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⁵⁶Fe Particle Exposure Results in a Long-Lasting Increase in a Cellular Index of Genomic Instability and Transiently Suppresses Adult Hippocampal Neurogenesis *in Vivo*

Nathan A. DeCarolisa^{a,1}, Phillip D. Rivera^{a,1}, Francisca Ahn^b, Wellington Z. Amaral^a, Junie A. LeBlanc^a, Shveta Malhotra^a, Hung-Ying Shih^b, David Petrik^a, Neal Melvin^a, Benjamin P.C. Chen^{*,b}, and Amelia J. Eisch^{*,a}

^aDept Psychiatry, UT Southwestern Medical Center, Dallas, TX, USA

^bDept Radiation Oncology, UT Southwestern Medical Center, Dallas, TX, USA

Abstract

The high-LET HZE particles from galactic cosmic radiation pose tremendous health risks to astronauts, as they may incur sub-threshold brain injury or maladaptations that may lead to cognitive impairment. The health effects of HZE particles are difficult to predict and unfeasible to prevent. This underscores the importance of estimating radiation risks to the central nervous system as a whole as well as to specific brain regions like the hippocampus, which is central to learning and memory. Given that neurogenesis in the hippocampus has been linked to learning and memory, we investigated the response and recovery of neurogenesis and neural stem cells in the adult mouse hippocampal dentate gyrus after HZE particle exposure using two nestin transgenic reporter mouse lines to label and track radial glia stem cells (Nestin-GFP and Nestin-CreER^{T2}/R26R:YFP mice, respectively). Mice were subjected to ⁵⁶Fe particle exposure (0 or 1 Gy, at either 300 or 1000 MeV/n) and brains were harvested at early (24h), intermediate (7d), and/or long time points (2–3mo) post-irradiation. ⁵⁶Fe particle exposure resulted in a robust increase in 53BP1+ foci at both the intermediate and long time points post-irradiation, suggesting long-term genomic instability in the brain. However, ⁵⁶Fe particle exposure only produced a transient decrease in immature neuron number at the intermediate time point, with no significant decrease at the long time point post-irradiation. ⁵⁶Fe particle exposure similarly produced a transient decrease in dividing progenitors, with fewer progenitors labeled at the early time point but equal number

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*Co-Corresponding Authors: Amelia J. Eisch, Department of Psychiatry, UT Southwestern Medical Center, Dallas, TX 75390-9070. amelia.eisch@utsouthwestern.edu. Benjamin P. C. Chen, Department of Radiation Oncology, UT Southwestern Medical Center, Dallas, TX 75390-9187. benjamin.chen@utsouthwestern.edu.

¹Authors contributed equally, and are listed alphabetically

Contributions: NAD, BPCC, and AJE conceived and designed the study. NAD, FA, HYS, SM, BPCC attended the BNL runs. NAD did mouse drug treatments. NAD and JAL spearheaded animal experiment logistics. NAD, FA, JAL, DP, NM sectioned tissue. PDR, NAD, FA, JAL, WZA, HYS performed immunohistochemistry. NAD, FA, and WZA collected data. PDR, NAD, and JAL complied and organized data. PDR, NAD, and WZA analyzed results. PDR and NAD made figures. PDR, NAD, BPCC, AJE interpreted results, wrote, and edited the manuscript.

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labeled at the intermediate time point, suggesting a recovery of neurogenesis. Notably, ^{56}Fe particle exposure did not change the total number of nestin-expressing neural stem cells. These results highlight that despite the persistence of an index of genomic instability, ^{56}Fe particle-induced deficits in adult hippocampal neurogenesis may be transient. These data support the regenerative capacity of the adult SGZ after HZE particle exposure and encourage additional inquiry into the relationship between radial glia stem cells and cognitive function after HZE particle exposure.

Keywords

subgranular zone; galactic cosmic radiation; transgenic mice; BrdU; adult neurogenesis; recovery

INTRODUCTION

Astronauts traveling to Mars during space flight missions will be exposed to damaging doses (~0.60 Sv/year) of galactic cosmic radiation (GCR) [1]. About half of this total annual dose is comprised of high linear energy transfer (LET) particles, including various high-energy, high-charge nuclei (HZE) that can damage cells and tissues due to their inherent properties. The damaging nature of these particles poses a serious risk to astronauts and therefore to mission success [1]. In particular, a major risk for mission success is the long-term effect of GCR on the adult brain [2]. Certain brain regions – like the hippocampus – continually give rise to new neurons throughout life [3], and the dividing cells in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) appear particularly vulnerable to the damaging effects of GCR [4–12]. It is notable that when GCR is mimicked using ground-based HZE particle radiation, deficits in DNA damage responses *in vitro*, DG SGZ adult neurogenesis *in vivo*, and cognition have been found [10, 11, 13–15]. However, studies have yet to examine the DNA damage response within the DG and compare various energies on SGZ adult neurogenesis. Therefore, it is important for the field of space research to assess the cellular response and array of energies that astronauts may receive during long-term space flight missions.

Currently, the literature supports a persistent deficit in hippocampal neurogenesis after HZE particle exposure. Many studies label exogenous (5-bromo-2'-deoxyuridine, BrdU) or endogenous (Ki67; doublecortin, DCX; and neuronal nuclei, NeuN) markers of adult neurogenesis to show HZE exposure-induced deficits in neurogenesis [4–6, 8, 10, 12]. In addition, studies have also used transgenic reporter mouse lines to quantify, label, and track adult-generated hippocampal neurons and their putative source, nestin-expressing radial glial-like Type-1 cells [9, 12]. Assessment of these different “stages” of neurogenesis are important given the distinct capabilities of cells in each stage. For example, Type-1 stem cells rarely divide, but are multipotent, giving rise to both neurons and astrocytes; progenitors divide frequently, but are unipotent, giving rise to only neurons [16–18]. Therefore, the resistance or susceptibility of Type-1 stem cell number to HZE particle irradiation (IR) may determine whether repopulation of hippocampal neurons is possible in the long-term, even if there is a decrease in hippocampal neurons (e.g. BrdU/NeuN immunoreactive [+] neurons) or even rapidly-dividing progenitors (e.g. Ki67+ cells). Two

recent studies examined the influence of HZE particles on Type-1 stem cells [9, 12]. While both studies showed radiation-induced deficits in neurogenesis, the one study that looked several months post-irradiation (post-IR) showed no change in the number of nestin-expressing radial glia Type-1 cells [12]. Given this persistence of radial glia Type-1 cells at a long time point post-IR, is it possible for adult neurogenesis to recover from ^{56}Fe particle exposure? This question of recovery has important implications for risk assessment for spaceflight missions but also is important in understanding the dynamic life-long process of adult neurogenesis.

The main goal of this study was to employ nestin transgenic reporter mouse lines to clarify to what extent neurogenesis deficits recover after ^{56}Fe particle exposure to various mission-relevant doses and LET. In addition, a secondary goal of this study was to examine the influence of energy of ^{56}Fe particle on neurogenesis and genomic instability. We exposed mice from two different nestin transgenic lines (one constitutive and one inducible reporter) to ^{56}Fe particles, and quantified the changes in neurogenesis at early (24 hours [h]), intermediate (7 days [d]), and long (2–3 months [mo]) time points post-IR. The constitutive Nestin-GFP mice allow quantification of adult neural stem cells (e.g. radial glia Type-1 cells [9, 12, 19], which are GFP+ with a radial morphology). The NestinCreER^{T2}/R26R:YFP mice allow inducible labeling of neural stem cells and their progeny [18]. We combine the use of these nestin transgenic lines with 2 more classic approaches to assess neurogenesis: 1) administration of a mitotic marker, BrdU, and quantification of cells at time points post-injection to assess proliferating, differentiating, and surviving cells; 2) quantification of immature neurons that express the endogenous protein DCX. Based on prior studies showing long-term deficits in adult hippocampal neurogenesis [10, 12, 14, 15] and behavior [14, 15, 20–22] after HZE particle exposure, we hypothesized there would be no recovery of neurogenesis after ^{56}Fe particle exposure. In keeping with our recent work [12], HZE particle exposure did not decrease the number of radial glia Type-1 cells. Consistent with prior work showing long-term deficits in neurogenesis [4, 10, 12] and *in vitro* work [13], HZE particle exposure induced long-term cellular evidence of genomic instability, as indicated by increase in 53BP1 puncta [23]. Surprisingly, HZE particle exposure resulted in only a transient decrease in the number of adult-generated hippocampal neurons. These data support the regenerative capacity of the adult SGZ after HZE particle exposure and encourage additional inquiry into the relationship between radial glia Type-1 cells and cognitive function after HZE particle exposure.

METHODS

Animals

Animal procedures and husbandry were in accordance with the National Institutes of Health and Guide for the Care and Use of Laboratory Animals, were approved by the UT Southwestern Medical Center (UTSW) Animal Care and Use Committee. Mice were group housed (n=2–5/cage) with *ad libitum* access to food and water on a 12h light-dark cycle. Homozygous Nestin-GFP mice [19] were generated and maintained on a C56BL/6J background. Homozygous Nestin-CreER^{T2} transgenic mice were also generated and maintained on a C57BL/6J background and were crossed to homozygous R26R:YFP knock-

in mice to produce Nestin-CreER^{T2}/R26R:YFP (“Nestin-Cre/YFP”) bitransgenic mice. Genotyping and characterization of both mouse lines have been described elsewhere [12, 18, 19, 24, 25]. At 5–6 weeks of age, Nestin-Cre/YFP mice were administered tamoxifen (TAM, 180 mg/kg/day, i.p.) for 5d to induce recombination in nestin-expressing cells and their progeny [16, 18]. For all studies, mice from both sexes were used since pilot studies showed no sex-dependent differences [18].

Particle Irradiation

Mice acclimated to Brookhaven National Laboratories (BNL) 2–5d before irradiation. Mice received whole-body irradiation with 1 Gy ⁵⁶Fe at 1000 MeV/n (LET: 148 keV/μm, Figs. 1–2) or 300 MeV/n (LET: 240 keV/μm, Figs. 1, 3–4) with a dose rate of 1 Gy/min at BNL NASA Space Radiation Laboratory (NSRL) as previously described [12]. We elected to use 1 Gy because this dose permits each cell in the hippocampus to have 1–3 hits per cell [9] and has been widely used for rodent studies [4, 7–9, 12, 15, 26]. We chose these energies because 1000 MeV/n is the highest energy available at BNL [NSRL Technical Note TN10-001] and has been commonly used for *in vivo* studies [4, 7, 9, 14, 15, 21, 26]; 300 MeV/n has also been used for *in vivo* studies [5, 12]. An additional reason to use these parameters is that we wanted to compare our findings in whole body irradiation non-anesthetized mice to prior work where cranially-directed 1000 MeV/n and 1Gy decreased nestin-GFP+ stem cells number in anesthetized mice [9], although the dose rate was not provided in that study. Sham mice were placed in the irradiation chambers for an equivalent period of time, but did not receive irradiation. Mice were 9–14 weeks of age at irradiation. Each cohort of animals included age-matched sham controls; cohorts were irradiated at a range of ages because of breeding and scheduling of BNL beam time. Cohorts (controls and age-matched irradiated animals) were examined, and no effect of cohort was seen. For the early time points, mice were sacrificed at BNL/NSRL. For survival experiments, mice were shipped back to UTSW 2–4 d post-IR.

BrdU and Tissue Collection

To assess cell proliferation and survival, mice were administered the thymidine analog BrdU (150 mg/kg, i.p., 10 mg/ml in 0.9% saline and 0.01M NaOH). BrdU was administered immediately (<5min), 22h, 7d, or 1mo post-IR, and tissue collected by transcardial perfusion or live decapitation. We administered a single injection of BrdU, consistent with previous studies in our laboratory [12, 16, 18, 27] and in the neurogenesis field [e.g. 28, 29]. All brains were post-fixed in 4% paraformaldehyde before cryoprotection in 30% sucrose/0.01% sodium azide in 1xPBS [27]. Brains were sectioned at 30μm in 1:9 series as described [18, 27].

Immunohistochemistry (IHC)

For staining of cells immunoreactive (+) for BrdU, GFP (e.g. Nestin-GFP), and YFP (e.g. Nestin-Cre/YFP), slide-mounted IHC was performed. Briefly, one series of sections were mounted onto glass slides (Fisher Superfrost/Plus, Hampton, NH). Slides were coded to ensure objectivity, and the code was not broken until after data collection was complete. For staining of cells immunoreactive (+) for DCX and 53BP1, free-floating IHC was performed.

For BrdU IHC, pretreatment consisted of: antigen retrieval (0.01M citric acid, pH 6.0, 95°C, 10min); membrane permeabilization (0.1% trypsin in 0.1M Tris and 0.1% CaCl₂, 10min); and DNA denaturation (2N hydrochloric acid in 1XPBS, 30min). For Nestin-GFP and YFP staining, slides underwent antigen retrieval alone. For both slide-mounted and free-floating IHC, nonspecific staining was blocked by incubation with 3% normal donkey serum (NDS) in 0.1% TritonX-100 in 1XPBS for 30–60min. Sections were incubated in primary antibodies overnight at room temperature in 3% NDS, 0.1% Tween-20 in 1XPBS. The following day, sections were incubated in secondary antibodies. Fluorophore-conjugated secondary antibodies (4h incubation) were used to visualize DCX, 53BP1, GFAP, or Nestin-GFP antigens. Signal for BrdU and YFP was amplified using biotinylated secondary antibodies (1:200, incubated for 1h in 1.5% NDS in 1XPBS), followed by incubation in 0.3% hydrogen peroxide (Sigma Laboratories) in 1XPBS for 30min, and avidin-biotin complex (1:50, Vector Laboratories) for 30min. Visualization of BrdU+ cells was achieved after incubation in DAB (Pierce, Rockford, IL) for 20–30min and counterstained with Nuclear Fast Red (Vector Laboratories). Visualization of YFP+ cells was achieved with TSA (1:50, Perkin-Elmer). Slide-mounted stained sections were dehydrated and coverslipped with DPX (Fisher Scientific). Free-floating stained sections were mounted onto uncharged slides, dried quickly, and coverslipped with glycerol/polyvinyl alcohol solution, as described [30]. The following primary antibodies were used: rat α -BrdU (Accurate, 1:400); rabbit α -GFP (Invitrogen, 1:3,000 amplified for detection of YFP in Nestin-Cre/YFP mice; 1:3,000 without amplification for GFP in Nestin-GFP mice); rabbit α -53BP1 (Bethyl Laboratories, 1:6,000); goat α -DCX (Santa Cruz, 1:200–500); mouse α -GFAP (Millipore, 1:3,000).

Cell Quantification

BrdU+, YFP+, Nestin-GFP+ Type-1, and Nestin-Cre/YFP+ Type-1 cells were quantified at 400x by an observer blind to experimental condition with a section-sampling fraction of 9 and the all cells were counted throughout the SGZ; no guard zones were used [27]. Quantification of stained cells was exhaustive through the septotemporal axis of the hippocampal dentate gyrus in a 1:9 series (bregma: –0.82 to –4.24), essentially as described [12, 16, 18, 25, 27]. In brief, bright-field or fluorescent staining of sections on coded slides was visualized and quantified with an Olympus BX-51 microscope. Staining was examined and quantified in the SGZ of the dentate gyrus; the SGZ was defined as a region straddling the border of the granular cell layer (GCL) and the hilus: 30 μ m into the hilus and the inner half of the GCL adjacent to the hilus. A cell on the border in the middle of the GCL was considered to be in the SGZ. The quantification of immunoreactive cells in the SGZ was performed using stereology by an examiner blinded to treatment. Using an Olympus BX-51 microscope at 400X magnification and continual adjustment through the depth of the section, immunoreactive cells in the SGZ were counted in every ninth section through the septotemporal axis of the hippocampus. The section sampling fraction was 1/9, and the resulting number for each region was multiplied by 9 to obtain an estimate of the total number of cells per dentate gyrus. Because the raw counts (before multiplication) for the immunoreactive cells counted here were low according to disector/fractionator standards, we used an area sampling fraction of 1 as is commonly used for counting rare populations of cells [16, 18, 27, 31, 32]; the height sampling fraction was set at 1. For Type-1 cell analysis,

GFP+ cells with a radial process perpendicular to the SGZ were similarly quantified by a single observer blinded to experimental condition [16, 27]. For DCX+ cells and 53BP1+ foci quantification, the optical fractionator method was used with section sampling fraction of 18, appropriate area sampling fractions, and guard zones to only quantify the inner 10µm of each sampling site, essentially as described [30]. Images were taken and imported into Photoshop (Adobe Systems) for composition purposes. In order to include an animal for histological quantification, all sections needed to be present and stained uniformly; in cases where the hippocampus was not sectioned through the posterior extent or where sections were lost or destroyed during staining, the animal was removed from analysis.

Statistical Analyses

Data are reported as mean±SEM. Statistical analyses were performed using either a Student's t-test, one- or two-way analysis of variance (ANOVA) followed by a Dunnett's (Fig. 1D–F, 2C, and 2E) or Sidak (Fig. 3G, and 4G) post-hoc analysis, respectively. The Student's t-test was used to either compare two energies, doses, or time points (Figs. 1C, 3B–D, and 4B). In two instances a Student's t-test was used after a one-way ANOVA (Fig. 2C) and a two-way ANOVA (Fig. 4G) to examine general differences in doses. All statistical analyses were performed using Prism GraphPad (version 6.0c) software. Genotypes were analyzed separately to account for slight age differences of the Nestin-GFP vs. Nestin-Cre/YFP available for the irradiation experiment at NSRL/BNL. Statistical significance was defined as $p<0.05$.

RESULTS

⁵⁶Fe particle exposure induces immediate deficits in DG proliferation

Prior work has shown decreased number of proliferating DG cells after HZE particle exposure when the cells are labeled soon after the irradiation [4]. To determine if deficits in dividing progenitors seen after ⁵⁶Fe exposure is energy-dependent, nestin transgenic mouse lines were irradiated with 1 Gy ⁵⁶Fe at 1000 or 300 MeV/n, administered with BrdU either immediately (<5min) or 22h post-IR, and killed at 24h post-IR (Fig. 1A–B). In Nestin-GFP mice, quantification of BrdU+ cells labeled immediately post-IR (Fig. 1C) revealed a significant interaction ($p<0.001$) between 300 MeV/n and sham mice. In addition, BrdU+ cell number labeled 22h post-IR in Nestin-GFP (Fig. 1D) revealed a significant effect of treatment ($F_{(2,10)}=12.97$, $p<0.0017$). Post-hoc analysis revealed that both irradiated groups had significantly fewer BrdU+ cells than sham (300 MeV/n vs. sham: $M=1159$, 95% CI [357.6,1961], $p<0.01$; 1000 MeV/n vs. sham: $M=1584$, 95% CI [503.2,2665], $p<0.01$). Similarly, in Nestin-Cre/YFP mice, quantification and analysis of BrdU+ cell number labeled immediately and 22h post-IR (Fig. 1B, E–F) revealed significant effects of treatment (Immed., $F_{(2,13)}=40.0$, $p<0.001$; 22h, $F_{(2,12)}=39.55$, $p<0.001$). Post-hoc analysis revealed that the both irradiated group had significantly fewer BrdU+ cells than sham controls in BrdU+ cells labeled immediately (Fig. 1E, 300 MeV/n vs. sham: $M=2851$, 95% CI [1952,3750], $p<0.001$; 1000 MeV/n vs. sham: $M=2582$, 95% CI [1721,3443], $p<0.001$) and 22h (Fig. 1F, 300 MeV/n vs. sham: $M=2470$, 95% CI [1542,3398], $p<0.001$; 1000 MeV/n vs. sham: $M=2678$, 95% CI [1750,3606], $p<0.001$) post-IR. Notably, both energies decreased BrdU+ cell numbers to a similar extent. Therefore, irrespective of energy (1000 or

300 MeV/n), ^{56}Fe particle exposure induces comparable deficits in adult generated progenitors immediately (<5min) and within 24h post-IR.

^{56}Fe particle exposure causes deficits in adult neurogenesis and increases an index of genomic instability in the DG

To examine the influence of ^{56}Fe particle radiation on adult neurogenesis as well as on a cellular index of genomic instability [23] at times post-IR, Nestin-Cre/YFP mice were irradiated with 0 or 1 Gy ^{56}Fe (1000 MeV/n) then killed 7d and 2mo post-IR (Fig. 2A). After stereological quantification of DCX+ cells in the dentate gyrus (Fig. 2B), there was a significant effect of treatment ($F_{(1,8)}=24.8$, $p<0.01$) and time ($F_{(1,8)}=9.1$, $p<0.05$) on DCX+ cell number, and a trend towards an interaction ($F_{(1,8)}=5.3$, $p=0.0505$; Fig. 2C). Post-hoc analysis revealed that the irradiated group had significantly fewer DCX+ cells at 7 days (1 Gy vs. 0 cGy: $M=15854$, 95% CI [7399,24309], $p<0.001$) and trend towards significance 2 months post-IR ($p=0.09$) compared to sham controls. The DNA damage response protein 53BP1 was used as a marker for complex DNA damage and genomic instability [13, 23, 33], and was assessed by stereological quantification of 53BP1+ foci in the dentate gyrus of the hippocampus post-IR (Fig. 2D–E). There was a significant effect of treatment on 53BP1+ foci number ($F_{(1,17)}=29.1$, $p<0.001$), but no effect of time or interaction (Fig. 2E). Post-hoc analysis revealed that the irradiated group had significantly more 53BP1+ foci in the DG than sham controls at 7 days (1 Gy vs. 0 Gy: $M=-83139$, 95% CI [-123006, -43273], $p<0.001$) and 2 months post-IR (1 Gy vs. 0 Gy: $M=-39129$, 95% CI [-77840, -418.1], $p<0.05$). This is consistent with our previous report that acute ^{56}Fe particle radiation posts long-term deficits in adult hippocampal neurogenesis [12].

^{56}Fe particle exposure decreases progenitor cell number without changing the number of radial glia Type-1 cells in DG

We returned to the question whether ^{56}Fe particle exposure affect the number of radial glia Type-1 cells after ^{56}Fe particle exposure. As we recently showed, ^{56}Fe particle exposure (1 Gy, 300 MeV/n, 238.8 keV/ μm) did not change radial glia Type-1 cell number 24h or 3mo post-IR in Nestin-Cre/YFP mice [12]. We wanted to test whether radial glia Type-1 cell number would be similarly resistant to decreases in Nestin-GFP mice. To examine the effect of ^{56}Fe particle exposure on early stages of neurogenesis (i.e. Type-1 and transiently amplifying progenitor cells) over time, Nestin-GFP mice were irradiated (^{56}Fe , 0 or 1 Gy, LET: 240 keV/ μm), administered BrdU 22h post-IR, and killed 24h, 7d, or 3mo post-IR (Fig. 3A). BrdU+ cell number quantification and analysis of proliferating (2h-old BrdU+ cells), differentiating (6d-old BrdU+ cells), and surviving (3mo-old BrdU+ cells) all revealed significantly fewer cells in irradiated vs. sham control mice in Nestin-GFP mice (2h-old Fig. 3B, 51% decrease, $p<0.01$; 6d-old Fig. 3C, 76% decrease, $p<0.001$; 3mo-old Fig. 3D, 87% decrease, $p<0.001$). Having established a long-term decrease in dividing cells when labeled 24h post-IR, we then turned to examine Nestin-GFP+ radial glia Type-1 cell number, utilizing phenotypic and morphological analysis as previously described ([12], Fig. 3E–F). However, quantification of radial glia Type-1 cell number revealed no significant difference at early, intermediate, or longer time points post-IR (Fig. 3G). Therefore, ^{56}Fe particle exposure induces a long-term decrease in the number of adult generated progenitors

labeled within 24h post-IR but does not change the number of radial glia Type-1 nestin-expressing neural stem cells.

⁵⁶Fe particle exposure transiently decreases neurogenic progenitors, but does not significantly change the proportion of YFP+ cells that are Type-1 radial glia-like cells

Thus far, we established that in two nestin transgenic mouse lines there exists a “critical period” within 24h after ⁵⁶Fe particle exposure during which labeled progenitors will have diminished proliferation and survival in the long-term, but that the number of radial glia Type-1 cell is unchanged by ⁵⁶Fe particle exposure. While assessment of general stages of neurogenesis are useful, the dynamic process of neurogenesis *in vivo* is best assessed via an inducible reporter mouse where one can quantify the contribution of stem cells and their progeny to neurogenesis over time [18]. To examine the effect of ⁵⁶Fe particle exposure on adult neurogenesis over time (i.e. Type-1 to mature neurons), Nestin-Cre/YFP mice were administered tamoxifen ~60d prior to irradiation. This time point was chosen as it is a time when YFP+ cells mainly consisted of YFP+ neurons and immature neurons (DCX), and a low percentage (~25%) of YFP+ radial glia Type-1 cells and progenitors (Ki67) [12, 16, 18]. Sixty days post-TAM, mice were then irradiated (⁵⁶Fe, 0 or 1 Gy, LET: 240 keV/μm), killed 24h, 7d, or 3mo post-IR (Fig. 4A), and YFP+ cells in the granule cell layer (GCL) were quantified. In Nestin-Cre/YFP mice examined 24h post-IR/~60d post-TAM, there were significantly fewer YFP+ cells in irradiated compared to sham mice (Fig. 4B, $p < 0.05$). In contrast, there were no significant differences in YFP+ cell number in irradiated compared to sham mice 7d post-IR/67d post-TAM (Fig. 4C) or 3mo post-IR/5mo post-TAM (Fig. 4D). These data suggest a recovery of neurogenesis by 7d post-IR. However, it is difficult to know from counting YFP+ total cell number since YFP+ cells consist of YFP+ radial glia Type-1 cells, YFP+ dividing transiently amplifying progenitors and immature neurons, and, at later time points, mature YFP+ neurons [16, 18]. Therefore we used phenotypic and morphological analysis to dissect what proportion of the total YFP+ cells were radial glia Type-1 cells (Fig. 4E–F). Phenotypic analysis revealed main effects of treatment ($F_{(1,34)} = 4.28$, $p < 0.05$) and time ($F_{(2,34)} = 6.4$, $p < 0.01$) but no significant interaction ($F_{(2,34)} = 1.0$, $p > 0.05$; Fig. 4G). However, there was a trend of having an increased proportion of YFP+ cells were radial glia Type-1 cells in irradiated mice compared to sham controls 24h post-IR (t -test, $p = 0.06$). There were no other significant differences between irradiated and sham controls 7d or 3mo post-IR. Therefore, relative to sham controls, irradiation of a labeled population of YFP+ stem cells and their neural progeny leads to an early, transient decrease in YFP+ cell number which is normalized by 7d post-IR. This early decrease in YFP+ cell number is accompanied by a transient increase in the proportion of YFP+ cells that are radial glia Type-1 cells. These data suggest that irradiation decreases the number of YFP+ progenitors or neurons, and reaffirms that YFP+ radial glia Type-1 cells are resistant to change in total number after HZE radiation.

DISCUSSION

Asymmetric division of adult radial glia stem cells after ground-based HZE particle exposure has been previously suggested as a potential mechanism for recovery of adult hippocampal neurogenesis [4, 8]. However, prior to our study presented here it was unclear

if recovery of adult hippocampal neurogenesis could occur after HZE particle exposure. The present study was designed to examine adult hippocampal neurogenesis at various times post-IR using BrdU to label dividing progenitor cells and two nestin transgenic reporter mouse lines to label nestin-expressing radial glia stem cells and early progenitors (Nestin-GFP) or nestin-expressing radial glia stem cells and their progeny throughout life (Nestin-Cre/YFP). Using both the Nestin-GFP and Nestin-Cre/YFP transgenic reporter mice to label adult-generated cells, we show that there is long-term cellular evidence of genomic instability and a transient suppression of adult-generated cells after ground based ^{56}Fe particle exposure. Below, we discuss how our results compare with published literature using other nestin transgenic reporter mice and what implications our results have on future space flight missions.

Transient suppression of adult neurogenesis occurs after ^{56}Fe particle exposure

The primary novel finding of our work is that adult hippocampal neurogenesis is transiently suppressed after ^{56}Fe particle exposure. Using our inducible Nestin-Cre/YFP mouse line, we show that ^{56}Fe particle exposure leads to a transient decrease in total YFP+ cell number 24h, which recovers at 7d and persists up to 3mo post-IR (Fig. 4B–D). There are at least two potential stages in adult neurogenesis that can contribute to the recovery in the DG, either the asymmetric division of radial glia Type-1 cells or the proliferation of transiently amplifying progenitors (TAPs) [12, 25]. Our lab has previously shown that YFP+ adult neural Type-1 stem cells with radial glia morphology from the same Nestin-Cre/YFP mouse line did not give rise to new TAPs after specifically suppressing only mitotic cells (i.e. maturing adult-generated cells) via AraC treatment (an anti-mitotic drug [12, 16, 34]). The transient depletion of TAPs suggested that any recovery that occurred in the Nestin-Cre/YFP mouse after loss of young mitotic granule cells would come from asymmetric division of radial glia stem cells [16]. Interestingly, our present work shows no change in the total number of GFP+ radial glia cells in the Nestin-GFP mouse (Fig. 3G); and a transient increase in the proportion of total YFP+ cells that have YFP+ radial glia Type-1 cell morphology 24h post-IR was observed in the Nestin-Cre/YFP mouse (Fig. 4G). Nestin-GFP proportional analysis was not preformed since GFP+ cells do not reflect the entire heterogeneous population observed during the process of adult neurogenesis (i.e. GFP+ cells only label Type-1 cells and TAPs of adult neurogenesis [3, 12]). Taken together, these data suggest that the recovery of adult-generated cells is based on the ability of the radial glia Type-1 cell to give rise to transiently amplifying progenitors and continually produce adult-generated cells after HZE particle radiation.

DCX+ cell number: differences with prior publications—Previous work has shown ^{56}Fe particle exposure to be damaging to DCX+ cells in the GCL of the dentate gyrus [4, 10, 12]. However, the work presented here shows significantly fewer total immature adult-generated (DCX+) cells 7d post-IR, while no significant difference in DCX+ cell number ($p=0.09$) was found 2mo post-IR compared to 0 Gy sham controls (Fig. 2). These data present a quandary in regards to our DCX+ data and previously published data: why do we see a transient decrease in DCX after ^{56}Fe particle exposure? Our lab has previously published work using the same Nestin-Cre/YFP, energy/dose, particle, and examined a similar time point post-IR and yet significant differences in DCX+ cell counts were found

[12]. One major difference between our works was the method used during irradiation. For instance, in Rivera, Shih, et al., animals were put in sham IR conditions once a day for 4d prior to irradiation on the fifth day. This paradigm likely compounded the deficits observed 3mo post-IR [12]. In the present work, mice were not exposed to such potentially stressful handling prior to irradiation, perhaps explaining the differences in DCX+ cell numbers. In addition to our previous work, there is literature also showing deficits in DCX+ cell number and other measures of neurogenesis after ^{56}Fe particle exposure. However, each has different variables that make it difficult to compare our present work. For example, differences between previous studies and our results presented here include differences in age at IR (8 weeks of age in [10] vs 9–14 weeks at IR here vs 10–12 weeks in [12] vs 3 mo in [4, 5]); dose (1 Gy here and in [4, 5, 12] vs 2 Gy [10]); energy (300 MeV/n used here and in [5, 12] vs 1000 MeV/n here and in [4, 5] vs 600 MeV/n in [8] vs 500 MeV/n in [10]); localization of irradiation (whole-body here and in [4, 5, 12] vs head-only in [8, 10]); and the method used to assess DCX+ cells (whole hippocampus here via stereological assessment here and in [12] vs morphometric sampling in 3 sections in [4, 5, 8, 10]). Taken together, given the variability between studies, it is possible that the observed transient decrease in DCX+ cells found in this present work can occur and lead to less severe deficits as a result of irradiation.

Role of BrdU injection paradigm?—Another difference between our current study and previous studies involves the injection paradigm for BrdU. BrdU is a common exogenous marker of dividing cells. Here we give one injection of BrdU at a dose that saturates the dividing cell pool [32], which labels all cells in S phase of the mouse SGZ. As described in detail elsewhere [32, 35], the interval between the injection and perfusion determines “how old” the BrdU+ cells will be at the time of death. For example, when animals are killed ~2h after BrdU (at a time less than the length of G2-M), the number of BrdU+ cells reflects proliferation, or the number of cells in S phase (Fig. 1: “22h”; Fig. 3: “24h”). When animals are killed at a slightly longer BrdU-perfusion interval (Fig. 1: “immediate”), cells will have gone through mitosis at least once, so this is a measure of both proliferation and differentiation. (While inexact, we selected this “immediate” time point in order to directly compare our data with previous findings [9]). When animals are killed at an even longer BrdU-perfusion interval, cells will have begun the process of neuronal differentiation and migration (Fig. 3: 7d) or incorporated as new neurons into the mossy fiber pathway (Fig. 3: 3mo; [36]), and thus the number of BrdU+ cells reflects the number of surviving BrdU cells. However, these longer BrdU-perfusion intervals are impossible to interpret unless data exist from a short BrdU-perfusion interval cohort. The use of one BrdU injection is common in the neurogenesis field, but differs from publications in the space radiation field, where BrdU is often given daily for one week or given in multiple injections over a single day. For example, Rola and others have traditionally used a 6d BrdU injection paradigm at a lower dose (50 mg/kg) [4, 5, 7, 8]. However, our laboratory has consistently used a single, high dose of BrdU (150 mg/kg) [12, 16, 18, 27]. We favor the single injection approach, particularly for the mouse SGZ where the cell cycle is 12h, as it minimizes DNA damage that may occur from accumulation of BrdU over the course of multiple injections. In addition, by providing a view of both “proliferating” and “surviving” BrdU+ cells, our work sheds light on how radiation differentially affects these processes. For example, ^{56}Fe

induced a 51% decrease in proliferating cells (Fig. 3B), but a greater influence of 76–87% decrease in surviving cells (Fig. 3C, 3D). It is unclear how differences in BrdU administration paradigms might account for differences in overall experimental results (considering the prudent use of controls). However, differences nonetheless exist between studies that require careful consideration and interpretation.

Functional/behavioral implications of transient decrease in hippocampal neurogenesis?—

While we did not assess hippocampal-related behaviors, the literature has conflicting results in regards to hippocampal-dependent behaviors after ^{56}Fe particle exposure. Previous work has shown that rodents irradiated with HZE particles were tested in the Morris water maze (rats, whole body, 1.5 Gy, 1000 MeV/n, ~1 month post-IR [21]) and Barnes maze (rats, whole body, 0.2 Gy, 1000 MeV/n, ~1 week post-IR [14]) show deficits in hippocampal-dependent behavior when compared to 0 Gy sham controls. This is in contrast to work that showed ~3 months after cranial ^{56}Fe particle exposure (mouse, whole body, 1 Gy to 2 Gy, energy not reported) no differences in percent freezing were found in both C57BL/6J males and females in the hippocampal-dependent behavior contextual fear conditioning when compared to 0 Gy sham controls [22]. In addition, 3.5 to 6 months after whole body ^{56}Fe particle exposure in a mouse model of Alzheimer's disease no difference in percent freezing was found in male (0.1 Gy) or female (1 Gy) mice compared to 0 Gy sham controls [15]. To further support the potential recovery of hippocampal function, rodents that were previously exposed to ^{56}Fe particles did not show deficits in radial arm maze [26] and Morris water maze [11] compared to 0 Gy sham controls. Taken together, in general, recovery of hippocampal-dependent behaviors appears to occur ~1 month after ^{56}Fe particle exposure. However, future experiments are still needed to determine if the recovery of hippocampal-related behaviors correlates to the recovery of adult neurogenesis and if there are dose-, particle-, and energy-dependent effects.

The relationship between adult hippocampal neurogenesis and hippocampal function in general remains a somewhat controversial topic in the field. Many researchers find a functional role for new neurons, and some do not (as reviewed in [37]). Within the field of space radiation, there is similar controversy. In brief, some papers suggest a correlation between space radiation-induced changes in neurogenesis and cognitive function [4, 5, 10], while others suggest a dose-dependent relationship or no correlation at all [6, 14]. However, there are few space radiation publications that examine neurogenesis and behavior in parallel groups of animals within the same paper, and no publications address whether decreased neurogenesis plays a causative role in space radiation-induced cognitive deficits. Therefore, more work is needed to clarify what, if any, role new neurons play in cognition. Recent works shows that new neurons buffer the stress response [28], raising the important issue of the stress state of the animal at the time of behavioral testing. Therefore, as the field of space radiation continues to address what role new neurons play in hippocampal function, it will be critical to keep the state of stress of the animals at time of testing in mind so as to avoid additional discrepancies across laboratories.

Various energies similarly decrease BrdU labeled cell number after ^{56}Fe particle exposure

Our second finding is that various energies of ^{56}Fe particles decrease BrdU cell number. A range of energies (e.g. 100–50,000 MeV/n) will intermittently and unpredictably bombard astronauts during interplanetary space flights [38]. Therefore, to address energy differences that may occur during space flight, we used two different high-LET energies (1000 or 300 MeV/n) to examine any differences in adult neurogenesis that would occur after ^{56}Fe particle exposure. We found similar deficits in proliferation (2h-old BrdU+ cells) using 300 and 1000 MeV/n in both nestin transgenic mouse lines (Fig. 1). This is consistent with previous publications examining proliferation of BrdU+ cells, but each publication only tested one specific energy, making it difficult to compare across the literature [9, 12]. As for survival of BrdU-labeled cells using only 300 MeV/n, we observed long-lasting deficits (24h to 3mo) in the survival of BrdU+ cells when labeled 22hr post-IR (Fig. 3B-D). This is consistent with the literature that ^{56}Fe particle exposure decreases BrdU+ cell number when administered either immediately [9] or 24h post-IR [12]. Other works examining survival (e.g. >24h-old BrdU+ cells) also found a decrease in 2 to 4wks old BrdU+ cells when labeled either 22hr [12] or 1 to 2mo post-IR [8]. While this is not our strongest finding (e.g. one group in Fig. 1 had only an n=2), we feel we can conservatively conclude that after ^{56}Fe particle exposure at the energies tested in an acute exposure decreases the proliferation and survival of BrdU-labeled adult-generated neurons within the GCL of the hippocampus. More work is needed to assess this in other energies, particles, and mouse lines (including nontransgenics), but taken with prior work our data suggest that the range of energies astronauts will be subjected to during interplanetary space flights will be similarly detrimental to actively dividing progenitors. An exciting aspect of the current work, though, is that it shows the potential for recovery still exists for some adult-generated neurons, particularly those generated after HZE exposure.

Long-lasting increase in a cellular index of genomic stability after ^{56}Fe particle exposure

Previous *in vitro* work has found persistence of the double strand break sensing protein 53BP1, which overlaps with -H2AX, to respond to DNA damage after ^{56}Fe particle exposure [13]. Based on this work we sought to determine if genomic stability of hippocampal GCL neurons was altered by ^{56}Fe particle exposure. Using 53BP1 as a marker for genomic instability [39, 40], we observed a long-lasting increase in 53BP1+ foci 7d to 2mo post-IR compared to 0 Gy sham controls *in vivo*. This suggests that the cells that survive after ^{56}Fe particle exposure are not free of DNA damage, and may have long-lasting deficits in their functionality. Future experiments using additional measures of genomic instability (such as -H2AX) and determining functionality of 53BP1+ neurons will be necessary to determine if cognition is affected after ^{56}Fe particle exposure.

Potential caveats and additional calls for future work

One might be concerned that the TAM used here may influence the results. Our past work shows no influence of a prior regimen of TAM on neurogenesis or gross development [18, 24] or on radiation-induced deficits in neurogenesis [12]. Therefore it is unlikely that TAM alone would influence the number of Type-1, proliferating progenitors, or surviving BrdU+ cells. However, it is possible that TAM may have some other unappreciated influence. For

example, TAM has been shown to alter radiation response [e.g. 41], and therefore it would be interesting in future experiments to consider the effects of TAM alone on radiation-induced changes in neurogenesis. However, because in most cases TAM is administered ~60d prior to HZE-particle exposure, it would be surprising if TAM alone had any long-term negative effects on these measures. Indeed, when we compare the number of BrdU cells between Nestin-GFP (which do not receive TAM, Fig 1C) and Nestin-Cre/YFP (which have received TAM 60d prior to irradiation, Fig 1E), both show similar number of basal BrdU+ cells, and similar decreases in BrdU+ cell number caused by irradiation. Also, both transgenic lines show no change in Type-1 radial glial stem cell number either here (Fig. 3 vs. 4) or in our recently published work (Rivera et al., 2013). Similarly, we have not observed any basal differences (e.g. in weight, number of DCX+ cells, etc.) between non-transgenic mice C57BL/6J mice compared to transgenic mice (e.g. Nestin-GFP and Nestin-Cre/YFP) on C57BL/6J background. While we do not think TAM is influencing the data we present here, the ability of TAM to potentially influence the radiation response means radiation researchers should approach inducible transgenic models with caution, and when possible include non-transgenic, wildtype mice for comparison.

Another potential concern is the way that we, like most neurogenesis researchers, report of the number of cells in each stage of neurogenesis without specifically addressing the reference volume of the GCL. It is reasonable to consider that IR may change the volume of a brain structure overtime, and thus influence the final interpretation of the data. However, the stereological approach used here indirectly takes into account the reference volume [42], and review of our data reveals no obvious difference in the size GCL between control and IR mice (data not shown). While a related concern is that we show cell number rather than cell density (thus directly taking into account the reference volume), we prefer the stereological approach used here. It allows sampling evenly throughout the entire longitudinal extent of the dentate gyrus, thus taking into account the heterogeneous nature of the dentate gyrus across the septal to temporal pole of the hippocampus [43]. While thorough assessment of the reference volume (e.g. via Cavalieri method) post-IR is an important measure for future studies [e.g. 44], the current data still support the conclusion that neurogenesis is transiently decreased post-IR.

Space radiation-induced decreases in hippocampal neurogenesis are often interpreted to mean decreased hippocampal-dependent function. This is understandable given the numerous correlative links between neurogenesis and hippocampal function. However, a caveat of the present work – and in fact all publications that assess the impact of space radiation on neurogenesis – is that they are correlative, not causative. Therefore, additional work is needed to specifically and inducibly alter adult hippocampal neurogenesis and assess the impact on space radiation-induced deficits in cognition. For example, recent work with neurogenic small molecules [45, 46] and inducible transgenic mouse lines that allow selective increase in adult-generated neurons [47, 48] might be employed to assess whether space radiation-induced deficits can be reversed or prevented by increased adult hippocampal neurogenesis.

A final caveat for the present work is the parameters selected. In order to compare our results with the existing literature and expand what is known about neurogenesis, we chose

common published parameters in mice. For example, we exposed 2–3 mon old mice to an acute dose of 1 Gy. While typical in the mouse HZE particle literature, this age is much younger than an equivalently aged astronaut (which would require 6mon+ old mice [49]), and this dose of HZE particles is much higher than the expected GCR HZE exposure on a trip to Mars (half of the total annual dose of 0.2 Gy at solar minimum and 0.1 at solar maximum; [1, 50]). Currently, most NSRL/BNL experiments in animals are now done with much lower doses (<0.5 Gy), and studies are starting to be published using mature mice that are astronaut-age equivalent at time of irradiation. One parameter that is more challenging to address in current ground-based space radiation experiments is dose rate. Here, we used a dose rate of 1Gy/min, which has been used in the literature but is much higher than the dose rate that may be experienced in transit to [1] or on the surface of Mars (~210 μ Gy/year outside of solar particle events [51]). Such mission-relevant dose rates are near impossible to employ on Earth (given the length of exposure needed and the high use of the facilities at NSRL/BNL). However, current studies with mice are using a range of doses [15] or fractionated exposure as reasonable compromises in order to allow researchers to help NASA estimate risk [12].

CONCLUSION

Here we show that ^{56}Fe particle exposure transiently increases the proportion of YFP+ adult-generated cells that are YFP+ radial glia Type-1 cells; ^{56}Fe particle exposure induces long-term cellular evidence of genomic instability; and there is a transient decrease in the number of adult-generated neurons. These data support the regenerative capacity of the adult SGZ after HZE particle exposure and encourage additional inquiry into the relationship between radial glia stem cells and cognitive function after HZE particle exposure.

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Abbreviations

LET	linear energy transfer
HZE	high-energy, high charge nuclei
GCR	galactic cosmic radiation
GFP	green fluorescent protein
YFP	yellow fluorescent protein
SGZ	subgranular zone
DG	dentate gyrus
BrdU	5-bromo-2'-deoxyuridine

DCX	doublecortin
NeuN	neuronal nuclei
IR	irradiation
BNL	Brookhaven National Laboratories

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Figure 1. ^{56}Fe particle exposure induces comparable deficits in proliferation immediately or within 1 day of irradiation, irrespective of energy
(A–B) Timeline of ^{56}Fe particle irradiation in Nestin-GFP **(A)** and Nestin-Cre/YFP **(B)** mice. Briefly, Nestin-Cre/YFP mice were given tamoxifen (TAM, arrow in **B**) to induce recombination in nestin-expressing stem/progenitor cells and their progeny. 60d later, Nestin-GFP and Nestin-Cre/YFP mice between 9–14 weeks of age were exposed to 0 Gy or 1 Gy ^{56}Fe (1000 or 300 MeV/n; grey lightning bolt). Mice were then given BrdU (arrow, **A**, **B**) either immediately (<5min) or 22h post-IR, and perfused (grey X) at an early time point (24h post-IR) in order to label 2h-old **(C)** or 22h-old **(D)** BrdU+ cells in Nestin-GFP mice and 2h-old **(E)** or 22h-old **(F)** BrdU+ cells in Nestin-Cre/YFP mice. *** $p < 0.001$, by two-tailed Student's t -test **(C)** or ** $p < 0.01$, *** $p < 0.001$, by Dunnett's post-hoc analysis following one-way ANOVA **(D–F)**. Mean ± SEM, $n = 2–6$ /group.

Figure 2. ^{56}Fe particle exposure induces cellular evidence of long-lasting genomic instability, but only induces transient deficits in adult neurogenesis

(A) Timeline of ^{56}Fe particle irradiation. Briefly, Nestin-Cre/YFP mice were administered TAM (arrow), exposed to 0 Gy or 1 Gy ^{56}Fe (grey lightning bolt, 1000 MeV/n) and were killed (grey X) at an intermediate (7d) or long time point (2mo) post-IR. (B) Representative photomicrograph of the dentate gyrus with 0 Gy ^{56}Fe 7d earlier and stained with DCX (red) and DAPI (blue). Arrow: DCX+ immature neuron soma, Bracket: DCX+ immature neuron dendrites extending into molecular layer. (C) Stereological assessment of DCX+ cells following 7d and 2mo post-IR. (D) Representative photomicrographs of the dentate gyrus with 1 Gy ^{56}Fe 7d earlier and stained with 53BP1 (red) and DAPI (blue). Arrowheads: 53BP1+ foci, a surrogate of DNA damage and genomic instability. (E) Stereological assessment of 53BP1+ foci following 7d and 2mo post-IR. * $p < 0.05$, *** $p < 0.001$, by Sidak's post-hoc analysis following two-way ANOVA (E); # $p = 0.09$, ** $p < 0.01$, by two-tailed Student's *t*-test (C). Mean $\pm$ SEM, $n = 3-7$ /group. Scale bars = (B) 50 and (D) 10 μm .

Figure 3. ^{56}Fe particle exposure decreases progenitor cell number without changing the number of radial glia-like cells

(A) Timeline of ^{56}Fe particle irradiation. Nestin-GFP mice were exposed to 0 Gy or 1 Gy ^{56}Fe (300 MeV/n; grey lightning bolt) and given BrdU 22h post-IR. Mice were killed at an early time point 2h later (24h post-IR), or at an intermediate (7d), or long time point (3mo) post-IR to label proliferating, differentiating, or surviving BrdU+ cells, respectively. (B–D) BrdU+ cell number after 0 or 1 Gy ^{56}Fe irradiation 24h (B, 2h-old BrdU+ cells), 7d (C, 6d-old BrdU+ cells), and 3mo post-IR (D, 3mo-old BrdU+ cells). (E) Representative photomicrographs of GFP+ Type-1 cells with radial glial morphology in 0 Gy (E) or 1 Gy (F) 24h post-IR labeled with antibodies against GFP (green), GFAP (red), and counterstained with DAPI (blue). Arrows: GFP+/GFAP+ radial cell bodies. (G) GFP+/GFAP+ cells with radial glial morphology in the SGZ at early, intermediate, and long time points post-IR. ** $p < 0.01$, *** $p < 0.001$, by two-tailed Student's t -test. (B–D) Mean $\pm$ SEM, $n = 4$ –8 mice/group. Scale bar (F) = 50 μm .

Figure 4. ⁵⁶Fe particle exposure transiently decreases neurogenic progenitors, but does not significantly change the proportion of YFP+ cells that are Type-1 radial glia-like cells (A) Timeline of ⁵⁶Fe particle irradiation. Nestin-Cre/YFP mice were administered TAM (arrow), exposed to 0 Gy or 1 Gy ⁵⁶Fe (300 MeV/n; grey lightning bolt), and perfused (grey X) at early (24h), intermediate (7d), or long time points (3mo) post-IR. (B–D) YFP+ cells in the SGZ at 24h (B, 60d post-TAM), 7d (C, 67d post-TAM), and 3mo (D, 150d post-TAM) post-IR. (E–F) Representative photomicrographs of YFP+ radial glia Type-1 cells with radial glial morphology (YFP, red; DAPI, blue) mice exposed to 0 Gy (E) or 1 Gy (F) ⁵⁶Fe particle radiation and collected 24h post-IR. Arrows: YFP+ radial glia Type-1 cells, enlarged in inset in (F). (G) Proportion of YFP+ SGZ cells with radial glial morphology. #*p*=0.06, **p*<0.01, by two-tailed Student's *t*-test. Mean±SEM, n=4–10 mice/group. Scale bar (E)=100 μm.