Effects of Acute Radiation Exposure on Extinction of Previously Conditioned Fear in Mice

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Abstract

Because of the use of radiation in cancer therapy, the risk of nuclear contamination from power plants, military conflicts, and terrorism, there is a compelling scientific and public health interest in the effects of environmental radiation exposure on brain function, in particular hippocampal function and learning and memory. Previous studies have emphasized changes in learning and memory following radiation exposure. These approaches have ignored the question of how radiation exposure might impact recently acquired memories, which might be acquired under traumatic circumstances (cancer treatment, nuclear disaster, etc.). To address the question of how radiation exposure might affect the processing and recall of recently acquired memories, we employed a fear conditioning paradigm wherein animals were trained, and subsequently irradiated (whole-body X-ray irradiation) 24 h later. Animals were given 2 weeks to recover, and were tested for retention and extinction of hippocampus-dependent contextual fear conditioning or hippocampus-independent cued fear conditioning. Exposure to irradiation following training was associated with reduced daily increases in body weights over the 22-days of the study and resulted in greater freezing levels and aberrant extinction 2 weeks later. This was also observed when the intensity of the training protocol was increased. Cued freezing levels and measures of anxiety 2 weeks after training were also higher in irradiated than sham-irradiated mice. In contrast to contextual freezing levels, cued freezing levels were even higher in irradiated mice receiving 5 shocks during training than sham-irradiated mice receiving 10 shocks during training. In addition, the effects of radiation on extinction of contextual fear were more profound than those on the extinction of cued fear. Thus, whole-body irradiation elevates contextual and cued fear memory recall.

Mice

One-month-old male C57Bl6/J wild-type mice purchased from the Jackson Laboratory (Bar Harbor, ME, USA) were used. The mice were housed under a constant 12 h light:12 h dark cycle. Food (PicoLab Rodent Diet 20, no. 5053; PMI Nutrition International, St. Louis, MO, USA) and water were provided *ad libitum*. As the mice were 1-month old at the time of training and irradiation and tested 2 weeks later, they were 1.5-month-old at beginning of extinction. All procedures conformed to the relevant regulatory standards and were approved by Institutional Animal Care and Use Committee at Oregon Health and Science University (OHSU, Portland, OR, USA).

Contextual Fear Conditioning

Experimental Groups

For all experiments, mice were assigned to experimental group (irradiated or non-irradiated) by repeated random sorting until all initial variables were equal between the groups. After fear conditioning training, and prior to irradiation, mice were randomly sorted until all initial values (body weight, baseline freezing, freezing levels after acquisition, etc.) were not significantly different between groups.

Experiment 1

Twenty mice were trained in a contextual fear conditioning paradigm, involving five 2-s 0.35 mA shocks, separated by 2-min inter-shock-intervals (ISI), with the first shock at 2 min from the beginning of the trail. The total length of the training session was 10 min. 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, USA) for whole-body irradiation exposure. Half of the mice (irradiation group) 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). The other half of the mice was placed in a new mouse cage and received a sham-irradiation procedure by being placed into a new cage, in a similarly confined and dark space, for the same duration of time. Fourteen days after training (or 13 days after irradiation or sham-irradiation), the mice were tested for recall and extinction of conditioned fear, over a period of 8 days. On day 9, the mice received a minimal reinstatement session: after a 2-min baseline period, one 2-s 0.35 mA shock was delivered. The mice remained in the testing chamber for an additional 8 min to maintain the same 10 min trial length in all trials. All freezing data in this paper were analyzed using Med Associates software. The software analyses freezing based on a proprietary algorithm scoring with freezing defined as no movement except respiration. The next day (day 10), recall of post-reinstatement hippocampus-dependent contextual fear recall was assessed by exposure to the training context. Mice were weighed the day after training (before irradiation), and every 3 days thereafter – for a total of eight measurements over the duration of the experiment.

Experiment 2

In order to control for differences in initial freezing to the context on day 14 (extinction trial 1) affecting extinction curves, Experiment 1 was repeated with another 20 mice, as described above with the following two exceptions. A 10 shock rather than a 5 shock paradigm was used during training and the shocks were separated by a 60-s ISI rather than a 120-s ISI. The pre-exposure period was therefore 60-s. The irradiation, sham-irradiation, and other experimental conditions of contextual fear conditioning testing were as described above in Experiment 1. The intent of this experiment was to ascertain whether differences in extinction could be due to reduced recall in one group compared to the other.

Experiment 3

To evaluate the contribution of non-hippocampus-dependent memory processes, 20 male mice were trained using a cued fear conditioning paradigm consisting of 5 shocks. A 60-s habituation period was followed by 30-s tones (2800 Hz, 80 dB) co-terminating with 2-s 0.35 mA shocks, and separated by 2-min ISI, and a final 2-min post-shock acquisition period. Twenty-four hours after training, 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 8 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-s baseline/habituation period was followed by five 60-s tone presentations separated by 60-s inter-stimulus-intervals. Mice were weighed the day after training and every 3 days thereafter.

Experiment 4

As in Experiment 2, in order to ascertain whether differences in extinction could be due to reduced recall in one group compared to the other, the cued experiment was repeated using 10 shocks, with the shocks separated by a 60-s ISI, and keeping all other experimental conditions as described as in Experiment 3.

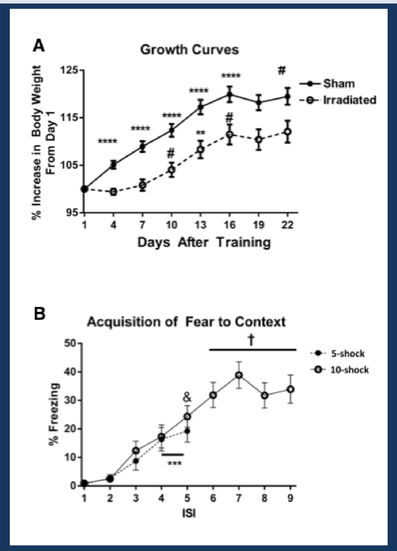
Experiment 5

Elevated zero maze. To determine whether potential differences in measures of anxiety might contribute to altered performance in fear conditioning tests, mice from Experiments 3 and 4 were 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, 1 day before the beginning of the extinction experiment. To assess the impact of irradiation on anxiety-like phenotypes in the absence of exposure to a fear-inducing event, a group of 20-animals who did not receive fear conditioning, and only received radiation treatment or were sham-irradiated, were also evaluated in the zero maze for anxiety-like behaviors. The elevated zero maze (Kinder Scientific, Poway, CA, USA) 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 min. Mice treated with a wide-range of anxiety reducing agents spend less time in the open areas. An automated photo beam detection method (Kinder Motor Monitor software, Kinder Scientific, Poway, CA, USA) was used to track mouse movements. Outcome measures were distance moved (centimeter), time spent in the open and closed areas (second) as well as crossings between the open and closed areas.

Western blot analysis. Mice from Experiments 1 and 2 were killed by cervical dislocation and their brains removed. The hippocampi were dissected and immediately frozen in liquid nitrogen for western blot analysis. Membranes were washed in TBST buffer (4× for 5 min) and incubated in 3% BSA TBST with one of the following primary antibodies for 12 h at 4°C: antibodies against MAP-2 (raised in mouse, 1 µg/ml, Millipore, Billerica, MA, USA) or β-actin antibody (raised in mouse, 0.5 µg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA). There were no effects of irradiation on β-actin levels. Membranes were washed in the TBST buffer (4× for 5 min) and were incubated in secondary antibody (Santa Cruz, goat anti-mouse-HRP, 1 µg/ml) in the 3% BSA TBST buffer for 1 h. Membranes were incubated in SuperSignal West Pico solution (Bio-Rad) for 5 min and pixel densities of specific bands of MAP-2 and β-actin for each sample were imaged and quantified using densitometry with Image Lab software (Image Lab™ Software, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Background levels were automatically determined by the software using upper- and lower-edge interpolation. The MAP-2 and β-actin bands were measured for each sample. Antibodies were stripped from the membranes using Restore Western Blot stripping buffer (Thermo Scientific) for 5 min at room temperature and re-blocked in 3% BSA TBST blocking buffer for 1 h. β-actin was used as a loading control for each membrane. Data were analyzed as a ratio between the MAP-2 and β-actin bands compared to sham-irradiated levels for a specific blot.

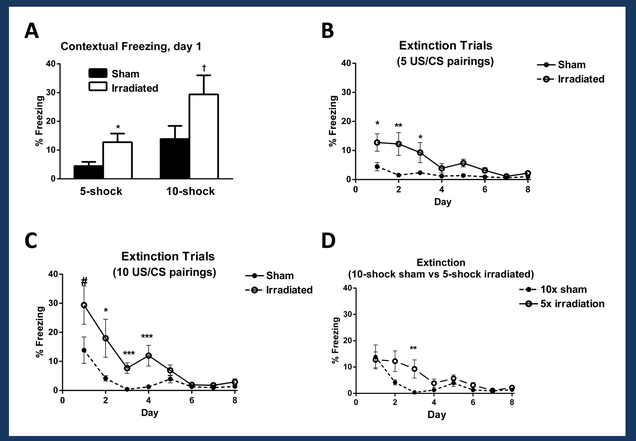
Statistical analyses. Analyses were conducted using SPSS 16.0 software (Chicago, IL, USA). Baseline measures between groups were analyzed by ANOVA, with treatment group as a between-subject variable. Comparisons of freezing, motion during shock, and body weight over time were performed as repeated measures ANOVA, with treatment as a between-subject variable and the time-unit as a within-subject variable. Data were evaluated as to their satisfaction of assumptions for parametric statistics. If the data were skewed or otherwise non-normally distributed or did not have equal variances, appropriate transformations were applied. For repeated measures analysis, if Mauchly's test of sphericity was violated, multivariate statistics reporting Welch's lambda (λ) were reported. For pairwise comparisons, Dunnett's *post hoc* were performed to compare selected values (between days and between groups). In the 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.

Effects of irradiation on body growth



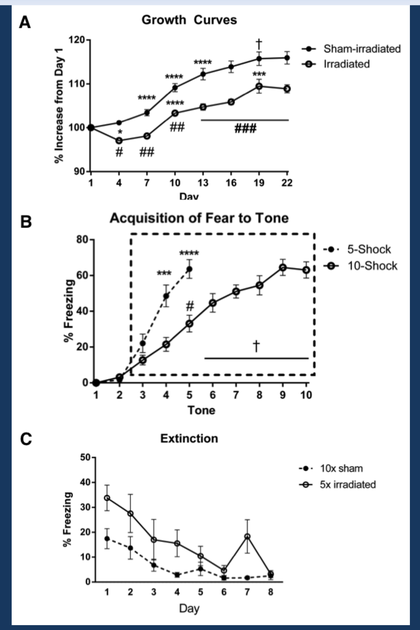
Effects of irradiation on body growth in mice trained and tested for contextual fear conditioning. *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001. (B) Acquisition of contextual fear conditioning, analyzed as immediate freezing during the ISI following a shock. ***p = 0.002 (vs. ISI 3), &p = 0.025 vs. ISI 3, +p < 0.0001 vs. ISI's 3, 4, and 5.

Contextual Extinction



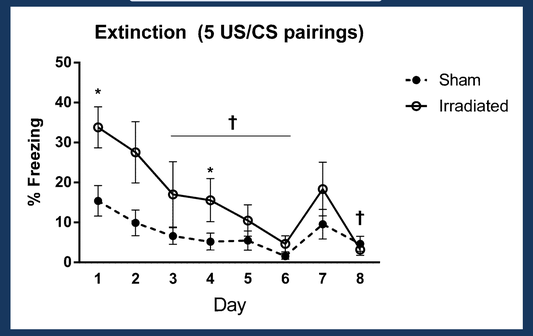
(A) Contextual freezing levels on day 1 (24 h after training) were higher in irradiated than sham-irradiated mice. *p = 0.021, +p = 0.083. **(B)** Extinction of contextual fear conditioning in sham-irradiated and irradiated mice that received five tone-shock pairings (Experiment 1) during training. *p < 0.05, **p < 0.01. **(C)** Extinction of contextual fear conditioning in sham-irradiated and irradiated mice that received 10 tone-shock pairings (Experiment 2) during training. *p < 0.01, ***p < 0.0001, #p = 0.083. **(D)** Extinction of contextual fear conditioning in sham-irradiated mice that received 10 tone-shock pairings during training and irradiated mice that received 5 tone-shock pairings during training. *p < 0.01.

Body Weights and Cued Fear Learning and Extinction



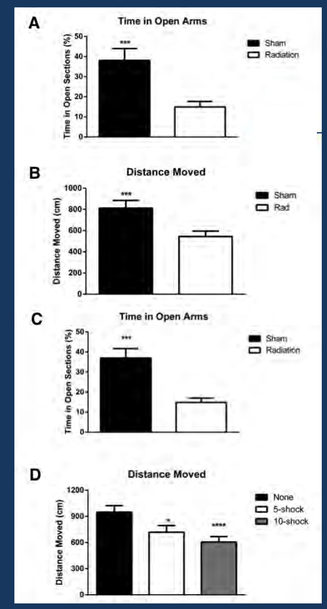
Effects of irradiation on body weights of mice trained and tested for cued fear conditioning. *Comparisons between days, #comparisons between groups. *p < 0.05, ***p < 0.001, ****p < 0.0001, #p < 0.01 = , ##p < 0.0001, +p = 0.090, &p = 0.076. **(B)** Acquisition of fear to the tone. ****p < 0.0001, #p = 0.005, +p < 0.0001. Freezing levels during ISI 6–10 were significantly greater than those during ISI 3 (ISI 6 vs. 3, p = 0.007; ISIs 7, 8, 10 vs. 3, p < 0.0001; ISI 9 vs. 3, p = 0.001), and freezing during ISIs 7, 8, 9, 10 were higher than those during ISI 4 (ISI 7 vs. 4, p = 0.001, ISI 8 vs. 4, p = 0.004, ISI 9 vs. 4, p = 0.012, and ISI 10 vs. 4, p = 0.028), ISI 5 (ISI 7 vs. 5, p = 0.002, ISI 8 vs. 5, p < 0.0001, ISI 9 vs. 5, p = 0.03, and ISI 10 vs. 5, p = 0.020). **(C)** Comparison of freezing levels between irradiated and sham-irradiated animals during extinction of cued fear. *p < 0.05.

Cued Extinction



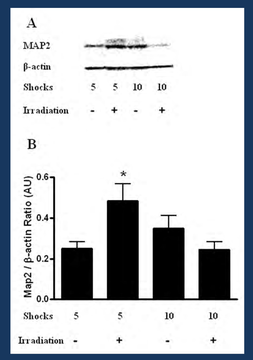
+Sham-irradiated mice showed decreases in freezing compared to day 1 from day 3 except for day 7 (day 3, p = 0.018; day 4, p = 0.004; day 5, p = 0.0033; day 6, p = 0.0002; day 8, p = 0.0022). Day 7 exhibited a trend toward a decrease compared to day 1 (p = 0.06). Irradiated animals showed decreases in freezing compared to day 1 on day 3 (p = 0.0137), with decreases occurring on days 5 (p = 0.0015), 6 (p = 0.0002), 7 (p = 0.0104), and 8 (p = 0.0001). Step-wise decreases in freezing were not observed in either group. *Freezing was elevated in the irradiated mice relative to sham on days 1 (p = 0.008) and 4 (p = 0.022).

Measures of Anxiety



(A) Effect of irradiation on measures of anxiety of mice trained and tested for cued fear conditioning in the elevated zero maze. Irradiated mice showed enhanced anxiety levels and spent less time in the more anxiety-provoking open areas. ***p < 0.001. **(B)** Effects of irradiation on activity levels of mice trained and tested for cued fear conditioning in the elevated zero maze. Irradiated mice moved less than sham-irradiated mice. ***p = 0.002. **(C)** Effects of irradiation on measures of anxiety of a combined group of behaviorally naïve mice and mice trained and tested for cued fear conditioning in the elevated zero maze. Irradiated mice showed enhanced anxiety levels and spent less time in the more anxiety-provoking open areas of the maze. ***p < 0.0001. **(D)** Effects of number of shocks on activity levels of mice in the elevated zero maze. Mice that had received shocks showed lower activity levels than those that did not and this was more pronounced in mice that had received 10 shocks than those that had received 5 shocks. *p = 0.001, ****p < 0.0001.

MAP-2 Western Blot



(A) Representative western blot of hippocampal MAP-2 levels in sham-irradiated and irradiated mice that received 5 or 10 shocks during training. **(B)** There was a radiation × shock interaction with higher hippocampal MAP-2 levels in irradiated mice that received 5 shocks than is sham-irradiated mice that received 5 shocks and irradiated mice received 10 shocks. *p < 0.05 vs. sham-irradiated mice that received 5 shocks and irradiated mice received 10 shocks.

Conclusions

- These data show enhanced fear memory, reduced extinction, and enhanced anxiety levels in mice that received whole-body irradiation following acquisition of fear conditioning 2 weeks earlier.
- Recently, we reported enhanced synaptic plasticity in the CA1 region of the hippocampus and enhanced contextual fear memory in mice trained and tested for contextual fear memory 3 months following ^{28}Si irradiation. Irradiated mice that received five shocks during trained showed increased hippocampal MAP-2 levels as compared to sham-irradiated mice. Consistent with these data, MAP-2 levels in the dentate gyrus were increased following brain only ^{56}Fe irradiation. In this study, the mice received two tone-shock pairings during fear conditioning training, performance was assessed in additional tests, and the brains were analyzed 3 months following irradiation. The radiation-induced increase in MAP-2 levels might be part of a compensatory change. Increased MAP-2 levels have been also seen in the hippocampus of non-human primates and brains of aged mice. These radiation-induced increased hippocampal MAP-2 levels were not seen in irradiated mice that received 10 shocks. Additional aversive stimuli during training might prevent this increase. The discrepancy in the pattern of acquisition and extinction and hippocampal MAP-2 levels suggests that changes in this marker of dendritic morphology cannot explain the effects of irradiation or the number of shock on cognitive function 2 weeks after irradiation.
- These behavioral and cognitive changes are pertinent to radiation exposure as part of a nuclear accident, military mission, or dirty bomb scenario, and reminiscent to symptoms seen in PTSD, a common and debilitating anxiety disorder (frequently comorbid with other mental disorders).
- Future efforts are warranted to determine the molecular mechanisms underlying these post-training radiation effects.

ACKNOWLEDGEMENTS

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