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Whole-Body Exposure to ^{28}Si -Radiation Dose-Dependently Disrupts Dentate Gyrus Neurogenesis and Proliferation in the Short Term and New Neuron Survival and Contextual Fear Conditioning in the Long Term

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Abstract

Astronauts traveling to Mars will be exposed to chronic low doses of galactic cosmic space radiation, which contains highly charged, high-energy (HZE) particles. ^{56}Fe -HZE-particle exposure decreases hippocampal dentate gyrus (DG) neurogenesis and disrupts hippocampal function in young adult rodents, raising the possibility of impaired astronaut cognition and risk of mission failure. However, far less is known about how exposure to other HZE particles, such as ^{28}Si , influences hippocampal neurogenesis and function. To compare the influence of ^{28}Si exposure on indices of neurogenesis and hippocampal function with previous studies on ^{56}Fe exposure, 9-week-old C57BL/6J and Nestin-GFP mice (NGFP; made and maintained for 10 or more generations on a C57BL/6J background) received whole-body ^{28}Si -particle-radiation exposure (0, 0.2 and 1 Gy, 300 MeV/n, LET 67 KeV/ μ , dose rate 1 Gy/min). For neurogenesis assessment, the NGFP mice were injected with the mitotic marker BrdU at 22 h postirradiation and brains were examined for indices of hippocampal proliferation and neurogenesis, including Ki67⁺, BrdU⁺, BrdU⁺NeuN⁺ and DCX⁺ cell numbers at short- and long-term time points (24 h and 3 months postirradiation, respectively). In the short-term group, stereology revealed fewer

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Ki67⁺, BrdU⁺ and DCX⁺ cells in 1-Gy-irradiated group relative to nonirradiated control mice, fewer Ki67⁺ and DCX⁺ cells in 0.2 Gy group relative to control group and fewer BrdU⁺ and DCX⁺ cells in 1 Gy group relative to 0.2 Gy group. In contrast to the clearly observed radiation-induced, dose-dependent reductions in the short-term group across all markers, only a few neurogenesis indices were changed in the long-term irradiated groups. Notably, there were fewer surviving BrdU⁺ cells in the 1 Gy group relative to 0- and 0.2-Gy-irradiated mice in the long-term group. When the short- and long-term groups were analyzed by sex, exposure to radiation had a similar effect on neurogenesis indices in male and female mice, although only male mice showed fewer surviving BrdU⁺ cells in the long-term group. Fluorescent immunolabeling and confocal phenotypic analysis revealed that most surviving BrdU⁺ cells in the long-term group expressed the neuronal marker NeuN, definitively confirming that exposure to 1 Gy ²⁸Si radiation decreased the number of surviving adult-generated neurons in male mice relative to both 0- and 0.2-Gy-irradiated mice. For hippocampal function assessment, 9-week-old male C57BL/6J mice received whole-body ²⁸Si-particle exposure and were then assessed long-term for performance on contextual and cued fear conditioning. In the context test the animals that received 0.2 Gy froze less relative to control animals, suggesting decreased hippocampal-dependent function. However, in the cued fear conditioning test, animals that received 1 Gy froze more during the pretone portion of the test, relative to controls and 0.2-Gy-irradiated mice, suggesting enhanced anxiety. Compared to previously reported studies, these data suggest that ²⁸Si-radiation exposure damages neurogenesis, but to a lesser extent than ⁵⁶Fe radiation and that low-dose ²⁸Si exposure induces abnormalities in hippocampal function, disrupting fear memory but also inducing anxiety-like behavior. Furthermore, exposure to ²⁸Si radiation decreased new neuron survival in long-term male groups but not females suggests that sex may be an important factor when performing brain health risk assessment for astronauts traveling in space.

INTRODUCTION

As the push for space exploration continues with plans by NASA to send humans to Mars by 2030 (1), understanding how high-linear energy transfer (LET) galactic cosmic radiation (GCR) effects the brain and behavior has become a top priority. Current shielding strategies are ineffective at blocking the high atomic number, high-energy (HZE) particles, including ⁵⁶Fe and ²⁸Si, which comprise space radiation (2–7). Thus, it is crucial to assess how exposure to HZE radiation influences the brain at both a cellular and behavioral level. Fortunately, ground-based accelerators have been used for many years to mimic space radiation, and recent developments in technology have allowed NASA's Space Radiation Laboratory [NSRL, Brookhaven National Laboratory (BNL); Upton, NY] to provide mixed beam radiation to better simulate the GCR spectrum (8). While these GCR simulation experiments are crucial to understanding the effects of HZE particles on brain and behavior, the effects of individual particles on the central nervous system (CNS) may vary depending on dose and energy (9). In fact, understanding how individual particles alter both brain and behavior will enhance efforts to accurately model and predict how the GCR spectrum encountered during deep space missions alters brain and behavior (10, 11), particularly in regards to experiments using mission-relevant doses of 0.25 Gy or less (12).

The vast majority of studies that examine the influence of HZE particles on the central nervous system (CNS) involve exposure of laboratory animals to ^{56}Fe . In contrast, there have been few studies examining the influence of smaller charged particles, such as ^{28}Si (12), on the CNS, particularly after low-dose exposure. As for CNS cellular analysis, ^{56}Fe exposure was shown to be detrimental to neurogenesis in the postnatal hippocampal dentate gyrus (DG) (13–18), a dynamic process closely linked to learning, memory and mood regulation. For example, 2-month-old mice exposed to 0.3 or 1 Gy ^{56}Fe radiation have fewer proliferating/differentiating DG cells shortly after irradiation (24–48 h). Also, using well-established indices of neurogenesis (19–24), published studies have shown that 2-month-old mice exposed to 1 Gy ^{56}Fe have fewer surviving bromodeoxy-uridine-immunoreactive (BrdU^+) cells and immature neurons, as labeled with doublecortin (DCX), at longer times postirradiation (1–3 months) (15, 16). In contrast to work in ^{56}Fe , to our knowledge there have been no published studies examining DG neurogenesis after ^{28}Si irradiation. As for CNS function, there is an ion-specific effect on the hippocampal-based learning task, contextual fear conditioning (CFC), where mice learn that a context is associated with a foot shock (25). For example, it has been reported that 2-month-old C57BL/6J mice exposed to ^{56}Fe (0.1–0.5 Gy) freeze as much as nonirradiated mice in a shock-associated context (26, 27). ^{56}Fe -exposed mice also freeze similarly to nonirradiated mice in a companion test to CFC, cued fear conditioning (FC), which is both hippocampal- and amygdalar-dependent and whose results can reflect anxiety-like behaviors (26). In contrast to the absence of ^{56}Fe -radiation effects on CFC and cued FC, ^{28}Si appears to have a dose-dependent effect on CFC. For example, 2-month-old C57BL/6J mice exposed to 1 Gy ^{28}Si learn CFC similar to nonirradiated mice, but mice exposed to 0.25 Gy ^{28}Si freeze more in a shock-associated context relative to control mice (28), suggesting improved hippocampal-dependent learning at this lower dose. It is unknown how ^{28}Si influences performance in the companion cued FC test. Thus, more information on how ^{28}Si -particle exposure influences these key indices of brain and behavior, DG neurogenesis, CFC and cued FC, is warranted to allow generalization across HZE particles, particularly in 2-month-old mice for which most data exist.

To fill these knowledge gaps, here we examined how ^{28}Si -particle exposure influences key measures of brain and behavior that have previously been evaluated after ^{56}Fe -particle exposure in 2-month-old mice. Specifically, we examined DG neurogenesis, including measures of neural progenitors, immature neurons and surviving cells, in male and female mice at short- and long-term time points (24 h and 3 months) after 0, 0.2 and 1 Gy whole-body ^{28}Si -particle exposure. In addition, CFC and cued FC were examined at the long-term time point. We observed that whole-body ^{28}Si radiation dose-dependently disrupts DG neurogenesis and proliferation in the short term and new neuron survival in the long term. In addition, male mice exposed to ^{28}Si radiation performed abnormally in a dose-dependent manner in CFC and cued FC in the long term, suggesting abnormal hippocampal and amygdalar function. These findings expand what is known about the influence of HZE particles on the brain and behavior in young adult mice, particularly in regards to mission-relevant doses, thus enabling better predictions about how single- and multiple-particle events may influence the astronaut brain and behavior during deep space missions.

METHODS

Animals

For the neurogenesis studies, Nestin-GFP (NGFP) male ($n = 39$) and female ($n = 32$) mice were used. NGFP mice were developed in C57BL/6 oocytes (29), and maintained on a C57BL/6J background for 10 or more generations at UT Southwestern Medical Center (UTSW) prior to their use in these studies (17, 22, 30, 31). For the behavioral studies, C57BL/6J males ($n = 21$) were used (Jackson Laboratory, Bar Harbor, ME). All mice were housed 4/cage, kept on a 12:12 h light-dark schedule (lights on 06:00) and given *ad libitum* access to food and water. At 9 weeks of age, all mice were shipped to BNL and were allowed to acclimate for five days prior to exposure (Fig. 1A). At 22 h postirradiation, all NGFP mice received a single intraperitoneal (i.p.) injection of the thymidine analog BrdU 150 mg/kg, 10 mg/ml in 0.9% saline and 0.001 M NaOH, consistent with our previously reported studies (16, 17, 32). One-half of the NGFP mice (for neurogenesis studies), identified as the “short-term” group, were sacrificed 2 h after BrdU injection and 24 h postirradiation (Fig. 1A) at BNL. The remaining half of the NGFP mice (for neurogenesis studies) and all of the C57BL/6J mice (for behavioral studies) were collectively identified as the “long-term” group, and were shipped back to UTSW for behavioral testing and sacrifice at a later time point (3 months after BrdU and irradiation; Fig. 1A). Short- and long-term animals were all weighed 24 h prior to exposure. Long-term mice were also weighed at 24 h and 3 months postirradiation to verify similarity with preirradiation weights and the lack of a long-term effect on body weight, respectively (data not shown). Experimental protocols were approved by the Animal Care and Use Committees of UTSW and BNL, and mice were treated in accordance with National Institutes of Health (NIH) guidelines.

Irradiations

Irradiations were performed at BNL, similar to our previously reported work (16, 17). ^{28}Si particles were produced at the Alternating Gradient Synchrotron Booster at BNL and transferred to the experimental beam line in the NSRL facility (33). The delivered beam was 20×20 cm with a uniformity of 5%. Mice were placed individually into clean, well-ventilated 50-ml conical tubes, and tubes were placed perpendicular to the beam such that the beam was centered with the mouse heads. The mice were then whole-body irradiated with 1 Gy ($n = 23$) or 0.2 Gy ($n = 25$) ^{28}Si particles (300 MeV/n, LET 67 KeV/ μ) at a dose rate of 1.0 Gy/min. Control animals were handled and placed in conical tubes for a similar amount of time as irradiated mice, but were not exposed to the beam. The NGFP mice (for neurogenesis data) were tested in the Fall 2011 NSRL campaign, while the C57BL/6J mice (for behavior data) were tested in the Summer 2013 NSRL campaign.

Immunohistochemistry

To quantify cells in the many stages of neurogenesis in the DG granule cell layer (34), the entire DG (including sections anterior and posterior to the DG) was processed for immunohistochemistry for neurogenesis-relevant markers (Fig. 1B). We opted not to quantify neural stem cells since they are unchanged after ^{56}Fe exposure (16, 17). Either 24 h or 3 months postirradiation (Fig. 1A), NGFP mice were anesthetized with chloral hydrate (250 mg/kg, i.p.) and underwent intracardial perfusion with ice-cold 0.1 M phosphate-

buffered saline (PBS) followed by 4% paraformaldehyde for fixation, as previously described elsewhere (16, 17). Extracted brains were immersed for 24 h in 4% paraformaldehyde in 0.1 *M* PBS at 4°C for post-fixation, followed by at least 3 days of immersion in 30% sucrose in 0.1 *M* PBS with 0.01% sodium azide for cryoprotection. For each animal, the entire brain was sectioned at 30 μ m in a 1:9 series (coronal plane) and stored in 1 $\times$ PBS with 0.01% sodium azide at 4°C until processing for immunohistochemistry.

Slide-mounted immunohistochemistry for BrdU⁺, Ki67⁺, DCX⁺ and NeuN⁺ cells in DG was performed as previously described elsewhere (16, 17, 35). Briefly, one entire series of the hippocampus (every ninth section) was slide-mounted onto charged slides. Antigen retrieval was performed using 0.01 *M* citric acid (pH 6.0) at 100°C for 15 min, followed by washing in PBS at room temperature. Next, endogenous peroxidase activity was inhibited by means of incubation with 0.3% hydrogen peroxide (H₂O₂) for 30 min. For BrdU immunohistochemistry, two additional steps were performed to allow the antibody access to DNA inside the cell nucleus: permeabilization and denaturation. Permeabilization was performed using 0.1% Trypsin in 0.1 *M* Tris and 0.1% CaCl₂, and denaturation was performed using 2 *N* HCl in 1 $\times$ PBS. Nonspecific binding was blocked with 3% serum (donkey) and 0.3% TritonTM X in PBS for 1 h.

After blocking and pretreatment steps, sections were incubated with rat- α -BrdU (1:400, Accurate[®] Chemical & Scientific Corp., Westbury, NY), rabbit- α -Ki67 antibody (1:500, Fischer ScientificTM, Freemont, CA), goat- α -DCX (1:8,000, Santa Cruz Biotechnology[®], Dallas, TX), or mouse- α -NeuN (1:500, EMD Millipore, Billerica, MA) in 3% serum and 0.3% Tween[®] 20 overnight. For single labeling immunohistochemistry, primary antibody incubation was followed by 1 $\times$ PBS rinses, incubation with biotinylated secondary antibodies [biotin-donkey- α -rat-IgG, biotin-donkey- α -rabbit-IgG or biotin-donkey- α -goat-IgG, all 1:200 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA)] for 1 h, 1 $\times$ PBS rinses, and incubation with an avidin-biotin complex for 90 min (ABC Elite, Vector[®] Laboratories, Burlingame, CA). After another set of rinses in 1 $\times$ PBS, immunoreactive cells were visualized with incubation with metal-enhanced diaminobenzidine (Fisher Scientific, Pittsburgh, PA) for 5–10 min. Lastly, slides were incubated for ~2 min in the nuclear counterstain, Fast Red (Vector Laboratories), dehydrated through a series of increasing ethanol concentrations and coverslipped using DPX. For NeuN⁺BrdU⁺ fluorescent double labeling, after BrdU pretreatment (above), slide-mounted tissue was incubated in BrdU and NeuN antibodies simultaneously overnight at room temperature, and in secondary antibodies [for BrdU, biotinylated donkey- α -rat; for NeuN, DyLight[®] 649 donkey- α -mouse; all 1:200 (Jackson ImmunoResearch)] simultaneously for 1 h. Tissue was then incubated in ABC, and the BrdU signal was amplified with Tyramide-Cy2 (PerkinElmer[®] Inc., Waltham, MA).

Stereological Cell Counts

BrdU⁺, Ki67⁺ and DCX⁺ DAB cells were quantified in line with stereology principles using an Olympus[®] BX-51 microscope at 400 $\times$ by an observer blind to experimental groups as described previously (22, 36, 37). Immunopositive cells were quantified in every ninth coronal section in the granule cell layer (GCL) in the DG, spanning the entire anterior-

posterior axis of the hippocampus (−0.82 mm to −4.33 mm from bregma). As the entire DG was examined using stereology, the number of sections per animals varied per stereology principles (38–40). However, the number of DG sections/mouse (mean ± SEM) for each group was as follows: for short-term, 11.03 ± 0.18 (0 Gy control: 11.55 ± 0.34 ; 0.2 Gy: 10.55 ± 0.27 ; 1 Gy: 11 ± 0.30); for long-term, 11.37 ± 0.13 (0 Gy control: 11.2 ± 0.20 ; 0.2 Gy: 11.23 ± 0.26 ; 1 Gy: 11.5 ± 0.19). Stereology was performed under brightfield microscopy, and total cell counts were multiplied by 9 to account for the whole DG. Data are presented as total GCL cell counts in all mice (Figs. 2–7A) and broken into group by sex (Figs. 2–7B). Data are also presented by sex along the longitudinal axis of the DG (distance from bregma, Figs. 2–7C, 2–7D). Photomicrograph images were captured using the Olympus DP Manager Program and were imported into software program Adobe Illustrator® for design of manuscript figures.

Confocal Phenotyping

NeuN⁺BrdU⁺ co-localization analysis of tissue from mice 3 months postirradiation (long-term cohort) was performed as previously described elsewhere (22, 41). Briefly, tissue was selected from mice whose BrdU⁺ DAB cell counts were representative of the mean [$n = 8$ (4 male, 4 female) per controls, 0.2 Gy and 1 Gy groups]. BrdU⁺ cells in the GCL were first identified under an epifluorescent microscope (Olympus BX51) with 10× and 40× objectives by an observer blind to treatment group. Using a Leica pinhole confocal microscope (TCS SP8, 40× oil objective; Buffalo Grove, IL), previously identified BrdU⁺ cells spanning the anterior-posterior axis of the DG (distance from bregma range: −1.63 to −3.79 mm) were examined via z-stacks through full-section thickness (~20 μm) by an additional observer blind to treatment. Step sizes of 0.5 μm were used, as were wavelengths of 489 and 654 nm laser lines to excite BrdU and NeuN signals with emission wavelengths of 506 and 673 nm, respectively. Scans were analyzed using Leica LAS X software, and BrdU⁺ cells were determined to be NeuN⁺ or NeuN[−] based on fluorescence intensity profiling (and histogram analysis) as well as orthogonal sectioning. Either 50 cells/group were examined, or in the case of 1 Gy dose, all BrdU⁺ cells were examined. Confocal phenotyping data are presented as percentage of BrdU⁺ cells that were also NeuN⁺ [calculated as $(\text{BrdU}^+\text{NeuN}^+)/(\text{BrdU}^+) \times 100$] (Fig. 6F). To calculate the number of BrdU⁺ neurons 3 months postirradiation (one way of assessing “new neurons” or “surviving neurons”; Table 2), the percentage of BrdU⁺ cells that were also NeuN⁺ was multiplied by the BrdU⁺ cell counts collected via stereology. For images, raw confocal scans were exported to Adobe Photoshop, and RGB channel adjustments were made by bringing white marker to the edge of the histogram. Images were then imported into Adobe Illustrator for cropping and placed into the figure. For clarity and presentation purposes, the pseudocolored NeuN channel (Cy5) was shifted to magenta (Fig. 6E).

Contextual Fear Conditioning and Cued Fear Conditioning

CFC and cued FC were performed as previously described elsewhere (42, 43) with the following modifications, male mice went through three phases of the test: training (day 1), CFC (day 2) and FC (day 2) (Fig. 8A). Mice were habituated to the behavior room for 1 h each day prior to training and testing. On day 1, mice were trained to associate a novel context (standard FC chamber, grid flooring, no odor, house lights on; Med Associates Inc.,

St. Albans, VT) with a shock. Specifically, 2 min after placement in the chamber, an auditory cue (80 db white noise, 30 s duration) was played (Med Associates Inc.), which co-terminated with a 0.5 mA shock (2 s duration). This cued-shock pairing was repeated two more times during the 6-min training session on day 1, with 1 min between cued-shock presentations. On day 2, mice were tested for CFC by being placed for 5 min in the same context as used for day 1 training, but no auditory cue or foot shock was presented. Also on day 2, but 2 h after CFC, mice underwent auditory cued FC testing where they were placed for 6 min in a novel context (plastic flooring, triangular roof, vanilla odor, house lights on). After 3 min, the tone from day 1 was presented for 3 min. For training and testing sessions, freezing behavior was assessed using Video Freeze[®] software (Med Associates Inc.), compiled for each phase of each session (e.g., pretone, during tone), and presented as percentage time freezing for each phase.

Statistical Analyses

Data are reported as mean $\pm$ SEM. Statistical analyses were performed using Prism software version 7 (GraphPad Software Inc., LaJolla, CA). Statistical significance was defined as $P < 0.05$. Statistical outcomes are reported in the Results section, as well as in Figs. 2–8, with details of statistical analyses provided in Table 1: short-term cohort (24 h postirradiation, Figs. 2–4); Table 2: long-term cohort and cellular phenotyping (3 months postirradiation, Figs. 5–7) and Table 3: CFC and cued FC data irrespective of gender (males and females combined) and behavioral data, statistical analyses were performed using one-way analysis of variance (ANOVA; variable of treatment: 0, 0.2 and 1 Gy), and Bonferroni multiple comparison post hoc tests were used (Figs. 2–7A and 8B–D). For total cell counts or phenotypic analysis considering treatment and sex, statistical analyses were performed using two-way ANOVA, and Bonferroni multiple comparison post hoc tests were used on male or female data, per our planned comparisons (Figs. 2–7B–D). BrdU⁺ cell survival rate was calculated as $(1 - \text{death}\%) \times 100$, where death percentage = $[(24 \text{ h BrdU}^+ \text{ group cell counts}) - (3 \text{ month BrdU}^+ \text{ group cell counts}) / (24 \text{ h BrdU}^+ \text{ cell counts})]$, similar to prior work (44). For cellular analysis, only animals with a full representative set of hippocampal sections were analyzed, per stereological principles. For the assessment of outliers, the ROUT test was performed with $Q = 1\%$. Three outliers were discovered and removed prior to subsequent analysis (2 animals from Ki67⁺ and 1 animal from DCX⁺ stains in the long-term group).

RESULTS

Proliferation and Neurogenesis are Dose-Dependently Reduced at 24 h Postirradiation in ²⁸Si-Irradiated vs. Control Animals

At 24 h postirradiation the effects of whole-body ²⁸Si exposure (Figs. 2–4) on DG proliferation and neurogenesis (Fig. 1B) were evaluated using stereological assessment of immunoreactive cells. As cells in distinct phases of the cell cycle can be differentially affected in regards to physiological manipulations, two markers were used to measure proliferation: the endogenous cell cycle marker Ki67, which labels the nucleus of cells in all phases of the cell cycle (Fig. 2) (45), and the exogenously-administered thymidine analog BrdU, which labels the nucleus of cells in S phase at the time of BrdU injection (Fig. 3) (44,

46). Ki67⁺ and BrdU⁺ cell counts in the short-term group reveal levels of DG proliferation at 24 h postirradiation. However, we also used a widely-accepted measure of neurogenesis for the 24 h postirradiation group (47–52). The number of cells immunoreactive for the neuronal fate commitment marker DCX (Fig. 4).

As previously shown elsewhere (22, 45), Ki67⁺ cells in the DG had intensely-stained, irregularly-shaped nuclei (Fig. 2A). In regards to Ki67⁺ cell numbers, ²⁸Si radiation had a dose-dependent effect on total Ki67⁺ cell numbers (Fig. 2A and Table 1). Post hoc analyses revealed that 0.2 and 1 Gy ²⁸Si exposure resulted in fewer Ki67⁺ cells relative to controls (20 and 61% reduction, respectively) (Fig. 2A and Table 1). Also, there were fewer Ki67⁺ cells after 1 Gy relative to 0.2 Gy ²⁸Si exposures (51% reduction; Fig. 2A). Subsequent analyses performed to assess possible differences between male and female mice revealed main effects of both sex and treatment and an interaction of sex and treatment (Table 1). Specifically, in males there were fewer Ki67⁺ cells after 0.2 and 1 Gy exposures relative to controls (22 and 64% reduction, respectively) (Fig. 2B and Table 1) and fewer Ki67⁺ cells after 1 Gy relative to 0.2 Gy exposures (54% reduction) (Fig. 2B and Table 1). In females, there were fewer Ki67⁺ cells after 1 Gy irradiation relative to controls (49% reduction) (Fig. 2B and Table 1). However, the lack of additional radiation effects on Ki67⁺ cells in female mice may be influenced by the lower control levels in female vs. male mice (Fig. 2B), as suggested by the interaction of sex and treatment (Table 1).

DG function varies with distance from bregma and indices of DG neurogenesis are often correlated with DG function (53–59). Therefore, in addition to total cell Ki67⁺ numbers (condensed from quantification throughout the longitudinal axis of the DG), we also analyzed cell counts for male and female mice across their respective distance from bregma to evaluate the sensitivity of proliferation throughout the coronal axis. For males and females, analyses of these data (by two-way ANOVA) revealed main effects of both bregma and radiation as well as an interaction (Table 1). Specifically, male mice that were exposed to 1 Gy had fewer Ki67⁺ cells at almost every bregma position relative to controls and animals that were exposed to 0.2 Gy (Fig. 2C). Male mice that received a 0.2 Gy dose had fewer Ki67⁺ cells at discrete bregma positions in the middle and posterior DG relative to controls (Fig. 2C). However, female mice that received a 1 Gy dose had fewer Ki67⁺ cells at only three bregma positions relative to controls and 0.2 Gy (Fig. 2D).

As with Ki67⁺ cells, DG BrdU⁺ cells examined at 24 h postirradiation (2 h after BrdU injection) presented intensely stained nuclei (Fig. 3A) clustered along the subgranular zone (SGZ) (45). As for BrdU⁺ cell counts, ²⁸Si exposure had an effect on total BrdU⁺ cell numbers (Fig. 3A and Table 1). Specifically, post hoc analyses revealed there were fewer BrdU⁺ cells after 1 Gy ²⁸Si irradiation relative controls (71% reduction) (Fig. 3A and Table 1). Also, there were fewer BrdU⁺ cells after 1 Gy relative to 0.2 Gy ²⁸Si irradiation (67%) (Fig. 3A). Subsequent consideration of treatment and sex (two-way ANOVA) revealed a main effect of treatment only, and not of sex (Table 1). This was somewhat surprising, as a main effect of sex was observed with the proliferation marker, Ki67 (Fig. 2B and Table 1) but not with this S-phase marker, BrdU (Fig. 3B and Table 1) (60), suggesting cell cycle stage-dependent susceptibility to HZE radiation. For BrdU⁺ cell numbers in males, there were fewer BrdU⁺ cells after 1 Gy irradiation relative to controls (74%; Fig. 3B, Table 1)

and fewer BrdU⁺ cells after receiving 1 Gy relative to a 0.2 Gy dose (64%) (Fig. 3B and Table 1). In females, there were fewer BrdU⁺ cells after receiving 1 Gy relative to controls (64%) (Fig. 3B and Table 1), and fewer BrdU⁺ cells after receiving 1 Gy relative to a 0.2 Gy dose (63%) (Fig. 3B and Table 1).

For males, consideration of treatment and bregma (two-way ANOVA) revealed main effects of both variables as well as an interaction effect (Table 1). Specifically, male mice that were exposed to 1 Gy had fewer BrdU⁺ cells at many bregma positions relative to 0.2-Gy-exposed animals and controls (Fig. 3C). Male mice that were exposed to 0.2 Gy had fewer BrdU⁺ cells at discrete posterior DG bregma positions relative to controls (Fig. 3C). For females, consideration of treatment and bregma (two-way ANOVA) also revealed main effects of both variables as well as an interaction (Table 1). However, female mice that were exposed to 1 Gy had fewer BrdU⁺ cells at only three bregma positions relative to controls and only five bregma positions relative to 0.2 Gy animals (Fig. 3D).

We next assessed neurogenesis in the 24 h postirradiation group using DCX cell counts, a widely-accepted measure of neurogenesis (47–52). As shown elsewhere (35, 61), DCX⁺ cells in the SGZ and granule cell layer (GCL) present a range of cellular morphologies, with approximately one-half presenting the morphology of an immature GCL neuron: an oval- or tear-drop-shaped soma (Fig. 4A) with a thin apical process, which branches after exiting the GCL and entering the DG molecular layer. As for DCX⁺ cell counts, ²⁸Si radiation had an effect on total DCX⁺ cell numbers (Fig. 4A and Table 1). Specifically, post hoc analyses revealed fewer DCX⁺ cells after 0.2 and 1 Gy ²⁸Si irradiation relative to controls (18 and 50% reduction, respectively) (Fig. 4A and Table 1). Also, there were fewer DCX⁺ cells after 1 Gy ²⁸Si irradiation relative to 0.2 Gy ²⁸Si irradiation (39%) (Fig. 4A). Subsequent consideration of treatment and sex (two-way ANOVA) revealed main effect of treatment only, with no effect of sex (Table 1). Specifically, in males there were fewer DCX⁺ cells after 1 Gy irradiation relative to controls (50%) (Fig. 4B and Table 1), and fewer DCX⁺ cells after 1 Gy irradiation relative to 0.2 Gy irradiation (41%) (Fig. 4B and Table 1). In females, there were fewer DCX⁺ cells after 1 Gy irradiation relative to controls (50%) (Fig. 4B and Table 1).

For males, consideration of treatment and bregma (two-way ANOVA) revealed main effects of both variables as well as an interaction effect (Table 1). Specifically, male mice exposed to 1 Gy had fewer DCX⁺ cells at many bregma positions relative to 0.2-Gy-exposed and control groups (Fig. 4C). Male mice exposed to 0.2 Gy had fewer DCX⁺ cells at discrete anterior and posterior DG bregma positions relative to controls (Fig. 4C). For females, consideration of treatment and bregma (two-way ANOVA) also revealed main effects of both variables, as well as an interaction effect (Table 1). However, female mice exposed to 1 Gy had fewer DCX⁺ cells at many bregma positions relative to nonirradiated controls, and only two bregma positions relative to 0.2-Gy-irradiated mice (Fig. 4D). Female mice exposed to 0.2 Gy had fewer DCX⁺ cells at discrete posterior DG bregma positions relative to controls (Fig. 4D).

Three Months after ^{28}Si Irradiation, Proliferation and Neurogenesis are Similar among Irradiated and Control Groups, whereas New Neuron Survival is Reduced, Particularly in Irradiated Male Mice

Three months after whole-body ^{28}Si exposure (Figs. 5–7), the effects on DG proliferation (Ki67^+ cells), neurogenesis (DCX^+ cells), surviving neurons (BrdU^+ , $\text{BrdU}^+\text{NeuN}^+$ cells) and cell survival rate (BrdU^+ cells 3 months vs. 24 h) were evaluated in the long-term group using stereological assessment of immunoreactive cells. As for Ki67^+ cell numbers between short- and long-term groups in control mice, those at 3 months postirradiation had approximately one-third of the Ki67^+ cell numbers compared to control animals at 24 h postirradiation, as expected, based on the age-induced decrease in proliferation (62, 63). However, in regards to the effect of radiation on Ki67^+ cell numbers, at 3 months after ^{28}Si irradiation, a similar total Ki67^+ cell numbers was observed in irradiated and control animals (Fig. 5A and Table 2). Subsequent consideration of treatment and sex (two-way ANOVA) revealed main effects of both variables as well as an interaction of sex and treatment (Table 2). Specifically, in male mice there were fewer Ki67^+ cells at 3 months after 1 Gy relative to 0.2 Gy irradiation (34% reduction) (Fig. 5B and Table 2), with no post hoc changes seen in female mice. However, the lack of radiation effects on Ki67^+ cells in female mice may be influenced by the lower control levels in female vs. male mice (Fig. 5B), as suggested by the interaction of sex and treatment (Table 2). In both males and females, two-way ANOVA revealed main effects of treatment and bregma on Ki67^+ cell numbers (Table 2). Specifically, at 3 months postirradiation, male mice that received 1 Gy irradiation had fewer Ki67^+ cells at three anterior bregma positions relative to controls and 0.2-Gy-irradiated mice (Fig. 5C). Female mice that were exposed to 1 Gy had fewer Ki67^+ cells at only one bregma position relative to controls (Fig. 5D), and female mice that were exposed to 0.2 Gy had fewer Ki67^+ cells at three bregma positions relative to controls, with two of them being posterior (Fig. 5D).

BrdU is bioavailable in the adult mouse for ~15 min (45). Thus, a BrdU injection, followed by a period of weeks, can be used to “birth date” cells and follow them into maturity. Therefore, unlike the BrdU^+ cells in the short-term group, which represent proliferating cells, BrdU^+ cells in the long-term group represent “surviving” cells. Qualitatively, DG BrdU^+ cells in the long-term group looked like neurons: solitary, round, intensely stained nuclei, many with a punctate staining pattern, in the SGZ and GCL (Fig. 6A). In regards to BrdU^+ cell counts in the long-term group, ^{28}Si radiation had a dose-dependent effect on total BrdU^+ cell numbers at 3 months postirradiation (Fig. 6A and Table 2). Specifically, post hoc analyses revealed fewer BrdU^+ cells at 3 months after exposure to 1 Gy ^{28}Si radiation relative to 0.2 Gy and control groups (57 and 71% reduction, respectively) (Fig. 6A and Table 2). Subsequent consideration of treatment and sex (two-way ANOVA) revealed main effects of both variables (Table 2). In males, post hoc analyses revealed fewer BrdU^+ cells at 3 months after exposure to 1 Gy relative to 0.2 Gy and control animals (71% and 64%, respectively) (Fig. 6B and Table 2). In contrast, exposure to radiation did not affect BrdU^+ cell numbers in this female long-term group. The lack of radiation effects on BrdU^+ cell numbers in female mice at 3 months postirradiation may be due to the low number of BrdU^+ cells in control female relative to control male groups (Fig. 6B), a difference that was not evident in the short-term control female vs. male groups (Fig. 3B).

In both males and females, a two-way ANOVA revealed main effects of both radiation and bregma position, with the males also having an interaction (Table 2). Specifically, male mice exposed to 1 Gy had fewer BrdU⁺ cells at numerous bregma positions relative to 0.2 Gy and control groups (Fig. 6C), while male mice exposed to 0.2 Gy had fewer BrdU⁺ cells at a single anterior bregma location relative to controls at 3 months postirradiation (Fig. 6C). Female mice exposed to 1 Gy had fewer BrdU⁺ cells at only one bregma location relative to 0.2 Gy and control groups (Fig. 6D).

While most surviving BrdU⁺ DG cells become neurons (64–67), manipulations can sometimes change the fate of the cells, for example, driving astrogenesis instead of neurogenesis (68). We tested the effect of ²⁸Si radiation on the fate of BrdU⁺ cells at 3 months postirradiation (and at 3 months after BrdU administration) by co-labeling with antibodies against BrdU and the neuronal nuclear protein NeuN and performing phenotypic analysis using confocal microscopy (Fig. 6E and F). As expected, the nuclei of BrdU⁺ cells in the long-term (3 months) postirradiation group co-labeled with NeuN (Fig. 6Ei and Eiii), even throughout the z plane of optical sectioning (Fig. 6Eiv). The percentage of BrdU⁺ cells that were also NeuN⁺ was collected to reflect the proportion of adult-generated cells that were neurons. A two-way ANOVA revealed no main effect of sex or treatment, and no interaction (Table 2), and the percentage of BrdU⁺ and NeuN⁺ co-labeled cells was approximately 100% for all treatment groups (Fig. 6F). Multiplication of the number of BrdU⁺ cells (Fig. 6B) by these percentages (Fig. 6F) (see Methods) resulted in a dose-dependent decrease of surviving adult-generated neurons in exposed animals relative to controls in males but not in females, strikingly similar to the BrdU⁺ cell numbers (Fig. 6B).

While these data show that the number of surviving, new neurons is decreased in male mice that were irradiated relative to control male mice, there is an additional consideration: perhaps this decrease is already evident in the “starting” value of BrdU cells in the 24 h group. Therefore, in addition to quantifying BrdU⁺ cells and neuron numbers at 3 months postirradiation in exposed vs. control animals, we also calculated the BrdU⁺ cell survival rate. The proportion of BrdU⁺ cells at 3 months postirradiation was represented as a fraction of the “starting” value of BrdU⁺ cells at 24 h postirradiation (see Methods). With this approach, BrdU⁺ cell survival rate was similar across groups (percentage surviving: controls, 18%; 0.2 Gy, 18%; 1 Gy, 23%). When sex was considered, exposed groups again had similar survival rates compared to their respective control groups (data not shown). Therefore, although the number of surviving BrdU⁺ cells and neurons is decreased in exposed vs. controls at 3 months postirradiation (Fig. 6A and Table 2) (see Results), the survival rate of BrdU⁺ cells (change in BrdU⁺ cell numbers in exposed and control groups between the 24 h and 3 month time points) was similar in exposed and control mice.

As for DCX⁺ cell count between short- and long-term control groups, at 3 months postirradiation controls, had approximately a quarter of DCX⁺ cells compared to controls at 24 h postirradiation, as expected, based on the age-induced decrease in neurogenesis (62, 63). For the long-term group, ²⁸Si radiation had an effect on total number of DCX⁺ cells (Fig. 7A and Table 2). Specifically, post hoc analyses revealed there were fewer DCX⁺ cells after ²⁸Si exposure to 1 Gy relative to 0.2 Gy (23% reduction) (Fig. 7A and Table 2). Neither 0.2 Gy nor 1 Gy exposure was significantly different from controls. Subsequent

consideration of treatment and sex (two-way ANOVA) revealed main effects of treatment only (Table 2), with post hoc analyses revealing no major changes within each sex (Fig. 7B).

For males, consideration of treatment and bregma (two-way ANOVA) revealed main effects of both variables (Table 2). Specifically, male mice exposed to 1 Gy had fewer DCX⁺ cells at one bregma position relative to controls, and a few bregma positions relative to 0.2 Gy exposure (Fig. 7C). For females, consideration of treatment and bregma (two-way ANOVA) only revealed main effects across bregma but not treatment (Table 2).

Three Months after ²⁸Si Irradiation, Male Mice Show a Dose-Dependent Disruption of Contextual Fear Memory and Increased Freezing in a Novel Environment

Since neurogenesis has been linked to disrupted performance on CFC, and since ²⁸Si exposure caused a long-term decrease in surviving BrdU cells and new neurons, we next examined whether ²⁸Si exposure caused a behavioral change. We focused on male mice, because they showed the greatest influence of ²⁸Si radiation on surviving new neurons in the long term. Specifically, 3 months after whole-body ²⁸Si irradiation, the radiation effects on hippocampal- and amygdala-dependent learning were evaluated in long-term mice using CFC and cued FC (Fig. 8A). While the C57BL/6J mice used for CFC and cued FC were treated the same as the NGFP mice used for the neurogenesis studies, the animals used for behavioral testing were irradiated two years after the NGFP mice and were not given BrdU injection after irradiation. Similar to our previously reported work (17), NGFP and C57BL/6J mice had body mass and weight gain throughout the experiment, and radiation exposure had no effect on weight gain in either mouse strain (data not shown).

Regarding CFC, ²⁸Si exposure had an effect on percentage freezing time in the context test (Fig. 8B and Table 3), with post hoc analyses revealing that mice exposed to 0.2 Gy froze less relative to controls (nearly relative to 1 Gy exposure; $P = 0.053$). Regarding the cued FC, ²⁸Si exposure had an effect on percentage freezing time in the pretone phase of the test (Fig. 8C and Table 3). Specifically, post hoc analyses revealed that mice exposed to 1 Gy spent more time freezing relative to 0.2 Gy and control animals when placed in a novel environment prior to hearing the tone (Fig. 8C and Table 3). In contrast, there was no radiation effect on percentage freezing time during tone (Fig. 8D and Table 3). Taken together, these data suggest a dose-dependent effect of ²⁸Si radiation on CFC and cued FC, where mice exposed to 0.2 Gy had worse hippocampal-dependent memory, but mice exposed to 1 Gy had increased freezing in general, indicating anxiety.

DISCUSSION

An important consideration for future travel into deep space is the potentially damaging effects of heavy particle space radiation to the CNS (12). A unique aspect of the current study is the focus on ²⁸Si effects on adult hippocampal DG neurogenesis and hippocampal-dependent behavior. Prior to this work, to our knowledge, nothing was known about how ²⁸Si exposure influenced neurogenesis, and little was known about how ²⁸Si exposure influenced behavior. Here we show that whole-body exposure to ²⁸Si radiation reduces mouse hippocampal DG proliferation and neurogenesis in the short term, and decreases new neuron survival in the long term. We also show that ²⁸Si radiation interferes with

performance on the hippocampal-dependent CFC task and a 1 Gy dose induces anxiety-like behavior. As discussed below, these findings are important additions to the growing body of knowledge on how HZE particles influence the brain and behavior.

While it is important to perform studies in fully mature, astronaut age-equivalent animals (69), we performed the current studies in young adult mice to enable a better comparison with the vast majority of published neurogenesis work, which focused on approximately 2-month-old rodents that are exposed to space radiation (15–18, 70). In our work presented here, ^{28}Si radiation was shown to dose-dependently decrease DG proliferation (Ki67^+ cell numbers) and neurogenesis (DCX^+ cell numbers) in the short term. Additionally, ^{28}Si radiation decreased the number of surviving new DG neurons in the long term. Specifically, we observed this decrease in surviving new DG neurons only in males, although the lack of decrease in females may be a consequence of a floor effect. These ^{28}Si radiation-induced decreases in DG neurogenesis are similar to, but less robust than, previously reported ^{56}Fe radiation-induced changes in neurogenesis (15–17, 71). For example, for DCX, which is a well-accepted marker of immature neurons and neurogenesis (49, 50), whole-body 1 Gy ^{28}Si exposure (current results) and 1 Gy ^{56}Fe (16, 17, 71) exposure were shown to decrease DCX^+ DG cell numbers in the short term. However, our current data show that ^{28}Si -irradiated and control mice have similar DCX^+ cell numbers in the long term, with just a decrease in DCX^+ cells in mice exposed to 1 Gy relative to 0.2 Gy. In contrast, it was previously shown that ^{56}Fe -exposed mice still have decreased DCX^+ cell numbers, compared to control mice, in the long term (16). Other published data, on 1 Gy ^{56}Fe -radiation exposure, shows a robust decrease in DCX^+ cells in the anterior DG at 1 month postirradiation (15), while our 1 Gy ^{28}Si -radiation exposure data presented in this work shows a modest decrease in DCX^+ cells in the anterior DG (bregma, -1.63) at 3 months postirradiation. In regards to proliferation, ^{28}Si -irradiated and control mice have similar Ki67^+ cell numbers in the long term. Thus, our data suggest ^{28}Si radiation has a more transient and less potent long-term influence on the process of DG neurogenesis than ^{56}Fe . The mechanism underlying the differential influence of ^{28}Si compared to ^{56}Fe on DCX^+ cell numbers is unknown. However, the larger initial track size and delta-ray damage produced by ^{56}Fe vs. ^{28}Si (9) may explain the greater long-term negative effect of ^{56}Fe on proliferation and this measure of neurogenesis.

In addition to looking at indices of neurogenesis in the overall DG, we also examined it in regards to distance from bregma. This is useful, since the hippocampus varies in function along its longitudinal axis, as in the rodent: the more anterior/dorsal/septal hippocampus is linked to spatial learning and memory, and the more posterior/ventral/temporal hippocampus is linked to emotion and mood regulation (72–78). Because neurogenesis is linked to both spatial memory and mood (65, 78, 79), examination along the septotemporal axis allows insight into DG neurogenesis that may be associated with specific changes in function (80–83). In fact, inducible manipulation of neurogenesis in the anterior compared to posterior DG disrupts spatial versus emotionally-linked hippocampal function, respectively (83). In the current work, surviving BrdU^+ cells at 3 months after ^{28}Si irradiation in male mice are dose-dependently decreased in the anterior DG, but decreased irrespective of dose in the posterior DG. This suggests that ^{28}Si radiation may dose-dependently impair spatial learning and memory, but also interfere with mood regulation irrespective of dose. While other work has examined the influence of ^{56}Fe radiation on neurogenesis via stereology of the entire

DG (16, 17), those data have been published as total immunoreactive DG cell numbers and not according to bregma. Consideration of this functional gradient of the hippocampus, and the biochemical, cellular and neuroanatomical gradients that accompany it, may enable more hypothesis-driven experiments for future work into the functional effect of space radiation on hippocampal function.

Indeed, our bregma analysis revealing fewer BrdU⁺ surviving cells in the anterior DG at 3 months after ²⁸Si irradiation (Fig. 6) fits with our reported ²⁸Si radiation-induced disruption of spatial learning and memory (CFC) and emotionally-linked memory (cued FC) (Fig. 8). Specifically, in the context test, 0.2-Gy-irradiated mice froze less relative to controls, suggesting decreased hippocampal-dependent function. However, in cued FC, 1-Gy-irradiated mice froze more during the pretone portion of the test relative to 0.2 Gy and controls, suggesting enhanced anxiety (84–86). These data raise several discussion points. First, while these data suggest a ²⁸Si radiation dose-dependent effect on memory and/or anxiety, a HZE-induced effect has also been observed at low doses with titanium-48 [⁴⁸Ti (0.3 Gy)] but not ⁵⁶Fe (0.1, 0.5 and 2 Gy) (87, 88). Importantly, these data indicate the utility of employing the cued FC as well as CFC testing, because without the cued FC we might have concluded that 1-Gy-irradiated mice had “normal” hippocampal-dependent function, while only the 0.2-Gy-irradiated mice had worse hippocampal function. A second point of discussion is that these data are notable in their contrast with prior behavioral testing with ²⁸Si radiation in young adult mice. For example, young adult mice exposed to a similar dose (0.25 Gy) of whole-body ²⁸Si radiation, also tested at 3 months postirradiation, displayed increased CFC freezing (28), in contrast to our decreased CFC freezing shown here. While increased freezing can be an index of anxiety, cued FC was not assessed. It is possible that this difference could be due to the lower energy used in our experiments [300 MeV vs. 600 MeV, as in ref. (28)], as lower-energy particles can be more damaging than higher-energy particles (9). Of course, age at time of exposure plays a role in the behavioral effects of ²⁸Si radiation, as has also been shown with other ions (87, 89). For example, mice exposed to ²⁸Si radiation at 6–7 months old were resistant to radiation-induced deficits in CFC at doses <1.6 Gy (90). However, our goal here was to work with young adult mice to enable comparison with prior published studies in which other ions were examined.

This raises our third discussion point: how does the ²⁸Si radiation-induced disruption of hippocampal function, assessed here using CFC, compare to that induced by other ions? Only a few studies have examined CFC in young adult mice after HZE-particle exposure, and it is challenging to compare these to our work. For example, young adult mice exposed to protons (150 MeV) have shown increased freezing at low dose (0.1 Gy) with no change at higher doses (0.5, 1 Gy) at 1 month postirradiation, and no change in any group at 3 months postirradiation (27). However, cued FC was not reported as being assessed, so it remains possible that increased freezing at 0.1 Gy proton irradiation was due to anxiety, not improved memory. In another study, mice exposed to ⁵⁶Fe (0.1–0.5 Gy) were reported to have no deficits in CFC at 2–4 weeks postirradiation (26), although the age at the time of exposure was not provided. Another way to answer this question is to widen the comparison to all studies investigating HZE-induced behavioral changes in young adult mice after exposure to mission-relevant doses (<1 Gy and ideally <0.2 Gy). From this perspective, there is increasing evidence that lower doses of ⁵⁶Fe (as low as 0.2 Gy), ²⁸Si (0.005 Gy),

^{16}O (0.01 Gy), ^{12}C (0.05 Gy), protons (0.1 Gy) or combined exposure (e.g., protons and ^{56}Fe) impede hippocampal, striatal and executive function in young adult rats and mice (9, 27, 91–100) in an energy-dependent manner. Although there is a relative paucity of behavioral studies using ^{28}Si radiation, our data show that exposure to 0.2 Gy ^{28}Si radiation induces deficits in hippocampal function even at 3 months postirradiation.

The neural mechanisms underlying the ^{28}Si -induced behavioral changes we report here are unknown. In other published work with these ^{28}Si doses, no change in key electrophysiological parameters in the mouse DG was reported at 3 months postirradiation (101), although those data were generated with more energetic (and perhaps less damaging) ^{28}Si particles than ours. Might these behavioral changes be due instead to ^{28}Si radiation-induced changes in neurogenesis? Certainly, DG neurogenesis is functionally important in hippocampal-dependent tasks (102–106). Notably, the mice in our current study have fewer surviving adult-generated DG neurons in both the anterior and posterior DG (Fig. 6A, E and F) as well as disrupted CFC performance at 3 months postirradiation (Fig. 8), a task that engages the posterior hippocampus. Since this is a correlation, it is not feasible or appropriate from our data to conclude that the ^{28}Si radiation-induced decrease in neurogenesis causes the disruption in CFC. More association studies may strengthen this correlation, e.g., if exposure to radiation disrupts other behaviors that specifically involve adult-generated neurons (54, 107, 108). Of course, only a causative study can truly reveal this relationship. For example, if selective and inducible elevation of the activity of adult-generated neurons rescues ^{28}Si radiation-induced disruption of CFC (109, 110), then a causative link may be inferred. In fact, one may not even expect a relationship between the number of new neurons and performance on hippocampal tasks (111), since this disconnect has already been shown after exposure to low-LET proton radiation (112). There are other measures relevant to neurogenesis, beyond new neurons, which are proposed to contribute to DG function (e.g., dendritic complexity), and these merit evaluations in HZE-exposed animals in future studies, along with consideration of the functional heterogeneity that is evident in the rodent hippocampus (72, 78).

One caveat of this study is that our neurogenesis findings (that ^{28}Si radiation damages DG newborn neuron survival), and our behavioral findings (that low doses of ^{28}Si induce abnormalities in hippocampal function, disrupting fear memory but also inducing anxiety) are collected from NGFP mice and C57BL/6J mice, respectively. Another caveat is that the mice were exposed to ^{28}Si during different NSRL campaigns. While we cannot say for certain that these differences do not have an effect on our conclusions, i.e., the behavior of NGFP would be different from C57BL/6J mice (or vice versa), several lines of evidence suggest that it is reasonable to generalize our conclusions across these mouse lines. First, the NGFP mouse line was created in C57BL/6 oocytes, and was maintained on a C57BL/6J background for 10 or more generations prior to use in this study. Supporting the broad utility of this mouse line for neurogenesis research, the NGFP mouse line is commonly used to explore fundamental and advanced concepts in neurogenesis research, and is often used interchangeably with C57BL/6 mice (29, 32, 51, 113–123), and such findings have informed seminal and widely-accepted models of the DG neurogenesis (124, 125). Also supporting the similarity of these mouse lines, NGFP mice are also commonly used in publications where neurogenesis data come from NGFP mice and behavior data come from C57BL/6

mice (122, 126, 127). There is also published work showing similar effects of HZE-induced changes in neurogenesis in NGFP and other mouse lines (16, 17). In addition, NGFP mice respond similarly to the contextual and cued fear conditioning paradigm used here (30), and have grossly similar measurements of anxiety, social avoidance and spatial learning as C57BL/6 mice (22, 30, 128, 129). Finally, many conclusions in the field on HZE-induced changes in the brain and behavior have been generalized across mutant mouse strains, rodents, different NSRL campaigns, to humans and even for computer modeling, as supported by the contents of review publications and even the titles of primary research works (12, 130–136). It is clearly important to acknowledge the potential differences in these mouse strains, as we do here, and future studies should strive to minimize variables of strain and irradiation date. However, by emphasizing both the differences and similarities in our mouse strains in this work, we feel it is reasonable to conclude that in our mice, whole-body exposure to ^{28}Si radiation dose-dependently disrupts DG neurogenesis and proliferation in the short term and new neuron survival and CFC in the long term.

In conclusion, this work was specifically done in young adult mice to facilitate comparison with the many prior studies exposing young adult mice to other ions. However, it is important that future work examine whether older mice, which are closer in age to that of astronauts, are similarly influenced by ^{28}Si (89). Indeed, as mentioned above, previous work with another mouse strain has revealed that these animals, which were 6–7 months old at time of exposure, were unimpaired in CFC at low doses of ^{28}Si and only showed impairment after 1.6 Gy ^{28}Si irradiation (90). Sex will be another important factor to assess as the number of female astronauts rises, since basic research has demonstrated sex-specific responses to space radiation (71, 137, 138). Here, we show robust and similar effects on neurogenesis indices in male and female mice in the short term, with more subtle sex-dependent differences in the long term. Whether these time-dependent effects are truly sex-dependent or merely less robust in females merit a study with larger cohort numbers per sex. In addition to the importance of sex, and the age at irradiation, it will be useful to assess the mechanisms underlying the changes in neurogenesis and hippocampal-dependent function reported here, such as radiation-induced changes in inflammation (12).

These findings expand what is known about the influence of HZE particles on the brain and behavior in young adult mice, particularly in regards to mission-relevant doses, thus enabling better predictions about how single- and multiple-particle events may affect the astronaut brain and behavior during deep space missions.

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FIG. 1.

Schematic of experimental design and stages of neurogenesis. Panel A: Timeline of experiment to examine short-term (24 h) and long-term (3 months) effects of ^{28}Si radiation on cellular indices of neurogenesis (short- and long-term) and behavior (long-term) in young adult mice (9 weeks old at exposure). Panel B: Schematic depicting the stages of neurogenesis in the DG granule cell layer (GCL) to emphasize that neurogenesis is a process, not a time point (34). Cells immunopositive for neurogenesis-relevant markers were quantified in the DG GCL (dotted rectangle) along the entire longitudinal axis of the hippocampus; three sample coronal sections along the bregma axis are shown. The

magnified GCL depicts cells in various stages of neurogenesis, from neural stem cell and progenitor, to immature and then mature DG granule cell (GC). Antibodies against the endogenous proteins Ki67, doublecortin (DCX) and NeuN label proliferating progenitors, immature neurons and mature neurons respectively. When injected i.p., the exogenous S-phase marker BrdU incorporates into the DNA of proliferating cells. Antibodies against BrdU label proliferating cells (2 h after BrdU injection) and surviving cells (3 months after BrdU injection).

24 h postirradiation

FIG. 2.

Twenty-four h after whole-body ^{28}Si irradiation, DG GCL proliferation was reduced, as measured by stereology of Ki67⁺ cells. Panel A: Stereological quantification of total GCL Ki67⁺ cells (inset: representative photomicrograph). Controls, $n = 11$; 0.2 Gy, $n = 11$; and 1 Gy, $n = 10$. Panel B: Stereological quantification of total GCL Ki67⁺ cells by sex. For males, $n = 7$ per group (controls, 0.2 Gy and 1 Gy). For females: controls, $n = 4$; 0.2 Gy, $n = 4$; and 1 Gy, $n = 3$. Panels C and D: Quantification of GCL Ki67⁺ cells across the bregma in male and female mice, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

^aControls vs. 0.2 Gy; ^bcontrols vs. 1 Gy; ^c0.2 Gy vs. 1 Gy. Scale bar = 10 μm .

24 h postirradiation

FIG. 3.

Twenty-four h after whole-body ^{28}Si irradiation, DG GCL proliferation was reduced, as measured by stereology of BrdU⁺ cells. Panel A: Stereological quantification of total GCL BrdU⁺ cells (inset: representative photomicrograph). Controls, $n = 11$; 0.2 Gy, $n = 11$; and 1 Gy, $n = 10$. Panel B: Stereological quantification of total GCL BrdU⁺ cells by sex. For males: $n = 7$ per group (controls, 0.2 Gy and 1 Gy). For females: controls, $n = 4$; 0.2 Gy, $n = 4$; and 1 Gy, $n = 3$. Panels C and D: Quantification of GCL BrdU⁺ cells across the bregma in male and female mice, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ^aControls vs. 0.2 Gy; ^bcontrols vs. 1 Gy; ^c0.2 Gy vs. 1 Gy. Scale bar = 10 μm .

FIG. 4.

Twenty-four h after whole-body ^{28}Si irradiation, DG neurogenesis was reduced, as measured by stereology of DCX⁺ cells. Panel A: Stereological quantification of total DCX⁺ cells (inset: representative photomicrograph). For controls, $n = 11$, 0.2 Gy = 11, 1 Gy = 10. Panel B: Stereological quantification of total GCL DCX⁺ cells by sex. For males: controls, $n = 7$; 0.2 Gy, $n = 7$; and 1 Gy, $n = 7$. For females: controls, $n = 4$; 0.2 Gy, $n = 4$; and 1 Gy, $n = 3$. Panels C and D: Quantification of GCL DCX⁺ cells across the bregma in male and female mice, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ^aControls vs. 0.2 Gy; ^bcontrols vs. 1 Gy; ^c0.2 Gy vs. 1 Gy. Scale bar = 10 μm .

3 months postirradiation

FIG. 5.

At three months postirradiation, proliferation in irradiated mice recovers compared to controls after whole-body exposure to ^{28}Si , as measured by stereology of Ki67⁺ cells. Panel A: Stereological quantification of total Ki67⁺ cells. For controls, $n = 10$; for 0.2 Gy, $n = 14$; and for 1 Gy, $n = 11$. Panel B: Stereological quantification of total GCL Ki67⁺ cells by sex. For males: controls, $n = 4$; 0.2 Gy, $n = 6$; and 1 Gy, $n = 7$. For females: controls, $n = 6$; 0.2 Gy, $n = 8$; and 1 Gy, $n = 4$. Panels C and D: Quantification of GCL Ki67⁺ cells across the bregma in male and female mice, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

^aControls vs. 0.2 Gy; ^bcontrols vs. 1 Gy; ^c0.2 Gy vs. 1 Gy.

FIG. 6.

Three months postirradiation, whole-body exposure to ^{28}Si reduces new neuron cells, as measured by stereology and confocal phenotypic analysis of BrdU⁺ cells. Panel A: Stereological quantification of total GCL BrdU⁺ cells (inset: representative photomicrograph). For control, $n = 10$; for 0.2 Gy, $n = 13$; and for 1 Gy, $n = 12$. Panel B: Stereological quantification of total GCL BrdU⁺ cells by sex. For males: controls, $n = 4$; 0.2 Gy, $n = 6$; and 1 Gy, $n = 8$. For females: controls, $n = 6$; 0.2 Gy, $n = 7$; and 1 Gy, $n = 4$. Panels C and D: Quantification of GCL BrdU⁺ cells across the bregma in male and female mice, respectively. Panel E i–iv: Representative confocal photomicrographs of BrdU⁺NeuN⁺

cells. Single channel photomicrographs are shown of BrdU⁺ cells (i: green, 489 nm excitation) and NeuN⁺ cells (ii: magenta, 654 nm excitation), with the merged image (iii). Orthogonal presentation is shown of BrdU⁺NeuN⁺ cells, along with xz and yz planes (iv). Panel F: Percentage of BrdU⁺ cells that are NeuN⁺ in male and female mice (subset of mice in panel A near mean). Males: controls, n = 4; 0.2 Gy, n = 4; 1 Gy, n = 4. Females: controls, n = 4; 0.2 Gy, n = 4; 1 Gy, n = 4. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

^aControls vs. 0.2 Gy; ^bcontrols vs. 1 Gy; ^c0.2 Gy vs. 1 Gy. Scale bar = 10 μ m.

FIG. 7.

Three months postirradiation, neurogenesis in irradiated mice recovers compared to controls after whole-body exposure to ^{28}Si , as measured by stereology of DCX⁺ cells. Panel A: Stereological quantification of total DCX⁺ cells. For controls, $n = 10$; for 0.2 Gy, $n = 14$; and for 1 Gy, $n = 11$. Panel B: Stereological quantification of total GCL DCX⁺ cells by sex. For males: controls, $n = 4$; 0.2 Gy, $n = 6$; and 1 Gy, $n = 7$. For females: controls, $n = 6$; 0.2 Gy, $n = 8$; and 1 Gy, $n = 4$. Panels C and D: Quantification of GCL DCX⁺ cells across the bregma in male and female mice, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ^bControls vs. 1 Gy; ^c0.2 Gy vs. 1 Gy.

FIG. 8.

Three months postirradiation, mice show a dose-dependent decrease in contextual fear conditioning (CFC) and dose-dependent appearance of anxiety-like trait. Panel A: Schematic of CFC pairing [pair a novel context with an auditory cue (speaker icon) that precedes a foot shock (lightning bolt)], CFC testing (context only, day 2) and cued fear conditioning (FC) (additional novel context with an auditory tone, day 2). Panels B–D: Percentage of time that mice froze in response to context (panel B), pre-tone in novel context (panel C) and cued FC (panel D). For controls, $n = 8$; for 0.2 Gy, $n = 7$; and for 1 Gy, $n = 8$. Mean $\pm$ SEM. $*P < 0.05$.

TABLE 1

Statistical Results, Short-Term Cohort (Figs. 2–4)

Antibody	Fig.	Bonferroni post hoc			
Ki67	2A	One-way ANOVA main effect F(2, 29) = 24.38, $P < 0.0001$	Controls vs. 0.2 Gy ns	Controls vs. 1 Gy $P < 0.0001$	0.2 Gy vs. 1 Gy $P < 0.0001$
	2B	Two-way ANOVA main effects Treatment F(2, 26) = 23.12, $P < 0.0001$ Sex F(1, 26) = 6.04, $P < 0.05$ Interaction F(2, 26) = 1.90, $P > 0.05$ Males $P < 0.0001$ Females $P < 0.05$	Controls vs. 0.2 Gy $P < 0.05$ ns	Controls vs. 1 Gy $P < 0.0001$ $P < 0.05$	0.2 Gy vs. 1 Gy $P < 0.0001$ ns
	2C	Two-way ANOVA main effects Treatment F(2, 233) = 78.06, $P < 0.0001$ Bregma F(12, 233) = 40.14, $P < 0.0001$ Males F(24, 233) = 3.42, $P < 0.0001$ Females F(26, 126) = 1.30, $P > 0.05$	See Fig. 2C		
	2D	F(2, 126) = 19.67, $P < 0.0001$ F(13, 126) = 19.37, $P < 0.0001$ Females	See Fig. 2D		
BrdU	3A	One-way ANOVA main effect F(2, 29) = 33.86, $P < 0.0001$	Controls vs. 0.2 Gy ns	Controls vs. 1 Gy $P < 0.0001$	0.2 Gy vs. 1 Gy $P < 0.0001$
	3B	Two-way ANOVA main effects Treatment F(2, 26) = 30.06, $P < 0.0001$ Sex F(1, 26) = 3.90, $P > 0.05$ Interaction F(2, 26) = 1.21, $P > 0.05$ Males $P < 0.0001$ Females $P < 0.01$	Controls vs. 0.2 Gy ns	Controls vs. 1 Gy $P < 0.0001$ $P < 0.01$	0.2 Gy vs. 1 Gy $P < 0.0001$ $P < 0.01$
	3C	Two-way ANOVA main effects Treatment F(2, 252) = 76.43, $P < 0.0001$ Bregma F(13, 252) = 24.80, $P < 0.0001$ Males F(26, 112) = 1.67, $P < 0.05$ Females	See Fig. 3C		
	3D	F(2, 112) = 20.56, $P < 0.0001$ F(13, 112) = 11.56, $P < 0.0001$ Females	See Fig. 3D		
DCX	4A	One-way ANOVA main effect F(2, 29) = 33.34, $P < 0.0001$	Controls vs. 0.2 Gy $P < 0.05$	Controls vs. 1 Gy $P < 0.0001$	0.2 Gy vs. 1 Gy $P < 0.0001$

Antibody	Fig.	Bonferroni post hoc					
4B	Two-way ANOVA main effects	Treatment	Sex				
			F(1, 26) = 0.20, $P > 0.05$				
			F(2, 26) = 26.24, $P < 0.0001$				
4C	Two-way ANOVA main effects	Interaction	Males	Controls vs. 0.2 Gy	Controls vs. 1 Gy	0.2 Gy vs. 1 Gy	
			Females	ns	$P < 0.0001$	$P < 0.001$	
				ns	$P < 0.001$	ns	
4D	Two-way ANOVA main effects	Interaction	Males	See Fig. 4C			
			Females	see Fig. 4D			

Note: Significance indicated in bold face; ns = not significant.

TABLE 2

Statistical Results, Long-Term Cohort (Figs. 5–7)

Antibody	Fig.	Bonferroni post hoc				
Ki67	5A	One-way ANOVA main effect $F(2, 32) = 0.4007, P > 0.05$		Controls vs. 0.2 Gy	Controls vs. 1 Gy	0.2 Gy vs. 1 Gy
				ns	ns	ns
	5B	Two-way ANOVA main effects				
		Treatment $F(2, 29) = 7.64, P < 0.05$	Sex $F(1, 29) = 75.17, P < 0.0001$	Controls vs. 0.2 Gy	Controls vs. 1 Gy	0.2 Gy vs. 1 Gy
			Interaction $F(2, 29) = 5.89, P > 0.01$	ns	ns	$P < 0.001$
			Males	ns	ns	
			Females	ns	ns	ns
	5C	Two-way ANOVA main effects				
		Treatment $F(2, 210) = 8.84, P > 0.05$	Bregma $F(13, 210) = 16.32, P < 0.0001$	See Fig. 2C		
			Interaction $F(26, 210) = 1.34, P > 0.05$			
			Males			
	5D	$F(2, 126) = 19.67, P < 0.0001$	$F(13, 126) = 19.37, P < 0.0001$	See Fig. 2D		
BrdU		One-way ANOVA main effect $F(2, 32) = 5.162, P < 0.05$		Controls vs. 0.2 Gy	Controls vs. 1 Gy	0.2 Gy vs. 1 Gy
				ns	$P < 0.05$	$P < 0.05$
	6A	Two-way ANOVA main effects				
		Treatment $F(2, 29) = 1.915, P < 0.001$	Sex $F(1, 29) = 16.72, P < 0.001$	Controls vs. 0.2 Gy	Controls vs. 1 Gy	0.2 Gy vs. 1 Gy
			Interaction $F(2, 29) = 1.915, P > 0.05$	ns	$P < 0.001$	$P < 0.01$
			Males	ns	ns	ns
			Females	ns	ns	ns
	6B	Two-way ANOVA main effects				
		Treatment $F(2, 29) = 1.915, P < 0.001$	Bregma $F(13, 210) = 11.8, P < 0.0001$	See Fig. 3C		
			Interaction $F(26, 210) = 2.151, P < 0.01$			
			Males			
	6D	$F(2, 195) = 4.67, P < 0.05$	$F(13, 195) = 4.09, P < 0.0001$	See Fig. 3D		
Percentage BrdU ⁺ and NeuN ⁺ /BrdU ⁺		Two-way ANOVA main effects				
		Treatment	Sex			
			Interaction			
	6F					

Antibody	Fig.	Bonferroni post hoc						
Surviving BrdU ⁺ neurons (total BrdU ⁺ and NeuN ⁺ cells) (Fig. not shown.)		F(2, 18) = 0.3144, <i>P</i> > 0.05	F(1, 18) = 0.9259, <i>P</i> > 0.05	F(2, 18) = 0.2526, <i>P</i> > 0.05	Controls vs. 0.2 Gy	Controls vs. 1 Gy	0.2 Gy vs. 1 Gy	
				Males	na	na	na	
				Females	na	na	na	
		Two-way ANOVA main effects		Interaction				
		Treatment	Sex					
		F(2, 18) = 12.75, <i>P</i> < 0.001	F(1, 18) = 21.09, <i>P</i> < 0.001	F(2, 18) = 2.437, <i>P</i> > 0.05	Controls vs. 0.2 Gy	Controls vs. 1 Gy	0.2 Gy vs. 1 Gy	
				Males	ns	<i>P</i> < 0.001	<i>P</i> < 0.05	
				Females	ns	ns	ns	
		7A	One-way ANOVA main effect			Controls vs. 0.2 Gy	Controls vs. 1 Gy	0.2 Gy vs. 1 Gy
			F(2, 32) = 4.359, <i>P</i> < 0.05			ns	ns	<i>P</i> < 0.05
DCX		Two-way ANOVA main effects						
		Treatment	Sex					
		F(2, 29) = 3.58, <i>P</i> < 0.05	F(1, 29) = 0.06, <i>P</i> > 0.05	F(2, 29) = 0.42, <i>P</i> > 0.05	Controls vs. 0.2 Gy	Controls vs. 1 Gy	0.2 Gy vs. 1 Gy	
				Males	ns	ns	ns	
				Females	ns	ns	ns	
				Interaction				
		7B						
		Two-way ANOVA main effects						
		Treatment	Bregma					
		F(2, 210) = 15.12, <i>P</i> < 0.0001	F(14, 210) = 52.80, <i>P</i> < 0.0001	F(28, 210) = 1.14, <i>P</i> > 0.05	Controls vs. 0.2 Gy	Controls vs. 1 Gy	0.2 Gy vs. 1 Gy	
				Males	ns	ns	ns	
				Females	ns	ns	ns	
				Interaction				
		7C						
		F(2, 225) = 2.67, <i>P</i> > 0.05	F(14, 225) = 40.53, <i>P</i> < 0.0001	F(28, 225) = 1.08, <i>P</i> > 0.05				
				Females	See Fig. 4C			

Note. Significance indicated in bold face; ns = not significant; na = not applicable.

TABLE 3

Statistical Results, Contextual Fear Conditioning (Fig. 8)

Phase	Fig.	One-way ANOVA main effect	Bonferroni post hoc		
			Controls vs. 0.2 Gy	Controls vs. 1 Gy	0.2 Gy vs. 1 Gy
Context	8B	F(2, 20) = 4.574, $P < 0.05$	$P < 0.05$	ns	ns, $P = 0.053$
Pretone	8C	F(2, 18) = 5.295, $P < 0.05$	ns	$P < 0.05$	$P < 0.05$
Cue	8D	F(2, 18) = 0.1505, $P > 0.05$	na	na	na

Note. Significance indicated in bold face; ns = not significant; na = not applicable.