Ionizing Radiation Impairs the Formation of Trace Fear Memories and Reduces Hippocampal Neurogenesis

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Long-term cognitive impairments are a feared consequence of therapeutic cranial irradiation in children as well as adults. Studies in animal models suggest that these deficits may be associated with a decrease in hippocampal granule cell proliferation and survival. In the present study the authors examined whether whole brain irradiation would affect trace fear conditioning, a hippocampal-dependent task. Preadolescent (postnatal Day 21, PD 21), adolescent (PD 50), and postadolescent (PD 70) rats received single doses of 0 Gray (Gy), 0.3 Gy, 3 Gy, or 10 Gy whole brain irradiation. Three months after radiation treatment, a significant dose-dependent decrease in bromo-deoxyuridine positive cells was observed. Irradiation produced a dose-dependent decrease in freezing in response to the conditioned stimulus in all age groups. Interestingly, the authors found no differences in context freezing between irradiated and control groups. Further, there were no differences in delay fear memories, which are independent of hippocampus function. Our results strongly indicate that irradiation impairs associative memories dependent on hippocampus and this deficit is accompanied by a decrease in granule cell neurogenesis indicating that these cells may be involved in normal hippocampal memory function.

*Keywords:* ionizing radiation, hippocampus, fear conditioning, neurogenesis, learning and memory

Following cranial radiation impairment of cognitive development is commonly observed in children and has also been described in adults. Currently, no “safe” whole brain radiation dose, yielding a zero probability of cognitive deficits, has been identified. Even at reduced total whole brain irradiation (WBI) doses, a negative effect on young children can be measured by neurocognitive testing procedures (Fuss, Poljanc, & Hug, 2000; Roman & Sperduto, 1995). Cognitive decline is mainly attributed to a diminished capability to learn and memorize new tasks and information, as well as to reductions in full-scale IQ (Macedoni-Luksic, Jereb, & Todorovski, 2003; Schatz, Kramer, Ablin, & Matthey, 2000).

Radiation-induced cognitive changes are often manifested as deficits in hippocampally dependent functions of learning and memory (Abayomi, 1996; Crossen, Garwood, Glatstein, & Neuweit, 1994; Lee, Hung, Woo, Tai, & Choi, 1989; Roman & Sperduto, 1995; Surma-aho et al., 2001). Doses of cranial radiation that are subthreshold for demyelinating pathology do cause delayed deficits in performance in hippocampal-dependent behavioral tasks (Hodges et al., 1998). These deficits are especially relevant regarding potential long-term consequences of prophylactic and lower dose of WBI prescribed to lower the incidence of brain involvement of hematological malignancies and the delayed occurrence of brain metastases in selective solid tumors. Both neurogenesis and performance in behavioral tasks that test hippocampal function decrease in a radiation dose-dependent manner (Sienkiewicz, Haylock, & Saunders, 1994). The underlying mechanisms for these effects have remained unknown, although it was suggested that changes in neuronal precursor cells in the dentate subgranular zone of the hippocampus might be involved. Evidence in animal models supports the importance of hippocampal neurogenesis to normal cognitive functioning (Madsen, Kristjansen, Bolwig, & Wortwein, 2003; Mizumatsu et al., 2003). Understanding how irradiation affects normal hippocampus functioning is of utmost importance in developing potential strategies to reduce cognitive impairments in humans. In this translational animal study we assessed the age dependent/dose dependent impact of ionizing radiation on hippocampus-dependent associative learning and memory and granule cell neurogenesis.

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We thank Dr. Steve Maren from the University of Michigan for the Excel file used to calculate freezing percentages in our behavioral analyses. We thank Dr. Heather Cameron (NIMH) for assistance with BrdU immunohistochemistry. We thank Dr. Angela Sikorski (UTSA) for assistance with statistical analysis, and Myra Guzman for her technical assistance.

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Materials and Methods

Subjects

The subjects were male Sprague Dawley (SD) rats from Charles River Labs, Indianapolis, IN. The rats were housed two per cage in clear Plexiglas cages maintained on a 12:12 hour light:dark cycle. Food and water were available ad libitum. All experiments except radiation exposure were performed at the University of Texas at San Antonio (UTSA). Animals received irradiation at the Cancer Therapy and Research Center (CTRC) at the University of Texas Health Science Center at San Antonio (UTHSCSA). All experimental procedures were approved by the Institutional Animal Care and Use Committees (IACUC) of UTHSCSA and UTSA. All experiments were performed during the light phase of the cycle.

Bromo-Deoxyuridine (BrdU) Administration

BrdU (Sigma) was diluted in sterile saline to a final concentration of 10 mg/ml with 1N NaOH/1-mL saline. A group of animals \( (n = 5 \) for each group; total \( n = 60 \) ) were given three daily i.p. injections of 200 mg/kg of BrdU a week prior to irradiation treatment and sacrificed three months later for BrdU immunohistochemistry.

Hippocampal Radiation

Hippocampal radiation was delivered on postnatal day (PD) 21, PD 50, and PD 70 rats in single doses of 0.3 Gy, 3 Gy, and 10 Gy, respectively. These doses were chosen since they reflect a single (3 Gy) dose of whole brain irradiation in humans, a dose that is a magnitude smaller (0.3 Gy) to assess the impact of very low radiation doses, and a dose that was most often used in animal cranial irradiation research (10 Gy). It is difficult to justify cranial irradiation doses in animal pilot research. Thus, we based our study on the doses commonly used in clinical treatment and animal research. These doses have been routinely used in animal experiments and do not cause apparent neurological or behavioral effects or necrosis in histopathological examination over observation periods much longer than 6 months. The animals were anesthetized with Nembutal Sodium solution intraperitoneal (i.p.), 25 mg/kg for PD 21 and 50 mg/kg for PD 50 and PD 70 for hippocampal radiation. Radiation exposure encompassed the entire brain while avoiding critical structures as eyes, ears, and snout (mainly the nasal cavity) to reduce radiation treatment related toxicity, by appropriate beam blocking. For sham irradiation, control animals were anesthetized, brought into the treatment room, and handled and positioned just as the animals that underwent irradiation, but no radiation was delivered. Besides physical shielding using both cerrobend blocking and linear accelerator integral tungsten multileaf collimator field shaping, the radiation was planned and delivered according to a three-dimensional radiation treatment plan based on computed tomography simulation, concordant with the current standard for human external beam radiation therapy. Both measures afforded homogeneous dose delivery, and allowed for conformal avoidance of mouth and pharynx, with the associated expectation of reducing the probability of normal tissue complication probability.

Histology

All the animals that received BrdU injections were anesthetized with Sodium Nembutal (50 mg/kg). Transcardial perfusion was first performed with ice-cold 0.9% saline containing 2 U/mL heparin (Sigma) until the liver cleared, followed by ice-cold fresh 4% paraformaldehyde (PF) in 0.1M phosphate buffer (PB), pH 7.4. The brains were removed from the skull, post fixed overnight in 4% PF/PB at 4 °C, and stored in 1 mg/mL sodium azide in PB until sectioned. The brains were equilibrated in 30% sucrose and sliced coronally at 50 μm using a freezing microtome. Sequential slices through the entire dentate gyrus were transferred to 12 wells containing chilled cryoprotectant (25% ethylene glycol, and 25% glycerol in 0.1M PB, pH 7.4). Series of every 12th section were processed for BrdU immunohistochemistry. The BrdU immunohistochemistry protocol was obtained from a previously published protocol (Cameron & McKay, 2001). Sections were mounted on superfrost glass slides, boiled in 0.01M citric acid pH 6.0 for 10 min using a microwave oven, permeabilized with trypsin for 10 min, and denatured with 2N HCl for 30 min. Sections were incubated in 0.2M phosphate buffer saline (PBS) containing 0.5% Triton-X-100, and 3% goat serum for 20 min, and then incubated with primary monoclonal mouse anti-BrdU antibody (1:100, Becton Dickinson) for 16 hrs at 4 °C. The sections were washed with PBS and were incubated with biotin conjugated secondary antibodies developed in goat (antimouse IgG 1:200, Sigma) for 1 hr at room temperature. The sections were treated with 0.3% hydrogen peroxide to block endogenous peroxidase activity for 30 min, incubated in Vector Elite peroxidase ABC reagent (Vector Laboratories) for 1 hr, and developed in cobalt-enhanced DAB (Sigma Fast Tablets). All sections were counterstained with cresyl violet, dehydrated, and cover slipped under Permount. For double labeling, every 24th section was immunostained for monoclonal rat anti-BrdU (1:50d, Santa Cruz) and monoclonal mouse anti-NeuN (1:100d, Chemicon) or monoclonal mouse anti-GFAP (1:200d, Chemicon) antibodies. Alexa 488–conjugated and Alexa 594–conjugated secondary antibodies (Molecular Probes, 1:500) were used. For Ki67 staining, the sections mounted on the glass slides were boiled in 0.01M citric acid pH 6.0 for 10 min for antigen retrieval. Sections were then incubated in 0.1M PBS containing 0.5% Triton-X-100, and 3% goat serum for 30 min, and then incubated with primary monoclonal rabbit anti-Ki67 antibodies (1:200, Neomarkers) overnight at 4 °C. Sections were washed with PBS and were incubated with 1:500 goat antimouse Alexafluor 488 conjugated (Millipore, Inc.) and nuclear stained with DAPI.

Behavioral Apparatus for Fear Conditioning/Testing

Conditioning and testing were conducted in a set of four identical modular chambers (30 × 24 × 21 cm, MED Associates, VT) constructed out of aluminum (two sidewalls) and Plexiglas (rear wall, ceiling, and hinged front door). The four chambers were housed individually within a sound-attenuating cubicule (56 × 38 × 36 cm) in an isolated room. The floor in each chamber consisted of 18 stainless steel rods (4 mm in diameter) spaced 1.5 cm apart that were wired to a shock generator and scrambler for the delivery of shock. A stimulus light, ventilation fan, and a speaker for delivering acoustic stimuli were mounted to a grating on the walls of the chamber. The light was on for the duration of
the experiment and the doors of the isolation cubicle were closed. Conditioning and tone testing were conducted in different chambers and were manipulated by olfactory, visual, and tactile cues. There was coconut oil scent in the conditioning chamber and food pellet scent in the testing chamber. To discriminate further the conditioning chamber from the testing chamber, the testing chamber was covered with black and white stripes surrounding the walls and a black Plexiglas grid floor (9 × 11 cm) was placed on top of the grid floor. In addition, individual rat bedding was placed into the chamber before testing to contribute to the novelty of the testing chamber. The chambers were cleaned between the conditioning and testing of each rat. All the movements of the rats were recorded beginning 2 min after placing the rat in the chamber.

 Trace Fear Conditioning/Testing

Rats received trace fear conditioning 90 days following radiation treatment. The numbers of animals for each group are shown in Table 1. For time line of behavioral experiments, see Figure 1. A day before conditioning each rat was acclimated to the training chamber for 30 min. During acclimation the animals were not presented with any stimuli and a baseline measurement of movement was recorded for 3 min. The rats were then returned to their home cages. During trace fear conditioning, each rat was placed inside the conditioning chamber, in which it was acclimated. The rats received 10 trials of paired stimuli with an intertrial interval (ITI) of 210 s. On each trial, a tone conditioned stimulus (CS; 82 dB, 15 s, 3000Hz, 5 ms rise/fall time) was followed by a 30-s silent trace interval followed by the footshock unconditioned stimulus (US) (0.5 s, 1 mA) delivered via the grid floor. The next day, the rats were tested for fear conditioning to tone 30 min after the context fear testing. For tone learning assessments, the rats were placed in a novel test chamber and allowed to acclimate for 5 min. Movements over 3 min was recorded and used as a baseline for testing. Ten tone trials without the footshock were given with an ITI of 240 s, and the freezing response was measured during this time.

 Context Fear Testing

Twenty-four hours after trace conditioning the rats were placed in the conditioning chamber for 5 min in the absence of any conditioning stimuli. Movements were recorded to measure fear associated with the training context. The rats were then returned to their home cage. An additional assessment of contextual fear conditioning was tested 2 weeks after trace conditioning (a day before delay fear conditioning). The context fear responses measured a day after trace training was referred to as context-1 and the responses measured 2 weeks after trace training was referred to as context-2.

 Delay Fear Conditioning/Testing

Twenty-four hours following context-2 fear testing, the rats were trained for delay fear conditioning. The conditioning and tone testing chambers and procedures used for the trace fear conditioning were same for delay fear conditioning except that there was no trace interval between the CS and US. Each rat received 10 delay fear conditioning trials with an ITI of 210 s, and each trial consisted of a tone CS (82 dB, 15 s, 3000Hz, 5 ms rise/fall time) immediately followed by a footshock US (0.5 s, 1 mA) through the grid floor. The onset of the US was immediately after the offset of the CS.

 Data Acquisition and Statistics

Each conditioning chamber was rested on a load-cell platform that was used to record chamber displacement in response to motor activity. The load cell amplifier output from each chamber was digitized at 5 Hz to yield one observation every 200 ms and acquired online using Threshold Activity software (MED Associates, VT). Freezing was defined as the animal not moving for at least one full second. We used an Excel template that transforms load cell output activity into freezing percentages. Freezing percentages of different dose and age groups for each time period during training and testing were analyzed using analysis of variance (ANOVA). Preplanned post hoc multiple comparisons using the least significant difference (LSD) test were used to determine significant differences between the groups. Further, context fear data was analyzed by univariate analysis of variance to see if there were time, dose, and age effects. All values in the text and figure legends are means ± SEM.

For histology data, cell counts were obtained from every 12th section through the entire dentate gyrus and the subgranular zone of the dentate gyrus. All positively labeled cells within and immediately adjacent to the granule cell layer (GCL) of both hemispheres were counted at 400X (Olympus, BX61). The total number of BrdU positive cells and Ki67 positive cells was estimated by multiplying the cell counts by 12 for each brain. The percentages of BrdU-positive cells colabeled for NeuN or GFAP were calculated for each brain. The cell counts for all animals in a given treatment group were averaged and the standard error of the means (SEM) were calculated. A one-way ANOVA was used to determine changes in total BrdU-positive cell counts with increasing doses of irradiation. Preplanned post hoc multiple comparisons using Student-Newman–Keuls method were used to determine significant differences between the groups.

Results

Ki67 was used as a proliferative marker to assess ongoing cell proliferation in the animals 3 months postradiation. As expected, Ki67 positive cells were found in the subgranular zone of the dentate gyrus and the hilus (Figure 2A–C). A statistically significant dose-dependent decrease in the number of Ki67 stained cells was observed three months after irradiation with doses ranging

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from 0.3 Gy to 10 Gy in all age groups, PD 21: $F(3, 20) = 9.98$, $p = .019$; PD 50: $F(3, 20) = 9.66$, $p = .022$; PD 70: $F(3, 20) = 21.55$, $p = <.001$), as shown in Figure 2D–F.

BrdU-positive cells were found along the suprapyramidal and infrapyramidal blades of the dentate gyrus, and in the subgranular zone of the dentate gyrus and the hilus (Figure 2G, H). A statistically significant dose-dependent decrease in BrdU-positive cells was observed 3 months after irradiation with doses ranging from 0.3 Gy to 10 Gy in all age groups, PD 21: $F(3, 20) = 43.49$, $p < .001$; PD 50: $F(3, 20) = 17.51$, $p < .001$; PD 70: $F(3, 20) = 273.05$, $p < .001$, as shown in Figure 2I–K. Subsequent pair wise comparisons using the Student-Newman–Keuls method showed a significant decrease in the total number of BrdU-positive cells in the 0.3, 3, and 10 Gy versus the 0 Gy dose groups ($p < .05$) in PD 21 (Figure 2I) and PD 50 (Figure 2J) groups of animals. In the PD 70 group of animals, there was a significant decrease in BrdU-positive cells in the 3, and 10 Gy dose groups compared to the 0 Gy group. ($p < .05$; Figure 2K). Though there was a decrease in BrdU-positive cells in 0.3 Gy dose group as compared to controls in PD 70 age group, the difference was not statistically significant (Figure 2K). The total BrdU-positive cells at the 0.3, 3, and 10 Gy dose level were reduced to 66%, 40%, and 14% respectively compared to 0 Gy in PD 21 group of animals. In the PD 50 group of animals, the BrdU-positive cells were decreased to 60%, 48%, and 4% in the 0.3, 3, and 10 Gy dose groups, respectively, compared to animals exposed to 0 Gy. In the PD 70 group of animals, the BrdU-positive cells were reduced to 90%, 56%, and 4% in the respective 0.3, 3, and 10 Gy groups, compared to the 0 Gy group. Further, the phenotype of these BrdU-positive cells was assessed by colabeling with the neuronal marker, NeuN or the glial marker, GFAP (Figure 2L, M). There was no difference in the percentage of BrdU-positive cells that colabeled for NeuN or GFAP across the three different age groups in our study. However, there was a significant reduction in the number of new neurons produced in 10 Gy irradiated animals compared to sham, $F(3, 35) = 20.522$, $p = .001$ (Figure 2M). Pairwise multiple comparisons using Student-Newman–Keuls method showed a significant decrease in 3 and 10 Gy group of animals compared to 0.3 Gy and sham treated animals ($p < .009$). Conversely, the generation of glial cells was not affected by radiation (data not shown) (Figure 2N).

**Trace Fear Conditioning/Testing**

A separate group of rats that received different doses of irradiation, along with their control animals were trained with 10 trace trials in the conditioning chamber. Freezing percentages obtained from baseline and tone, trace, shock, and ITI of the last trial of trace fear conditioning are shown in Figure 3A–C. There were no significant differences in conditioning between the groups. The following day, rats were tested in a novel chamber for fear conditioning to the CS. Irradiation produced a dose-dependent decrease in freezing in response to the CS in all age groups (Figure 4A–C). The PD 21 group of animals (Figure 4A) had a significant dose effect in the 3 and 10 Gy compared to the 0 Gy group during the trace, $F(3, 43) = 6.53$, $p = .001$, and during the ITI, $F(3, 43) = 7.20$, $p = .001$. The PD 50 group of animals (Figure 4B) showed a significant dose effect in the 10 Gy group compared to 0 Gy group during the trace, $F(3, 42) = 3.96$, $p = .015$, and in the 0.3 and 10 Gy groups versus the 0 Gy group during the ITI, $F(3, 42) = 3.82$, $p = .017$. In the PD 70 group of animals (Figure 4C), there was a significant dose effect in the 10 Gy group versus 0 Gy group during the trace, $F(3, 39) = 2.88$, $p = .049$, and during the ITI, $F(3, 39) = 4.16$, $p = .013$. The effect of radiation on trace fear conditioning was significant only in the first trial of testing. Though there was a slight decrease in freezing in the subsequent trials, the effect between the groups was not significant. There were no differences during baseline and tone between the groups. Further, there were no significant age effects between the groups.

**Context Fear**

Both the irradiated and non irradiated groups of rats froze to the context-1 when the animals were placed in conditioning chamber (Figure 5A) indicating no deficits in context fear memories 3 months after irradiation. There was no significant dose effect in any age group, $F(3, 115) = 1.66$, $p = .18$, and no significant age effect, $F(2, 115) = 0.78$, $p = .46$. Animals tested for context fear memories two weeks after training showed no deficits in context fear. There was no significant dose, $F(3, 115) = 1.59$, $p = .2$, or age $F(2, 115) = 0.38$, $p = .68$, effects in context-2 (Figure 5B).

**Delay Fear Conditioning/Testing**

Animals were subjected to 10 trials of delay fear conditioning one day after context-2. We found no differences in delay fear conditioning (Figure 6A–C) and delay fear testing (Figure 7A–C), which are independent of hippocampus function.
Figure 2. Ionizing radiation decreases hippocampus neurogenesis. A–C shows Ki67 immunostained cell in the SGZ of the hippocampus. BrdU-positive cells detected by immunohistochemistry in the granule cell layer (GCL) of the rat Dentate Gyrus at 40× and 400× magnification. Arrows show BrdU-positive cells with punctate staining. Dose dependent decrease in the mean (±SEM) total number of Ki67-positive cells (D–F) and BrdU-positive cell (I–K) was observed in all three age groups following irradiation. There was a decrease in the percentage of BrdU-positive cells that colabeled for NeuN, shown in L and M, but not in BrdU-positive cells colabeled for GFAP, shown in N. * indicates p < .05 versus 0 Gy.
Discussion

Previous studies addressed the effects of ionizing irradiation on hippocampal neurogenesis in prenatal or neonatal animals showing irreversible depletion of neural precursor cells (Mizumatsu et al., 2003; Tada, Parent, Lowenstein, & Fike, 2000). Here in this study we investigated the effects of irradiation on dentate gyrus neurogenesis in rats at preadolescent, adolescent and post adolescent ages of development. The results showed a dose-dependent decrease in the total number of BrdU-positive cells three months after irradiation in all the three age groups. Preadolescent animals (P21) showed a relatively higher number of BrdU-positive cells compared to PD 50 and PD 70 group of animals, as the rate of dentate granule cell neurogenesis during early postnatal life is greater than in the adult. The higher number in the P21 rats may be due to the fact that the BrdU injections were given during the second week after birth a time when the postnatal granule cell production was occurring in PD 21 group of animals. It is known that about 80% of the granule cells are produced after birth in rats (Bayer, 1982). Granule cell genesis peaks shortly after birth and continues during the first and second week (Altman & Das, 1965; Schlessinger, Cowan, & Gottlieb, 1975). Thereafter, the production of new granule cells slows down with age, but continues in the subgranular zone of the dentate gyrus (Bayer, 1980). We show a slight decrease in BrdU-positive cells in the sham irradiated PD 70 group of animals compared to PD 50 group of animals, however

Figure 3. Mean (±SEM) percentage freezing during baseline and the last tone, trace, shock, and ITI (10th trial) presentation of trace fear conditioning for P21 (A), P50 (B), and P70 (C) rats. Freezing levels were similar among the groups.

Figure 4. Mean (±SEM) percent freezing during baseline and first tone, trace, and ITI (1st trial) presentation for P21 (A), P50 (B), and P70 (C) with different doses of irradiation. Low levels of freezing after tone were observed in a dose dependent manner. * indicates p < .001 in 3, 10 Gy versus 0 Gy, # indicates p < .01 in 10 Gy versus 0 Gy, and $ indicates p < .05 in 0.3, 10 Gy versus 0 Gy.
This decrease was not statistically significant. Post hoc multiple comparisons showed a significant decrease in BrdU-positive and Ki67-positive cells in animals exposed to 0.3 Gy compared to 0 Gy, in the PD 21 and PD 50 groups of animals but not in PD 70 group. These results suggest that younger rats are more sensitive to low doses of irradiation than adult rats. The decrease in the generation of new neurons, but not in glial cells, three months post irradiation indicate that radiation affects neurogenesis but not gliogenesis (Mizumatsu et al., 2003; Monje, Toda, & Palmer, 2003).

Further we show that WBI impaired the formation of hippocampus-dependent trace fear memories, but not delay fear memories which are independent of the hippocampus. The most important finding of this part of our study was a dose dependent decrease in freezing during the trace and the ITI in all of the age groups. In the trace fear conditioning animals were given tone and shock separated by a trace interval. Therefore, during testing the animals expect shock after the tone. Our results showed no significant differences during the baseline and tone presentation between the groups. PD 50 and PD 70 age group animals showed significant decreases in freezing with higher doses of irradiation (10 Gy), P21 group of animals however, showed significant decrease in freezing with 3 Gy and 10 Gy doses of irradiation. This suggests that younger animals exhibit hippocampal dependent impairment at a lower dose of radiation compared to more mature, and adult animals. The low levels of freezing seen in PD 50 Gy group of animals following 0.3 Gy dose during ITI is interpreted as an increased sensitivity to irradiation in the adolescent rats, as we did not find impairments during the trace and in PD 21 and PD 70 age groups. Delay fear conditioning was not affected in the present study. These results strongly indicate that irradiation affects the associative memories dependent on hippocampus.

Both fear conditioning and the conditioned eyelink response using trace paradigms require the hippocampus (Huerta, Sun,
Wilson, & Tonegawa, 2000; McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998; Phillips & LeDoux, 1992; Weiss, Bouwmeester, Power, & Disterhoft, 1999) and hippocampal neurogenesis (Leuner et al., 2004; Shors et al., 2001; Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002). Though increased neurogenesis is observed following a number of tasks that depend on hippocampal function, recent studies suggest immature neurons are necessary for acquisition of only some learning tasks dependent upon the hippocampus. Blocking proliferation of neurons by systemic methylacrilimide (MAM) injection impaired trace fear memories (Shors et al., 2002) but had no effect on spatial memories. The requirement of new neurons only for some hippocampal tasks may be explained by prior studies suggesting that some tasks do not absolutely require hippocampal participation. Further, MAM treatment appears to have no effect on contextual fear conditioning consistent with our results (Shors et al., 2002). However, these results are in contrast to findings of Winocur et al. (2006) and Saxe et al. (2006). These authors showed impaired contextual fear conditioning four weeks, and three months after irradiation, respectively. These differences may be attributable to the differences in the complexity of the context which was comprised of multiple environmental cues in those studies that found an effect of irradiation on context fear conditioning, as opposed to simple context used in our study and to the training paradigm. Our interpretation is strengthened by those studies that dispute the notion that the hippocampus is even required for contextual fear conditioning (Gerlai, 2001; Gewirtz, McNish, & Davis, 2000), because single cues within a given context may become associated with fear, and such “cue learning” is sensitive to damage of the amygdala but not the hippocampus (Gerlai, 2001). Further, studies based on the subregional lesions of hippocampus showed that dorsal and ventral hippocampus were critical in different aspects of event representation and trace fear conditioning (Esclassan, Coutureau, Di Scala, & Marchand, 2009; Rogers, Hunsaker, & Kesner, 2006). At this point, it is unclear if contextual fear conditioning is dependent on hippocampus and it appears that the hippocampus cannot be treated as a single structure with regard to classical conditioning. It may be that different functions with regard to trace and context conditioning are associated with the different subregions of the hippocampus and the pathways that directly connect each of the subregions as well as their connections with the entorhinal cortex (Kesner, Lee, & Gilbert, 2004).

The step-by-step mechanisms associated with delayed and irreversible radiation-induced cognitive impairments remain unknown. Radiation-induced cognitive impairments are seen months to years after whole brain irradiation in humans although there is an increased apoptosis and decreased cell proliferation and survival within hours after irradiation (Mizumatsu et al., 2003). The delayed appearance of the deficits is likely due to the fact that the cells that are born prior to the treatment are still present and are recruited into memory circuits in the hippocampus. Adult generated neurons migrate into the granule cell layer and extend axons into the CA3 by 2 weeks following differentiation (Hastings & Gould, 1999; Zhao, Teng, Summers, Ming, & Gage, 2006). Two-week-old immature neurons at this stage receive GABA-mediated excitatory synaptic inputs and by 4 weeks these neurons receive glutamatergic input from the perforant path (Coderre et al., 2006). By the time new neurons are 4–8 weeks old, they may be capable of being recruited into hippocampus-based memory circuits (Kee, Sivalingam, Boonstra, & Wojtowicz, 2002). Further, new neurons are competitively advantaged compared to existing granule cells, by having a lower threshold of activation, and a lower threshold for inducing long-term potentiation (Schmidt-Hieber, Jonas, & Bischofberger, 2004; Wang, Scott, & Wojtowicz, 2000) and this competitive advantage is maintained for several months (Ramirez-Amaya, Marrone, Gage, Worley, & Barnes, 2006). The effect of irradiation on neurogenesis is a continuous and interactive process leading to the persistent decrease in the production of new neurons. The significant dose-dependent decrease in the production of new

**Figure 7.** Mean (±SEM) percent freezing during baseline and first tone, and ITI (1st trial) presentation for P21 (A), P50 (B), and P70 (C) with different doses of irradiation. No significant dose and age effects were observed between the groups.
neurons, as observed three months after irradiation could result in a lack of pool of younger neurons being available to be recruited into a hippocampally dependent learning and memory circuit after irradiation. However, not all the groups in our study that had a significant decrease in neurogenesis exhibited impairments in trace fear conditioning, indicating that impairments in trace fear conditioning are observed only when the decrease in BrdU + cells in the DG is greater than 60%. Thus, our results show a threshold for the number of new neurons that have to exist in the pool by continuing hippocampal neurogenesis and development of cognitive impairments following irradiation should the number fall below 60%.

Here we developed an animal model of irradiation induced deficits in hippocampal dependent learning and neurogenesis that are both age and dose dependent. Our results could be used to enhance those studies focused on neuroprotective strategies to potentially reverse the effects of the radiation-induced brain damage. Such studies include: Intravenous administration of basic fibroblast growth factor (bFGF) immediately prior to radiation therapy inhibits endothelial cell apoptosis in the central nervous system (CNS; Pena, Fuks, & Kolesnick, 2000); intrathecal administration of other growth factors such as platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF) are known to protect neurons from radiation damage (Andratschke et al., 2004; Nieder, Andratschke, Price, Rivera, & Ang, 2002); inhibiting acid sphingomyelinase activity using phosphatidylinositol-3, 5-biphosphate is also extensively studied, which is known to decrease apoptosis (Kolzer et al., 2003); intraperitoneal administration of erythropoietin (EPO) 1 hr after radiation treatment in animals blocked impairments in spatial memory, and reduced progenitor cell death by caspase inhibition (Fukuda et al., 2004); indomethacin given daily after radiation treatment decreased microglial activation and restored partially neurogenesis within the dentate gyrus (Mizumatsu et al., 2003); housing irradiated animals in enriched environments enhanced the number of new neurons and also improved spatial learning and memory compared to the irradiated animals housed in standard cages (Fan, Liu, Weinstein, Fike, & Liu, 2007; Meshi et al., 2006). All these studies contributed to the development of pharmacological and biological methods to protect against radiation-induced CNS damage. It remains to be seen if a decrease in apoptosis, an increase in neurogenesis, and an improvement in hippocampal dependent learning can be achieved without affecting the ability of radiation to kill tumor cells, using the animal model developed in this study.

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Radiation Impairs Trace Fear Memories


Received November 3, 2008
Revision received November 3, 2008
Accepted April 22, 2009

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