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C-FOS EXPRESSION AND CELL PROLIFERATION IN MICE AFTER EXPOSURE TO NANOSECOND REPETITIVELY-PULSED X-RAYS

Nanosecond repetitively-pulsed X-ray irradiation is expected to produce different effects on biological systems compared to continuous irradiation even in extremely lower cumulative doses. Present work aimed to study the effect of nanosecond repetitively-pulsed X-rays (4000 impulses per day, frequency 13 Hz during 10 days, impulse duration of 4 nsec, accelerating voltage of 300 kV, photon's energy spectra with maximum of 100 kEv, absorbed dose of 10 Gy) on neural activation in the reticular formation, hypothalamus, and motor cortex in mice. Also the levels of cell proliferation in the hypothalamus and dentate gyrus of hippocampus were estimated. Neural activation and cell proliferation in the brain structures were assessed by immunochemistry labeling with c-fos proteins and bromodeoxiuridine injected immediately after irradiation. A significant increase of neural activation in the motor cortex and hypothalamus and a significant decrease of the number of activated cells in the reticular formation after exposure to nanosecond repetitively-pulsed X-rays were identified. No significant effect on cell proliferation in the dentate gyrus of hippocampus and hypothalamus were observed.

Keywords: *nanosecond repetitively-pulsed X-rays, low doses, mice, c-fos, neuronal activation, Brdu, cell proliferation, immunochemistry*

Introduction

A negative effect of high dose (20-60 Gy) radiation on the central nervous system is well known [1]. At the same time, effects of extremely low doses (1 Gy or less) of radiation are less documented in the literature. Besides, several studies have shown the difference between the effects of pulsed [2-4] and continuous [5] X-ray irradiation on cancer, liver, and blood cells. It was also demonstrated that X-rays in low doses (1 Gy or less) applied in the pulse mode affects generation of the action potential of neurons [1].The advent of sources of nanosecond periodical pulsed irradiation [6] enables the possibility of their use as a new tool for remote control of brain activity.

Recent studies showed the significant effect of nanosecond repetitively-pulsed X-rays in low doses (0.2 and 1 Gy) on the animal activity [7, 8]. Investigations of the impact of similar irradiation regimen on neural activity in specific brain areas, such as hypothalamus, cortex and reticular formation, could clarify these behavioral and metabolic effects at the cellular level. The immediate early genes such as *c-fos*, which is one of the most popular neurobiological tools for mapping functional activity of brain [9], represents a particular interest in association with the effect of X-ray irradiation

Another known important effects of nanosecond repetitively-pulsed X-rays is their influence on cell proliferation, which was found for cancer cells [2] Over past decades, numerous studies have directly shown the ability of the adult brain to proliferate in several brain areas including the dentate gyrus of hippocampus, subventricular zone of lateral ventricles [10], and, possibly, hypothalamus [11]. Due to the ability to stop cell proliferation, X-rays irradiation is widely used for preventing or decelerating neurogenesis even in low doses, beginning from 0.5 Gy [12]. However, the effect of pulsed X-rays may be substantially different then the effect of X-rays in continuous mode.

We present the pilot study aimed to characterize the effect of nanosecond repetitively-pulsed X-rays on cell proliferation and neural activity in the mouse brain.

Methods

The study was carried out on 8 C57B1/6 male mice (m=25-30 g) bred at the Research Institute of Phfarmacology RAMS (Tomsk). For local exposure of the brain to irradiation, a mouse body (except head) was placed into leaden screen. The head was irradiated by 4000 pulses with frequency 13 Hz during 10 days. Every day a mouse was irradiated with a dose of 0.1 Gy, resulting in the total cumulative dose of 1 Gy per 10 days. As source of pulse-periodic X-rays irradiation was the proton accelerator «Sinus 150» (Russia) with the pulse duration of 4 nsec, accelerating voltage of 300 kV, and photon energy spectra with

the maximum at 100 kEv. Sham-irradiated animals were subjected the same experimental procedures as irradiated ones except for irradiation. Three hours after irradiation or sham-irradiation the animals received bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) in the dose of 200 mg/kg to evaluate neural cell proliferation [13]. In 24 hours after the last irradiation session, the animals were euthanized, and the brains were frozen in liquid nitrogen steam.

Frozen brain sections (10 μm) were obtained using cryostat HM 250 (*Thermo Scientific*, Germany). The neuronal activation [9] was evaluated by the immunohystochemical label to the early response proteins c-fos (rabbit anti-c-fos, Santa Cruz sc–52; donkey anti-rabbit IgG (H+L), conjugated with Alexa Fluor® 488, Invitrogen, A-21206). C-fos+ cells were counted in the dorsal and ventral hypothalamus, motor cortex, and reticular formation. The level of neuronal activation in the above brain areas was estimated as a percentage of activated (expressing c-fos proteins) neurons relative to the total neuron count in the area of 500×500 μm. The number of newborn cells in the dentate gyrus and hypothalamus was evaluated by immunohystochemical label to BrdU (mouse anti-BrdU, Santa Cruz sc-32323; donkey anti-mouse IgG (H+L), conjugated with Alexa Fluor® 594, Jackson Laboratory 715-585-150). The microphotographs of brain sections were analyzed using the *Axio Imager Z2* (Carl Zeiss, Germany) fluorescent microscope and *AxioVision 4.0* (Carl Zeiss, Germany) software.

Statistical analyses were carried out using *Statistica 6.0* software (*Statsoft*, USA). Immunohistochemical data were analyzed using Mann–Whitney U test and analysis of variance (ANOVA). The effects of the group factor (sham-irradiated control and irradiated groups), the factor of anatomic structure (four locations for c-fos+ cells – dorsal and ventral hypothalamus, motor cortex, and reticular formation; two locations for BrdU+ cells – dentate gyrus and hypothalamus), and their interactions were estimated. For multiple comparisons, the Geenhouse-Gesser correction procedure was used. Post hoc analysis was performed using the Mann–Whitney U test.*P* values <0.5 were considered statistically significant.

Results

The significant main effect of irradiation on the number of c-fos expressed cells was found across all explored brain structures (F(1, 39)=9,7800, p=0.00333) (fig. 1).

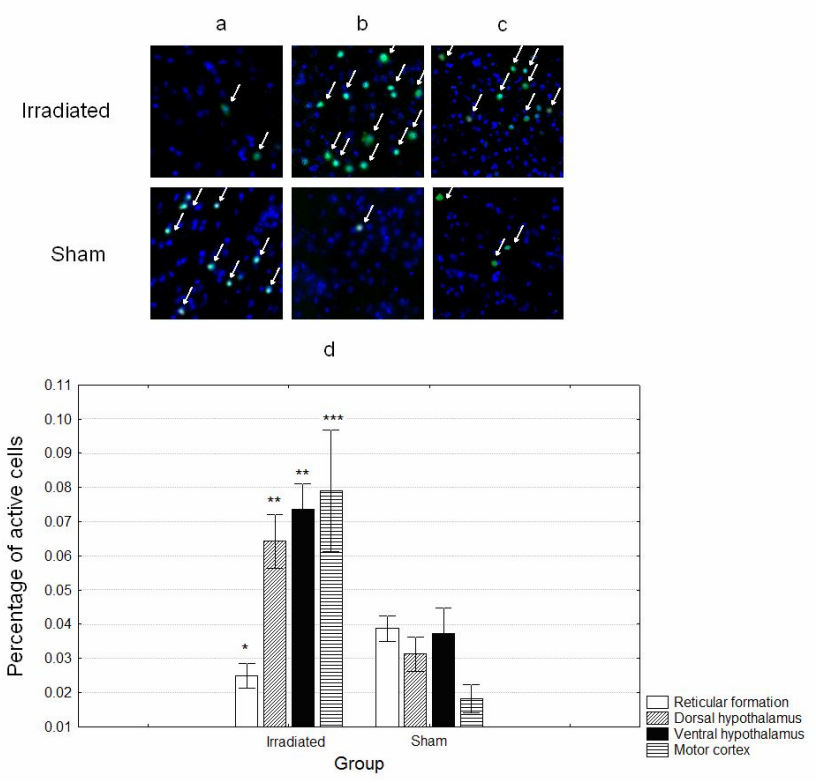

Fig. 1. Representative microphotographs of c-fos labeling in the reticular formation (a), motor cortex (b), and hypothalamus (c) of the murine brain. d - Significant effect of nanosecond repetitively-pulsed X-rays radiation in dose of 1 Gy to neuronal activation in motor cortex, hypothalamus, and reticular formation (* – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$).

The interaction of the factors of irradiation and structure also was significant ($F(3, 117)=8.0370$, $p=0.00006$). Irradiated animals had significantly higher level of c-fos expression in motor cortex and hypothalamus ($p \leq 0.001$), and lower level in reticular formation ($p < 0.05$), compared to sham-irradiated animals. The number of active cells in dorsal and ventral hypothalamus did not differ significantly.

No significant differences between cell proliferation (the number of BrdU+ cells) in irradiated and sham-irradiated animals both in dentate gyrus and hypothalamus was observed (fig. 2).

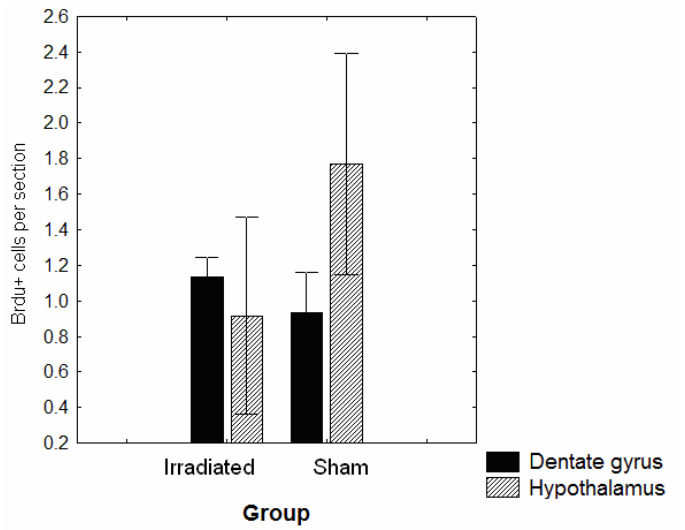

Fig. 2. No differences between cell proliferation of irradiated and sham-irradiated animals both in dentate gyrus and hypothalamus.

Discussion

The key finding of this study is that significant changes of neuronal activation occur in all investigated brain areas after exposure to nanosecond repetitively-pulsed X-rays. Except for the reticular formation, all brain structures demonstrated an increase of neural activity.

The reticular activating system (RAS) is composed of several neuronal circuits connecting the brainstem to the cortex and regulates arousal and sleep-wake cycle [14]. A key component of RAS is the reticular formation which mediates the arousal pathway bifurcated at the diencephalon into two branches that run into the thalamus and hypothalamus, respectively [14].The thalamic branch has been thought to play a critical role in regulating thalamo-cortical transmission and associated with sleep and wakefulness. A second branch runs through the lateral hypothalamus and basal forebrain, where it is augmented by additional neurons that project directly to the cerebral cortex [15]. Due to functional differences of the reticular formation nuclei, the relationships between cortical and brainstem arousal are complex and do not correlate directly. Castro-Alamancos and Oldford [16] showed that sensory-evoked responses were suppressed in the neocortex by activating the brainstem reticular formation during natural arousal. Thus, a decrease of activation in the reticular formation and it's increase in the motor cortex could cause depressive behavior rather than stimulation effects. These results are in agreement with previous studies where reduced behavior activity was revealed in the open field test after an exposure to the same irradiation regime in mice [7].

We have also found no significant effect of irradiation on cell proliferation in the dentate gyrus and hypothalamus. This result contradicts to an earlier established impact of nanosecond repetitively pulsed X-rays on proliferation of cancer cells [2]. One possible explanation of this observation is a short-term effect of irradiation in the pulsed mode, because we estimated only proliferation after irradiation but not during it. Another reason may be associated with a higher radiation sensitivity of cancer cells in the above study [2] compared to neural progenitor cells in vivo.

Conclusion

The study demonstrated the significant effect of nanosecond repetitively-pulsed X-rays in extremely low dose of 1 Gy on neuronal activation in the mouse brain. Neuronal activation in irradiated mice was

higher in the motor cortex and hypothalamus, and lower in the reticular formation compared to the control group. The effect on cell proliferation in the dentate gyrus and hypothalamus after 10 days of irradiation was not revealed.

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