immediately after collection, blood samples were centrifuged, and the fraction of an anticoagulated blood sample that contained most of the white blood cells and platelets, known as the buffy coat, was isolated for analysis. Number of cells was determined using a Countess™ cell counter (Thermo Fisher Scientific).

Dendritic Morphology Quantification

The explored morphological characteristics included Sholl analysis, total dendritic length, number of branch points and dendritic complexity index (DCI); these characteristics were analyzed with the use of the Neuroexplorer component of the NeuroLucida software. First, we obtained Sholl analysis, which is used to assess the amount and distribution of the arbor at increasing distances from the cell soma (25). The distance between each radius was set to 10 µm for our experiments. The length of dendritic branches within each progressively larger circle was counted from the soma. This provides information concerning the amount and distribution of dendritic material. Next, we performed branch point analysis. A branch point represents a bifurcation of a dendrite when a branch divides into two sub-branches. Branch point analysis is based on the number of bifurcations and the order of the points (26). Lower branch point orders represent proximal regions of the tree, whereas larger orders characterize distal regions. The branch point analysis was used to determine the complexity of the dendritic arborization. The complexity of the dendritic tree is an important phenotypic component of the branching analysis. DCI was determined by the following equation: $DCI = \sum (\text{branch tip orders} + \text{number of branch tips}) \times (\text{total dendritic length} / \text{total number of primary dendrites})$. In the CA1 and area, apical and basal dendrites were analyzed separately.

Data Analysis

Data were expressed as means $\pm$ the standard error of the mean (SEM). All statistical analyses were performed using Prism software version 6.0 (GraphPad Software Inc., LaJolla, CA), and $P < 0.05$ was considered significant. For measures of dendritic intersections, a mixed-factors analysis of variance (ANOVA) tested for the effects of radiation (between-subjects variable) and distance from the cell soma (Sholl radius, repeated-measures variable); Fisher's least significant difference (LSD) post hoc tests followed, when appropriate. A one-way ANOVA followed by Holm's post hoc test was used to evaluate statistical differences between sham-irradiated and irradiated groups in the Y-maze. Visible- and hidden-platform water maze learning curves were analyzed by two-way repeated-measures ANOVA. The Holm's correction was used to control for multiple comparisons. Separate

analyses were performed for the visible- and hidden-platform learning curves. For analysis of performance in the water maze probe trials, one-way ANOVAs were used along with Holm's post hoc test, when appropriate. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Spatial Memory and Exploratory Activity Assessment

Y-maze. Figure 1 shows the time spent in the novel arm compared with the familiar and the start arms of the Y-maze. Sham-irradiated mice exhibited a significant preference for the novel arm over the familiar and the start arms, which indicated normal spatial recognition ($F_{(2,18)} = 25.25$, $P < 0.0001$). In contrast, the 10 Gy ($F_{(2,21)} = 0.005$, $P = 0.99$) and 20 Gy ($F_{(2,21)} = 1.19$, $P = 0.32$) irradiated mice spent similar amounts of time in both the familiar and the novel arms, which suggests that they did not recognize the novel arm as a result of impaired spatial recognition memory.

Discrimination ratios can be interpreted as the animals' "forgetting" which arm was encountered during the familiarization phase; a decrease in the discrimination ratio can occur either as a result of increased exploration of the familiar arm or reduced exploration of the novel arm (22). A group-difference effect on recognition memory was expected, and, indeed, the results showed a statistically significant treatment effect on the discrimination ratios ($F_{(2,20)} = 3.96$, $P < 0.05$). Both 10 and 20 Gy irradiated animals spent equal amounts of time exploring the familiar, novel and start arms (Fig. 2). As for locomotion activity of animals, the percentage of entrances into the novel arm was significantly higher for sham-irradiated mice compared to 20 Gy irradiated mice (Fig. 2).

Morris water maze tests. Cognitive testing using the water maze was performed after the Y-maze test. In this test, a decrease in path length (distance) to the platform indicated an improvement in spatial learning and memory. Swim velocity can influence latency to the target during training sessions. The repeated-measures ANOVA of distance traveled between sham-irradiated and 10 Gy irradiated mice revealed no significant differences in interactions ($F_{(4,56)} = 0.37$, $P = 0.83$) or treatment ($F_{(1,14)} = 0.03$, $P = 0.87$). However, we did see a significant difference in time ($F_{(4,56)} = 22.87$, $P < 0.0001$), meaning that these animals performed better as testing progressed. Next, we compared latency between sham-irradiated and 10 Gy irradiated animals and observed no significant difference in interactions ($F_{(4,56)} = 0.48$, $P = 0.75$) or treatment ($F_{(1,4)} = 0.005$, $P = 0.95$; Fig. 3A and B). Finally, we assessed the mean latency to the platform in 10 vs. 20 Gy irradiated mice and found significant differences in interactions ($F_{(4,56)} = 4.65$, $P < 0.01$), time ($F_{(4,56)} = 26.91$, $P < 0.0001$) and treatment ($F_{(1,14)} = 14.12$, $P < 0.01$). Multiple comparisons revealed that 20 Gy irradiated mice spent more time locating the platform on day 3 and 4 ($P < 0.05$) and day 5 ($P < 0.0001$). In addition, we examined distance moved, and found significant

FIG. 1. Y-maze testing shows that radiation impaired short-term memory. Sham-irradiated mice (panel A) spent more time exploring the novel arm than start of familiar. Irradiated mice (panels B and C) could not distinguish among the three Y-maze arms and spent the same proportion of time exploring the familiar arm during the retention trial as the novel and start arms. These data indicate that the irradiated mice did not recognize the novel environment when exposed to it 4 h later. Each datum point represents the mean of 9–10 mice; error bars are standard error of the mean (SEM). **** $P < 0.05$.

differences in interactions ($F_{(4,56)} = 4.65$, $P < 0.01$), time ($F_{(4,56)} = 26.91$, $P < 0.0001$) and treatment ($F_{(1,14)} = 14.12$, $P < 0.01$) (Fig. 3C and D). Multiple comparisons revealed that 20 Gy irradiated mice swam long distances, locating the platform on day 3 ($P < 0.05$) and day 5 ($P < 0.0001$).

Probe trials. To assess spatial memory, a probe trial was administered on training days 3–5. Sham-irradiated animals showed spatial memory retention by spending more time in the target quadrant on days 3–5 (Fig. 4A–C). In contrast, 10 Gy irradiated animals did not show memory retention on day 3 ($F_{(2,27)} = 1.84$, $P = 0.16$) or day 4 ($F_{(3,27)} = 1.43$, $P = 0.25$). However, by the end of day 5 training, 10 Gy irradiated mice showed memory retention ($F_{(3,27)} = 16.80$, $P < 0.001$) (Fig. 4B). The 20 Gy irradiated mice did not show memory retention on days 3–5 (Fig. 4A–C).

Decreased Leukocyte Counts

After spatial memory and exploratory activity assessment, circulating leukocyte counts were measured. Overall, radiation significantly decreased leukocyte counts ($F_{(2,20)} = 3.58$, $P < 0.05$). Leukocyte counts (leukocytes per ml of blood) were 1.9×10^7 (sham), 1.5×10^7 (10 Gy, single dose) and 5.5×10^6 (20 Gy, fractionated doses). Post hoc analysis revealed that there was a significant decrease in leukocytes in the 20 Gy, fractionated doses group compared to sham-irradiated group (Fig 5).

Changes in Spine Density and Spine Morphology

Quantitative analysis showed that overall spine density in the DG after cranial irradiation was not significantly changed ($F_{(2,21)} = 2.61$, $P = 0.09$; Table 1). Next, we analyzed the density of different types of dendritic spines, finding that the density of neither thin spines ($F_{(2,21)} = 1.35$, $P = 0.28$; Table 1) nor stubby spines ($F_{(2,21)} = 0.23$, $P = 0.79$; Table 1) was significantly modulated. However, there was a significant decrease in mushroom spine density after 20 Gy cranial irradiation but not after sham or 10 Gy exposure ($F_{(2,21)} = 5.61$, $P < 0.05$; Table 1) (Fig. 10).

In CA1 apical pyramidal dendrites, differences in overall spine density were observed; however, these changes failed to reach significance ($F_{(2,9)} = 4.05$, $P = 0.06$ Table 2, top). Next, analyzing spine types, we found no differences in thin ($F_{(2,9)} = 1.54$, $P = 0.27$), stubby ($F_{(2,9)} = 0.97$, $P = 0.42$) or mushroom spine density ($F_{(2,9)} = 0.93$, $P = 0.43$; Table 2). In basal pyramidal dendrites of the CA1 region, overall spine density was also unchanged after irradiation ($F_{(2,9)} = 0.43$, $P = 0.66$; Table 2). Likewise, we did not observe any changes in the density of spine types.

Changes in Dendritic Morphology

DG granule neurons. To further investigate the effects of cranial irradiation on neuronal morphology, a segmental Sholl analysis was performed to examine the changes in dendritic length as a function of radial distance from the cell

FIG. 2. Irradiated mice showed significantly decreased discrimination ratios and exploratory activity in the Y-maze test. Panel A: Sham-irradiated animals spent more time exploring the novel arm than the familiar and start arms. Irradiated animals spent equal amounts of time exploring the familiar, novel and start arms. Panel B: In mice that received 20 Gy there was also a significantly decreased percentage of entries into the novel arm compared to sham-irradiated animals. Each datum point represents the mean of 9–10 mice; error bars are SEM. * $P < 0.05$.

soma. In the DG, there was a significant interaction between treatment and segmental dendritic length after 10 Gy ($F_{(25,150)} = 2.46$, $P < 0.001$) and 20 Gy ($F_{(25,150)} = 1.81$, $P < 0.01$), indicating that the effects of radiation were associated with a different distribution of dendritic branches over the entire tree. The ANOVA also detected significant main effects of treatment after 10 Gy ($F_{(1,6)} = 7.72$, $P < 0.05$) and 20 Gy ($F_{(1,6)} = 7.82$, $P < 0.05$). Post hoc analysis revealed that radiation reduced dendritic arborization compared to that in sham-irradiated controls. This decreased arborization was particularly evident after 10 Gy at 60 μm from the soma (Fisher's LSD, $P < 0.05$) and again at 70–140 μm (Fisher's LSD, $P < 0.01$; Fig. 6A). Mice that received 20 Gy also showed a decrease in arborization at a distance of 50–60 μm from the soma (Fisher's LSD, $P < 0.05$) and again at 70–120 μm from the soma (Fisher's LSD, $P < 0.01$; Fig. 6B).

In addition, after irradiation, differences were found in total dendritic length ($F_{(2,9)} = 73.25$, $P < 0.0001$), number of branch points ($F_{(2,9)} = 226.3$, $P < 0.0001$) and

number of branch tips ($F_{(2,9)} = 124.7$, $P < 0.0001$; Fig. 6C). Accordingly, the DCI of the DG, which is calculated from the parameters, was significantly different among the groups after irradiation ($F_{(2,9)} = 184.7$, $P < 0.0001$; Fig. 6C).

CA1 pyramidal neurons. Similar analyses were performed on the apical and basal regions of CA1 pyramidal neurons. The interaction between radiation and segmental dendritic length in the CA1 apical area was significant in the groups exposed to 10 Gy ($F_{(24,144)} = 7.92$, $P < 0.0001$) and 20 Gy ($F_{(24,144)} = 11.17$, $P < 0.0001$). Two-way ANOVA also revealed significant main effects of radiation at 10 Gy ($F_{(1,6)} = 174.4$, $P < 0.0001$) and 20 Gy ($F_{(1,6)} = 625.2$, $P < 0.0001$) and an effect of length at 10 Gy ($F_{(24,144)} = 39.20$, $P < 0.0001$) and 20 Gy ($F_{(24,144)} = 41.62$, $P < 0.0001$). Further analysis with Fisher's LSD showed that radiation induced alterations of apical dendrite morphology, depending on the radial distance from the soma. Indeed, analysis of dendritic arborization using Sholl analysis revealed that 10 Gy exposure significantly decreased dendritic length in animals at distances of 70–150 μm (Fisher's LSD, $P < 0.0001$; Fig.

FIG. 3. Distances moved and mean latency to the target platform during visible- and hidden-platform training sessions. Panels A and B: There were no significant differences in latency or distance moved between the control and 10 Gy irradiated groups throughout testing. Panel C and D: Significant differences were detected in latency and distance moved on hidden-platform days (days 3–5) in 20 Gy irradiated mice compared to 10 Gy irradiated mice. Compared to 10 Gy irradiated mice, the 20 Gy group ($^{\dagger}P < 0.0001$, day 5; $*P < 0.05$, days 3 and 4; Holm's) swam longer escape distances. Each datum point represents the mean of 9–10 mice; error bars are SEM.

7A) and 160–170 μm (Fisher's LSD, $P < 0.01$; Fig. 6A) from the soma. In 20 Gy irradiated mice, arborization was significantly decreased at 60 μm (Fisher's LSD, $P < 0.01$; Fig. 7B), 70–150 μm (Fisher's LSD, $P < 0.001$; Fig. 6B) and 160–170 μm (Fisher's LSD, $P < 0.01$; Fig. 7B) from the soma, compared to sham-irradiated mice.

In the basal dendrites of CA1 pyramidal cells, interaction between exposure and segmental dendritic length was significant after 10 Gy ($F_{(26,156)} = 9.62$, $P < 0.0001$) and 20 Gy ($F_{(26,156)} = 21.22$, $P < 0.0001$). The ANOVA also

detected main effects of length after 10 Gy ($F_{(26,156)} = 102.6$, $P < 0.0001$) and 20 Gy ($F_{(26,156)} = 113.80$, $P < 0.0001$). Post hoc analysis revealed that in 10 Gy irradiated animals, dendritic arborization length was decreased at distances of 50–60 μm (Fisher's LSD, $P < 0.01$; Fig. 8A), 70–120 μm (Fisher's LSD, $P < 0.0001$; Fig. 8A) and 130 μm (Fisher's LSD, $P < 0.01$; Fig. 8A) from the soma. In 20 Gy irradiated mice, arborization was significantly decreased at distances of 50–120 μm (Fisher's LSD, $P < 0.0001$; Fig. 8B) and 130 μm (Fisher's LSD, $P < 0.01$; Fig. 8B) from the soma,

TABLE 1
Effects of Radiation on Spine Morphology in Hippocampal DG

Cell types and measurements	Sham ^a	10 Gy ^a	20 Gy ^a	<i>P</i> value
Thin spines (no.)	56.99 $\pm$ 1.47	57.68 $\pm$ 1.55	59.97 $\pm$ 0.90	0.28
Stubby spines (no.)	34.18 $\pm$ 1.33	33.28 $\pm$ 1.57	32.95 $\pm$ 1.0	0.79
Mushroom spines (no.)	8.83 $\pm$ 0.52	9.04 $\pm$ 0.43	7.08 $\pm$ 0.40	<0.05^b
Overall density (no.)	21.64 $\pm$ 0.40	21.06 $\pm$ 0.72	19.88 $\pm$ 0.04	0.098
Total dendritic length (μm)	1297 $\pm$ 57.75	604.4 $\pm$ 46.58	633.0 $\pm$ 27.91	<0.0001
Total branch points (no.)	11.39 $\pm$ 0.35	3.750 $\pm$ 0.23	4.56 $\pm$ 0.24	<0.0001
Total branch tips (no.)	13.40 $\pm$ 0.57	5.25 $\pm$ 0.31	6.06 $\pm$ 0.26	<0.0001
Dendritic complexity	54492 $\pm$ 2980	7652 $\pm$ 1051	10763 $\pm$ 1077	<0.0001

^a Mean $\pm$ SEM.

^b Significant values are indicated in boldface type.

FIG. 4. Spatial memory retention in mice during the Morris water maze probe trial after the first day of hidden-platform training. Panels A, B and C: Sham-irradiated animals spent significantly more time in the target quadrant than the right, opposite or left quadrants. Panels A and B: The 10 and 20 Gy irradiated animals showed impairment of hippocampus-dependent spatial memory during day 3 and 4 probe trials. Panel C: In day 5 training, 10 Gy irradiated mice showed memory retention and spent significantly more time in the target quadrant than all other quadrants; the preference was significant ($P < 0.05$). The 20 Gy irradiated animals did not show memory retention on days 3–5. Each bar represents the mean of 9–10 mice; error bars are SEM.

compared to sham-irradiated mice. As in the CA1 apical region, these data showed that 10 and 20 Gy cranial exposure decreased the complexity of dendritic morphology in CA1 basal dendrites at proximal and distal distances from the soma.

Juvenile mice live blood leukocyte counts

FIG. 5. Circulating leukocyte counts are decreased in irradiated mice. Leukocyte counts were significantly decreased in 10 and 20 Gy groups compared to sham-irradiated controls. Each datum point represents the mean of 5 mice; error bars are SEM. * $P < 0.05$.

As was observed in the DG, after irradiation differences were found in the CA1 apical and basal areas for total dendritic length (apical $F_{(2,9)} = 173.6$, $P < 0.0001$; basal $F_{(2,9)} = 17.13$, $P < 0.001$; Table 2), number of branch points (apical $F_{(2,9)} = 51.15$, $P < 0.0001$; basal $F_{(2,9)} = 15.89$, $P < 0.01$; Table 2), number of branch tips (apical $F_{(2,9)} = 47.43$, $P < 0.0001$; basal $F_{(2,9)} = 10.82$, $P < 0.005$; Table 2) and dendritic complexity (apical $F_{(2,9)} = 87.34$, $P < 0.01$; basal $F_{(2,9)} = 9.33$, $P < 0.01$; Fig. 8C).

Further analysis of dendritic length across the observed groups showed differences in the CA1 apical and basal regions between the 10 Gy and 20 Gy irradiated mice. From this analysis, it was determined that dendritic length was significantly decreased in 20 Gy irradiated mice at 40 and 90 μm away from the soma ($F_{(22,144)} = 45.27$, $P < 0.0001$; Fig. 8A) of the apical region of CA1. In the basal region of CA1, significant decreases were found in the dendritic length from 10–30, 50–90 μm away from the soma ($F_{(26,156)} = 80.22$, $P < 0.001$; Fig. 9B).

DISCUSSION

These studies show that cranial irradiation in juvenile mice impairs hippocampus-dependent spatial memory consolidation and decreases dendrite arborization and spine density in the hippocampus. In a Y-maze task, animals exposed to either a 10 Gy single dose or a 20 Gy fractionated doses spent an equal proportion of time exploring the familiar and the start arms during testing, suggesting that they did not recognize the novel arm. Juvenile sham-irradiated controls showed spatial memory retention during all Morris water maze (MWM) probe trials. Mice that received a 10 Gy single dose showed spatial memory retention during the third probe trial, whereas animals that received 20 Gy fractionated doses never

FIG. 6. Sholl analysis and dendrite complexity of DG neurons. Panel A: Dendritic length measured by Sholl analysis revealed a decrease in arborization that was particularly evident after 10 Gy irradiation at 60 μm from the soma and again at 70–140 μm . Panel B: Mice that received 20 Gy showed a decrease in arborization at 50–60 μm from the soma and again at 70–120 μm . Panel C: Overall dendrite complexity in the DG is greatly decreased after 10 and 20 Gy irradiation. Average $\pm$ SEM ($n = 4$); * $P < 0.05$; **** $P < 0.0001$; † $P < 0.001$.

FIG. 7. Sholl analysis of CA1 apical pyramidal neurons and dendrite complexity. Panel A: Sholl analysis revealed that exposure to 10 Gy significantly decreased arborization in CA1 apical pyramidal dendrites at a distance of 70–170 μm from the soma. Panel B: Exposure to 20 Gy decreased arborization at a distance of 60–170 μm from the soma. Panel C: Overall, both 10 and 20 Gy exposure significantly decreased apical dendrite complexity compared to that in sham-irradiated controls. Average $\pm$ SEM ($n = 4$); * $P < 0.01$; ** $P < 0.01$; † $P < 0.0001$.

FIG. 8. Sholl analysis of CA1 basal pyramidal neurons and dendrite complexity. Panel A: Sholl analysis revealed that 10 Gy exposure significantly decreased arborization in CA1 basal pyramidal dendrites at a distance of 50–130 μm from the soma. Panel B: Exposure to 20 Gy decreased arborization at distances of 50–120 μm and 130 μm from the soma. Panel C: 10 Gy and 20 Gy radiation significantly decreased basal dendrite complexity. Average $\pm$ SEM ($n = 4$); * $P < 0.05$; ** $P < 0.01$; † $P < 0.0001$.

showed spatial memory retention during the MWM task. From our Golgi-staining studies, we observed decreased dendritic arborization and dendritic length in hippocampi from irradiated animals compared to sham-irradiated animals. These findings demonstrate that radiation causes alterations in dendritic morphology that may contribute to the impairment of cognitive function, as shown in the Y-maze and MWM paradigms.

Cognitive dysfunctions are common sequelae in patients who undergo cranial exposure, manifesting months to years after the event, and include decreases in spatial and short-term memory (23). The Y-maze is a simple two-trial recognition test for measuring spatial recognition memory in animal experiments. The Y-maze test is based on the instinctive curiosity of rodents to explore novel areas without negative or positive reinforcements to the animals (24). The irradiated animals' lack of curiosity about the novel arm implies the inability to remember the start or the familiar arms. Our data significantly demonstrate that animals receiving 10 and 20 Gy cranial irradiation failed to distinguish the novel arm from the familiar arm in the Y-maze paradigm, suggesting deficits in the hippocampus-dependent process of short-term recall (25).

The MWM cognitive training and testing paradigm used in our experiments employed three probe trials, one of which was performed at the end of each day of multiple hidden-platform training sessions (26). The MWM task makes it possible to determine hippocampus-dependent spatial memory in mice. Our MWM data are consistent with previously reported data by Rola *et al.* (27), who conducted similar tests in 21-day-old mice that received whole-brain X-ray irradiation. In their studies, exposed mice demonstrated significant deficits in cognitive tests, specifically MWM tasks. They found that mice receiving whole-brain irradiation at doses of 2, 5 and 10 Gy were unable to remember platform placement after training. Raber *et al.* (5) found that impairments in hippocampus-dependent spatial learning and memory were induced by a 10 Gy single dose, which was equivalent to 2 Gy $\times$ 10 fractionated doses, without structural changes in the hippocampus.

Dendritic spines can be defined as growths extending from dendrites that are important for neurotransmitter exchange between neurons (28, 29). The number of dendritic spines can be an indicator of the amount of information that is being passed between neurons. Changes in spine density or length are thought to represent morphological correlates of altered brain functions associated with learning and memory (30). Thin dendritic spines are much more plastic and transient than stubby spines and are referred to as “learning” spines, while stubby spines are “memory” spines, which are more stable. Mushroom spines represent more stable memory spines because they have greater postsynaptic density than thin spines (31). Mice that received 20 Gy displayed a spatial learning memory deficit during testing, while sham-irradiated and 10 Gy irradiated mice were able to remember the platform

FIG. 9. Comparison of dendritic length between 10 and 20 Gy irradiated mice. Panel A: Mice exposed to 10 Gy show significantly greater dendritic length than mice that received 20 Gy at 40 and 90 μm from the soma in the CA1 region of the hippocampus. Panel B: Mice exposed to 10 Gy show significantly greater dendritic length from 10–30, 50–90 μm from the soma. Average $\pm$ SEM ($n = 4$); * $P < 0.01$; † $P < 0.001$.

location. In the DG, we found significant decreases in mushroom spine density in animals receiving 20 Gy cranial irradiation compared to sham-irradiated controls. The proportional decrease of mushroom spines could hamper the management of previously acquired information. It is plausible that the structural changes observed in dendritic spine morphology after 20 Gy irradiation led to disruption of neural circuitry with consequent cognitive dysfunction. Taken together, these data indicate a dose-dependent response, where 20 Gy exposure is more detrimental to learning and memory than 10 Gy.

Dendrites are protrusions that branch off from the soma of neurons and enable formation of synapses (32). Similar to the changes observed in dendritic spines, changes in the Sholl analysis of dendrites can be indicative of brain health. Decreased dendritic complexity can have implications for disruption of memory formation. Our data showed that cranial exposure significantly decreased dendritic length in the DG and CA1 regions of the hippocampus. Decreased dendritic length has previously been associated with neuronal damage after irradiation because dendritic morphology has been implicated in the health of neurons (33). This is consistent with other published studies in which

FIG. 10. Analysis of the percentage of mushroom spines in the dentate gyrus of mice treated with 0 Gy, 10 Gy or 20 Gy cranial irradiation. Mice exposed to 20 Gy had significantly fewer mushroom spines compared to sham-irradiated and 10 Gy irradiated mice. * $P < 0.05$. Average $\pm$ SEM.

dendritic pathology was found in neurodegenerative diseases. Spires *et al.* (34) reported the finding of decreased dendritic length, as well as decreased synaptic plasticity, in a Huntington's disease mouse model. Although the findings were similar, their model of neurodegeneration is remarkably different. There is a paucity of published studies examining changes in dendritic arborization after cranial irradiation in juvenile models. The current study provides

critical information regarding the mechanism of disruption of neural circuitry after radiation exposure.

Dendrite branching and morphology facilitate and allow individual neurons to carry out specialized brain functions and cognitive behaviors, such as learning and memory (35). We found significant decreases in the number of dendritic branch points and in branch point complexity in the DG and CA1 apical and basal regions after 10 and 20 Gy cranial irradiation. Changes in branch points refer to decreased branching or diverging of dendritic arbors. Dendritic branch point complexity refers to the number of arbors that branch off at each point of divergence. Increased branch point complexity usually indicates the number of arbors that branch from each dendrite. Increased branching increases the surface area where spines can grow (36). Our findings of changes in dendritic morphology are aligned with findings in the literature showing abnormal morphology and decreased complexity associated with impaired learning and memory on behavioral testing (37). In addition, from our Sholl analysis we observed significant decreases in the dendritic length when comparing 10 and 20 Gy exposed mice. Interestingly, these data are consistent with the findings in the MWM probe trial tests. In the MWM, we observed that 10 Gy irradiated mice were able to retain spatial memories by the end of testing while the 20 Gy irradiated mice were not. This is indicative of a dose-dependent effect on memory retention as well as dendritic complexity in the juvenile mouse hippocampus after focal irradiation of the brain.

Another prevalent side effect of radiotherapy is lymphopenia. Lymphopenia, or leukopenia, is characterized by marked decreases in the leukocyte counts (38). It has been demonstrated in patients who have undergone radiotherapy for different cancer types. Patients treated for small cell lung

TABLE 2
Morphological Analysis of Apical and Basal Dendrites in CA1

Cell types and measurements	Sham ^a	10 Gy ^a	20 Gy ^a	P value
CA1 apical				
Thin spines (no.)	59.35 $\pm$ 1.08	55.11 $\pm$ 2.30	56.58 $\pm$ 1.60	0.27
Stubby spines (no.)	32.82 $\pm$ 1.35	35.45 $\pm$ 2.23	35.65 $\pm$ 0.98	0.42
Mushroom spines (no.)	7.83 $\pm$ 0.34	9.44 $\pm$ 0.16	7.76 $\pm$ 1.66	0.43
Overall density (no.)	22.12 $\pm$ 0.15	20.7 $\pm$ 0.44	19.33 $\pm$ 1.11	0.06
Total dendritic length (μ m)	41.32 $\pm$ 7.20	17.93 $\pm$ 4.69	14.85 $\pm$ 4.22	<0.0001^b
Total branch points (no.)	12.63 $\pm$ 0.16	7.06 $\pm$ 0.60	6.60 $\pm$ 0.52	<0.0001
Total branch tips (no.)	13.69 $\pm$ 0.12	8.125 $\pm$ 0.66	7.67 $\pm$ 0.51	<0.0001
Dendritic complexity	92587 $\pm$ 7679	17629 $\pm$ 1921	15269 $\pm$ 1939	<0.0001
CA1 basal				
Thin spines (no.)	56.24 $\pm$ 2.09	58.38 $\pm$ 1.99	56.26 $\pm$ 1.87	0.69
Stubby spines (no.)	35.88 $\pm$ 1.81	32.01 $\pm$ 1.21	36.18 $\pm$ 1.96	0.21
Mushroom spines (no.)	7.89 $\pm$ 0.76	9.61 $\pm$ 1.25	7.56 $\pm$ 0.61	0.28
Overall density (no.)	20.28 $\pm$ 0.99	19.40 $\pm$ 0.64	19.41 $\pm$ 0.61	0.66
Total dendritic length (μ m)	1314 $\pm$ 115.9	741.7 $\pm$ 103.8	573.7 $\pm$ 46.54	<0.001
Total branch points (no.)	13.19 $\pm$ 1.17	7.69 $\pm$ 0.84	6.94 $\pm$ 0.033	<0.001
Total branch tips (no.)	16.25 $\pm$ 1.02	11.38 $\pm$ 1.07	10.31 $\pm$ 0.77	<0.01
Dendritic complexity	34860 $\pm$ 8762	8040 $\pm$ 1276	7178 $\pm$ 1108	<0.01

^a Mean $\pm$ SEM.

^b Significant values are indicated in boldface type.

cancer as well as juvenile patients who received cranial radiotherapy for acute leukemias have shown decreased leukocyte counts after treatment (39, 40). In many instances, these decreased leukocyte counts can be evident for up to one year after treatment (38). It has been shown that different radiation types are responsible for lymphopenia. It has also been demonstrated that numbers of leukocytes have been greatly reduced after total-body proton and gamma irradiation (41). In a study by Sanzari *et al.*, immune function as well as circulating leukocyte counts were measured in total-body irradiated (TBI) mice. They found significantly decreased numbers of leukocytes as well as decreased immune function in these rodents. Lymphopenia does not occur only as a result of TBI; it has also been shown to occur after focal irradiation as well. A study by Harisiadis *et al.* showed that those who received craniospinal radiotherapy had decreased WBC counts after treatment (39). While increased lymphocyte counts (42) can be indicative of an inflammatory response, which can be detrimental to cognitive function, lymphopenia can also be harmful to the cognitive functioning of individuals. Published studies of elderly patients who were admitted to psychiatric facilities demonstrating severe dementia also had lymphopenia (43). In the current study, leukocyte counts show dose-dependent decreases in peripheral leukocytes. Animals that received 10 Gy, on average, had fewer circulating leukocytes than sham-irradiated controls. Interestingly, animals that received 20 Gy fractionated doses had significantly fewer numbers of circulating leukocytes compared to the animals that received 10 Gy and sham-irradiated controls. This suggests that higher doses of radiation lead to greater severity of lymphopenia. Also, this dose dependence appears to align with our behavioral findings. In this study, we observed that the 10 Gy irradiated animals recovered some memory retention by day 5 of MWM testing. However, animals that received 20 Gy fractionated doses did not recover during testing. The decreased leukocyte count correlates with decreased memory capabilities in juvenile mice after irradiation; however, further studies using this animal model of juvenile cranial irradiation are needed to certify these findings.

CONCLUSION

Cranial irradiation decreases dendrite arborization and impairs spatial memory consolidation in juvenile mice. The functional implications of these observed radiation effects on dendritic complexity and morphology in subregions of the hippocampus are not yet clear. Thus, further studies are needed to examine these functional implications, as well as whether these alterations occur and/or persist beyond the time frame of our current study. However, it is also imperative that researchers attempt to elucidate the pathology and underlying mechanisms to potentially find ways to prevent or ameliorate radiation treatment side

effects, which can last for years and significantly affect the quality of life of pediatric cancer survivors (6).

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