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Enhanced cued fear memory following post-training whole body irradiation of 3-month-old mice

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Abstract

Typically, in studies designed to assess effects of irradiation on cognitive performance the animals are trained and tested for cognitive function following irradiation. Little is known about post-training effects of irradiation on cognitive performance. In the current study, 3-month-old male mice were irradiated with X-rays 24 hours following training in a fear conditioning paradigm and cognitively testing starting two weeks later. Average motion during the extinction trials, measures of anxiety in the elevated zero maze, and body weight changes over the course of the study were assessed as well. Exposure to whole body irradiation 24 hours following training in a fear conditioning resulted in greater freezing levels 2 weeks after training. In addition, motion during both contextual and cued extinction trials was lower in irradiated than sham-irradiated mice. In mice trained for cued fear conditioning, activity levels in the elevated zero maze 12 days after sham-irradiation or irradiation were also lower in irradiated than sham-irradiated mice. Finally, the trajectory of body weight changes was affected by irradiation, with lower body weights in irradiated than sham-irradiated mice, with the most profound effect 7 days after training. These effects were associated with reduced c-Myc protein levels in the amygdala of the irradiated mice. These data indicate that whole body X ray irradiation of mice at 3 months of age causes persistent alterations in the fear response and activity levels in a novel environment, while the effects on body weight seem more transient.

Keywords

cued fear; body weight; zero maze; irradiation; post-training

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1. Introduction

Consequences of radiation exposure are perhaps most typically thought of in terms of cancer risk [1]. However, a growing body of evidence supports a pernicious influence of irradiation on neurobiological systems with profound behavioral and cognitive consequences [2]. As environmental whole body irradiation exposure might occur as part of a natural disaster, an accident at a nuclear facility, a military mission, or radiological terrorism, it is important to establish a body of knowledge from which to predict and anticipate important consequences and outcomes [3]. This is also important absent catastrophe when considering the incidence of radiological exposure of cancer patients. Radiological effects on behavioral and cognitive performance appear to be long lasting. Following total body irradiation (8 Gy) of 8-week-old mice, activity in the open field was reduced at 3 hours following irradiation, partially recovered within 24 hours, followed by a later decrease 4 days after irradiation with a slow recovery starting at 17 days [4]. Locomotion and body temperature, as assessed by telemetry, decreased rapidly following irradiation reaching a minimum between 13 and 17 days following irradiation and full recovery starting at 27 days [4]. Radiation effects on learning and memory have been reported 30 days or more after whole body irradiation of 3- or 8-week-old male mice (4 Gy) [5, 6]. Diffusion tension imaging (DTI) and *in vivo* proton nuclear magnetic resonance spectroscopy (MRS) performed 2 or more days following irradiation showed that the hippocampus and frontal cortex are especially sensitive to these early radiation effects [7] [8] thus implicating potentially retrograde mnemonic processes as being particularly sensitive. These effects might be age-dependent, as activity in the open field was not altered in 6-month-old mice 24 hours following receiving whole body irradiation at 2, 5, or 8 Gy [9].

In all these studies, the animals were trained and tested for cognitive function following irradiation. Thus, neurobehavioral processes reliant on previous experiences, conditioning, and memory were not assessed in isolation. Post-training irradiation is translational relevant. For example, soldiers, physicians and nurses, astronauts, emergency responders, and cancer patients can be exposed to irradiation exposure following learning experiences and formation of memories. Therefore, it would be important to assess whether memory alterations due to radiation exposure could be retrograde. Previously, we showed enhanced contextual and cued fear memory prior to and during extinction following post-training irradiation of one-month-old male mice [10]. Due to the established age-sensitivity of radiation effects in mice with increased sensitivity in younger mice, the prior observed enhanced contextual and cued fear memory prior to and during extinction following post-training irradiation following post-training irradiation of one-month-old male mice could be age-dependent. Thus, more adult mice might be conferred relative protection against effects of irradiation on contextual and cued fear memories. This could have significant consequences when anticipating potential human corrolaries, with possible focus on specific sequelae and subsequent intervention in younger or older populations. Therefore, in the current study 3-month-old male mice were irradiated with X-rays 24 hours following training in a fear conditioning paradigm and tested two weeks later for retention and extinction of hippocampus-dependent contextual fear memory or hippocampus-independent cued fear memory. Average motion during the extinction trials was analyzed as well, to

determine whether these measures of the fear (escape) response were affected by post-training irradiation. As measures of anxiety might affect retention and extinction of fear, performance in the elevated zero maze was assessed as well. Finally, effects of irradiation on body weight changes over the course of the study were assessed.

Irradiation affects the levels of the v-myc avian myelocytomatotsis viral oncogene homolog (c-Myc) protein [11]. Recently, we reported that c-Myc plays an important role in the response of the brain in substance abuse [12]. Therefore, in this study we also assessed whether the behavioral changes were associated with alterations of c-Myc protein levels in pertinent brain regions.

2. Material and Methods

2.1. Animals

Three-month-old male C57Bl6/J wild-type mice ($n = 70$) purchased from the Jackson Laboratory (Bar Harbor, ME) were used for the experiments of this study, as described below in detail. 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 the Institutional Animal Care and Use Committee of Oregon Health & Science University (OHSU), Portland, Oregon.

2.2. Experiment 1: *Contextual fear conditioning*

Forty mice were cognitively trained in a contextual fear conditioning paradigm, involving a ten-shock paradigm, consisting of 2-second 0.35 mA shocks, separated by 60-second inter-shock-intervals (ISI), with the first shock at 60 seconds from the beginning of the trial. The total length of the training session was 10 minutes. Twenty-four hours after training, all mice were brought to a room within the animal facility containing an X-ray irradiator (Rad Source RS2000 Biological Research Irradiator, Suwanee, GA). Half of the mice (randomly sorted into two groups until any differences in baseline measurements were non-significant) were placed in a new mouse cage fitting in the irradiator and received whole body irradiation at a dose of 4 Gy (dose rate: 1.25 Gy/min). This dose was selected as that was the dose we used previously to assess post-training irradiation effects on cognitive performance in one-month-old mice [10]. This dose could be relevant in the context of radiation therapy in cancer patients, nuclear contamination from power plants, military conflicts, and bioterrorism. The other half of the mice was placed in a new cage and received a sham-irradiation procedure by being placed into a new cage for the same duration of time. Fourteen days after training (or thirteen days after irradiation or sham-irradiation), the mice were tested for recall and extinction of conditioned fear, over a period of six days. The mice remained in the testing environment for an additional eight minutes to maintain the same 10-minute trial length in all trials. For assessment of contextual fear memory, the same environment was used as during training. For assessment of cued fear memory, a different environment was used compared to the one used during training. On day ten, recall of post-reinstatement hippocampus-dependent contextual fear memory was assessed by exposure to the training context. Mice were weighed the day after training (before irradiation), and every three days thereafter.

2.3. Experiment 2: *Cued fear conditioning*

In a separate experiment, to evaluate the contribution of non-hippocampus-dependent memory processes, thirty male mice were cognitively trained using a cued fear conditioning paradigm consisting of ten shocks. A 60-second habituation period was followed by 30-second tones (2800 Hz, 80 dB), co-terminating with 2-second 0.35 mA shocks, separated by 60-second inter-shock-intervals (ISI), and with a final 2-minute post-shock acquisition period. Twenty-four hours after training, the mice were irradiated with 4 Gy or sham-irradiated as described in Experiment 1. Two weeks (14 days) after training (13 days after irradiation), the mice were tested for recall and extinction of cued fear over eight days. Cued extinction trials consisted of the mouse being placed into an environment distinct from the one used during training (rounded walls, novel floor texture, cleaning with a 10% isopropanol solution). A 60-second baseline/habituation period was followed by five 60-second tone-presentations separated by 60-second inter-stimulus-intervals. Mice were weighed the day after training, and every three days thereafter.

2.4. Elevated Zero Maze

To determine whether potential differences in measures of anxiety might contribute to altered performance in fear conditioning tests, a subgroup of 20 mice from the cued fear experiment was tested for anxiety-like phenotype in the elevated zero maze. Because the potential anxiety phenotype in question required a temporal proximity to the fear conditioning extinction testing, mice were tested 12 days after irradiation or sham-irradiation, one day before the beginning of the extinction experiment.

The elevated zero-maze (Kinder Scientific, Poway, CA) consisted of four sections (6 cm wide), alternating between open and closed sections. Mice were placed into an open area of the maze and allowed to explore the maze for 10 minutes. An automated photo beam detection method (Kinder Motor Monitor software, Kinder Scientific, Poway, CA) was used to track mouse movements. Outcome measures were distance moved (cm) and percent time spent in the open areas.

2.5. Experiment 3: *Western blot analysis*

Amygdala and hypothalamus mouse tissues were homogenized in RIPA lysis buffer [0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 150 mM NaCl, 50 mM Tris pH 8.0] containing the phosphatase inhibitor sodium vanadate [NaV, 1mM]. Protein lysates were extracted by centrifugation and protein concentrations were calculated using the MicroBCA protein assay. Equal amounts of protein were separated on sodium dodecyl sulfate polyacrylamide (4–12% SDS-PAGE) gradient gels and transferred to a polyvinylidene difluoride (PVDF) membrane. Membrane blots were then blocked in tris-buffered saline and tween 20 (TBST) [1 × TBS, 0.1% Tween 20] containing 5% bovine serum albumin (BSA). The membranes were then incubated with c-Myc primary antibody diluted in TBST containing 1% bovine serum albumin (BSA) overnight at 4°C. Membranes were then washed in TBST [3×10min] before being incubated in secondary antibody [1:10,000 dilution] for one hour. Membranes were incubated in enhanced chemiluminescence (ECL) reagent before being exposed to CL-XPosure Film to detect protein changes. We used total protein as loading control, as

described [13], as we noticed that c-Myc protein levels did not correlate well with the levels of the house keeping protein β-actin.

2.6. Statistical analyses

Analyses were conducted using SPSS 22.0 software (Chicago, IL). Baseline measures between groups were analyzed by ANOVA, with treatment group as a between-subject variable. Comparisons of freezing, motion during shock, and body weight over time were performed as repeated measures ANOVA, with treatment as a between-subject variable and the time-unit as a within-subject variable. Cohort was added as a covariate to all analyses. Data were evaluated as to their satisfaction of assumptions for parametric statistics. For repeated measures analysis, if Mauchly's test of sphericity was violated, Greenhouse-Geisser corrections were used and reported. For pairwise comparisons, Bonferroni's posthoc tests were performed to compare selected values (between days and between groups). In case of a between-subject-variable interaction with a within-subject variable, the between-subject groups were separately analyzed to evaluate the potential difference in within-subject effects being mediated by the between-subject variable.

3. Results

3.1. Effects of irradiation on contextual fear

Baseline activity levels (prior to the first shock) were similar in both groups (Fig. 1A). To determine potential pre-existing group differences in sensitivity or perception to the aversive stimuli, average motion during the shocks was analyzed. The average motion during the shocks was also comparable and not significantly different in both groups (Fig. 1B). Acquisition of conditioned fear was assessed as immediate freezing during the inter-shock-interval (ISI) following a shock. Freezing levels during the ISIs on the training day increased with shock number in each group with no pre-existing group differences prior to radiation (Fig. 1C). As nearly all mice did not freeze immediately following the first shock, the first inter-shock interval was excluded from the analysis, as zero values can obscure parametric analysis of repeated measures.

Our study focused on between-trial extinction. Therefore, we analyzed the first five minutes of the extinction trials, to avoid the influence of habituation. Four animals (4 irradiated mice) were removed from the extinction analysis due to recording errors with the software. Two animals (1 sham-irradiated mouse and 1 irradiated mouse) were removed from the analysis based on outlier analysis (more than two standard deviations from the mean). Both groups showed extinction of contextual fear (effect of day: (F(2.118,65.651) = 3.743, p = 0.027, Fig. 2A). However, there was no radiation × day interaction, indicating that radiation did not affect the extinction of contextual fear per se. A multivariate analysis showed only a significant effect of irradiation on day 6 (F(1,31) = 4.545, p = 0.041).

We analyzed motion during the extinction trials as well (Fig. 2B). There was an effect of irradiation on average motion during the extinction trials (F(1,31) = 6.570, p = 0.015), with lower motion levels in irradiated than sham-irradiated mice. The multivariate analysis revealed effects of irradiation on days 2 (F(1,31) = 4.469, p = 0.043), 4 (F(1,31) = 6.777, p =

0.014), and 5 ($F(1,31) = 4.933$, $p = 0.034$), with a trend towards an effect of irradiation on day 3 ($F(1,31) = 4.172$, $p = 0.05$), and 6 ($F(1,31) = 3.803$, $p = 0.060$). On day ten, recall of post-reinstatement contextual fear memory was assessed. Sham-irradiated and irradiated mice showed comparable recall of hippocampus-dependent contextual fear.

3.2. Effects of irradiation on cued fear

Baseline activity levels (prior to the first shock) were similar in both groups (Fig. 3A). There were no group differences in motion during the shock, and the average motion during the shocks was comparable in both groups (Fig. 3B). Acquisition of cued fear was assessed as freezing during the inter-shock-interval (ISI) following a tone-shock pairing. Freezing levels during the ISIs during training increased with shock number ($F(7,196) = 16.767$, $p < 0.0001$) and was comparable in both groups (Fig. 3C).

During extinction, there was no difference in motion prior to the first tone during the extinction trials (not shown), which suggests that the mice did not experience any generalized anxiety differences. As our study focused on between-trial extinction, we analyzed freezing during the first three tones of the extinction trials. There was an effect of radiation ($F(1,27) = 8.146$, $p = 0.008$), with higher freezing levels in irradiated than sham-irradiated mice (Fig. 4A). Multivariate analysis revealed effects of irradiation on days 2 ($F(1,27) = 5.928$, $p = 0.022$) and 3 ($F(1,27) = 7.32$, $p = 0.012$). Sham-irradiated and irradiated mice showed similar freezing levels the day following re-instatement (not shown).

We next analyzed motion during the extinction trials (Fig. 4B). There was an effect of irradiation ($F(1,27) = 15.069$, $p = 0.001$) with lower motion levels in irradiated than sham-irradiated mice. The multivariate analysis revealed effects of irradiation on days 1 ($F(1,27) = 7.483$, $p = 0.011$), 2 ($F(1,27) = 13.614$, $p = 0.001$), 3 ($F(1,27) = 4.566$, $p = 0.042$), and 6 ($F(1,27) = 5.689$, $p = 0.0024$), with a trend towards an effect of irradiation on day 4 ($F(1,27) = 4.119$, $p = 0.052$).

3.3. Effects of irradiation on body weights and on performance in the elevated zero maze

Mice were weighed the day after training (before irradiation), and every three days thereafter. The trajectory of body weight change was significantly altered in irradiated animals, as shown by a day × irradiation interaction ($F(3.393,213.743) = 8.556$, $p = 0.000$). Multivariate analysis showed lower body weights in irradiated than sham-irradiated mice on day 7 ($p = 0.002$) and day 10 ($p = 0.036$), with a trend towards lower body weights on day 13 ($p = 0.063$, Fig. 5). Comparing the body weights on the last day with those on day 1, there was a significant group × time interaction ($F(1,38) = 10.51$, $p = 0.0025$). The effect of time was more pronounced in the irradiated ($p = 0.0065$) than the sham-irradiated ($p = 0.03$) mice. At both time point, there was no difference between the two groups (beginning weights: sham-irradiated: 25.46 g, irradiated: 25.89 g; $t(76) = 0.8031$, $p = 0.8489$; end weights: sham-irradiated: 25.62 g; irradiated: 24.97 g; $t(76) = 1.223$, $p = 0.4500$).

Anxiety and activity levels were assessed in the elevated zero maze. The percent time spent in the more anxiety-provoking open areas of the maze was not significantly different between sham-irradiated and irradiated mice ($t(14) = 1.166$, $p = 0.2632$), indicating similar

anxiety levels in the groups (Fig. 6A). However, irradiated mice showed lower overall activity levels than sham-irradiated mice ($t(14) = 3.763$, $p = 0.0021$, Fig. 6B).

3.4. Effect of irradiation on c-Myc protein in the brain

Western blot analysis was used to detect changes in c-Myc protein in irradiated animals. In the amygdala, we observed a significant decrease in c-Myc protein levels in mice that were irradiated when compared to amygdala of mice that were sham treated (Fig. 7). This decrease in c-Myc protein levels was not detected in the hypothalamus of mice that were irradiated (Fig. 7).

4. Discussion

The data of the current study shows that exposure to whole body irradiation 24 hours following cued fear conditioning training results in greater freezing levels 2 weeks after training. In addition, motion during both contextual and cued extinction trials was lower in irradiated than sham-irradiated mice. In mice trained for cued fear conditioning prior to sham-irradiation or irradiation, activity levels in the elevated zero maze 12 days after irradiation were also lower in irradiated than sham-irradiated mice. Finally, the trajectory of body weight changes was affected by irradiation with lower body weights in irradiated than sham-irradiated mice, with the most profound effect 7 days after training. These data indicate that whole body X ray irradiation causes persistent alterations in the fear response and activity levels in a novel environment, while the effects on body weight seem more transient. These data are pertinent to human radiation exposure related to nuclear accidents or subsequent cleanup efforts at nuclear power plants [14–17], military missions, and dirty bomb scenarios [3, 18, 19].

Irradiation of 3-month-old mice in the current study did not affect anxiety-like behavior two weeks after training. Therefore, it is unlikely that measures of anxiety modulate the observed effects on the fear response during the extinction trials. In contrast, enhanced measures of anxiety were observed following post-training irradiation of 1-month-old mice [10]. The enhancement in freezing levels during extinction trials of cued fear but not contextual fear suggests that limbic areas like the amygdala might be more important for this effect than the hippocampus. However, the effect of irradiation on average motion during the contextual extinction trials suggests that the hippocampus might be involved as well. Interestingly, the reverse pattern was seen in 1-month-old mice with more profound effects of post-training irradiation on freezing levels during contextual than cued extinction trials [10]. The effect of post-training irradiation on body weights was more pronounced in 1-month-old [10] than 3-month-old mice. While the effect of irradiation on body weights was more transient at 3 months of age, this effect was persistent at 1 month of age. These data indicate that the age at irradiation critically modulates the effects of post-training irradiation.

Effects of post-training irradiation on memory might not be limited to X ray or ^{137}Cs irradiation and also occur following space irradiation. Recently, detrimental effects of irradiation with ^{56}Fe (25 cGy, 600 MeV/n) or ^{16}O (5 cGy, 600 MeV/n) 2–4 hours after the training session in an object recognition test was shown to impair novelty preference in rats 18 hours after irradiation [20]. Besides the age at radiation exposure, the effects of post-

training irradiation on memory might be dependent on the cognitive task, radiation type, dose, and energy, and the interval between irradiation and memory assessment. Genetic factors might be important modulators as well. For example, training and memory testing three months following ^{28}Si irradiation showed impaired contextual fear memory in B6D2F1 mice [21] but enhanced contextual fear memory in C57BL/6J mice [22].

Our data show reduced c-Myc protein levels in the amygdala of irradiated mice that show enhanced cued fear memory and reduced activity levels in the elevated zero maze. These reduced c-Myc protein levels in the amygdala might relate to the behavioral and cognitive alterations seen in these post-training irradiated mice. Cued fear memory involves the amygdala [23–28] and the amygdala plays an important role in regulating anxiety levels [28, 29] as well. Future efforts are warranted to determine the mechanism underlying the role of c-Myc in amygdala-related behavioral and cognitive performance.

The reduced c-Myc protein levels in the amygdala of irradiated mice might be relevant to cancer as well. Pathways downstream of receptor tyrosine kinases such MAPK/ERK and PI3K/Akt/mTOR that indirectly control c-Myc protein stability are often overexpressed or mutated in brain tumors. MAPK/ERK kinase inhibitors have been shown to dephosphorylate c-Myc and reduce cell proliferation and anchorage-independent growth of rhabdomyosarcoma [30], a soft tissue sarcoma in children. mTOR occurs in a complex, mTORC1, that directly phosphorylates and inhibits PP2A which can lead to sustained c-Myc protein activity by dephosphorylating serine-62 within c-Myc's transactivation domain. Consequently, clinical inhibitors of PI3K/mTOR are used to degrade c-Myc in neuroblastoma. In gliomas where suppressor genes like p53 and Pten are inactivated, c-Myc is critical for tumor maintenance. c-Myc inhibition will suppress tumors by promoting differentiation of the glioma cells [31] and inhibiting glioma cancer stem cells [32]. While the c-Myc protein is a validated target for cancer therapies, targeting a transcription factor that lack clear binding domains is challenging. Based on our data, irradiation could play a key role in combating brain tumors by reducing c-Myc protein levels.

In summary, the data of the current study show enhanced freezing during cued fear extinction and reduced motion during contextual and cued fear extinction in mice that received whole body irradiation following acquisition of fear conditioning two weeks earlier. Activity levels in the elevated zero maze were reduced as well following post-training irradiation. More transient effects on body weight changes following post-training irradiation were observed as well. These behavioral and cognitive changes are pertinent to radiation exposure as part of a nuclear accident, military mission, or dirty bomb scenario. The mechanism underlying these post-training radiation effects might involve c-Myc protein levels in the amygdala. Future efforts are warranted to determine the detailed mechanisms underlying these post-training radiation effects.

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Highlights

- Post-training irradiation increases freezing levels
- Post-training irradiation reduces motion in extinction trials
- Post-training irradiation reduces activity levels in the zero maze
- Post-training irradiation transiently reduces body weights

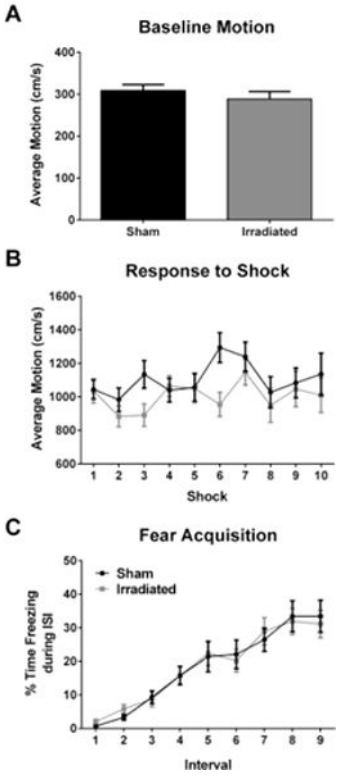

Fig. 1.

No effect of irradiation on acquisition of contextual fear. **A.** Activity during training at baseline, prior to the first shock. **B.** Average motion during the shocks during training. **In panels B and C, the black line represents sham-irradiated mice and the grey line irradiated mice. C.** Acquisition of contextual fear, analyzed as immediate freezing during the ISI following a shock. $N = 20$ mice/group.

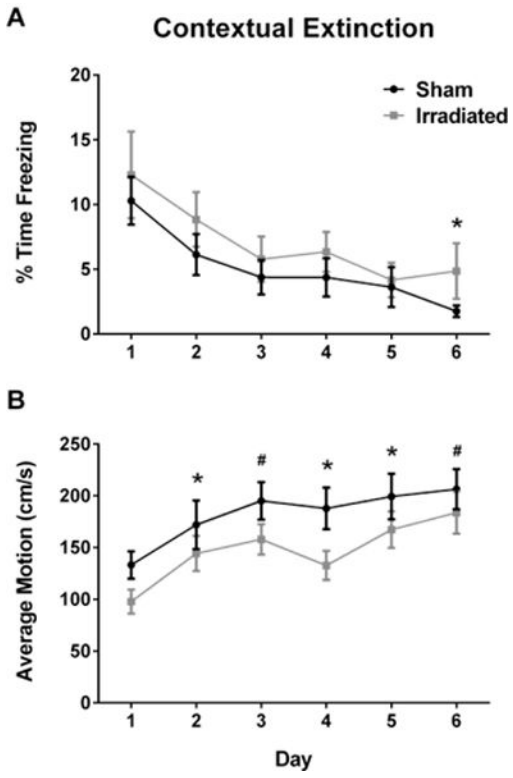

Fig. 2.

A. Effect of irradiation on extinction of contextual fear memory. **B.** Effect of irradiation on average motion during the contextual extinction trials. **In panels A and B, the black line represents sham-irradiated mice and the grey line irradiated mice. The X-axis in both panels indicate the day of measurement**. $^{*}p < 0.05$; $^{\#}p = 0.05$. $N = 16–19$ mice/group.

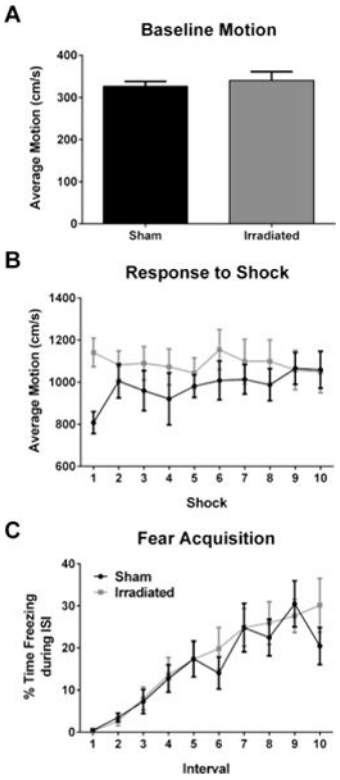

Fig. 3.

No Effect of irradiation on acquisition of cued fear. **A.** Activity during training at baseline, prior to the first shock. **B.** Average motion during the shocks on the training day. **C.** Acquisition of cued fear, analyzed as freezing during the ISI following tone-shock pairings. **In panels B and C, the black line represents sham-irradiated mice and the grey line irradiated mice.** $N = 15$ mice/group.

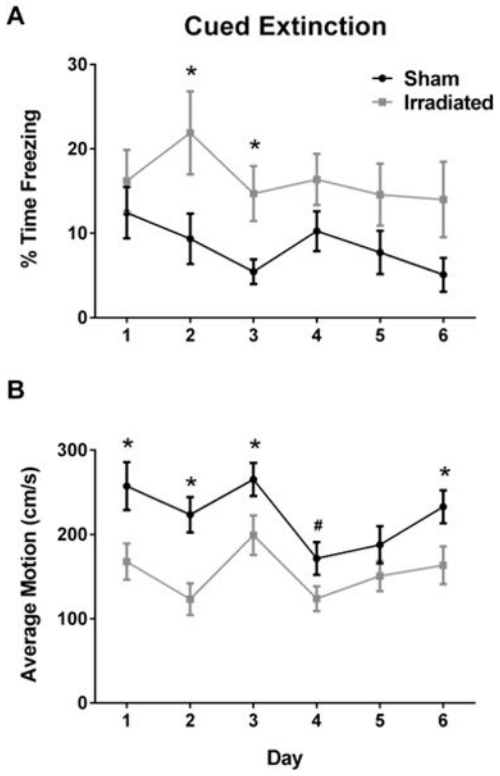

Fig. 4.

A. Effect of irradiation on extinction of cued fear memory. **B.** Effect of irradiation on average motion during the cued extinction trials. **In panels A and B, the black line represents sham-irradiated mice and the grey line irradiated mice. The X-axis in both panels indicate the day of measurement**. *$p < 0.05$; #$p = 0.05$. $N = 15$ mice/group.

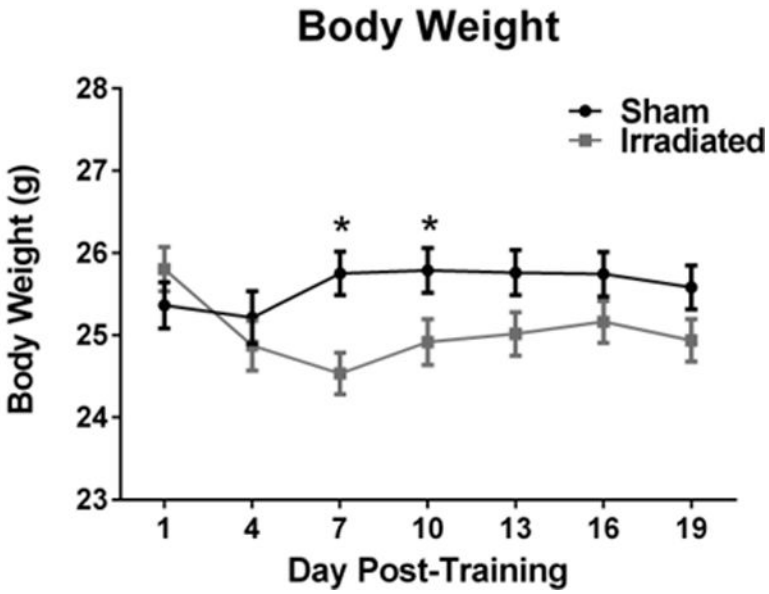

Fig. 5.

Effects of irradiation on body weights of mice trained and tested for contextual and cued fear conditioning. There was a day × irradiation interaction with significantly lower body weights in irradiated mice than sham-irradiated mice on days 7 and 10. ***p* < 0.05 versus irradiated mice on the same day**. *N* = 31–34 mice/group.

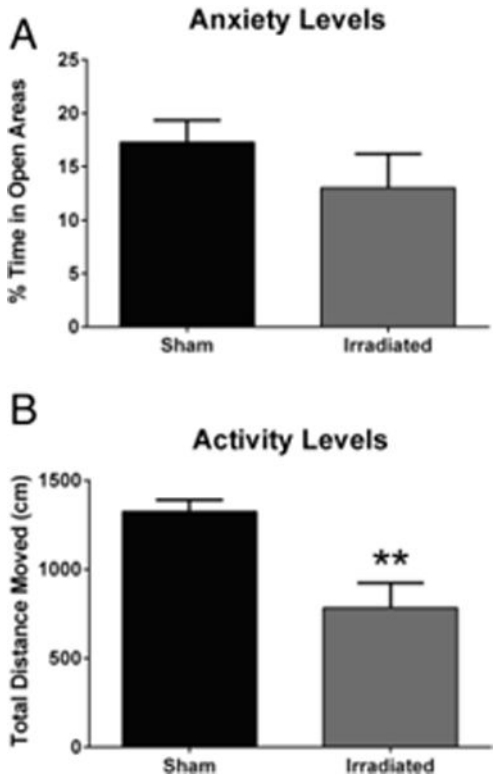

Fig. 6.

A. No effects of irradiation on measures of anxiety in the elevated zero maze. $N = 10$ mice/group. B. Effect of irradiation on activity levels in the elevated zero maze. $^{**}p < 0.01$ versus sham-irradiated mice. $N = 10$ mice/group.

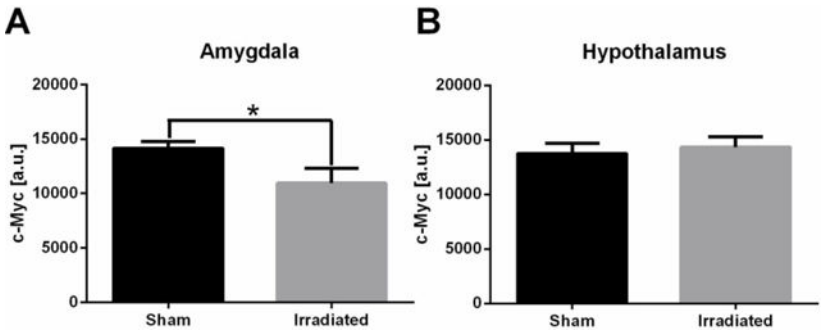

Fig. 7.

C-Myc protein levels in the amygdala (A) and hypothalamus (B) of sham-irradiated and irradiated mice. *N* = 8 mice/brain region/treatment.