Published in final edited form as:

Hippocampus. 2010 January ; 20(1): 19–23. doi:10.1002/hipo.20659.

Sex-Dependent Effects of ^{56}Fe Irradiation on Contextual Fear Conditioning in C57BL/6J Mice

Laura Villasana¹, Jenna Rosenberg¹, and Jacob Raber^{1,2,3,*}

¹Department of Behavioral Neuroscience, Oregon Health and Science University, Portland, Oregon

²Department of Neurology, Oregon Health and Science University, Portland, Oregon

³Division of Neuroscience, ONPRC, Oregon Health and Science University, Portland, Oregon

Abstract

Effects of irradiation on hippocampal function have been mostly studied in male rodents and relatively little is known about potential effects of irradiation on hippocampal function in female rodents. Moreover, although the long-term effects of clinical radiation on cognitive function have been well established, the effects of other forms of irradiation, such as high charged, high energy radiation (HZE particles) that astronauts encounter during space missions have not been well characterized. In this study we compared the effects of ^{56}Fe irradiation on fear conditioning in C57BL/6J female and male mice. Hippocampus-dependent contextual fear conditioning was impaired in female mice but improved in male mice following ^{56}Fe irradiation. Such impairment was not seen for hippocampus-independent cued fear conditioning. Thus, the effects of ^{56}Fe irradiation on hippocampus-dependent contextual fear conditioning are critically modulated by sex.

Keywords

fear conditioning; contextual; cued; hippocampus; mice

INTRODUCTION

The brain is exposed to irradiation under various conditions, including therapy for clinical conditions and in the space environment. A potential adverse effect of cranial irradiation is cognitive dysfunction involving the temporal lobes (Caveness, 1977; Sheline et al., 1980). Consistent with the human data, impairments in hippocampus-dependent learning and memory in rodents are seen following cranial irradiation with X-ray (Raber et al., 2004; Rola et al., 2004; Saxe et al., 2006), $^{137}\text{Cesium}$ (Villasana et al., 2006), or ^{56}Fe (Shukitt-Hale et al., 2000; Higuchi et al., 2002), which is part of the space environment.

Women might be more susceptible to impairments in hippocampal function than men. Age-related cognitive decline (ACD) is associated with a decline in spatial and nonspatial learning and memory (Small et al., 1995) with marked impairments in executive function such as working memory and attention (Tisserand and Jolles, 2003) and decline in regional brain volume and cortical activation in the prefrontal cortex and medial-temporal lobe, including the hippocampus (Resnick et al., 2003). ACD is more profound in women than men. Women show a faster rate of decline with age in visual-spatial skills than men (Small et al., 1995) and are

more sensitive to the effects of apolipoprotein (apo) E4, a risk factor for ACD, on cognition (Mortensen and Hogh, 2001). Finally, women are three to four times more likely to develop Alzheimer's disease (AD) than men (Gao et al., 1998).

Consistent with these human studies, female mice are more sensitive than male mice to effects of age (Benice et al., 2006) and apoE4 (Raber et al., 1998, 2000) on hippocampus-dependent cognitive function. In addition, apoE3 and apoE4 female mice are more susceptible than genotype matched male mice to ¹³⁷Cesium radiation-induced impairments in hippocampus-dependent cognitive function (Villasana et al., 2006). In this study, we assessed whether female C57BL/6J wild-type mice are also more susceptible than strain-matched male mice to detrimental effects of ⁵⁶Fe irradiation on hippocampal function. All procedures were according to the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were in compliance with institutional IACUC approval at OHSU and BNL. Behavioral tests were conducted by an experimenter blinded to the treatment of the mice. Results are expressed as mean $\pm$ standard error of the mean (standard error of mean (SEM)) and are considered significant at $P < 0.05$.

To assess the effects of HZE irradiation on hippocampal function, 2-month-old female and male C57B/6J mice ($N = 6-8$ mice/dose/sex) were cranially irradiated with ⁵⁶Fe at a dose of 0, 1, 2, or 3 Gy at Brookhaven National Laboratories (BNL), Upton, NY. The mice were shipped from OHSU to BNL and irradiated 1 week after arrival. Four days after irradiation, the mice were shipped back to OHSU and cognitively tested 12 weeks after irradiation. A 12-week-period between irradiation and testing was chosen because inflammatory responses increase for at least 2 months following irradiation (Monje et al., 2003; Rola et al., 2008) and to allow sufficient time for new cells to become functionally integrated into a neuronal network.

Because irradiation could potentially affect the physical health of mice, the body weights of mice were compared before irradiation and following behavioral testing. The change in body weight (grams) was compared between groups using sex and dose as between-subject factors in a two-way analysis of variance (ANOVA) (2×4).

Anxiety can influence behaviors related to fear conditioning. Therefore, an open field test was first conducted to assess potential effects of sex and irradiation on measures of anxiety. In the open field test, mice were placed in a brightly lit (800 lux) open field chamber for 10 min as previously described (Villasana et al., 2006). The percent time spent in the center of the open field, which is a measure of anxiety, and distance moved were analyzed for potential group differences using a two-way ANOVA with sex and dose as between-subject factors.

Novel location and novel object recognition were used to assess hippocampus-dependent and independent learning and memory. Each mouse was first habituated to an enclosure (2 days, 10 min/day). On the fourth day, mice were placed again in the enclosure containing three different objects. These objects were selected based on preliminary data showing that the mice explored these objects equally. After three familiarization trials (10 min/trial, 8-min inter-trial interval (ITI)), one of the objects was moved to a different corner. The time the mice spent exploring each of the three objects in the five trials was recorded. Mice that recognize the change in spatial configuration will spend more time exploring the object in the novel location than old location (Trial 4 vs. Trial 3). The percent time the mice spent exploring the object in the novel vs. old location was calculated (novel location recognition; paired t test). In the fifth trial, one of the familiar objects was replaced by a novel object and the percent time the mice spent exploring the novel object and the familiar objects calculated. Mice that recognize the novel object will spend more time exploring the novel object than the two familiar objects. Novel object recognition data were analyzed by comparing the percent time exploring of the objects using a three-way ANOVA with sex, dose, and object as between-subject factors.

Exploration of the objects was defined by the mice approaching the objects with their nose within 2–4 cm and was scored using a multiple body point video tracking system as previously described (Benice et al., 2008; Ethovision XT, Noldus Information Technology, Wageningen, Netherlands).

In contextual fear conditioning, mice learn to associate the environmental context (fear conditioning chamber) with a mild foot shock (US). Contextual fear conditioning is hippocampus- and amygdala-dependent. Therefore, the mild foot shock was also paired with a tone for cued fear conditioning, which is sensitive to amygdala function. When mice are reexposed to the context or tone, conditioned fear results in freezing behavior, which is cessation of all movement except respiration. On Day 1, each mouse was placed in a fear conditioning chamber and allowed to explore for 2 min before delivery of a 30-s tone (3.8 kHz, 80 db) which was immediately followed by a 2-s foot shock (0.35 or 0.9 mA) (Fig. 1D). Two min later, a second CS-US pair was delivered. On Day 2, each mouse was first placed in the fear conditioning chamber containing the exact same context with the exception of the tone or foot shock. Freezing was scored for 3 min. One hour later, the mice were placed in a new context (containing a different odor, cleaning solution, floor texture, walls, and shape) where they were allowed to explore for 3 min before being re-exposed to the fear conditioning tone for a duration of 3 min. Freezing was measured using the Ethovision XT video tracking system (Ethovision 5.0) as previously described (Pham et al., 2009). Detection parameters included: six samples/s as sample rate of processing; static subtraction detection method; object is always darker than background as object intensity; a 3% immobility threshold; a 60% mobile threshold; a mobility averaging interval of 1; erosion and dilation filters of 1 pixel. Contextual and cued fear conditioning trials from the shock intensity experiment were hand scored (every 5 s) to validate proper detection parameters using the Ethovision automated system. There was a significant correlation between the automated and hand scored freezing analysis in the context trials ($r = 0.80$; $P < 0.001$; $N = 46$; hand-score mean and SEM = $33.32\% \pm 3.68\%$, Ethovision mean and SEM = $33.75\% \pm 2.95\%$, Pearson correlation) and in the cued fear conditioning trials ($r = 0.91$; $P < 0.001$; $N = 46$; hand-score mean and SEM = $49.57\% \pm 3.68\%$, Ethovision mean = $49.81\% \pm 3.73\%$, Pearson correlation). To exclude potential effects of anxiety-like or locomotor behavior contributing to group differences in freezing behavior of ^{56}Fe irradiated mice, we analyzed mobility and distance moved during the first 2 min of fear conditioning on Day 1 prior to the tone/shock exposure. To determine whether there are potential shock intensity-dependent sex differences in fear conditioning, we first determined the effects of different shock intensity (0, 0.35, and 0.9 mA) in a separate group of 2-month-old C57BL/6J male and female mice ($n = 6\text{--}9$ mice/sex/shock intensity). Potential group differences in this experiment were assessed by comparing the percent freezing between groups using a two-way ANOVA with sex and shock intensity as between-subject factors. For the ^{56}Fe dose experiment, sex and dose were used as between-subject factors and performance measures included percent freezing, mobility, distance moved, and velocity. To ensure that group differences in freezing behavior were not due to potential differences in shock reactivity, velocity in response to the first shock exposure on Day 1 was analyzed as this has been shown to provide a sensitive index of shock reactivity (Godsil et al., 1997; Wiltgen et al., 2001).

Analysis of weight changes revealed that females gained more weight than males ($F(1,49) = 4.78$; $P < 0.05$) (females 1.23 ± 0.26 g; males 0.44 ± 0.25 g) but there was no sex $\times$ dose interaction ($F(3,49) = 2.08$; $P = 0.12$) or effect of dose ($F(3,49) = 0.92$; $P = 0.54$). In female mice, the weight gains for 0, 1, 2, and 3 Gy ^{56}Fe irradiated mice were (in mean $\pm$ SEM) 0.87 ± 0.33 , 0.63 ± 0.33 , 1.68 ± 0.36 , and 1.73 ± 0.31 , respectively. In male mice, the weight changes for 0, 1, 2, and 3 Gy ^{56}Fe irradiated mice were 0.43 ± 0.59 , 0.78 ± 0.68 , 1.03 ± 0.64 , and -0.49 ± 0.59 , respectively.

In the open field test, females moved more than males ($F(1,49) = 9.06$; $P < 0.01$) but there was no effect of irradiation or a sex $\times$ irradiation interaction (Fig. 1A). There was no effect of sex, irradiation or a sex $\times$ irradiation interaction on anxiety-like behavior as assessed by the percent time spent in the center of the open field (Fig. 1B).

In the novel location recognition task, none of the groups of mice explored the object in its new location more than in its old location (data not shown). The lack of novel location recognition may have been due to the longer ITI used in this study (8 min) than in our previous studies (3 min), making this test much more challenging. This increase in ITI was related to the expansion of the number of object recognition test chambers simultaneously used from 1 to 4. In the novel object recognition task, there was a sex $\times$ dose $\times$ object interaction ($F(6,18,611) = 2.44$; $P < 0.05$); female mice irradiated with ^{56}Fe at a dose of 3 Gy and male mice irradiated with ^{56}Fe at a dose of 2 Gy did not show novel object recognition and did not explore the novel object more than the two familiar ones (Fisher's protected least significant differences or PLSD, $P > 0.05$, Fig. 1C). All other groups of mice showed novel object recognition. In our previous experiments, although mice did show novel location recognition, recognition for a novel object was more robust than novel location recognition, indicating that novel location recognition is a more challenging task. This might explain why in the current study the longer ITI affected novel location recognition but not novel object recognition in sham-irradiated mice.

In the shock intensity fear conditioning experiment, males showed more contextual freezing than females ($F(1,40) = 21.58$; $P < 0.001$, Fig. 1E). Differences in contextual fear conditioning to shock intensity were also observed (effect of shock intensity, $F(2,40) = 25.06$; $P < 0.001$); Compared to 0 mA, exposure to 0.35 or 0.9 mA increased hippocampus-dependent contextual freezing (PLSD, $P < 0.01$ and $P < 0.001$, respectively). There was no sex $\times$ dose interaction ($F(2,40) = 0.11$; $P = 0.89$). A set of planned comparisons were conducted to determine whether sex differences in freezing existed within specific shock intensities. Compared to females, males showed greater freezing at the 0.90 mA shock (effect of sex, $F(1,14) = 8.76$; $P < 0.01$) but not at the 0.35 mA shock exposure ($F(1,12) = 3.79$; $P > 0.05$). In cued fear conditioning, a similar effect of sex ($F(1,40) = 12.49$; $P < 0.01$) and shock intensity ($F(2,40) = 39.41$; $P < 0.001$) were observed (Fig. 1F).

Based on these data, the 0.35 mA intensity was selected to compare fear conditioning in ^{56}Fe irradiated mice. There was a sex $\times$ dose interaction in contextual fear conditioning ($F(3,49) = 3.31$; $P < 0.05$). An effect of dose was observed in the female ($F(3,25) = 3.05$; $P < 0.05$) but not in the male ($F(3,24) = 2.36$; $P = 0.1$) mice (Fig. 1G). Compared to sham-irradiated female mice, female mice irradiated at a dose of 1 Gy showed a nonsignificant reduction in contextual freezing (PLSD, $P = 0.09$). Female mice irradiated with ^{56}Fe at a dose of 2 Gy did not show impairments in contextual fear conditioning (PLSD, $P = 0.8$). However, female mice irradiated at a dose of 3 Gy showed impairments in contextual fear conditioning as compared to sham-irradiated female mice (PLSD, $P < 0.05$). In contrast to female mice, irradiation at any dose did not impair contextual fear conditioning in male mice. Although the overall effect of irradiation was not significant in the male mice, male mice irradiated at a 1 and 2 Gy ^{56}Fe dose froze significantly more than sham-irradiated male mice (PLSD, $P < 0.05$, Fig. 1G). The fact that this inverse dose effect was seen in male, but not female mice, indicates that irradiation dose-response effects need to be carefully analyzed in both sexes. These effects might also depend on the source of irradiation. X ray irradiation at a dose of 15 Gy impaired contextual, but not cued fear conditioning in male mice (Saxe et al., 2006).

The sex-dependent dose effect of irradiation on contextual fear conditioning was unlikely due to anxiety-like or locomotor behavior during fear conditioning as analysis of mobility and distance moved during the first 2 min of training showed no effect of sex or dose on mobility

(effect of sex: $F(1,49) = 1.22$; $P = 0.28$; effect of dose: $F(1,49) = 0.59$; $P = 0.62$) or distance moved (effect of sex: $F(1,49) = 0.89$; $P = 0.35$; effect of dose: $F(1,49) = 0.24$; $P = 0.87$; data not shown). Furthermore, analysis of velocity of movement in response to the shock showed no effect of sex ($F(1,49) = 0.14$; $P = 0.71$), dose ($F(3,49) = 0.64$; $P = 0.60$) or a sex $\times$ dose interaction ($F(3,49) = 0.266$; $P = 0.85$; data not shown). Thus, the sex-dependent dose effect of ^{56}Fe irradiation on contextual fear conditioning cannot be explained by sex differences in shock reactivity.

In cued fear conditioning, there was an effect of sex in ^{56}Fe irradiated mice ($F(1,49) = 6.82$; $P < 0.05$); male mice froze more than female mice ($P < 0.05$, Fig. 1H). This sex difference may have been driven by the lower freezing in the irradiated female than male mice. However, there was no sex $\times$ dose interaction ($F(3,49) = 1.66$; $P = 0.19$). There was no effect of dose on cued fear conditioning ($F(3,49) = 0.99$; $P = 0.41$). In the female mice, it is unlikely that the trend towards reduced freezing in the cued fear conditioning test contributed to the reduced contextual freezing following irradiation at 2 Gy. First, all mice acquired cued fear conditioning, suggesting no gross impairments in amygdala function. Second, the pattern of changes in cued fear conditioning did not correspond to those in contextual fear conditioning. Compared to sham-irradiated female mice, female mice irradiated with ^{56}Fe at a dose of 2 Gy did not show reduced contextual fear conditioning, yet they did show reduced cued fear conditioning. Similarly, male mice irradiated at a dose of 1 or 2 Gy showed enhanced contextual fear conditioning but similar cued fear conditioning compared to sham-irradiated male mice.

In summary, hippocampus-dependent contextual fear conditioning is sensitive to the effects of ^{56}Fe irradiation in a sex-dependent fashion. In agreement with our previous study using ^{137}Cs irradiation, the present study indicates that female mice are more sensitive than male mice to the effects of irradiation. These effects cannot be explained by potential differences in the overall health or weight of the mice, anxiety-like behavior or shock reactivity. However, we cannot rule out the possibility that differences in the estrous cycle phase between the female groups may have influenced contextual freezing. Sex differences in hippocampal neurogenesis following irradiation might have contributed to the differential effects of ^{56}Fe irradiation on contextual fear conditioning in female and male mice. Inhibition of neurogenesis was shown to be associated with both impaired (Raber et al., 2004; Rola et al., 2004) and enhanced hippocampus-dependent memory (Saxe et al., 2007) in male mice. Future studies are warranted to determine the mechanisms underlying the sex-dependent effects of HZE irradiation on hippocampus-dependent and hippocampus-independent learning and memory.

Finally, irradiation at a dose of 3 Gy impaired novel object recognition in the female mice, and at a dose of 2 Gy impaired novel object recognition in male mice suggesting the radiation-induced deficits are not limited to the hippocampus but affect the cortex as well.

Acknowledgments

Grant sponsor: NIA; Grant number: T32 NS007466-05; Grant sponsor: NASA; Grant number: NNJ05HE63G; Grant sponsor: Alzheimer's Association; Grant number: IIRG-05-14021.

REFERENCES

- Benice T, Rizk A, Pfankuch T, Kohama S, Raber J. Sex-differences in age-related cognitive decline in C57BL/6J mice associated with increased brain microtubule-associated protein 2 and synaptophysin immunoreactivity. *Neuroscience* 2006;137:413–423. [PubMed: 16330151]
- Benice TS, Bader T, Raber J. Object Recognition analysis in mice using nose-point digital video tracking. *J Neuroscience Methods* 2008;168:422–430.
- Caveness WF. Pathology of radiation damage to the normal brain of the monkey. *NCI Monogr* 1977;46:57–76.

- Gao S, Hendrie HC, Hall KS, Hui S. The relationships between age, sex, and the incidence of dementia and Alzheimer disease: A meta-analysis. *Arch Gen Psychiatry* 1998;55:809–815. [PubMed: 9736007]
- Godsil BP, Spooner JR, Anagnostaras SG, Gale GD, Fanselow MS. Quantification of the unconditional response to footshock in the rat: Measurement of the activity burst with the use of computer-based image analysis. *Soc Neurosci (Abstracts)* 1997;23:1612.
- Higuchi Y, Nelson GA, Vazquez M, Laskowitz DT, Slater JM, Pearlstein RD. Apolipoprotein E expression and behavioral toxicity of high charge, high energy (HZE) particle radiation. *J Radiat Res* 2002;43(Suppl):S219–S224. [PubMed: 12793762]
- Monje ML, Toda K, Palmer TD. Inflammatory blockade restores adult hippocampal neurogenesis. *Science* 2003;302:1760–1765. [PubMed: 14615545]
- Mortensen EL, Hogh P. A gender difference in the association between APOE genotype and age-related cognitive decline. *Neurology* 2001;57:89–95. [PubMed: 11445633]
- Pham J, Cabrera SM, Sanchis-Sequra C, Wood MA. Automated scoring of fear-related behavior using Ethovision software. *J Neurosci Methods* 2009;178:323–326. [PubMed: 19150629]
- Raber J, Wong D, Buttini M, Orth M, Bellosta S, Pitas RE, Mahley RW, Mucke L. Isoform-specific effects of human apolipoprotein E on brain function revealed in *ApoE* knockout mice: Increased susceptibility of females. *Proc Natl Acad Sci USA* 1998;95:10914–10919. [PubMed: 9724804]
- Raber J, Wong D, Yu G-Q, Buttini M, Mahley RW, Pitas RE, Mucke L. Alzheimer's disease: Apolipoprotein E, cognitive performance. *Nature* 2000;404:352–354. [PubMed: 10746713]
- Raber J, Rola R, LeFevour A, Morhardt D, Curley J, Mizumatsu S, VandenBerg S, Fike JR. Radiation-induced cognitive impairments are associated with changes in indicators of hippocampal neurogenesis. *Radiat Res* 2004;162:39–47. [PubMed: 15222778]
- Resnick S, Pham D, Kraut M, Zonderman A, Davatzikos C. Longitudinal magnetic resonance imaging studies of older adults: A shrinking brain. *J Neurosci* 2003;23:3295–3301. [PubMed: 12716936]
- Rola R, Raber J, Rizk A, Otsuka S, VandenBerg S, Morhardt D, Fike J. Radiation-induced impairment of hippocampal neurogenesis is associated with cognitive deficits in young mice. *Exp Neurol* 2004;188:316–330. [PubMed: 15246832]
- Rola R, Fishman K, Baure J, Rosi S, Lamborn KR, Obenaus A, Nelson GA, Fike JR. Hippocampal neurogenesis and neuroinflammation after cranial irradiation with (56)Fe particles. *Radiat Res* 2008;169:626–632. [PubMed: 18494546]
- Saxe M, Battaglia F, Wang J-W, Malleret G, David D, Monckton J, Garcia A, Sofroniew M, Kandel E, Santarelli L, Hen R, Drew M. Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proc Natl Acad Sci USA* 2006;14:17501–17506. [PubMed: 17088541]
- Saxe MD, Malleret G, Vronskaya S, Mendez I, Garcia AD, Sofroniew MV, Kandel ER, Hen R. Paradoxical influence of hippocampal neurogenesis on working memory. *Proc Natl Acad Sci USA* 2007;104:4642–4648. [PubMed: 17360577]
- Sheline GE, Wara WM, Smith V. Therapeutic irradiation and brain injury. *Int J Radiat Oncol Biol Phys* 1980;6:1215–1228. [PubMed: 7007303]
- Shukitt-Hale B, Casadesus G, McEwen J, Rabin B, Joseph J. Spatial learning and memory deficits induced by exposure to iron-56- particle radiation. *Radiat Res* 2000;154:28–33. [PubMed: 10856962]
- Small G, La Rue A, Komo S, Kaplan A, Mandelkern M. Predictors of cognitive change in middle-aged and older adults with memory loss. *Am J Psychiatr* 1995;152:1757–1764. [PubMed: 8526242]
- Tisserand D, Jolles J. On the involvement of prefrontal networks in cognitive ageing. *Cortex* 2003;39:1107–1128. [PubMed: 14584569]
- Villasana L, Acevedo S, Poage C, Raber J. Sex- and ApoE Iso-form-dependent effects of radiation on cognitive function. *Radiat Res* 2006;166:883–891. [PubMed: 17149978]
- Wiltgen BJ, Sanders MJ, Behne NS, Fanselow MS. Sex differences, context preexposure, and the immediate shock deficit in Pavlovian context conditioning with mice. *Behav Neurosci* 2001;115:26–32. [PubMed: 11256449]

FIGURE 1.

Locomotor activity (A) and anxiety-like behavior (B) in the open field test are not altered by ^{56}Fe irradiation; however females ($N = 29$) moved more than males ($N = 28$) (** $P < 0.01$). (C) 3Gy irradiated females and 2Gy irradiated males did not show novel object recognition ($P > 0.05$, novel object vs. the two familiar objects, PLSD); however, all other groups of mice did explore the novel object more than the two familiar ones (* $P < 0.05$). (D) Diagram of the fear conditioning paradigm. (E and F) Males showed more contextual and cued freezing than females in the fear conditioning test (effect of sex, $F(1,40) = 21.58$; $P < 0.001$). In contextual freezing (E), both males and females showed greater freezing to the 0.35 and 0.90 mA shock intensity compared to the no-shock group (* $P < 0.05$, *** $P < 0.001$, no shock group vs. 0.35 and 0.90 mA, respectively). (G) 3Gy irradiated females showed deficits in contextual fear conditioning ($P < 0.05$ vs. sham-irradiated females). (H) Cued fear conditioning was not impaired by irradiation. (For A–C and G–H, $N = 6$ –8 mice/sex/dose).