Title

More May Not be Better: Enhanced Spacecraft Shielding May Exacerbate Cognitive Decrements by Increasing Pion Exposures during Deep Space Exploration

Journal

Radiation Research, 201(2)

ISSN

0033-7587

Authors

Vozenin, Marie-Catherine

Alaghband, Yasaman

Drayson, Olivia GG

et al.

Publication Date

2024-02-01

DOI

10.1667/rade-23-00241.1.s1

Peer reviewed

RADIATION RESEARCH **201**, 93–103 (2024)
0033-7587/24 $15.00

DOI: 10.1667/RADE-23-00241.1.S1

More May Not be Better: Enhanced Spacecraft Shielding May Exacerbate Cognitive Decrements by Increasing Pion Exposures during Deep Space Exploration

Marie-Catherine Vozenin,[a,1] Yasaman Alaghband,[b] Olivia G. G. Drayson,[b] Filippo Piaget,[a] Ron Leavitt,[a] Barrett D. Allen,[b] Ngoc-Lien Doan,[b] Tigran Rostomyan,[c] Alberto Stabilini,[c] Davide Reggiani,[c] Wojciech Hajdas,[c] Eduardo G. Yukihara,[c] John W. Norbury,[d] Claude Bailat,[e] Laurent Desorgher,[e] Janet E. Baulch,[b] Charles L. Limoli[b,2]

[a] Laboratory of Radiation Oncology, Department of Radiation Oncology, Lausanne University Hospital and University of Lausanne, Switzerland; [b] Department of Radiation Oncology, University of California, Irvine, California 92697-2695; [c] Paul Scherrer Institute (PSI), Villigen, Switzerland; [d] NASA Langley Research Center, Hampton, Virginia 23666; [e] Institute of Radiation Physics, Lausanne University Hospital and University of Lausanne, Switzerland

Vozenin M-C, Alaghband Y, Drayson OGG, Piaget F, Leavitt R, Allen BD, Doan N-L, Rostomyan T, Stabilini A, Reggiani D, Hajdas W, Yukihara EG, Norbury JW, Bailat C, Desorgher L, Baulch JE, Limoli CL. More May Not be Better: Enhanced Spacecraft Shielding May Exacerbate Cognitive Decrements by Increasing Pion Exposures during Deep Space Exploration. Radiat Res. 201, 93–103 (2024).

The pervasiveness of deep space radiation remains a confounding factor for the transit of humans through our solar system. Spacecraft shielding both protects astronauts but also contributes to absorbed dose through galactic cosmic ray interactions that produce secondary particles. The resultant biological effects drop to a minimum for aluminum shielding around 20 g/cm^2 but increase with additional shielding. The present work evaluates for the first time, the impact of secondary pions on central nervous system functionality. The fractional pion dose emanating from thicker shielded spacecraft regions could contribute up to 10% of the total absorbed radiation dose. New results from the Paul Scherrer Institute have revealed that low dose exposures to 150 MeV positive and negative pions, akin to a Mars mission, result in significant, long-lasting cognitive impairments. These surprising findings emphasize the need to carefully evaluate shielding configurations to optimize safe exposure limits for astronauts during deep space travel.

INTRODUCTION

The space radiation environment includes highly energetic galactic cosmic rays (GCR) that must be shielded against to protect astronauts. In this context, many different mesons (two quarks) and baryons (three quarks) are produced by the interactions of GCR with spacecraft shielding materials. The lightest baryons are the protons and neutrons, which are produced abundantly. The lightest mesons are the pions (π), which are also produced abundantly. The importance of pions to absorbed dose in space radiation shielding studies was first illustrated by Aghara et al. (*1*), who showed that pion contributions to dose could be as large as 20%, depending on the shield thickness. Importantly, any contribution to absorbed dose (Gray, Gy) does not equate to dose equivalent (sieverts, Sv), which informs on biologic effect. Moreover, after pions are produced, they can continue to interact with other nucleons, producing more mesons, baryons and leptons. The pions also decay, producing an electromagnetic cascade of electrons, positrons, and photons. All of these secondary particles contribute to the 20% dose referred to above. The direct pion contribution to dose, ignoring the secondary particles, is lower and varies between 10–15% depending on the shield thickness (10% for 20 g/cm^2 Al, and 15% for 100 g/cm^2). More direct pions and secondary particles are produced as the shield thickness gets larger, so that from a radiation point of view, "more" is not "better". This theme of "more may not be better" was especially highlighted in the work of Slaba et al. (*2*), where it was shown that there is a minimum in the dose equivalent versus depth curve at approximately 20 g/cm^2, beyond which the dose equivalent keeps increasing, mainly due to nucleon-induced production of secondary neutrons. Recent modeling efforts, using space radiation transport codes, have demonstrated the crucial importance of including pion interactions for correctly predicting absorbed radiation dose compared to data collected on balloon flights (*3*, *4*) and the International Space Station (ISS) (*5*).

Health risks associated with deep space travel are multifaceted, and involve multiple stressors including microgravity, fluid shifts, isolation, sleep deprivation, and radiation exposure (*6*). The consequences of exposure to these individual and combined stressors on humans and animal

[1] Current address and contact information: Radiotherapy and Radiobiology Sector, Radiation Therapy Service, University Hospital of Geneva, Geneva, Switzerland; email: marie-catherine.vozenin@hcuge.ch.
[2] Corresponding author: Charles L. Limoli, Dept. of Radiation Oncology, University of California, Irvine, CA 92617-2695; email: climoli@uci.edu.

models have been studied extensively, and paramount among these are the adverse effects of radiation exposure on the brain (*6*, *7*). Rodent studies using single ion and mixed ion fields delivered at various space relevant doses, energies and dose rates have consistently uncovered persistent neurocognitive deficits and changes in mood behaviors that track with electrophysiological changes in neurotransmission, altered neuronal structure and elevated neuroinflammation (*8–15*). The implications of these findings suggest that radiation injury or the imprint of that injury on the brain is permanent, never resolving to basal levels of homeostatic signaling over study timeframes. More confounding is the evidence that central nervous system (CNS) changes induced by such low dose exposures ($\leq$500 mGy) transpire in the relative absence of cell loss. Much of what is known regarding the response of the brain to cranial irradiation has come from the radiotherapeutic management of brain tumors, where much higher radiation doses ($\sim$60 Gy) and different types of radiation are used and known to elicit cognitive dysfunction and necrosis (*16*). While the latter is not observed following simulated space radiation exposures, equivalent cognitive deficits have been reported routinely using various charged particle exposures at doses 120 times lower than those delivered in the clinic (*16*). These data highlight the need to protect astronauts from exposure to these deleterious space radiation fields.

Deep space radiation is composed of complex mixtures of charged particle types, mainly protons and helium nuclei, with heavier nuclei found within the isotropic field of GCR (*17*). To date, and given the high particle energies involved, complete protection by shielding is impractical if not impossible, and while shielding configurations and materials can be optimized to minimize radiation doses, certain levels of exposure are inevitable. Interaction of these particles with the spacecraft leads to nuclear fragmentation products, generating lighter ions, neutrons, photons, and other subatomic particles including positive ($\pi+$) and negative ($\pi-$) pions (*18*).

Nuclear transport codes developed by physicists at NASA and other agencies provide the means to calculate the dose contribution derived from these interactions, based on shielding design, composition and thickness, such that local radiation fields within the spacecraft can be estimated (*18*, *19*). With these approaches, a surprising revelation has emerged indicating that the relative pion contribution to the total radiation dose on a deep space mission can be $\sim$10% of the total absorbed dose or higher, equating to 15–25 mGy/year, based on a shielding thickness of 20–50 g/cm^2 Al. Detailed results are given in Table 1, which were calculated using the On-Line Tool for the Assessment of Radiation in Space (OLTARIS) (*20*, *21*). The input GCR spectrum was the 2010 solar minimum spectrum with slab geometry. Depending on mission duration and activities, total absorbed radiation doses are not expected to exceed 500 mGy for a round trip mission to Mars lasting around three years (*20*, *22*, *23*), placing the total fractional absorbed dose from pions at $\sim$50–75 mGy. While this is clearly an estimate, actual pion doses could vary substantially within the spacecraft, depending on occupant location in relation to specific materials and interior configurations.

TABLE 1
Predicted Pion Doses Based on Spacecraft Shielding Configuration and Pion Doses Utilized in This Study

Cumulative space radiation exposures			
Spacecraft shielding (g/cm^2 Al)	20	50	100
Total dose/day (mGy)	0.45	0.52	0.63
Total dose/year (mGy)	164	190	230
Fractional $\pi+$ dose (%)	5	7	8.1
Fractional $\pi-$ dose (%)	4	6	6.2
Annual total π dose (mGy)	15	25	32.9
3 Year total π dose (mGy)	45	75	99
Study pion exposures			
$\pi+$ Dose	42 mGy		
	160 mGy		
$\pi-$ Dose	160 mGy		

Furthermore, while absorbed dose is an absolute quantity, it does not reveal information referred to as dose equivalent, that considers the biological impact of an isodose that depends on the energy and mass of a given particle (*24*). These factors define the microdosimetric properties specific to each particle, that can be gauged by their stopping power in terms of energy loss per unit thickness, or in terms of track length defined by the linear energy transfer (LET; keV/μm) (*24*). Generally, most physical models that have been developed to address carcinogenic risk assessment, accurately predict that dose equivalents will increase with the LET of a particle, where higher LET values associated with more densely ionizing particles elicit more deleterious damage to target molecules in the cells and tissues that they traverse.

However, one of the more confounding features to emerge from recent CNS radiobiology studies has been the lack of correlation between radiation LET and the induced effects observed in an intact rodent brain. Functional CNS deficits measured more than one year after exposure to low dose and low dose rate space-relevant radiation paradigms indicate little reliance on radiation type or dose, with dose thresholds for observing specific decrements likely at or below 50 mGy (*9–12*, *15*). Thus, model-based expectations predicted that the consequences of pion exposures would resemble photon dose equivalents and have little impact on biological outcome based on their similar and relatively low-LET values ($\leq$2 keV/μm) (*17*, *24–26*). Here, we challenged that tenet and conducted the first series of rigorous studies designed to evaluate functional CNS outcomes following space-relevant pion exposures. Our findings yielded some unexpected if not remarkable revelations and provide robust evidence that space-relevant pion exposures of $\leq$ 60 mGy exhibit unusually high-relative biological effects (RBE). These data suggest that areas of increased shielding may present greater risks to astronauts for eliciting mission

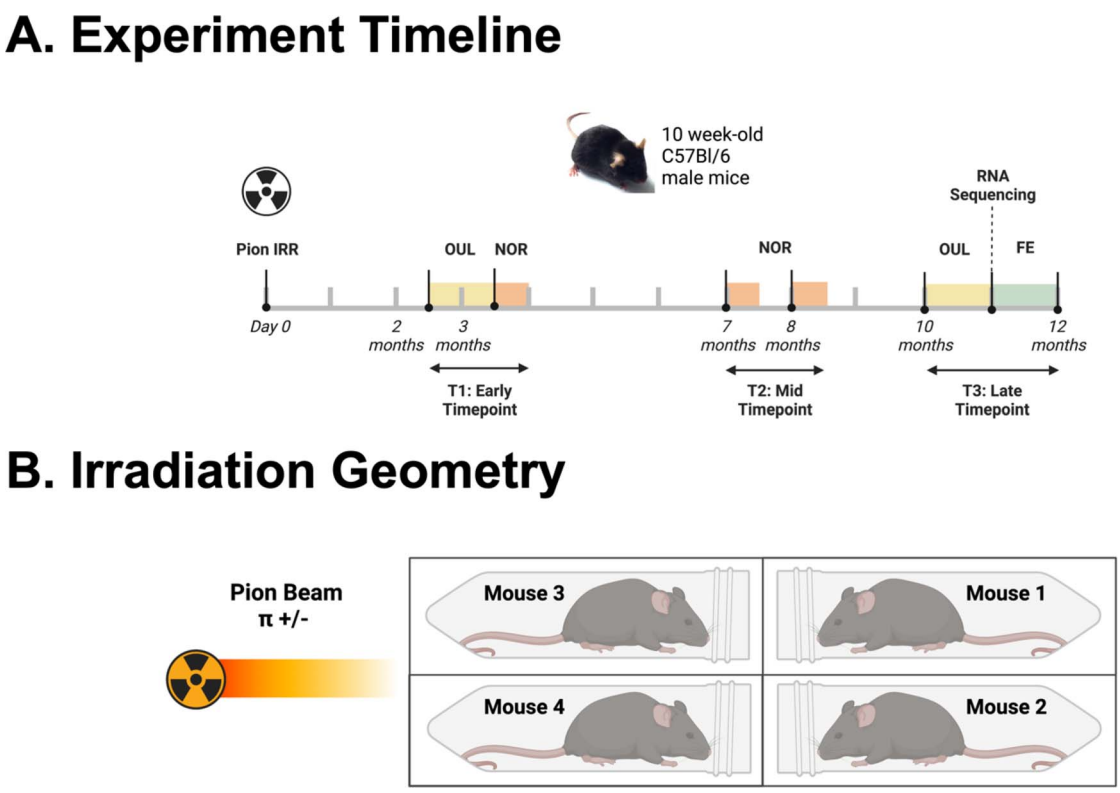

FIG. 1. Study design. Panel A: A single cohort of 48 wild-type male C57BL/6 mice were randomly divided into four experimental groups: sham irradiated controls, and mice irradiated using 42 mGy π+, 160 mGy π+ or π–. Within one-week postirradiation mice were shipped from PSI to UC Irvine. Animals were acclimated at least two months prior to behavior testing. Panel B: The irradiation geometry used for all mouse experimentation at the PSI is shown.

critical cognitive performance decrements. Thus, a need to consider the impact of pions more carefully on space radiation-induced CNS decrements and the internal shielding configurations of spacecraft seems warranted.

MATERIALS AND METHODS

Animals and Irradiations

Animal experiments were approved by the Swiss (VD3459) and University of California, Irvine ethics committees for animal experimentation and performed within institutional guidelines. A single cohort of wild-type C57Bl/6 male mice (Charles River Laboratory, France) were acclimated, and group housed at Paul Scherrer Institute (PSI) under standard conditions (20°C ± 1°C; 70% ± 10% humidity; 12 h:12 h light and dark cycle) and provided ad libitum access to food and water.

Mice received whole-body irradiation with 150 MeV positive (π+) or negative (π–) pions at absorbed doses of 40–45 mGy or 160–170 mGy using the PIM1 beam line at the PSI. Interaction of the high intensity (590 MeV) proton beam with a 2-mm graphite target generates secondary pions, muons, electrons, and protons. These particles are then guided to the target area where dipole magnets are used to select for the desired pion momentum and quadrapole magnets are used to focus the charged beam depending on magnet polarity (*27*, *28*). The configuration used for the calibration measurement and the mouse irradiation is shown [Fig. 1B (*29*)]. The beam was characterized by a plastic scintillator (PIL detector) set on a X-Y motorized stage. We measured the contamination level of the beam by muons and electrons using a time-of-flight analysis of the PIL detector signal relative to a pulsed signal with a 20 ns cycle, synchronized with the main proton beam. Delivered absorbed doses were verified using thermoluminescence dosimeters and optically stimulated luminescence detectors, as described by Desorgher et al. (*29*).

Cognitive Testing

To determine the effects of pion exposure on cognitive function, mice were subjected to behavioral testing (Fig. 1). The object in updated location (OUL) task was performed at early (2–3 month) and late (10–11 month) postirradiation. Novel object recognition (NOR) was conducted at early (3–4 month) and mid (7–8 month) postirradiation. Fear extinction (FE) testing, the most invasive behavioral test, was the final task performed at 11 months postirradiation. Except for the mid-timepoint NOR task, all mice from all four experimental groups were tested concurrently (i.e., control, 42 mGy π+, 160 mGy π+, π–). Data analyses were conducted independently and blindly and are presented as the average of all trials scored for each task. All behavioral testing was conducted following previously published and carefully controlled protocols (*30*).

RNAseq Analysis

RNA was isolated from micro-dissected hippocampi from four mice in each group at 11 months postirradiation and samples processed at GTF/UNIL. Raw FASTQ files were uploaded to the European Galaxy server [Galaxy | Europe (usegalaxy.eu)] for further manipulation and processing. Read quality was assessed using FastQC [version 0.73 + galaxy0 (*31*)]. The RNA STAR aligner [version 2.7.8a + galaxy0 (*32*)] was used to align the reads to mouse genome (mm10). Binary alignment (BAM) files from all sequencing lanes for each sample were merged at this point using the Samtools merge tool (version 1.13). These merged alignments were then counted for annotated genes using featureCounts in Galaxy [version 2.0.1 + galaxy1 (*33*)]. Raw count tables were imported back into RStudio for differential gene expression analysis using the DESeq2

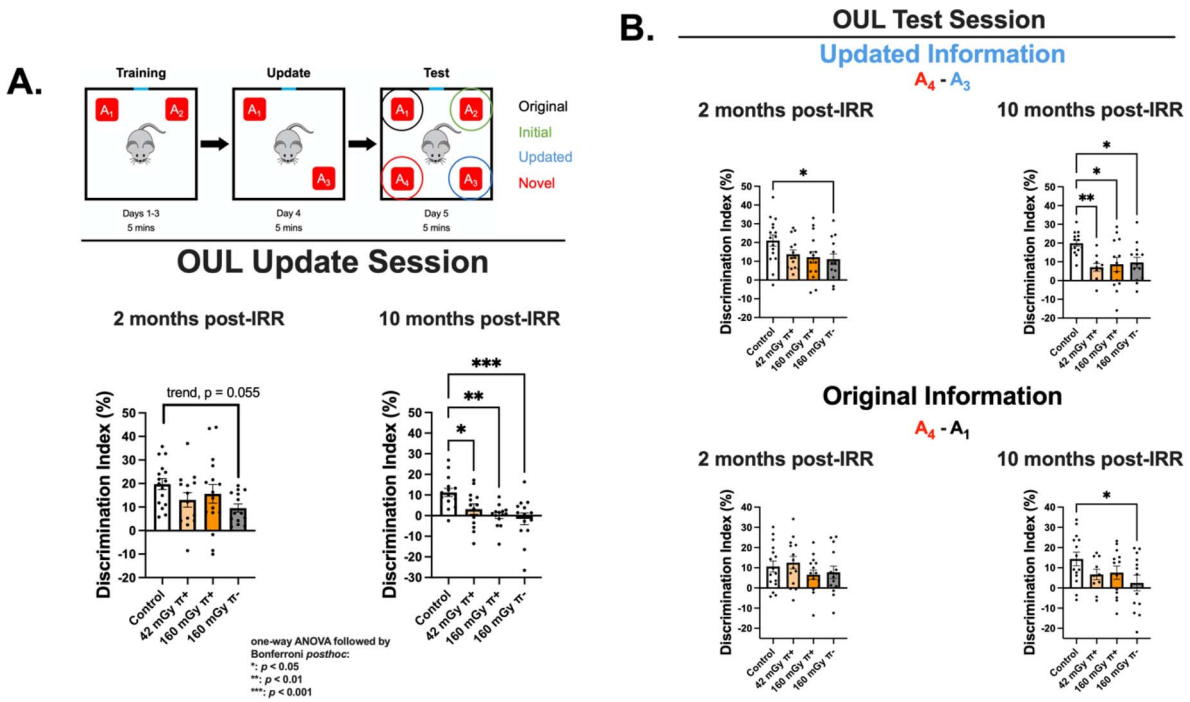

FIG. 2. Pion exposure elicits impairments in memory formation and updating. Panel A: While mice exposed to pions exhibited discrimination indices similar to that of controls during the update session at two months postirradiation (A_3 updated vs. A_1 fixed location; left panel), at 10 months postirradiation all irradiated mice were impaired, demonstrating no preference for the object in the updated location as compared to the fixed location object (right panel). Panel B: During the early time point test session, 160 mGy π– irradiated male mice were impaired on the memory of the updated location (A_3) relative to the novel location (A_4), while all irradiated mice retained the original information (A_4 vs. A_1) (upper and lower left panels). At 10 months postirradiation, all pion-exposed mice were impaired on the memory of the updated location relative to the novel location (A_3 vs. A_4), and the 160 mGy π– irradiated mice were also impaired in recall of the original information (A_4 vs. A_1) (upper and lower right panels). Data are mean ± SEM (N = 13–16 per group); P values derived from one-way ANOVA followed by a Bonferroni's multiple comparisons test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

(version 1.30.1) packages (*34*, *35*). The principal component analysis (PCA) plot was generated using the ggplots2 package [version 3.3.5 (*35*)] and the heatmap was generated by the pheatmap package [version 1.0.12 (*36*)].

Statistical Analyses

Statistical analyses for behavioral testing were carried out using GraphPad Prism (v8) software. Analyses for the OUL and NOR utilized one-way ANOVA to assess significance between control and irradiated groups, and when an overall group effect was found to be statistically significant, a Bonferroni's post hoc test was used to compare irradiated groups against the control group. An outlier was defined as a mouse whose behavior was outside of two standard deviations of the mean. Unless stated otherwise, behavior data were expressed as mean ± SEM and all analyses considered a value of $P < 0.05$ to be statistically significant.

For the FE test, conditioning Day 1 (T_1–T_3) and extinction training days were analyzed using two-way ANOVA followed by the Bonferroni's post hoc test with the percent time spent freezing as within-subjects variables and radiation treatment vs. control. This statistical test was used to make specific comparisons when significant interactions and/or main group effects were observed.

For RNAseq analysis, all adjusted P values had FDR correction applied using the Benjamini-Hochberg procedure (*37*).

RESULTS

Pion Irradiation

Dosimetric characterization of the PIM1 beam line used for these studies has been described elsewhere (*29*). Space radiation parameters relevant to this study are detailed in Table 1. Mice were then evaluated behaviorally and molecularly over the subsequent year. The irradiation geometry and experimental timeline are shown (Fig. 1) with the timing of the OUL, NOR, and FE tests identified.

Behavioral Testing. Radiation effects in the brain, including space radiations, take time to manifest, and to capture the development of these projected deficits it was necessary to conduct a long-term longitudinal behavioral assessment. Behavioral testing was conducted at early (2–4 month), mid (7–8 month) and late (10–12 month) postirradiation. Testing was initiated with the OUL task, subsequent testing involved the NOR task and finished with a FE test (Fig. 1A).

At the early and late postirradiation times mice underwent OUL testing (Fig. 2A), and the discrimination index (DI) was scored. This task uses a memory updating paradigm that assesses both the original memory and the updated information in a single test session (*38*, *39*). Further, the OUL task uses incidental learning that takes advantage of the innate preference of rodents for novelty. After an initial habituation to the arena, mice learned the locations of two identical objects in the fixed A_1 and the initial A_2 locations of the arena during training sessions on Days 1–3. During the following Day 4 update session, each mouse was exposed to the familiar A_1 fixed object location

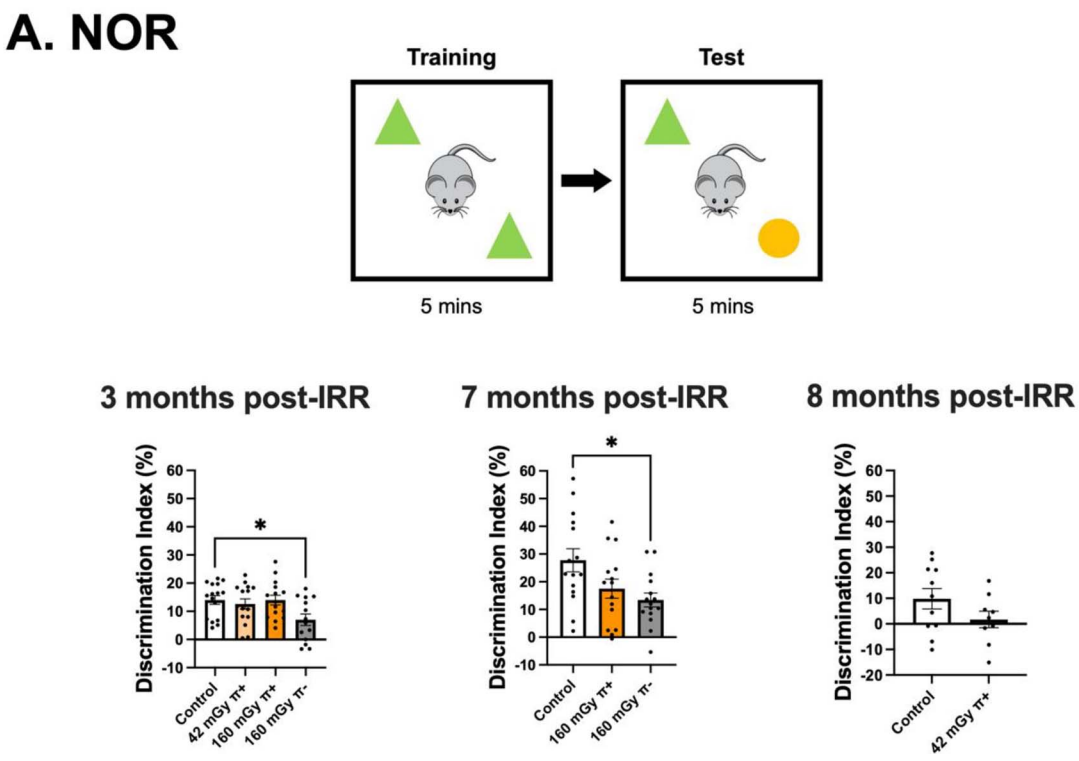

FIG. 3A. Pion exposures induces memory impairments and extinction. At the early three month postirradiation time, NOR testing indicated that 160 mGy π exposed mice had reduced discrimination index scores relative to controls, indicating no preference for the novel object (left panel). At the seven month mid time point only the 160 mGyπ – irradiated mice were impaired on NOR (center panel), while at month 8, mice exposed to 42 mGy π + exhibited a reduced DI that did not reach significance (right panel). These data are the mean ± SEM (N = 14–16 mice/group); P values derived from one-way ANOVA followed by a Bonferroni's multiple comparisons test. *$P < 0.05$.

and one identical object moved to a new updated A_3 location. Control animals were shown to have successfully acquired the original object location memory (OLM) during this update session, recognizing the A_3 location as novel. While not significant, all groups of irradiated male mice showed similar interest in the novel place as illustrated by their discrimination indices (DI; see Methods section) (Fig. 2A left panel; one-way ANOVA: $F_{(3,56)} = 2.11$; $P = 0.109$).

Interestingly, when this test was re-administered to mice eight months later, all irradiated cohorts exhibited significant decrements on the recognition of novelty. During the Day 4 update session, all four groups of irradiated mice were impaired in acquiring OLM as measured by DI for recognition of the novel A_3 object location as compared to the fixed A_1 object location (Fig. 2A right panel; one-way ANOVA $F_{(3,52)} = 6.87$; $P = 0.0006$; post hoc comparison of control vs. 42 mGy π+, 160 mGy π+ and 160 mGy π–, $P = 0.041$, $P = 0.0019$ and $P = 0.0004$, respectively).

One distinct advantage of the OUL task is the capability to increase cognitive load, by challenging mice to discriminate between multiple overlapping associative memories. This in fact may well be more representative of the multitasking activities asked of astronauts, providing more translationally relevant behavioral outcomes. During the training and update phases of the OUL task, and during the subsequent Day 5 test session, memory for the updated information was examined via comparison between exploration of the object in the novel A_4 location to exploration of the fixed A_1 location and the updated A_3 location. Cognitively intact mice exhibit preferential exploration of the object in the novel A_4 location compared to each of the other objects and is reflected by a higher DI score on this task.

At the early postirradiation time, most of the irradiated mice exhibited only a trend for a group effect in differentiation of the updated A_3 location object relative to the novel A_4 location relative to controls. Impairments however, were found to be significant for the 160 mGy π– irradiated mice (Fig. 2B upper left panel; one-way ANOVA: $F_{(3,55)} = 2.64$, $P = 0.058$; post hoc comparison of control vs. 160 mGy π–, $P = 0.046$). All irradiated mice retained a preference similar to control mice for the object in the A_4 location relative to the fixed A_1 location object (Fig. 2B lower left panel; one-way ANOVA: $F_{(3,55)} = 9.26$; $P = 0.43$).

Irradiated mice re-tested eight months later were again found to exhibit more extensive decrements on the test phases of this task. During the subsequent Day 5 test session at this late postirradiation time point, all irradiated mice exhibited a significantly impaired ability to differentiate between the updated A_3 location object relative to the

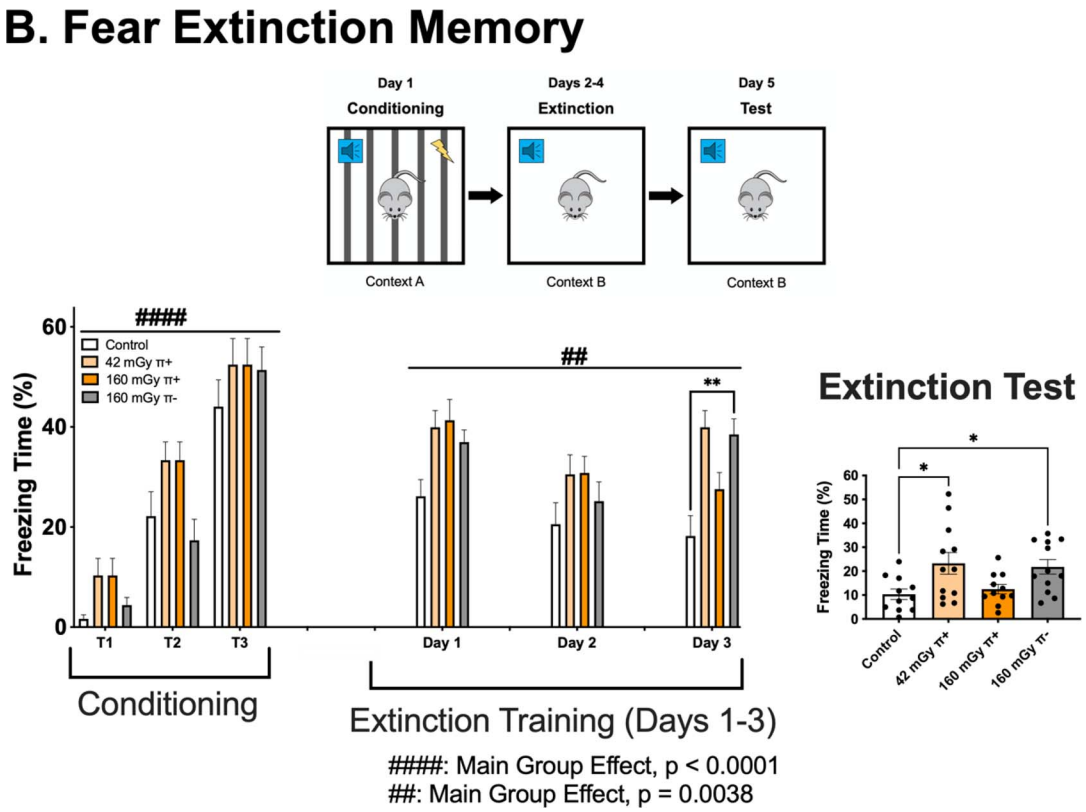

FIG. 3B. Pion exposures induces impairments in extinction memory. Exposure of mice to pions did not impair the acquisition of conditioned fear memories on FE testing, evaluated at 11 months postirradiation, however a significant main group effect was observed (T_1–T_3, tone-shock pairings, left panel). The time spent freezing over the extinction sessions on Days 1 and 2 were similar across groups, but on Day 3 mice exposed to 160 mGy π+ exhibited increased freezing relative to controls (tone only, center panel). In this case a significant main group effect was also observed. During the Fear Extinction Test the 42 mGy π+ and 160 mGy π– exposed groups were unable to abolish fear memories (right panel). These data are mean ± SEM (N = 11–12 mice per group); P values derived from two-way repeated measures ANOVA followed by the Bonferroni's multiple comparisons test. *P < 0.05, **P < 0.01, ##P < 0.01, ###P < 0.0001.

novel A_4 location compared to controls (Fig. 2B upper right panel; one-way ANOVA: $F_{(3,46)} = 4.79$, P = 0.0055; post hoc comparison of control vs. 42 mGy π+, 160 mGy π+ and 160 mGy π–, P = 0.0071, P = 0.012 and P = 0.024, respectively). Interestingly, when updated memory was again examined via comparison between exploration of the object in the novel A_4 location to exploration of the fixed A_1 location and the updated A_3 location, only the 160 mGy π– irradiated mice exhibited significant impairments in discrimination between the novel A_4 and fixed A_1 locations relative to control mice (Fig. 2B lower right panel; one-way ANOVA: $F_{(3,46)} = 2.24$; P = 0.096; post hoc comparison of control vs. 160 mGy π–, P = 0.043).

Immediately after the completion of the early postirradiation time point OUL task all four groups of mice were tested on the NOR test which depends on both the hippocampus and perirhinal cortex to test the mouse's ability to discriminate novelty (*40*, *41*). In the NOR task, an overall group effect was observed where the irradiated mice were impaired in their ability to discriminate the novel object compared to controls, again reaching significance for only the 160 mGy π– irradiated mice (Fig. 3A left panel; ANOVA: $F_{(3,56)} = 3.39$; P = 0.024; post hoc comparison of control vs. 160 mGy π–, P = 0.021).

To evaluate the temporal progression of deficits on this task at a mid postirradiation time, we split the testing regimen between two times, one at seven months for the higher dose of 160 mGy, and another at eight months for the lower dose of 42 mGy, to allow more time for the manifestation of latent impairments. For the 160 mGy cohort re-tested on the NOR task at seven months postirradiation a new set of objects was used. In each instance, only the 160 mGy π– irradiated mice exhibited significant impairments relative to controls (Fig. 3A center panel; one-way ANOVA: $F_{(2,42)} = 4.59$; P = 0.016; post hoc comparison of control vs. 160 mGy π+, P = 0.083; control vs. 160 mGy π–, P = 0.011). One month later, similar testing conducted for the 42 mGy π+ mice vs. controls showed trends towards impairments but did not reach significance (Fig. 3A right panel).

The inability to actively process dissociated learned responses to prior adverse events, similar to a post-traumatic stress-like behavior, can be detrimental to long term CNS function (*42*, *43*). Further, to examine the impact of pion exposures on non-exploratory behavior, we also

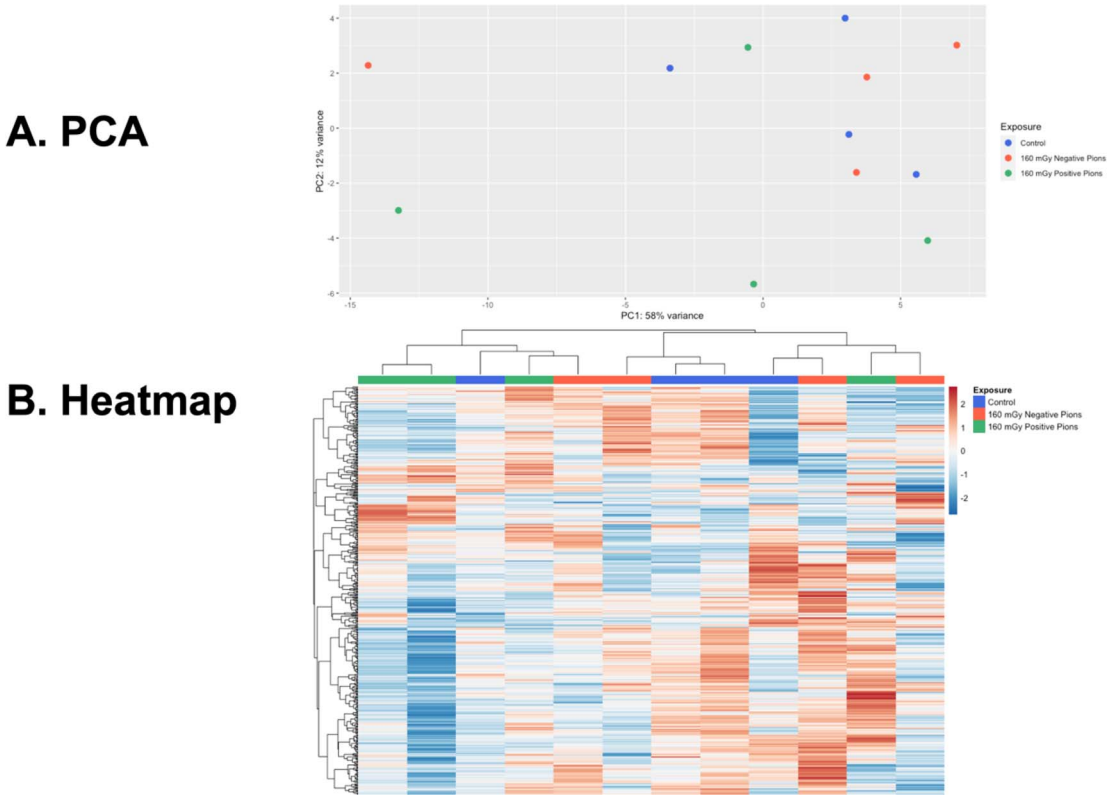

FIG. 4. RNAseq analysis. Panel A: PCA was used to visualize sample-to-sample distances and group clustering based on the top two principal components (PC1 and PC2), with PC1 accounting for 58% of the total variance seen between samples. Panel B: Unsupervised heatmap for the top 500 genes was used to visualize and identify gene clusters and association with exposure groups - columns (samples) and rows (genes, Z-score) clustered hierarchically.

examined how the extinction of fear memory might be altered by pion exposure (Fig. 3B). At 11 months postirradiation, mice were subjected to a rigorous protocol designed to elucidate whether irradiated mice could extinguish the learned behavior of associating a tone with a mild foot shock, an indicator of memory consolidation (*43*). On the Day 1 conditioning phase, all groups were given three tone-shock pairings and no interaction or main group effect of treatment was observed, indicating that all mice learned the association irrespective of radiation exposure (Fig. 3B left panel; T1-T3; two-way ANOVA: $F_{(2,\ 132)} = 1.15$, $P < 0.0001$). During the subsequent Day 1–3 fear extinction trials in a new context, a main treatment group effect was observed for the irradiated mice relative to the control with the 160 mGy π– mice exhibiting significant impairments relative to controls on Day 3 (Fig. 3B center panel; Day 1–3; two-way ANOVA: $F_{(2,\ 132)} = 5.81$, $P = 0.0038$; post hoc comparison of control vs. 160 mGy π– on Day 3, $P = 0.004$). On the final day, an extinction test was given in which only three tones were administered at 2 min intervals. Differences based on pion exposure were observed relative to controls (Fig. 3B right panel; one-way ANOVA: $F_{(3,\ 42)} = 4.19$, $P = 0.011$). Post hoc comparison of the irradiated mice revealed significant differences among groups (control vs. 42 mGy π+ and 160 mGy π–, $P = 0.019$ and 0.044, respectively).

Gene Profiling

To explore the molecular impact of the higher 160 mGy π– and π+ exposures against controls and each other, whole brain samples were isolated at the conclusion of cognitive testing (∼1-year postirradiation) for RNA sequence analysis. The PCA showed that the control group (blue) clustered well, as expected (Fig. 4A). However, samples exposed to π– (red) and π+ (green) were more dispersed, with singular samples from each group that did not cluster with the others. The heat map did not enable any clear clustering amongst groups (Fig. 4B). While the limited number and protracted nature of the sample collection precluded a robust statistical analysis, the difficulty of obtaining replicate cohorts justified our gene profiling approach in efforts to establish potential molecular links to our strong functional results.

Several cross-analyses were subsequently performed comparing the transcriptomic profiles obtained from the brains of animals exposed to π– and π+ vs. control and π– vs. π+. We report in Table 2 the top genes that reach an adjusted P value (Padj) < 0.09 for 160 mGy π– vs. 160 mGy π+ and Padj < 0.9 for 160 mGy π– vs. control and 160 mGy π+ vs. control. Note that the data in Table 2 have been sorted by their false discovery rate (FDR) Padj value.

TABLE 2
A. The Transcriptomic Profile Obtained in the Brain of Animals Exposed to 160 mGy Negative Pions (π–) vs. Control. B. 160 mGy Negative Pions (π–) vs. 160 mGy Positive Pions (π+). C. 160 mGy Positive Pions (π+) vs. Control

ENTREZID	Gene	Stat	*P* value	Padj
A. 160 mGy negative pions (π–) vs. control				
14735	**Gpc4**	–4.18	2.87E-05	0.314
224792	**Adgrf5**	4.11	3.88E-05	0.314
13876	Erg	3.96	7.48E-05	0.404
14268	Fn1	3.82	1.35E-04	0.438
224093	Fam43a	3.82	1.34E-04	0.438
140792	Colec12	3.72	2.02E-04	0.546
14254	Flt1	3.52	4.32E-04	0.875
67425	Eps8l1	–3.53	4.09E-04	0.875
17116	Mab21l1	3.48	5.07E-04	0.906
237400	Mex3d	–3.44	5.92E-04	0.906
B. 160 mGy negative pions (π–) vs. 160 mGy positive pions (π+)				
12227	**Btg2**	–5.07	3.95E-07	0.003
70615	Ankrd24	5.06	4.25E-07	0.003
19941	Rpl26	–4.7	2.58E-06	0.012
13123	Cyp7b1	–4.63	3.62E-06	0.013
22658	Pcgf2	4.57	4.98E-06	0.014
14268	Fn1	4.51	6.58E-06	0.016
224792	**Adgrf5**	4.47	7.70E-06	0.016
620678	Gm12174	–4.39	1.15E-05	0.021
13527	Dtna	–4.23	2.32E-05	0.032
17116	Mab21l1	4.22	2.49E-05	0.032
75744	Svip	–4.24	2.24E-05	0.032
11838	**Arc**	–4.09	4.29E-05	0.047
C. 160 mGy positive pions (π+) vs. control				
327747	Mettl24	–4.22468	2.39E-05	0.388
100502895	Unknown	3.8741	1.07E-04	0.578
18760	Prkd1	3.91595	9.00E-05	0.578
71753	Tmprss6	3.68515	2.29E-04	0.926

Notes. The boldfaced genes are possible candidates, selected through literature search, to explain the effect of pion exposure shown by cognitive and behavior testing. In red if over-expressed, whereas in blue if under-expressed. Genes are sorted by their FDR-adjusted *Padj* value.

DISCUSSION

NASA has funded ground-based radiobiology studies in efforts to both understand and develop strategies to mitigate he potential adverse health effects of space radiation exposure. Findings from numerous investigations have convincingly demonstrated that the risks of cognitive impairments resulting from such exposures are significant (*6*). While scaling and translating rodent-based studies to human flight scenarios remains a challenge, current findings add to the growing body of literature suggesting that traditional mitigation efforts involving spacecraft shielding may need to be re-evaluated. NASA clearly wants to minimize the risk for unexpected or adverse radiation-induced health effects during any mission scenario and has funded space radiobiology studies to model those risks with the goal of protecting against catastrophic surprises. While current findings increase our knowledge of CNS risks from pion exposures, they should not be construed as overly alarming. Nonetheless, they do highlight gaps in knowledge, and the need to reconsider the effects of pion exposures that will depend on the configuration of internally shielded regions of spacecraft.

Striking was the observation that exposure to both positive and negative pions caused significant cognitive decrements that increased over an extended postirradiation time. The observations obtained collectively from the OUL and NOR open arena behavioral tasks at early and later postirradiation times suggest that pion exposures initially induced more subtle impairments in cortical and hippocampal-based learning and memory. The intermediate NOR testing at 7–8 months postirradiation was implemented to assess the progression of pion-induced decrements and suggested that longer term follow up might reveal enhanced impairments. Interestingly, and as demonstrated in previous studies, later postirradiation times revealed increased cognitive deficits, effects that could adversely impact astronaut performance on a long term, deep space Mars mission. While animals exposed to 160 mGy negative pions generally showed earlier onset and more robust deficits, animals exposed to doses of both 42 mGy and 160 mGy positive pions also exhibited significant impairments, arguing that localized energy deposition from spallation products and the possibility of resultant cell kill is not a plausible explanation for our collective results. It was interesting and uncertain why fear memories were abolished at the higher positive pion dose of 160 mGy as opposed to the 42 mGy positive and 160 mGy negative pion exposures. Different cross-sectional interactions between pions and select circuitry in the brain can elicit ionizations in critical synaptic elements mediating feedback between the prefrontal cortex and the amygdala that could lead to offsetting changes in extinction memory. Importantly, the inability to extinguish fear memories one year after exposure to 42 and 160 mGy of positive and negative pions respectively, portends potential problems for astronauts, and underscores certain uncertainties in our predictive ability to estimate space radiation risks and behavioral outcomes for the multifaceted CNS space radiation response.

A direct RBE estimation is confounded in our study by the lack of directly comparative photon studies under similar conditions. However, if we consider that the functional decline in cognition observed here with pions was equivalent to past studies performed with x-ray doses from 8,000–20,000 mGy (*44–46*), RBE can be calculated to range between 50 and 475. This is unrealistically high and argues that RBE comparisons for functional CNS endpoints are relatively uninformative and calls to question concepts of target theory on which microdosimetry and fluence-based models are based. While current data indicate that higher pion doses caused more significant deficits than lower doses, additional

work will need to be undertaken to further define possible dose thresholds for pion-induced cognitive decrements. Nonetheless, our findings suggest a previously unrecognized possibility that certain exposures to both positive and negative pions do pose an added element of risk from long-term spaceflight. In many respects, the preponderance of data suggest that the whole brain may define the critical target for space radiation-induced cognitive decline and indicates the need to further evaluate the mechanistic basis of these outcomes.

To address the foregoing, RNAseq was initiated after the cessation of behavioral testing, to glean possible pathways that altered network level connectivity. Despite some inherent limitations, RNA profiling identified specific alterations in gene expression including two receptors GPC4, ADGRF5, and two regulators of synaptic function BTG2 and ARC, each, or all of which could play a role in pion-induced neurocognitive decrements. Our results show a decrement in Gpc4 gene expression, a receptor belonging to glypicans family, known to regulate the postsynaptic expression levels of ionotropic glutamate receptors (iGluRs), controlling the electrophysiological properties of synapses. Its dysfunction could lead to failures in neuronal network formation, malfunction of synapses, and abnormal behaviors (*47*). ADGRF5 codes for a G protein-coupled receptor known to regulate blood-brain barrier (BBB) development and maintenance. Its relevance in CNS function and response to damages is therefore essential (*48*). Our results show that π– exposure increased the expression of this gene, suggesting they could damage the BBB. Studies on btg2 expression have demonstrated that the protein it encodes acts as a transcription co-regulator able to enhance or inhibit the activity of transcription factors, controlling cell cycle progression, pro-neural genes expression and neuronal differentiation (*49*). Depletion of BTG2 interferes with the formation of contextual memories (*49*) and suggests that reduced expression btg2 after π– exposure, might compromise behavioral performance. Interestingly, RNA-seq data showed decreased expression of activity regulated cytoskeletal-associated protein (arc) after π– exposure. Since arc is a member of the immediate-early gene family that plays a fundamental role in the stabilization of activity-dependent hippocampal plasticity, reduced levels of arc may adversely impact learning and memory-related molecular processes (*50*) in the pion irradiated brain.

Present studies were initiated to address the unknown dose equivalent of pion exposures to the brain, with relevance in the context of deep space exploration. Dose selection was based on both practicalities of extracting positive and negative pions from the PIM1 beam line along with providing NASA data based on estimated pion exposures calculated from OLTARIS. Thus, the absorbed dose of 42 mGy was selected based on estimates that placed the total pion absorbed dose at ~50 mGy for a round trip to Mars. However, the focus of this study was also to provide NASA with an upper bound to a possible "worst case scenario" for how a higher dose of pions might contribute to potential neurological impairments. Nonetheless, higher pion doses clearly emanate from thicker regions of shielding, so given uncertainties in dose estimation, we evaluated the consequences of 160 mGy exposures.

For the estimation of CNS risks, past work has emphasized caution against over-reliance on a single behavioral task, the need to engage rodents on more demanding tasks and stressed the need for more cross-species relevant behavioral paradigms to facilitate translation to human activities. Here we addressed each of these points by undertaking a rigorous longitudinal assessment of mice on multiple behavioral tasks, including the OUL and FE tasks that exhibit strong parallels between rodents and humans. While rodent testing outcomes and extrapolation to human performance metrics during space travel will always remain an imperfect science, it remains difficult to dismiss the implications of these data. Numerous in-depth literature reviews have evaluated the risks of combined spaceflight stressors and have correctly concluded that the risk from radiation is both the most problematic and uncertain (*6*, *7*, *51*), owing in large part to the small number of astronauts exposed to the deep space radiation environment.

Terrestrial experiences involving radiation exposure to the brain, either through accidental, occupational, or medical routes are poor surrogates of the exposures encountered in space, and as such, definitive biomarkers, cellular/structural targets, and mechanisms of action able to account for the functional decline in neurological health observed from space radiation exposure remain incompletely understood. In terms of astronaut relevant behaviors, data derived from the OUL task are likely to be more relevant. The OUL task elevates task rigor by analyzing multiple associative memory traces in similar but distinct ways from other testing paradigms such as the Attentional Set Shifting (ATSET) and Associative Recognition Memory and Interference Touchscreen (ARMIT) tasks implemented by other groups (*14*, *52*). The foregoing platforms can scrutinize radiation-induced behavioral outcomes under elevated cognitive load and indicate that, should an astronaut be faced with an unexpected situation requiring on the spot problem solving, they may be at increased risk for manifesting mission critical performance decrements. Nonetheless, until the number of humans returning from deep space travel increases, we will be reliant on animal models and terrestrial space simulations to provide reasonable limits to the uncertainties associated with radiation exposure in space. Notwithstanding, current data sets do indicate that we have much to learn and that further surprises are likely as we delve into the expanse of space, with ground-based efforts focused on averting what is reasonably possible.

ACKNOWLEDGMENTS

The authors would like to thank the collaborators from PSI who helped to make this study possible: Cristina Müller and Susan Cohrs for their assistance with animal housing and logistics; Radoslaw Marcin Marcinkowski and

Patryk Socha for help operating the beam. We are very grateful to Drs. Tony Slaba and Charles Werneth (NASA Langley Research Center) for reviewing the manuscript prior to journal submission.

This work was supported by the NASA Specialized Center of Research (NSCOR) grant NNX15AI22G (CLL, JEB) and by a generous beamtime allotment from the PSI. JWN was supported by the RadWorks project within Exploration Capabilities of the Mars Campaign Development Division in the Exploration Systems Development Mission Directorate of NASA. Authors declare that they have no competing interests. Where applicable, data and materials used in this manuscript will be made available upon request.

Received: November 23, 2023; accepted: December 15, 2023; published online: January 4, 2024

REFERENCES

1. Aghara SK, Blattnig SR, Norbury JW, Singleterry RC, Monte Carlo analysis of pion contribution to absorbed dose from Galactic cosmic rays. Nuclear Instruments & Methods in Physics Research Section B-Beam Interactions with Materials and Atoms 2009; 267, 1115-24.
2. Slaba TC, Bahadori AA, Reddell BD, Singleterry RC, Clowdsley MS, Blattnig SR, Optimal shielding thickness for galactic cosmic ray environments. Life Sci Space Res (Amst) 2017; 12, 1-15.
3. Norman RB, Blattnig SR, De Angelis G, Badavi FF, Norbury JW, Deterministic pion and muon transport in Earth's atmosphere. Adv Space Res 2012; 50, 146-55.
4. Norman RB, Slaba TC, Blattnig SR, An extension of HZTERN for cosmic ray initiated electromagnetic cascades. Adv Space Res 2013; 51, 2251-60.
5. Slaba TC, Wilson JW, Werneth CM, Whitman K, Updated deterministic radiation transport for future deep space missions. Life Sci Space Res (Amst) 2020; 27, 6-18.
6. Desai RI, Limoli CL, Stark CEL, Stark SM, Impact of spaceflight stressors on behavior and cognition: A molecular, neurochemical, and neurobiological perspective. Neurosci Biobehav Rev 2022; 138, 104676.
7. Britten RA, Wellman LL, Sanford LD, Progressive increase in the complexity and translatability of rodent testing to assess space-radiation induced cognitive impairment. Neurosci Biobehav Rev 2021; 126, 159-74.
8. Parihar VK, Allen B, Tran KK, Macaraeg TG, Chu EM, Kwok SF, et al., What happens to your brain on the way to Mars. Sci Adv 2015; 1.
9. Parihar VK, Allen BD, Caressi C, Kwok S, Chu E, Tran KK, et al., Cosmic radiation exposure and persistent cognitive dysfunction. Sci Rep 2016; 6, 34774.
10. Parihar VK, Maroso M, Syage A, Allen BD, Angulo MC, Soltesz I, et al., Persistent nature of alterations in cognition and neuronal circuit excitability after exposure to simulated cosmic radiation in mice. Exp Neurol 2018; 305, 44-55.
11. Acharya MM, Baulch JE, Klein PM, Baddour AAD, Apodaca LA, Kramar EA, et al., New concerns for neurocognitive function during deep space exposures to chronic, low dose-rate, neutron radiation. eNeuro 2019; 6.
12. Klein PM, Parihar VK, Szabo GG, Zoldi M, Angulo MC, Allen BD, et al., Detrimental impacts of mixed-ion radiation on nervous system function. Neurobiology of disease 2021; 151, 105252.
13. Kiffer FC, Luitel K, Tran FH, Patel RA, Guzman CS, Soler I, et al., Effects of a 33-ion sequential beam galactic cosmic ray analog on male mouse behavior and evaluation of CDDO-EA as a radiation countermeasure. Behav Brain Res 2022; 419, 113677.
14. Britten RA, Fesshaye A, Ihle P, Wheeler A, Baulch JE, Limoli CL, et al., Dissecting Differential Complex Behavioral Responses to Simulated Space Radiation Exposures. Radiat Res 2022; 197, 289-97.
15. Alaghband Y, Klein PM, Kramar EA, Cranston MN, Perry BC, Shelerud LM, et al., Galactic cosmic radiation exposure causes multifaceted neurocognitive impairments. Cell Mol Life Sci 2023; 80, 29.
16. Limoli C, Can a comparison of clinical and deep space irradiation scenarios shed light on the radiation response of the brain? Br J Radiol 2020; 93, 20200245.
17. Nelson GA, Space radiation and human exposures, a primer. Radiat Res 2016; 185, 349-58.
18. Norbury JW, Slaba TC, Aghara S, Badavi FF, Blattnig SR, Clowdsley MS, et al., Advances in space radiation physics and transport at NASA. Life Sci Space Res (Amst) 2019; 22, 98-124.
19. Norbury JW, Sobolevsky N, Werneth CM, SHIELD and HZETRN comparisons of pion production cross sections. Nuclear Instruments & Methods in Physics Research Section B-Beam Interactions with Materials and Atoms 2018; 418, 13-17.
20. Ehresmann B, Hassler DM, Zeitlin C, Guo J, Kohler J, Wimmer-Schweingruber RF, et al., Charged particle spectra measured during the transit to Mars with the Mars Science Laboratory Radiation Assessment Detector (MSL/RAD). Life Sci Space Res (Amst) 2016; 10, 29-37.
21. Ehresmann B, Zeitlin CJ, Hassler DM, Matthia D, Guo J, Wimmer-Schweingruber RF, et al., The charged particle radiation environment on Mars measured by MSL/RAD from November 15, 2015 to January 15, 2016. Life Sci Space Res (Amst) 2017; 14, 3-11.
22. Hassler DM, Zeitlin C, Wimmer-Schweingruber RF, Ehresmann B, Rafkin S, Eigenbrode JL, et al., Mars' surface radiation environment measured with the Mars Science Laboratory's Curiosity rover. Science 2014; 343, 1244797.
23. Zeitlin C, Hassler DM, Cucinotta FA, Ehresmann B, Wimmer-Schweingruber RF, Brinza DE, et al., Measurements of energetic particle radiation in transit to Mars on the Mars Science Laboratory. Science 2013; 340, 1080-4.
24. Cucinotta F, Alp M, Sulzman F, Wang M, Space radiation risks to the central nervous system. Life Sci Space Res *(Amst)* 2014; 2, 54-69.
25. Kronenberg A, Cucinotta FA, Space radiation protection issues. Health physics 2012; 103, 556-67.
26. Chancellor JC, Scott GB, Sutton JP, Space Radiation: The number one risk to astronaut health beyond low Earth orbit. Life (Basel) 2014; 4, 491-510.
27. Deiters K, piM1 Beamline. https://wwwpsich/en/sbl/pim1-beamline 2013.
28. Hajdas W, Desorgher L, Deiters K, Reggiani D, Rauber T, Tulej M, et al. High energy electron radiation exposure facility at PSI. J Applied Math Phys 2014; 2, 910-17.
29. Desorgher L, Stabilini A, Rostomyan T, Reggiani D, Hajdas W, Marcinkowski R, et al., Dosimetry of the PIM1 pion beam at the Paul Scherrer Institute for radiobiological studies of mice. Radiat Res 2023; Submitted.
30. Drayson O, Vozenin M-C, Limoli C, A rigorous behavioral platform for the assessment of radiation-induced neurological outcomes. Method Molec Biol 2023; In Press.
31. de Sena Brandine G, Smith AD, Falco: high-speed FastQC emulation for quality control of sequencing data. F1000Res 2019; 8, 1874.
32. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al., STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013; 29, 15-21.
33. Liao Y, Smyth GK, Shi W, featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 2014; 30, 923-30.
34. Love MI, Huber W, Anders S, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014; 15, 550.
35. Wickham H, Navarro D, Pedersen TL, *ggplot*: Springer New York, NY; 2016.

36. Kolde R, pheatmap:Pretty heatmaps. 2019.

37. Benjamini Y, Hochberg Y, Controlling the false discovery rate: A practical and powerful approach to multiple testing. J Royal Statist Soc 1995; 57, 289-300.

38. Kwapis JL, Alaghband Y, Keiser AA, Dong TN, Michael CM, Rhee D, et al., Aging mice show impaired memory updating in the novel OUL updating paradigm. Neuropsychopharmacology 2020; 45, 337-46.

39. Wright DS, Bodinayake KK, Kwapis JL, Investigating memory updating in mice using the objects in updated locations task. Curr Protoc Neurosci 2020; 91, e87.

40. Barker GR, Bird F, Alexander V, Warburton EC, Recognition memory for objects, place, and temporal order: a disconnection analysis of the role of the medial prefrontal cortex and perirhinal cortex. J Neurosci 2007; 27, 2948-57.

41. Barker GR, Warburton EC, When is the hippocampus involved in recognition memory? J Neurosci 2011; 31, 10721-31.

42. Milad MR, Quirk GJ, Neurons in medial prefrontal cortex signal memory for fear extinction. Nature 2002; 420, 70-4.

43. Quirk GJ, Mueller D, Neural mechanisms of extinction learning and retrieval. Neuropsychopharmacology 2008; 33, 56-72.

44. Montay-Gruel P, Acharya MM, Petersson K, Alikhani L, Yakkala C, Allen BD, et al., Long-term neurocognitive benefits of FLASH radiotherapy driven by reduced reactive oxygen species. Proc Natl Acad Sci U S A 2019; 116, 10943-51.

45. Alaghband Y, Cheeks SN, Allen BD, Montay-Gruel P, Doan NL, Petit B, et al., Neuroprotection of radiosensitive juvenile mice by ultra-high dose rate FLASH irradiation. Cancers (Basel) 2020; 12.

46. Allen BD, Alaghband Y, Kramar EA, Ru N, Petit B, Grilj V, et al., Elucidating the neurological mechanism of the FLASH effect in juvenile mice exposed to hypofractionated radiotherapy. Neuro-oncology 2022.

47. Kamimura K, Maeda N, Glypicans and heparan sulfate in synaptic development, neural plasticity, and neurological disorders. Frontiers in neural circuits 2021; 15, 595596.

48. Pluimer BR, Colt M, Zhao Z, G Protein-coupled receptors in the mammalian blood-brain barrier. Front Cell Neurosci 2020; 14, 139.

49. Farioli-Vecchioli S, Saraulli D, Costanzi M, Leonardi L, Cina I, Micheli L, et al., Impaired terminal differentiation of hippocampal granule neurons and defective contextual memory in PC3/Tis21 knockout mice. PLoS One 2009; 4, e8339.

50. Guzowski J, Lyford G, Stevenson G, Houston F, McGaugh J, Worley P, et al., Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. J Neurosci 2000; 20, 3993-4001.

51. Britten RA, Limoli CL, New Radiobiological Principles for the CNS Arising from Space Radiation Research. Life (Basel) 2023; 13.

52. Britten RA, Duncan VD, Fesshaye A, Rudobeck E, Nelson GA, Vlkolinsky R, Altered Cognitive Flexibility and Synaptic Plasticity in the Rat Prefrontal Cortex after Exposure to Low (</=15 cGy) Doses of (28)Si Radiation. Radiat Res 2020; 193, 223-35.