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Sex-Specific Effects of a Wartime-Like Radiation Exposure on Cognitive Function

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Evaluating the risk for central nervous system (CNS) effects after whole-body or partial-body irradiation presents challenges due in part to the varied exposure scenarios in the context of occupational, accidental or wartime releases. Risk estimations are further complicated by the fact that robust changes in brain function are unlikely to manifest until significantly late post exposure times. Collectively, the current data regarding CNS radiation risk are conflicting in humans and a survey of the animal model data shows that it is similarly inconsistent. Due to the sparseness of such data, the current study was conducted using male and female mice to evaluate the brain for the delayed effects of a 2 Gy whole-body exposure to γ rays starting six months postirradiation. Behavioral testing indicated sex-specific differences in the induction of anxiety-like behaviors and in the ability to abolish fear memories. Molecular analyses showed alterations in post-synaptic protein levels that might affect synaptic plasticity and increased levels of global DNA methylation, suggesting a potential epigenetic mechanism that might contribute to radiation-induced cognitive dysfunction. These data add to the understanding of the CNS response to whole-body irradiation and may lead to improved risk assessment and provide guidance in the development of effective radiation countermeasures to protect military personnel and civilians alike.

INTRODUCTION

The age of the atomic bomb ushered in many changes for humankind, and certain concerns regarding the potential risks of adverse health effects after exposure to ionizing radiation persist to this day. Since the advent of the splitting, then fusing, of atomic nuclei, we have learned to capitalize on the ionizing properties of select radiation types for energy production, industrial applications and medical diagnostics and therapeutics. Unfortunately, many of these benefits come at a cost, exemplified by accidental industrial releases such as that experienced at Fukushima Daiichi in 2011 or through the realistic threats of a terrorist-mediated radiologic attack. Whether accidental or intentional, establishing the health risks associated with radiation exposures is extremely complicated, and confounded by a variety of biological and sex-specific factors that affect radiosensitivity, as well as dosimetry, and the difficulties of accounting for differences in radiation quality, dose rate and absorbed dose-depth profiles in an exposed population (*1–4*). The varied exposure scenarios that include whole- or partial-body irradiation in the context of occupational, accidental or wartime releases, or cranial irradiation in the context of medical procedures, underscore the complexities of risk estimation.

It has been well documented that clinically relevant radiotherapy paradigms used in the treatment of glioma and secondary malignancies of the brain induce persistent and progressive cognitive impairments that manifest long after the cessation of treatment (*5–7*). These clinical fractionated irradiation paradigms typically employ relatively high doses (~60 Gy) of low-linear energy transfer (LET) X rays or protons (*8*), which induce cognitive impairments that adversely affect quality of life for many cancer survivors. Similarly, whole-body radiation exposures also raise concerns for health risks. For many clinical occupations, including radiologists, radiology technicians and imaging practitioners, as well as power plant workers and even airline pilots and flight attendants, it is possible to be exposed to radiation doses that can affect brain function (*9*). However, the majority of humans, though, the greatest risks of radiation exposure would involve accidental releases or terrorist-mediated radiological events able to expose large numbers of military personnel and civilians to a range of whole- and partial-body exposures. In the event of nuclear fallout or a dirty bomb blast, radiation types are likely to include gamma rays, X rays, α- and β particles or neutrons emitted from nuclear interactions and isotopic decay. It is well documented that relatively high-dose exposures (≥6

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Gy) in such scenarios have the potential to induce acute radiation syndromes that affect the hematopoietic system or gastrointestinal tract and require immediate medical intervention (*10*). A less tangible, but problematic outcome of such exposures may also include an increased lifetime risk of secondary radiogenic cancers (*11*). A third, but largely undefined late-risk of non-lethal exposures is injury to the central nervous system (CNS) that results in cognitive impairments. While therapeutic cranial radiation exposures have consistently been demonstrated to elicit impairments in learning and memory, and increased anxiety- or depression-like behaviors (*12*), lower-dose diagnostic and/or occupational radiation exposures to the cranium and/or whole body have led to inconsistent CNS results, largely owing to differing mammalian model systems, variable behavioral testing paradigms and inappropriate times of postirradiation analyses (i.e., too early). These realities have led to a misconception that CNS deficits only manifest after lethal irradiation, which is inaccurate.

Evaluating the CNS effects after lower dose whole- and/or partial-body irradiation scenarios presents challenges, in particular due to the lack of robust changes that transpire early after exposure. Possibly for this reason, there is little information regarding the risk of adverse effects on the brain after exposures used to simulate an occupational, deliberate or accidental radiation release. In addition, because a significant number of first responders, cleanup workers and civilians could also be at risk, this scenario takes on even greater importance. If cognitive impairments manifest at lower doses and progress over time they could clearly impact an individual's ability to adapt and respond in stressful situations. As impairments continue to manifest or worsen, they present a clear and adverse effect on longer-term quality of life, including increased risks of developing mood disorders, learning and memory impairments, as well as progressive neurodegenerative disease-like symptoms (*13–15*). For these reasons adult male and female mice were used in this study to evaluate the brain for the delayed effects of a whole-body gamma-ray exposure that would not induce acute radiation syndromes. Understanding the CNS response to this radiation exposure scenario will improve risk assessment and provide guidance in the development of effective radiation countermeasures to protect military personnel and civilians alike.

MATERIALS AND METHODS

Animals and Irradiation

All animal experimental procedures were conducted in accordance with the guidelines provided by the National Institutes of Health and approved by the University of California Irvine Institutional Animal Care and Use Committee. Animals were maintained in standard housing conditions (20°C ± 1°C; 70% ± 10% humidity; 12:12 h light-dark schedule) and provided *ad libitum* access to standard rodent chow (Teklad 2020x; Envigo RMS Inc. Indianapolis, IN) and water. Single cohorts of 2-to-2.5-month-old wild-type male mice or female mice (C57BL/6J; Jackson Laboratory, Bar Harbor, ME) were randomly assigned to either 2 Gy irradiated (N = 12 females, N = 14 males) or sham-irradiated concurrent control (N = 12 females, N = 12 males) experimental groups and acclimated in the vivarium for approximately two weeks prior to irradiation. Mice were lightly restrained in a well-ventilated Lucite® irradiator pie cage for whole-body 2 Gy exposure using a ^{137}Cs gamma-ray irradiator at a dose rate of 2.07 Gy/min (JL Shepherd & Associates, San Fernando, CA). Concurrent control mice were placed into the pie cage and irradiator for the same length of restraint and exposure time as that required to deliver the 2 Gy dose.

Behavioral Testing

Behavioral studies were initiated at 6–7 months postirradiation where male mice were tested separately from female mice. For one week prior to behavioral studies, the lead investigator, blinded to the animal grouping, handled all mice for habituation. Testing occurred over a two-week period and included the following paradigms in the following order: elevated plus maze (EPM), light-dark box (LDB), forced swim test (FST) and fear extinction (FE). Independent investigators, blinded to the experimental groups, scored all behavior videos. These behavioral testing paradigms measure anxiety and despair, as well as fear learning and memory and fear consolidation, which involve interplay between cellular circuits of various brain regions, including frontal cortex, hippocampus and amygdala (*16, 17*).

The EPM and LDB tests are based on the tendency of anxious rodents to avoid open or brightly-lit areas and to exhibit reluctance to explore open environments, resulting in reduced amounts of time spent in the open arms of the EPM or in reduced numbers of transitions between the dark and light compartments of the LDB testing arena (*18, 19*). The EPM consists of two open arms and two closed arms arranged such that the two open arms are opposite each other and at 90 degrees to the closed arms in the shape of a plus sign, elevated 40 cm off the floor, placed in a brightly-lit (915 lux) room (*18*). Each mouse was placed in the neutral center zone of the plus maze and allowed to explore for 5 min. Anxiety-like behavior was scored as the percentage time spent in the open arms of the maze compared to the closed arms. After EPM, on the next day, mice were tested on LDB, where anxiety was measured by a mouse's willingness to transition freely between a large well-lit chamber (30 × 20 × 27 cm, 915 lux) through a 7.5 × 7.5 cm opening to a smaller, dimly-lit chamber (15 × 10 × 27 cm, 4 lux). Fewer transitions during the 10-min test would suggest increased anxiety-like behavior (*19*).

Mice were next tested for depression-like or despair-like behavior using the FST, where the mouse was placed in a beaker of water such that the mouse could not escape from the beaker, nor touch the bottom (15 cm diameter × 20 cm, 22°C). Each mouse underwent a 5-min test that was scored for the time climbing or swimming as opposed to the time immobile or floating where the mouse moved only enough to keep the nose above water. An increase in the percentage test time engaged in floating suggested increased despair-like behavior (*20*). For all of these tests, N = 12–14 mice/group, as described in the ''Animals and Irradiation'' section.

The final behavior test administered was the FE test where two contexts (A and B) were used to determine whether mice could learn and then extinguish conditioned fear responses (*21*). The conditioning test chamber (context A; 17.5 × 17.5 × 18 cm; Coulbourn Instruments, Holliston, MA) had a steel grid floor and the scent of 10% acetic acid in water, while the extinction chamber (context B) had a smooth Plexiglas® floor, additional stimulus lighting and the scent of 10% almond extract in water. Digital cameras were mounted in the ceiling of each chamber and connected via a quad processor for automated scoring of freezing (FreezeFrame, Coulbourn Instruments). For each mouse, the fear conditioning protocol started with a 120-s pre-fear conditioning incubation followed by three pairings of a 120 s, 80-dB, 16-kHz white noise conditioned stimulus (CS) co-terminating with a 1 s, 0.6-mA foot shock (US), presented at 2-min intervals (day 1, T_1–T_3). For extinction, each mouse was exposed to context B where they were

allowed to acclimate for 2 min, and then, extinction training comprised 15 non-reinforced 120 s CS presentations at 5 s intervals. Fear extinction data are presented as the average of five tones. Extinction training was repeated on each of three days. Subsequently, retention testing was performed on day 5 at which time each mouse was returned to context B where, after a 2-min acclimation, freezing was assessed during three non-US reinforced CS tones (16 kHz, 80 dB, lasting 120 s) at 2-min intervals. Extinction memory was calculated as the percentage of time spent freezing during the tests. For this test, N = 8–12 mice/group.

Immunohistochemistry

Immediately after behavior studies were completed, immunohistochemical analyses were performed on a subset of the same mice that had been used in the behavior studies. Mice were deeply anesthetized with isoflurane and euthanized via intracardiac perfusion using 4% paraformaldehyde (Acros Organics™, NJ) in 100 m*M* phosphate buffered saline (PBS, pH 7.4; Gibco®, Grand Island, NY). Brains were cryoprotected (10–30% sucrose gradient) and sectioned coronally into 30 μm using a cryostat (Leica Microsystems, Nussloch, Germany). For each end point, 3–4 representative coronal brain sections from each of 3–4 animals per experimental group were selected at approximately 15 section intervals and stored in Tris-buffered saline (TBS, 100 m*M*, pH 7.4, Sigma-Aldrich®, St. Louis, MO). For the immunofluorescence labeling of PSD-95 and microglial activation marker CD68 mouse anti–PSD-95 (1:1,000; Thermo Scientific™, Waltham, MA) and rat anti-mouse CD68 (1:500; AbD Serotec, Raleigh, NC) primary antibodies were used with Alexa Fluor® 594 secondary antibody (1:1,000). Tissues were then DAPI nuclear counterstained and sealed in slow-fade/antifade mounting medium (Life Technologies, Grand Island, NY). Similarly, for the immunofluorescence of 5mC and 5hmC, mouse monoclonal anti-5mC (1:2,000; EpiGentek Group Inc., Farmingdale, NY) and rabbit polyclonal anti-5hmC (1:5,000; Active Motif®, Carlsbad, CA) primary antibodies were used with Alexa Fluor 594 secondary antibody, respectively, at a dilution of 1:750.

Confocal Microscopy, Image Processing and Three-Dimensional Quantification

The immunostained brain sections were scanned using a confocal microscope (Nikon Eclipse Ti C2) equipped with a 40× PlanApo oil-immersion lens (1.3 NA, Nikon® Instruments Inc., Melville, NY) and a NIS-Elements AR interface version 4.30 (Nikon Instruments). A total of 30 z stacks (1,024-bit depth) at 0.5 μm from three different fields (318 × 318 × 24 μm) in each section were imaged from the dentate gyrus (DG) or from the amygdala. The digitized z stacks were deconvoluted using AutoQuant software version X3.0.4 (Media Cybernetics Inc., Rockville, MD). An adaptive, 3D blinded method was used to create deconvoluted images for direct import into the Imaris module, version 8.1.2 (Bitplane AG, Zurich, Switzerland). The 3D algorithm-based surface rendering and quantification of fluorescence intensity for each fluorescently labeled marker was performed in Imaris at 100% rendering quality. Each channel was analyzed separately. The 3D surface rendering detects immunostained puncta or nuclear staining (DAPI) satisfying pre-defined criteria, for the puncta size (0.5–1 μm) and verified visually for accuracy. Using deconvoluted confocal z stack volume from the control group (nonirradiated) as a baseline for the minimum thresholding, a channel mean intensity filter was applied and used for all the experimental groups for each batch of molecular markers. The pre-set parameters were kept constant throughout the subsequent analysis of immunoreactivity for each antigen. To maintain uniformity among the varying number of puncta for each individual time point and/or antigen analyzed, the number of puncta per 318 × 318 × 24 μm was normalized to control and data were expressed as a mean immunoreactivity (percentage) relative to nonirradiated controls.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 6 (LaJolla, CA). Unpaired Student's *t* tests were used to determine significance between control and irradiated groups of mice where $P \leq 0.05$ was considered statistically significant. Given that the distribution of behavior data is gamma distributed, we also applied a non-parametric approach, kernel density estimation (KDE), to calculate the proportion of mice that exhibited impaired performance within each cohort for a given behavior task (*22*). Analogous to the Z-score principle, a performance level that encompassed 95% of the probability density of the control cohort's performance data was selected as the threshold level, below which an individual animal's performance would be classified as ''severely impaired''.

RESULTS

Radiation has been shown with some consistency to induce impairments in learning and memory, and increased anxiety- or depression-like behaviors after charged particle or clinically relevant exposures (*12, 23*). Similarly, differences in the sensitivity of the CNS to such exposures have been reported between males and females (*4, 24, 25*). Conversely, even relatively higher whole-body or head-only exposures to X rays or gamma rays have been inconsistent in eliciting CNS responses (*26–28*). To determine whether 2 Gy whole-body gamma-ray irradiation might also trigger long-term anxiety- and depression-like behavior, male and female mice were administered the elevated plus maze, light-dark box and forced swim tests at 6–7 months postirradiation (EPM, LDB and FST, respectively; Fig. 1). The EPM and LDB tests are based on the tendency of anxious rodents to avoid open or brightly-lit areas and to exhibit reluctance to explore those open environments. These tendencies result in reduced amounts of time spent in the open arms of the EPM or in reduced numbers of transitions between the dark and light compartments of the LDB testing arena (*19*). Similarly, the FST provides a measure of despair- or depression-like behavior where the animal stops trying to escape the aversive environment of the water-filled beaker and spends an increased percentage of the test time immobile. While EPM and LDB testing showed no effect of irradiation for the male mice, the irradiated female mice exhibited significantly increased anxiety-like behavior on both tests ($P < 0.05$; Fig. 1A and B). Conversely, no differences between irradiated and control mice of either sex were observed on the FST where the analysis of time spent floating is an indicator of depression-like behavior (Fig. 1C). These data demonstrate that female mice may be at greater risk for exhibiting anxiety-like behavior at delayed times after exposure to a military relevant 2 Gy dose of gamma rays.

Fear extinction memory refers to an active process of dissociating a learned response to a prior adverse event that facilitates coping in unpleasant or stressful situations. During the day-1 conditioning phase of FE, control and irradiated mice were administered three tone-shock pairings. All groups of mice showed comparable learning as demonstrated by similar amounts of time spent freezing

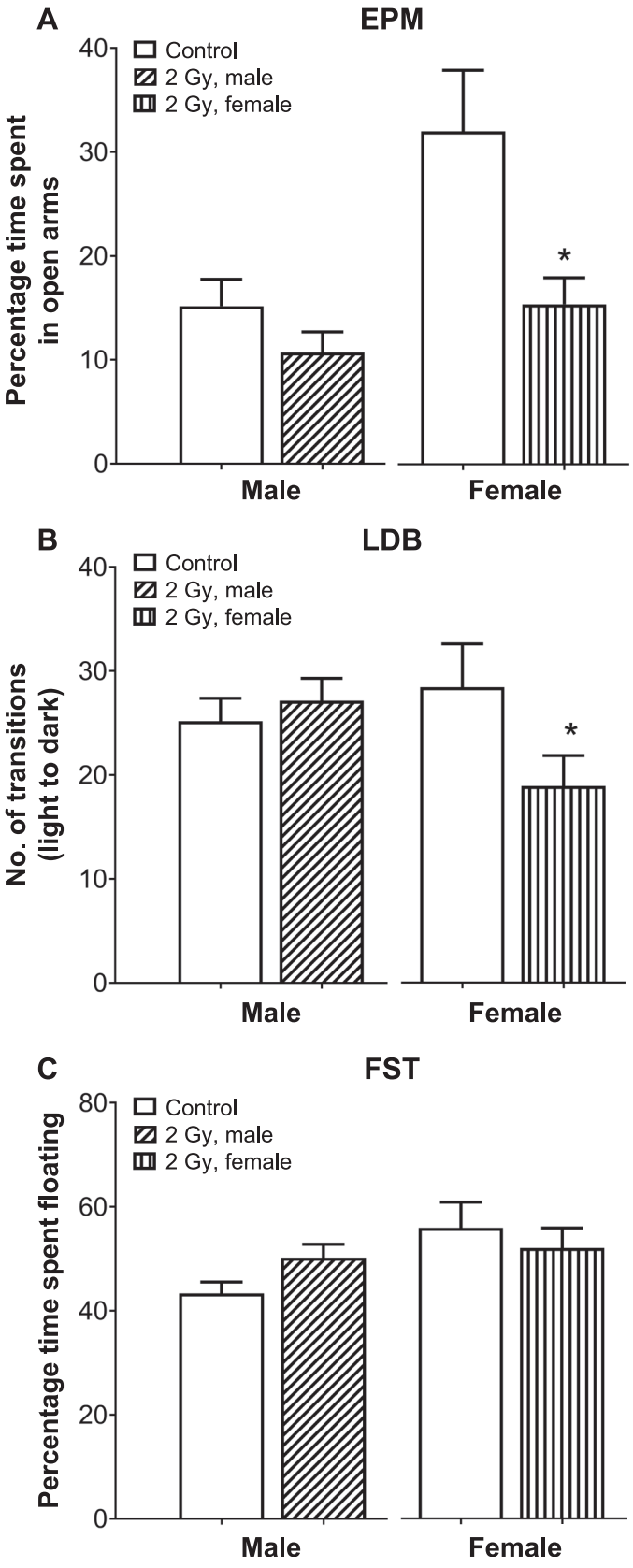

FIG. 1. Whole-body exposure to 2 Gy ^{137}Cs gamma rays elicits anxiety-like behavior in female mice 6–7 months postirradiation. Increased anxiety-like behavior was observed in irradiated female mice, but not male mice, as demonstrated by (panel A) reduced time spent in the open arms of the EPM and (panel B) reduced numbers of transitions between the light and dark chambers in the LDB test. Panel C: Neither male nor female irradiated mice showed depression-like behavior on the FST. Data are presented as mean ± SEM (N = 12 mice/group for male and female controls and for the irradiated female mice; N = 14 mice/group for the irradiated male mice). Unpaired Student's *t* test, $*P < 0.05$.

during this tone-shock conditioning phase (Fig. 2A and C; T_1–T_3). Subsequently, the mice exhibited a gradual decrease in freezing behavior over the three extinction training days (15 tones/day, data shown as average of 5 tones). However, both male and female mice that had been irradiated maintained relatively higher freezing levels on extinction training days 2 and 3 compared to their respective nonirradiated controls ($P < 0.05$). At 24 h after completion of extinction training, the male and female mice underwent extinction testing (three tones only, spaced by 120 s). Male mice that had been irradiated months earlier exhibited abolished fear memory as demonstrated by freezing behavior that was indistinguishable from that of the control males (Fig. 2B). The irradiated female mice, however, demonstrated an inability to abolish fear memories during this retrieval testing and exhibited increased freezing again ($P < 0.05$; Fig. 2D). This freezing behavior during the extinction test is reflective of elevated anxiety or an almost post-traumatic stress disorder (PTSD)-like behavior.

Together, these behavioral testing data indicate alterations in learning, anxiety and the ability to extinguish fear memories in the female mice at delayed times postirradiation that are reflective of PTSD-like symptoms. Outcomes such as these would clearly be detrimental to military personnel, first responders and civilians with regards to on-the-job performance and in the long term with regards to quality of life. However, cohort-averaged data do not indicate how frequently or dramatically cognitive performance is affected in a given population. Therefore, the individual mouse behavior data were also analyzed using kernel density estimation (KDE) to generate a performance probability profile that was then used to determine the percentage of irradiated mice that exhibited significantly altered behavior, defined as performance in a task $\geq$5th percentile of the performance observed for the control mice (*22*). Analyses of EPM, LDB and FST data by KDE did not show significant changes. Strikingly however, KDE demonstrated that 33.6% of irradiated female mice were impaired on the FE test relative to the 5% impairment threshold applied to control mice ($P < 0.05$, Fisher's exact test; Fig. 2D) and the 8.9% impairment exhibited by irradiated male mice (Fig. 2B). These data suggest an absolute relative risk for the irradiated females of 28.6% compared to 3.9% for the irradiated male mice. The numbers-needed-to-harm (NNH) algorithm was then applied to the KDE data (*22*) and predicted that one in every four irradiated females would exhibit difficulties with this

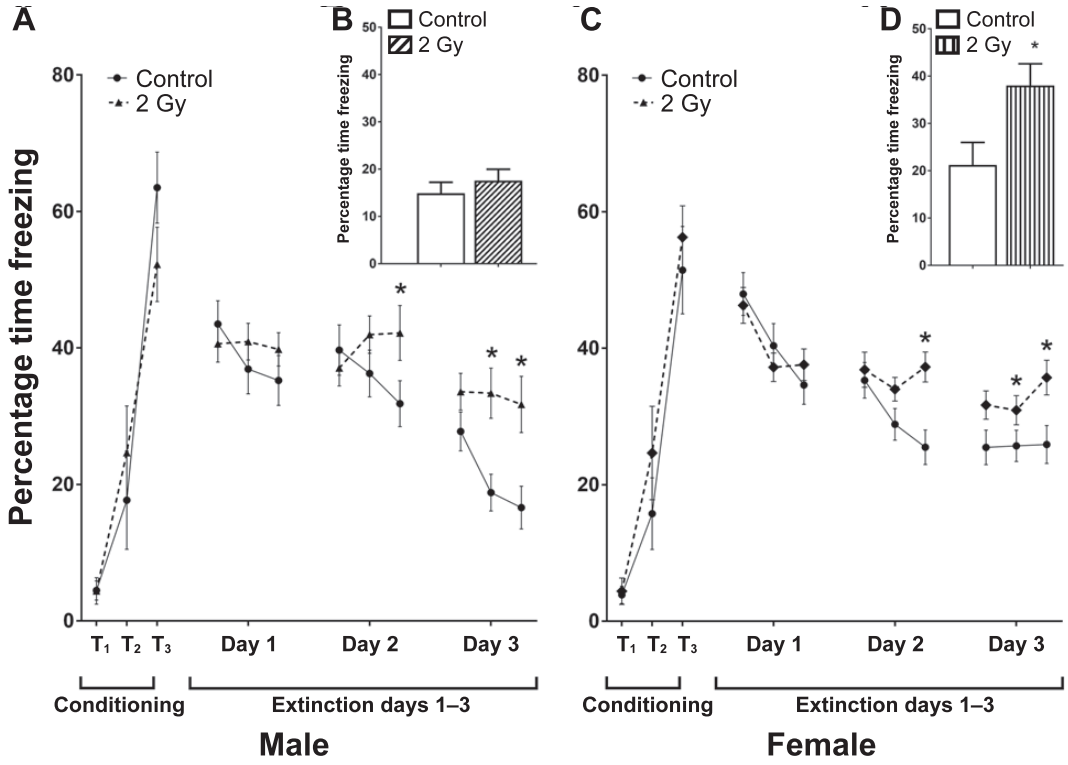

FIG. 2. Whole-body irradiation compromised fear extinction memory and enhanced memory recall in female mice. All mice showed elevated freezing after a series of three tone-shock pairings (0.6 mA, T_1–T_3). Subsequently, fear extinction training was administered every 24 h (15 tones) for 3 days. These data are presented as the average of five tones (panels A and C). Both male and female mice showed a gradual decrease in freezing behavior (days 1–3); however, irradiated mice spent significantly more time freezing compared to controls on extinction training days 2 and 3. At 24 h after extinction training male mice (panel B) exhibited successful extinction on the fear test, while irradiated female mice (panel D) exhibited enhanced fear recall. Data are presented as mean ± SEM (N = 8–12 mice/group). Unpaired Student's *t* test, $*P < 0.05$.

PTSD-like behavior compared to one in every 26 irradiated males.

Post-synaptic density protein 95 (PSD-95) is an excitatory-associated synaptic protein involved in plasticity, the level of which can be influenced by radiation exposure (*29, 30*). Because PSD-95 is responsible for recruiting receptors and other proteins to the synaptic cleft, changes in expression level of PSD-95 may disrupt neurotransmission in a manner that could contribute to cognitive dysfunction. After completion of behavior testing, PSD-95 protein levels were analyzed in the DG region of the hippocampus and in the amygdala. These analyses showed that whole-body, 2 Gy γ irradiation induced decreased protein levels in the DG at 7–8 months postirradiation in both irradiated male mice (Fig. 3A–C; $P < 0.05$) and irradiated female mice (Fig. 3A, D and E; $P < 0.01$). Conversely, whole-body, 2 Gy gamma-ray irradiation induced significantly increased protein levels in the amygdala at 7–8 months postirradiation in irradiated male mice (Fig. 3F–H; $P < 0.001$) and irradiated female mice (Fig. 3F, I and J; $P < 0.01$). The functional relevance of altered PSD-95 protein levels in the irradiated brain has not been determined, but it may suggest a possible loss of synaptic integrity. Given that the learning and memory necessary for fear conditioning and extinction rely upon functional neural circuitry between the hippocampus and amygdala (as well as the medial prefrontal cortex) (*31*), the observed dysregulation of PSD-95 in both of these regions of the brain may contribute to the impairments observed on fear extinction testing in this study (Fig. 2). Barring direct evidence for that, changes in PSD-95 protein levels appear to provide a biomarker for irradiation even at very late postirradiation times.

Microglial activation has been used as a marker for neuroinflammation in multiple irradiation scenarios, and that neuroinflammation is thought to contribute to radiation-induced cognitive dysfunction. Using CD68 as a marker of microglial activation, a modest, but statistically significant increase in CD68 protein level was observed in the DG region of the irradiated hippocampus of male mice at 7–8 months postirradiation (Fig. 4A–C; $P < 0.01$), which was not observed in the DG of irradiated female mice (Fig. 4A, D and E). Similar analysis of the amygdala indicated no increase in CD68 immunoreactivity in the irradiated male mice compared to controls (Fig. 4F–H), but a significant increase in CD68 immunoreactivity was observed in the amygdala of irradiated female mice (Fig. 4F, I and J; $P < 0.05$). These findings reinforce the general supposition that elevated inflammation contributes to radiation-induce

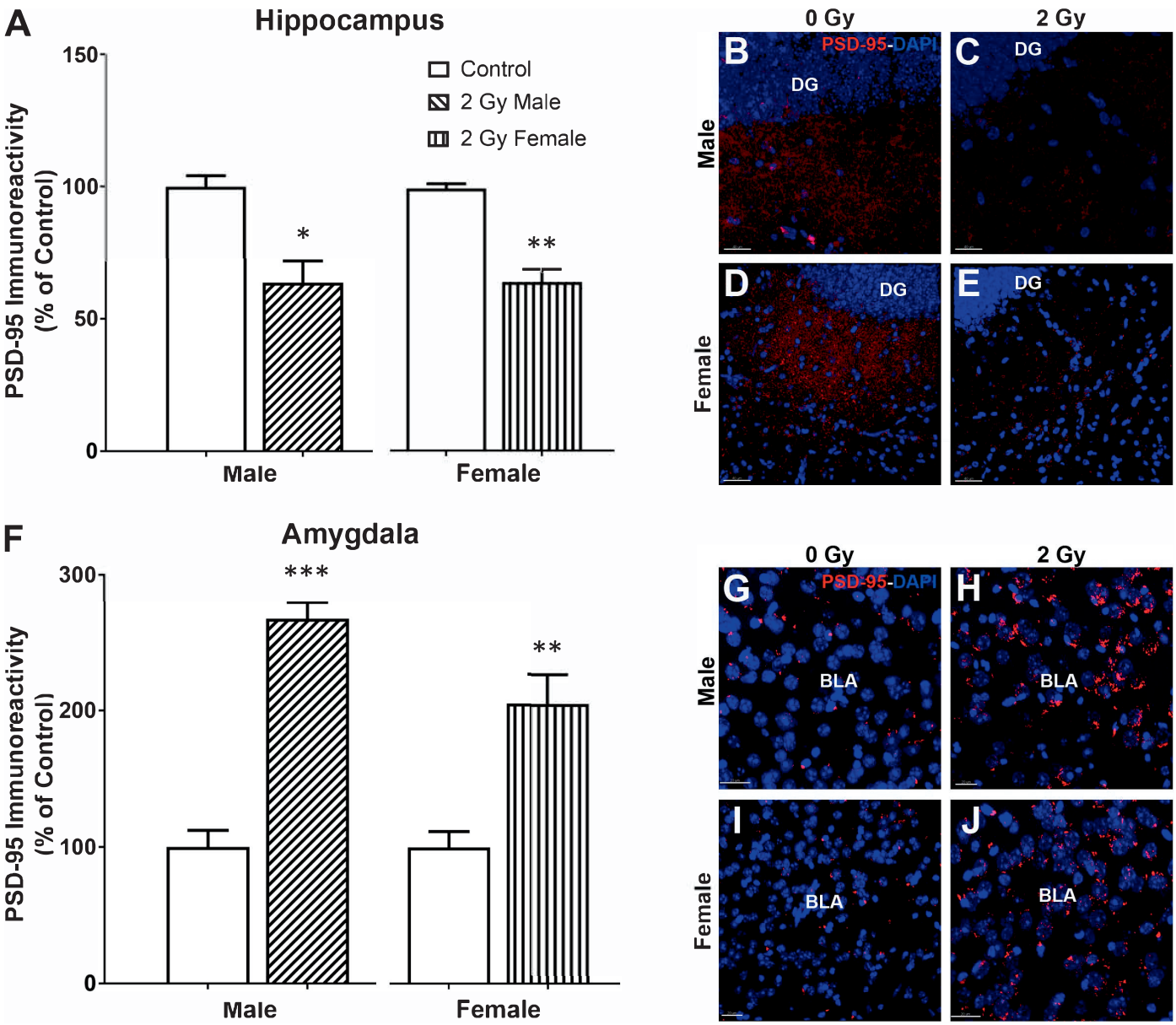

FIG. 3. Whole-body ^{137}Cs gamma-ray irradiation (2 Gy) significantly altered the number of PSD-95 puncta. Panel A: Quantification of PSD-95 from deconvoluted confocal images demonstrated significant reductions in the number of PSD-95 puncta in the hippocampus of the irradiated mouse brain for both male and female mice. Panels B–E: Representative high-resolution confocal micrographs of PSD-95 immunohistochemical staining in the dentate gyrus (DG) molecular layer of control and irradiated male mice (panels B and C) and female mice (panels D and E) (red, PSD-95; blue, DAPI nuclear counterstain). Panel F: Conversely, quantification of PSD-95 in the amygdala demonstrated significant increases in the number of PSD-95 puncta for both male and female irradiated mice. Representative high-resolution confocal micrographs of PSD-95 immunohistochemical staining in the basal lateral amygdala (BLA) of control and irradiated male mice (panels G and H) and female mice (panels I and J). All data are presented as mean ± SEM (N = 3–4 mice/group). Unpaired Student's *t* tests, $*P < 0.05$, $**P < 0.01$, $**P < 0.001$. Scale bar = 20 μm.

cognitive impairments (*32, 33*). More specifically, the elevated levels of activated microglia in the female amygdala suggest that persistent neuroinflammation may have played a disruptive role in fear memory consolidation in the irradiated female mice as reflected by their increased freezing during the fear extinction trial (Fig. 2D).

Previously published work performed by others and by us has suggested that radiation affects changes in DNA methylation profiles that correlate with cognitive impairments (*34–36*). In some cases, these changes have been implicated not just as biomarkers of exposure, but also as a functionally relevant epigenetic mechanism underlying the CNS effects of irradiation. Based on these observations, global levels of both 5-methylcytocine (5mC) and 5-hydroxymethylcytosine (5hmC) were evaluated in the hippocampus of the irradiated mice (Fig. 5). Analysis of 5mC, typically a gene silencing epigenetic modification, showed significantly increased levels in the DG and CA1

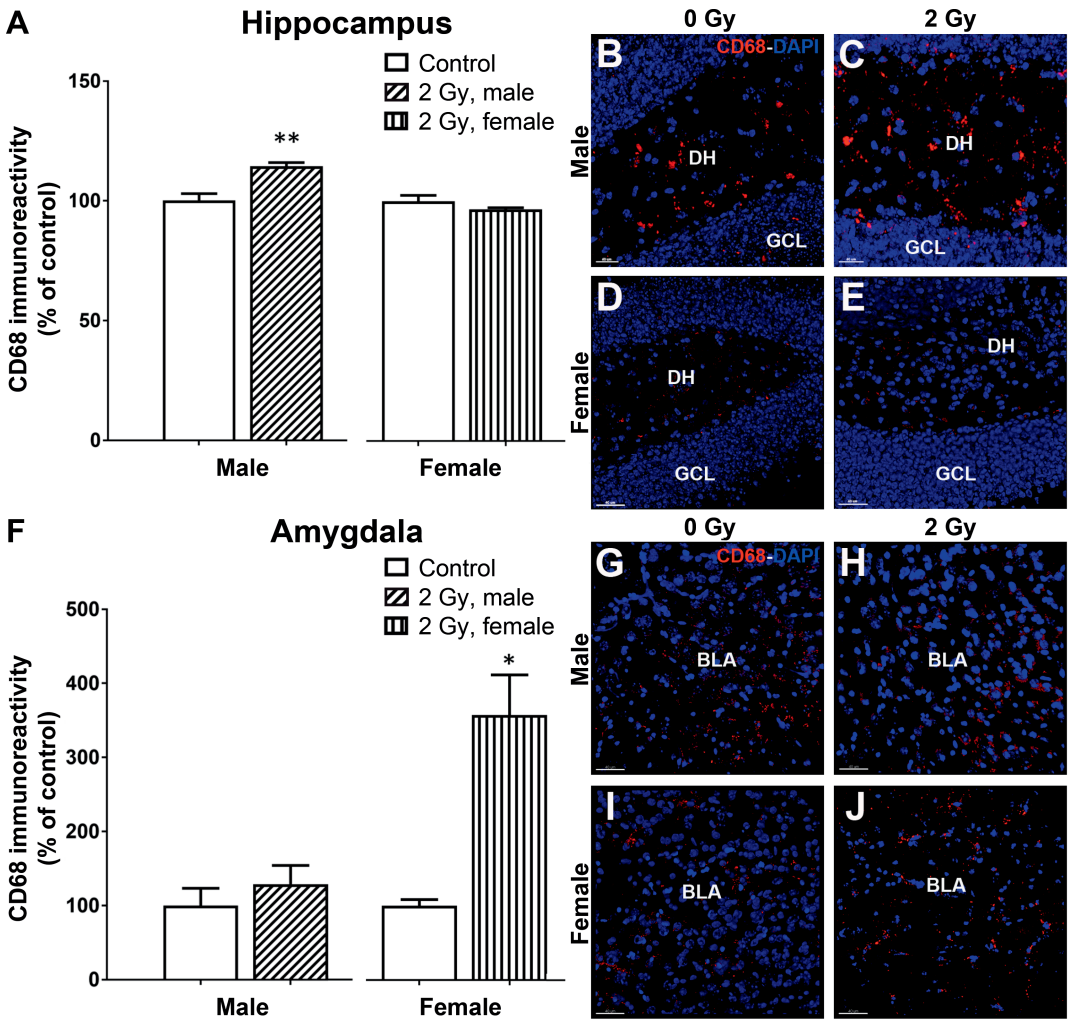

FIG. 4. Effect of 2 Gy whole-body irradiation on microglial activation. Representative (panel A) quantification of CD68 immunohistochemical staining of the DG region of the hippocampus demonstrated that compared to controls, irradiated male mice had increased microglial activation 7–8 months, but that females did not. Panels B–E: Representative high-resolution confocal micrographs of CD68 immunohistochemical staining in the dentate hilus (DH) and granular cell layer (GCL) of control and irradiated male mice (panels B and C) and female mice (panels D and E) (red, CD68; blue, DAPI nuclear counterstain). Panel F: Conversely, quantification of CD68 in the amygdala region of the brain demonstrated significant increases in the number of activated microglia in irradiated female mice that were not observed in the irradiated male mice. Representative high-resolution confocal micrographs from the basal lateral amygdala (BLA) of control and irradiated (panels G and H) male mice and (panels I and J) female mice. Data are presented as mean ± SEM (N = 3–4 mice/group). Unpaired Student's *t* test, $*P < 0.05$, $**P < 0.01$. Scale bar = 20 μm.

regions of the brain for both male and female irradiated mice relative to their concurrent controls (Fig. 5A and B; $P < 0.05$ and $P < 0.01$ for males and females, respectively). Evaluation of 5hmC, thought to be a gene activating epigenetic modification, was also elevated in the DG region of the hippocampus of the irradiated male mice (Fig. 5C and D; $P < 0.05$) that was not observed in the CA1 region of those same mice. No increases above control levels of 5hmC were observed in either region of the brain for the irradiated female mice. Together, these data suggest the possibility that a whole-body exposure to ^{137}Cs gamma rays induces epigenetic dysregulation, observed 7–8 months later, which may cause adverse changes in gene expression that contribute to cognitive dysfunction.

DISCUSSION

While whole-body exposure to low dose, low-LET radiation has been shown to induce cognitive dysfunction, there remains little information to allow for accurate risk assessment. The numerous exposure scenarios include those involving military personnel, first responders, cleanup workers and civilians in the context of a large-scale accidental or terrorist-mediated release, in addition to other occupational exposures. Manifestation of impairments such as mood disorders, learning and memory impairments or progressive neurodegenerative-like changes can adversely affect long-term quality of life. In contrast to what has been demonstrated after clinical exposures, occupational and/or

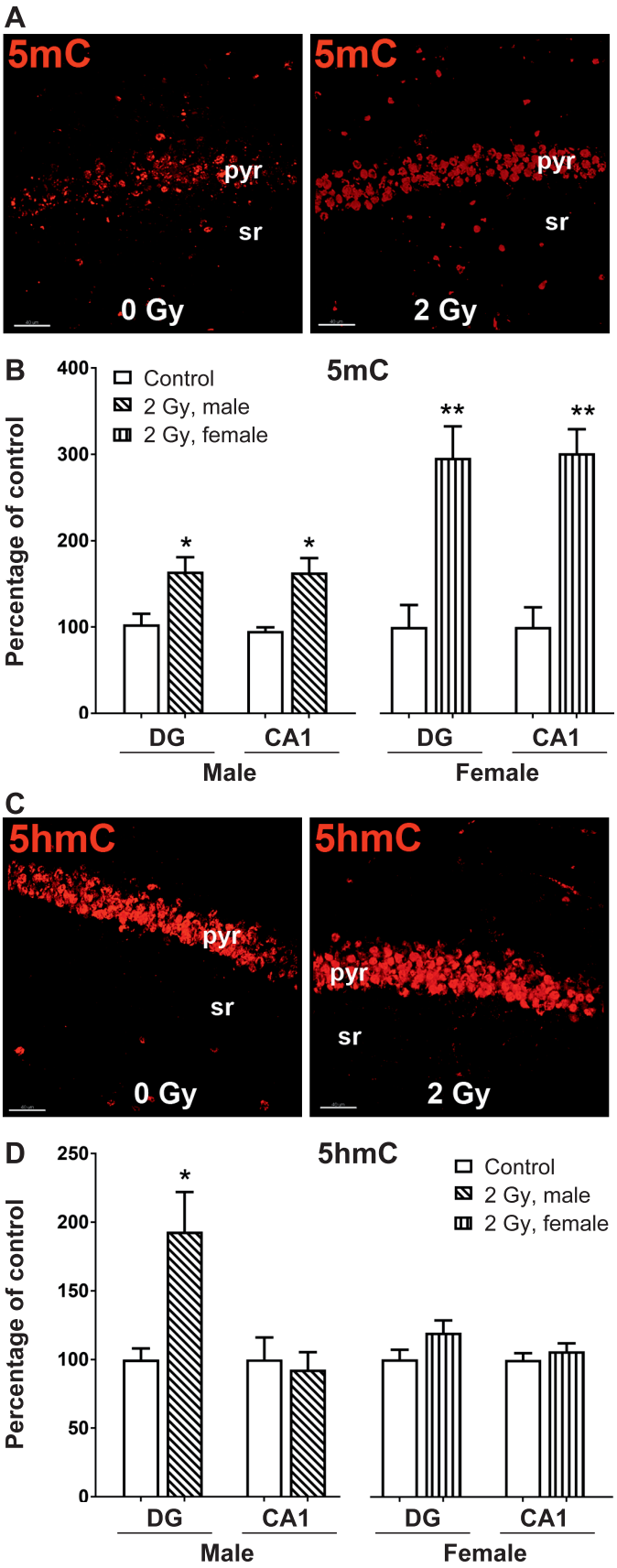

FIG. 5. Exposure to 2 Gy of ^{137}Cs γ rays results in altered global DNA methylation in the hippocampus. Representative images show that radiation exposure increased levels of 5mC (panel A) and 5hmC (panel C) in the DG region of the hippocampus (pyr, pyramidal cell layer; sr stratum radiatum). Panel B: Quantification of immunohistochemical staining demonstrates that compared to controls, irradiated male and female mice have increased 5mC in the DG and CA1 regions of the hippocampus and that (panel D) irradiated male mice have increased 5hmC in the DG region of the hippocampus (red; 5mC, 5hmC). Data are presented as mean ± SEM (N = 3–4 mice/group). Unpaired Student's *t* test, $*P < 0.05$, $**P < 0.01$. Scale bar = 40 μm.

accidental exposure scenarios are projected to elicit more subtle cognitive changes that manifest over protracted postirradiation intervals, if not a lifetime. These factors have confounded rigorous risk assessments in humans.

The human data regarding CNS risk after radiation exposure are somewhat conflicting. In published studies of Chernobyl cleanup workers receiving occupational radiation exposures, cognitive impairments, as well as other adverse health outcomes have been reported (*37, 38*). Those findings have been supported by three published studies of female nuclear workers, which suggested that occupational radiation exposure was linked to increased risk of mortality from dementia (*39–41*). Conversely, in a published study of more than 2,000 atomic bomb survivors there was no link found between radiation exposure and dementia irrespective of dose (*42*). While prenatal exposures have defined risks for microcephaly and mental retardation (*43*), CNS risks are more difficult to define after exposures to adult populations, prompting further work in non-human primates (NHP) and other mammalian species.

Non-human primate models provide distinct advantages for radiation-induced cognitive studies due to their close match to human behavioral traits and complexity, but such studies routinely suffer from smaller sample sizes. Rhesus macaques that received clinically relevant whole-brain fractionated doses of 8 × 5 Gy (40 Gy), exhibited significant neuropathology and cognitive decline 14 months later (*44*). Rhesus macaques that received whole-body gamma-ray irradiation at a lower dose of 6.75–8.05 Gy were evaluated 3.1–4.3 years later. Those irradiated animals were less likely to engage in behavior testing, suggested to be a result of attention deficits. Otherwise, the irradiated NHP were slower to learn and complete the task, and had reduced cognitive flexibility (*45*). Furthermore, these animals exhibited evidence of persistent inflammation, expression of complement proteins and T-cell activation, as well as impaired glutamatergic neurotransmission and signal transduction within the white matter of the brain (*46*). While additional evidence in NHP has pointed to the promise of using circulating biomarkers for radiation exposures and lethality, they have not been linked to neurobehavioral outcomes (*47–49*). As a result, much of what is known and can be projected regarding risks to the CNS after whole- or partial-body irradiation comes from the rodent literature.

As described by others, even relatively higher whole-body doses of low-LET radiation have been inconsistent in eliciting CNS responses in rodent studies (*50–52*). This lack of consistency may be attributed to differences in exposure paradigms, postirradiation analysis times and the types of behavioral testing paradigms used. Head-only irradiations using 2 Gy of either X rays or gamma rays were demonstrated to induce behavioral decrements and decreased neurogenesis in two published rodent studies (*26, 53, 54*). A comparison of rats exposed to either ^{4}He or gamma rays demonstrated consistent decrements after ^{4}He irradiation that did not manifest after gamma-ray irradiation, where 50–200 cGy doses of gamma rays elicited anxiety-like behavior on the EPM, but no decrements on the novel object and novel place recognition tests that measure perirhinal cortex- and hippocampal-dependent learning and memory, respectively (*50*). While certain findings point to the ability of lower, whole-body low-LET radiation exposures to elicit functional CNS decrements, the data remain inconclusive in that definitive dose thresholds and time to onset for neurocognitive deficits has remained difficult to elucidate.

The scarcity of carefully controlled neurocognitive studies after low-dose, whole-body irradiation prompted the current study where it was anticipated that lower dose exposures would require longer times for behavioral decrements to manifest. For that reason, male and female mice were used in the current study to evaluate the brain for the delayed CNS effects beginning 6–7 months after whole-body low-dose gamma irradiation, which would not induce acute radiation sickness. Behavioral testing indicated sex-specific differences in the induction of anxiety-like behaviors, in which females were much more sensitive than males. It was noted that for the first behavior test, EPM, control male mice spent significantly less time in the open arms of the maze than did the females. This discrepancy was not evident in subsequent behavior tests, suggesting that more pre-test handling might have been needed for the male mice than the females in this particular study.

The fact that male mice exhibited relatively normal cognitive function while exhibiting similar or worse molecular pathology compared to the females was somewhat surprising. However, it is challenging to correlate this behavioral testing data with tangible estimates of radiation-induced CNS impairments, particularly given the lack of epidemiological data. KDE provides a means to define a level of behavioral performance that might represent significant cognitive deficits and to estimate the number of severely affected individuals within a study cohort (*22*). In this case, the control cohort was assigned a 5% level of performance, below which the irradiated animals were considered severely impaired. By entering the numbers derived from KDE into the NNH algorithm, an estimate of absolute relative risk and of the potential frequency of impaired behavior outcomes for the FE test was obtained (Fig. 2A and D). Dramatically, this analysis predicted that at least 1 in 4 women would exhibit anxiety- or PTSD-like impairments at delayed times after a 2 Gy whole-body exposure, while such symptoms would manifest in only 1 in 26 men.

While cognitive impairments were not significantly evident in the male mice, molecular analyses showed alterations in post-synaptic protein levels in both male and female mice that might affect synaptic plasticity, and showed increased indications of neuroinflammation in male mice as measured by CD68^{+} microglia staining. Significant alterations in global levels of DNA methylation were also observed in the hippocampus of both the male and female irradiated mice. Altogether, these data suggest several conclusions. The first is that more sensitive and/or rigorous behavioral testing might be required to uncover more subtle effects in animals exposed to whole-body, low-LET radiation. The data also suggest that 2 Gy may be near the dose threshold for eliciting radiation-induced behavioral impairments in this paradigm. Furthermore, estrus cycle is a factor that could contribute to the observed sex-specific differences in the CNS radiation response and cognitive dysfunction, although studies of high-LET radiation exposures have suggested the possibility that females might be more resistant to radiation-induced cognitive impairments (*4, 55*). However, systematic studies need to be conducted to critically evaluate the link between radiation-induced CNS effects and hormone cycles. Finally, the observed epigenetic changes have been posited to be a potential epigenetic mechanism that ultimately contributes to radiation-induced cognitive dysfunction (*34–36*). If a causative link could be established between alterations in DNA methylation and cognitive changes, it may provide a logical avenue for the development of radiation protection and mitigation strategies.

Due to global tensions and proliferation of nuclear arms, as well as accidents such as Chernobyl and Fukushima, the evaluation of radiation-induced cognitive impairments will remain a priority. A better understanding the CNS response to such radiation exposure scenarios will improve risk assessment and provide guidance in the development of effective radiation countermeasures to protect military personnel and civilians alike.

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