JOINT INSTITUTE FOR NUCLEAR RESEARCH

Laboratory of Radiation Biology

FINAL REPORT ON THE

START PROGRAMME

“The effect of gamma radiation on the morphometry of the hippocampus cells”

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Content

Annotation

In this study, the effect of radiation exposure on the brain cells of the hippocampus of laboratory animals was conducted. The influence of gamma radiation on the morphometry of the neuron nucleus was studied on the mice in one month after irradiation. The results showed that the area of hippocampal cells in experimental animals exposed to radiation was statistically significantly larger than in the control group.

Introduction

The effect of ionizing radiation on the central nervous system (CNS) is an main task of space radiobiology, neuroradiobiology. It has been shown that damage to the central nervous system by corpuscular radiation is the leading risk factor for the health of astronauts during interplanetary flights (Grigoriev et al., 2017; Patel et al., 2017). The results of radiobiological experiments on laboratory animals demonstrate the radiation influence on the CNS in the form of impaired behavioral and cognitive functions.

People face ionizing radiation sources one way or another when working in mixed radiation fields in the nuclear industry, in medical and biological practice, in the agricultural sector, as well as in research activities. Radiobiological studies allow us to obtain data on the specifics and severity of lesions when exposed to ionizing radiation.

The clinical effects of radiation exposure depend on many variables: the type of exposure, the type of affected tissue (tissue sensitivity), the type of radiation, the depth of penetration of radiation into the body, the total absorbed dose, its power and many other factors. Therefore, understanding the radiobiological effects of ionizing radiation with different physical characteristics on the body is an essential thing to use in clinical practice.

Gamma radiation is a type of electromagnetic radiation that has an extremely short wavelength and is characterized by pronounced corpuscular and weakly pronounced wave properties. It refers to ionizing radiation, that is, to such radiation, the interaction of which with matter can lead to the formation of ions of different signs. Gamma radiation is used in the treatment of brain tumors as one of the most commonly used types of radiation for this treatment (Acharya et al., 2010; Meyers et al., 2004). With radiotherapy, there is always a risk of damage to healthy tissues, which, naturally, can affect the functioning of the central nervous system. It has been shown that radiation damage can alter cognitive functions, as well as cause neuroinflammation and other adverse side effects (Meyers et al., 2004; Wang et al., 2017; Greene-Schloesser et al., 2012; Yang et al., 2017; Gondi et al., 2013; Parihar et al., 2015).

In this work, the object of the study was the brain part- the hippocampus - a paired formation in the brain in the temporal lobes of both hemispheres. Its parts are connected by nerve fibers of the cerebral vault. In mammals, the hippocampus is a part of the old cortex (archicortex) and the center of the structure of the limbic system. A typical neuron consists of soma, dendrites, and one axon. Neurons can connect to each other, forming neural networks. The nucleus of a neuron is the most radiosensitive object and therefore it is the critical structure during irradiation.

The purpose of the project

The aim of the work was to study the effects of gamma-rays exposure 60Co at a dose of 2 Gy on the CNS of experimental animals in one month after irradiation.

The tasks of the study included the following points:

- mastering the standard histological technique,
- to study the methods of histological and cytological analysis,
- to study the specialized softwares to take the photos of histological slides, to prepare the image dataset of hippocampus pics and work in it,
- to study the method of morphometry in the analysis of photographic images of histological slides of the brain,
- work in programs for the analysis of photographs of histological slices of nervous tissue (ImageJ, ICY),
- studying methods of statistical data processing,
- working in programs for statistical analysis (Past, Excel).

Materials and methods

To perform this work, materials of the conducted experiment in the Laboratory of Radiation Biology were used. The study was carried out on male ICR mice at the age of seven months, the average weight was 45.7 grams. The animals were purchased from the Pushchino laboratory animal nursery. During the entire period of detention, the animals had unlimited access to water and food. The mice were divided into two groups of 10 animals by randomization: the irradiated group and the control group. The first group of animals was irradiated totally with 60Co gamma rays at a dose of 2 Gy, a dose rate of 0.505 Gy/min, an isodose of 90%, RIP = 75 cm, at the Rokus-M installation of the JINR medical and technical complex. A month after the irradiation, biological material was taken. The brain was fixed by immersion in 10% formalin (for 24 hours) and embedded into paraffin. Histological

slices with a thickness of 7 microns were prepared using a Thermo Fisher Scientific HM 340E (ZEISS) rotary microtome. They were placed on slides (Heinz Herenz, Hamburg, Germany) using a distilled water bath (GFL 1052, Germany) and then dried on a heating plate designed to dry sections of fabric (Medite OTS 40, Germany). Histological sections were stained with hematoxylin-eosin (H&E). To obtain digital images of hippocampus, a BiOptic B-200 light microscope, a 5.1MP 1/2.5” industrial digital camera with a USB2.0 Aptina color cmos sensor and the ImageView computer program were used. The images of the brain slices were taken with magnification of the objective × 40, eyepiece × 10. The images were saved in png/tiff format (2048×1536 pixels). The obtained photos of the hippocampus formed the basis of this work to study the morphometric method of analyzing neurons.

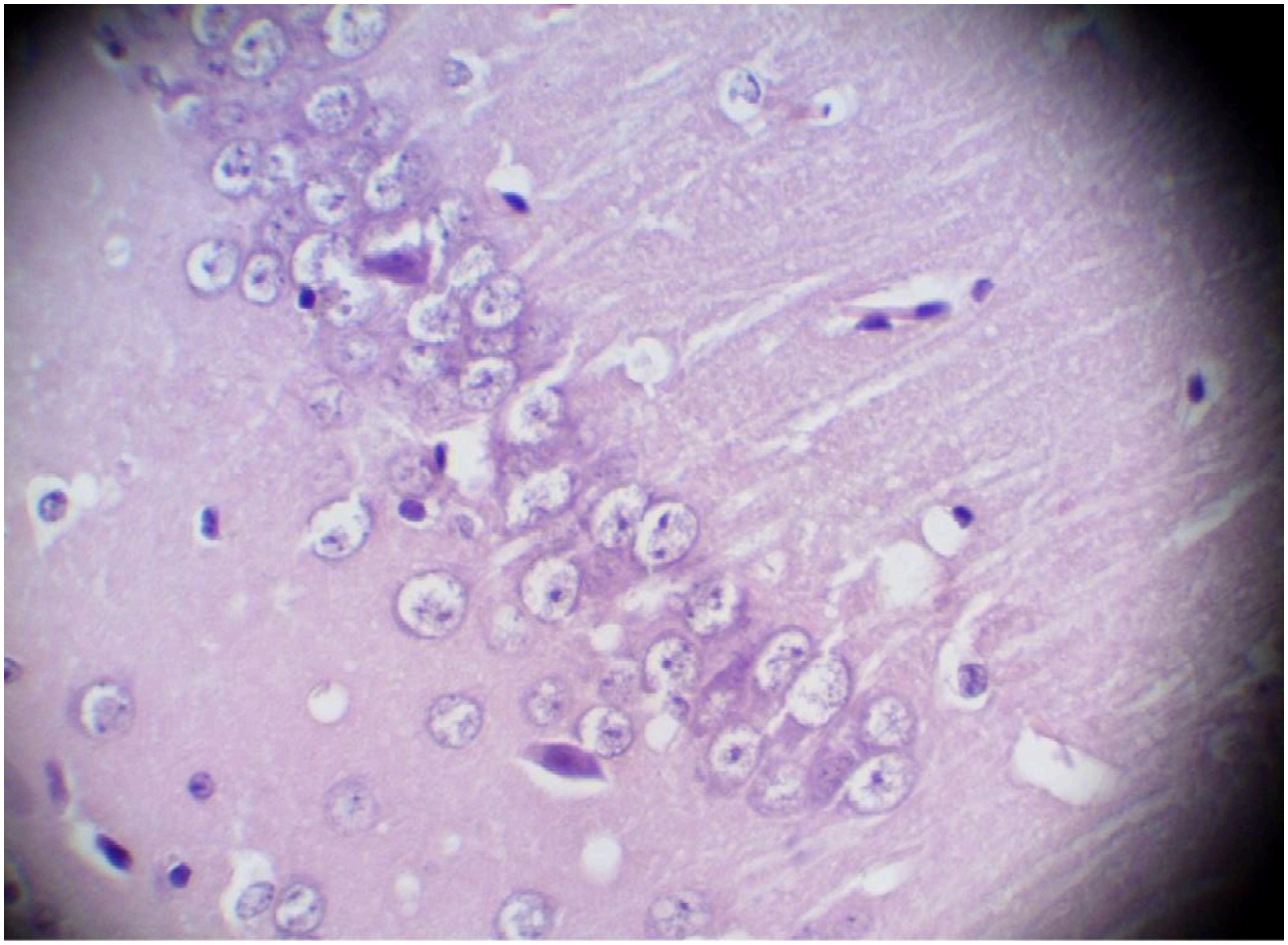


Figure 1. The photo of H&E-stained section of the hippocampus. Zoom in 40x10

Morphometric analysis

To determine the morphometric parameters of brain cells, the area of each neuron of the fields CA1-CA3/4 of the hippocampus was allocated at the obtained images using the ICY program function - ellipse selection. This figure was chosen for selection, since most normal neurons have a nucleus shape close to a circle or ellipse, which allows them to be approximated in this way for the convenience of subsequent statistical processing. Figure 2. a) shows the outline for selecting an object. Figure 2. b) demonstrates the interface of the ICY program.

a)

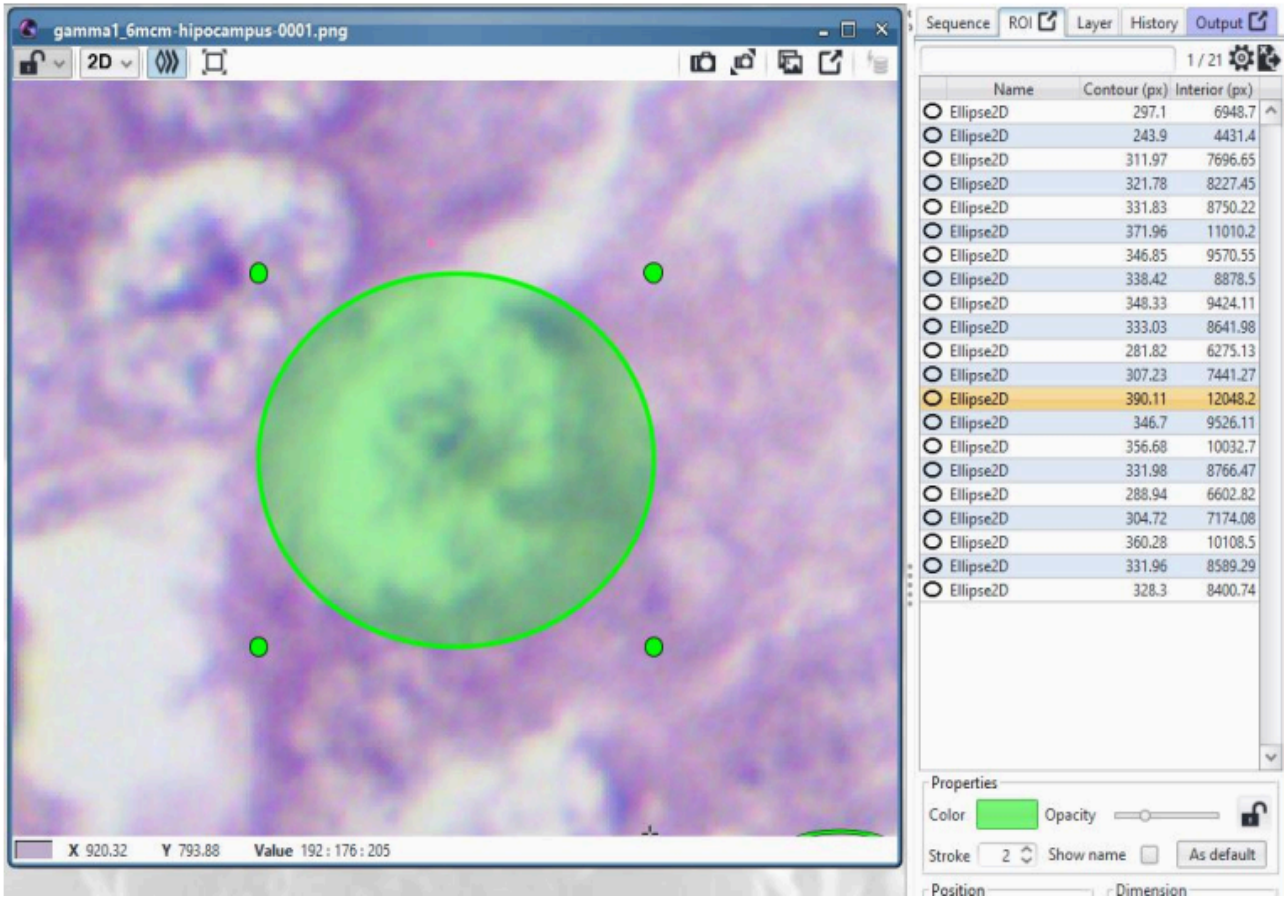


Figure 2. a) Outline selection of the object

b)

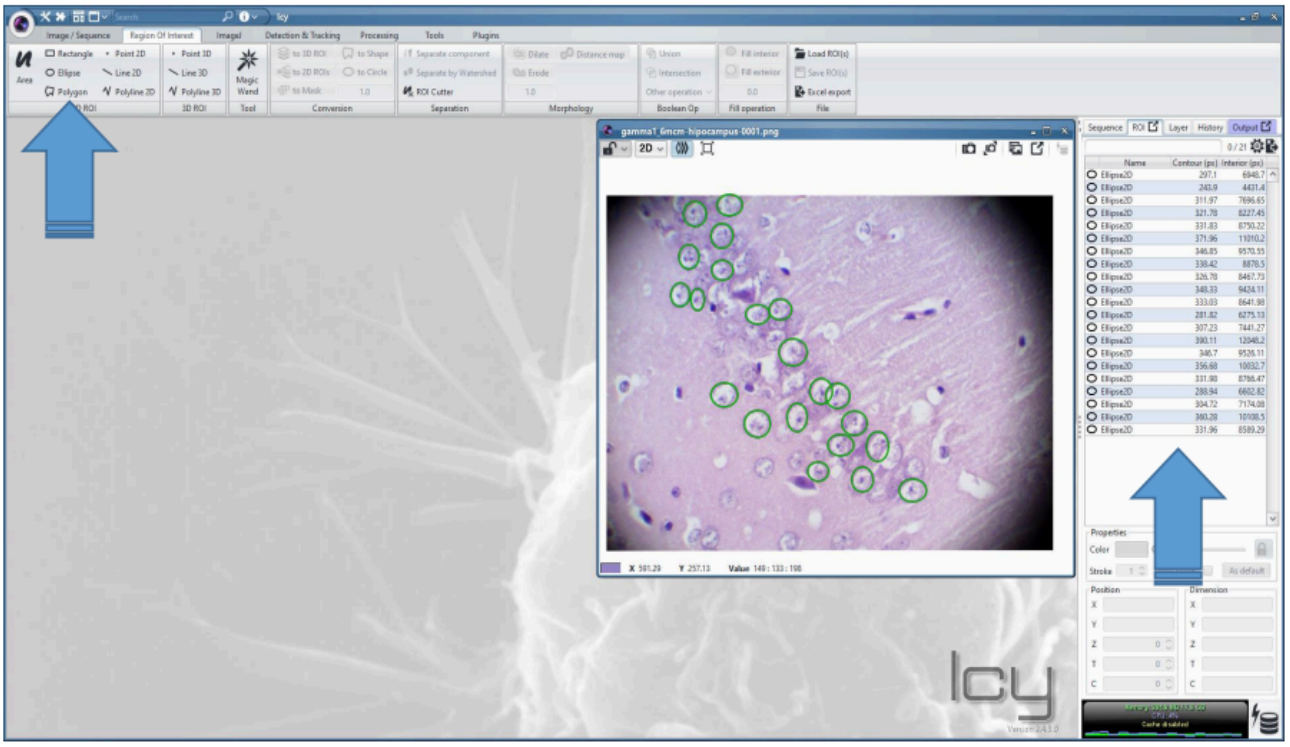


Figure 2. b) ICY interface

The obtained data as a result of the measurements are stored in special tables for subsequent statistical processing.

The shape of the cells was determined through eccentricity, a numerical characteristic of a conical section showing the degree of its deviation from the circle. The choice of eccentricity for this task is explained by the fact that it is convenient to describe the shape of cells using an ellipse (or circle) approximation, which is a special case of a conical section. Also, eccentricity is a convenient assessment of the shape of cells, as it reflects the change in the ratio of the length of the large and small axes of the cell. The eccentricity was calculated using the following formula (1).

$$E = \sqrt{1 - \frac{a^2}{b^2}} \quad (1)$$

where “a” is the length of the major axis, “b” is the length of the minor axis.

The resulting value is also added to the table. As a result, after analyzing all the images, two tables were obtained: containing information about the hippocampal

neurons of the irradiated group and the control one.

The second characteristic of the cells was the area of the ellipse used for approximation. This value was obtained in pixels. The following formula (2) was used to convert pixels to micrometers:

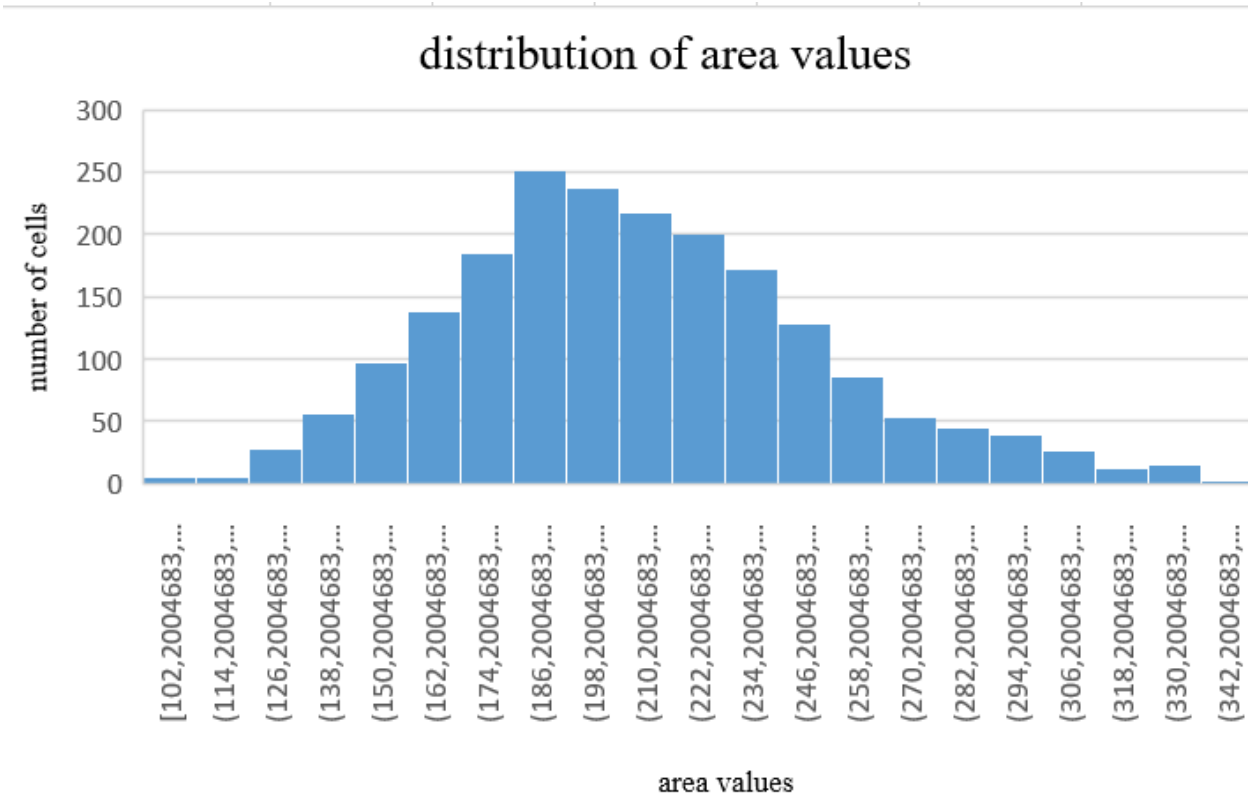
(2) $$5pix = 1\mu m$$

Statistical processing

Statistical data processing was carried out using Excel and PAST programs. The eccentricity of the ellipse and its area in pixels were used for statistical processing.

The first stage of statistical processing included the construction of a histogram of the distribution of values for these samples. Thus, the specified distributions were checked for their normality. The data are presented in Figure 3 - for a sample of the values of the control group and in Figure 4 - for the irradiated group.

a)



b)

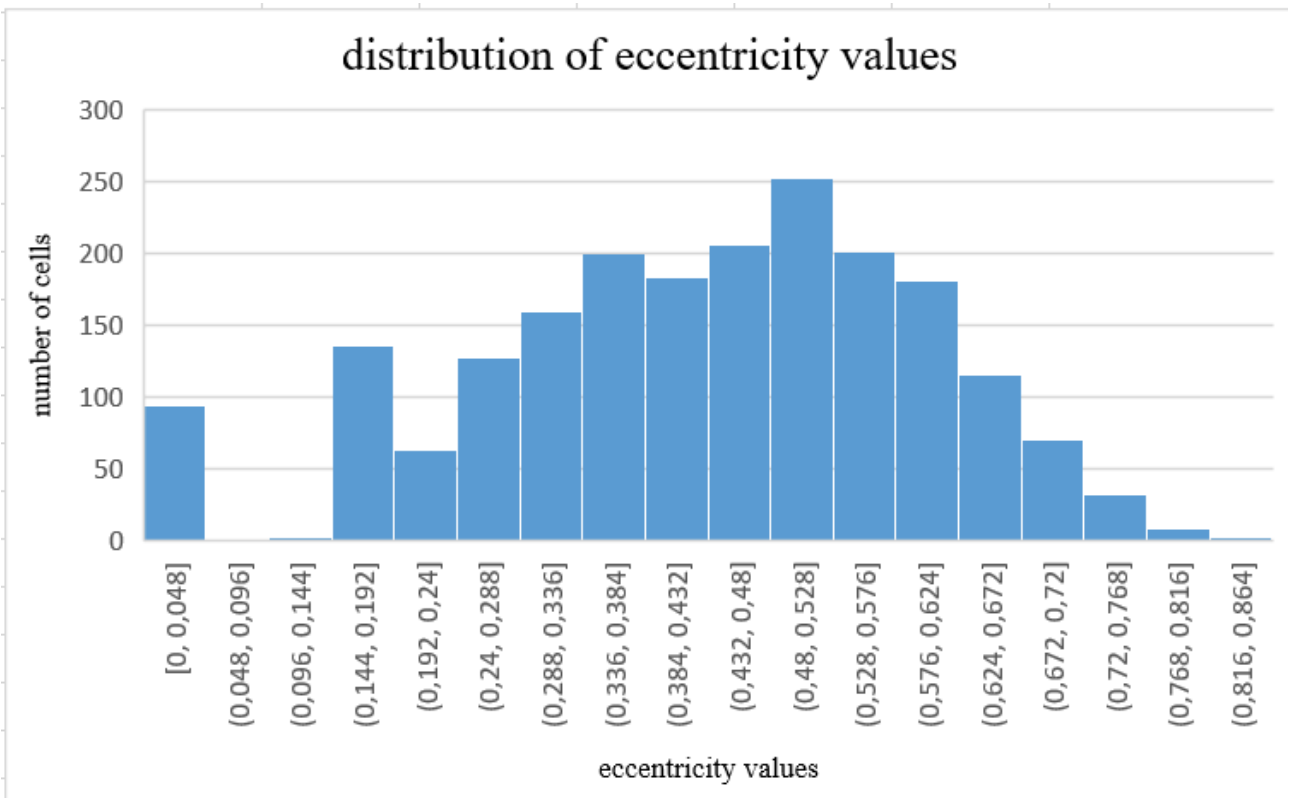
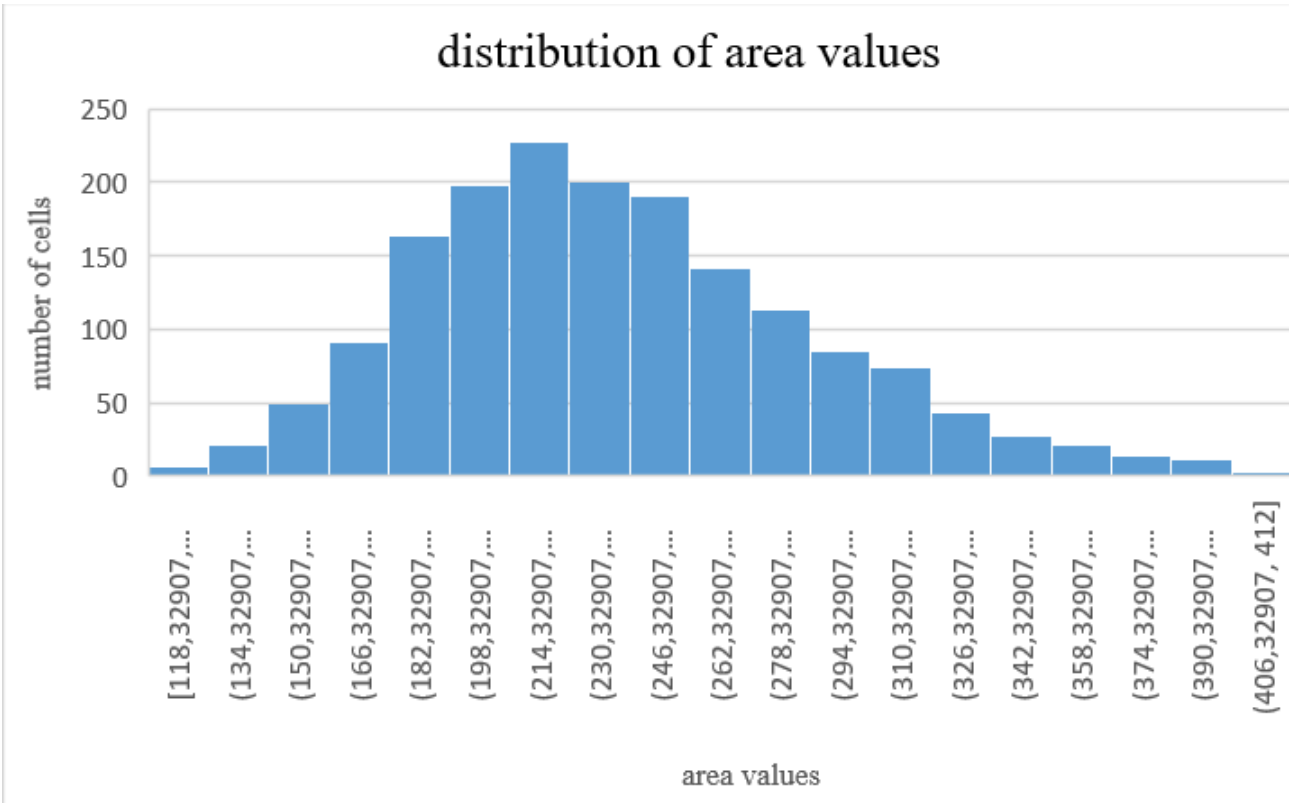


Figure 3. Distribution of values of a) area, b) eccentricity

a)



b)

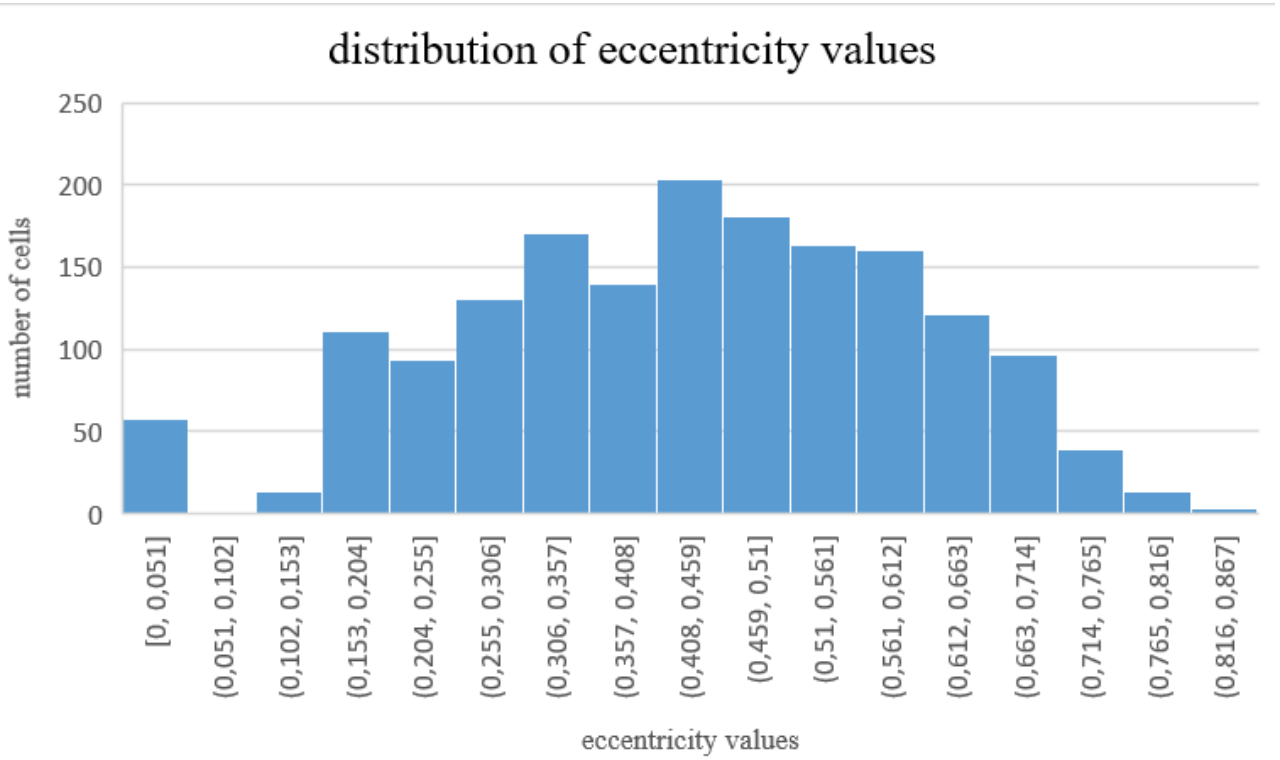


Figure 4. Distribution of values of a) area, b) eccentricity

From the obtained histograms, it becomes clear that the distributions for characteristics such as eccentricity and neuron area are normal.

Then a criterion was chosen to assess the statistical significance of the data. The data have been defined as quantitative, as they can be expressed in numbers. The samples were determined to be unrelated, since the groups are independent of each other. As it was shown earlier, all distributions are normal, therefore, it was concluded that the Student's t-criterion should be used for the reliability of differences in average values. The criterion was determined based on table 1.

Table 1. Methods of statistical analysis

Signs and distribution	**Determining the validity of differences between features**				**The relationship of signs**
	Independent groups		**Dependent groups (repeated**		
	Two groups	*More than two groups*	*One group before and after treatment*	*One group, several types of treatment*	
Quantitative, normal distribution	unpaired Student's t-tests	Analysis of variance. Then the Student's criterion is corrected Bonferroni or criterion Newman-Kales	paired Student's t-tests	The analysis of variance of repeated changes, then the criterion Student or criterion Newman-Keils for repeated measurements	Regression analysis, correlation coefficient Pearson's.
Quantitative, abnormal distribution or ordinal feature	Criteria Manna-Whitney, White's criterion, Van der Waerden, Kolgorova-Smirnova	Criteria Kruskal-Wallis, then nonparametric variants of the criteria Newman-Kales, Dannet or Dann	Criteria Wilkinson's criterion of signs	Friedman's criterion, then nonparametric variants of the criteria Nyman-kales or Danneta	The coefficient of rank correlation Spearman, Correlation coefficient Kendall
A qualitative sign	A qualitative sign Criterion of squared X, criterion Z, Fischer's exact criterion	Criterion of squared X then four-field tables with an estimate of the differences in squared X	Criteria Mcnimara	The Kohren criterion, then four-field tables with an estimate of the differences in squared X	The coefficient of conjugacy, Yula Association Coefficient

This criterion was applied using the PAST software for data on cell area (Fig. 5a) and their eccentricity (Fig. 5b).

a)

Tests for equal means

gamma		***control***	
N:	1696	**N:**	2026
Mean:	6122	**Mean:**	5391,4
95% conf.:	(6055,7 6188,3)	**95% conf.:**	(5342,5 5440,3)
Variance:	1,9395E06	**Variance:**	1,2591E06

Difference between means:	730,6
95% conf. interval (parametric):	(649,77 811,43)
95% conf. interval (bootstrap):	(648,74 814,11)

***t*:**	17,721	***p* (same mean):**	1,5966E-67
Uneq. var. *t*:	17,39	***p* (same mean):**	7,9569E-65
Monte Carlo permutation:		***p* (same mean):**	0,0001

Bootstrap N: 9999 Permutation N: 9999 Recompute

b)

Tests for equal means

excentrisetet control		***excentrisetet gamma***	
N:	2026	**N:**	1696
Mean:	0,42463	**Mean:**	0,43119
95% conf.:	(0,41712 0,43215)	**95% conf.:**	(0,4229 0,43949)
Variance:	0,029742	**Variance:**	0,030365

Difference between means:	0,0065633
95% conf. interval (parametric):	(-0,004618 0,017745)
95% conf. interval (bootstrap):	(-0,0046815 0,017683)

***t*:**	-1,1509	***p* (same mean):**	0,24987
Uneq. var. *t*:	-1,1498	***p* (same mean):**	0,2503
Monte Carlo permutation:		***p* (same mean):**	0,2476

Bootstrap N: 9999 Permutation N: 9999 Recompute

Figure 5. Statistical analysis of a) area, b) eccentricity

When using this criterion, the obtained parameter “p” determines the statistical significance of the data presented. If the p-value is less than the accepted significance level ($p \leq 0.05$), it can be assumed that the data differ significantly. As can be seen from Figure 6, for eccentricity, the p-value is 0.25, which exceeds the significance level ($p = 0.05$). The obtained result means that there are no statistically significant differences in the studied parameter. However, for area values, the p-value is $1.6 * 10^{-67}$, which is significantly less than the accepted significance level ($p \leq 0.05$). Therefore, it can be assumed that the values of the area of hippocampal neurons in animals of the irradiated group statistically significantly differ from the values of the control group.

Using functions in Excel, arithmetic averages, standard deviations and mean errors were determined for each of the parameters (area and eccentricity) in the specified groups. The data are presented in the tables: for the control group (Table 2a) and the irradiated group (Table 2b).

Table 2a. Statistical processing of the results of the control group

	eccentricity	area, µm
average	0,4246	215,6566
standard deviation	0,1725	44,8844
error of mean	0,0075	1,9551
t-test coefficient	1,9614	

Table 2b. Statistical processing of the results of the irradiated group

	eccentricity	area, µm
average	0,4246	244,8806
standard deviation	0,1725	55,7068
error of mean	0,0075	2,4268
t-test coefficient	1,9614	

Results

The dataset of photographic images of hippocampal microslides was prepared for this project. A total of 138 images were analyzed, 69 in the control group and the same number in the irradiated one. In total, 2026 cells of the control group (neurons of the fields CA1-CA3/4) and 1696 of the irradiated were analyzed, respectively. Eccentricity and area were determined for all hippocampal cells from the specified groups. During statistical processing, the data for both samples were checked for normality. Further, based on the types of parameters studied and the obtained normal distributions, a criterion for evaluating the statistical significance of eccentricity and area for two samples was chosen. The average values, standard deviations and errors of the mean for each parameter in two samples were calculated (Table 3).

Table 3. Statistical processing of the results of parameters

	control group	gamma group
area	215,66±1,96*	244,88±1,96
eccentricity	0,42±1,96	0,42±1,96

As a result, a statistically significant difference in the area of neurons was found: in irradiated animals, neurons were larger in size compared with controls (Figure 7). The average value of the area of neurons is plotted along the y axis.

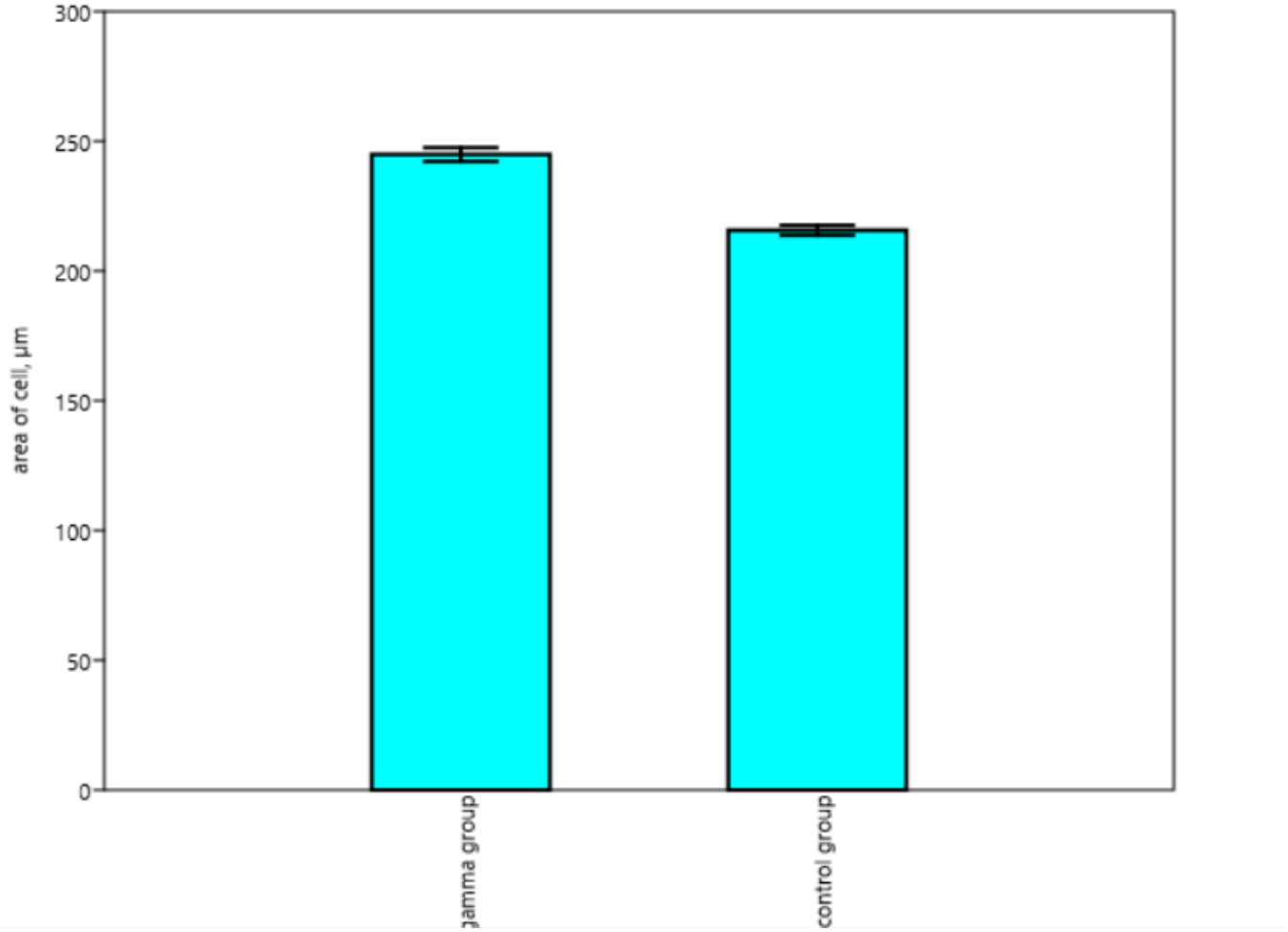


Figure 7. Results of statistical processing for the control and irradiated groups

Conclusions

The theoretical aspects of the structure of the brain of small laboratory animals, brain anatomy, hippocampus structure, structure of nerve cells, histology of nervous tissue have been studied. Practical skills such as: methods of histological and cytological analysis, cell morphometry, statistical processing of experimental results have been mastered. Experience was gained in working with histological samples, photographic images of microslides of nervous tissue, with subsequent processing of these data using software for data analysis and visualization: ImageJ, ICY, PAST and Excel. The obtained results allow us to conclude that the area of brain cells in the hippocampus of experimental animals after total gamma radiation is statistically significantly larger in comparison with the control group. Thus, the revealed differences in the size of neurons indicate the presence of a cell response to gamma-rays exposure in one month after influence.

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