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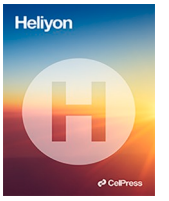

Research article

Fractionated brain X-irradiation profoundly reduces hippocampal immature neuron numbers without affecting spontaneous behavior in mice

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ABSTRACT

Whole brain radiotherapy (WBRT) is used to improve tumor control in patients with primary brain tumors, or brain metastasis from various primary tumors to improve tumor control. However, WBRT can lead to cognitive decline in patients. We assessed whether fractionated WBRT (fWBRT) affects spontaneous behavior of mice in automated home cages and cognition (spatial memory) using the Barnes maze.

Male C57Bl/6j mice received bi-lateral fWBRT at a dosage of 4 Gy/day on 5 consecutive days. In line with previous reports, immunohistochemical analysis of doublecortin positive cells in the dentate gyrus showed a profound reduction in immature neurons 4 weeks after fWBRT. Surprisingly, spontaneous behavior as measured in automated home cages was not affected. Moreover, learning and memory measured with Barnes maze, was also not affected 4–6 weeks after fWBRT. At 10–11 weeks after fWBRT a significant difference in escape latency during the learning phase, but not in the probe test of the Barnes maze was observed.

In conclusion, although we confirmed the serious adverse effect of fWBRT on neurogenesis 4 weeks after fWBRT, we did not find similar profound effects on spontaneous behavior in the automated home cage nor on learning abilities as measured by the Barnes maze. The relationship between the neurobiological effects of fWBRT and cognition seems more complex than often assumed and the choice of animal model, cognitive tasks, neurobiological parameters, and experimental set-up might be important factors in these types of experiments.

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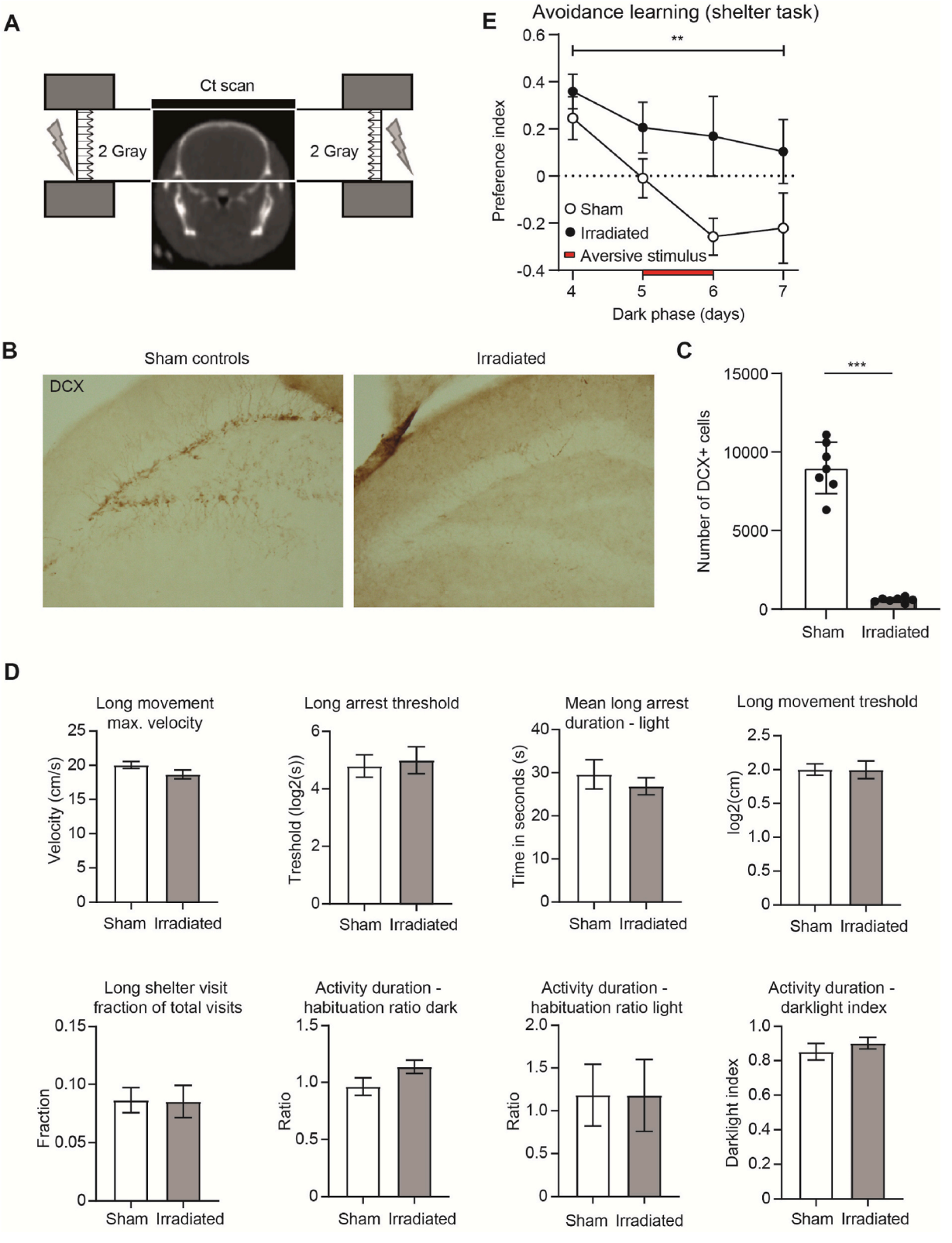

(caption on next page)

Fig. 1. Fractionated brain irradiation reduces the number of immature neurons, but does not change spontaneous behavior in the automated home cage

A. Scheme for setup of the image-guided radiation therapy: Radiation beams are delivered daily for 5 days in 2 fractions of 2 Gy from both contralateral sides. B. Representative images of DCX staining in the hippocampus. C. Total number of DCX positive cells in the dentate gyrus of the hippocampus from sham-control mice (white bar) and irradiated mice (grey bar). Irradiated mice had significantly less DCX (***$p < 0.001$) positive cells than sham-control mice. Each dot represents the results from one animal, error bars represent standard deviation, unpaired students *t*-test. D. Graphs showing a selection of behavioral readouts from the automated home cage, showing no differences between sham-control mice (white bars) and irradiated mice (grey bars) on different parameters. Bars represent the mean with error bars representing the standard error of the mean, unpaired students *t*-test were used for statistical analysis. E. Preference index during the dark phase of day 4–7 of the avoidance learning (shelter task) in the automated home cage showing a preference for the entrance at day 4. At day 5 the aversive stimulus is introduced (red bar), showing that sham-controls do not show a preference anymore which continues to decline at day 6 and 7 whereas the irradiated mice retain a preference for the entrance with the aversive stimulus ($F = 10.6794$, **$p = 0.002$). Dots represent the mean, error bars represent the standard error of the mean, 2-way ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

1. Introduction

Whole brain radiotherapy (WBRT) is often used to treat patients with primary brain tumors and patients with brain metastasis from primary tumors of various origin. Where WBRT used to be the standard treatment for brain metastases, current practice increasingly advises the use of stereotactic radiosurgery (SRS) to reduce radiation-induced cognitive impairment [1]. From 6 months onwards following WBRT, patients can experience irreversible and progressive cognitive decline [2]. Long-term sequelae of WBRT include a slower speed of information processing, memory retrieval deficits, and decreased executive functioning and attention [3,4]. Many risk factors are associated with these complications following radiotherapy, including age, dose per fraction, cumulative dose, volume of the irradiated brain region and overall treatment time [2,5]. However, it remains difficult to predict which patients are at risk to develop cognitive decline [6].

Pre-clinical studies have been performed in order to understand the effects of radiotherapy on cognition using various cognitive tasks, however, with varying results [7]. Analysis of the brain of irradiated rodents revealed alterations including reduced neurogenesis, loss of endothelial cells, loss of oligodendrocytes and microglial activation [2,8–10]. Overall, the relation between radiation-associated cognitive impairment and radiation-induced neurobiological changes still remains poorly understood, partly due to the varying outcomes of cognitive tests using rodents. Automated home cages serve as a sensitive measurement of subtle behavioral changes, independent of confounding effects such as frequent animal handling [11]. Previous studies with automated home cages showed that hippocampal lesions resulted in changes in spontaneous behavior, without effects on learning simple spatial memory tasks [12]. In addition, we previously showed that treatment with several chemotherapeutic agents affects spontaneous behavior in automated home cages, sometimes in the absence of deviant performance on traditional cognitive tasks [13]. Preclinical studies examining both biological effects, spontaneous behavior in automated home cages and performance on traditional cognitive tests after irradiation may therefore increase our understanding of the relation between irradiation and cognitive impairment.

To study the cognitive problems after fractionated WBRT (fWBRT), male C57Bl/6j mice received 4 Gy per day (2 × 2 Gy bilaterally) on 5 consecutive days. Separate groups of mice were tested starting at 3 and 10 weeks after fWBRT using automated home cages, the open field task and a Barnes maze. In parallel, a group of mice was irradiated and their brain was assessed to study the loss of neurogenesis.

2. Material and methods

2.1. Animal maintenance

Adult male C57BL/6j mice of 11 weeks of age (Charles River, France) were housed in groups of 4–5 mice in clear Plexiglas cages on a layer of wood shavings with a fixed 12:12 h light:dark cycle (lights on at 07.00 a.m.) and food and water *ad libitum*.

2.2. Ethical approval and guideline adherence

Experiments were approved by the Animal Experimentation Committee of the Netherlands Cancer Institute (NKI hereafter) and the Vrije Universiteit (VU) and all were carried out in accordance with the approved protocols. The principles of laboratory animal care, Dutch law and the guidelines for care and use of mammals in Neuroscience and Behavioral Research were followed. The number of mice per group were determined based on previous experience with immunohistochemistry and behavioral analysis. All methods are reported in accordance with ARRIVE guidelines.

2.3. Irradiation

Experiments started 2 weeks after arrival of the mice from Charles river (age 13 weeks). Mice were randomly included in the sham-irradiation or irradiation groups (investigator could not be blinded to treatment at this stage of the experiment). Irradiation was performed using image-guided radiotherapy on the X-rad 225 μ-IGRT system (Precision X-Ray, North Branford, CT, USA) with a square beam of 7 × 7 mm. Mice were anesthetized using a mixture of 2.5 % isoflurane and air. A sham-irradiated animal and an irradiated

animal were paired, so that both mice received equivalent anesthesia to avoid this confounder. The sham mice (later referred to as sham-control) were left in the induction chamber, whereas the irradiation mice received treatment. First, a CT scan was made to correctly adjust the position of the irradiation beam for each animal. The upper part of the head and the skull was irradiated sparing the skull base, the pituitary gland, the parotid and mandibular salivary glands, the jaw muscles, most of the olfactory bulb and cerebellum (see Fig. 1A for the setup of the cone beam). The prefrontal cortex, midbrain regions were fully irradiated. The animal received 4 Gy, of which 2 Gy per hemisphere, once a day for 5 consecutive days (total dose of 20 Gy) (Fig. 1A). Subsequently, mice were relocated to the animal facility of the Vrije Universiteit Amsterdam (VU) according to the timelines described below and in Tables 1 and 2. The mice tolerated the X irradiation well and all mice gained weight over time (Figure S1).

2.4. Immunohistochemistry

To explore the effects of irradiation on the brain, 7 control and 7 irradiated mice were relocated to the VU 2 weeks after irradiation started and were sacrificed by perfusion under anesthesia 4 weeks after the start of irradiation. From the serial coronal sections, every sixth section from each animal was selected and immunocytochemically stained with markers doublecortin (DCX) using a slightly adapted standard protocol [14,15] as described previously [16]. Primary antibody (DCX: goat-*anti*-DCX, Santa Cruz, 1:2500), secondary antibody (rabbit-*anti*-goat, 1:400, Jackson) and avidin biotinylated peroxidase complex were used (1:400, ABC Elite Kit, Vector Laboratories).

Counting of DCX positive cells in both hemispheres of the dentate gyrus was performed by an experimenter that was blinded to the experimental group under a light microscope with a magnification of 400×. Quantification was performed in the subgranular layer of the dentate gyrus and counts in both blades were summed. The border of the area that was quantified was defined as the subgranular layer having a thickness of two cell diameters. All cells were counted in the subgranular layer of the dentate gyrus from top to bottom of the 40 μm thick section. Because every sixth section of the brain was stained, the number of positive cells was multiplied by 6 to get the estimated total number of DCX positive cells in the hippocampus. Pictures taken with Zeiss LSM Meta 510 confocal microscope were analyzed using ImageJ, as described previously [16].

2.5. Timing of behavioral and cognitive assessments

Early-stage observations and cognitive performance (3–6 weeks): Ten days after the last irradiation, 11 control and 11 irradiated mice were relocated to the VU University and behavioral and cognitive experiments started 1 week after relocation, which is 3 weeks after irradiation (Table 1). At the VU University, mice were housed individually to minimize the stress induced before and after the cognitive tasks (reduces handling and relocation of cages). Behavioral studies and the analyses thereof were performed by an experimenter that was blinded to the experimental group.

Later-stage cognitive performance (10–11 weeks): Five days after the last irradiation, 12 control and 12 irradiated mice were relocated to the VU for cognitive testing. The mice were subjected to the Barnes maze starting at 10 weeks after irradiation (Table 2). Behavioral studies and the analysis thereof were performed by an experimenter that was blinded to the experimental group.

2.6. Automated home cage

Measurements in a home cage environment (PhenoTyper model 3000, Noldus Information Technology, Wageningen, the Netherlands) [17,18] were performed as previously described [13] and started 22 days after irradiation (short-term) and lasted 7 days. The automated home cages (L = 30 × W = 30 × H = 35 cm) consisted of transparent Perspex walls and an opaque Perspex floor covered with cellulose-based bedding. On two adjacent walls, there was a feeding station attached to one wall and a water bottle to the other wall. The cage contained a triangular shaped shelter compartment (height: 10 cm; non-transparent material) equipped with two entrances. The shelter compartment was fixed in the corner. At the top unit of each cage an infrared-sensitive video camera and an array of infrared LEDs was positioned and used for video-tracking [18]. Sampling resolution was 15 coordinates every second, where the X–Y coordinates of the center of gravity of mice was used to trace them. These samplings were acquired and smoothed using EthoVision software (EthoVision HTP 2.1.2.0, based on EthoVision XT 4.1, Noldus Information Technology, Wageningen, The Netherlands) and further processed to extract behavioral parameters by AHCODA analysis software (Synaptologics BV, Amsterdam, The Netherlands). The first 3 days were used to analyze spontaneous home cage behavior where 28 measurements of kinematics, 14 measurements of sheltering, 28 measurements of habituation, 15 measurements of DarkLight index, 16 measurements of activity pattern and 14 measurements of activity give a good insight in changes in spontaneous movement in the home cage [18]. After the spontaneous behavior the avoidance learning paradigm was started. In this task, the preference for one of the two entrances to the shelter is measured. After establishing this, when the mouse uses the preferred entrance a bright light in the shelter will be turned on (day 5–6). If mice are able to learn, they will lose their preference for this entrance to avoid this bright light by either not using any

Table 1
Time schedule of behavioral and cognitive assays for the short-term effects of irradiation. Day 0 is the first day of irradiation.

Day	−14	0–4	14	22–24	25–29	31	35–39
Activity	Arrival NKI	Irradiation	Transport VU	Automated home cage spontaneous behavior	Automated home cage avoidance learning	Open field	Barnes maze

Table 2
Time schedule of cognitive assays for the long-term effects of irradiation. Day 0 is the first day of irradiation.

Day	−14	0–4	9	69–73
Activity	Arrival NKI	Irradiation	Transport VU	Barnes maze

entrances (sleeping outside of the shelter) or using the other entrance (output measurement). Detailed descriptions of the measures are listed in supplementary date file 2. Twenty cages were available for the analysis, mice were arbitrarily selected for the automated home cage analysis. One animal in the control group (sham) was excluded from analysis by the quality control after testing, resulting in 9 mice in the control group and 10 mice in the irradiated group.

2.7. Open field

The open field task was executed 31 days after irradiation. The set-up consisted of a white Polyvinylchloride box (50 × 50 × 50 cm), which was illuminated with 60 lux of white fluorescent light from above. The animal was placed in the center and was allowed to explore the arena freely for 10 min. Between each mouse cleaning of the box using 70 % alcohol was performed. The box was divided into an outer zone and an inner zone (25 × 25 cm). Time spent in the inner zone (in seconds) was analyzed with Biobserve (Biobserve GmbH). The time in the inner zone was taken as measure of anxiety as described previously [13].

2.8. Barnes maze

Spatial learning and memory in the Barnes maze were assessed 35 days (short-term) or 69 days (longer-term) after irradiation as described previously [13]. The Barnes maze consisted of a large round platform (122 cm diameter, 80 cm above the floor) with 24 holes (9 cm from the edge of the maze). One hole served as the 'escape hole' and had an escape box hanging underneath (15 × 5.5 × 5.5 cm), whereas the other holes contained round cylinders of 8 cm by 4.5 cm diameter. Illuminated of the room was done at 1000 Lux and the four surrounding walls contained large external cues. Barnes maze training consisted of 2 sessions per day for 5 days. At the start of the experiment the mouse was placed in a cylinder located in the center of the maze, where it was rested for 30 s before the cylinder was lifted via a pulley system. Free exploration was allowed for a maximum of 5 min or until the escape hole was found. If the animal did not find the escape hole within these 5 min, it was guided by hand. For the short-term experiment 15 out of 22 animals needed guidance on at least one of the trials on day 1 or 2. At day 3 one animal needed guidance at one of the trials. From these in total 16 animals that needed at least one time guidance 7 were in the radiated group. For the long term experiment 11 out of 24 animals needed guidance on at least one of the trial on day 1 or 2. At day 3, 4 and 5 none of the animals needed guidance. From these 11 animals 7 animals were in the radiation group. At the final session on day 5 the escape hole was removed and replaced by a cylinder equal to the others. The time spent (in seconds) in the escape zone was used as a readout on spatial learning. Data from the first minute of the probe trial was used for analysis. After the behavioral experiments mice were sacrificed using CO_2 inhalation.

3. Statistics

The automated home cage spontaneous behavior readouts were analyzed using unpaired *t*-tests and Mann-Whitney U tests, while the avoidance learning paradigm was analyzed using a 2-way ANOVA. The learning phase of the Barnes maze was analyzed using repeated measures ANOVA with a Geisser-Greenhouse correction. The probe trial of the Barnes maze, open field and DCX quantification were analyzed using an unpaired *t*-test. For all statistical tests, a $p < 0.05$ was considered to be statistically significant. For statistical analysis Graphpad Prism 9 and R (v 4.3.0) were used.

4. Results

4.1. Immunohistochemistry shows loss of neurogenesis after irradiation

To determine the presence of immature neurons in the hippocampus, DCX positive cells in the dentate gyrus were visualized and quantified 4 weeks after fWBRT. Irradiated mice showed significant fewer DCX positive cells than sham-control mice, indicative of severe radiotherapy-induced loss of neurogenesis in the dentate gyrus ($p < 0.001$, Fig. 1B and C).

4.2. Early effects of irradiation on behavior and cognition

At 3 weeks after fWBRT the sham-control and irradiated mice were first subjected to automated home cages and subsequently to the open field task and Barnes maze, which started at 4 weeks after irradiation (Table 1). None of the parameters of spontaneous behavior as measured in the automated home cage (kinematics, sheltering, habituation, DarkLight index, activity pattern or activity) were altered in irradiated mice compared with sham-control mice (Fig. 1D; supplemental data file 1). This means there were no differences in the velocity of movements, time of movement and arrests, time spent in the shelter compartment, response to the light/dark phase, anticipation to light/dark phase, or activity during the light/dark phase. After the spontaneous behavior measured in the first three

days, an avoidance learning paradigm was started from day 4. The behavior during this task is visualized as a multi-day preference index curve during the dark phase, where the sham-controls show a reduction in their preference index upon the presence of the aversive stimulus (red bar) and maintained this at day 7 (Fig. 1E). While sham-control mice reduce their entrances through the preferred entrance and develop a preference for the other entrance, the irradiated mice retain a preference for the aversive entrance (2-way ANOVA, main effect of genotype $p = 0.0017$, main effect of time $p = 0.0017$)(Fig. 1E).

Following the automated home cage experiments mice were allowed to acclimate for one day before being subjected to traditional cognitive tasks (Table 1). In the open field test no difference was seen in anxiety, measured by the time spent in the inner zone of the open field between the 2 groups (Fig. 2A). The Barnes maze is used to measure spatial learning, as contextual cues can be used to learn the location of the target hole. The escape latency during the learning phase and time spent in the escape zone during the probe phase did not differ between sham-controls and irradiated mice in the Barnes maze (Fig. 2B and C), suggesting there is no impairment in spatial memory. During the learning phase there was a significant time effect (repeated measures ANOVA $F_{4,80} = 39.83$, $p < 0.0001$), showing improvement of escape latencies over time (Fig. 2B).

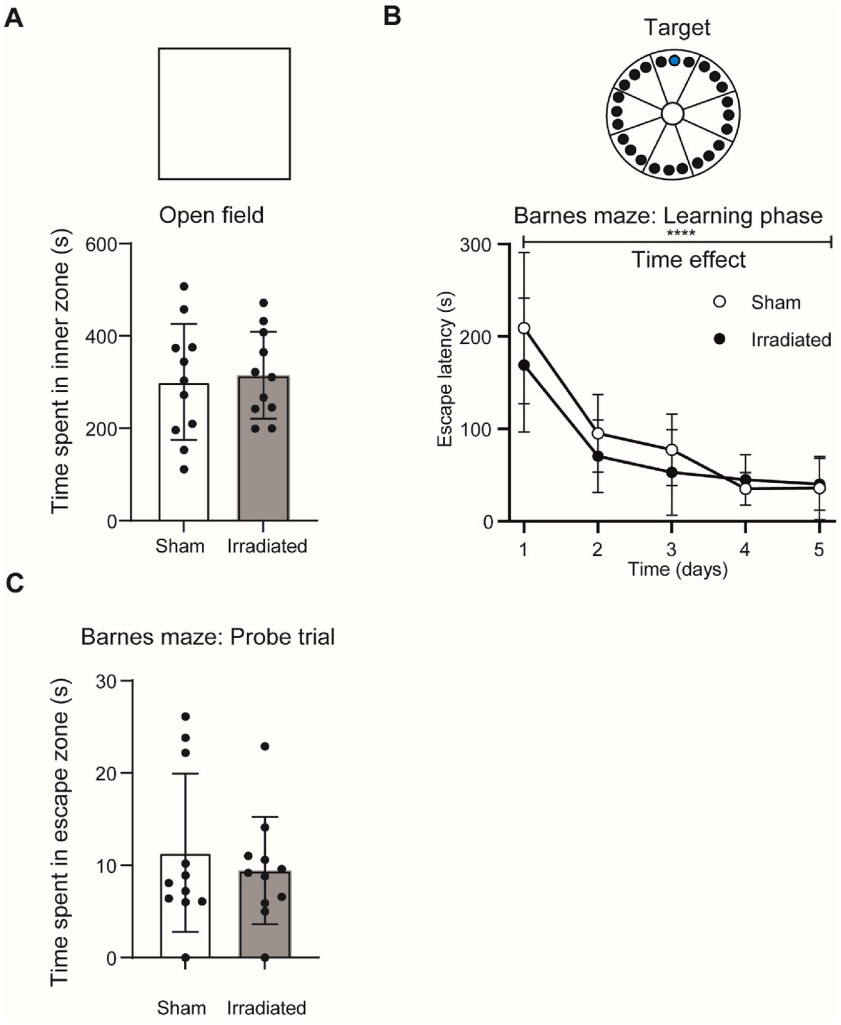

Fig. 2. Fractionated whole brain irradiation did not result in measured cognitive deficits from 4 to 6 weeks after irradiation
A. Time spent in the inner zone of the open field for sham-control mice (white bar) and irradiated mice (grey bar) 31 days after irradiation did not significantly differ between groups. B. Escape latencies during the learning phase in the Barnes maze 35–39 days after irradiation. The escape latencies of the 2 trials per animal per day are set as average. No "group effect" (sham-control mice (open circles) and irradiated mice (black circles), but a significant overall "time effect" was found (repeated measures ANOVA $F_{4,80} = 39.83$, ****$p < 0.0001$). Each dot represents the mean of the mice per group, error bar represents standard deviation. C. Probe trial showing the time spent in the zone were the escape hole used to be during the first minute of the probe trial for sham-control mice (white bar) and irradiated mice (grey bar) at 39 days after irradiation. A, C. Each dot represents one animal, error bars represent the standard deviation. Unpaired students *t*-test.

4.3. Longer-term effects of irradiation on cognition

Since cognitive decline after irradiation appears to be a delayed effect in patients, often presenting from 6 months after fWBRT onwards [2], we performed spatial learning starting at 10 weeks after irradiation (Table 2). There was an overall significant group effect (repeated measures ANOVA: $F_{1,22} = 7.406$, $p = 0.0125$, Fig. 3A) and time effect (repeated measures ANOVA: $F_{4,88} = 17,96$, $p < 0.0001$, Fig. 3A) on escape latency during the learning phase of the Barnes maze. It was observed that the irradiated animals consistently showed a longer escape latency on the Barnes maze (worse performance). During the probe trial no difference was observed in the time spent in the correct quadrant between sham-controls and irradiated mice (Fig. 3B). However, assessing time spent in each target area showed that whereas the sham control mice spent relatively most of their time in the target quadrant, this was not the case for the irradiated mice (Fig. 3C).

5. Discussion

In this study we observed loss of neurogenesis after fWBRT accompanied by worse performance on avoidance learning in an automated home cage 3 weeks after irradiation and worse performance on the learning phase of the Barnes maze at 10 weeks, but not 5 weeks after irradiation. Importantly, we did not find any changes in spontaneous behavior using an automated home cage, neither did we detect changes on the other cognitive tests measured. It has to be noted that the overall performance of both groups of mice on the probe trial of the Barnes maze was poor since the animals spent less than 1/6th of their time in the target quadrant during the first minute, therefore the effects during the learning phase at 10 weeks after radiation are more reliable. In addition, during the probe trial the irradiated animals did not spent most of their time in the target area, whereas the sham control mice did. Therefore, irradiation

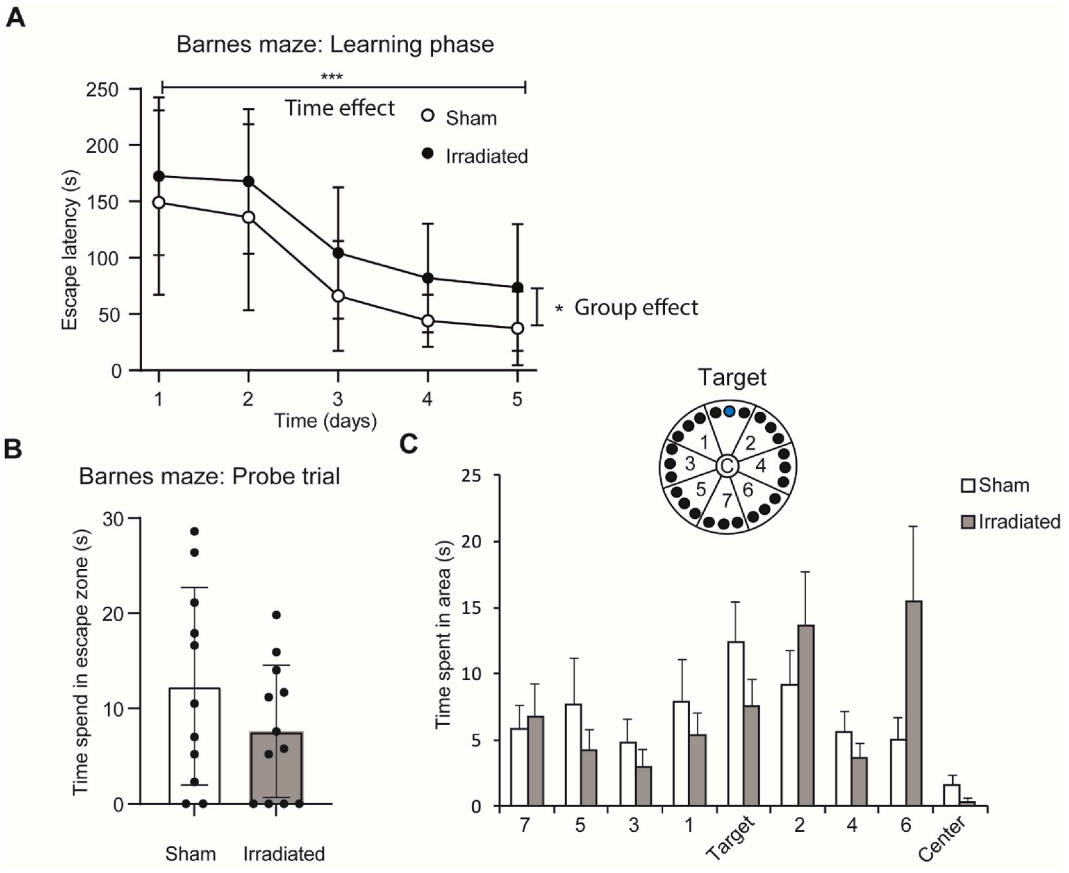

Fig. 3. Cognitive assays on mice 10 weeks after fractionated irradiation
A. Escape latency in the Barnes maze for sham-control mice (open circles) and irradiated mice (black circles) 10 weeks after irradiation. The escape latencies of the 2 trials per animal per day were set as average. There was an overall significant "group effect" (repeated measures ANOVA: $F_{1,22} = 7.406$, *$p = 0.01$) and "time effect" (repeated measures ANOVA: $F_{4,88} = 17,96$, ***$p < 0.0001$) in learning phase. Each dot represents the mean and error bars represent the standard deviation. B. Time spent in the zone were the escape hole used to be during the first minute of the probe trial for sham-control mice (white bar) and irradiated mice (grey bar) 10 weeks after irradiation. Each dot represents one animal, error bars represent the standard deviation. No statistical differences were found, students *t*-test. C Time spent in each zone during the first minute of the probe trial, bars represent mean error bars represent standard deviation.

seems to impair escape latency in the Barnes maze which might reflect a defect in learning and memory acquisition. Neurogenesis has repeatedly shown to be decreased after brain irradiation independent of age, sex or species used [19–29], which is in line with our findings showing a reduction of immature neurons in the dentate gyrus. However, we did not detect changes in spontaneous behavior, in contrast to our previous findings using chemotherapeutics [13] and that of others that reported changes in spontaneous behavior during natural aging [30]. The effect of fWBRT on cognition is also limited in our experiments, since only defects in avoidance learning in the automated home cage 3 weeks after fWBRT and subtle differences in the learning phase of the Barnes maze at 10 weeks after fWBRT were detected. A recent study corroborates this pattern of findings as they reported significant loss of neurogenesis but also found no changes in contextual fear, spatial memory (Morris water maze), NOR and open field task [31].

Overall, whereas effects of WBRT on neurobiology seem clear and robust, the results from cognitive studies using WBRT in rodents are inconclusive. Some studies failed to detect changes in spatial memory (mainly Morris water maze) upon irradiation [19,20,32,33], whereas others could find changes [21,34–40]. Some interesting observations were previously made with respect to (1) the use of deviating spatial strategies in spatial memory tasks by irradiated mice [41], (2) enhanced extinction of memory upon irradiation and (3) deficits in cognitive flexibility in irradiated mice [42]. However, these types of changes might not be measured by the cognitive tasks we used and therefore these specific effects of irradiation on cognition might not always be detected. More complex learning/cognitive flexibility tasks including for example reversal learning might give better insight in impairments after WBRT.

5.1. Strength and limitations

The strength of our study lies in the use of automated home cages to assess spontaneous behavior, which is independent of the investigator. Using this model we previously identified alterations in spontaneous behavior, in particular at two weeks after various chemotherapeutics using 8 to 15 mice per group, showing that it is possible to detect differences in spontaneous behavior after cancer therapy using similar numbers of mice.

Our study also contains limitations: first, our behavioral and cognitive assessments were performed between 3 and 11 weeks after irradiation, which might be too short after irradiation to develop behavioral and cognitive abnormalities, since effects are usually seen only after 6 months in patients [2]. Second, the radiation effect in avoidance learning task in the automated home cage requires confirmation in a separate cohort of mice, since from previous experiments it is known that group differences might be induced by spurious non-cognitive factors. For example, the bright light that shines upon entry through the preferred entrance, might interfere with the sleeping patterns of the mice and their circadian rhythm on the days after the test. The bright light might also reduce the use of the shelter to sleep altogether. Third, only one sex namely male mice were used in this study and therefore possible sex effects could not be assessed. Last, the sample sizes of 9–12 mice per group might not give sufficient power due to individual variation.

6. Conclusion

In our animal model the number of immature neurons in the dentate gyrus was strongly reduced 4 weeks after fWBRT. Spontaneous behavior and cognition were not affected at any time point, except for avoidance learning 3 weeks after fWBRT and the learning phase of the Barnes maze at 10 weeks after fWBRT where the irradiated mice performed worse compared to the controls. The data in this paper underscores the complexity of the relation between irradiation, neurogenesis and cognition.

Ethics statement

Experiments were approved by the Animal Experimentation Committee of the Netherlands Cancer Institute (NKI hereafter) and the Vrije Universiteit (VU) and all were carried out in accordance with the approved protocols (DEC number 13.003).

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Data availability

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials. Raw research data are stored in an institutional repository and will be shared upon request to the corresponding author, data has not been deposited into a publicly available repository.

CRediT authorship contribution statement

L.E. Kuil: Writing – review & editing, Writing – original draft, Visualization. **R. Seigers:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **M. Loos:** Writing – review & editing, Methodology, Investigation, Formal analysis. **M.C. de Gooijer:** Writing – review & editing. **A. Compter:** Writing – review & editing. **W. Boogerd:** Writing – review & editing, Supervision, Formal analysis. **O. Van Tellingen:** Writing – review & editing, Supervision, Conceptualization. **A.B. Smit:**

Writing – review & editing, Investigation, Conceptualization. **S.B. Schagen:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Sanne Schagen reports financial support for the study was provided by KWF. However, the KWF did not influence the design, execution or data analysis performed in this study. All other authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29947.

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