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Research report

Effect of behavioral testing on spine density of basal dendrites in the CA1 region of the hippocampus modulated by ^{56}Fe irradiation

Jacob Raber[a,b,*,1], Antiño R. Allen[c,1], Sydney Weber[a], Ayanabha Chakraborti[d,e], Sourabh Sharma[b], John R. Fike[b,f]

[a] Department of Behavioral Neuroscience, ONPRC, Oregon Health and Science University, Portland, OR 97239, United States
[b] Departments of Neurology, Radiation Medicine and Division of Neuroscience, ONPRC, Oregon Health and Science University, Portland, OR 97239, United States
[c] Division of Radiation Health, Department of Pharmaceutical Sciences, University of Arkansas for Medical Sciences, Little Rock, AR 72205, United States
[d] Brain and Spinal Injury Center, Department of Neurological Surgery, University of California, San Francisco, CA 94110, United States
[e] The Brain Research Institute at Monash Sunway, Selangor Darul Ehsan, Malaysia
[f] Department of Radiation Oncology, University of California, San Francisco, CA 94110, United States

HIGHLIGHTS

- Exploratory behavior increases basal spine density in the CA1 hippocampal region.
- Exploratory behavior increases basal spine density in the enclosed blade of the DG.
- These effects were not seen in ^{56}Fe-irradiated mice.
- ^{56}Fe irradiation affects the ability of exploratory behavior to alter spine measures.

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ABSTRACT

A unique feature of the space radiation environment is the presence of high-energy charged particles, including ^{56}Fe ions, which can present a significant hazard to space flight crews during and following a mission. ^{56}Fe irradiation-induced cognitive changes often involve alterations in hippocampal function. These alterations might involve changes in spine morphology and density. In addition to irradiation, performing a cognitive task can also affect spine morphology. Therefore, it is often hard to determine whether changes in spine morphology and density are due to an environmental challenge or group differences in performance on cognitive tests. In this study, we tested the hypothesis that the ability of exploratory behavior to increase specific measures of hippocampal spine morphology and density is affected by ^{56}Fe irradiation. In sham-irradiated mice, exploratory behavior increased basal spine density in the CA1 region of the hippocampus and the enclosed blade of the dentate gyrus. These effects were not seen in irradiated mice. In addition, following exploratory behavior, there was a trend toward a decrease in the percent stubby spines on apical dendrites in the CA3 region of the hippocampus in ^{56}Fe-irradiated, but not sham-irradiated, mice. Other hippocampal regions and spine measures affected by ^{56}Fe irradiation showed comparable radiation effects in behaviorally naïve and cognitively tested mice. Thus, the ability of exploratory behavior to alter spine density and morphology in specific hippocampal regions is affected by ^{56}Fe irradiation.

1. Introduction

During space travel, astronauts are exposed to irradiation from solar particle events (SPE), galactic cosmic radiation (GCR), and the earth's magnetosphere (Van Allen belt) [1]. The space radiation environment includes heavy ions, such as ^{56}Fe, ^{28}Si, ^{16}O, and ^{12}C, which are the major contributors to the dose in GCR. Among these heavy ions, ^{56}Fe ions are of particular concern, because they are the most densely ionizing particles present in relatively large amounts in GCR [2].

^{56}Fe irradiation-induced cognitive changes often involve alterations in hippocampal function [3–17]. The alterations in hippocampal function following ^{56}Fe irradiation might involve

* Corresponding author at: Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239, United States. Fax: +1 503 494 6877.
E-mail address: raberj@ohsu.edu (J. Raber).
[1] Equal contribution.

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changes in spine morphology and density. These changes might include alterations in spine morphology, number, and density, as well as retraction or loss of dendrite branching patterns and fragmentation of dendrites. Dendrites (the branched projections of a neuron) and dendritic spines are involved in synaptic transmission and learning and memory [18,19]. Changes in spine morphology and density are important for cognitive performance as they can be long-lasting [20] and correlate with synaptic plasticity and memory formation (for reviews, see Refs. [21,22] and are associated with neurological conditions.

The morphology of dendritic spines can be categorized into three main classes: the stubby spine, which lacks an apparent neck; the thin spine, which contains a small bulbous head and a thin, long neck; and the mushroom spine, which contains a large mushroom-shaped head [23,24]. Thin spines are transient spines that emerge and disappear over a few days. In contrast, mushroom spines persist for months. The numbers of spines are thought to reflect the amount of connectivity between neurons and because spines are sites of glutamatergic synapses, they can regulate the amount of excitatory neurotransmission in a particular region [19]. Changes in spine densities or length are thought to represent a morphological correlate of altered brain functions associated with learning and memory [25]. Such data might provide critical information regarding the mechanism of disruption of neural circuitry following high LET radiation exposure.

In addition to irradiation, performing a cognitive task can also affect spine morphology [26]. For example, exploratory behavior in a novel environment for 60 min can increase spine density [27]. This ability of exploratory behavior to increase specific measures of hippocampal spine morphology and density might be affected by space irradiation. In most studies, spine morphology is analyzed in either only behaviorally naïve animals or only behaviorally tested animals. As a result, it is often hard to determine whether changes in spine morphology and density are due to an environmental challenge or group differences in performance on cognitive tests. Interestingly, alterations in spine morphology are seen in aged Fisher female rats [28] and aged Long–Evans rats that show impaired hippocampus-dependent memory [29], but not in handled but behaviorally naïve aged Long–Evans rats [30], or aged vehicle-treated behaviorally naïve Sprague-Dawley rats [31]. However, the situation might be more complicated. Alterations in spine morphology were also reported in aged behaviorally naïve female and male [25]. The ability of cognitive testing to alter spine morphology is also important with regard to ability to cope with subsequent environmental challenges. For example, positive alterations in spine morphology following environmental enrichment might protect against detrimental effects of environmental challenges on cognitive performance [32].

In this study, we tested the hypothesis that the ability of exploratory behavior to increase specific measures of hippocampal spine morphology and density is affected by ^{56}Fe irradiation. Therefore, we compared hippocampal dendritic spine density and morphology in behaviorally naïve and cognitive tested mice three months following a relatively low dose of ^{56}Fe irradiation.

2. Materials and methods

2.1. Animals and study design

Two-month-old male C57Bl6/J wild-type mice ($n = 36$) purchased from the Jackson Laboratory (Bar Harbor, ME) were used in this study. The mice were housed under a constant 12 h light: 12 h dark cycle. Food (PicoLab Rodent Diet 20, no. 5053; PMI Nutrition International, St. Louis, MO) and water were provided ad libitum. All procedures were approved by Institutional Animal Care and Use Committee at OHSU and BNL.

Mice were shipped from the Jackson Laboratory to Brookhaven National Laboratory (Long Island, NY) and group housed at 4/cage. The mice were randomly assigned to two treatment groups that received either sham irradiation ($n = 18$) or whole body exposure of 0.5 Gy ^{56}Fe (600 MeV) irradiation ($n = 18$). One week following irradiation or sham-irradiation, the mice were shipped to the Oregon Health & Science University. Three months after irradiation, 12 sham-irradiated and 12 irradiated mice were tested for exploratory behavior and habituation, while the other 12 mice served as behaviorally naïve sham-irradiated and irradiated cage controls ($n = 6$ mice/group). The three-month time point was selected as that time point was shown in earlier studies to show radiation effects on neurogenesis [33], the immediate early gene encoding activity-regulated cytoskeleton-associated protein Arc [10,34], and measures of inflammation [35]. The mice were killed by cervical dislocation and their brains quickly removed at the same time as the mice that were cognitively tested. The brains of all mice were processed and analyzed for measures of spine morphology and density at the same time. From all experimental conditions, $n = 6$ mice per group or $n = 24$ mice in total, were analyzed for spine morphology. The spine morphology and behavioral performance of the cognitively tested mice have been reported separately [3].

2.2. Cognitive testing

On two subsequent days, mice were individually placed in enclosures containing grid floors in sound attenuated boxes for 5 min, as described previously [34]. Thirty minutes following the behavioral testing on the second day, the mice were killed by cervical dislocation and their brains quickly removed.

2.3. Golgi staining

For spine analyses, Golgi staining was performed using the FD Rapid Golgi Stain Kit (FD Neurotechnologies, Baltimore, MD), following the manufacturer's guidelines. Briefly, freshly removed brains were immersed in a proprietary impregnation solution and stored at room temperature for 2 weeks in the dark. Next, the brains were transferred to a second impregnation solution and incubated for 48 h at 4 °C. Finally, the tissue was shipped to FD Neurotechnologies, where they were sectioned to a thickness of 120 mm, stained and then mounted on gelatin coated slides.

2.4. Spine density and spine morphology

Spine analyses were conducted blind to the experimental conditions on coded Golgi impregnated brain sections containing the dorsal hippocampus, as described previously [3]. Spines were examined on dendrites of DG granule neurons as well as apical (stratum radiatum) and basal (stratum oriens) dendrites of CA1 and CA3 pyramidal neurons. The neurons that satisfied the following criteria were chosen for analysis in each of the experimental groups: (i) presence of untruncated dendrites; (ii) consistent and dark Golgi staining along the entire extent of the dendrites; and (iii) relative isolation from neighboring neurons to avoid interference with analysis. 3–5 dendritic segments, each at least 15 mm in length were analyzed per neuron, and 10–11 neurons were analyzed per brain. Neurons that met staining criteria were traced using a 63 × oil objective, a computerized stage, and Neurolucida software (Ver. 11, Microbrightfield, Inc., Williston, VT).

To acquire images for spine analysis, the dendritic segments were imaged under brightfield illumination on a Zeiss Axioimager microscope with a 63× oil immersion objective. This method does not assess spine density in a 3 dimensional manner but focuses on

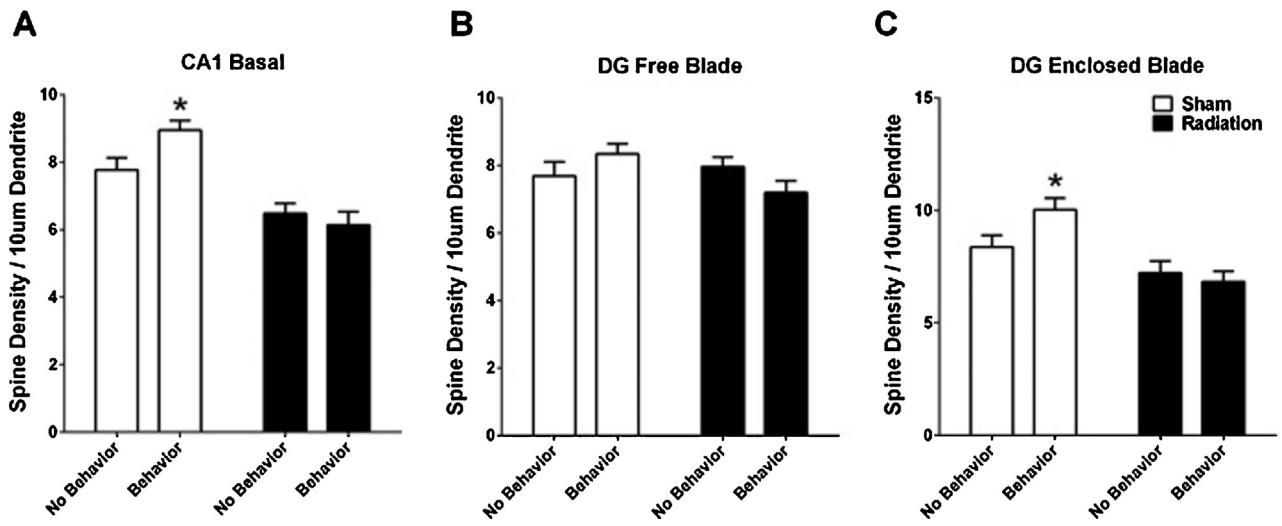

Fig. 1. (A) Cognitive testing increased the basal dendritic spine density in sham-irradiated mice in the CA1 region of the hippocampus, but it did not increase them in irradiated mice. There was a significant radiation × testing interaction for density of basal dendritic spines in the CA1 region of the hippocampus ($F = 5.012$, $p = 0.037$). Cognitive tested increased the basal dendritic spine density in sham-irradiated, but not irradiated, mice ($t = 2.567$, $p = 0.0299$, 2-tailed). (B) There was a significant radiation × testing interaction for spine density in the free blade of the dentate gyrus ($F = 4.412$, $p = 0.049$) but exploratory behavior did not significantly increase spine density in sham-irradiated mice or significantly decrease it in irradiated mice. (C) Cognitive testing increased the dendritic spine density in sham-irradiated mice in the enclosed blade of the dentate gyrus ($p < 0.05$, 2-tailed, C), but it did not increase them in irradiated mice. There was a trend toward a radiation × testing interaction in the enclosed blade of the dentate gyrus ($F = 4.063$, $p = 0.058$). *$p < 0.05$. $n = 5$–6 mice/radiation condition/testing condition.

spines that are parallel to the plane of section. Although the method may underestimate the total number of spines, it facilitates a direct comparison of treatment groups when they are analyzed in an identical manner. Image J software was used to calculate linear spine density, which was presented as the number of spines per 10 μm of dendrite length.

2.5. Statistical analysis

Data are expressed as mean and SEM. All statistical analyses were conducted using SPSS (Chicago, IL) software. As we a priori hypothesized that exploratory behavior would affect spine measures differently in sham-irradiated and irradiated mice, analyses were used to determine the effect of behavioral testing on spine measures in behaviorally naïve and cognitively tested mice separately. In addition, analysis of variance (ANOVA) was used with behavioral testing and irradiation as between subject factors to determine whether were radiation × behavioral testing interactions. All figures were generated using GraphPad Prism 6.0 software (La Jolla, CA). A $p < 0.05$ was considered significant.

3. Results

3.1. ^{56}Fe irradiation reduces exploratory behavior

On Day 1, sham-irradiated mice explored the novel environment more than irradiated mice (sham-irradiated: 307 ± 14 a.u.; irradiated: 271 ± 8 a.u.; $F = 5.103$, $p = 0.034$; 2-tailed t-test, $t = 2.259$, $p < 0.05$, $n = 12$ mice/group). When placed again in the same environment 24 h later, both groups showed reduced exploratory behavior compared to day 1 (sham-irradiated: 192 ± 16; irradiated: 176 ± 11; $n = 12$ mice/group) without an effect of irradiation, indicating that ^{56}Fe irradiation did not affect spatial habituation learning.

3.2. Ability of exploratory behavior to increase distinct measures of hippocampal spine morphology and density affected by ^{56}Fe irradiation

In this study, we tested the hypothesis that the ability of exploratory behavior to alter hippocampal spine morphology and density is affected by ^{56}Fe irradiation. Confirming our hypothesis, there was a significant radiation × testing interaction for density of basal dendritic spines in the CA1 region of the hippocampus ($F = 5.012$, $p = 0.037$, Fig. 1A) and spine density in the free blade of the dentate gyrus ($F = 4.412$, $p = 0.049$, Fig. 1B), and there was a trend toward a radiation × testing interaction for spine density in the enclosed blade of the dentate gyrus ($F = 4.063$, $p = 0.058$, Fig. 1C). While cognitive testing increased the basal dendritic spine density in sham-irradiated mice in the CA1 region of the hippocampus ($p < 0.05$, 2-tailed, Fig. 1A) and the enclosed blade of the dentate gyrus ($p < 0.05$, 2-tailed, Fig. 1C), it did not increase them in irradiated mice. As we a priori hypothesized that exploratory behavior would affect spine measures differently in sham-irradiated and irradiated mice, analyses were used to determine the effect of behavioral testing on spine measures in the enclosed blade of the dentate gyrus of behaviorally naïve and cognitively tested mice separately although the p values for the radiation × testing interaction was 0.058. In addition, following exploratory behavior, there was a trend toward a decrease in the percent stubby spines on apical dendrites in the CA3 region of the hippocampus in ^{56}Fe-irradiated, but not sham-irradiated, mice ($p = 0.087$, 2-tailed, Fig. 2).

There was specificity to the differential ability of exploratory behavior to affect spine measures in sham-irradiated and irradiated mice described above. Effects of ^{56}Fe irradiation only were seen for the density of apical and basal spines in the CA3 region of the hippocampus (Table 1). In addition, effects of ^{56}Fe irradiation only were seen for the percent thin spines in the enclosed blade of the dentate gyrus, the percent mushroom spines for basal spines in the CA1 and CA3 regions of the hippocampus, and the enclosed blade of the dentate gyrus, and the percent stubby spines for basal spines in the CA3 region of the hippocampus and in the enclosed blade of the dentate gyrus (Table 1). No other effects of ^{56}Fe irradiation were seen.

Table 1
Effects of ^{56}Fe irradiation only on hippocampal spine measures.[a]

Region	Sham		Radiation		Radiation effect (p-value)
	No Behavior	Behavior	No Behavior	Behavior	
Spine density					
Apical CA3	8.32 ± 0.38	8.54 ± 0.33	7.63 ± 0.39	7.32 ± 0.38	$p < 0.001$
Basal CA3	8.19 ± 0.54	8.11 ± 0.41	7.50 ± 0.36	7.30 ± 0.35	$p = 0.087$
% Thin spines					
DG Enclosed Blade	43.63 ± 0.89	43.73 ± 1.33	33.05 ± 2.21	31.64 ± 1.48	$p < 0.001$
%Mushroom spines					
Basal CA1	34.76 ± 0.68	30.77 ± 0.89	27.09 ± 1.38	26.89 ± 1.77	$p < 0.001$
Basal CA3	36.63 ± 1.82	34.79 ± 1.83	27.22 ± 1.70	26.61 ± 1.53	$p < 0.001$
DG Enclosed Blade	29.85 ± 0.85	28.96 ± 1.25	31.66 ± 1.43	33.81 ± 1.79	$p = 0.030$
% Stubby spines					
Basal CA3	18.57 ± 2.13	21.74 ± 1.57	25.86 ± 1.33	28.2 ± 2.77	$p = 0.003$
DG Enclosed Blade	26.53 ± 1.41	27.31 ± 1.77	35.29 ± 2.39	34.55 ± 2.25	$p = 0.001$

[a] n = 5–6 mice/radiation condition/testing condition.

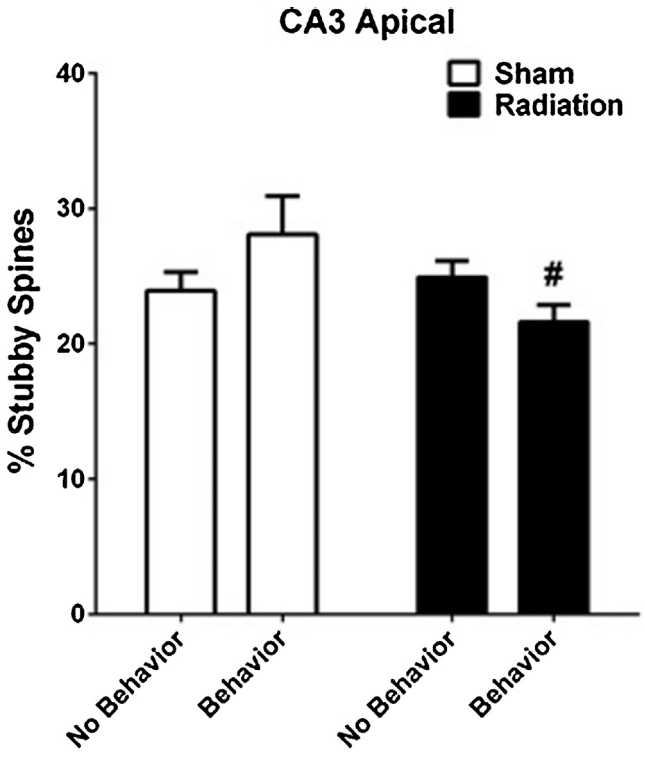

Fig. 2. Following exploratory behavior, there was a trend toward a decrease in the percent stubby spines on apical dendrites in the CA3 region of the hippocampus in ^{56}Fe-irradiated mice. This was not seen in sham-irradiated mice. There was a trend toward a radiation × testing interaction for the percent stubby spines on apical dendrites in the CA3 region of the hippocampus ($F = 4.041$, $p = 0.059$). n = 5–6 mice/radiation condition/testing condition. $^{\#}p = 0.087$.

4. Discussion

There are two main conclusions based on this study: (1) the ability of the brain to respond to a novel environment is affected by ^{56}Fe irradiation; and (2) when behaviorally naïve mice are not included in radiation studies and only brains of cognitively tested mice are analyzed, group differences in measures of spine morphology might be due to effects of irradiation or in response to the environment. As a result, group differences in response of the brain to the environment the animal is placed in during behavioral testing might contribute to effects attributed to irradiation.

This study shows that the ability of exploratory behavior to increase the density of basal dendritic spines in the CA1 region of the hippocampus and the density of spines in the enclosed blade of the dentate gyrus is affected by ^{56}Fe irradiation. In addition, there was a radiation × testing interaction for spine density in the free blade of the dentate gyrus. Other hippocampal regions and spine measures affected by ^{56}Fe irradiation showed comparable radiation effects in behaviorally naïve and cognitively tested mice. These data show that the ability of exploratory behavior to alter spine density and morphology in specific hippocampal regions is affected by ^{56}Fe irradiation.

Behavioral testing increased basal dendritic spine density in sham-irradiated, but not irradiated, mice in the CA1 region of the hippocampus. In our study, this effect was associated with reduced exploratory behavior of ^{56}Fe-irradiated mice in a novel environment. This indicates that exploratory behavior and curiosity might be affected by space irradiation. Other cognitive domains might be affected as well. For example, environmental enrichment affected spatial memory and synaptic plasticity in the CA1 region [36]. As the mice were irradiated three months prior to behavioral testing, these data indicate that ^{56}Fe irradiation has long-term detrimental effects on the brain. With the CA1 region as the output structure of the hippocampus [37,38], these data indicate the significance of these finding and suggests that extra hippocampal sites might be affected as well by these behavioral testing × radiation interactions.

In addition to the CA1 region of the hippocampus, there was a significant behavioral testing × radiation interaction in the free blade of the dentate gyrus. Interestingly, in a previous study we showed that mice that received head-only ^{56}Fe (1 Gy) irradiation showed a lower fraction of neurons expressing activity-regulated cytoskeleton-associated protein (Arc) in the free blade of the dentate gyrus than sham-irradiated mice [34]. Together, these data suggest that neurons normally showing increased spine density following exploratory behavior and having this ability being affected by ^{56}Fe irradiation might be expressing the immediate early gene Arc that is enriched in neuronal dendrites [39] and affected by cranial irradiation [40]. The trend toward a behavioral testing × radiation interaction for apical dendrites in the CA3 region of the hippocampus is remarkable based on the retraction and simplification of apical dendrites in the CA3 region of the hippocampus following chronic restraint stress [41,42].

The difference in the density of basal dendritic spines in the CA1 region of the hippocampus and the density of spines in the free blade of the dentate gyrus of sham-irradiated and ^{56}Fe-irradiated mice is more pronounced in behaviorally tested than behaviorally naïve mice. This is perhaps not a surprising result, as performing a cognitive task [26], including exploratory behavior for 60 min [27], can alter spine morphology and density. However, as in most studies spine morphology is analyzed in either only behaviorally naïve animals or only behaviorally tested animals it is hard to determine

the contribution of cognitive testing. It is conceivable that effects of space irradiation on the brain are underestimated as behaviorally naïve mice were used in the analysis. The inability of the irradiated brain to appropriately respond to a cognitive experience as seen in the brain of sham-irradiated mice suggests a functional impairment that can easily be missed pending the experimental paradigm used.

We recognize that as part of the experimental paradigm used, all mice were shipped from BNL to the home institution. Therefore, we cannot determine how this transport might have modulated effects of ^{56}Fe ion irradiation on spine measures of behaviorally naïve or cognitively tested mice three months later. To the best of our knowledge, there are no published reports directly comparing effects of space irradiation at BNL three months following exposure without shipping the mice following exposure with the effects of space irradiation at BNL following shipment of the mice to the home institution following radiation exposure. Housing and behaviorally testing of mice at BNL three months following irradiation might only partially address this possibility, as there are likely differences in housing conditions and animal handling at BNL and OHSU as well that might differentially affect spine measures. In addition, even if such differences were to be found, shipping of mice following irradiation would still be a relevant stressor, as astronauts in space also experience environmental stressors in addition to radiation exposure.

In summary, the ability of exploratory behavior to increase the density of basal dendritic spines in the CA1 region of the hippocampus and the density of spines in the enclosed blade of the dentate gyrus is affected by ^{56}Fe irradiation. These effects on spine morphology are not limited to ^{56}Fe irradiation and are also seen at shorter intervals following irradiation. For example, proton irradiation (0.1 or 1 Gy) reduced spine density in the dentate gyrus one month following exposure [43]. The effects of irradiation on spine morphology are likely indicative of cognitive injury to the CNS, as dendritic spines are receiving excitatory input and changes in their morphology are associated with functional synaptic alterations [24]. Consistent with this notion, abnormalities in dendritic spines morphology have been described in several neurological disorders associated with cognitive impairments, including age-related cognitive decline in the absence of frank dementia, Alzheimer's disease, Huntington's disease, schizophrenia, and neurodevelopmental disorders, including autism spectrum disorders [25,44–46]. Increased efforts are warranted to determine the molecular mechanisms underlying the reduced responsiveness of spines of the irradiated brain to cognitive tasks.

Competing interest

None of the authors has competing financial interests or other conflicts of interest.

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