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^{16}O Oxygen irradiation enhances cued fear memory in B6D2F1 mice



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ABSTRACT

The space radiation environment includes energetic charged particles that may impact cognitive performance. We assessed the effects of ^{16}O ion irradiation on cognitive performance of C57BL/6J × DBA/2J F1 (B6D2F1) mice at OHSU (Portland, OR) one month following irradiation at Brookhaven National Laboratory (BNL, Upton, NY). Hippocampus-dependent contextual fear memory and hippocampus-independent cued fear memory of B6D2F1 mice were tested. ^{16}O ion exposure enhanced cued fear memory. This effect showed a bell-shaped dose response curve. Cued fear memory was significantly stronger in mice irradiated with ^{16}O ions at a dose of 0.4 or 0.8 Gy than in sham-irradiated mice or following irradiation at 1.6 Gy. In contrast to cued fear memory, contextual fear memory was not affected following ^{16}O ion irradiation at the doses used in this study. These data indicate that the amygdala might be particularly susceptible to effects of ^{16}O ion exposure.

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1. Introduction

The space radiation environment includes energetic charged particles from protons to uranium that may pose a significant risk to the central nervous system during and following missions. In addition to irradiation, the environmental conditions astronauts experience during space missions include psychological and physical stressors. Therefore, it is important to consider controlled environmental emotional stressors in assessments of effects of space irradiation on cognition. Fear conditioning allows assessments of learning and memory in the context of emotional stressors. Translational fear conditioning tests, which involve aversive stimuli, are being used to assess fear memory in humans (Milad et al., 2011).

Contextual fear conditioning, involving re-exposure to an environment in which an aversive stimulus was received previously during training, is often used to assess hippocampus-dependent memory (Anagnostaras et al., 2001) and is sensitive to detect effects of gamma (Saxe et al., 2006; Olsen et al., 2014), ^{56}Fe (Villasana et al., 2010b), and ^{28}Si irradiation (Raber et al., 2015). Cued fear conditioning involves exposure to a novel environment and re-exposure of the conditioned stimulus (e.g., white noise)

that co-terminated with the aversive stimulus during training. This test is used to assess hippocampus-independent memory and more amygdala-dependent memory (Carmack et al., 2010; Phillips and LeDoux, 1992). More specifically, for the acquisition of cued fear, the basolateral amygdala is especially important (Maren, 1996; Rogan et al., 1997). Different brain regions are involved in contextual and cued fear memory, and as a result one form of memory can be affected while another remains intact (Sangha et al., 2009).

While effects of gamma irradiation on cued fear memory have been reported (Olsen et al., 2014), less is known about effects of space irradiation on cued fear memory. ^{56}Fe irradiation was shown to affect hippocampus-dependent cognitive performance, but not hippocampus-independent cognitive performance, in C57BL/6J wild-type mice (Haley et al., 2013) and in targeted replacement human apoE mice on a C57BL/6J background (Haley et al., 2012) assessed within 5 weeks of irradiation. ^{28}Si irradiation also was shown to affect hippocampus-dependent contextual fear memory without affecting hippocampus-independent cued fear memory in hybrid C57BL/6J × DBA/2J F1 (B6D2F1) mice three months after radiation exposure (Raber et al., 2015).

To date, the most comprehensive set of data regarding the behavioral effects of space flight radiation have come from studies in rats, including effects from low to moderate doses of various ions, including ^{16}O ions (Poulose et al., 2011; Rabin et al., 2011; Rabin et al., 2014). In rats, ^{16}O ion exposure (600 MeV/amu) at two months of age impaired novel object recognition two months

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later for doses between 0.5 and 5 cGy, but not at 0.1 or 10 cGy (Rabin et al., 2014). ^{16}O ion irradiation (1000 MeV/amu) at two months of age impaired novel object recognition four months later at doses of 5 and 10 cGy, but not at 25 cGy (Rabin et al., 2014). ^{16}O ion exposure also reduced operant responding in rats 6–8 weeks following exposure (Rabin et al., 2011). Finally, ^{16}O ion irradiation (1000 MeV/amu) decreased autophagy and increased inflammation and oxidative stress (Poulose et al., 2011). The effects of ^{16}O ion irradiation on cognitive performance of mice are not known. In the present study, we tested the effects of ^{16}O ions (250 MeV/amu) on hippocampus-dependent contextual fear memory and hippocampus-independent cued fear memory in B6D2F1 mice exposed at six months of age (a mouse age pertinent to the age of most astronauts) one month following irradiation.

2. Materials and methods

2.1. Animals and study design

Breeder animals for this study were obtained from Jackson Laboratories, Bar Harbor Maine. The experimental mice were 6-month-old B6D2F1 female and male mice ($n = 87$ mice in total, December 2013; 1 month time interval between irradiation and cognitive testing: sham-irradiation ($n = 18$ mice; 9 female and 9 male mice); ^{16}O ; 250 MeV/amu O ions (25 keV/ μm): 0.4 ($n = 29$ mice; 14 female and 15 male mice), 0.8 ($n = 23$ mice; 11 female and 12 male mice), 1.6 Gy ($n = 18$ mice; 9 female and 9 male mice)). The radiation doses were selected and approved by NASA based on a funded kidney mutagenesis study in the same animals and therefore the dose range for this study is higher than for studies planned solely to assess brain function. To enhance the chances to detect effects of irradiation at a lower dose, a larger number of mice were included for the lowest radiation dose (0.4 Gy) and a slightly larger number of mice were included for the middle radiation dose (0.8 Gy). The mice were shipped from Oregon Health and Science University (OHSU) to Brookhaven National Laboratory (BNL) and allowed to accommodate to the housing facility there for one week. The mice in each cohort were randomly assigned to the treatment groups described above. The mice were housed under a constant 12 hr light: 12 hr dark cycle. Food (PicoLab Rodent Diet 20, no. 5053; PMI Nutrition International, St. Louis, MO) and water were provided *ad libitum*. All procedures were approved by Institutional Animal Care and Use Committees at OHSU and BNL.

For irradiation, mice were loaded into $8 \times 3 \times 3$ cm plastic enclosures with air holes and placed in a foam fixture in the beam line of the NASA Space Radiation Laboratory (NSRL). They were exposed to a square beam of approximately 20×20 cm. Dose calibration was performed using three parallel plate ion chambers that were positioned upstream of target and a NIST traceable Far West thimble chamber. The values of the thimble chamber were then compared with the upstream ion chambers so that the desired dose could be delivered to the samples, as described (Kronenberg et al., 2009). Sham-irradiated mice were placed into the plastic enclosures for the same time as the irradiated mice, as described (Raber et al., 2015).

One week following irradiation or sham-irradiation at BNL, the mice were shipped back to OHSU for cognitive testing as described in detail below. All protocols were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of OHSU and BNL and were in compliance with all Federal regulations.

2.2. Cognitive testing

Investigators at OHSU involved with the cognitive testing of this study group of ^{16}O ion-irradiated and sham-irradiated mice were blinded to the dose levels until after completion

of the cognitive testing. Cognitive testing was performed during the light phase one month following irradiation or sham-irradiation, as described (Raber et al., 2015). Briefly, the mice were tested for hippocampus-dependent contextual fear conditioning and hippocampus-independent cued fear conditioning using Med Associates NIR Video and automated analysis (Med Associates, St. Albans, VT, USA) utilizing Med Associates Video Freeze automated scoring system. Pavlovian fear conditioning is a versatile and well-understood method of assessing associative learning and memory. In this task, mice learned to associate a conditioned stimulus (CS, e.g. a tone) with an unconditioned stimulus (US, e.g. foot shock). CS-US pairings were preceded by a short habituation period, from which a baseline measure of locomotor activity is derived. Freezing is defined as the absence of motion with the exception of respiration. The freezing response is a widely used indicator of a conditioned fear response.

On day 1 training, each mouse was placed inside a white LED lit (100 lux) fear conditioning chamber (Context A). Context A consisted of a metal grid floor with gray and white walls. There was a 90 second baseline followed by five CS-US pairings. During acquisition, the 30-second tone (CS) (80 db, 2800 Hz) co-terminated with 2-second foot shocks (0.7 mA) (US). The inter-tone interval (ITI) was 90 seconds. Motion during shock (arbitrary units from proprietary index) was measured to evaluate potential treatment-induced differences in response to the aversive stimulus. Percent time freezing during each subsequent ITI and tone presentation was measured to assess acquisition of the fear response. On day 2, mice were initially exposed to the training environment (Context A), for 300 seconds. Four hours later, the mice were exposed to a new environment, Context B. Context B consisted of a smooth white plastic floor, with a “tented” black plastic ceiling and scented with a 10% isopropanol solution. There was a 90 second baseline followed by a 180 second tone.

2.3. Statistical analyses

All data are shown as mean \pm standard error of the mean (SEM). Statistical analyses were performed using SPSS™ (Chicago, IL) and GraphPad Prism™ (San Diego, CA) software packages. The baseline data, the immobility during the tones and between the tone-shocks during the training day, and the contextual fear memory data were each analyzed using a one-way ANOVA. Cued fear memory data were analyzed using a two-way ANOVA. All figures were generated using GraphPad Prism software. ANOVA was used with radiation dose and sex as the between factors, followed up by Dunnett's or Tukey–Kramer posthoc tests when appropriate, as indicated. We considered $p < 0.05$ as statistically significant.

3. Results

3.1. Assessments of cognitive performance one month following ^{16}O ion or sham irradiation at BNL

The radiation doses delivered were well tolerated by the animals and no adverse effects were observed during the post-irradiation follow-up and testing periods. No effects of sex or of a radiation \times sex interaction were observed for any outcome measure. On the training day, there were no effects of irradiation on activity or immobility during the baseline period (prior to the first tone-shock pairing) (Table 1, third column). In addition, there were no effects of irradiation on freezing levels between the five tone shock pairings (Table 1, columns 5–8).

The day following the training day, the mice were first returned to the training day environment to assess hippocampus-dependent contextual fear memory. There were no tone or shock presented

Table 1
Cognitive performance during training one month following ¹⁶O ion or sham irradiation at BNL.

Cohort radiation type	Dose (Gy) [n]	Average motion baseline (AU)	Average motion during shocks (AU)	% Time freezing ITI 1	% Time freezing ITI 2	% Time freezing ITI 3	% Time freezing ITI 4
¹⁶ O	Control [18]	245 ± 12	1565 ± 42	30 ± 5	37 ± 4	53 ± 5	64 ± 5
	0.4 [29]	263 ± 8	1705 ± 19	31 ± 4	40 ± 3	50 ± 4	60 ± 4
	0.8 [22]	232 ± 14	1587 ± 33	28 ± 5	48 ± 5	61 ± 5	70 ± 3
	1.6 [18]	239 ± 12	1671 ± 41	28 ± 5	34 ± 5	54 ± 5	55 ± 5

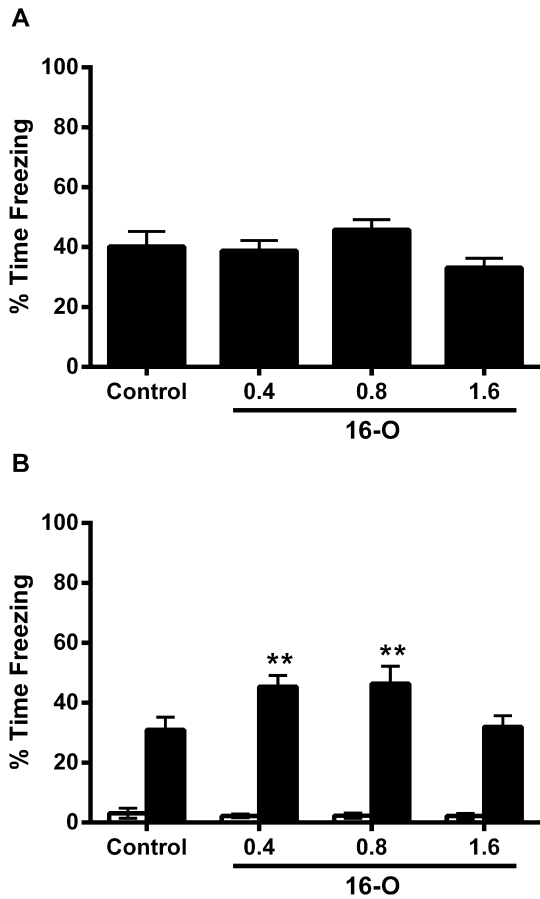


Fig. 1. Effects of ¹⁶O ion irradiation on contextual and cued fear memory. **A.** ¹⁶O ion irradiation did not affect contextual fear memory. **B.** There was no effect of radiation on freezing levels during the pre-tone condition (white bars, $F = 0.1905$, $p = 0.9026$) but there was an effect of radiation on freezing levels during the tone condition (black bars, $F = 3.262$, $p = 0.0255$). Cued fear memory was significantly stronger in mice irradiated with ¹⁶O ions at a dose of 0.4 ($p = 0.0056$) or 0.8 ($p = 0.0053$) Gy than in sham-irradiated mice (Dunnett’s test). Doses in Gy are indicated under the bars. Error bars represent the standard error of the mean. ** $p < 0.01$ versus control. $N = 18–29$ mice/dose.

during this memory trial. ¹⁶O ion irradiation did not affect contextual fear memory at the doses used in this study ($F = 1.733$, $p = 0.1666$, Fig. 1A).

In contrast to the contextual fear memory test, the cued fear memory test involves freezing in the novel environment prior (pre-tone, white bars) and during the tone (black bars), which is associated with a shock during the training. Including all experimental groups in the analysis, there was a trend towards an overall effect of irradiation on freezing levels during the cued test ($F = 1.972$, $p = 0.05$). There was no overall effect of irradiation on freezing levels during the pre-tone condition ($F = 0.1905$, $p = 0.9026$), and pre-tone condition freezing for each dose tested was not different from what was measured in sham-irradiated mice (Tukey’s test). However, an overall effect of irradiation was observed for freezing levels during the tone condition (Fig. 1B; $F = 3.262$, $p = 0.0255$).

Cued fear memory was significantly stronger in mice irradiated with ¹⁶O ions at a dose of 0.4 Gy ($p = 0.0056$) or 0.8 Gy ($p = 0.0053$) than in sham-irradiated mice (Dunnett’s test). Alterations in cued fear memory showed a bell-shaped dose response curve and no change was measured following a high dose of 1.6 Gy.

4. Discussion

The results of the current study show that ¹⁶O ion exposure affected cued fear memory in B6D2F1 mice irradiated with a 0.4 Gy or 0.8 Gy dose at six months of age. Charged particle effects on the hippocampus have been studied extensively, due to the effects on neurogenesis in the dentate gyrus (Saxe et al., 2006; Raber et al., 2004a, 2004b; Villasana et al., 2010a) and the fact that the hippocampus is frequently used for electrophysiological experiments to determine effects of irradiation on synaptic plasticity (Raber et al., 2014; Vlkolinsky et al., 2008, 2012; Rudobeck et al., 2014). However, much less is known about effects of charged particle exposure on the regulation of cued fear (Olsen et al., 2014), which does not involve the hippocampus but instead other brain areas such as the amygdala (Phillips and LeDoux, 1992). The data of the current study indicate that space radiation effects are not limited to the hippocampus, and in the case of ¹⁶O ion irradiation the amygdala may be more susceptible than the hippocampus to radiation-induced cognitive changes.

In contrast to the detrimental effects of ¹⁶O ion irradiation on cued, but not contextual, fear memory seen one month following irradiation in B6D2F1 mice in the current study, ²⁸Si irradiation affected contextual fear memory without affecting cued fear memory of B6D2F1 mice three months after radiation exposure (Raber et al., 2015). Based on the different time intervals between irradiation and cognitive testing used in the ¹⁶O ion and ²⁸Si ion studies, it is not clear whether these distinct cognitive effects are due to the different radiation exposures and/or the different time intervals involved in these studies. Clearly, future studies are warranted to compare different radiation exposure at one and three months following irradiation.

While the amygdala receives input from the hippocampus (Kim and Jung, 2006), hippocampal lesions affect contextual but not cued fear conditioning (Phillips and LeDoux, 1992). Similarly, input from the hippocampus to the basolateral amygdala and ventromedial prefrontal cortex has been proposed to be essential for contextual modulation of fear acquisition and extinction (Moustafa et al., 2013). In contrast, amygdala lesions may interfere with both contextual and cued fear conditioning. Only alteration in cued fear conditioning was noted in the present study. Consistent with the rodent data, cued fear conditioning in humans does not involve the hippocampus while contextual fear conditioning does (Maren et al., 2013). Taken together, our data suggest that the amygdala might be particularly susceptible to ¹⁶O ion irradiation.

In the present study, memory of cued fear was enhanced following ¹⁶O ion irradiation at doses of 0.4 and 0.8 Gy. This enhancement was not seen following irradiation at a high dose of 1.6 Gy. A pattern of cognitive effects at lower doses, but not at a higher dose, challenges the prevailing theory that the relationship between dose and effect is linear. The possibility exists that

compensatory mechanisms are recruited at the higher dose, but not elicited at the two lower doses, which could explain this dose response curve. Similar nonlinear dose responses were obtained for contextual fear memory in mice exposed to ^{28}Si ions (600 MeV/amu) at two months of age (Raber et al., 2014) and in rats exposed to ^{16}O ions (600 MeV/amu and 1000 MeV/amu) at two months of age (Rabin et al., 2014). The age of the animal at the time of irradiation is likely to be important. In the present mouse study, the choice of six-month-old mice was based on the typical age range of astronauts exposed to space radiation. This pattern does not seem restricted to radiation exposure. The toxic response following exposure of zebrafish embryos/larvae to alcohol shows a nonlinear dose response curve with a reduction in toxicity at moderate doses (Loucks and Carvan, 2004).

Effects of ^{16}O ion irradiation on arousal and subsequent effects of heightened arousal on cued fear memory might contribute to the effects observed in the ^{16}O ion exposed mice. Heightened arousal generated by exposure to novel environments is shown to enhance cued fear memory in rats through an interaction between peripheral adrenergic and brainstem glutamatergic systems (King and Williams, 2009). Consistent with the rodent data, presentation of arousal associated with novel visual slides (Fenker et al., 2008) or earlier slides (Cahill and Alkire, 2003) enhances verbal memory in humans and β -adrenergic mediated signaling is critical for enhanced emotional memory (Cahill et al., 1994). The combination of novelty and aversive environmental stimuli is pertinent to conditions experienced by astronauts during space missions.

In mice irradiated with gamma rays shortly after training for fear conditioning, contextual and cued fear conditioning were enhanced two weeks following irradiation (Olsen et al., 2014). These radiation effects are associated with impaired extinction of fear, even if compared with extinction of a sham-irradiated group with matching freezing levels due to enhanced training during the first memory session, (Olsen et al., 2014). Future studies are warranted to determine if extinction of cued and contextual fear memory are impaired following ^{16}O irradiation. Altered fear memory, especially when associated with impaired extinction of this memory, might negatively affect operational performance of astronauts during space missions and increase the risk of developing stress disorders. Stress disorders are associated with impaired extinction of fear memories (Michael et al., 2007, Johnson et al., 2015).

One motivation to use B6D2F1 mice for risk assessment studies is that they have a longer life span than C57Bl6/J mice (Lesniewski et al., 2009; Turturro et al., 1999). Therefore, this mouse strain is particularly suitable for follow up studies to assess long-term effects of space radiation on cognitive performance starting with irradiation at an age that reflects the age range of astronauts and continuing throughout the lifespan. This would allow a direct comparison with cognition studies performed in other mouse strains, wherein the radiation exposures were performed at a very young age and with relatively limited follow-up post-exposure.

In summary, the results of the present study show an effect of charged particle exposure on cued fear memory. This finding suggests that the amygdala is particularly susceptible to effects of ^{16}O ion irradiation. Future studies are warranted to determine the mechanisms underlying these effects.

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