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MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

Sex-dependent differences in behavior and hippocampal neurogenesis after irradiation to the young mouse brain

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Abstract

Cranial radiotherapy in the treatment of pediatric malignancies may lead to cognitive deficits, and girls suffer more severe deficits than boys. However, most experimental studies are performed on male animals only. Our aim was to investigate possible long-term gender differences in response to cranial irradiation (IR). Basal neurogenesis in non-irradiated mice was higher in females but this was not apparent until the animals were adult. Male and female C57BL/6J mice received a single dose of 8 Gy to the whole brain on postnatal day 14 and were killed 6 h or 4 months later. Proliferation in the subgranular zone of the dentate gyrus in the hippocampus, as judged by the number of phosphohistone H3-positive cells, was reduced by half 6 h after IR in both males and females. The reduced proliferation was still obvious 4 months after IR. Consequently, the continuous addition of new neurons to the granule cell layer (GCL) during brain growth was reduced in irradiated mice, and the reduction was more pronounced in females. This resulted in hampered growth of the GCL, reduced bromodeoxyuridine incorporation in adulthood, and severely reduced adult neurogenesis, as judged by the number of doublecortin-positive cells in the GCL. In an open-field test, locomotor activity was increased in both males and females after IR and anxiety levels were increased, more so in females. In an IntelliCage test, place learning was impaired by IR in females but not males.

Introduction

Of all pediatric malignancies, almost one-third are brain tumors and the incidence has increased over recent decades (Dreifaldt *et al.*, 2004; Smith *et al.*, 2010; Rosychuk *et al.*, 2011). Improved treatment protocols have considerably increased survival and today > 70% survive their disease (Armstrong *et al.*, 2009). Treatment strategies for pediatric malignancies are associated with adverse late effects such as perturbed growth, hormonal imbalances, learning difficulties and cognitive decline (Lannering *et al.*, 1990; Han *et al.*, 2009). At greatest risk of cognitive decline are children who receive radiotherapy to the CNS (Packer *et al.*, 2003; Lahteenmaki *et al.*, 2007; Mueller & Chang, 2009). Young age at diagnosis and female gender are associated with a greater risk of cognitive decline (Ris *et al.*, 2001; Fouladi *et al.*, 2005; Lahteenmaki *et al.*, 2007). Lahteenmaki *et al.* (2007) reported a Finnish, nation-wide, register-based study where cranial radiotherapy (CRT) patients had lower overall grade averages than their peers, and young girls treated with CRT showed greater differences than their male counterparts. Injury caused by irradiation (IR) affects many regions and cell types, but the underlying pathogenesis is not well understood. It has been suggested that injury to neural stem and progenitor cells in the hippocampus contributes to learning deficits after IR. Neurogenesis occurs throughout life in the subventricular zone and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus (Kempermann *et al.*, 2004). These regions are susceptible to irradiation, especially in the developing brain (Fukuda *et al.*, 2004, 2005; Rola *et al.*, 2004). It has been shown that IR perturbs the microenvironment in the brain and that part of the damage seen after IR could be explained by inflammation (Monje *et al.*, 2002; Hellstrom *et al.*, 2009, 2011; Kalm *et al.*, 2009).

Irradiation has been shown to alter locomotor behavior in mice subjected to IR early in life (Naylor *et al.*, 2008), but voluntary running could ameliorate these IR-induced changes, at least in male mice (Naylor *et al.*, 2008). Gender differences have been reported in behavior tests, such as spatial learning, where males outperformed females. This difference has been documented in both rodents and humans (Cimadevilla & Arias, 2008; Woolley *et al.*, 2010). To our knowledge, gender and behavior after irradiation to the developing brain have not been studied in the IntelliCage platform before. This behavioral test minimizes handling of the animals and allows learning and memory to be studied in a home cage environment over a longer period of time (Galsworthy *et al.*, 2005; Knapska *et al.*, 2006; Karlsson *et al.*, 2011).

This study aimed to investigate differences between genders in hippocampal neurogenesis after IR to the developing brain. We further

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aimed to investigate possible long-term behavioral differences after IR early in life.

Materials and methods

Animals

Male and female C57BL/6J mice were used (Charles River Laboratories, Sulzfeld, Germany). The animals were kept on a 12-h light–dark cycle. Food and water were provided *ad libitum.* Animals used in the IntelliCages were at the time of weaning anesthetized, and microtransponders were implanted (Datamars; Petlink, Youngstown, OH, USA). All experiments were approved by the Gothenburg committee of the Swedish Animal Welfare Agency (46-2007, 30-2008, 423-2008 and 326-09).

Irradiation procedure

For the irradiation procedure, a linear accelerator (Varian Clinac 600 CD; Radiation Oncology Systems LLC, San Diego, CA, USA) with a 4-MV nominal photon energy and a dose rate of 2.3 Gy/min was used. Male and female littermates were anesthetized on postnatal day (P)14 with an intraperitoneal tribromoethanol injection (50 mg/kg) and then placed in the prone position on a polystyrene bed. The head was covered with a 1 cm tissue equivalent material to obtain an even irradiation dose throughout the underlying tissue. The whole brain was irradiated with an irradiation field of 2 × 2 cm and the source to skin distance was ~ 99.5 cm. An absorbed dose of 8 Gray (Gy) was administered. A single dose of 8 Gy is equivalent to 18 Gy delivered in 2-Gy fractions, according to the linear quadratic formula and an alpha/beta ratio of 3 for late effects in the normal brain tissue (Fowler, 1989). Control animals were anesthetized but not subjected to irradiation. After irradiation, the pups were returned to their dams. Animals were killed 6 h post-IR (20 males and 20 females) or 4 months post-IR (70 males and 70 females). The timeline of the study is represented in Fig. 1.

Bromodexoyuridine (BrdU) labelling

Animals used in the IntelliCage experiment were given one daily injection of BrdU (50 mg/kg) for three consecutive days after the IntelliCage experiment (3 months of age; Fig. 1). All BrdU injections were given at the beginning of the active period. Four weeks later the animals were killed.

Blood collection and preparation

Animals used for blood sampling were anesthetized with isoflurane in a mixture of 50% oxygen and air: 4–5% for induction and 2.5–3.5% for maintenance. The chest was opened and blood was drawn from the left heart ventricle using a syringe. The blood samples were centrifuged at 2000 ***g*** for 10 min. The serum obtained was transferred to new tubes and stored at −80 °C. Blood was always collected at the same time of day (09.00–10.00 h) to minimize circadian rhythm variations.

Tissue preparation and cutting

Animals were deeply anesthetized with sodium pentobarbital (Pentothal®; Electra-box Pharma, Tyresö, Sweden) before being transcardially perfused with a 6% formaldehyde solution buffered with sodium phosphate at pH 7.4, and stabilized with methanol (Histofix™; Histolab Products AB, Sweden). The brains were immersion-fixed in Histofix for 24 h after perfusion; this was changed to 30% sucrose solution containing 100 mM phosphate buffer, pH 7.5. The right hemisphere was cut into 25-μm sagittal sections in a series of 12, using a sliding microtome. The sections were stored in a cryoprotection solution, containing 25% ethylene glycol and 25% glycerol, at 4 °C until staining.

Immunohistochemistry

All the immunohistochemistry was performed using free-floating staining protocols. For antigen retrieval, sections to be stained for phosphohistone H3 were treated with 10 mM sodium citrate at 80 °C for 30 min and then rinsed (3 × 10 min) with Tris-buffered saline (TBS; Tris-HCl in 150 mM NaCl, pH 7.5). To block endogenous peroxidases the sections were treated with 0.6% hydrogen peroxide for 30 min and then rinsed (3 × 10) min in TBS. Sections stained for BrdU were treated with 2 M HCl at 37 °C for 30 min, followed by 10 min in 100-mM borate buffer (pH 8.0). After rinsing with TBS, nonspecific binding was blocked by treating the sections with 3% donkey serum in TBS with 0.1% Triton X-100 for 30 min (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA). Sections were then immediately incubated at 4 °C overnight [sections stained for doublecortin (DCX) were incubated for three nights] with primary antibodies against phosphohistone H3 (rabbit polyclonal anti-phosphohistone H3, 1 : 1000; 06-570; Millipore/Chemicon, Billerica, MA, USA), BrdU (rat monoclonal anti-BrdU, 1 : 500, OBT0030; AbD Serotec, Kidlington, UK) or DCX (goat polyclonal anti-DCX, 1 : 125, sc-8066; Santa Cruz Biotechnology, Inc., CA, USA) diluted in 3% donkey serum in TBS containing 0.1% Triton X-100. The following day the sections were rinsed in TBS (3 × 10 min) and a biotinylated secondary antibody was added for 1 h at room temperature (an average of 20 °C; donkey anti-rabbit IgG, 1 : 1000; donkey anti-rat IgG, 1 : 1000; or donkey anti-goat IgG, 1 : 1000, all from Jackson Immunoresearch Laboratories Inc., West Grove,

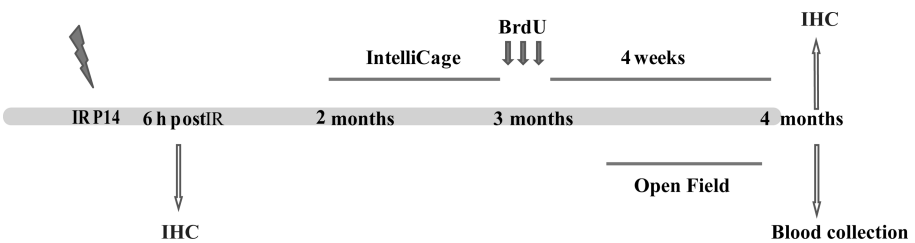

FIG. 1. The timeline of the study. Animals were subjected to IR on P14. One group was killed 6 h post-IR and the tissue was used for immunohistochemistry (IHC). The second group was subjected to IntelliCage followed by three injections of BrdU and then killed 4 weeks later and used for IHC. The third group was placed in an open-field arena prior to blood collection and euthanasia.

PA, USA). The sections were rinsed in TBS (3 × 10 min) before a biotin–avidin solution was added for 1 h (Vectastain ABC Elite kit; Burlingame, CA, USA). The staining was developed using 3–3′-diaminobenzindine tetrahydrochloride (DAB; Saveen Werner AB, Malmö, Sweden) diluted in TBS containing hydrogen peroxide and nickel chloride to enhance the reaction. The reaction was stopped using tap water for several rinses and then placed in TBS before mounting was performed in 0.1 M phosphate buffer, pH 7.5. All three primary antibodies used in this report have been well validated in our lab (Naylor *et al.*, 2008; Hellstrom *et al.*, 2009; Zhu *et al.*, 2010) and they are included in the Neuroscience Information Framework (http://www.neuinfo.org) list of thoroughly characterized antibodies (numbers 310177, 609566 and 2088494, respectively). Omission of the primary antibodies yielded only very weak nonspecific staining. Furthermore, identification of cells immunopositive for phosphohistone H3, BrdU or DCX is facilitated by their characteristic morphology and specific localization.

Cell counting and volume assessment

BrdU-positive ($^+$) cells were counted in the granule cell layer (GCL) on serially cut sagittal sections using stereological principles (Stereoinvestigator; MicroBrightField, Colchester, VT, USA). All immunopositive cells in the SGZ were counted in every 12th section throughout the GCL in the right hemisphere, resulting in analysis of 5–7 sections per animal (n = 9–13). Total volumes were calculated according to the Cavalieri principle, using the following formula: $V = \mathrm{SA} \times P \times T$, where V is the total volume, SA is the sum of area measurements, P is the inverse of the sampling fraction and T is the section thickness. The total number of cells was obtained by multiplying the number of cells with the sampling fraction. The inner length of the GCL was measured as the border between the GCL and the hilus (Fig. 2). Phosphohistone $H3^+$ and DCX^+ cells were counted only in the SGZ, defined as ranging from three cell layers into the GCL to two layers into the hilus ($n_{(6\ \mathrm{h\ post\text{-}IR})}$ = 8–10, $n_{(4\ \mathrm{months\ post\text{-}IR})}$ = 9–13).

IntelliCage

The IntelliCage experiment lasted for 21 days, starting when animals were 2.5 months old. Males and females were tested separately, on different occasions, as the cages are not ventilated. To minimize social stress in the IntelliCages, mice were kept in the same groups of 8–10 animals per group from weaning until the end of the experiments. In each cage, half of the animals were randomized to IR and half to control treatment. Four cages were used in parallel ($n_{\mathrm{females/IR}}$ = 15, $n_{\mathrm{females/Control}}$ = 19, $n_{\mathrm{males/IR}}$ = 17, $n_{\mathrm{males/Control}}$ = 17). IntelliCage is a behavioral assessment method in a home-cage environment where the animals' ability to learn a task can be measured. An IntelliCage is equipped with four conditioning corners, each containing an antenna to register the implanted microtransponder in each mouse entering the corner. Each corner is equipped with two water bottles and, to be able to drink from these, the animal must perform a nose poke to open a door (one door to each water bottle). In each corner there are sensors that register entering (a rise in temperature), nose pokes (crossing a light beam) and drinking (a lick sensor). The software was programmed to open the doors and give access to the water bottles only when an animal with a certain microtransponder enters the corner and performs a nose poke. The doors in the other corners will then be ''incorrect'' for that particular mouse and remain closed when the mouse tries to open them. The experimental set-up has been described earlier, using males only (Karlsson *et al.*, 2011). The experiment started with an introduction period of 6 days, to allow the animals to explore their new environment and learn to perform nose pokes to get access to water. This was followed by a corner-training period, when the animals were randomized to one corner (excluding the most visited corner from the introduction period) and when they had to learn to perform nose pokes in this particular corner to access the water bottles. Nose pokes in the other corners did not open the doors to the water bottles. After 5 days, the correct corner was randomized to another one, excluding the corner which had been assigned previously. The corner-training periods lasted for 5 days each and no more than three mice were assigned to the same correct corner. During the first corner period, place learning is assessed and, during the second and third corner periods, reversal learning is assessed.

Data from the IntelliCages were analyzed using the INTELLICAGE software (IntelliCage Plus, version 2.4; NewBehavior AG, Zurich, Switzerland), Microsoft Excel 2007 and SPSS 17.0 (SPSS Inc, Chicago, IL, USA). The light (inactive) period, 06.00–18.00 h, was not included in the analysis. Visits lasting longer than 180 s were excluded from the analysis to avoid including visits during which the animals rested or slept in the corners (Karlsson *et al.*, 2011).

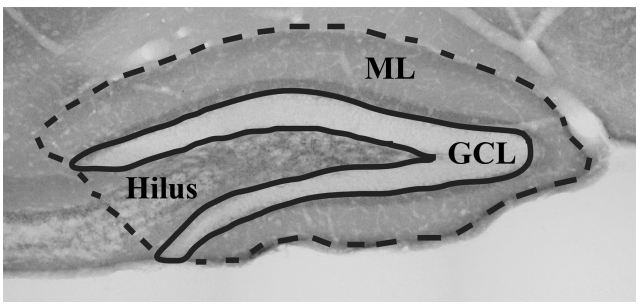

FIG. 2. A representative picture that shows the dentate gyrus. The GCL volume was measured as the area between the hilus and the molecular layer (ML). The inner length of the GCL was measured as the border between the GCL and the hilus.

Open field

Open-field testing was performed when animals had reached the age of 4 months. General locomotor activity was assessed in an open-field arena (50 × 50 cm) for 30 min. Total distance moved and percentage distance moved in the central vs. the entire zone was measured using VIEWER 3.0 Software (Biobserve GmbH, Bonn, Germany). The middle of the body of the animal was defined as the point for tracking zone entries. The central zone was defined as a 30 × 30 cm area in the center of the arena. The animals were introduced to this unfamiliar arena individually (n = 7–9). Four arenas were used simultaneously and recorded from above using a CCD camera. The arenas were made of gray plexiglass and the floors were covered with sawdust which had earlier been exposed to other mice. The walls were cleaned with 70% alcohol and the sawdust was moved around between trials.

Enzyme-linked immunosorbent assay (ELISA)

ELISAs were used to measure hormones that may be affected by irradiation to the hypothalamic–pituitary axis. Duplicate samples were

analyzed from each animal (n = 9–10). The hormones investigated were IGF-1 (IGF-1-R20; Mediagnost, Reutlingeen, Germany), estrogen (TKE21; Siemens Healthcare Diagnostics Ltd, Camberley, UK), testosterone (cat. no. 189102; MP Biomedicals, Costa Mesa, CA, USA), free triiodothyronine (T3; ELISA TF E-3100; LDN Am Eichenhain, Nordhorn, Germany) and free thyroxine (T4; Amerlex-MAB, Trinity Biotech Co., Wicklow, Ireland). The ELISA analyses were performed according to the instructions of the manufacturer.

Statistical analysis

Cell-counting data were analyzed using two-way ANOVA. Treatment and gender, and possible interaction between these two, were considered the main effects. For the IntelliCage and the open-field tests, generalized estimating equations analysis was used as described earlier (Karlsson *et al.*, 2011), If the interaction (time × treatment) showed no significant effect, the interaction term was excluded and the model was re-fitted. The effects of treatment and time were then considered independent, and were evaluated separately from each other in the final results. Statistical analysis was performed using SPSS 17.0 (SPSS, Chicago, IL, USA) and significance was assumed when $P < 0.05$. All data are shown as mean ± SEM.

Results

Proliferation and growth of the GCL after IR

Proliferating cells are particularly susceptible to IR and, when the growing DG is subjected to IR, loss of proliferating neural stem and progenitor cells results in impaired growth and a smaller GCL volume than in non-irradiated brains (Fukuda *et al.*, 2004, 2005). Proliferation was measured by quantifying phosphohistone $H3^+$ ($PHH3^+$) cells in the SGZ of the DG in the hippocampus 6 h and 4 months after IR, respectively. Phosphorylation of the serine 10 residue of histone 3 takes place during the late G2 and M phases of the cell cycle, thereby offering more specific temporal resolution than Ki-67, which is expressed during the S/G2/M phases, and than PCNA, which is expressed during the G1/S/G2/M phases of the cell cycle (Hendzel *et al.*, 1997; Mandyam *et al.*, 2007). At both time points there was a decrease in $PHH3^+$ cell numbers in the irradiated animals (Fig. 3B). At the 6-h time point there was a 44% decrease in males and 46% in females (main effect of treatment, $F = 28.13$, $P < 0.0001$, interaction n.s.). No difference between genders was seen at this early time point. Four months after IR there was still a lower number of $PHH3^+$ cells (35% in males and 31% in females; main effect of treatment, $F = 9.21$, $P = 0.001$, interaction n.s.). In the adult brains, a gender difference was observed: non-irradiated

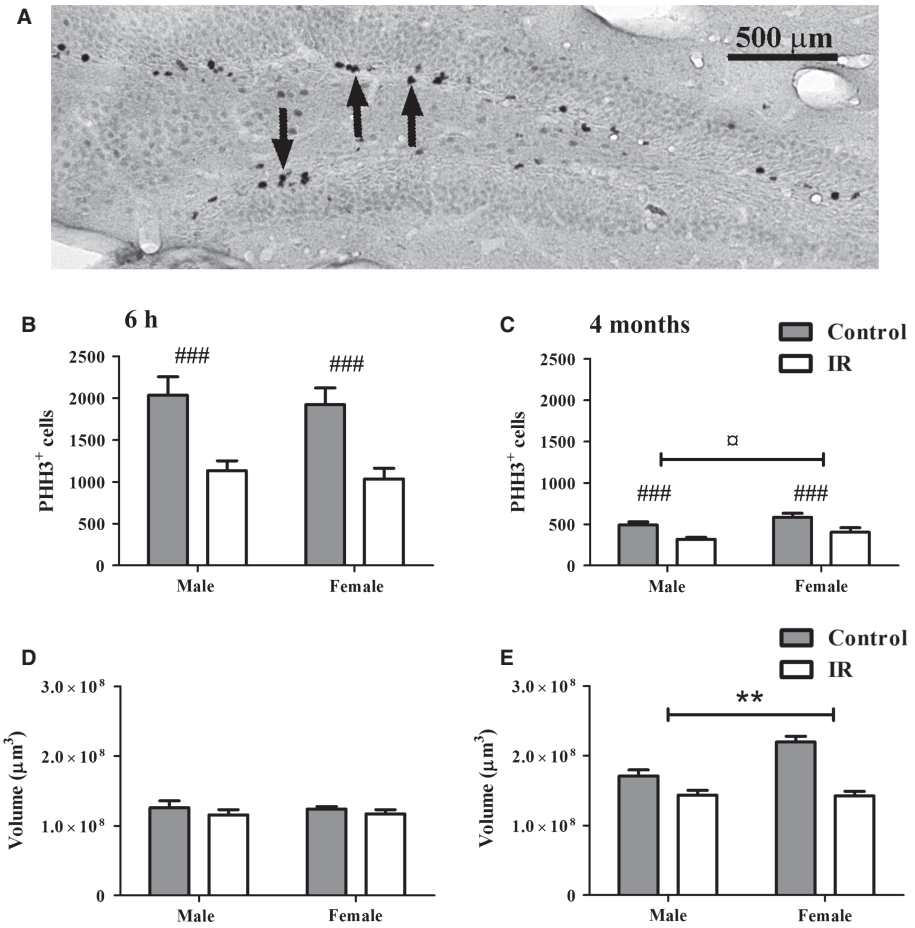

FIG. 3. (A) A representative microphotograph of $PHH3^+$ cells in the GCL from control mouse brain at P14. (B)The number of proliferating $PHH3^+$ cells decreased 6 h post-IR in both males and females. No gender differences were observed at this timepoint. (C) Four months after IR there was still an IR-induced decrease in proliferation in the SGZ in both genders. An interaction between treatment and gender was observed at this timepoint, showing that irradiated females had a greater decrease in proliferation than did males. (D) There was no difference in GCL volumes 6 h post-IR, nor between genders, nor between treated animals. (E) Four months after IR a significant interaction was seen in GCL volume, where females showed a greater lack of growth caused by IR than did male mice. Further on, females that was not subjected to IR showed a larger GCL volume than did control male mice. Data shown as mean ± SEM. $^{¤}P < 0.05$ for gender; $^{**}P < 0.01$ for interaction between treatment and gender; $^{###}P < 0.001$ for treatment.

females had 18% more proliferating cells than males (Fig. 3C; main effect of gender, $F = 4.39$, $P = 0.044$), but the relative loss after IR was not different between males and females (the interaction treatment × gender was not significantly different in the two-way ANOVA).

The GCL volume 6 h after IR did not differ between males and females or between IR and control brains (Fig. 3D). This is not surprising as the time after IR is too short to affect the volume in GCL. When the animals were adult, however, the GCL volume was smaller (growth-retarded) in irradiated males and females (16% smaller in males and 35% smaller in females; Fig. 3E; main effect of treatment, $F = 46.98$, $P < 0.0001$). This can be seen as a result of reduced proliferation from the day of IR. Non-irradiated control females showed a larger GCL volume than their male counterparts (Fig. 3E; main effect of gender, $F = 10.34$, $P = 0.002$). There was an interaction between treatment and gender in GCL volume, where the IR-induced lack of growth was more pronounced in females (Fig. 3E; interaction between gender and treatment, $F = 9.39$, $P = 0.004$). The inner length of the GCL (the SGZ; Fig. 2) was 19% shorter in females and 6% shorter in males after IR (92.7 ± 5.1 μm in control males and 87.0 ± 3.5 μm after IR; 105.8 ± 4.2 μm in control females and 85.3 ± 3.4 μm in females; main effect of treatment, $F = 10.03$, $P = 0.003$; not shown).

Neurogenesis and cell survival in the GCL in the adult mouse brain

Bromodeoxyuridine (BrdU)

BrdU 3 months after IR, followed by quantification of the number of labelled surviving cells in the GCL 4 weeks later (4 months after IR), revealed a lower number of labelled surviving cells in the GCL in irradiated males and females (75% fewer in males, 87% fewer in females: main effect of treatment, $F = 107.69$, $P < 0.0001$; Fig. 4C). There was also a difference between genders, where non-irradiated females showed a higher number of BrdU-labelled cells compared to non-irradiated males (main effect of gender, $F = 8.62$, $P = 0.005$; Fig. 4C). In addition, the relative IR-induced reduction in cytogenic capacity was greater in females than in males (interaction between treatment and gender, $F = 9.19$, $P = 0.004$; Fig. 4C).

Doublecortin (DCX)

Doublecortin was used as a marker of immature neurons in the GCL. While BrdU labelling reflected the total cytogenic capacity, DCX labelling reflected the neurogenic capacity, revealing a virtually complete loss of neurogenesis after IR in both males and females. The absolute numbers of DCX$^+$ cells in the controls correspond well with what others have found (Ben Abdallah *et al.*, 2010). The numbers in

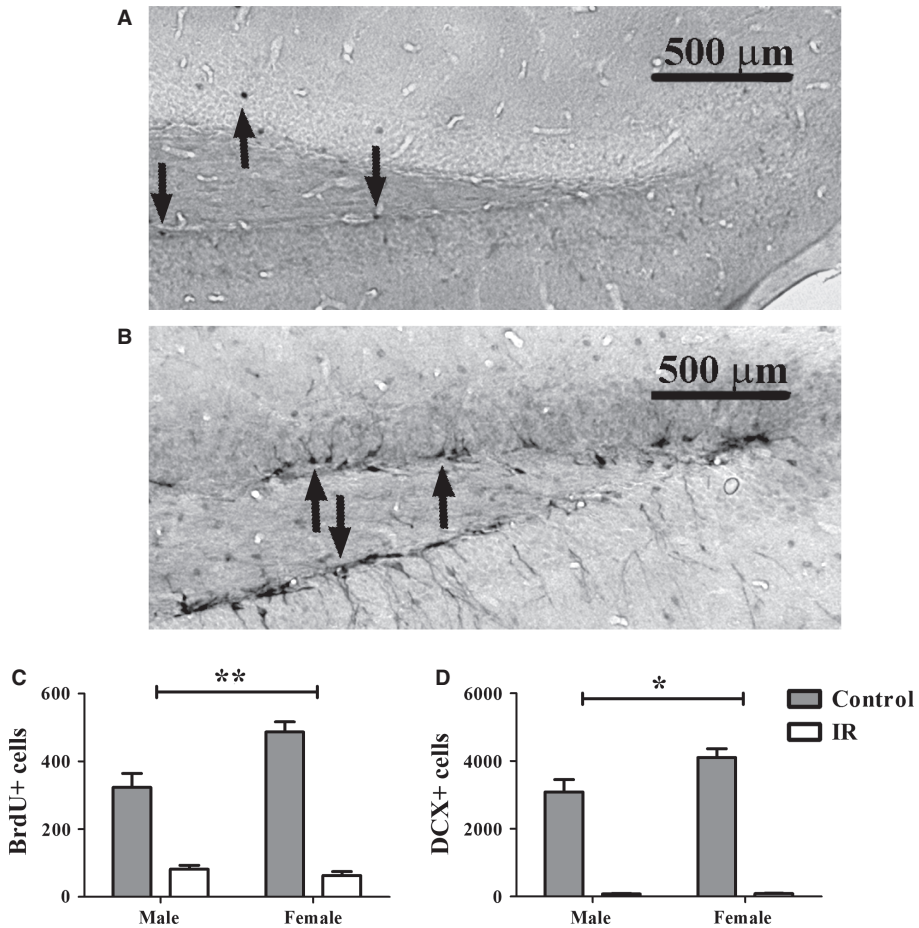

FIG. 4. A representative microphotograph of (A) BrdU$^+$ cells and (B) DCX$^+$ cells in the GCL from adult control mouse brain. (C) The number of BrdU$^+$ cells in the GCL 4 months after IR. There was a significant interaction between gender and treatment showing that BrdU incorporation was more affected in irradiated females than males. Females not subjected to IR showed higher BrdU incorporation than males not subjected to IR. (D) Total number of DCX$^+$ cells in the GCL 4 months after IR. An interaction between gender and treatment was observed in the total number of DCX$^+$ cells, where females showed a greater IR-induced decrease in DCX positive cells than did males. Control females showed a higher total number of DCX$^+$ cells in the GCL than did control males. Data shown as mean ± SEM. *$P < 0.05$, **$P < 0.01$ for interaction between treatment and gender.

males were 3085 ± 369 cells per hippocampus in controls vs. 79 ± 15 cells after IR. In female mice the numbers were 4102 ± 260 vs. 85 ± 12 DCX^+ cells per hippocampus (Fig. 4D; main effect of treatment, $F = 223.40$, $P < 0.0001$). A gender difference was observed in the DCX counts, where non-irradiated females showed higher numbers of immature neurons than did males (main effect of gender, $F = 4.78$, $P = 0.034$). We detected an interaction between gender and treatment: irradiated females displayed a greater reduction in the number of DCX^+ cells than did males (interaction between gender and treatment, $F = 4.58$, $P = 0.038$).

Activity: open field

To investigate locomotor activity we analyzed the distance moved, revealing that irradiated males and females were more active than controls. This was shown as an interaction between treatment and distance moved in the open-field arena (Fig. 5A; interaction between treatment and time, $P = 0.048$ in males, $P = 0.022$ in females).

We also analyzed the ratio between distance moved in the central vs. the entire zone of the open-field arena. Irradiated females, but not males, spent less time in the center zone (main effect of treatment: females, $P = 0.025$; males, n.s.), which may indicate a higher level of anxiety. Both irradiated and non-irradiated males and females showed an adaptation over time, moving around less in the arena (Fig. 5B; main effect of time, $P = 0.001$ in females, $P < 0.001$ in males).

Learning: IntelliCage

To investigate whether learning was affected by irradiation we used the IntelliCage platform, as described earlier (Karlsson *et al.*, 2011). The incorrect visit ratio (ratio of visits to the incorrect, non-allocated corners to the total number of visits), incorrect nose-poke ratio (ratio of performed nose pokes in the incorrect, non-allocated corners to the total number of nose pokes), and nose pokes per incorrect visit (i.e. attempts to open the door in incorrect, non-allocated corners) were used as measures of the animals' ability to learn.

The incorrect visit ratio showed no differences between male treatment groups, but they did improve over time, which is interpreted as learning (Fig. 6A). The females showed an interaction between treatment and time (interaction between treatment and time, $P = 0.049$) during the first corner period (place learning). Females in the control group decreased their ratio (improved) 7.5% per day, whereas irradiated females showed a 3.9% increase (worsened) per day (Fig. 6A). The same was seen for the incorrect nose-poke ratio, where the males showed an improvement over time for all three corner periods but no differences between irradiated and control males. The control females, however, showed a 13.7% improvement over the 5 days, whereas irradiated females did not improve (0.01%) (interaction between treatment and time, $P = 0.012$; Fig. 6B). The third parameter, nose pokes per incorrect visit, revealed that irradiated males performed more nose pokes when entering an incorrect corner during the first and second corner periods (Fig. 6C). During the first corner period the control male mice showed a 35% improvement per day compared to the irradiated group (main effect of treatment, $P = 0.044$; Fig. 6C). During the second corner period the difference between the groups was even bigger (51%; main effect of treatment, $P = 0.008$). No difference was seen during the third corner period. The females improved over time but no difference between groups was seen (Fig. 6C). These data show a dissociation between learning (improvement over time) and perseverance (persisting in trying to open a door by performing nose pokes). In summary, place learning, i.e. learning where to find water when this task was new to the animals (tested in corner 1) was impaired by IR in females but not in males. Reversal learning, i.e. learning where the new correct corner is while not persisting in trying to drink from the previously correct corner (tested in corners 2 and 3), was not significantly affected by IR in either males or females. Interestingly, perseverance was increased by IR in males but not in females, as

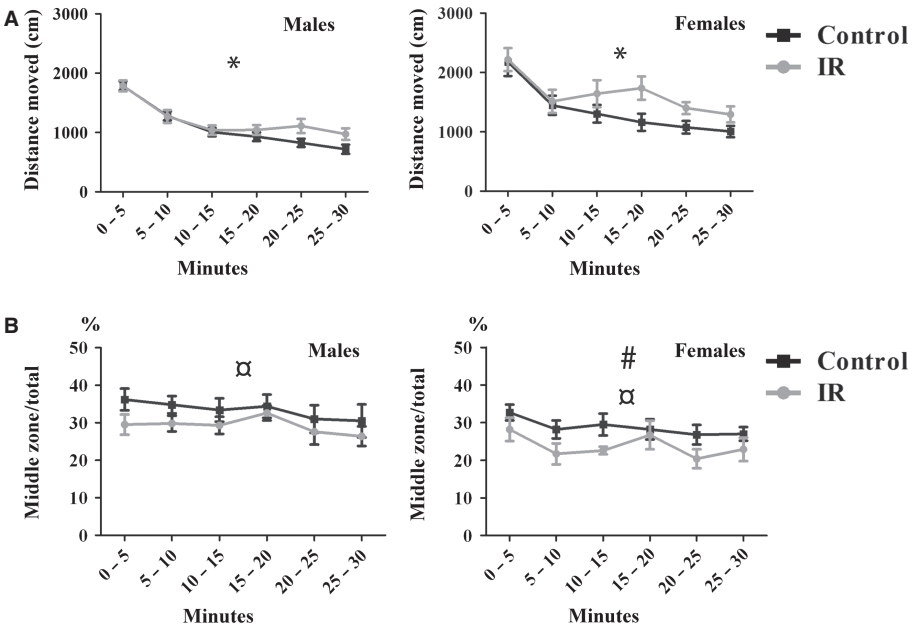

FIG. 5. (A) Total distance moved (in cm) in the whole arena in males (left panel) and females (right panel). An interaction between treatment and time was observed in both genders, showing that there was an IR-induced difference in the distance moved that decreased over time in both genders, more so in the control animals than the irradiated animals. (B) Percentage distance moved in the middle zone (30 × 30 cm area in the middle) vs. the entire arena in males (left panel) and females (right panel). A treatment effect was observed in females only, showing that IR females had a lower percentage distance moved in the central arena, indicating a more anxious behavior caused by IR. Data shown as mean ± SEM. Significance symbols: *interaction between treatment and gender; #treatment; ¤gender, $^{*,\#,¤}P < 0.05$.

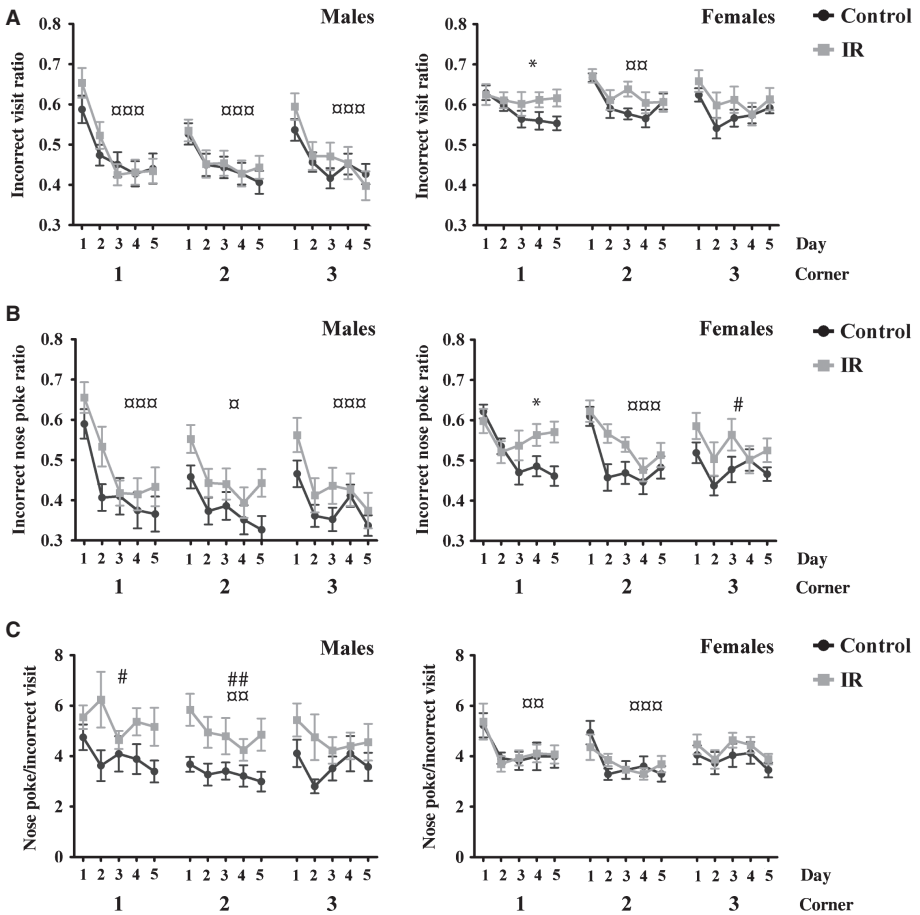

FIG. 6. (A) Incorrect-visit ratio (visits in incorrect corner divided by total visits) in males (left panel) and females (right panel). In males both IR and non-IR animals showed an improvement over time in all three corner periods. In females an interaction between treatment and time was observed in the first corner period only. An improvement over time was observed in both IR and non-IR females in the second corner but not in the third corner period. (B) Incorrect nose-poke ratio (the ratio of performed nose pokes in incorrect corners divided by total nose pokes) in males (left panel) and females (right panel). An improvement over time was seen in males regardless of IR in all three corner periods. In females an interaction between time and treatment was observed in the first corner period, but an improvement over time was shown in the second corner period and a treatment difference in the third corner period. (C) Nose pokes per incorrect visit (number of performed nose pokes per visit in incorrect corners) in males (left panel) and females (right panel). In males a treatment effect was observed in the first and second corner period. Males also showed an improvement over time in both IR and non-IR group in the second corner period. Females showed an improvement over time in the first and second corner period regardless of subjection to IR. Data shown as mean ± SEM. Significance symbols: *interaction between treatment and gender; #treatment; ¤gender; $^{*,\#,¤}P < 0.05$, $^{\#\#,¤¤}P < 0.01$, $^{¤¤¤}P < 0.001$.

judged by the animals' higher numbers of nose pokes performed in non-allocated corners.

We measured how long it took for the animals to visit any corner during the introduction period. It took almost four times longer for the males to visit their first corner compared to the females, and this was not affected by IR, possibly indicating stronger exploratory behavior in females (main effect of gender, $F = 8.77$, $P = 0.004$; not shown).

Hormone serum concentrations

We did not detect any differences in the measured hormone levels in irradiated animals vs. controls. Females showed higher levles of estrogen than did males (main effect of gender, $F = 7.38$, $P = 0.01$) and males higher levels of testosterone (main effect of gender, $F = 8.54$, $P = 0.006$), as expected (Table 1).

Discussion

Differences in neurogenesis between genders have been studied previously (Kavaliers *et al.*, 1996; Westenbroek *et al.*, 2004; Galea *et al.*, 2006; Lagace *et al.*, 2007; Galea, 2008), but to our knowledge gender has not been taken into consideration in studies of neurogenesis and IR to the young brain. This is an important issue, as it is well known empirically that girls suffer more severe late effects than boys after CRT (Bleyer *et al.*, 1990; Christie *et al.*, 1995; Lahteenmaki *et al.*, 2007) but the neurobiological basis for this gender difference is unknown. We used an IR dose of 8 Gy, which is equivalent to 18 Gy administered in 2-Gy fractions, according to the linear quadratic formula and an alpha/beta ratio of 3 for late effects in the normal brain tissue (Fowler, 1989). Prophylactic cranial irradiation (12–18 Gy) is given to patients with acute lymphoblastic leukemia (ALL) who have an increased risk of CNS relapse (such as T-cell ALL, overt CNS involvement, high-risk cytogenetic features or poor response to remission induction treatment), and also for patients with acute myeloid leukemia (AML) who present with overt CNS disease at diagnosis. The dose used for treatment of pediatric brain tumors such as medulloblastoma is higher, up to 55 Gy to the tumor bed, combined with 35 Gy craniospinal IR. A moderate dose of 8 Gy to the brains of young rats or mice has been shown to cause long-lasting and drastically decreased neurogenesis and disrupted growth of the GCL

TABLE 1. Serum hormone levels measured using ELISA 4 months after IR

	IGF-1 (ng/mL)	Estrogen (pg/mL)	Testosterone (ng/mL)	FT3 (pM/L)	FT4 (pM/L)
Male					
Control	221.3 ± 9.2	8.2 ± 0.8	1.9 ± 0.8	4.3 ± 0.4	15.6 ± 0.7
IR	217.3 ± 8.2	8.1 ± 0.5	1.2 ± 0.7	4.6 ± 0.3	15.5 ± 0.5
Female					
Control	230.1 ± 17.9	10.4 ± 0.5	0.0 ± 0.0	4.0 ± 0.5	15.4 ± 0.6
IR	204.4 ± 9.0	10.2 ± 1.1	0.0 ± 0.0	4.4 ± 0.3	15.4 ± 0.9

No gender- or IR-induced differences were observed in measured IGF-1, FT3 or FT4. As expected we detected a gender difference in the levels of testosterone, where males showed a higher level in both the IR and non-IR group than did females, and of estrogen, where females showed a higher level in both the IR and non-IR group than did males. Data shown as mean ± SEM.

(Fukuda *et al.*, 2004, 2005; Naylor *et al.*, 2008; Hellstrom *et al.*, 2009).

In this study we irradiated animals on P14, before sexual maturity, so the estrous cycle should not be a confounding factor at the time of treatment. Estradiol has been shown to have neuroprotective properties in, for example, models of ischemic injury (Hurn & Macrae, 2000) and also to affect hippocampal neurogenesis (Ormerod *et al.*, 2003). Neuroprotection through estradiol has been shown to fluctuate with the estrous cycle (Tanapat *et al.*, 1999). Tanapat *et al.* (1999) reported that female rats injected with BrdU in their proestrous phase of the estrous cycle showed a greater number of $BrdU^+$ cells in the DG than did males and also females in their estrous or diestrous phase. Perfilieva *et al.* (2001) showed that proliferation in the DG was higher in males than females in two different rat strains. In this study the animals were younger than the ones in the study by Tanapat *et al.* (1999) and had not reached sexual maturity at the time of BrdU administration. In mice, however, Lagace *et al.* (2007) did not observe any differences between males and females: neither proliferation nor survival of cells in the DG differed between genders. They also reported that ovariectomized mice were not different from control females with respect to proliferation or formation of new neurons in the DG and that proliferation in females did not fluctuate with the endogenous levels of estradiol, as previously reported to be the case in rats. The finding in our study that proliferation and neurogenesis were higher in females than in males only in adult animals indicates that the normal decline in hippocampal neurogenesis occurred at a slower rate in females. In the young animals, when the normal rate of proliferation was four times higher than in the adults, we did not find any gender differences, but 3–4 months later neurogenesis, as judged by BrdU incorporation and by quantification of DCX^+ cells, revealed higher levels in females. This is in agreement with a study by Ben Abdallah *et al.* (2007) where they found more DCX^+ cells in young adult females. Irradiation reduced proliferation, BrdU incorporation and DCX numbers to virtually identical levels in males and females, so the relative decrease was greater in females. We measured thyroid hormones (FT3, FT4), IGF-1 (indirectly assessing growth hormone levels), estrogen and testosterone but could not detect any irradiation-induced differences. These results argue against a loss of function in the hypothalamus and/or the pituitary, at least not after the moderate dose of 8 Gy. Endocrine deficiencies are common late effects in children treated with cranial radiotherapy (Duffner, 2004). The results from our study are in agreement with those of Clark *et al.* (2008) who showed a higher rate of neurogenesis in the DG of adult female mice. They further showed that running improved neurogenesis and increased DG volume. Females were more active compared to males, ran longer distances and had larger GCL volumes. This agrees with the current study, where we observed larger GCL volumes in control females than males and also observed that females moved greater distances in the open field after IR. Altogether, it appears that female mice suffer more from cranial IR, not because their hippocampal neurogenesis is more severely damaged but because they start from a higher level and are depleted to the same level as the males.

It is important to investigate behavioral changes after IR to the developing brain and, if possible, correlate them to age, gender and morphological changes. It is important to keep in mind that the whole brain is irradiated in this animal model, to mimic the clinical situation, so some of the functional changes we see can be due to extrahippocampal changes, for example impaired myelination (Fukuda *et al.*, 2005). The open-field test showed higher locomotor activity in irradiated animals, more so in females, and irradiated females also displayed increased anxiety as judged by the decreased time spent in the middle of the arena. Previously we observed an increased number of stops and increased rearing when male mice were irradiated on P9 (Naylor *et al.*, 2008), i.e. a younger age at the time of IR than in the current study. However, using a slightly different open-field paradigm at a different age we previously failed to see differences in locomotor activity in male mice after 8 Gy IR on P14 (Karlsson *et al.*, 2011). In a study of heavy ion ^{56}Fe IR in adult mice the investigators found that females moved more than males in the open-field test, in agreement with our results, but they did not find any effect of irradiation (Villasana *et al.*, 2010). Unlike distance moved in the open field, wheel running, another aspect of locomotor activity, was not increased by cranial IR, either in males irradiated at young age (Naylor *et al.*, 2008) or in adult males and females (Clark *et al.*, 2008). IntelliCage is a method by which memory and learning can be assessed in a social context with a minimum of interference by the investigator. We have previously found the IntelliCage useful in assessing place learning and reversal learning (Barlind *et al.*, 2010; Zhu *et al.*, 2010; Karlsson *et al.*, 2011). Both place and reversal learning can be claimed to be, at least partly, hippocampal-dependent (Colgin *et al.*, 2008). The production of new neurons in the hippocampus is thought to play a role in hippocampus-dependent learning (Shors *et al.*, 2001; Voss *et al.*, 2011). Male mice showed IR-induced perseverance, as judged by their persistence in performing nose pokes in non-allocated corners. This was the only parameter where males performed worse than females after IR and, interestingly, it seemed to be independent of the learning that occurred (Fig. 6C). Perseverance was found as a feature of altered search strategies in adult female mice treated with temozolomide to suppress neurogenesis. The treated mice did learn how to find the hidden platform in the Morris water maze, but were more limited in their search strategies (Garthe *et al.*, 2009). IR of adult mice was found to impair the performance of females more than males in a contextual fear conditioning paradigm (Villasana *et al.*, 2010) and in a Morris water maze test (Villasana *et al.*, 2006). In tasks such as the radial arm maze and Morris water maze, males perform better, at least in rats (Cimadevilla & Arias, 2008). However, if the animals are

given the opportunity to familiarize themselves with the test arena before the actual test, females can outperform males in a spatial learning task such as the Morris water maze. Perrot-Sinal *et al.* (1996) showed that female rats performed better in the Morris water maze once pre-trained (without visual cues) in the test arena. This holds true in humans as well, where a study in a human virtual water maze revealed that males outperformed females during training, but no gender difference was detected during pre-training (Woolley *et al.*, 2010). However, a study using trace eyeblink conditioning when investigating learning and neurogenesis in rats showed that females learned this task faster than males. The authors further reported that females, after several trials, showed a larger incorporation of new cells per tissue volume in the ventral hippocampus than did males (Dalla *et al.*, 2009).

In conclusion, we found that the normal decline in neurogenesis (measured as DCX^+ cells) that occurs when the brain stops growing is more pronounced in male mice, leaving females with a 33% higher level of neurogenesis. A single moderate dose of IR administered to the growing P14 mouse brain caused a profound and long-lasting decrease in neurogenesis, to the same low levels in both males and females. Hence, the rates of neurogenesis after IR were the same in males and females but, as females have a higher rate under normal conditions, the decrease was greater. This was reflected in increased locomotor activity, increased anxiety and impaired learning, more so in females than in males. The situation is the same in patients, where girls suffer more severe late effects after cranial radiotherapy than boys. One has to bear in mind that the mechanisms of injury may be different between mice and humans and that comparisons should be made with caution, but clinical experience and our studies both show that females are more susceptible to cranial irradiation. This underscores the importance of taking gender into consideration when investigating irradiation-induced injury, in both the human and the rodent brain.

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Abbreviations

$^+$, positive; BrdU, bromodeoxyuridine; CRT, cranial radiotherapy; DCX, doublecortin; DG, dentate gyrus; ELISA, enzyme-linked immunosorbent assay; GCL, granule cell layer; IR, irradiation; P, postnatal day; PHH3, phosphohistone H3; SGZ, subgranular zone.

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