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Grafting of neural stem and progenitor cells to the hippocampus of young, irradiated mice causes gliosis and disrupts the granule cell layer

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Ionizing radiation persistently reduces the pool of neural stem and progenitor cells (NSPCs) in the dentate gyrus (DG) of the hippocampus, which may explain some of the learning deficits observed in patients treated with radiotherapy, particularly pediatric patients. A single dose of 8 Gy irradiation (IR) was administered to the brains of postnatal day 14 (P14) C57BL/6 mice and 1.0×10^5 bromodeoxyuridine-labeled, syngeneic NSPCs were injected into the hippocampus 1 day, 1 week or 6 weeks after IR. Cell survival and phenotype were evaluated 5 weeks after grafting. When grafted 1 day post-IR, survival and neuronal differentiation of the transplanted NSPCs were lower in irradiated brains, whereas the survival and cell fate of grafted cells were not significantly different between irradiated and control brains when transplantation was performed 1 or 6 weeks after IR. A young recipient brain favored neuronal development of grafted cells, whereas the older recipient brains displayed an increasing number of cells developing into astrocytes or unidentified cells. Injection of NSPCs, but not vehicle, induced astrogliosis and reduced thickness of the dorsal blade of the GCL after 5 months. In summary, we demonstrate that age and interval between IR and grafting can affect survival and differentiation of grafted NSPCs. The observed long-term gliosis and degeneration warrant caution in the context of NSPC grafting for therapeutical purposes.
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In pediatric hematology and oncology almost one-third of the cases are brain tumors and the incidence has increased over the past decades.[1–3] Approximately 70% of pediatric brain tumor patients survive, and the survival rate has improved greatly in the past decades.[4,5] Despite improved techniques in neurosurgery and advances in chemotherapy, radiation therapy (RT) remains an essential treatment modality for malignant brain tumors, as well as for central nervous system (CNS) involvement of leukemia and lymphoma. However, RT is also one of the major causes of long-term complications seen in survivors of pediatric brain tumors. Cognitive impairments, secondary malignancies as well as perturbed growth and puberty are some of the so-called late effects seen after RT, and cognitive deficits have been shown to be more severe in younger children after RT.[6,7] Even low doses of ionizing radiation to the CNS are believed to cause cognitive impairment,[8] and the impairments increase over time after RT. Hence, ameliorating the late effects of radiotherapy would greatly improve the quality of life of the increasing number of cancer survivors, particularly in children where the remaining life expectancy is long.

The injury caused by irradiation (IR) affects many brain regions and cell types, but the underlying pathogenesis is not well understood. Neurogenesis occurs throughout life in the subventricular zone and in the dentate gyrus (DG) of the hippocampus, making these regions harboring proliferating cells particularly susceptible to IR.[9,10] It has been suggested that injury to neural stem and progenitor cells (NSPCs) in the hippocampus contributes to some of the late effects seen after IR,[11–15] and IR-induced depletion of neural stem cells appears to be long-lasting, if not permanent, even after a single, moderate dose of IR.[16,17] Very few potential interventions after RT have been investigated, but memory training was shown to improve the attention and memory performance of children treated for medulloblastoma.[18] Interestingly, voluntary physical exercise was shown to increase the number of stem cells and the rate of neurogenesis after IR of the young mouse brain, and, furthermore, at least partly normalize the IR-induced behavior changes.[13] Grafting of human embryonic stem cells into the hippocampus of irradiated adult immune-deficient rats improved their performance in a memory task.[19] Grafting NSPCs into the

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Abbreviations: BrdU, bromodeoxyuridine; CNS, central nervous system; DG, dentate gyrus; GCL, granule cell layer; NSPC, neural stem/progenitor cells; SGZ, subgranular zone
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hippocampus may be a promising therapy after IR also at young age. The host tissue properties can change dramatically after IR. We have shown that IR elicits a transient inflammatory reaction in the immature brain,[20,21] which is different in nature from the more long-lasting inflammation seen after IR to the adult brain.[10,22] In the adult rat brain, it has been shown that the inflammatory reaction to IR caused the remaining neural precursors to adopt glial fates, and transplants of non-irradiated neural precursor cells failed to differentiate into neurons in the irradiated hippocampus caused NSPCs to adopt a glial phenotype instead of a neuronal one to a higher extent.[10] The aim of this study was to explore the effects of age and interval between IR and grafting on survival and differentiation to investigate the optimal time-point for transplantation therapy after IR.

Results

Effects of age and interval between IR and grafting on survival. Postnatal day 14 (P14) mice were subjected to IR of one hemisphere using a single dose of 8 Gy or sham-IR, followed by injection of bromodeoxyuridine (BrdU)-labeled NSPCs into the hippocampus 1 day, 1 week or 6 weeks after IR (Figure 1). At 5 weeks after grafting, we counted the number of surviving BrdU-positive cells in the granule cell layer (GCL) of the DG in the hippocampus. Regardless of IR or the interval between IR and grafting, the majority of the grafted, surviving cells were found in the dorsal blade of the GCL (Figures 2a–f). When grafted 1 day post-IR, survival was 52% lower in irradiated brains (Figure 3; $P<0.05$). When NSPCs were grafted 1 or 6 weeks post-IR, survival was not significantly different between irradiated and non-irradiated brains (Figure 3).

Effects of age and interval between IR and grafting on differentiation. The phenotype of the surviving, BrdU-labeled cells was evaluated. Representative confocal photomicrographs of the GCL are shown in Figure 4, demonstrating how Z-stacks were utilized to phenotype the BrdU-positive cells, to avoid confusing, for example, juxtaposed satellite cells with truly BrdU-positive neurons (Figure 4a) or astrocytes (Figure 4b). When grafting was performed 1 day after IR, neuronal differentiation (BrdU+/NeuN+ cells) of the transplanted NSPCs was 37% lower (Figure 5; $P<0.01$), whereas differentiation into astrocytes (BrdU+/S100β+ cells) was 294% higher in irradiated *versus* non-irradiated brains (Figure 5; $P<0.01$) and the ratio of unidentified cells was unchanged (Figure 5). The fate of grafted cells was not significantly different between irradiated and control brains when transplantation was performed 1 or 6 weeks after IR (Figure 5). The age of the recipient brain also affected differentiation of the grafted

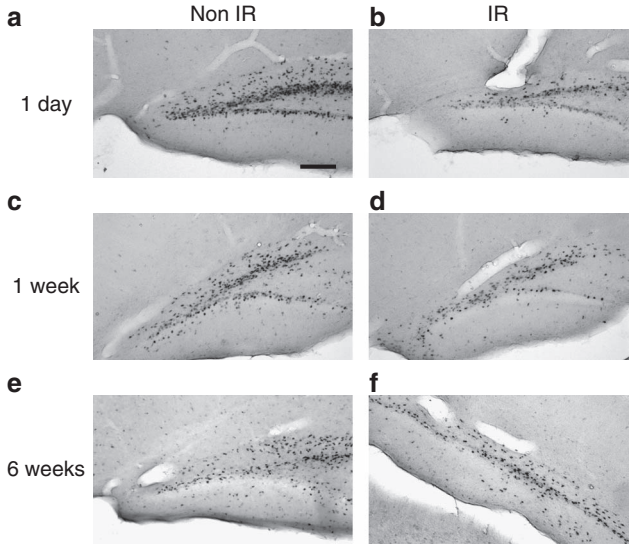

Figure 2 Representative microphotographs of the DG of the hippocampus stained for BrdU. A single IR dose of 8 Gy or sham-IR was administered to the brains of P14 mice and BrdU-labeled NSPCs were injected into the hippocampus 1 day (P15) (**a** and **b**), 1 week (P21) (**c** and **d**) or 6 weeks (P56) (**e** and **f**) after IR. At 5 weeks after grafting, we evaluated the surviving BrdU-positive cells in the GCL of the DG in the hippocampus. It was apparent that the majority of the grafted, surviving cells were found in the dorsal blade of the GCL, and that survival was lower in the irradiated GCL 1 day after IR. Scale bar = 100 μm

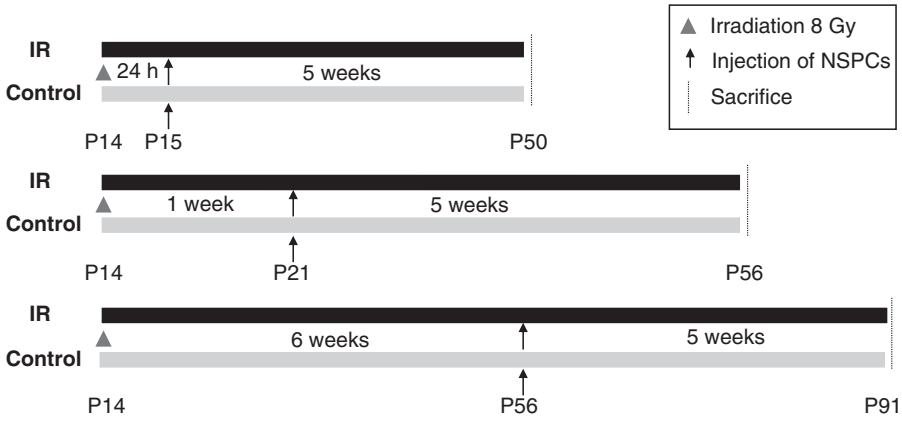

Figure 1 Timeline of the study. The left hemisphere of C57BL/6 mice was irradiated with 8 Gy on (P14), and 1.0×10^5 BrdU-labeled, syngeneic NSPCs were injected into the left hippocampus 1 day, 1 week or 6 weeks after IR. Cell survival and phenotype were evaluated 5 weeks after grafting

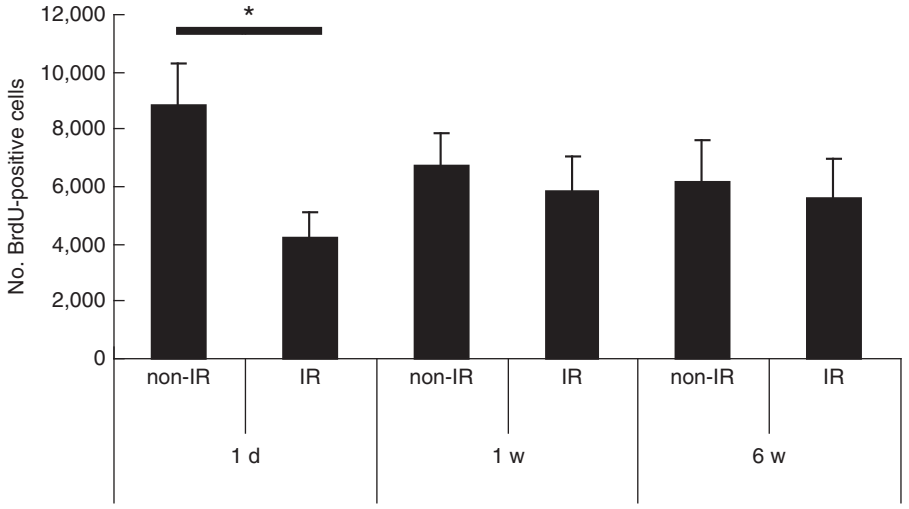

Figure 3 Effects of age and interval between IR and grafting on survival. Survival was evaluated 5 weeks after grafting. When grafting 24 h after IR, survival was reduced. When grafting 1 or 6 weeks after IR, survival was not different between irradiated and non-irradiated brains. Data are shown as mean ± S.E.M. *$P<0.05$

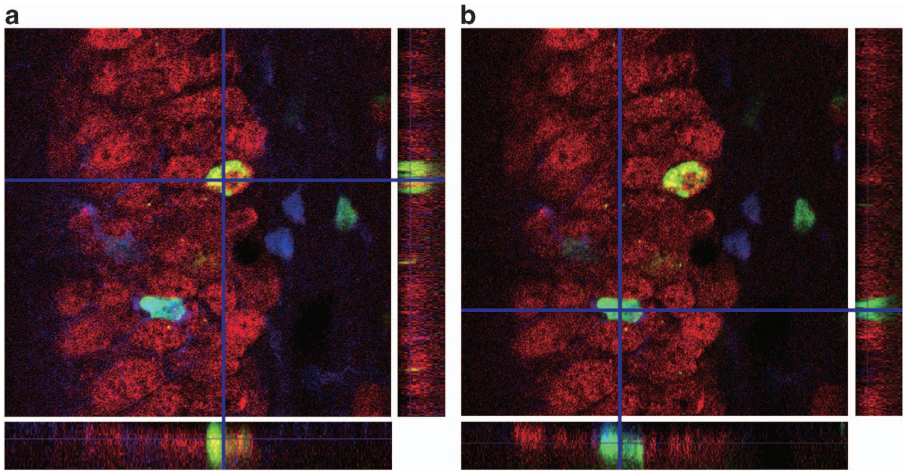

Figure 4 Representative microphotographs of the DG of the hippocampus stained for BrdU, NeuN and S100β. At 5 weeks after grafting, we evaluated the phenotype of surviving BrdU-positive cells in the GCL of the DG in the hippocampus. Cells were stained for BrdU (green), NeuN (red) and S100β (blue). Z-stacks were created to analyze BrdU+/NeuN+ cells (**a**) and BrdU+/S100β+ cells (**b**) to verify that they were truly double-positive

cells. A young (P15) recipient brain favored neuronal development of grafted cells, as the BrdU+/NeuN+ cells constituted 84% at P15, 66% at P21 ($P<0.05$) and 38% at P56 ($P<0.001$) (Figure 5). Older recipient brains favored an astroglial phenotype, as the BrdU+/S100β+ cells constituted 9% at P15, 29% at P21 ($P<0.05$) and 44% at P56 ($P<0.01$) (Figure 5). The number of unidentified grafted cells was higher in the adult recipient brains, as the ratio of BrdU+/NeuN−/S100β− cells was 6% at P15, 5% at P21 (NS) and 18% at P56 ($P<0.05$) (Figure 5). The number of unidentified cells in the subgranular zone (SGZ) and GCL, not having adopted a neuronal or astroglial phenotype, was not affected by IR at any age (Figure 5).

Reduced thickness of the GCL and astrogliosis. Reduced thickness of the dorsal blade of the GCL, defined as a thickness of four granule cells or less, was observed in 42% of all injected brains, irrespective of prior IR, and was more likely to develop in older brains (Table 1). The fate of grafted, ectopic cells in the needle tract was evaluated. Cells were injected on P15 and evaluated 5 weeks later. Some of the grafted, BrdU+ cells remained in the needle tract and virtually all of these were astrocytes (S100β+) (Figure 6). Under normal conditions, very few astrocytes are found in the GCL, but a surprisingly high number of the grafted cells in the GCL developed into astrocytes, as judged by the number of BrdU+/S100β+ cells, (Figure 5) and by far, most of these were found in the dorsal blade (Figure 7). Dorsal blades containing astrocytes in multiple layers of the blade invariably also displayed reduced thickness. To distinguish between the effects of the injections *per se* and the grafted cells, the dorsal blades of the GCL were evaluated long term, 5 months after vehicle or NSPC injections. Astrogliosis developed in the dorsal blade of brains injected with NSPCs (Figure 8).

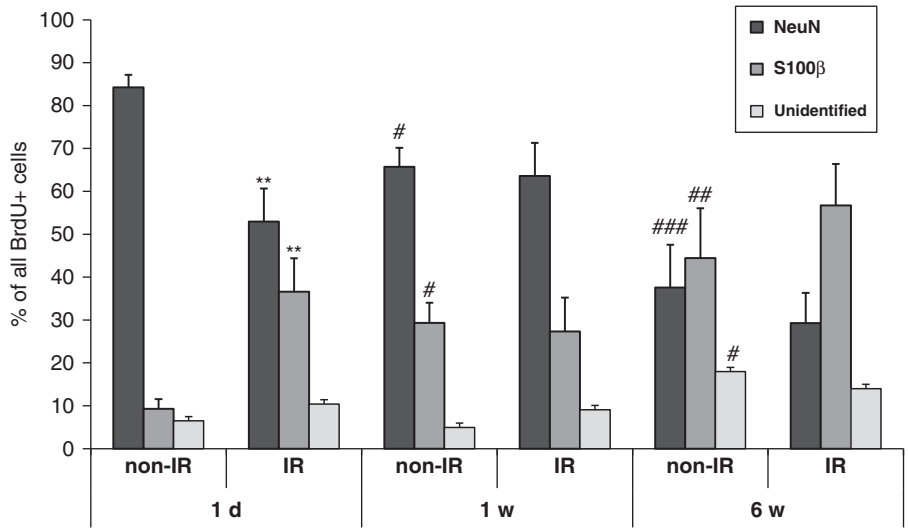

Figure 5 Effects of age and interval between IR and grafting on phenotype. Cell phenotype was evaluated 5 weeks after grafting. When grafting 1 day after IR, neuronal differentiation (BrdU + /NeuN + cells) was lower, whereas differentiation into astrocytes (BrdU + /S100β + cells) was higher in the irradiated *versus* non-irradiated brains. The phenotype of grafted cells was not significantly different between irradiated and control brains when transplantation was performed 1 or 6 weeks post-IR. In non-irradiated, control brains, lower age at the time of grafting favored neuronal differentiation and higher age favored astroglial differentiation. The ratio of unidentified cells was three times higher in the older recipient brains, but not different between irradiated and non-irradiated brains. Data represent the mean ± S.E.M. $^{**}P<0.01$ when comparing irradiated with non-irradiated brains. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ when comparing 1 week (P21) or 6 weeks (P56) brains with 1 day (P15) brains

Table 1 Reduced thickness of the dorsal blade of the GCL

Grafting	IR/non-IR	GCL reduction	
		+	−
1D	Non-IR	2	10
	IR	2	5
1W	Non-IR	7	4
	IR	5	4
6W	Non-IR	3	3
	IR	3	4

The number of brains displaying reduced thickness of the GCL, as defined in the Materials and Methods section. Mice were irradiated on P14, and NSPCs were injected 1 day (1D), 1 week (1W) or 6 weeks (6W) after irradiation. Evaluation was performed 5 weeks after grafting

All nine brains injected with NSPCs displayed astrogliosis, but only 2 out of 17 brains injected with vehicle (Table 2). Out of the vehicle-injected brains, only 1 in 10 control brains (non-irradiated) and 1 in 7 irradiated brains displayed astrogliosis. As for the earlier time points, in the brains injected with NSPCs, IR did not appear to predispose to astrogliosis in the GCL (Table 2).

Discussion

In this study, we demonstrated that grafted, syngeneic NSPCs (derived from the same strain of inbred C57BL6/J mice) injected into the young brain can survive in the GCL for at least 5 months without immunosuppressive treatment. We also show that IR-induced changes in the brain transiently create an environment that hampers survival and alters differentiation of grafted NSPCs from a neuronal to an astroglial fate.

It is known that inflammatory mechanisms affect survival, migration and differentiation of NSPCs,[10,23–25] so we wanted to avoid immunosuppressive treatment or the use of immunodeficient animals. We showed that the survival of transplanted NSPCs was significantly impaired and the neuronal differentiation was significantly lower in irradiated brains than in non-irradiated brain when we transplanted 24 h after IR, whereas there were no significant differences when NSPCs were transplanted 1 week or 6 weeks after IR. These results support our earlier findings that the inflammatory reaction in the young, still growing brain is transient,[20,21] unlike the adult rodent brain.[10] From a therapeutical point of view, this indicates that grafting of NSPCs should not be performed soon after IR, but that this non-permissive phase is short in the young brain. As the younger brains appear to be more permissive to cell survival and neuronal differentiation, it is likely an advantage to graft cells as early as possible. In this particular paradigm, when P14 mice were irradiated, 1 week after IR was apparently safe, but it is possible that an even shorter interval (shorter than one week, longer than one day) would yield higher survival without affecting differentiation. It remains to be determined if the survival and differentiation rates are different in adult mouse brains after IR, given the protracted inflammatory reaction.

In a model of adult spinal cord injury, it has also been shown that transplantation of NSPCs during the acute phase, in which inflammatory chemical mediators and cytokines were increased,[26] cause the grafted cells to differentiate into astrocytes to a higher extent, and reduce the beneficial treatment effects.[27] Grafting of neural stem cells has been utilized also in models of stroke in adult rodents. For example, early transplantation of neural stem cells (6 and 24 h after injury) resulted in higher rates of astroglial differentiation, whereas late transplantation (7 and 14 days after injury), after the peak of microglia activation

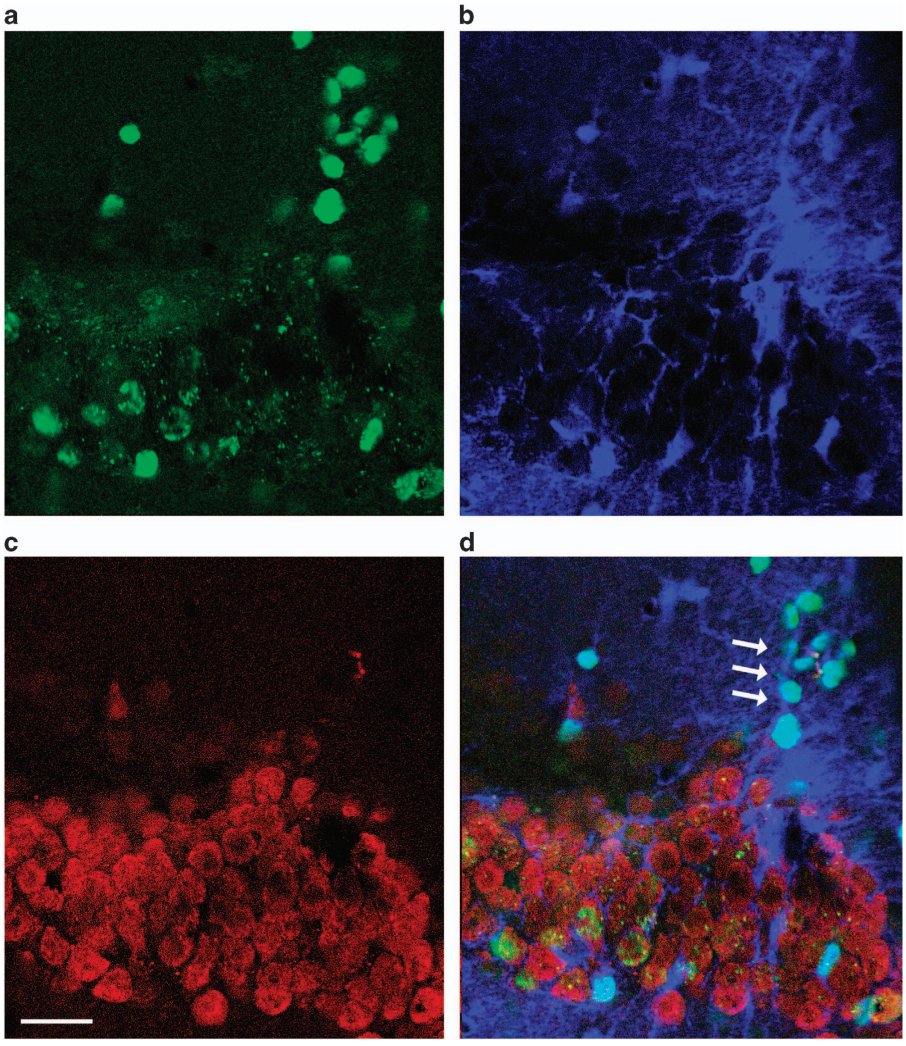

Figure 6 Ectopic, grafted cells in the needle tract. Representative microphotographs showing BrdU-labeled NSPCs grafted on P15 and evaluated 5 weeks after grafting. Cells were stained for BrdU (**a**, green), S100β (**b**, blue) and NeuN(**c**, red). Virtually all BrdU + grafted cells in the needle tract (arrows) were positive for S100β (**d**). Scale bar = 25 μm

and chemokine expression, including CCL2, resulted in higher neuronal differentiation.[28]

Transplanted cells home to the neurogenic niche of the SGZ, but a small number of BrdU-positive cells, were found outside the SGZ and GCL, mainly in the hilus and the molecular layer, very few cells were found outside the hippocampus (Figure 2). Grafted cells can sometimes also be found in areas with tissue damage, such as the needle track (Figure 6), and to some extent also in other hippocampal areas and the corpus callosum. Cells in extra-hippocampal locations more often adopt a glial phenotype, whereas cells displaying neuronal characteristics virtually only are found in the GCL.[29] Vietje and Wells[30] reported that fluid injection could induce granule cell degeneration. Injections of 2–10 μl into the GCL of adult rats caused almost complete loss of granule cells, usually in the dorsal blade. This unexpected morphological observation was corroborated by Cassel *et al*.[31] Both studies observed increased degeneration of the GCL and the molecular layer over time. The underlying mechanisms of the cell loss remain unknown, but presumably involve axotomy of granule cells.[30] Our observations also revealed that grafting induced reduced thickness of the dorsal blade after 5 weeks, and concurrent astrogliosis with almost complete loss of granule cells after 5 months in all mice. Unlike the observations of Vietje and Wells,[30] we did not find that injection of fluid only caused disruption of the GCL. This discrepancy might be explained by the difference in age at the time of grafting. In support of this, we observed the dorsal blade reduction was more likely to develop in older mice (Table 1). Although the morphological alterations induced by the injection were extensive, the degeneration had limited effects on behavior (locomotor activity, spontaneous alternation and spatial learning).[32] Grafting of NSPCs, despite the degeneration of granule cells, could even improve behavioral impairments (unpublished observations). The reason why predominantly the dorsal blade is affected remains to be explained.

Considering that proliferating NSPCs are highly susceptible to IR,[9,10,15,16,33,34] and that the reduced numbers of proliferating NSPCs in the GCL of the hippocampus most likely contribute to the memory and learning deficits observed later

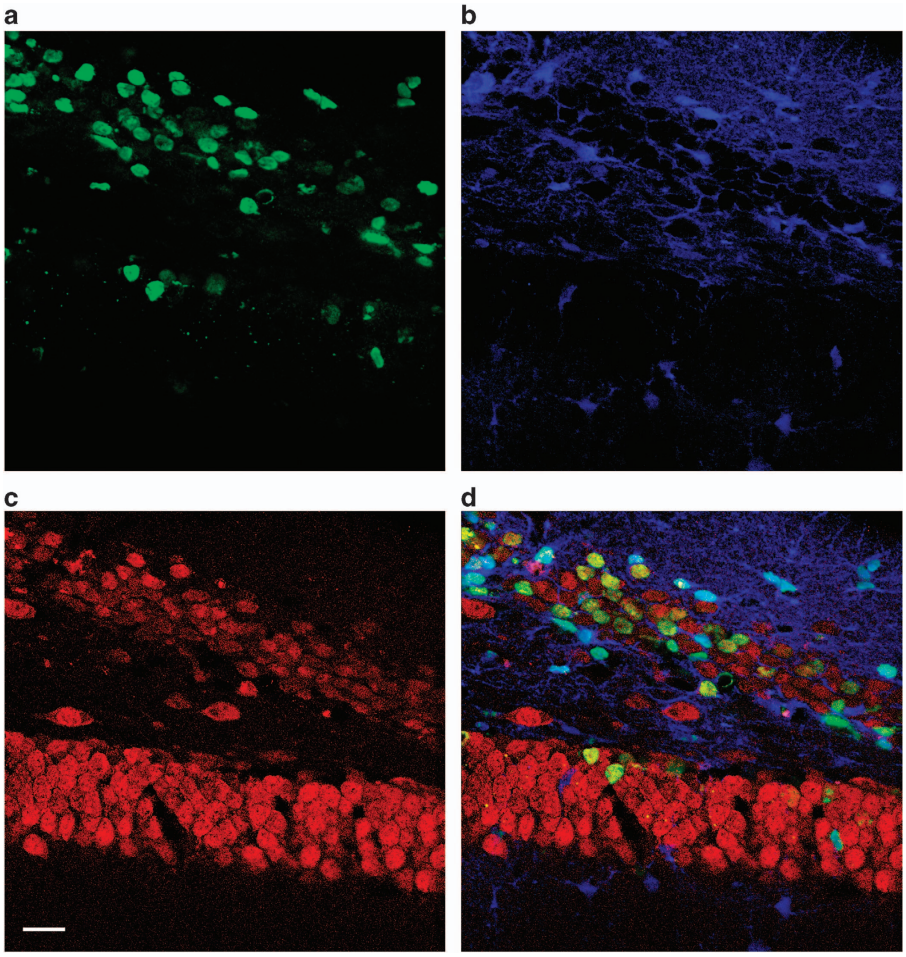

Figure 7 Astrogliosis and reduced thickness of the dorsal GCL blade. Representative microphotographs showing BrdU-labeled NSPCs grafted on P15 and evaluated 5 weeks after grafting. Cells were stained for BrdU (**a**, green), S100β (**b**, blue) and NeuN (**c**, red). An overlay of the three stainings is shown in (**d**). As seen in Figure 3, the majority of the grafted BrdU + cells were found in the dorsal blade. Phenotyping revealed that approximately half of the BrdU + cells developed into S100β + astrocytes, depending on the time of grafting (Figure 6), and these BrdU + /S100β + were almost exclusively found in the dorsal blade. The reduced thickness of the dorsal blade is most easily appreciated in the NeuN staining (**c**). Scale bar = 25 μm

in life, it is plausible that exogenous NSPCs could ameliorate the so-called late effects of radiation therapy. Furthermore, the younger the recipient brain, the higher the survival and neuronal differentiation, indicating that this strategy may be more fruitful in a pediatric setting. Further studies are needed to demonstrate possible functional benefits of NSPC grafting and the underlying mechanisms. For safety reasons, the source of cells and the risk of tumor formation must be considered. The observed astrogliosis and reduced thickness of the GCL warrant caution and the apparent discrepancy between the astrogliosis and degeneration on the one hand and the lack of functional deficits on the other hand remain to be investigated. If we can mitigate the adverse side effects of radiation therapy in the increasing number of survivors of childhood cancer, we could improve their quality of life and reduce the economic burden on society.

Materials and Methods

Animals. All animal experimental protocols in this study were approved by the Gothenburg committee of the Swedish Animal Welfare Agency (326-2009). C57BL/6J male pups were from Charles River (Sulzfeld, Germany), and maintained under a 12 h light/dark cycle with access to food and water *ad libitum*.

Experimental procedures. A single IR dose of 8 Gy was administered to the brains of P14 C57BL/6J mice as described earlier[9] and 1.0×10^5 BrdU-labeled NSPCs, derived from the same mouse strain, were injected into the hippocampus 1 day (P15), 1 week (P21) or 6 weeks (P56) after IR. No immunosuppression was used. Cell survival, phenotype and morphology of the GCL were evaluated 5 weeks after grafting (Figure 1). The NSPCs were prepared from whole mouse brain (without the cerebellum or olfactory bulb)[35] and kindly provided by Gage FH. To evaluate long-term effects of vehicle and NSPC injections on GCL morphology, a separate series of mice were irradiated on P9, followed by vehicle or NSPCs injections into the hippocampus on P21, and evaluation of astrogliosis 5 months after grafting.

IR procedure. For IR, a linear accelerator (Varian Clinac 600CD; Radiation Oncology Systems LLC, San Diego, CA, USA) with 4 MV nominal photon energy and a dose rate of 2.3 Gy/min was used. Mice (9 or 14 days old) were anesthetized with an intraperitoneal injection of tribromoethanol (Sigma-Aldrich, Stockholm, Sweden), placed in prone position (head to gantry) on an expanded polystyrene bed. The left cerebral hemisphere of each animal was irradiated with an asymmetrical radiation field of 1×2 cm^2. The source to skin distance was approximately 99.5 cm. The head was covered with a 1 cm tissue equivalent. A single absorbed dose of 8 Gy was administered and the dose

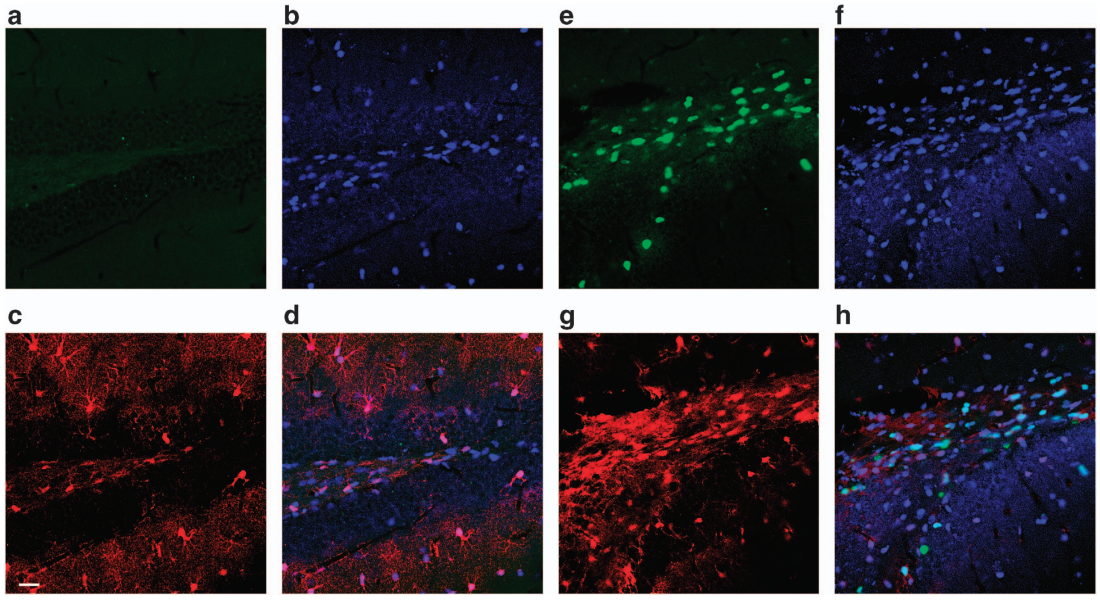

Figure 8 Astrogliosis. Representative microphotographs showing the DG after injection of vehicle (**a**–**d**) or BrdU-labeled NSPCs (**e**–**h**) on P9, evaluated 5 months after the injection. Cells were stained for BrdU (**a** and **e**; green), Sox2 (**b** and **f**; blue) and S100β (**c** and **g**; red). An overlay of the three stainings is shown in (**d**) and (**h**). Astrogliosis in the dorsal blade was found only in the brains injected with NSPCs, not vehicle, at this late time point. Scale bar = 25 μm

Table 2 Astrogliosis of the dorsal blade of the GCL

IR/non-IR	Grafting	Gliosis	
		+	−
Non-IR	Veh	1	9
IR	Veh	1	6
IR	NSPC	9	0

The number of brains displaying astrogliosis in the GCL, as defined in the Materials and Methods section. Mice were irradiated on P9, neural stem and progenitor cells (NSPCs) or Vehicle (Veh) was injected on P21. Astrogliosis was evaluated 5 months after grafting

variation within the target volume was estimated to be ±5%. The entire procedure was completed within 10 min. After IR, the pups were returned to their biological dams until weaning. The sham control animals were anesthetized but not subjected to IR. Using the LQ model[36] and an α/β ratio of 3 for late effects in the normal brain tissue, an acute exposure of 8 Gy is equivalent to approximately 18 Gy when delivered in repeated 2 Gy fractions, as in patients. This dose represents a clinically relevant dose, equivalent to the total dose used in treatment protocols for prophylactic cranial IR in selected cases of childhood acute lymphatic leukemia. The dose used for malignant brain tumors is higher, usually 24–35 Gy craniospinal IR combined with 55–60 Gy to the tumor bed.

Culture of neural stem/progenitor cells. NSPCs were plated in uncoated (10 cm) tissue culture plastic plates (SARSTEDT, Neumbrecht, Germany), and were cultured and expanded as a monolayer in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (1 : 1) (Invitrogen Corporation, San Diego, CA, USA) containing 1% N2 (Invitrogen), 20 ng/ml epidermal growth factor (Sigma-Aldrich, Saint Louis, MO, USA), 20 ng/ml basic fibroblast growth factor-2 (bFGF; BD Biosciences, Franklin Lakes, NJ, USA), and 5 μg/ml heparin (Sigma-Aldrich). The cells were passaged when they reached 60–70% confluency. At 2 days before transplantation, BrdU was added to the medium at a final concentration of 1.25 μM. After collecting and washing, the cells were suspended in DMEM containing 300 μg bFGF for transplantation.

Transplantation of neural stem/progenitor cells. Mice were immobilized and mounted in a stereotactic head holder (Kopf Instruments, Tujunga, CA, USA) under anesthesia with isoflurane (Isoba vet.; Schering-Plough Corporation, Kenilworth, NJ, USA; 5% for induction, and 2–3% for maintenance) in the flat skull position. To transplant NSPCs into the hippocampus, a 5 μl 26-gauge syringe (Innovative Labor Systeme, Stuetzerbach, Germany) was inserted using the following coordinates: 0.45 × (distance from lambda to bregma) mm posterior and ±1.2 mm lateral to bregma, and 3.0 mm deep from the skull surface for P15 mice; 0.42 × (distance from lambda to bregma) mm posterior and ±1.3 mm lateral to bregma, 3.2 mm deep from the skull surface for P21 mice; and 0.42 × (distance from lambda to bregma) mm posterior and ±1.5 mm lateral to bregma, 3.2 mm deep from the skull surface for P56 mice. These coordinates were determined in preliminary experiments. Before the needle was inserted, a small hole was drilled in the proper position according to the above coordinates for P21 and P56 mice. A total of 1×10^5 NSPCs in 2 μl DMEM were injected slowly, over 2 min, with a 4 min delay before removal of the syringe, allowing 2 min for syringe removal. For an overview of the experimental design, see Figure 1.

Tissue preparation. Mice were deeply anesthetized 5 weeks or 5 months after transplantation, and intracardially perfusion-fixed with 0.9% NaCl, followed by a 6% formaldehyde solution buffered with sodium phosphate at pH 7.4 and stabilized with methanol (Histofix; Histolab Products AB, Gothenburg, Sweden). The brains were removed and immersion-fixed in the same solution at 4 °C for 24 h, and then immersed in 30% sucrose for at least 2 days. The brains were cut into 30 μm sagittal sections in a series of 12, using a sliding microtome and dry ice.

Microscopy and immunohistochemistry. The following antibodies and final dilutions were used: anti-BrdU (1 : 500; AbD Serotec, Martinsried, Germany), mouse anti-NeuN (1 : 250; Millipore Corporation, Billerica, MA, USA), rabbit anti-S100β (1 : 1000; Swant, Bellinzona, Switzerland), goat anti-Sox2 (1 : 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Detection of BrdU-labeled cells was performed as follows: free-floating sections were rinsed in Tris-buffered saline: 0.1 M Tris-HCl, pH 7.4/0.9% NaCl (TBS), sections were then treated with 0.6% H_2O_2/TBS for 30 min, followed by incubation for 2 h in 50% formamide/2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) at 65 °C, rinsed in 2 × SSC, incubated for 30 min in 2 N HCl at 37 °C and rinsed in 0.1 M boric acid, pH 8.5. Incubation in TBS/3% donkey serum/0.1% Triton X-100 (TBS + +) for 30 min was followed by overnight incubation with mouse anti-BrdU. After rinsing in TBS, sections were incubated for 1 h with donkey anti-mouse-biotin

(1 : 1000 biotinylated donkey anti-mouse; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and then avidin–biotin–peroxidase complex (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA, USA), followed by peroxidase detection for 5 min (0.25 mg/ml DAB, 0.01% H_2O_2, 0.04% NiCl).

Triple immunofluorescence including anti-BrdU was performed as follows: free-floating sections were rinsed in TBS, incubated for 30 min in 2 N HCl at 37°C, and rinsed in 0.1 M boric acid, pH 8.5. After several rinses in TBS, sections were incubated in TBS + + for 30 min, followed by a primary antibody cocktail for 24 h at + 4 °C. The sections were rinsed in TBS, incubated with a cocktail of fluorochrome-labeled secondary antibodies for 2 h (all secondary antibodies, 1 : 1000; Invitrogen Corporation), rinsed again in TBS and mounted on glass slides.

Stereological quantification of cells. Every 12th section containing the dorsal hippocampus was used to determine the total number of BrdU-positive cells in the GCL under light microscopy in each animal. The number was counted using the Stereo Investigator (version 6) software (MBF Bioscience, Williston, VT, USA). Cell counts were then multiplied with the series factor (12) and represent the total number of cells per GCL. For triple immunofluorescence, at least 50 BrdU-positive cells per animal were analyzed. The percentages of neurons (BrdU + /NeuN +), astrocytes (BrdU + /S100β +) and unidentified cells (BrdU + /NeuN − / S100β −) were assessed using a confocal microscope (Leica TCS SP2; Leica Microsystems, Wetzlar, Germany), followed by multiplication with the absolute number of BrdU-positive cells, thereby yielding the absolute numbers of newborn neurons, astrocytes and unidentified cells.

Reduced thickness of the GCL and astrogliosis. The number of layers of granule cells in the dorsal blade of the GCL was counted. Reduced thickness of the blade was arbitrarily defined as a thickness of four granule cells or less. Astrogliosis, defined as astrocytes observed in multiple layers throughout the blade, was only found in blades with reduced thickness.

Statistics. All data are presented as means ± S.E. Student's *t*-test was used when comparing two groups, whereas ANOVA followed by the PLS *post hoc* test was used when comparing three or more groups. A *P*-value < 0.05 was considered to indicate significant differences between the compared mean values.

Conflict of Interest

The authors declare no conflict of interest.

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