

## Transient Impairment of Hippocampus-dependent Learning and Memory in Relatively Low-Dose of Acute Radiation Syndrome is Associated with Inhibition of Hippocampal Neurogenesis

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### Hippocampus/Neurogenesis/Acute radiation syndrome/Cognitive impairment.

Neurogenesis in the adult hippocampus, which occurs constitutively, is vulnerable to ionizing radiation. In the relatively low-dose exposure of acute radiation syndrome (ARS), the change in the adult hippocampal function is poorly understood. This study analyzed the changes in apoptotic cell death and neurogenesis in the DGs of hippocampi from adult ICR mice with single whole-body gamma-irradiation using the TUNEL method and immunohistochemical markers of neurogenesis, Ki-67 and doublecortin (DCX). In addition, the hippocampus-dependent learning and memory tasks after single whole-body gamma-irradiation were examined in order to evaluate the hippocampus-related behavioral dysfunction in the relatively low-dose exposure of ARS. The number of TUNEL-positive apoptotic nuclei in the dentate gyrus (DG) was increased 6–12 h after acute gamma-irradiation (a single dose of 0.5 to 4 Gy). In contrast, the number of Ki-67- and DCX-positive cells began to decrease significantly 6 h postirradiation, reaching its lowest level 24 h after irradiation. The level of Ki-67 and DCX immunoreactivity decreased in a dose-dependent manner within the range of irradiation applied (0–4 Gy). In passive avoidance and object recognition memory test, the mice trained 1 day after acute irradiation (2 Gy) showed significant memory deficits, compared with the sham controls. In conclusion, the pattern of the hippocampus-dependent memory dysfunction is consistent with the change in neurogenesis after acute irradiation. It is suggested that a relatively low dose of ARS in adult ICR mice is sufficiently detrimental to interrupt the functioning of the hippocampus, including learning and memory, possibly through the inhibition of neurogenesis.

### INTRODUCTION

Neurogenesis in the dentate gyrus (DG) of the mammalian hippocampus occurs constitutively throughout life,<sup>1)</sup> and new neurons are derived from progenitor cells located in the subgranular zone (SGZ) between the hilus and granular cells. The rate of neurogenesis may be altered by age,

hormonal status, excitatory input, growth factors, chemicals, physiologic stimuli and environmental effectors (e.g., tumor radiation therapy and accidental cases of nuclear power plants).<sup>1–7)</sup>

There is increasing clinical interest in acute whole-body exposure from nuclear accidents and the use/testing of atomic weapons, as well as in significant partial-body irradiation from radiosurgery, which is a promising new therapy for cancer patients. Individuals that become sick after a single exposure to ionizing irradiation (broad dose range) are said to have acute radiation syndrome (ARS),<sup>8)</sup> the major cause of which is the direct (DNA damage) or indirect (oxidative stress and inflammatory response) depletion of immature stem cells in specific tissues.<sup>9)</sup> However, a relatively low-dose (~2 Gy) of whole body irradiation in experimental animals showed transient damage to immature stem cells in specific tissues, including the skin, intestine and testis.<sup>10–12)</sup>

Irradiation has a variety of effects on both the developing and adult brain, even though the adult brain is less vulnerable to irradiation than other organs. While macroscopic changes

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involving brain tissue destruction are generally associated with high irradiation doses,<sup>13</sup> less histological injury can occur after relatively low doses, resulting in variable degrees of cognitive impairment in adult mammals.<sup>6,14</sup> Several studies have suggested that radiation-induced cognitive deficits are associated with the inhibition of hippocampal neurogenesis in adult animals.<sup>14–16</sup> They showed the prolonged neurogenesis depression and cognitive impairment in adult experimental animals with irradiation. Moreover, Mizumatsu *et al.*<sup>17</sup> suggested that the activation of microglia may be a critical factor in the chronic depression of neurogenesis. Therefore, it is important to clarify the time- and dose-dependent vulnerability of neural stem cells in the SGZ of the adult hippocampus to acute irradiation exposure and the precise correlation between hippocampal neurogenesis and cognitive impairment caused by irradiation.

This study examined the apoptotic cell death and neurogenesis in the DGs of hippocampi from adult ICR mice with single whole-body gamma-irradiation using in situ DNA nick end-labeling (TUNEL) and immunohistochemical markers of neurogenesis, including Ki-67 (proliferating cell marker) and doublecortin (DCX; immature progenitor cell marker), in order to detect the time- and dose-dependent changes in hippocampal neurogenesis in gamma-irradiated adult mice. In addition, the hippocampus-dependent learning and memory tasks after single whole-body gamma-irradiation (2 Gy) were examined in order to evaluate the hippocampus-related behavioral dysfunction in relatively low dose exposure of ARS.

## MATERIALS AND METHODS

### *Animals*

Eight-week-old male ICR mice were obtained from the animal center at the Korea Research Institute of Bioscience and Biotechnology. The mice were fed a standard animal diet. All animal experiments followed a protocol approved by the Committee for Animal Experimentation at the Chonnam National University.

### *Irradiation and tissue sampling*

The time-dependent effect of gamma-irradiation on neurogenesis in the adult mouse hippocampus were observed after whole-body irradiating the mice with 0, 0.5, 2, or 4 Gy of <sup>60</sup>Co gamma-rays (Gamma-cell Elan 3000; Nordion International, Kanata, ON, Canada) at a dose rate of 8.2 Gy/min. The mice were then sacrificed 6, 12, or 24 h, and 3, 7, or 14 days ( $n = 3$  mice/group) later and the hippocampi were dissected from each group. The sham control mice were also transported to the irradiation facility but did not receive radiation.

In order to observe the dose-dependent effects of gamma-irradiation on neurogenesis in adult hippocampi, the mice were whole-body irradiated with 0, 0.5, 2, or 4 Gy of

gamma-irradiation. The mice were sacrificed 24 h later and the hippocampi from each group were dissected ( $n = 3$  mice/group).

The samples were processed for embedding in paraffin wax after fixation in 10% buffered formalin, and stored at  $-70^{\circ}\text{C}$  for biochemical analysis.

The behavioral dysfunction in the mice after relatively low-dose of ARS was evaluated after whole-body irradiation with 2 Gy of <sup>60</sup>Co gamma-rays (Gamma-cell Elan 3000; Nordion International) at a dose rate of 8.2 Gy/min by open field analysis ( $n = 8$  mice/group), passive avoidance ( $n = 10$  mice/group), and object recognition memory test ( $n = 9$  mice/group) at 1, 3 and 7 days after irradiation, respectively. The sham control mice were also transported to the irradiation facility but were not irradiated.

### *Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL)*

DNA fragmentation was detected using in situ nick end-labeling (Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling, TUNEL), which was performed according to the manufacturer's instructions (ApopTag<sup>®</sup> In Situ Apoptosis Detection Kit; Intergen, Purchase, NY, USA).

### *Immunohistochemistry*

Five-micron-thick coronal sections of paraffin wax-embedded brain tissue was deparaffinized and allowed to react with the immunohistochemical markers of neurogenesis, including monoclonal rabbit anti-Ki-67 (DRM004; 1:500; Acris Antibodies GmbH, Hiddenhausen, Germany) and polyclonal rabbit anti-DCX (1:400; Cell Signaling Technology, Beverly, MA, USA) antibodies. The immunoreactions were visualized using avidin–biotin peroxidase complexes (Elite Kit; Vector Laboratories, Burlingame, CA, USA), and the peroxidase reaction was developed using a diaminobenzidine substrate kit (Vector Laboratories). As a control, the primary antibodies were omitted for a few test sections in each experiment. No specific labeling of the cells was observed in any of the sections (data not shown). The sections were counterstained with hematoxylin prior to mounting.

### *Determination of cell number*

The number of cells with apoptotic nuclei (TUNEL-positive), proliferating cells (Ki-67-positive) and immature progenitor cells (DCX-positive) were scored blind using a histomorphometric approach.<sup>14,17,18</sup> The brain from each mouse was sampled at level  $\sim 2.12$  mm behind the bregma. A standardized counting area, which contained 5- $\mu\text{m}$ -thick coronal sections in a one-in-ten series of sections representing the rostral/mid-hippocampus, was used. For each mouse, three non-overlapping sections were analyzed, one each from the three regions of the hippocampus ( $\sim 50$   $\mu\text{m}$  apart).

All positively labeled cells within the SGZ of the supra- and infra-pyramidal blades of the DG were quantified. The number of positive cells was determined by averaging the values from each hemisphere in three tissue sections, and is expressed as the mean  $\pm$  SEM for each group.

#### *Western blot analysis*

The hippocampus from each mouse was immersed quickly in buffer H (50 mM  $\beta$ -glycerophosphate, 1.5 mM EGTA, 0.1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM DTT, 10  $\mu\text{g/ml}$  aprotinin, 2  $\mu\text{g/ml}$  pepstatin, 10  $\mu\text{g/ml}$  leupeptin, 1 mM PMSF, pH 7.4), and sonicated for 10 sec. A sodium dodecyl sulfate (SDS) sample buffer (4 $\times$ ) was added to each homogenized sample, and the samples were heated to 100°C for 10 min. The samples were then separated by 7% SDS-PAGE, transferred to a PVDF membrane, and blocked with 5% skim milk in PBS-T (PBS, 0.1% tween 20) for 30 min at room temperature. The membranes were then incubated with monoclonal rat anti-mouse F4/80 (Cl:A3-1; 1:100; AbD Serotec, Oxford, UK) antibody in PBS-T overnight at 4°C. After extensive washing and incubation with horseradish peroxidase (HRP)-conjugated rabbit anti-rat antibody (1:10,000, Vector Laboratories), the signals were visualized using chemiluminescence methods (SuperSignal<sup>®</sup> West Pico, Pierce, Rockford, IL). For normalization purposes, the membranes were re-probed with the antibodies for beta-actin (1:20,000, Sigma). Several exposure times were used to obtain the signals in the linear range. The bands were quantified using Scion Image Beta 4.0.2 for Windows XP software (Scion Corp. Frederick, Maryland).

#### *Open-field test*

Open-field analysis was used to measure the activity of the mice in a novel environment. The 8-week-old mice were placed individually in brightly lit arenas (40 cm  $\times$  40 cm, 250 lux), which were equipped with automated infrared photocells to measure the activity. Parameters, including the total moving distance (cm), resting time (sec), ambulatory movement time (sec), and ambulatory movement episodes, were determined over a 10 min period using the Activity Monitor (MED-associates Inc, VT, USA).

#### *Passive avoidance*

The passive avoidance paradigm was used to examine the hippocampus-dependent association memory,<sup>19–21</sup> in which the animals learned to associate the aversive unconditioned stimulus (mild electric foot shock) with the conditioned stimulus (the dark chamber). During training, a mouse (8-week-old) was introduced to the lit half of the training chamber (Ugo Basile, Italy), and allowed 1 min to explore the area before the trap door was opened. The trap door was then closed, and a mild foot shock (0.5 mA for 1 sec) was delivered immediately after the mouse had entered the darkened half. The trained mouse remained in the dark chamber

for 20 sec after the shock, and was then returned to their home cage. When tested 24 h after training, the trained mouse was re-introduced to the lit chamber. The time spent in the lit half before entering the darkened half was scored as the cross-over latency, and used as an index for memory formation. Five hundred seconds was chosen as the cut off value for the cross-over latency. The mice were removed manually from the lit chamber when the cut-off value was reached.

The sensitivity to the electric foot-shock was further tested in the mice. The electric shock thresholds (mA) to elicit stereotypic responses (flinch, vocalization, and jump/vocalization) were measured.

#### *Object recognition memory test*

Another hippocampus-dependent learning paradigm was used.<sup>20–22</sup> The mice (8-week-old) were first habituated in the training/testing chamber (41.6 cm L, 27.6 cm W, 17.8 cm H) for 24 h. The objects for recognition to be discriminated, which were made from plastic, had three different shapes: cubes, pyramids and cylinders that were 3.5 cm high and they could not be displaced by the mice. The chamber arena and objects were cleaned with 75% ethanol between trials to prevent the build-up of olfactory cues. During training, two objects selected randomly with different shapes were presented to each mouse for 15 min. Twenty-four hours after training, another set of objects (one old object and one novel object) was presented to the trained mice. e.g. If the cube- and pyramid-shaped objects were presented during training, the cylinder-shaped object was used as a novel object during testing. The interaction of the mouse with each object, including approaches and sniffing was scored. If the mouse had memory retention for an old object, it would show preference to the novel object during testing. The percentage of preference was defined as the “number of interactions for a specific object” divided by the “total number of interactions for both objects”.

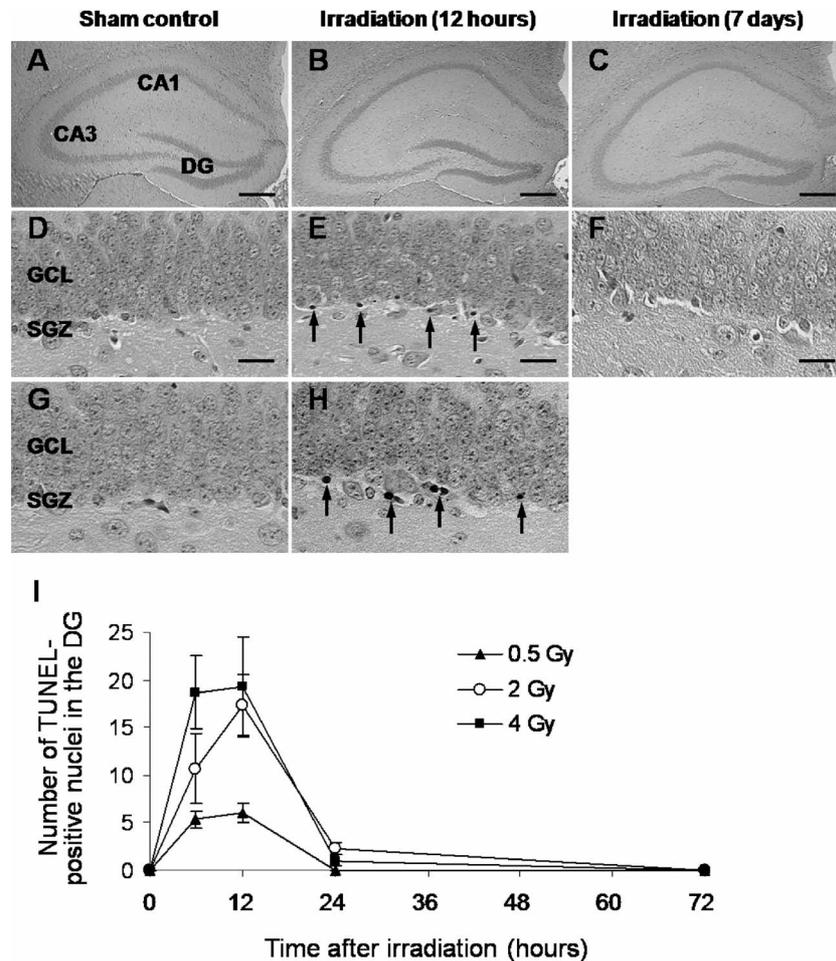
#### *Statistical analysis*

The data is reported as the mean  $\pm$  SEM and were analyzed using one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls post hoc test for multiple comparisons. In all cases, a *P* value < 0.05 was considered significant.

## RESULTS

#### *Detection of apoptotic cells in the hippocampal DGs of adult mice following acute gamma-irradiation at 0.5, 2, and 4 Gy*

No unusual hippocampal structure was observed in the mice after irradiation (Fig. 1, A–C). However, hematoxylin and eosin (H&E) (Fig. 1, D–F) and TUNEL staining (Fig. 1, G and H) revealed the presence of apoptotic nuclei in the hippocampal DGs of the mice. TUNEL-positive apoptotic



**Fig. 1.** Histological results for the sham controls (12 h after sham irradiation of 0 Gy, A and D) and irradiated mouse hippocampus at 12 h (B and E) and 7 days (C and F) after acute gamma-irradiation. **A–C.** No unusual hippocampal structure was observed in sham controls and irradiated mice. **D–F.** Apoptotic nuclei (E, arrows) were detected in the SGZ of the DG in irradiated mice by hematoxylin and eosin staining. **G and H.** TUNEL staining of the sham controls (G) and irradiated mouse hippocampus at 12 h (H) after acute gamma-irradiation (2 Gy). The arrows in H indicate TUNEL-positive apoptotic nuclei. **I.** The number of TUNEL-positive nuclei in the DGs of adult mouse hippocampi changed in a time-dependent manner following acute gamma-irradiation. The number of TUNEL-positive nuclei increased significantly 6–12 h postirradiation ( $P < 0.05$  vs. sham controls). The cells in G and H were counterstained with hematoxylin. GCL, granular cell layer; SGZ, subgranular zone; DG, dentate gyrus. Scale bars in (A–C) = 200  $\mu$ m. Scale bars in (D–H) = 20  $\mu$ m. The data is reported as the mean  $\pm$  SEM (for I).

nuclei were detected primarily in the neural precursor cells of the SGZ along the supra- and infra-pyramidal blades of the DG (Fig. 1H). All histological examinations in this study showed similar findings, which were largely consistent with a previous report.<sup>23)</sup>

Increasing numbers of TUNEL-positive, apoptotic cells began to accumulate in the DGs of the gamma-irradiated mice within 6 h after irradiation at 0.5, 2, and 4 Gy; the number of TUNEL-positive nuclei peaked within 12 h postirradiation [0.5 Gy:  $6 \pm 1$  nuclei/DG ( $n = 3$ ,  $P < 0.01$  vs. control), 2 Gy:  $17.3 \pm 3.3$  nuclei/DG ( $n = 3$ ,  $P < 0.01$  vs. control), and 4 Gy:  $19.3 \pm 5.2$  nuclei/DG ( $n = 3$ ,  $P < 0.05$  vs.

control); Fig. 1I]. However, the number of TUNEL-positive nuclei declined sharply between 12 and 24 h postirradiation (Fig. 1I). This suggests that single-dose irradiation at 0.5, 2, and 4 Gy has transient detrimental effects on the neural stem cells of the DG in adult mouse hippocampi.

#### *Acute gamma-irradiation (2 Gy) transiently decreases Ki-67 and DCX expression in the hippocampal DGs of adult mice*

Ki-67- (Fig. 2A) and DCX-positive cells (Fig. 2C) were consistently observed in the DGs of the hippocampus in adult control mice.

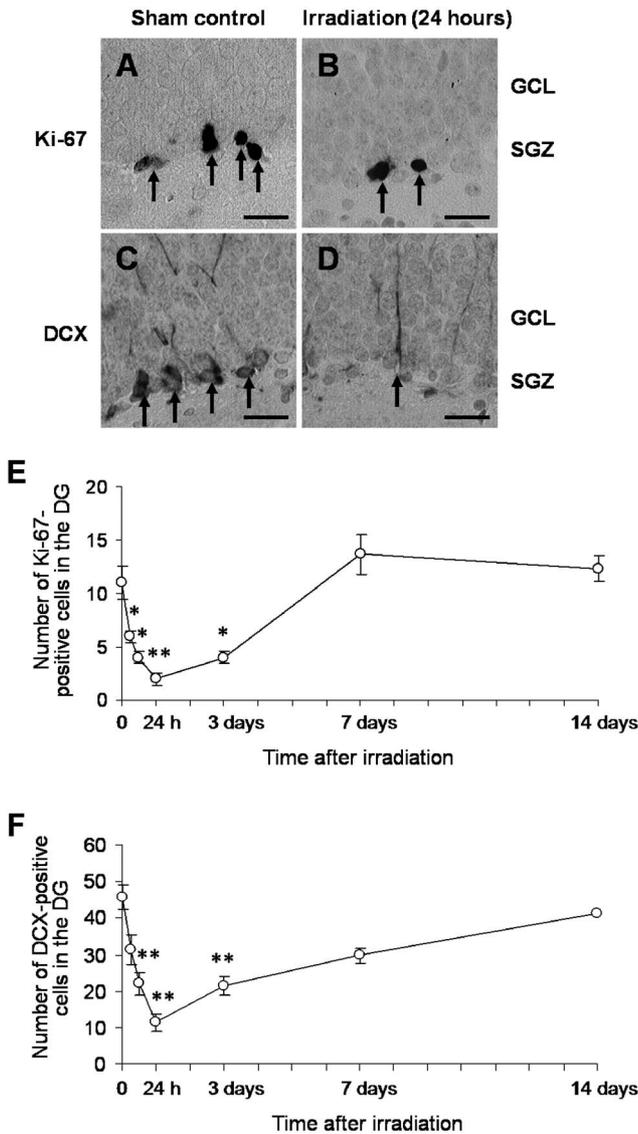
At 2 Gy, between 0 and 24 h postirradiation, the number of Ki-67-positive cells in the DG markedly declined ( $2 \pm 0.6$  cells/DG,  $n = 3$ ,  $P < 0.01$  vs. control; Fig. 2, B and E); however, it gradually increased to the sham control level ( $11 \pm$

1.5 cells/DG,  $n = 3$ ) between 3 and 7 days (Fig. 2E).

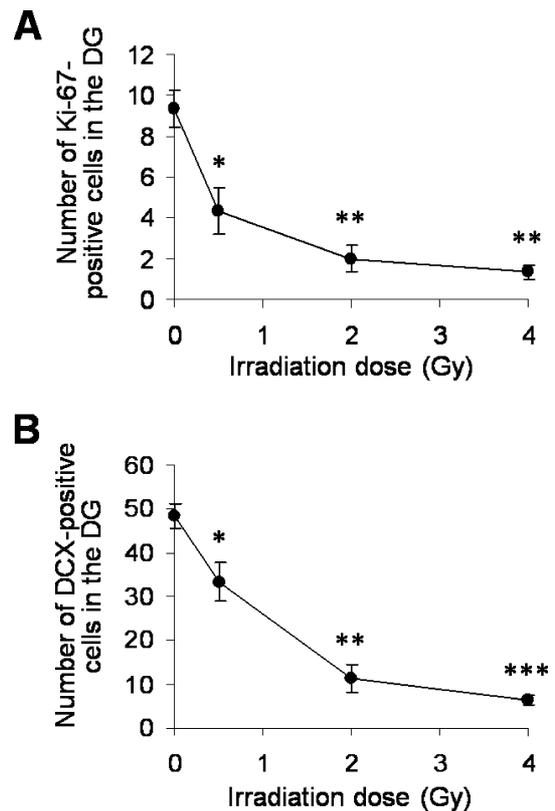
In comparison, the number of DCX-positive cells in the DG declined sharply between 0 and 24 h postirradiation ( $11.3 \pm 2.4$  cells/DG,  $n = 3$ ,  $P < 0.01$  vs. control; Fig. 2, D and F), which then increased gradually to the sham control level ( $45.7 \pm 3.3$  cells/DG,  $n = 3$ ) between 3 and 14 days postirradiation (Fig. 2F). However, after 4 Gy of irradiation, decrease in either type could not be returned (at least, up to 14 days; data not shown). This suggests that in the short term, neurogenesis in the DGs of adult mice with low-level ARS is reversible (exposure range 0.5–2 Gy).

*Dose-dependent changes in Ki-67 and DCX immunoreactivity in the hippocampal DGs of adult mice following acute gamma-irradiation*

Ki-67 immunoreactivity in the SGZ of the DG decreased progressively with increasing dose of gamma-irradiation (0–4 Gy; Fig. 3A). In contrast to the pattern of increase



**Fig. 2.** Temporal changes in the number of proliferating cells (Ki-67, A and B) and immature progenitor cells (DCX, C and D) in the DG after acute gamma-irradiation (single dose of 2 Gy). **A–D.** Representative images showing the Ki-67- and DCX-positive cells in the DGs of adult hippocampi taken from the sham controls (24 h after sham irradiation of 0 Gy) and mice 24 h after irradiation (2 Gy). **E and F.** The number of Ki-67- and DCX-positive cells in the DG markedly decreased 12–24 h after 2 Gy of irradiation but it increased gradually to the control level. The cells were counterstained with hematoxylin (A–D). GCL, granular cell layer; SGZ, subgranular zone; DG, dentate gyrus. Scale bars = 20  $\mu$ m. The data is reported as the mean  $\pm$  SEM (for E and F). \*  $P < 0.05$  vs. sham controls, \*\*  $P < 0.01$  vs. sham controls, \*\*\*  $P < 0.001$  vs. sham controls.



**Fig. 3.** Dose-dependent changes in Ki-67 and DCX immunoreactivity in the DGs of adult mouse hippocampi at 24 h after acute gamma-irradiation. **A.** The number of Ki-67-positive cells in the DGs. **B.** The number of DCX-positive cells in the DGs. Ki-67 and DCX immunoreactivity in the SGZ of the DG decreased as the dose of gamma-irradiation increased (0–4 Gy). The data is reported as the mean  $\pm$  SEM. \*  $P < 0.05$  vs. sham controls (24 h after sham irradiation of 0 Gy), \*\*  $P < 0.01$  vs. sham controls, \*\*\*  $P < 0.001$  vs. sham controls.

observed in the TUNEL-positive apoptotic cells, the number of Ki-67-positive proliferating cells declined steeply at 0 to 1 Gy of irradiation but leveled off slowly as the dose was increased.

A decrease in the number of DCX-positive cells in the DG was also observed with increasing irradiation dose (0–4 Gy; Fig. 3B). An obvious decrease was observed in the number of DCX-positive cells between 0 and 0.5 Gy but the change leveled off at higher doses.

#### *Immunoreactivity of F4/80 in adult mouse hippocampi following acute gamma-irradiation*

F4/80, an immunochemical marker of microglia,<sup>24)</sup> was used to assess the degree of tissue inflammation in the DG.<sup>17,25,26)</sup> When the immunoreactivity of F4/80 was assessed semiquantitatively ( $n = 3$  per group) using Western blot analysis, no significant difference in the immunoreactivity of F4/80 was observed between the groups (Fig. 4).

#### *Mice with acute whole body irradiation (2 Gy) showed normal locomotor activity in open field test*

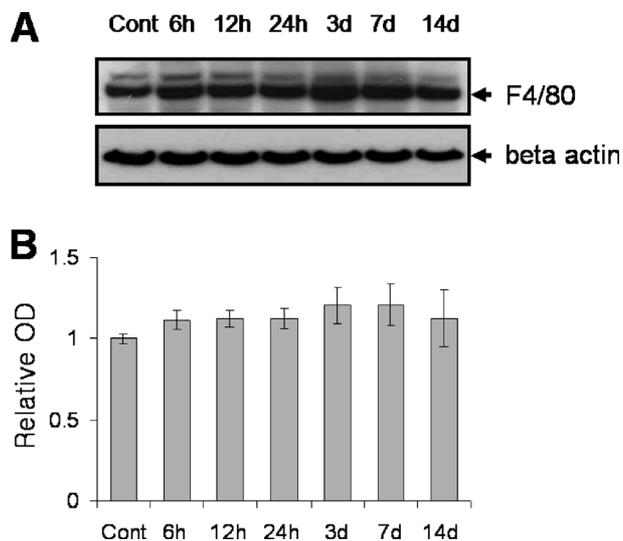
The basal locomotor activity of sham controls and mice 1, 3 and 7 days after irradiation was examined in a novel environment by open field analysis ( $n = 8$  for each group), because different levels of anxiety can affect the motivation and performance in the learning and memory tests. The sham controls and mice 1, 3 and 7 days after irradiation showed comparable moving distances, ambulatory movement times and episodes, and resting times (see Table 1). This suggests that acute whole body irradiation of 2 Gy did not alter basal locomotor activity that can contribute to potential differences in hippocampus-dependent learning and memory behavior tests.

#### *Mice with acute whole body irradiation (2 Gy) showed transient memory deficit in passive avoidance*

The mice were examined by passive avoidance ( $n = 10$  for

each group), which is one of the sensitive hippocampus-dependent learning and memory tests.<sup>19–21)</sup> All mice subjected to this task stepped through the door into a dark compartment for a short period during the acquisition trial ( $39.6 \pm 6.7$ ,  $34 \pm 11.1$ ,  $36.4 \pm 4.9$ , and  $38.2 \pm 7.5$  sec for the sham controls and mice 1, 3 and 7 days after irradiation, respectively), when they had received an electric foot-shock (Fig. 5A).

The memory retention trials (testing) were carried out 24 h after the acquisition trial (Training). The sham-irradiated

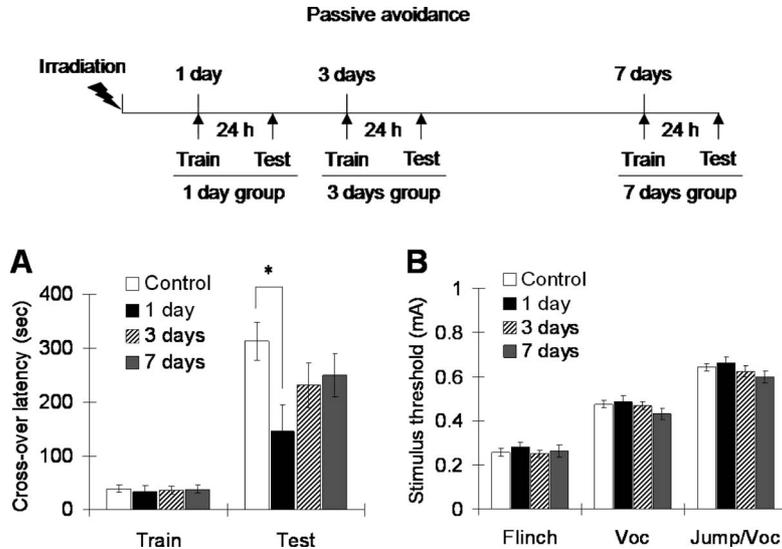


**Fig. 4.** Western blot analysis of F4/80 in adult hippocampus of the sham control (lane 1) and experimental mice at 6 (lane 2), 12 (lane 3), 24 h (lane 4), 3 (lane 5), 7 (lane 6) and 14 days (lane 7) postirradiation (2 Gy). **A.** Representative photographs of the Western blots for F4/80 (~160 kDa) and beta actin (~45 kDa). **B.** Bar graphs of the densitometric data analyses of the Western blots. The data is presented as the mean  $\pm$  SEM ( $n = 3$  mice/group) for the protein level of each group relative to the level of the sham control (arbitrarily defined as 1), after normalization against the beta actin in each sample.

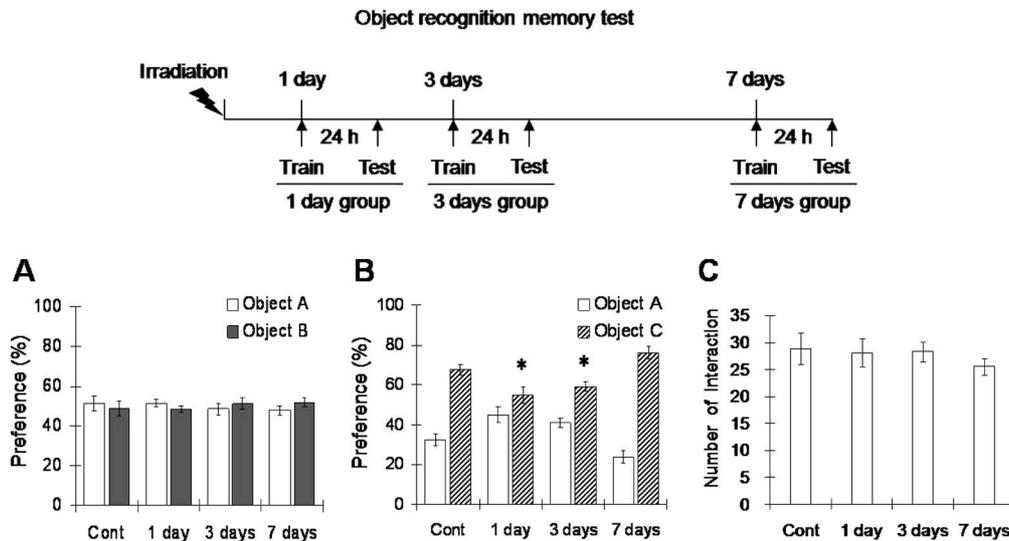
**Table 1.** Open-field analysis of mice 1, 3 and 7 days after acute gamma-irradiation in a novel environment.

	Distance (cm)	Movement time (sec)	Movement episodes	Resting time (sec)
Sham control	1553 $\pm$ 150	103 $\pm$ 10.9	1245 $\pm$ 168	317.1 $\pm$ 13.4
1 day after irradiation	1443 $\pm$ 127	97.2 $\pm$ 8.7	1160 $\pm$ 134	327 $\pm$ 9.1
3 days after irradiation	1616 $\pm$ 117	106.1 $\pm$ 8.8	1281 $\pm$ 137	307.1 $\pm$ 11.4
7 days after irradiation	1401 $\pm$ 136	97.6 $\pm$ 9.4	1199 $\pm$ 137	329.9 $\pm$ 9.8

The data for sham controls (1 day after sham irradiation of 0 Gy), and mice 1, 3 and 7 days after 2 Gy of acute gamma-irradiation were monitored, respectively ( $n = 8$  for each group). There were no significant differences in the movement distance ( $P = 0.584$  [1 day],  $P = 0.336$  [3 days] and  $P = 0.465$  [7 days] vs. sham controls, respectively), ambulatory movement time ( $P = 0.685$  [1 day],  $P = 0.485$  [3 days] and  $P = 0.717$  [7 days] vs. sham controls, respectively), ambulatory movement episodes ( $P = 0.699$  [1 day],  $P = 0.537$  [3 days] and  $P = 0.834$  [7 days] vs. sham controls, respectively), and resting time ( $P = 0.551$  [1 day],  $P = 0.195$  [3 days] and  $P = 0.452$  [7 days] vs. sham controls, respectively) in the sham controls and mice 1, 3 and 7 days after acute gamma-irradiation. The data is reported as the mean  $\pm$  SEM.



**Fig. 5.** Mice with acute gamma-irradiation showed a transient deficit in passive avoidance. **A.** Sham controls (1 day after sham irradiation of 0 Gy) and mice 1, 3 and 7 days after 2 Gy of acute gamma-irradiation were trained by passive avoidance, respectively ( $n = 10$  for each group). The trained mice were tested 24 h after training. The cross-over latency was recorded. Mice trained 1 day after irradiation showed weaker learning and memory formation than the sham controls, as indicated by the significantly lower cross-over latency on testing ( $* P < 0.05$ ). **B.** The electric shock thresholds (mA) to elicit stereotypic responses (flinch, vocalization, and jump/vocalization) were measured for sham controls (1 day after sham irradiation of 0 Gy), and mice 1, 3 and 7 days after 2 Gy of acute gamma-irradiation ( $n = 8$  for each group). The threshold current to elicit stereotypic responses was similar in sham controls and mice 1, 3 and 7 days after irradiation. The data is reported as the mean  $\pm$  SEM.



**Fig. 6.** Acute gamma-irradiation transiently decreases object recognition memory in mice with acute gamma-irradiation. The sham controls (1 day after sham irradiation of 0 Gy), and mice 1, 3 and 7 days after 2 Gy of acute gamma-irradiation were examined ( $n = 9$  for each group). During training, two objects were presented to each mouse for 15 min. After 24 h, one of the old objects was replaced with a novel object (testing). If the mouse remembered the old object, it would spend more time with the novel object during testing, as indicated by the higher percentage of object preference. **A.** The sham controls and mice 1, 3 and 7 days after irradiation showed equal preference to the two objects during training. **B.** During testing, sham control and mice trained 1, 3 and 7 days after irradiation showed significant preference to the novel object, but there is no significant preference for the novel object in the mice trained 1 day after irradiation. There was a significant difference in the novel object preference between the sham controls and mice trained 1 day after irradiation during testing. **C.** There were no significant differences in the interaction with the two training objects during training between the sham controls and irradiated mice groups (1, 3 and 7 days after irradiation). The data is reported as the mean  $\pm$  SEM.  $* P < 0.05$  vs. sham controls.

mice showed a significant increase in cross-over latency when tested 24 h after training ( $313.1 \pm 34.7$  sec, Fig. 5A). The mice trained 1 day after irradiation showed significantly lower cross-over latency during the test ( $145.9 \pm 49.6$  sec,  $P = 0.013$  vs. sham control, Fig. 5A). However, there were no significant differences in the cross-over latency (testing) between either mice trained 3 ( $231.2 \pm 37.3$  sec) or 7 days ( $249.5 \pm 41.1$  sec) after irradiation and the sham controls (Fig. 5A).

Their sensitivity to the electric foot-shock was further tested ( $n = 8$  for each group), and there were no significant differences in the threshold current to elicit stereotypic responses, including flinch, vocalization, and jump/vocalization, between sham controls and irradiated mice (Fig. 5B). This suggests that they had comparable sensitivity to the electric foot-shock.

#### *Acute gamma-irradiation (2 Gy) transiently decreases object recognition memory in mice*

We further examined mice ( $n = 9$  for each group) by another sensitive hippocampus-dependent paradigm, object recognition memory.<sup>20–22</sup> The sham controls and mice 1, 3 and 7 days after acute irradiation displayed an equal preference to the two objects during training (Fig. 6A). During testing, the mice trained 1 and 3 days after irradiation showed memory deficits ( $P < 0.05$  vs. sham controls), whereas the mice trained 7 days after irradiation did not show a deficit in object recognition memory (Fig. 6B). There was no significant difference in the total time spent exploring both objects during the training trial between the groups. During the test, the preferences (mean  $\pm$  SE) to a novel object were  $67.68 \pm 2.7\%$  in the sham controls,  $54.9 \pm 4.2\%$  in the mice trained 1 day,  $59.1 \pm 2.2\%$  in mice trained 3 days, and  $76.1 \pm 3.1\%$  in mice trained 7 days after irradiation.

There was no significant difference in total number of interactions during training between the sham control ( $28.9 \pm 2.9$ ) and mice 1 day ( $28.1 \pm 2.7$ ,  $P = 0.845$  vs. sham controls), 3 days ( $28.3 \pm 1.9$ ,  $P = 0.873$  vs. sham controls) and 7 days ( $25.6 \pm 1.5$ ,  $P = 0.319$  vs. sham controls) after irradiation (Fig. 6C). This suggests that they had comparable attention, motivation and visual perception.

## DISCUSSION

This study detected an increase in apoptosis between the SGZ cells of the DG 6–12 h after acute gamma-irradiation (2 Gy), which then declined to the control level after 24 h. In contrast, after 24 h, the level of neurogenesis in the DG decreased markedly but returned to the sham control level 7–14 days after irradiation. In addition, there was a dose-dependent increase in apoptosis and decrease in neurogenesis after gamma-irradiation.

Neurogenesis gives rise to new neural cells in the adult brain of various species. The adult brain retains at least two

active germinal zones: the SGZ in the DG, which generates new granular cells in the adult hippocampus, and the fore-brain subventricular zone, which gives rise to granular cells in the olfactory bulb.<sup>27,28</sup> The hippocampus is involved in cognitive processing, including learning and memory, and neurogenesis in the DG of adults has received widespread attention as it may be associated with learning and memory.<sup>15,16,29</sup> The progenitor neural cells in the DG of the adult hippocampus are particularly vulnerable to ionizing radiation,<sup>23,30</sup> which may cause hippocampus-dependent learning and memory impairment.<sup>26,31</sup> However, little is known regarding the temporal and dose-dependent effects of irradiation on hippocampal neurogenesis in adult mice and the precise correlation between hippocampal neurogenesis and cognitive impairment caused by acute irradiation.

There is consensus exists as to the irradiation doses needed to kill proliferating progenitor cells in an adult brain without inducing serious short-term side effects.<sup>16,26</sup> Several studies have reported the effects of irradiation on the hippocampal structure and/or function in pre-/postnatal and adult animals. In pre-/postnatal animals, irradiation causes variable amounts of morphological changes in the brain structure. However, fewer structural changes are associated with irradiation in adult brains.<sup>13,16,26,32–34</sup> In the brains of adult rats, a single 10 Gy dose induces apoptosis in the proliferating stem cells in the DG of the hippocampus, while the remaining cells are unaffected.<sup>30</sup> Similarly, a single 5 Gy dose was reported to block adult neurogenesis.<sup>4</sup> In a mouse brain, a 10 Gy X-ray dose resulted in the significant loss of proliferating cells ( $\sim 90\%$  relative to control cells 3–4 months after irradiation) in the DG.<sup>14</sup> In this study, it was found that gamma-irradiation at 0.5 to 4 Gy produced few changes in the hippocampal structure in adult mice but the amount of gamma-irradiation used altered hippocampal neurogenesis in the mice in a time- and dose-dependent manner. This suggests that the range of gamma-irradiation used in this study is sufficiently detrimental to inhibit neurogenesis in the hippocampi of mice without causing changes in hippocampal structure, and can be applied in future studies on the hippocampal function.

Previously, Raber *et al.*<sup>14</sup> reported that a 10 Gy X-ray dose resulted in the significant and prolonged loss of proliferating cells and their progeny 3–4 months after irradiating the DG in adult mice. However, a recent report<sup>35</sup> showed the acute effect of 4 Gy of cranial X-irradiation on adult rat neurogenesis, which was fully reversed 1 month later. In this study, it was found that irradiation has a reversible effect on hippocampal neurogenesis in adult mice. The immunohistochemical markers for microglia have been used to monitor the degree of tissue inflammation in the DG and elsewhere.<sup>17,25,26</sup> Furthermore, during inflammation, activated microglia may prolong the reduction in adult rat neurogenesis.<sup>25,26</sup> These results show the restoration of adult hippocampal neurogenesis in the short term (approximately 1

week) without changes in immunoreactivity upon microglial activation, even though the mice had been irradiated.

This study examined two hippocampus-dependent learning paradigms, passive avoidance and object recognition memory test, in mice 1, 3 and 7 days after acute irradiation, in order to determine the precise correlation between hippocampal neurogenesis and the cognitive impairment caused by acute whole body irradiation. Previously, it was reported that low-dose of gamma-ARS (1.5 Gy) does not affect the passive avoidance tasks in Swiss albino CD1 mice.<sup>36)</sup> In contrast, these results showed that acute whole body irradiation of 2 Gy induced transient memory impairment in mice trained 1 day after irradiation. Therefore, this study showed that reduced hippocampal neurogenesis correlated in time with the deficit of hippocampal-dependent memory retention, and the recovered hippocampal neurogenesis correlated with the recovery of hippocampal-dependent memory retention. However, more studies will be needed to establish an unequivocal link between the changes in hippocampal neurogenesis and behavior. Furthermore, since other regions were not included in this analysis, the possibility remains that neural changes in other regions may contribute to the radiation-induced cognitive impairment observed in this study.

As shown in this study, reversible alteration of adult hippocampal neurogenesis in the DGs with relatively low-dose of ARS (exposure to 2 Gy) is associated with the transient impairment of hippocampus-dependent learning and memory in adult ICR mice. This suggests that it may be a good model for examining the short-term cognitive impairment caused by the transient inhibition of hippocampal neurogenesis.

In conclusion, the pattern of the hippocampus-dependent memory dysfunction is consistent with the change in neurogenesis after acute irradiation. This suggests that a relatively low-dose of ARS in adult ICR mice can interrupt the functioning of the hippocampus, including learning and memory, possibly through the inhibition of neurogenesis.

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