Suppressed neurogenesis without cognitive deficits: effects of fast neutron irradiation in mice

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This study assessed the effects of combined low-dose neutron and γ-ray irradiation on hippocampal neurogenesis and hippocampal-dependent memory. Neural progenitor cell division and survival were evaluated in brain sections and whole hippocampal preparations following head irradiation at a dose of 0.34 Gy for neutron radiation and 0.36 Gy for γ-ray radiation. Hippocampal-dependent memory formation was tested in a contextual fear conditioning task following irradiation at doses of 0.4 Gy for neutron radiation and 0.42 Gy for γ-ray radiation. Cell division was suppressed consistently along the entire dorsoventral axis of the hippocampus 24 h after the irradiation, but quiescent stem cells remained unaffected. The control and irradiated mice showed no differences in terms of exploratory behavior or anxiety 6 weeks after the irradiation. The ability to form hippocampus-dependent memory was also unaffected. The data may be indicative of a negligible effect of the low-dose of fast neutron irradiation and the neurogenesis suppression on animal behavior at 6 weeks after irradiation. ***NeuroReport*** **30:538–543**

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Introduction

Design of long-range space flights, radiotherapy protocols, and nuclear pollution liquidation measures calls for studies on the potential cognitive deficits following irradiation from various energy spectrums. γ-Ray and X-ray irradiations are used as diagnostic and therapy tools, and are most commonly encountered by humans; thus, there is a considerable body of data on their effects on body tissues and the brain [1–4]. However, the effects of other types of irradiation, particularly fast neutron irradiation, on the mammalian brain and cognitive functions, remain understudied [5,6]. Fast neutrons naturally appear as the result of unstable isotope decay and as a product of an interaction between primary cosmic rays and the magnetosphere. Thus, cosmonauts, liquidators of nuclear disasters, and frequent flyers are all in a higher risk group.

Irradiation is widely known to target proliferating cells [2,4]. The effects of these types of irradiation on neurogenesis are of particular interest because hippocampal neurogenesis continues in mammals throughout the life [7–10] and is also present in humans at least at a young age and possibly during the entire life [11–13]. Newly generated hippocampal neurons were shown to participate in learning and memory [7] as well as in emotional behavior [14,15]. Therefore, some of the cognitive effects of irradiation can be associated with disrupted neurogenesis [1–3].

Fast neutron irradiation is a more potent damaging factor than γ-rays or X-rays because of higher energy and more severe DNA damage [5,16]. Consequently, even low doses may inhibit neurogenesis. Therefore, the aim of this study was to investigate the effects of low-dose fast neutron irradiation on hippocampal neurogenesis and hippocampus-dependent memory.

Materials and methods

Mice

Two-month-old homozygous nestin-cyan fluorescent protein (CFP)nuc male mice were used for neurogenesis assessment ($N = 14$) and 2-month-old hemizygous male and female nestin-GFP mice were used for behavioral training ($N = 46$) [17]. Nestin-CFPnuc homozygous mice were chosen for cell counting experiments to allow automatic counting of nestin-positive cells by virtue of nuclear localization of the marker protein. For behavioral experiments, hemizygous nestin-GFP mice were chosen to better relate to our previous data on γ-irradiation [18]. Both mouse strains had the same C57BL/6 background.

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The irradiated mice were subjected to combined fast neutron radiation and γ-radiation; the control mice were treated in the same manner, but without irradiation. Before and after the irradiation treatment, the mice were housed in standard home cages (40 × 25 × 19 cm) with food and water *ad libitum* and a normal 12/12 h light–dark cycle. The study was carried out in accordance with the law of the Ministry of Health of the Russian Federation no. 267 from 19 June 2003 and according to protocol no. 1 from 3 September 2005 of the P.K. Anokhin Research Institute of Normal Physiology guidelines for animal experiments.

Irradiation

Irradiation was performed using the U-150 cyclotron from the Kurchatov Institute. The animals were anesthetized with an injection of Zoletil 100 (40 mg/kg; Virbac, Carros, France) and Rometar (5 mg/kg; Bioveta, Ivanovice na Hané, Czech Republic) 15 min before irradiation. The animals were irradiated in two sessions. Mice were placed horizontally in individual polyethylene cylinders and fixed in a foam block, and this block was installed along the beam axis. The distances from the edge of the target to the animal's heads were 80 cm in the neurogenesis experiment and 73 cm in the behavioral experiment, resulting in the absorbed doses of 0.34 Gy for neutron and 0.36 Gy for γ-radiation in the neurogenesis experiment and 0.40 Gy for neutron and 0.42 Gy for γ-radiation in the behavior experiment. A beryllium target, irradiated by a 32 MeV proton beam with an intensity of up to 30 μA, was used to obtain a flux of fast neutrons. The irradiation lasted for 10 min. The fast neutron flux spectrum was measured on a spectrometer using a transit technique, and the uniformity of the flux of fast neutrons was determined by an activation method using test indium foils. The dose of γ-radiation was measured with a DKP-50A dosimeter. The flux of fast neutrons at the neutron source reached values of 3×10^{16} neutrons/sterad in the energy range of 0.5–30 MeV. The absorbed neutron dose calculation was performed on the basis of the assumption that for protons with a fixed energy, the neutron yield per coulomb to steradian for the $^{9}Be(p,n)^{9}B$ reaction does not change. The absorbed dose in the neurogenesis analysis experiment was 0.34 and 0.36 Gy for neutron and γ-radiation, respectively. In the behavioral experiment, the absorbed dose was 0.40 Gy for the neutron radiation and 0.42 Gy for the γ-radiation.

Analysis of hippocampal neurogenesis

To analyze cell division in the hippocampus at the time when apoptosis has already subsided [19], the animals were injected with a synthetic thymidine analogue 5-ethinyl-2′-deoxyuridine (EdU) 22 h after irradiation and 2 h before euthanasia. The time between the injection of the label and euthanasia was chosen as an often-used time interval to detect cells that have currently entered the S-phase of the cell cycle. The mice were anesthetized with chloral hydrate (10 mg/kg; Sigma-Aldrich, St.Louis, Missouri, USA) and then perfused with 1% paraformaldehyde, pH 7.4. After perfusion, the samples were fixed for 18 h in 1% paraformaldehyde at 4° C. The brain was divided into two hemispheres. Whole hippocampi preparations were made from the left halves; serial sagittal sections 50 μm thick with a step of 300 μm were made from the right halves. Dividing cells were detected by a click reaction in sections and in the whole hippocampi. Progenitor cells were detected immunohistochemically and analyzed only in sections.

Staining of preparations of the whole hippocampus

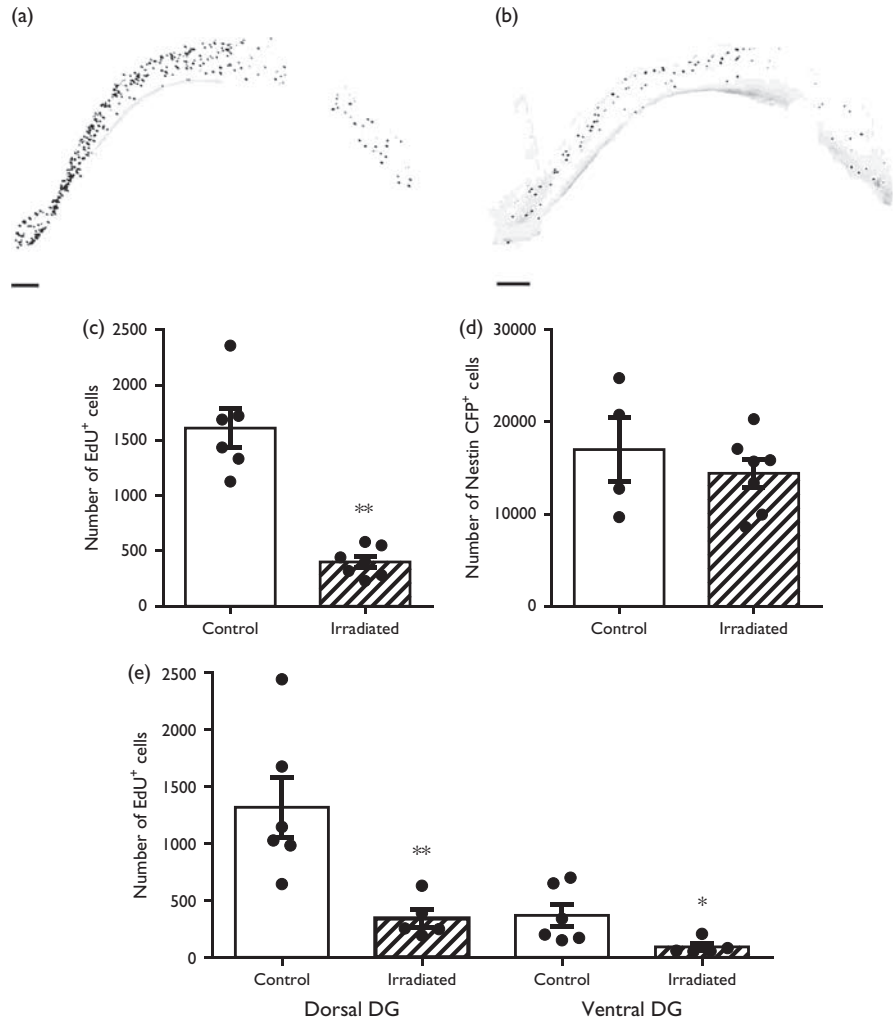
All procedures were performed with constant stirring and 20°C, unless otherwise specified. The preparations were treated for 2 h in a dent solution (100% methanol/DMSO, 4 : 1, 4°C), bleached for 2 h in a dent bleach solution (100% methanol/DMSO/30% H_2O_2, 4 : 1 : 1) in bright light, and washed three times for 1 h in 100% methanol. The preparations were rehydrated for 60 min in 50, 25, and 12.5% methanol, washed twice for 90 min with buffer, and permeabilized for 2 h in 2% Triton X-100 and 5% DMSO solution. The preparations were washed for 30 min in a buffer with 0.2% Triton X-100 and stained for 2 h in 100 mM Tris-HCl buffer (pH 8.0) with 5% DMSO, 100 mM sodium ascorbate, 1 mM $CuSO_4$, and 10 μM azide (A20012; Invitrogen, Waltham, Massachusetts, USA). The click reaction was stopped by three incubations of the samples for 60 min in 0.1 M EDTA. The preparations were washed, dehydrated for 60 min in alcohols (50, 96, and 100% ethanol) and in 2-butoxyethanol overnight, and clarified for 12 h in BBBA solution. 3D reconstruction, segmentation, and automatic cell counting were performed in Imaris 6.0 (Bitplane). Examples of original 3D reconstructions are shown in Supplementary Videos 1–4 (Supplemental digital content 1–4, *http://links.lww.com/WNR/A507*, *http://links.lww.com/WNR/A508*, *http://links.lww.com/WNR/A509*, *http://links.lww.com/WNR/A510*). Snapshots of the same samples in negative grayscale pallet are used in Fig. 1a and b. The detailed method is described by Lazutkin *et al.* [20].

Brain sections staining

Sections were first permeabilized for 1 h in a 2% Triton X-100 solution with 5% normal goat serum, and then washed with 0.2% Triton X-100 and incubated with the primary antibodies for GFP (1 : 500, GFP-1020; Aves Labs Inc., Davis, California, USA) dissolved in 0.2% Triton X-100 and 5% normal goat serum solution for 12 h at 4°C. After three washes, the sections were incubated with the secondary antibodies (1 : 400, A11039; Invitrogen), washed again, and stained by the click reaction as described above. Finally, the sections were washed with PBS and mounted on slides.

The preparations of the whole hippocampus and sections were imaged using an Olympus FluoView 1000 scanning

Fig. 1



Irradiation effects on early neural precursors and cell division in the dentate gyrus (DG) 24 h after exposure. Examples of the 3D reconstructed DG from the whole-mount stained hippocampi. (a) a control animal, (b) an irradiated animal. 3D reconstructions are presented in negative grayscale pallet. Scale: (a) 200 μm, (b) 200 μm. (c) Results of the automated count for EdU^+-cells, **Mann–Whitney test, $P < 0.005$; (d) results of the automated count for early neural precursors expressing CFP in nuclei. (e) Dividing cells in the dorsal and ventral hippocampus. Mann–Whitney test, *$P < 0.05$, **$P < 0.005$. CFP, cyan fluorescent protein; EdU, 5-ethinyl-2′-deoxyuridine.

confocal microscope (Olympus, Tokyo, Japan). Stitching, 3D reconstruction, segmentation, and automatic cell counting were performed in Imaris 6.0 (Bitplane AG, Zurich, Switzerland). No sections were excluded from the analysis. Approximately 8–10 sections for each brain were analyzed, and the estimations were extrapolated to the whole hippocampus.

Animal training

We estimated the contextual memory in a weak contextual fear conditioning task. Training and tests were performed 6 weeks after the irradiation. Mice were placed in a chamber fitted with a metal grid floor and located in a sound-attenuating cubicle (Fear Conditioning Package for Mouse; Med Associates Inc., Fairfax, Vermont USA). Animals explored the chamber freely for 30 s. The 0.3 mA footshock was delivered during the last 2 s of exploration. After the training session, animals were returned to their home cages. Mice were then tested in the training (familiar) context for 5 min at 24 h. Memory was assessed by freezing duration, which was defined as no movement except breathing for 0.5 s (Video Fear Conditioning 'Video Freeze' Software; Med Associates Inc.). To assess the level of generalization, the test in the unfamiliar chamber was performed for 5 min at 48 h. The unfamiliar context differed from the training context in chamber walls (an A-frame insertion instead of regular walls), the floor (plastic floor covered with standard home cage bedding instead of a grid floor), and lighting (switched off).

Exploratory activity and anxiety were tested in the open field and the elevated plus maze before the contextual fear conditioning task. The open field had a 120 cm

diameter, was illuminated with ~150 lx, and was divided into three zones, that is, a central zone with a 20 cm radius, a border (20 cm near the wall), and an intermediate zone between the previous two. The track length, average velocities, and times spent in the zones were measured during a 10-min interval. The elevated plus maze was placed 80 cm above the floor. Two of the four arms were equipped with nontransparent walls of 30 cm height. The time spent in the arms was measured during a 5 min interval. Behavior was analyzed in EthoVision 8.5 software (Noldus, Wageningen, The Netherlands).

Statistical analysis

The data are presented as mean ± SE. Results were analyzed using GraphPad Prism software version 6.0 (GraphPad Software Inc., San Diego, California, USA). Two-way analysis of variance (ANOVA) with Sidak post-hoc *k*-test or the Kruskal–Wallis test were used in behavioral experiments where appropriate. For comparison of histological data, the Mann–Whitney test was performed.

Results and discussion

We first estimated the effect of combined low-dose neutron and γ-ray irradiation on cell proliferation in the dentate gyrus (DG). To label the dividing cells, we injected a nucleotide analogue EdU 22 h after the irradiation and analyzed the brains 2 h later. EdU-labeled cells mostly represented dividing stem cells and intermediate progenitors in their S-phase or immediately after its completion [8]. In serial brain sections, there was a 4.0-fold decrease in the number of EdU $^{+}$ cells in the irradiated animals compared with the control group (Mann–Whitney test, $P < 0.005$): 1611 ± 174 ($N = 6$) for the controls and 402 ± 51 ($N = 7$) for the irradiated mice. Similar results were obtained on whole-mount preparations (3.8-fold decrease, 1682 ± 343 ($N = 6$), for the controls and 445 ± 110 ($N = 5$) for the irradiated mice, Mann–Whitney test, $P < 0.01$, Fig. 1c). The results and examples of the whole-mount click reaction stained hippocampi are presented in (Fig. 1a, b) and Supplementary Videos 2 and 3 (Supplemental digital content 2 and 3, *http://links.lww.com/WNR/A508* and *http://links.lww.com/WNR/A509*). In addition, the survival of early neural progenitors was estimated using the expression of CFP under the nestin promoter, which thus traced neural stem cells and their immediate progeny [6]. The control and irradiated mice had similar numbers of nestin-CFP^{+} cells in the DG (17 003 ± 3488, $N = 4$ in the controls and 14 426 ± 1547, $N = 7$ in the irradiated mice, Mann–Whitney test, $P = 0.65$; Fig. 1d). The labeled dividing cells accounted for ~10% of all CFP-expressing precursors, and the observed reduction in EdU-labeled cell number by 1200 cells was similar to the scatter of the nestin-CFP^{+} cell counts (1500–3500 cells), suggesting almost no effect on the total number of hippocampal progenitor cells, the majority of which are quiescent stem cells.

The neurogenesis rate and function are different in the dorsal and ventral hippocampus [10,15]. In particular, cell proliferation may disappear in one of the hippocampal areas because of a pre-existing nonuniform density of the dividing cells. Alternatively, the neurogenesis decline rate may be different in the dorsal and ventral hippocampus because of potential differences in the precursor cell cycle parameters of different areas. Both possibilities are important for assessing and understanding neurogenesis-related cognitive deficits. Whole-mount preparations have advantages over the serially sectioned specimens in terms of nonuniformity in the cell type distribution. We thus analyzed acute irradiation effects on cell proliferation in the DG using whole-mount stained hippocampi. The DG was then subdivided into dorsal and ventral portions, where the EdU^{+} cells were counted. The segmentation examples and the cell counts are represented in Fig. 1e and Supplementary Video 4 (Supplemental digital content 4, *http://links.lww.com/WNR/A510*). The dorsal DG had more EdU-labeled cells than the ventral DG: 1321 ± 263 ($N = 6$) dorsal versus 369 ± 101 ($N = 6$) ventral cells in the controls; 343 ± 79 ($N = 5$) dorsal versus 98 ± 35 ($N = 5$) ventral cells in the irradiated animals. The reduction in EdU-labeled cell counts was similar for both areas: 3.9× in the dorsal hippocampus ($P < 0.05$, Mann–Whitney test) and 3.8× in the ventral hippocampus ($P < 0.05$, Mann–Whitney test). Thus, the irradiation effect on cell proliferation was similar in these two main hippocampal subdivisions. However, because of initial unevenness in dividing cell density, it resulted in an almost complete absence of dividing cells in the ventral portion.

In summary, the combined neutron and γ-ray irradiation at doses of 0.34 and 0.36 Gy, respectively, suppressed hippocampal cell proliferation (3.8–4.0-fold) 24 h after the irradiation, but had no effect on the majority of the progenitors, which were primarily comprised of neural stem cells. A negative effect of fast neutron irradiation on neurogenesis was reported previously only in two studies. Yang *et al.* [5,6] showed a 2.5-fold decrease in Ki-67^{+} cells and a 1.5-fold decrease in DCX^{+} cells 12 h after fast neutron irradiation at a dose of 0.4 Gy [5]. They also reported a five-fold decrease in hippocampal Ki-67^{+} cells and a 3.5-fold decrease in hippocampal DCX^{+} cells 24 h after fast neutron irradiation at a dose of 0.8 Gy [6]. The curve for the proliferation decline in response to the 0.8 Gy irradiation from Yang *et al.* [5], if approximated up to 0.4 Gy and 24 h, will correspond to a 3.9-fold decrease, which closely matches our data. It is noteworthy that the same level of proliferation decline was observed using different cell division markers (Ki-67 in Yang *et al.* [5] and EdU in this study), reflecting the dynamics of apoptosis in response to irradiation.

Several studies have shown that irradiation-induced cognitive deficits can be associated with suppressed hippocampal neurogenesis [1,3]. Because the decrease in the number of dividing cells was 3.9-fold, we anticipated significant cognitive effects in the irradiated animals.

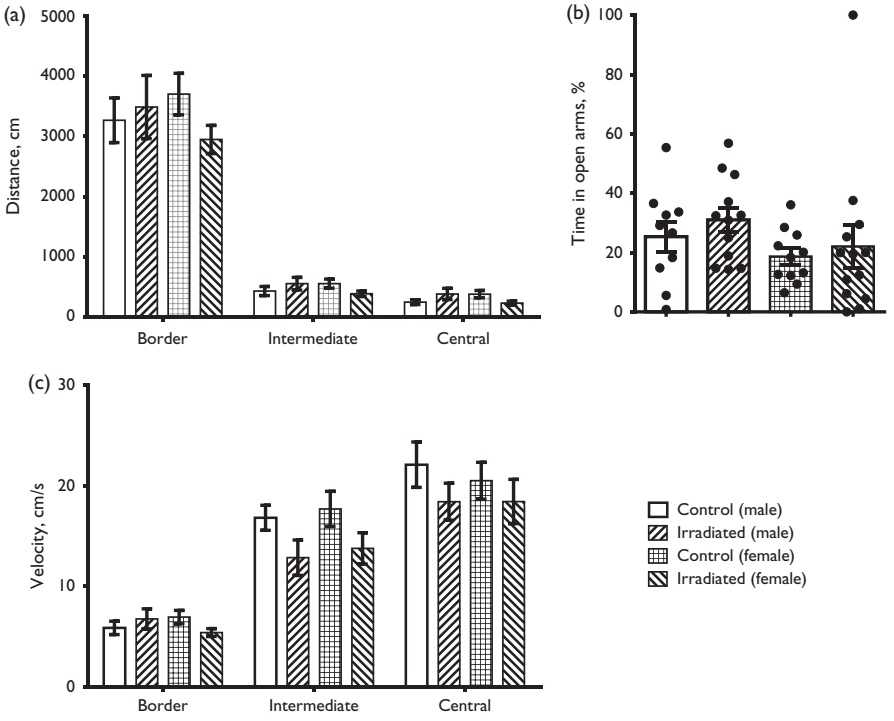
Behavioral testing was carried out 6 weeks after the irradiation because of the following reasons. First, the irradiation-induced inflammation should be alleviated by this time. Second, the delay is necessary to deplete the pool of highly plastic young neurons that were generated before the irradiation [9].

To test the overall exploratory activity and anxiety, we performed the open-field test and the elevated plus maze test. The irradiated and control males and females showed no significant differences in the open-field test in terms of the distance traveled [two-way ANOVA, $F(3, 42) = 1.283$ $P = 0.2927$], velocity [two-way ANOVA, $F(3, 42) = 1.354$ $P = 0.2700$] (Fig. 2a–c) and thus the time spent in each of the zones. Males and females of both groups traveled longer distances and spent more time near the borders (two-way ANOVA, with Tukey post-hoc). All animal groups also showed no differences in terms of the arm preferences in the elevated plus maze (Fig. 2d, Kruskal–Wallis test $P = 0.1197$). Thus, despite the disrupted neurogenesis in the ventral hippocampus, the irradiation exerted no effects on the overall exploratory activity or anxiety in the open field or elevated plus maze.

The formation of hippocampal-dependent memory was assessed in a contextual fear conditioning task. On the day of training, the percentage of freezing was predictably low: $1.4 \pm 1.0\%$ ($N = 10$) in the control and $1.7 \pm 0.8\%$ ($N = 12$) in the irradiated males; $2.4 \pm 1.4\%$ ($N = 11$) in the control and $0.6 \pm 0.4\%$ ($N = 13$) in the irradiated females. No significant differences were detected between the groups. The memory test at 24 h after training showed similar results for the control and irradiated animals in the familiar context: 31.9 ± 3.1 and $29.1 \pm 4.9\%$ for males; 28.6 ± 6.5 and $33.6 \pm 3.7\%$ for females. The animals were tested in the safe context at 24 h after the first test. The freezing percentage was $13.6 \pm 2.3\%$ in the control and $16.4 \pm 2.5\%$ in the irradiated males, and $9.9 \pm 3.2\%$ in the control and $14.8 \pm 3.4\%$ in the irradiated females. No significant differences were detected between the groups. Notably, the within-group differences between the dangerous and safe contexts were significant in both males and females (Fig. 3). Collectively, the results indicate that irradiation with fast neutrons and γ-rays at the doses of 0.40 and 0.42 Gy, respectively, does not affect memory formation in a contextual fear conditioning model.

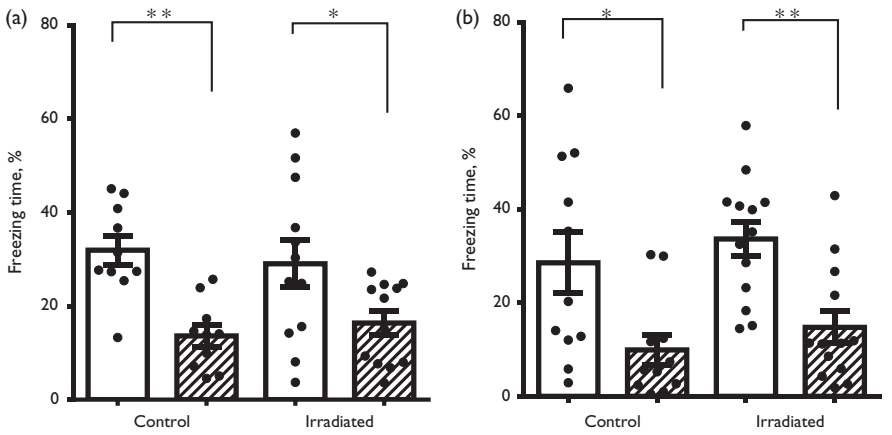
Previously, the effects of low-dose neutron irradiation on hippocampus-dependent memory in mice were only evaluated in a single study with head irradiation at a dose of 0.8 Gy [6]. Behavioral testing was performed after 24 h, 7, and 14 days. The testing involved open-field recordings, object-recognition testing, and training in a contextual fear conditioning task. The open-field test showed no irradiation effects at all times tested. However, the irradiated

Fig. 2



Long-term effects of neutron irradiation in the open-field test (a, b) and in the elevated plus maze (c). (a) The distance traveled, (b) animal velocity in different arena zones averaged for the 10-min interval, (c) percentage of time spent in the open arms. No significant differences were found.

Fig. 3



Hippocampus-dependent memory in mice 6 weeks after neutron irradiation. Freezing of males (a) and females (b) in familiar (white bar) and unfamiliar (dashed bar) contexts. *$P < 0.05$; **$P < 0.005$, Sidak.

mice underperformed in the object-recognition task at 1 and 7 days after the irradiation. The irradiated mice also showed significantly lower freezing times than the controls at 1 and 7 days after the irradiation. The described deficits were transient and were not observed 14 days after the irradiation. The authors attributed the deficits to the specific effect of a lack of newly generated neurons. However, an acute inflammatory process can also be a cause of the disturbed behavior after such a short time following the irradiation [1]. Our data indirectly support this hypothesis by showing a lack of the irradiation-induced effects on hippocampus-dependent behavior 6 weeks after irradiation.

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Conflicts of interest

There are no conflicts of interest.

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