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Acute and Fractionated Exposure to High-LET ⁵⁶Fe HZE-Particle Radiation Both Result in Similar Long-Term Deficits in Adult Hippocampal Neurogenesis

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Abstract

Astronauts on multi-year interplanetary missions will be exposed to a low, chronic dose of high-energy, high-charge particles. Studies in rodents show acute, nonfractionated exposure to these particles causes brain changes such as fewer adult-generated hippocampal neurons and stem cells that may be detrimental to cognition and mood regulation and thus compromise mission success. However, the influence of a low, chronic dose of these particles on neurogenesis and stem cells is unknown. To examine the influence of galactic cosmic radiation on neurogenesis, adult-generated stem and progenitor cells in Nestin-CreER^{T2}/R26R-YFP transgenic mice were inducibly labeled to allow fate tracking. Mice were then sham exposed or given one acute 100 cGy ⁵⁶Fe-particle exposure or five fractionated 20 cGy ⁵⁶Fe-particle exposures. Adult-generated hippocampal neurons and stem cells were quantified 24 h or 3 months later. Both acute and fractionated exposure decreased the amount of proliferating cells and immature neurons relative to sham exposure. Unexpectedly, neither acute nor fractionated exposure decreased the number of adult neural stem cells relative to sham exposure. Our findings show that single and fractionated exposures of ⁵⁶Fe-particle irradiation are similarly detrimental to adult-generated neurons. Implications for future missions and ground-based studies in space radiation are discussed.

INTRODUCTION

Astronauts travelling through interplanetary space will be exposed to a chronic low dose (~60 cGy per year) of galactic cosmic radiation (GCR) (1–4). Galactic cosmic radiation contains penetrating high-energy, high-charge nuclei (HZE) that are damaging to cells and tissues due to the inherent high-linear energy transfer (LET) properties these particles possess (4, 5). Small mammals sent into space subsequently show deficits in their immune, hematopoietic, reproductive and central nervous systems (CNS) (6–10). Experiments simulating GCR on Earth strongly suggest HZE particles themselves are damaging to these systems (11–13), particularly the CNS (14). However, it is unclear whether the results from ground-based experiments (which are primarily done with a single acute exposure) reflect the chronic or “fractionated” exposure that would be experienced in space (15, 16).

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Therefore, an important challenge for the field is to directly compare acute exposure versus fractionated whole-body exposures to high-LET particles. This would enable optimal translation of findings from ground-based HZE particle experiments into relevant risk assessment for spaceflight.

In regards to the CNS, evidence suggests high-LET particles encountered during spaceflight may damage brain regions critical for mission success. For example, ground-based HZE-particle exposure (100 cGy) leads to short- and long-term deficits in hippocampal-dependent spatial learning (17–19). Even lower doses of HZE particles (10–100 cGy) lead to persistent deficits in hippocampal-dependent spatial learning (20) and the premature appearance of neurodegeneration in a mouse model of Alzheimer's disease (21). One cellular explanation for these acute HZE-particle-induced functional deficits is a reduction in adult hippocampal neurogenesis (17, 19, 22–26), a dynamic process where stem cells and neuroblasts/immature neurons give rise to fully mature neurons (27, 28). While the negative effects of acute HZE-particle exposure on neurogenesis are clear, it is unknown if and how adult neurogenesis is influenced by a fractionated exposure. Such information would be important as it may either mitigate or exacerbate the CNS risk assessment for extended missions in space.

The purpose of this study was to determine how acute exposure versus fractionated HZE-particle exposures differentially influences adult hippocampal neurogenesis. Using transgenic mice in which the dynamic process of adult neurogenesis can be inducibly labeled and tracked (29), hippocampal stem cells and adult-generated neurons were quantified at 24 h and 3 months after acute or fractionated whole-body exposures of 100 cGy ^{56}Fe particles. Based on prior studies comparing the effect of whole-body acute exposure versus fractionated exposures on other CNS and non-CNS measures (15, 16), we hypothesized that fractionated exposures would be less damaging to adult hippocampal neurogenesis when compared to acute exposure. However, here we report that both acute and fractionated exposure cause long-term decreases in the number of immature and mature adult-generated neurons, but neither exposure scheme changes the number of putative hippocampal neural stem cells. These data highlight striking similarities between acute and fractionated HZE-particle exposure and their influence on adult hippocampal neurogenesis and therefore are important to consider for risk assessment for spaceflight on extended missions.

MATERIALS AND METHODS

Animals

All animal procedures and husbandry were in accordance with the National Institutes of Health, Guide for the Care and Use of Laboratory Animals, and performed in IACUC-approved facilities at UT Southwestern Medical Center (UTSW) and Brookhaven National Laboratories' animal facility. Experiments were designed to minimize animal number, pain and suffering. Male and female Nestin-CreER^{T2}/R26R-YFP mice were used (n = 68; 34 male, 34 female) (29). Mice were housed at UTSW (2–4/cage, 12-h light/dark cycle) with *ad libitum* access to food and water and moderate housing enrichment (Mouse Igloo®, Bio-Serv). To generate the mice for these studies, homozygous Nestin-CreER^{T2} transgenic mice on a C57BL/6 background [(29) generated by the Eisch Laboratory and bred at UTSW, but also available by Jackson Laboratories, stock #016261] were crossed with homozygous R26R-YFP knock-in mice (Jackson Laboratories, stock #006148) to produce the heterozygous mice (Nestin-CreER^{T2} hemizygous, R26R-YFP heterozygous, designated Nestin-CreER^{T2}/R26R-YFP) that were genotyped as previously described (29).

Drug Treatment

To induce recombination and YFP expression in nestin-expressing stem cells and their progeny (29), Nestin-CreERT²/R26R-YFP mice were given tamoxifen (TAM, 150–180 mg/kg, i.p., 30 mg/mL in 10% EtOH/sunflower oil, all from Sigma Aldrich) during young adulthood (5–6 weeks of age) for 5 days (29) (Fig. 1). To label proliferating cells in the S phase of the cell cycle (30), all mice received the thymidine analog bromodeoxyuridine (BrdU, 150 mg/kg, i.p., 10 mg/mL in 0.9% saline, Boehringer Mannheim) 2 h prior to being euthanized (Fig. 1) (30).

Particle Irradiation

Adult Nestin-CreERT²/R26R-YFP mice (8.6–11.7 weeks of age, 28–35 days post-TAM) were shipped from UTSW to Brookhaven National Laboratory (BNL; Upton, NY) and allowed to acclimate 4 days prior to irradiation (IR) (Fig. 1). The reference number for this NASA-sponsored experimental campaign was NSRL-09C. The 5 day experiment began on November 2, 2009 by bringing mice from Brookhaven Laboratory Animal Facility (BLAF) to the NASA Space Radiation Laboratory (NSRL). For each of the 5 days of the experiment, mice in all groups were placed into IR “hotels” (polypropylene, 50 mL conical centrifuge tubes, 30 × 115 mm, Fisher Scientific, ~6–8 5-mm air holes drilled throughout length of the tube) for ~20 min (Fig. 1, gray arrow) between 9–11 a.m. Mice were split across 3 groups (balanced by weight, birthdate and gender) to receive different treatments (Fig. 1): “Sham” mice (n = 21; 9 male, 12 female) received 5 exposures of 0 cGy; “Acute” mice (n = 25; 11 male, 14 female) received 4 exposures of 0 cGy and 1 exposure of 100 cGy (5th day, Fig. 1, one black arrow); “Fractionated” mice (n = 22; 14 male, 8 female) received 5 exposures of 20 cGy (Fig. 1, five black arrows). The ⁵⁶Fe²⁶⁺ ion beam was produced by the AGS Booster Accelerator at BNL and transferred to the experimental beam line in the NSRL. Irradiation at NSRL consisted of whole-body exposure to ⁵⁶Fe²⁶⁺ ions at 300 MeV/nucleon (⁵⁶Fe, LET, 238.8 KeV/micron). A ⁵⁶Fe beam was delivered at an average dose rate of 20 cGy/min for the fractionated group and 100 cGy/min for the acute group. Delivered doses were ±0.5% of the requested value.

Body Weight Measures and Tissue Collection

Weights were recorded for mice 1 month prior to irradiation, 24 h postirradiation and 3 months postirradiation. According to UTSW and BNL IACUC-approved policies, mice were euthanized by live decapitation 24 h or 3 months postirradiation and brains were rapidly extracted. Brains were cut on the midsagittal sulcus and immersion-fixed in 4% paraformaldehyde (PFA). PFA was changed twice daily for 2 days before brains were placed in cryoprotectant [30% sucrose in 0.1 M PBS and 0.1% sodium azide (NaN₃)]. After brains sank – an indicator of cryoprotection – brain hemispheres were sectioned coronally on a freezing microtome (Leica) at a thickness of 30 μm through the entire longitudinal extent of the hippocampus plus immediately anterior and posterior regions (distance from Bregma –0.82 to –4.24) in a 1:9 series and stored in 0.1% NaN₃ in 1×PBS at 4°C until processed (31).

Immunohistochemistry (IHC)

Immunohistochemistry was performed generally as previously described (29, 31). For staining, one hemisphere from the series of 1:9 sections was mounted onto glass slides (Superfrost/Plus, Fisher) in rostral to caudal order and allowed to dry for 2 h. For immunohistochemistry to visualize BrdU-immunoreactive (BrdU⁺) cells, pre-treatment consisted of antigen retrieval (0.01 M citric acid, pH 6.0, 95 °C, 15 min), membrane permeabilization (0.1% trypsin in 0.1 M Tris and 0.1% CaCl₂, 10 min), DNA denaturation (2N hydrochloric acid in 1×PBS, 30 min) and quenching of endogenous peroxidases (0.3%

hydrogen peroxide in 1×PBS, 30 min). For immunohistochemistry for visualization of doublecortin (DCX)⁺ and yellow fluorescent protein (YFP)⁺ cells, pretreatment consisted of only antigen retrieval. For all immunohistochemistry, nonspecific staining was blocked by incubation with 3% normal donkey serum (NDS) in 0.1% Triton X-100 in 1×PBS for 60 min. Sections were incubated in their respective primary antibody [rat- α -BrdU, 1:400, Accurate; goat- α -DCX, 1:500, Santa Cruz; rabbit- α -GFP (used to detect YFP), 1:3000, Invitrogen] overnight at room temperature in 3% NDS, 0.1% Tween-20 in 1×PBS. The following day, sections were incubated in their respective biotinylated antibody (donkey anti-rat, donkey anti-goat or donkey anti-rabbit, 1:200 in 1.5% normal donkey serum in 1×PBS, Jackson ImmunoResearch) for 60 min followed by rinses and then avidin-biotin complex (ABC, 1:50, Vector Laboratories) for 60 min. Staining of BrdU⁺ and DCX⁺ cells was visualized using 3,3-Diaminobenzidine (DAB, Thermo Scientific Pierce) and counterstained with Nuclear Fast Red (Vector Laboratories). Staining of YFP⁺ cells was visualized with CY3-conjugated Tyramide Signal Amplification Systems (TSA, Perkin-Elmer) and counterstained with DAPI (1:5000, Roche Applied Science). Based on parameter optimization, the quenching (H₂O₂ step) was performed for BrdU staining after Trypsin and HCl steps; for DCX prior to secondary incubation; and for YFP between the secondary and ABC incubations. Tissue for all staining was then dehydrated and cover slipped (VWR, no. 1.5, 24 × 60 mm) with DPX Mountant (Sigma-Aldrich).

Cell Quantification and Subregional Volume Determination

Using an Olympus BX-51 microscope (Tokyo, Japan), BrdU⁺, YFP⁺ and DCX⁺ cells were quantified using stereological principles. BrdU⁺ and YFP⁺ cells are rare populations, and therefore were assessed stereologically by exhaustive counting of cells in the subgranular zone (SGZ) of the hippocampus at 400× magnification (NA = 0.90) as previously described (29, 32). Briefly, stereological parameters of BrdU⁺ and YFP⁺ cells used a section sampling fraction of 1/9, an area sampling fraction set to 1 to account for rare populations of cells and a height sampling fraction of 1 (32, 33). The raw counts were multiplied by 18 to estimate the total number of BrdU⁺ or YFP⁺ cells in the entire SGZ of both hemispheres. The SGZ was defined as the inner half of the dentate gyrus granule cell layer (GCL) and two cell-widths into the hilus (34). BrdU⁺ cells presented darkly stained or punctated irregular shaped nuclei (Fig. 2A). Total YFP⁺ cells were quantified, but YFP⁺ Type-1 cells were also quantified based on their irregularly shaped soma and a characteristic central meandering projection with many branching processes and fine filigree-tufted ends of the molecular-layer-leading process [Fig. 4A, boxed (33, 35)]. DCX⁺ cells were quantified by Stereo-Investigator software (MBF Bioscience, Williston, VT) at 400×magnification (NA = 0.70) using the optical fractionator. The section sampling fraction was 1/18 [grid size: 100 × 100, frame size: 80 × 80, estimated mean Schmitz-Hoff CE values were between 0.09 and 0.16 with an averaged group mean of 0.12 (36), modified from ref. (37)]. Raw DCX⁺ counts were multiplied by 2 to give the total number of DCX⁺ cells in the subgranular zone.

Subregional volume determination was made for three discrete regions of the dentate gyrus (DG): hilus, GCL and molecular layer (Mol). The volume of each was calculated using Cavalieri's principle within Bregma -0.82 to -4.24 using Stereo-Investigator software (29, 38). The hilus was defined as the region within the infra- and suprapyramidal blades of the GCL and enclosed at the caudal ends of the pyramidal blades (Fig. 5A). The molecular layer was defined as the area between the GCL and the hippocampal fissure. Pilot experiments revealed that reliable and repeatable molecular layer measurements were achieved by enclosing the molecular layer dorsal to the suprapyramidal blade by connecting the most caudal point of the suprapyramidal blade to the hippocampal fissure (Fig. 5A). After collecting hilus, GCL and molecular layer volumes, the volumes were summed to generate the DG volume. Images were imported into Photoshop (Adobe Systems) and adjustments

were made using the “level” function, in accordance with published guidelines for image presentation (39).

Statistical Analyses

Data are reported as mean $\pm$ SEM for sham, acute and fractionated groups. Statistical analyses were performed using a two-way analysis of variance (ANOVA) followed by a Bonferroni’s post-hoc (brain region volume) or one-way ANOVA followed by a Tukey’s multiple comparison post-hoc test (neurogenesis measures). All statistical analyses were performed using Prism GraphPad (version 5.0d) software. Statistical significance was defined as $P < 0.05$, but values of $P < 0.01$ and $P < 0.001$ are shown as well to indicate level of confidence.

RESULTS

Acute and Fractionated Irradiation both Result in Fewer Proliferating (BrdU⁺) Cells 24 h and 3 Months after Exposure

Nestin-CreER^{T2}/R26R-YFP mice in the sham, acute and fractionated groups were in good health throughout the experiment, gaining weight at a normal rate at the short time point (24 h) postirradiation. However, relative to acute and sham, fractionated mice appeared to gain more weight at the long time point (3 months) postirradiation, but neither the acute or fractionated groups were significantly different from the sham group when expressed as percentage sham (data not shown). To examine the influence of ⁵⁶Fe-particle irradiation on the number of proliferating hippocampal cells, mice received BrdU 2 h prior to sacrifice 24 h and 3 months postirradiation (Fig. 1) (40). BrdU⁺ cells were easily identified in the hippocampal SGZ of all mice, presenting typical darkly stained nuclei with immunoreactive puncta (Fig. 2A inset). At both the 24 h and 3 months time points, there was a significant effect of treatment on BrdU⁺ SGZ cell number (24 h: $F_{(2,29)} = 103.6$, $P < 0.001$; 3 months: $F_{(2,27)} = 13.49$, $P < 0.001$). At the 24 h time point postirradiation (Fig. 2B), post-hoc analysis revealed that mice in both acute and fractionated groups had fewer BrdU⁺ SGZ cells when compared to the sham group [acute vs. sham: $M = 3118$, 95% CI (2559, 3678), $P < 0.001$; fractionated vs. sham: $M = 2455$, 95% CI (1866, 3043), $P < 0.001$]. Acute and fractionated groups were also significantly different from each other [$M = -663.8$, 95% CI (-1239, -88.22), $P < 0.05$], with a greater decrease seen in the acute group (74%) than in the fractionated group (58%) when compared to sham group. At the 3 months time point (Fig. 2C), post-hoc analysis revealed that mice in both acute and fractionated groups had fewer total BrdU⁺ cells when compared to mice in the sham group [acute vs. sham: $M = 730.0$, 95% CI (375.0, 1085), $P < 0.001$; fractionated vs. sham: $M = 570.2$, 95% CI (161.3, 979.1), $P < 0.01$]. At the 3 months time point there were moderately fewer BrdU⁺ cells in the acute group (46%) than the fractionate group (36%) compared to the sham group; however, acute and fractionated groups were not significantly different from each other [$M = -159.8$, 95% CI (-530.2, 210.6), $P < 0.05$]. Therefore, relative to the sham exposure, both acute and fractionated ⁵⁶Fe-particle exposures result in immediate and long-term deficits in the number of proliferating cells in the hippocampal SGZ.

Acute and Fractionated ⁵⁶Fe-Particle Exposures both Result in Fewer Neuroblasts/Immature Neurons (DCX⁺) Cells 24 h and 3 Months Postirradiation

While a decrease of proliferating SGZ cells is often reflective of a decrease in adult-generated neurons, the processes of proliferation and neurogenesis/survival can also be differentially regulated (41). Therefore, we also quantified the number of DCX⁺ cells (Fig. 3), a widely used measure of neurogenesis levels (42). DCX⁺ cells were evident in the SGZ and GCL of all mice, presenting their characteristic range of morphologies indicative of mitotic neuroblasts and post-mitotic immature neurons (Fig. 3A) (43). Similar to the BrdU

results, at both 24 h and 3 months postirradiation there was a significant effect of treatment on the number of DCX⁺ GCL cells [24 h: Fig. 3B, $F_{(2,23)} = 29.54$, $P < 0.001$; 3 months: Fig. 3C, $F_{(2,26)} = 12.43$, $P < 0.001$]. At the 24 h time point postirradiation (Fig. 3B), post-hoc analysis revealed that mice in both acute and fractionated groups had fewer DCX⁺ GCL cells when compared to the sham group [acute vs. sham: $M = 23113$, 95% CI (13661, 32566), $P < 0.001$; fractionated vs. sham: $M = 29271$, 95% CI (19018, 39523), $P < 0.001$]. Further post-hoc analysis revealed that while there appeared to be a greater decrease in the fractionated group (54%) than in the acute group (43%) when compared to sham group, acute and fractionated groups were not significantly different from each other [$M = 6157$, 95% CI (-3295, 15609), $P < 0.05$]. At the 3 months time point postirradiation (Fig. 3C), post-hoc analysis revealed that mice in both acute and fractionated groups had fewer DCX⁺ cells when compared to the sham group [acute vs. sham: $M = 6970$, 95% CI (3275, 10664), $P < 0.001$; fractionated vs. sham: $M = 6190$, 95% CI (2048, 10333), $P < 0.01$]. While there appeared to be a slightly greater decrease in the fractionated group (39%) than in the acute group (44%) when compared to the sham group, further post-hoc analysis revealed that acute and fractionated groups were not significantly different from each other [$M = -779.5$, 95% CI (-4754, 3195), $P < 0.05$]. Therefore, relative to sham exposure, both acute and fractionated ⁵⁶Fe-particle exposures result in immediate and long-term deficits in the number of immature neurons in the hippocampal GCL.

Acute and Fractionated ⁵⁶Fe-Particle Exposures both Result in Fewer YFP⁺ Cells but Not Type-1 YFP⁺ Cells 24 h and 3 Months Postirradiation

The decrease in both BrdU⁺ and DCX⁺ cell numbers (Figs. 2 and 3) suggested that irradiation induced a decrease in adult hippocampal neurogenesis. However, BrdU and DCX IHC analyses alone do not allow assessment of the dynamic process of neurogenesis. In addition, neither BrdU nor DCX label the putative local source of adult-generated progenitors and neurons, the Type-1 neural stem cell (29). Therefore, to gain more clarity on how ⁵⁶Fe particle irradiation influences neurogenesis, we examined the number of YFP⁺ cells in our TAM-injected Nestin-CreER^{T2}/R26R-YFP mice.

As previously shown (29), TAM induced YFP labeling of Type-1 cells with radial glial morphology in the SGZ as well as the derivative progenitors and mature granule neurons (Fig. 4A). Stereological quantification of total YFP⁺ cell number revealed that, as with the BrdU⁺ and DCX⁺ cell counts, there was a significant effect of treatment on the number of total YFP⁺ cells [24 h: Fig. 4B, $F_{(2,25)} = 5.712$, $P < 0.01$; 3 months: Fig. 4C, $F_{(2,30)} = 4.213$, $P < 0.05$]. At the 24 h time point postirradiation (Fig. 4B), post-hoc analysis revealed that mice in both acute and fractionated groups had fewer total YFP⁺ cells in the SGZ when compared to the sham group [acute vs. sham: $M = 4385$, 95% CI (585, 8185), $P < 0.05$; fractionated vs. sham: $M = 4800$, 95% CI (914.2, 8686), $P < 0.05$], with a similar level of decrease seen in both acute (47%) and fractionated groups (52%) compared to sham group [$M = 415.2$, 95% CI (-3128, 3958), $P < 0.05$]. At the 3 months time point postirradiation (Fig. 4C), acute and fractionated groups also had fewer total YFP⁺ cells in the SGZ when compared to the sham group [acute vs. sham: $M = 3580$, 95% CI (138.6, 7020), $P < 0.05$; fractionated vs. sham: $M = 3667$, 95% CI (81.43, 7252), $P < 0.05$], with a similar level of decrease seen in both acute (40%) and fractionated (41%) groups compared to the sham group [$M = 87.30$, 95% CI (-3254, 3428), $P < 0.05$].

Total YFP⁺ cell number in the Nestin-CreER^{T2}/R26R-YFP mouse is often used as the initial indicator of the influence of a manipulation on neurogenesis (35). Further determination of Type-1 cell number directly by stereology allows direct assessment of the influence of ⁵⁶Fe particles on the nestin-lineage neural stem cells (29). The YFP⁺ Type-1 cells can be identified by their characteristic irregularly-shaped soma, distinctive meandering and

branching process, and fine filigree-tufted ends of the molecular-layer-leading process (Fig. 4A, see boxed area). There was no significant effect of IR on Type-1 YFP⁺ cell number at either the 24 h (Fig. 4D) or 3 months time points postirradiation [Fig. 4E; 24 h: $F_{(2,23)} = 2.829$, $P < 0.05$; 3 months: $F_{(2,30)} = 0.529$, $P < 0.05$]. Therefore, relative to sham exposure, both acute and fractionated ⁵⁶Fe-particle exposures result in immediate and long-term deficits in the total number of YFP⁺ cells, but not the Type-1 YFP⁺ cells in the hippocampal SGZ.

Neither Acute nor Fractionated ⁵⁶Fe-Particle Exposures Result in Changes in Dentate Gyrus or Hippocampal Volume 3 Months Postirradiation

One potential reason for the significantly decreased indices of neurogenesis seen in acute and fractionated ⁵⁶Fe-particle exposed mice is that irradiation may have decrease the overall size of the hippocampus. To address this, we stereologically quantified the volume of hippocampal subregions (molecular layer, GCL, hilus and DG; Fig. 5A) at the 3 months time point postirradiation (Fig. 5B). As expected given the different volumes of these subregions (Fig. 5A), there was a main effect of subregion ($F_{(3,55)} = 2220.89$, $P < 0.001$), with the dentate gyrus and molecular layer being larger than the GCL and hilus. However, there was no effect of treatment [$F_{(2,55)} = 2.16$, $P < 0.05$] or interaction of treatment and subregion [$F_{(6,55)} = 0.58$, $P < 0.05$]. These data show that acute and fractionated irradiation had no effect on hippocampal subregional volume.

DISCUSSION

Ground-based studies widely support that acute HZE particle exposure damages the CNS, leading to cellular and structural deficits (e.g., decreased adult hippocampal neurogenesis) and functional deficits (e.g., diminished performance on hippocampal-dependent tasks). These changes underscore the potential risk of HZE particle exposure to mission success (23, 44, 45). However, prior to our study presented here, it was unclear if fractionated and acute exposure to HZE particles had similar deleterious effects on adult hippocampal neurogenesis. Fractionated exposure is presumed to more closely mimic the chronic low exposure that astronauts receive in space (46). Therefore, the current study was designed to directly compare acute and fractionated HZE particle exposure in an effort to clarify the risk of short- and long-term space flight in regards to adult hippocampal neurogenesis.

Using an inducible transgenic mouse model to label nestin-expressing stem cells and their progeny, we demonstrate that both acute (e.g., nonfractionated) and fractionated ⁵⁶Fe-particle exposures produce similar negative effects on adult hippocampal neurogenesis. Specifically, both acute and fractionated HZE-particle exposure decreased the number of progenitors and neuroblast/immature neurons in the adult mouse hippocampus. In contrast to the negative effects on these later stages of adult hippocampal neurogenesis, neither acute nor fractionated HZE-particle exposure changed the number of putative hippocampal neural stem cells. As discussed below, our acute findings are generally consistent with prior work, with the intriguing difference that in our current study we show no decrease in the number of adult neural stem cells. In addition, our fractionated findings are highly novel, showing robust short- and long-term decreases in adult hippocampal neurogenesis. Taken together, these data provide evidence that ground-based studies in space radiation using acute and fractionated HZE-particle exposure are similarly detrimental, at least in regards to adult hippocampal neurogenesis.

Decreased Adult Hippocampal Neurogenesis after Acute or Fractionated ^{56}Fe -Particle Exposure

Our data on acute ^{56}Fe -particle exposure have similarities to previously published data. For example, our data are consistent with the literature that ^{56}Fe -particle exposure decreases adult hippocampal neurogenesis (19, 22–26). Methodological differences make it difficult to make precise comparisons in the magnitude of the effects presented here versus those in prior results. However, even with these differences it is useful to note that both our stereological assessment here (seen after exposure to 100 cGy, 300 MeV/n ^{56}Fe , LET = 238 keV/ μM) and prior results with quantitative but non-stereological assessment [1,000 MeV/n ^{56}Fe , LET = 148 keV/ μM (23, 24)] show ~40% decrease in DCX⁺ cells in the SGZ 3 months postirradiation. Therefore, a central consistency of our current work with prior work is confirmation that acute exposure to ^{56}Fe particles results in relatively long term reductions in adult hippocampal neurogenesis. These may indeed contribute to the hippocampal functional deficits seen in various models (18–21, 45, 47).

In addition to this confirmation that acute ^{56}Fe -particle exposure decreases adult hippocampal neurogenesis, our data also provide several novel insights into the effects of fractionated ^{56}Fe -particle exposure. Specifically, our work shows that both acute and fractionated ^{56}Fe -particle exposure result in 40–50% in DCX⁺ cell number 24 h and 3 months postirradiation. It is interesting to consider our results using a Poisson distribution, which gives the probability a cell will be hit at a given fluence (48). For example, the estimated average hits a neuron (10 μm diameter) would receive in our acute and fractionated groups are 2.0 and 0.4 per exposure, respectively. This can be interpreted to mean that a neuron on average is hit twice with one exposure (acute) or a neuron is hit 0.4 times per day over the course of 5 days of exposure (fractionated). Although the overall total number of HZE particles that hit a neuron is predicted to be the same (2 hits per neuron after 5 days) in both acute and fractionated groups, in the fractionated ^{56}Fe -particle group there may be a time interval between 2 different hits in the same neuron. The interval between fractions presumably allows DNA repair and possible recovery from radiation-induced damage, as observed after low-LET radiation exposure. Here our results demonstrate that ^{56}Fe -particle exposure – irrespective of acute or protracted fractionated exposure – results in a long lasting decrease in the process of adult hippocampal neurogenesis (Figs. 2 and 3). Therefore, fractionated ^{56}Fe -particle exposure is likely equally damaging as acute ^{56}Fe -particle exposure, suggesting minimum repair activity could be carried out during the short interval between fractions. It agrees with the general notion that complex HZE-particle induced DNA lesions are difficult or impossible to repair properly (5, 49, 50).

Neural Stem Cell Number Is Unchanged after Acute or Fractionated ^{56}Fe -Particle Exposure

While there is an equivalent loss of immature neurons and neuroblasts after both acute and fractionated irradiation (Fig. 3B, C), there is no change in stem cell numbers after either acute or fractionated irradiation (Fig. 4D, E). This is intriguing, as an intact neural stem cell pool is thought to be an ongoing source of new neurons throughout life (35, 51, 52). Therefore, the maintenance of the stem cell pool observed here suggests that the irradiated brain still maintains the potential to regenerate the lost adult-generated neurons.

An important difference between our work and the published literature is that we report no decrease in the number of neural stem cells in the SGZ after acute or fractionated exposure. This differs from published work showing that acute 100 cGy ^{56}Fe -particle radiation leads to a 45% reduction in the number of the quiescent neural stem cells in the SGZ (25). Both our work and this prior work share similar experimental design, including the irradiation of 2-month-old mice, and both use transgenic mice to label nestin-expressing stem cells in DG. However, there are key differences. For example, Encinas *et al.* used head-only exposure,

while we used whole-body exposure. It is possible that head-only exposure induces a distinct physiological response than whole-body exposure, leading to an immune response that diminishes the adult neural stem cell population (22, 53, 54), a concept that warrants future study.

There are other important differences between Encinas *et al.* and our study. For example, Encinas *et al.* used a lower LET ^{56}Fe particle (1,000 MeV/n, LET = 148 keV/ μM), examined only a short 24 h time point, used a constitutive nestin “reporter” mouse coupled with immunostaining to identify and quantify certain cell types (e.g., neural stem cells and transiently amplifying progenitor cells) and employed a 24 h delay between BrdU and kill. In contrast, our study used: a higher LET ^{56}Fe particle (300 MeV/n, LET = 238 keV/ μM) and evaluated both 24 h and 3 months time points postirradiation; an inducible nestin transgenic mouse to examine stem cells and their progeny using YFP⁺ immunostaining and morphological analysis; used immunostaining for markers of stages of neurogenesis (e.g., DCX to infer neurogenesis); and employed a 2 h delay between BrdU and kill. These differences make it challenging to directly compare the studies. Our study assesses a broader span of the dynamic process of neurogenesis, since we assess DCX⁺ cell number (42) and proliferating cells by exogenous S-phase marker BrdU, as well as the number of YFP⁺ stem cells and their YFP⁺ progeny. However, it is important to note that our results may be influenced by the fact that although mice were “adult” at time of irradiation, our mice were young adult (5–6 weeks of age) at the time that recombination was induced. Regardless, a point of agreement in both studies is that ^{56}Fe -particle exposure decreases the number of dividing SGZ cells. Aside from this common result, additional studies are needed to assess which of these individual or combined experimental and methodological differences contribute to the maintenance of adult neural stem cell number reported here.

Conclusions, Limitations and Future Directions

Our data show robust short- and long-term decrease in adult hippocampal neurogenesis after acute or fractionated ^{56}Fe -HZE-particle exposure. Thus, these data provide a direct comparison of how acute vs. fractionated exposure influence a cellular measure linked to hippocampal function. As such, these cellular data support the appropriateness of using ground-based acute-only exposure to assess CNS risk in astronauts during long-term interplanetary space travel. These data are also important as they represent the first step towards modeling the influence of a very low-dose rate on hippocampal neurogenesis. However they are only the first step. Below we discuss the limitations and future directions of this work in the hope that they will be considered in regards to future experiments.

For example, one limitation of our study is the dose rate used and duration of exposure. Our dose rate (20 cGy/day) and duration (5 days) are 100×higher and 40×shorter than what is estimated for a mission to Mars (~0.2 cGy/day, 200 days) (4). While our dose rate and duration were close to the limits currently feasible with ground-based simulation of GCR, future studies should use an even lower dose rate and even longer duration when possible. It is difficult to predict what influence lower dose rates would have on the CNS, but published works using low acute doses provide some insight. For example, some work already shows a negative influence of acute 20 cGy exposure on CNS function (20, 55), suggesting even low-acute doses can be damaging. However, other work comparing acute exposure of 10 vs. 100 cGy (21) or 5 vs. 50 and 100 cGy (56) reveal mixed results; low and high doses cause equal deficits in some behavioral and/or cellular assays, but the low doses do not cause deficits in other measures (21, 56). Based on these CNS data and other non-CNS data showing a sparing effect after fractionation (15, 16), one might hypothesize that low dose rates may not be as detrimental as higher dose rates. However, it is also possible that low dose rates over a longer period would be more detrimental as the cells have less time to

recover between exposures. Finally, it is possible that the repeated exposure at a low dose rate has some stimulatory influence, resulting in a positive influence on some cellular or behavioral measures (57). Clearly more work with low dose rates (0.2 cGy/day) and longer durations (minimum 5 days) is warranted.

Another limitation of the current study – and many other studies of “adult” neurogenesis – is the age of the mice at the time of recombination (5–6 weeks of age) and irradiation (~9–11 weeks of age). Our study spans a dynamic period of hippocampal neurogenesis, when levels of neurogenesis are decreasing rapidly to reach a lower steady state by 2 months of age (58, 59). Focus on this developmental period was useful for this first study, as it matched the age of HZE-particle irradiation in other studies (25), thus allowing comparison across publications. However, it would be more relevant for spaceflight to use older mice whose age is a rough equivalent of that of astronauts [6 months old mouse is roughly equivalent to a 30-year-old human (60)]. This is particularly pertinent in regards to our data showing maintenance of adult neural stem cell number after ^{56}Fe -particle exposure (i.e., no change in adult neural stem cell number between sham and irradiated groups was observed 24 h and 3 months postirradiation). The persistence of the Type-1 stem cell pool suggests that the irradiated hippocampus may have the potential for regeneration of adult neurogenesis (61). However, as stem cell characteristics change with age (62, 63), additional studies are warranted to assess the “functionality” of the stem cell pool at even longer time points postirradiation. In addition, given the existence of numerous sources of neural stem cells in the adult mouse SGZ (33), future studies would also ideally utilize different inducible transgenic models to label and track stem cells arising from discrete sources to assess their contribution to adult neurogenesis in both young and older animals. The influence of age and time postirradiation is particularly important to assess in future studies exploring the mechanisms underlying decreased neurogenesis, as the influence of irradiation on the neurogenic milieu, including the vasculature, likely changes over time (24, 64).

Our findings show that a single exposure, as well as fractionated exposure, of particle radiation are both similarly detrimental to the dynamic process of adult hippocampal neurogenesis. Loss of neurogenesis is often linked to deficits in learning, memory and mood regulation (65). However, the influence of altered neurogenesis on hippocampal function is also state-dependent (66); for example, the requirement of neurogenesis for mood regulation is only seen under conditions of stress. Therefore, it would be of great utility to assess whether the loss of adult neurogenesis reported here results in functional deficits in hippocampal-dependent tasks, and to further assess whether the state-dependency of hippocampal function on neurogenesis is influenced by particle radiation.

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FIG. 1.

Time line of experiment and schematic of experimental groups. Nestin-CreER^{T2}/R26R-YFP mice received Tamoxifen (TAM) for 5 days at ~5–6 weeks of age, ~30 days prior to irradiation (IR). During the 5 day experiment at Brookhaven National Laboratories/NSRL, mice in the sham (S, n =21), acute (A, n =25) and fractionated (F, n = 22) groups were placed in irradiation chambers for equivalent periods of time (gray arrow). Sham exposed mice did not receive radiation while in irradiation chambers. Mice in the acute group received one exposure of 100 cGy on day 5 (one black arrow) and mice in the fractionated group received 20 cGy on days 1–5 (five black arrows). All groups received BrdU 2 h prior to sacrifice, either at 24 h or 3 months time points postirradiation.

FIG. 2.

Acute and fractionated ^{56}Fe -particle exposure result in fewer proliferating (BrdU^+) cells at 24 h and 3 months postirradiation compared to sham exposure. Panel A: Photomicrograph of BrdU^+ cells in the SGZ (400 $\times$); inset: cluster of BrdU^+ cells (1,000 $\times$) seen at lower power in dotted box (400 $\times$). Scale bar: (panel A) 20 μm ; inset 10 μm . Panels B and C: Stereological quantification of BrdU^+ cells at 24 h (panel B) or 3 months (panel C) postirradiation. GCL = granule cell layer; H =hilus; Mol =molecular layer; SGZ =subgranular zone. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; $n = 7\text{--}13/\text{group}$.

FIG. 3.

Acute and fractionated ^{56}Fe -particle exposure result in fewer neuroblasts/immature neurons (DCX⁺) cells at 24 h and 3 months postirradiation compared to sham exposure. Panel A: Photomicrograph of DCX⁺ cells in the SGZ extending their processes through the SGZ into the molecular layer (400 $\times$). Scale bar: (panel A) 20 μm . Panel B–C: Stereological quantification of DCX⁺ cells at 24 h (short, panel B) or 3 months (long, panel C) time points postirradiation. GCL = granule cell layer; H = hilus; Mol = molecular layer; SGZ = subgranular zone. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; $n = 6\text{--}12/\text{group}$.

FIG. 4.

Acute and fractionated ^{56}Fe -particle exposure result in fewer YFP⁺ cells but not Type-1 YFP⁺ cells. Panel A: Photomicrograph of YFP⁺ cells in the GCL of a sham exposed mouse 24 h postirradiation (400 $\times$); white arrows: YFP⁺ cells with the morphology of mature GCL neurons; boxed area: two YFP⁺ cells with the morphology of a Type-1. Scale bar: (panel A) 20 μm . Panels B–E: Stereological quantification of YFP⁺ cells (panels B and C) and YFP⁺ Type-1 cells (panels D and E) at 24 h (panels B and D) and 3 months (panels C and E) time points postirradiation. GCL = granule cell layer; H = hilus; Mol = molecular layer; SGZ = subgranular zone. * $P < 0.05$; $n = 7\text{--}12/\text{group}$.

FIG. 5.

⁵⁶Fe-particle irradiation has no effect on hippocampal volume 3 months after exposure compared to Sham exposure. Panel A: Photomicrograph of the hippocampus (40×) depicting the dentate gyrus subregional analysis used for volume analysis via stereology. Scale bar: (panel A) 500 μm. Panel B: Stereological analysis of volume in hippocampal dentate gyrus subregions at 3 months (n = 4–6/experimental condition). GCL = granule cell layer; Mol = molecular layer; H = hilus; DG = dentate gyrus.