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Loss of hippocampal neurogenesis, increased novelty-induced activity, decreased home cage activity, and impaired reversal learning one year after irradiation of the young mouse brain

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ABSTRACT

Radiotherapy is a major cause of long-term complications in survivors of pediatric brain tumors. These complications include intellectual and memory impairments as well as perturbed growth and puberty. We investigated the long-term effects of a single 8 Gy irradiation dose to the brains of 14-day-old mice. Behavior was assessed one year after irradiation using IntelliCage and open field, followed by immunohistochemical investigation of proliferation and neurogenesis in the dentate gyrus of the hippocampus. We found a 61% reduction in proliferation and survival (BrdU incorporation 4 weeks prior to sacrifice), 99% decrease in neurogenesis (number of doublecortin-positive cells) and gliosis (12% higher astrocyte density) one year following irradiation. Irradiated animals displayed increased activity in a novel environment but decreased activity in their home cage. Place learning in the IntelliCage was unaffected by irradiation but reversal learning was impaired. Irradiated animals persevered in visiting previously correct corners to a higher extent compared to control animals. Hence, despite the virtual absence of neurogenesis in these old mice, spatial learning could take place. Reversal learning however, where a previous memory was replaced with a new one, was partly impaired. This model is useful to study the so called late effects of radiotherapy to the young brain and to evaluate possible interventions.

Introduction

Radiotherapy (RT) is one of the major causes of long-term complications seen in survivors of pediatric brain tumors. Intellectual and memory impairments as well as perturbed growth and puberty are some of the so called late effects seen after RT (Hoffman and Yock, 2009; Lannering et al., 1990a, 1990b, 1995; Roman and Sperduto, 1995; Spiegler et al., 2004). These impairments have been shown to be more severe in children younger than 3 years of age at the time of RT (Chin and Maruyama, 1984; Duffner et al., 1985; Fouladi et al., 2005; Packer et al., 1987). Irradiation (IR)-induced damage to the growing brain involves dose-dependent loss of cells in the surrounding healthy brain tissue and has been reported in the immature, juvenile and adult rodent brain (Andres-Mach et al., 2008; Fukuda et al., 2004, 2005a, 2005b; Hellstrom et al., 2009; Naylor et al., 2008). An increasing number of studies have investigated the molecular mechanisms of injury to the healthy tissue, aiming to develop novel strategies to ameliorate the late effects after RT. Importantly, pre-oligodendrocytes and proliferating cells in the neurogenic regions appear to be particularly susceptible to IR (Fukuda et al., 2004, 2005a, 2005b; Monje et al., 2002). Functional outcomes after IR to the brain have been studied using e.g. Morris water maze, fear conditioning and open field (Naylor et al., 2008; Raber et al., 2011; Rola et al., 2004; Wojtowicz et al., 2008). The IntelliCage platform was designed to minimize interaction between the experimenter and the animals and is therefore an attractive tool for any brain injury paradigm (Karlsson et al., 2011).

The aim of this study was to investigate long-term effects of a single dose of IR to the young brain by using open field and IntelliCage, followed by immunohistochemical analysis of the hippocampus. The high cure rates obtained with current treatment protocols and the particularly long remaining life expectancy of children have generated a population of young adult childhood cancer survivors. In patients, cranial radiotherapy is known to cause long-term cognitive decline, often aggravated over time (Dietrich et al., 2008; Spiegler et al., 2004). To our knowledge, there are very few animal studies with a follow-up as long as one year after IR. If cognitive decline could be ameliorated, the quality of life of the survivors would increase and

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the socioeconomic burden would be reduced. It is therefore important to develop relevant experimental models of treatment-induced late effects.

Materials and methods

Animals

Male C57BL/6 pups from Charles River Laboratories (Sulzfeld, Germany) were kept on a 12-hour light cycle. Food and water were provided ad libitium. At the time of weaning, mice were anesthetized with isoflurane and injected subcutaneously with microtransponders (DATAMARS, PetLink, Youngstown, OH, USA) for identification purposes. The animals were kept in groups of up to 10. All animal experiments were approved by the regional ethical committee (46/2007 and 326/2009).

Irradiation

The irradiation procedure has been described previously (Fukuda et al., 2004; Kalm et al., 2009a, 2009b; Karlsson et al., 2011). Briefly, on postnatal day 14, the whole brain was irradiated with a 2×2 cm field and a radiation source-to-skin distance of approximately 99.5 cm. The dose variation within the tissue was estimated to ±5%. A single 8 Gy dose (2.3 Gy/min) was administered and after irradiation the animals were returned to their dams for recovery. Control animals were anesthetized but not exposed to irradiation. A dose of 8 Gy is equivalent to approximately 18 Gy when delivered in repeated 2 Gy fractions, as in clinical practice, according to the linear-quadratic formula (Fowler, 1989) and an α/β-ratio of 3 for late effects in the normal brain tissue. A total dose of 18 Gy is a moderate dose to the brain, equivalent to the doses used in selected cases of CNS involvement of leukemia or lymphoma. The doses used for brain tumors are higher. In the case of medulloblastoma, the most common malignant brain tumor in children, current treatment protocols recommend 23–35 Gy to the whole brain and spinal cord, followed by a boost to the tumor bed, resulting in a total dose of 55–68 Gy to that area.

IntelliCage

The IntelliCage platform enables monitoring of activity and learning in a home cage environment over extended periods of time (Barlind et al., 2009; Jaholkowski et al., 2009; Karlsson et al., 2011; Onishchenko et al., 2007; Zhu et al., 2010). The protocol used in this study has been described in detail (Karlsson et al., 2011). Animals were placed in the IntelliCages at an age of 57 weeks (55 weeks post IR). The experiment started with an introduction period (6 days) followed by three corner training periods (each lasting 5 days). During the corner training, each mouse was assigned to a corner (the correct corner). Care was taken to not assign the animals to a corner they had previously been assigned to. Animals that were not registered at all or failed to drink in the IntelliCages were removed from the cages. Food was provided ad libitum during the IntelliCage experiment and animals were allowed to drink freely from their assigned corners. At the time of IntelliCage 30% of the animals had been lost to follow-up due to fighting (14%), death (8%), disease (4%), or microtransponder malfunction (4%). The dropout rates were not different between the control group and the irradiation group (not shown). Immediately after the IntelliCage experiment the animals were injected with 5-bromo-2′-deoxyuridine (BrdU, 50 mg/kg, Roche Applied Science) for three consecutive days. Data from the inactive period, from visits lasting longer than 180 s and from visits where no nose pokes were performed, were excluded from the analysis (Karlsson et al., 2011). In addition, animals were required to perform at least 6 visits and at least 6 nose pokes/licks per day to be included in the analysis. Failure to fulfill these criteria indicated either microtransponder detection problems or that the mice had problems finding water. When analyzing the first hour during the introduction period all visits and nose pokes where included (mice that did not display any activity during this time period were treated as missing values).

Open field

When the control and IR animals were 65 weeks old their activity pattern was analyzed by video tracking (sampling frequency of 12.5 Hz). This experimental procedure has previously been described elsewhere (Nilsson et al., 2006). Data were analyzed with respect to distance moved, stops, exploratory rearing, spatial variability in the movement path and percent time spent in the middle of the arena. The variables were summarized for 30 min and are presented in Table 1.

Tissue processing and cell counting

When sacrificed (one month after BrdU injections), animals were deeply anesthetized with sodium pentobarbital (1.2 g/kg intraperitoneally) and transcardially perfused with phosphate-buffered saline (PBS) followed by Histofix (buffered 6% formaldehyde solution; Histolab Products AB, Sweden). The brains were removed and immersion-fixed in the same solution at 4 °C for 24 h and then transferred to 30% sucrose in 0.1 M phosphate buffer (pH 7) for storage at 4 °C. The right hemisphere was cut in 25 μm sagittal sections on a sliding microtome. The sections were collected in series of 12 and stored in a cryoprotection solution containing 25% glycerine and 25% ethylene glycol in 0.1 M phosphate buffer (pH 7).

Sections were rinsed with Tris-buffered saline (TBS) before incubating with 0.6% H_2O_2 in TBS for 30 min. Non-specific binding was blocked with 3% donkey serum (Jackson ImmunoResearch Laboratories Inc., Cambridgeshire, UK) in 0.1% Triton X-100 and TBS. The tissue was then incubated with primary antibodies, goat anti-doublecortin C-18 (DCX, 1:125, Santa Cruz Biotechnology, Inc.), mouse anti-glial fibrillary acidic protein (GFAP, 1:1000, Millipore) and rat anti-BrdU (1:500, Nordic Biosite), in 3% donkey serum, 0.1% Triton X-100 and TBS over night at 4 °C and subsequently incubated with biotinylated donkey anti-goat or anti-rat secondary antibody (1:1000 Jackson ImmunoResearch, West Grove, PA) in 3% donkey serum, 0.1% Triton X-100 and TBS for 1 h. After further incubation with an avidin–biotin–peroxidase solution (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA), stainings were developed with 3.3-diaminobenzidine.

Table 1
A list of the open field variables analyzed.

%IZm	Percent time in middle zone
%MV	% time in motion
%MVm	% time spend in motion in middle zone
AM	Mean absolute meander
AMm	Mean absolute meander in middle zone
AM0.5	Mean absolute meander with filter 0.5 cm
sdAM.05	Standard deviation of AM0.5
AM0.5 m	AM0.5 in middle zone
sdAM0.5 m	Standard deviation of AM0.5 m
DM	Distance moved
DMm	Distance moved in middle zone
DM1.5	Distance moved calculated with a filter that does not measure the distance until the animal has moved at least 1.5 cm.
DM1.5 m	DM1.5 in middle zone
NIZm	Number of entries in middle zone
R	Rearings
Rm	Rearings in middle zone
ST	Number of changes between moving and not moving states (Stops, defined as velocity below 5 cm/s)
STm	Stops in middle zone