

# Effect of Low-Dose Irradiation on Proliferation Dynamics in the Rostral Migratory Stream of Adult Rats

(ionizing radiation / rat brain / rostral migratory stream / cell proliferation)

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**Abstract.** Ionizing radiation as one of the strongest cytogenetic factors can induce significant injury to adult brain. In the present study, adult male Wistar rats were exposed to single whole-body gamma irradiation with the dose of 3 Gy. One, 5, 10, 25, 40 or 80 days after irradiation, proliferating cells were labelled using BrdU immunohistochemistry. BrdU-positive cells were counted individually in the three anatomical parts of the brain RMS: the vertical arm, elbow, and horizontal arm. The number of BrdU<sup>+</sup> cells decreased rapidly during the 1<sup>st</sup> day after exposure in the whole extent of the RMS. In course of the following days, considerable increase was observed in the elbow and vertical arm of the RMS with the maximal values on the 5<sup>th</sup> and 10<sup>th</sup> days, respectively; until the 40<sup>th</sup> day after irradiation, the numbers of BrdU<sup>+</sup> cells returned to the control level. Contrary to the two previous parts of the RMS, in the horizontal arm, no statistically significant increase was found and the decrease under control values occurred at the longest survival time. Our results suggest that the whole-body irradiation of rats with the sublethal dose of gamma irradiation can induce acute as well as long-lasting changes in the brain regions where proliferation activity is retained during adulthood.

The subventricular zone (SVZ) is the largest germinal zone of the mammalian brain, which persists until adulthood and retains the capacity to give rise to both neuronal and glial cells. In adult brain, the fate of pro-

genitors of neurons and glia cells depends on their location. Progenitor cells of the hippocampal subgranular zone (SGZ) migrate into the granular cell layer of the dentate gyrus, progenitor cells of the SVZ migrate along the restricted pathway called the rostral migratory stream (RMS) to the olfactory bulb (OB). In the OB they migrate radially and those that survive differentiate into granular and periglomerular interneurons (Alvarez-Buylla et al., 2002; Doetsch, 2003; Suzuki and Goldman, 2003), which can establish some functional connections (Zigova and Sanberg, 1999; Carleton et al., 2003). The SVZ of adult mammalian brain therefore serves as an important source of progenitors that could be used for neuroregenerative therapy. Thus, the SVZ-RMS-OB system represents an interesting experimental model for the study of the origin, migration and integration of new neurons into functional brain circuits.

The SVZ progenitors are vulnerable to damage, whereas the neural stem cells are resilient and after moderate injury (but not after more severe injury) the SVZ can recover (Tada et al., 1999; Romanko et al., 2004). Therefore, after transient decrease, brain lesions generally induce an increase in SVZ neurogenesis or gliogenesis and cause cell emigration to ectopic location (Sundholm-Peters et al., 2005). Perinatal as well as adult SVZ not only have the capacity to replenish their own numbers, but also the ability to replace neurons and glia after different models of brain injuries (Kirschenbaum et al., 1999; Romanko et al., 2004; Ramaswamy et al., 2005).

Ionizing radiation, currently used in the radiotherapy, can cause significant damage to normal mammalian brain especially in prenatal age. Local irradiation of the juvenile rat brain with the doses of 1–3 Gy caused expressive reduction in the number of neural stem and progenitor cells in the SVZ and increased apoptosis, but these changes were reversible (Amano et al., 2002). Whole-brain irradiation with large-scale doses (2–10 Gy) also caused a decrease in the number of SGZ cells and their progeny in the hippocampus of young mice in a dose-dependent fashion (Rola et al., 2004). To deter-

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Abbreviations: ABC – avidin-biotin complex, BrdU – 5-bromo-2'-deoxyuridine, BrdU<sup>+</sup> – BrdU-positivity, DAB – diaminobenzidine, OB – olfactory bulb, PBS – phosphate-buffered saline, RMS – rostral migratory stream, SGZ – subgranular zone, SVZ – subventricular zone.

mine whether acute changes can be converted into long-term alterations, the brain tissues were examined in various intervals after exposure (Shinohara et al., 1997; Peissner et al., 1999; Tada et al., 2000; Mizumatsu et al., 2003; Raber et al., 2004). The most marked reduction in the number of proliferating cells and increase in apoptosis were found several hours post-irradiation. After temporal increase in cell proliferation, the production of immature neurons was frequently reduced several months after irradiation (Mizumatsu et al., 2003; Raber et al., 2004; Rola et al., 2004).

In the present study we investigated the proliferating activity of cells in the RMS of the brain of adult rats exposed to a whole-body sublethal dose (3 Gy) of gamma rays at various intervals of post-irradiation survival.

## Material and Methods

**Animals.** Male rats of the Wistar strain at the age of 4–7 months at the beginning of the experiment were used. The control rats ( $n = 3$ ), similarly as irradiated rats ( $n = 18$ ), were kept under standard conditions (temperature of 22–24 °C, natural light rhythm) and provided with food and water *ad libitum*. All animal procedures were conducted in accordance with the requirements for ethical standards of welfare and treatment of animals.

**Irradiation.** Experimental animals were irradiated with a single whole-body dose of 3 Gy of gamma rays using a  $^{60}\text{Co}$  source (apparatus Chisostat, Chirana, Prague, Czech Republic) at a dose rate of  $0.077 \text{ Gy}\cdot\text{min}^{-1}$ . The animals were investigated 1, 5, 10, 25, 40 or 80 days after exposure (three animals at each time interval).

**Immunohistochemistry.** The thymidine analogue 5-bromo-2'-deoxyuridine (BrdU, Sigma, St. Louis, MO), proliferative marker which incorporates into the DNA of dividing cells, was used to label the proliferating cells. The animals were injected i.p. at a dose of 100 mg/kg body weight two times in the course of 24 hours. Four hours after the second injection, the rats received inhalation anaesthetic halothan, then were injected i.p. ketamine/xylazine (0.2 and 0.1 ml/100 g b.w.) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS).

After sacrifice the brains were immediately removed from the skull, postfixed in the same fixative overnight at 4°C and cryoprotected with 30% sucrose for at least 24 h before sectioning. Serial sagittal 42  $\mu\text{m}$  thick sections were cut using a cryostat. Immunostaining was performed on floating sections and washing with PBS was used after each handling step. For detection of BrdU<sup>+</sup> nuclei, DNA was denatured to expose the antigen by incubating the sections with 2 N HCl at 60 °C for 30 min and then neutralized with 0.1 M borate buffer. To suppress endogenous peroxidase activity, the sections were incubated with 0.3%  $\text{H}_2\text{O}_2$  in 20% methanol and with blocking buffer (10% normal horse serum in

TBS-T). The next incubation was carried out by diluted rat anti-BrdU antibody (1 : 500, Oxford Biotechnology, Kidlington, UK) overnight at 4°C. The secondary incubation was performed with goat anti-rat IgG biotinylated antibody (1 : 200, Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Sections were finally incubated with avidin-biotin complex (ABC-kit, Vector Laboratories) and immunoreactivity was revealed using sensitive chromogene diaminobenzidine (DAB, Sigma, St. Louis, MO).

**Computer image analysis.** Counting of BrdU immunopositive cells was performed on cryostat sections, which were viewed by microscope Olympus BX51 (Tokyo, Japan) under a 40x oil immersion objective; serial digital images were obtained by a digital camera (DP50) linked to DP Imaging software, version 3.0 and displayed in a computer. The number of BrdU-positive cells was counted in each stained section with the whole extent of the RMS (4–6 sections per animal) in three different standardized counting areas along the subventricular zone – olfactory bulb axis. The three areas included: the vertical arm, corresponding to the caudal half of the RMS, the elbow, occurring about half of the distance from the anterior SVZ to the OB, and the horizontal arm, corresponding to the rostral part of the RMS.

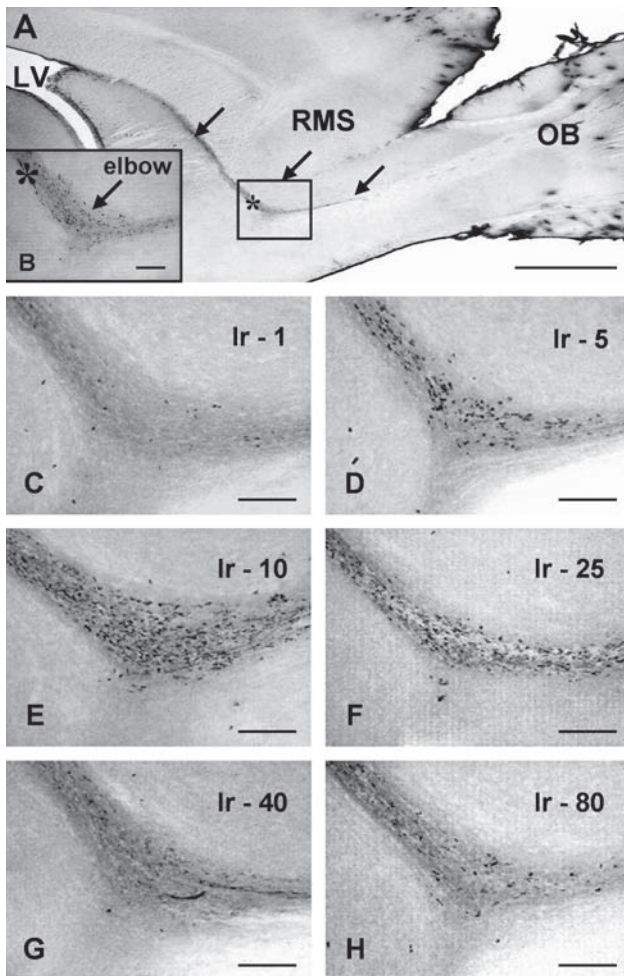
All cells labelled by BrdU were determined using the Dissector program, version 2.0 (Tomori et al., 2001), point-counting method for unbiased estimation of the particle number in three-dimensional space.

**Statistical analysis.** The values were analysed by the ANOVA one-way test and results are presented as the mean  $\pm$  S.E.M. Statistical significance was assumed for  $P \leq 0.05$ ;  $P \leq 0.02$  and  $P \leq 0.01$ .

## Results

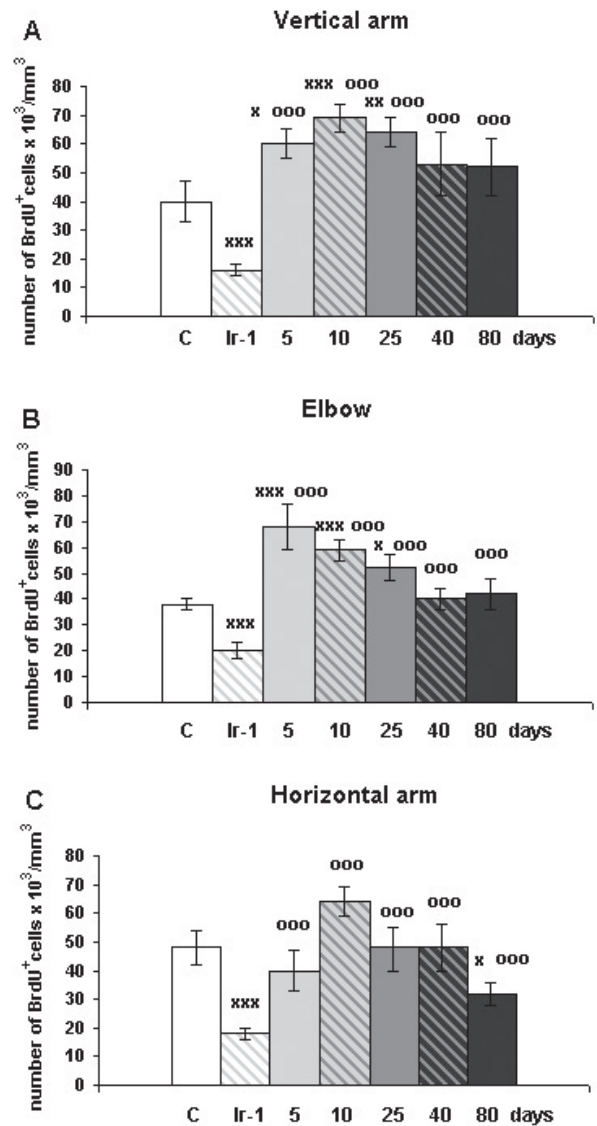
BrdU immunohistochemistry demonstrates an overview of the control animals' RMS, composed of highly packed proliferating cells that are clearly distinguishable from the surrounding brain parenchyma (Fig. 1A, B). Light microscopic examination of sagittal sections through the forebrain of rats irradiated with the dose of 3 Gy of gamma rays showed initial depletion of BrdU<sup>+</sup> cells in the rostral migratory pathway (Fig. 1C). This decrease was followed by transient over-accumulation of cells (Fig. 1D, E, F). After long-term survival, BrdU<sup>+</sup> cells displayed a similar pattern of density as in control animals (Fig. 1G, H). Results of quantitative computer image analyses in individual parts of the RMS are given in Fig. 2.

In the vertical arm of control animals, the values of BrdU<sup>+</sup> cells averaged  $40 \pm 7 \times 10^3/\text{mm}^3$ . One day after exposure, a sharp decrease was seen to the level of  $15 \pm 2$  (Fig. 2A). In the course of the following post-irradiation days, a strong increase was observed. The proliferating activity peaked on the 10<sup>th</sup> day after irradiation ( $69 \pm 5$ ); after this striking increase, a partial decrease



**Fig. 1.** BrdU immunohistochemistry. Photomicrographs of the sagittal sections through the forebrain of adult male rats showing: (A) whole extent of the RMS (arrows) and the RMS elbow (asterisk). Details of labelled (dark brown) nuclei of BrdU-positive cells in the RMS elbow of control (B) and experimental rats (C–H) 1, 5, 10, 25, 40 and 80 days after irradiation (Ir) with the dose of 3 Gy of gamma rays. LV – lateral ventricle; OB – olfactory bulb; RMS – rostral migratory stream. Calibration bars: A = 1000  $\mu\text{m}$ ; B – H = 100  $\mu\text{m}$ .

occurred until the 40<sup>th</sup> day after exposure ( $52 \pm 10$ ). In comparison to control animals ( $38 \pm 2 \times 10^3/\text{mm}^3$ ), the number of proliferating cells in the RMS elbow was significantly reduced on the 1<sup>st</sup> day after exposure ( $20 \pm 3$ ) (Fig. 2B). Rapid increase to the maximum ( $68 \pm 9$ ) on the 5<sup>th</sup> day was followed by gradual decrease to the control level until the 40<sup>th</sup> day. Similarly as in the two more caudal parts, the number of BrdU<sup>+</sup> cells sharply decreased (from  $48 \pm 6$  in controls to  $18 \pm 2$ ) on the 1<sup>st</sup> day after irradiation also in the rostral part of the RMS, in the horizontal arm (Fig. 2C). However, the following increase was gradual and the maximal value ( $64 \pm 5$ ) on the 10<sup>th</sup> day was not significantly higher comparing to controls. Until the 80<sup>th</sup> day, the values decreased below the control level (to  $32 \pm 4$ ).



**Fig. 2.** Distribution of BrdU-positive cells in the forebrain of adult male rats. Total number of BrdU<sup>+</sup> cells in the vertical arm (A), elbow (B) and horizontal arm (C) of the RMS of control and experimental rats 1, 5, 10, 25, 40 and 80 days after irradiation with the dose of 3 Gy of gamma rays (Ir-1 – 80d;  $\bar{x} \pm \text{SEM}$ ). Statistical significance of differences between experimental and control groups: \*  $P \leq 0.05$ ; \*\*  $P \leq 0.02$ ; \*\*\*  $P \leq 0.01$ ; between Ir-1d and the following experimental groups: °°°  $P \leq 0.01$ .

## Discussion

In the present study, alterations in the proliferation dynamics, induced by the sublethal dose of 3 Gy of gamma radiation in adult rat brain, were studied on the basis of the number of BrdU-positive cells quantified within the vertical arm, elbow, and horizontal arm of the RMS at various time intervals after exposure. In all three parts of the RMS, the time course of changes showed biphasic response to radiation; it was characterized by steep decreases in the numbers of BrdU<sup>+</sup> cells until 24

hours post-irradiation and subsequent transient increases. This finding in the RMS is in accordance with the results of Uberti et al. (2001), who revealed a similar response to radiation in the dentate gyrus of mice, characterized by an early inhibition and delayed stimulation of cell proliferation. On the basis of determination of cell type-specific gene expression, distribution of neuronal phenotype in the OB with injured RMS or after OB removal and after transplantation of SVZ progenitor cells, several authors proposed that in the injured migratory pathway just neuronal differentiation was altered (Zigova et al., 1998; Kirschenbaum et al., 1999; Law et al., 1999; Fukushima et al., 2002).

In addition to inhibition of cell proliferation, exposure to single or fractionated doses of ionizing radiation significantly decreases the numbers of stem or precursor cells via apoptosis (Shinohara et al., 1997). In our pilot study, aimed to analysis of dying precursor cells in the RMS following irradiation, evidence for apoptosis was obtained by the use of Fluoro-Jade B. Recently, this fluorescent dye has been confirmed to label cells undergoing apoptosis under physiological conditions in the RMS of neonatal rats (Martoncikova et al., 2003) as well as adult animals (Mitruskova et al., 2005). Our preliminary results showed that the reduction of BrdU<sup>+</sup> cells 24 h after the irradiation was accompanied by a simultaneous striking increase of apoptotic Fluoro-Jade B-positive cells. In the course of the five days following the exposure, the number of apoptotic cells significantly decreased but it was still higher than in control animals.

Several studies reported that apoptotic index peaked between 6–12 hours post-irradiation, according to the dose rate and corresponding brain region (Shinohara et al., 1997; Mizumatsu et al., 2003; Rola et al., 2004). Using the phenotypic markers, Amano et al. (2002) found that reduction in cell numbers in the SVZ was accompanied by a steep decrease in the number of neural stem or progenitor cells. In contrast, there was no evident effect of lower radiation doses (< 5 Gy) on the production of glial components, such as astrocytes and oligodendrocytes (Mizumatsu et al., 2003; Rola et al., 2004). Another example of such specificity is illustrated in the study dealing with the function of adult neurogenesis in hippocampus, where the neuronal precursors expressing double-cortin were almost completely eliminated, but the glial precursors were spared (Snyder et al., 2005). The marked ability of recovery of the neural progenitor pool after initial depletion, which was also found after fractionated irradiation, may represent the recruitment of a relatively quiescent stem cell population (Shinohara et al., 1997; Philippo et al., 2005). However, repopulation of the SVZ was impaired in a dose-dependent fashion and stem cells, surviving high doses of irradiation, were unable to regenerate the SVZ (Shinohara et al., 1997; Tada et al., 1999).

Comparison of experimental data obtained in various parts of the RMS showed that the initial decrease was similar in the whole extent of the RMS (by 50–63%), suggesting similar radiosensitivity of BrdU<sup>+</sup> cells along the migratory pathway. In later time intervals, however, the highest and most long-lasting increase was seen in the caudal part of the RMS, in the vertical arm (by 50–72% on the 5<sup>th</sup>–25<sup>th</sup> days), and the smallest and shortest increase in the rostral part of the RMS, in the horizontal arm (by 33% on the 10<sup>th</sup> day). Moreover, the secondary decrease under the corresponding control values was found only in the rostral part (by 33% on the 80<sup>th</sup> day). These findings suggest that the BrdU-labelled cells originate mainly from the SVZ and during their migration from the caudal to the rostral part of the RMS they die or cease to proliferate, or, most probably, they accumulate in the caudal parts of the RMS due to slackening of migration. The results of our investigation correspond with the findings of Smith and Luskin (1998) concerning the different cell cycle length of neuronal progenitors in the RMS. This idea, beside many other questions on what influences cell division and inhibition in the RMS, awaits further confirmation and correlated studies.

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