JOINT INSTITUTE FOR NUCLEAR RESEARCH
Laboratory of Radiation Biology

FINAL REPORT ON THE
SUMMER STUDENT PROGRAM

Modifications of the nervous system in rats after proton irradiation

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Content

Abstract

Our research aims to identify the changes that have occurred in the behavior of rats 450 days after irradiation. Short-term memory and anxiety level are tested with T-maze and Open Field Test. Besides the macroscopic analysis of behavior our study also looks at the changes induced by radiation at the cell level by histological methods both in the brain and in the eyes. The purpose of study is to analyze and understand the changes made to nervous system by radiation to prevent or treat astronauts involved in space programs. Our research highlights the effects of radiation affecting neurons in the nervous system and changes the behavior of exposed rats.

Introduction

One of the challenges of long lasting space missions is the exposure of the astronauts to galactic cosmic rays (GCR) during the mission [1]. The component of cosmic radiation can be expressed as a percentage as follows: 86-91% protons, 8-13% helium nuclei, and 1% heavy energetic nuclei (HZE) [2]. In low-orbit missions astronauts are protected from exposure to charged particles due to the Earth's magnetic field except the areas where we meet the Van Allen belts. The mission to Mars would require between 800-1100 days of which about 500 days are on the Martian surface. These details depending on how mission is designed [3]. The equipment for space missions also provides radiation shield but an amount of the radiation passes the shield. Behind the shielding provided by Mars Science Laboratory and en cruise to Mars, the GCR dose rate was approximately 0.481 ± 0.080 mGy/day [4]. Mission Curiosity on the Martian surface indicated a GCR dose rate of 0.210 ± 0.040 mGy/day [5].The shield used in space missions provides protection on the martian surface the problem remains the radiation dose received on the way to Mars.

The body of literature examining animal behavioral outcomes in response to high-energy charged-particle radiation suggests differential effects in response to different particles and energies. Our study looks at behavioral changes in adult rats 450 days after proton irradiation. To investigate behavioral changes the T-maze method and Open Field Test were used. These tests allow the analysis of different disorders as an example the disease of hippocampus are analyzed using T-maze: rats and mice naturally tand to alternate the chosen arm in the T-maze test. This involves short-term memory or "working memory". Alternation reflects the motivation of the animal to explore its environment and locate the presence of resources as food, water etc. [6] the phenomenon is called spontaneous alternation. Our measurements showed that rats that have been irradiated have difficulty in remembering the arm of the maze or avoid choosing, this is due to the level of anxiety. To quantify the level of anxiety the study analyzes anxiety level with Open Field Test. For a correct and rigorous interpretation of the experimental data for both T-maze and Open Field Test, we statistically processed and analyzed the recorded rats behaviors and the frequency of their occurrence.

The destructive effects of ^{1}H radiation on brain cells have been observed since the very early days of particle accelerators [7]. To analyze the effect of radiation at the cellular level we performed histological measurements on the brain and eyes tissue. The microscopy measurements revealed damage caused by radiation at the cellular level.

Measurements and method

1. The irradiation process

For our study 11 SD rats were used (males, age at the time of irradiation - 12 weeks), acquired in the Pushchino Laboratory animal nursery. A month before irradiation, the animals were divided into two groups: the control group (5) and the group of irradiated animals (6). The animals were kept on a standard diet with free access to water and feed. Keeping and all animal procedures were carried out in accordance with the "International Recommendations for Biomedical Research Using Animals" of the Council of International Medical Organizations (CIOMS), Geneva 1985.

The rats of the control group underwent the same procedures (transportation, placement in containers, stress) as the animals of the experimental group, with the exception of the radiation itself.: 5 control and 6 rats irradiated with 5 Gy proton radiation.

In order to be irradiated the rats were placed in containers (Fig. 1a) and the irradiation process was performed in the position as in the Figure 1b to radiate only the head of rats. The energy of proton irradiation was 170 MeV performed with the proton beam of the phasotron of the DLNP JINR. Dose rate - 1 Gy/min. Particle flow on the path from the collimator was equal to 1, 276 × 109 particles/cm². Dosimetric calibration of the observed ionization chamber TM30013 was done with the clinical dosimeter PTW UNIDOS-E. No anesthesia was used.

a)  **b)** 

Figure 1 a) rats in plastic cages for transport and irradiation; **b)** rats in plastic fixing containers for head irradiation procedure

2. Behavioral analysis

To measure anxiety level Open Field Test [8] was used. The purpose in this test is to count the following activities in rats: rearing (Fig. 2a), climbing wall (Fig. 2b), hole dipping (Fig. 2c), short grooming, long grooming (Fig. 2d), freezing, defecation, urination and center crossing. Each rat was recorded 6 minutes, the time interval being divided into halves and analyzed separately. The data obtained from Open Field Test are presented in Table 1 and Table 2.

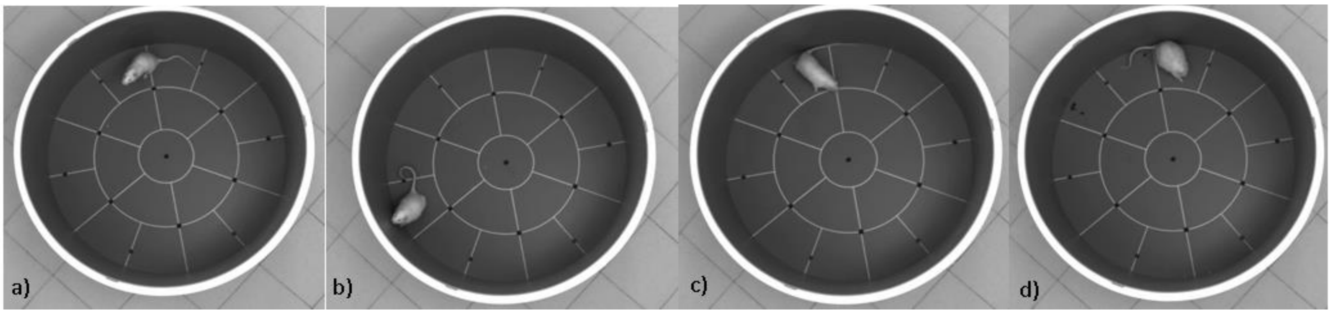

Figure 2 a) rearing; **b)** rat climbing wall; **c)** hole dippimg; **d)** grooming;

Grup	Number	Rearing	Climbing walls	Hole dipping	Short Grooming	Long Grooming	Freezing	Defecation	Urination	Center
Control	1	0	1	0	0	3	3	4	0	0
Control	2	2	2	1	0	0	0	0	0	2
Control	3	2	6	2	0	0	0	2	0	1
Control	4	0	0	0	0	0	9	0	0	0
Control	5	0	0	0	0	0	4	0	0	0
Proton_5Gy_iradiation	1	0	0	1	0	1	3	0	0	2
Proton_5Gy_iradiation	2	0	3	5	1	0	1	0	0	1
Proton_5Gy_iradiation	3	0	0	1	0	1	2	2	0	0
Proton_5Gy_iradiation	4	0	0	0	0	3	3	4	0	0
Proton_5Gy_iradiation	5	0	1	3	0	0	8	3	0	0
Proton_5Gy_iradiation	6	0	3	3	0	0	4	1	0	0

Table 1 activities in Open Field Test in the first half time , t< 3min;

Grup	Number	Rearing	Climbing walls	Hole dipping	Short Grooming	Long Grooming	Freezing	Defecation	Urination	Center
Control	1	0	5	2	0	1	9	3	0	0
Control	2	7	1	0	0	0	5	0	0	0
Control	3	0	4	1	1	0	6	0	0	0
Control	4	0	0	1	0	1	9	2	0	0
Control	5	0	0	0	0	0	7	1	0	0
Proton_5Gy_iradiation	1	0	2	2	0	0	10	1	0	0
Proton_5Gy_iradiation	2	0	1	2	0	2	5	0	0	0
Proton_5Gy_iradiation	3	2	10	1	0	1	0	1	0	0
Proton_5Gy_iradiation	4	0	0	0	0	0	6	1	0	0
Proton_5Gy_iradiation	5	0	6	2	0	1	5	0	0	0
Proton_5Gy_iradiation	6	0	1	1	0	2	5	0	1	0

Table 2 activities in Open Field Test in the second half time , t> 3min;

To see if the behavioral differences are statistically relevant the study uses the Mann-Whitney Test [9] a mathematical support that allows us to see if two independent samples have the same distribution. The analysis of the main parameters: weight of the animals, number of rearing, number of climbing walls, number of holes dipping, number of short and long grooming, number of freezing and number of defecation did not present statistically relevant differences not even in the first half of time (Figure 3) nor in the second half (Figure 4).

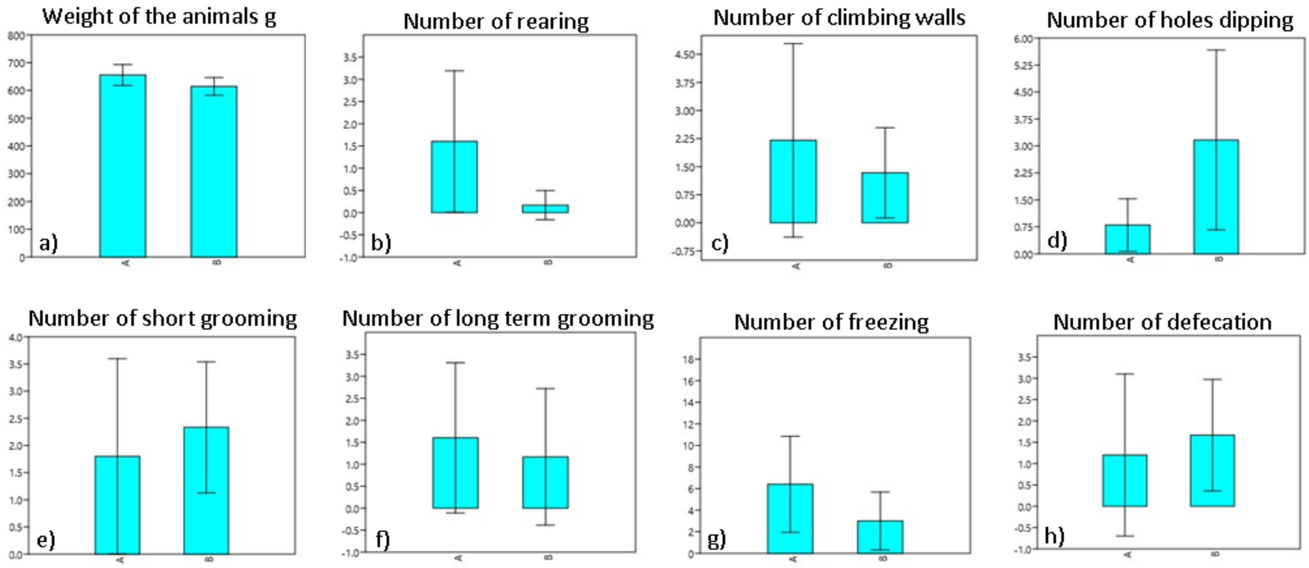

Figure 4 a) weight of animals A control group, B irradiated group, p=0.171; **b)** number of rearing behavior p=0.137; **c)** climbing walls behavior p=0.771; **d)** number of holes dipping behavior p=0.219; **e)** short grooming p=0.516; **f)** number of long term grooming p=0.490; **g)** number of freezing behavior p=0.260; **h)** number of defecation p=0.565;

As p is greater than 0.05 between the two groups there are no statistical differences at least not in the first 3 minutes of Open Field Test. The results for the last 3 minutes, after Mann-Whitney test, are shown in Figure 5.

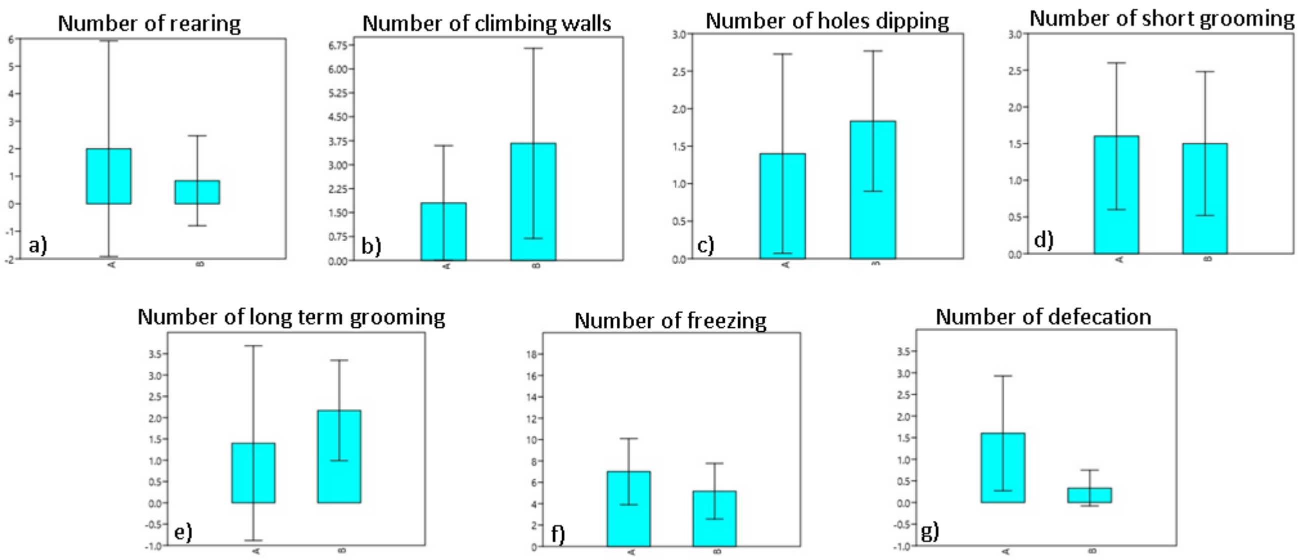

Figure 5 A control group, B irradiated group **a)** number of rearing behavior p=0.892; **b)** climbing walls behavior p=0.459; **c)** number of holes dipping behavior p=0.704; **d)** short grooming p=1.0; **e)** number of long term grooming p=0.160; **f)** number of freezing behavior p=0.519; **g)** number of defecation p=0.193;

Open Field test result reveal that there are no significant statistical differences between groups of control and irradiated animals 450 days after cranial irradiation with protons at a dose of 5 Gy. With the help of Etho Vision XT software the track of each animal in the Open Field test was recorded (Figure 6). With its help we could calculate and compare the distance traveled by rats in each group, the average speed of rats and the time spent in the center of the area (Figure 7).

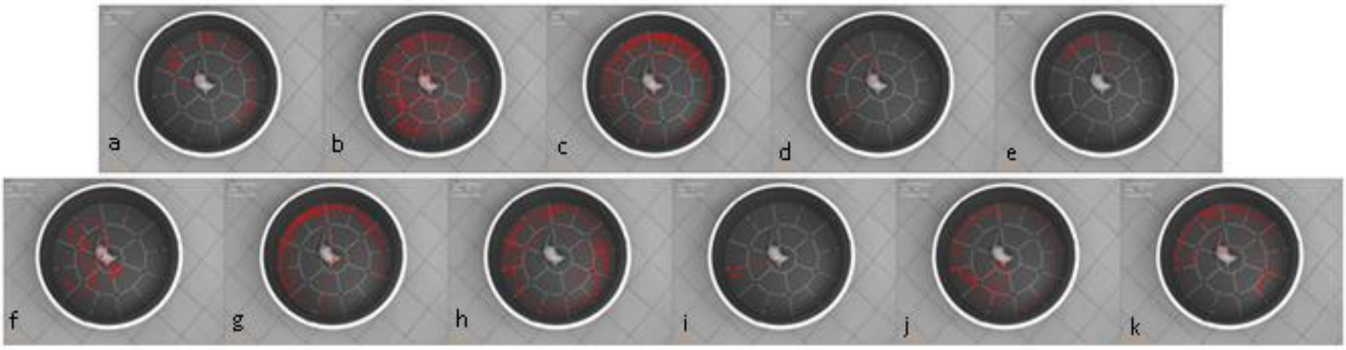

Figure 6 a-e the track of control rats group; **f-k** the track of irradiated rats group

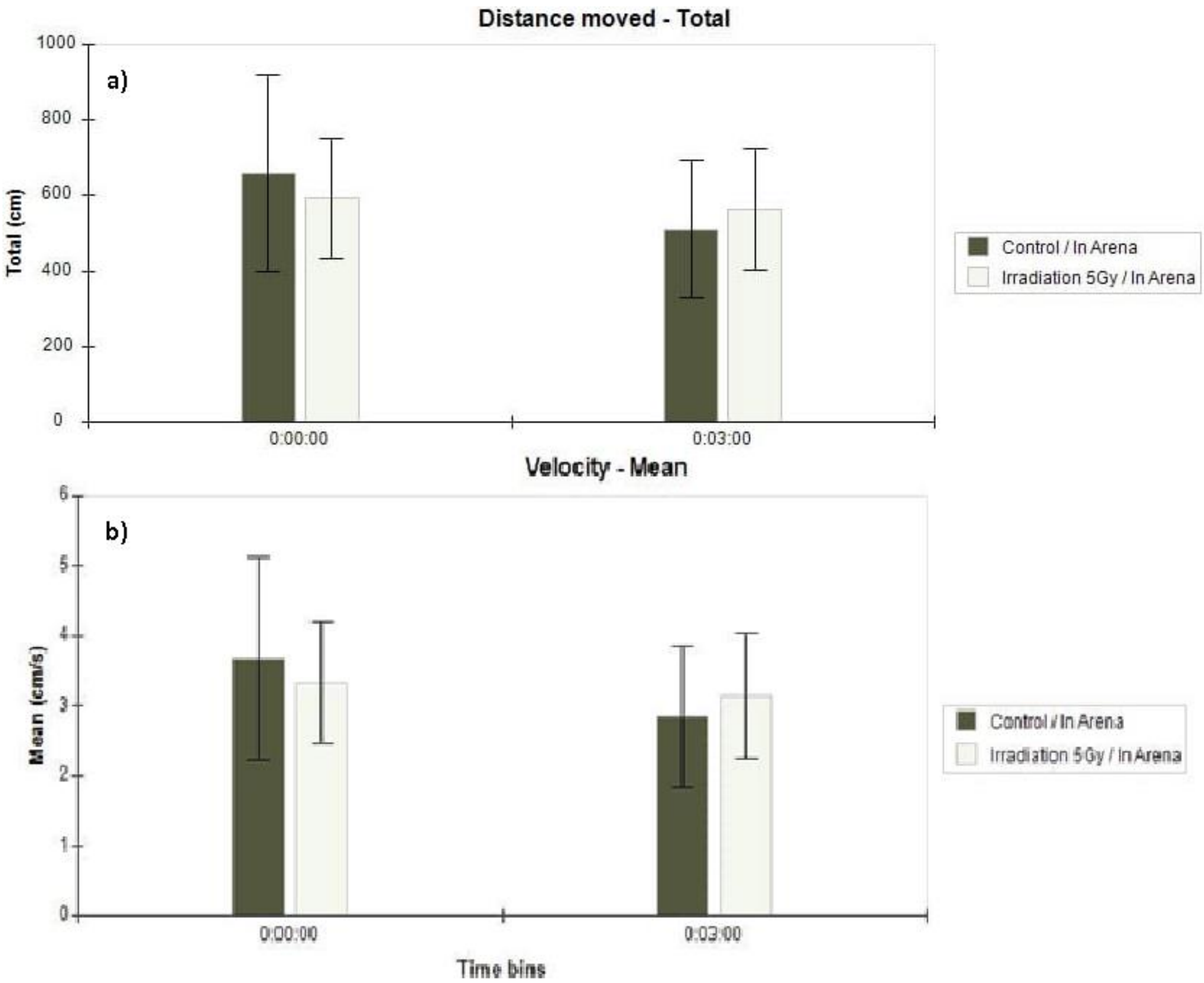

Figure 7 a) total distance traveled by the two groups in the first and last 3 minutes of Open Field test; **b)** average speed of rats groups in the two halves of the experiment;

The results obtained indicate the development of compensatory mechanisms a long time after irradiation with respect to basic behavioral reactions (level of locomotion, anxiety, research activity). When compared with younger animals, there is a tendency to a decrease in the total number of reactions everywhere. Which makes it difficult to find statistically significant differences. In the future, it is important to consider tests with long term training of animals, identifying more complex behavioral functions, and establishing correlations with morphological changes in the brain.

Short-term memory was investigated using the T-maze test. The experimental data obtained are presented in Table 3. In normal conditions a healthy rat alters the arm choises [6]. If it first chose the right one it tends to choose left but if working memory is affected rats chooses the same arm successively (right-right, left-left or the same sequence of arms right-left and agin right-left for example). Figure 8 a and b shows the behavior of rats in T-maze. Due to anxiety and age some rats miss the test because they are freeze in starting position (Figure 8 c).

Group	No.	First Day		Second Day		Third day	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Control	Rat 1	Fail	x	L	R	R	L
	Rat2	L	L	R	L	L	L
	Rat 3	Fail	x	R	L	L	R
	Rat 4	Fail	x	L	Fail	Fail	x
	Rat 5	L	Fail	Fail	x	Fail	x
Iradiated	Rat 1	Fail	x	L	R	R	R
	Rat 2	Fail	x	Fail	x	L	L
	Rat 3	Fail	x	Fail	x	Fail	x
	Rat 4	R	L	R	L	R	L
	Rat 5	L	R	R	L	L	R
	Rat 6	Fail	x	Fail	x	Fail	x

Table 3 T-maze test results for groups of rats in each of the 3 days of test;

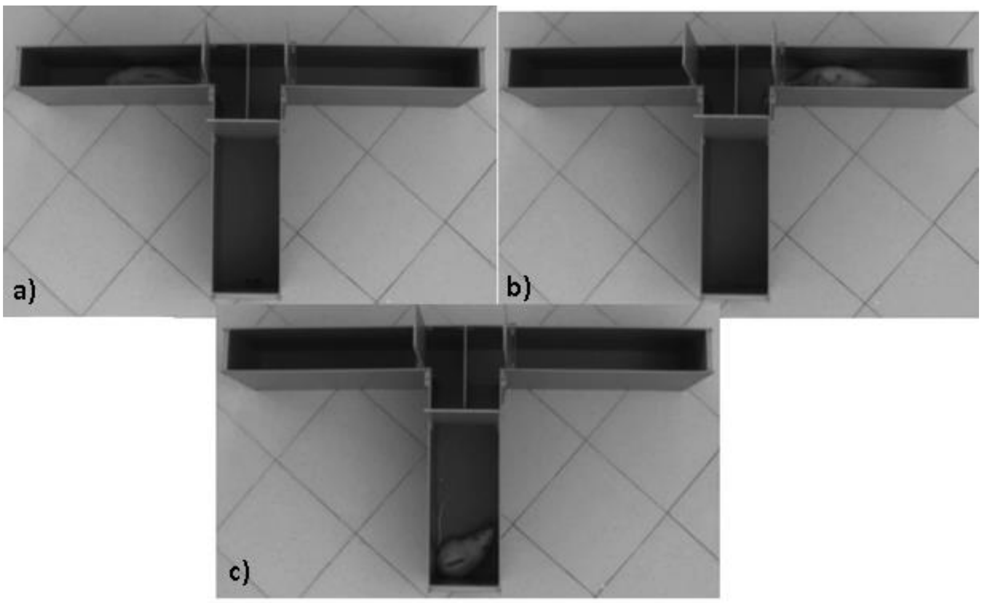

Figure 8 T-maze test **a)** chose left; **b)** chose right; **c)** failed the test because he chose nothing

Analysing the number of failures and the frequency of choice alternation there are no significant differences between the trends of the two groups. The results obtained indicate the development of compensatory mechanisms a long time after irradiation with respect to basic behavioral reactions (level of locomotion, anxiety, research activity). When compared with younger animals, there is a tendency to a decrease in the total number of reactions everywhere. Which makes it difficult to find statistically significant differences. In the future, it is important to consider tests with long term training of animals, identifying more complex behavioral functions, and establishing correlations with morphological changes in the brain.

3. Histological research

To study the effects of radiation on the nervous system of irradiated rats and to learn about the changes that take place at the cell level the study subjected the histological analysis the brains and eyes of rats from both groups. After guillotining the rats and after extracting the brains and eyes the organs were held 24h in Formaline 10% for process of fixation. To integrate organs into paraffin and to be able to perform microscope samples the following protocol was used:
1. Dist. water (2 h)
2. Ethanol 70% (1 h)
3. Ethanol 80% (2 h) (1h for eyes)
4. Ethanol 96% (12 h) (1 h for eyes)
5. Ethanol 100% (1h)
6. Chloroform 1 (30 min)
7. Chloroform 2 (30 min)
8. Chloroform + Paraffin mix (30 min)
9. Paraffin 1 (1h)
10. Paraffin 2 (1 h)

After fixing the organs in paraffin (Figure 9 a) semi-automated microtome HM 340E was used to produce the samples (Figure 9 b).

a) 

b) 

Figure 9 a) the organs fixed in paraffin; **b)** cutting the samples to the microtome;

For histological analysis of the samples we used two contrast procedures. The first: contrast method used was Hematoxylin and Eosin, one of the principal tissue stains used in histology [10]. The idea is that hematoxylin stains cell nuclei blue and eosin stains the extracellular matrix and cytoplasm pink. The protocol used for this method followed a sequence of steps:
1. Xylen 3 min;

2. Xylen 3 min;
3. 96% ethanol;
4. 80% ethanol;
5. 60% ethanol;;
6. 40% ethanol;
7. d. H_2O 2min;
8. Hematoxylin 2 min;
9. d. H_2O+ NaOH;
10. d. H_2O;
11. Eosin 1 min;
12. d. H_2O 1 min;
13. 40% ethanol;
14. 60% ethanol;
15. 80% ethanol;
16. 90% ethanol;
17. Xylen 2 min;
18. Xylen 2 min;

Images of brain tissue and retina were acquired using the microscope (Figure 10).

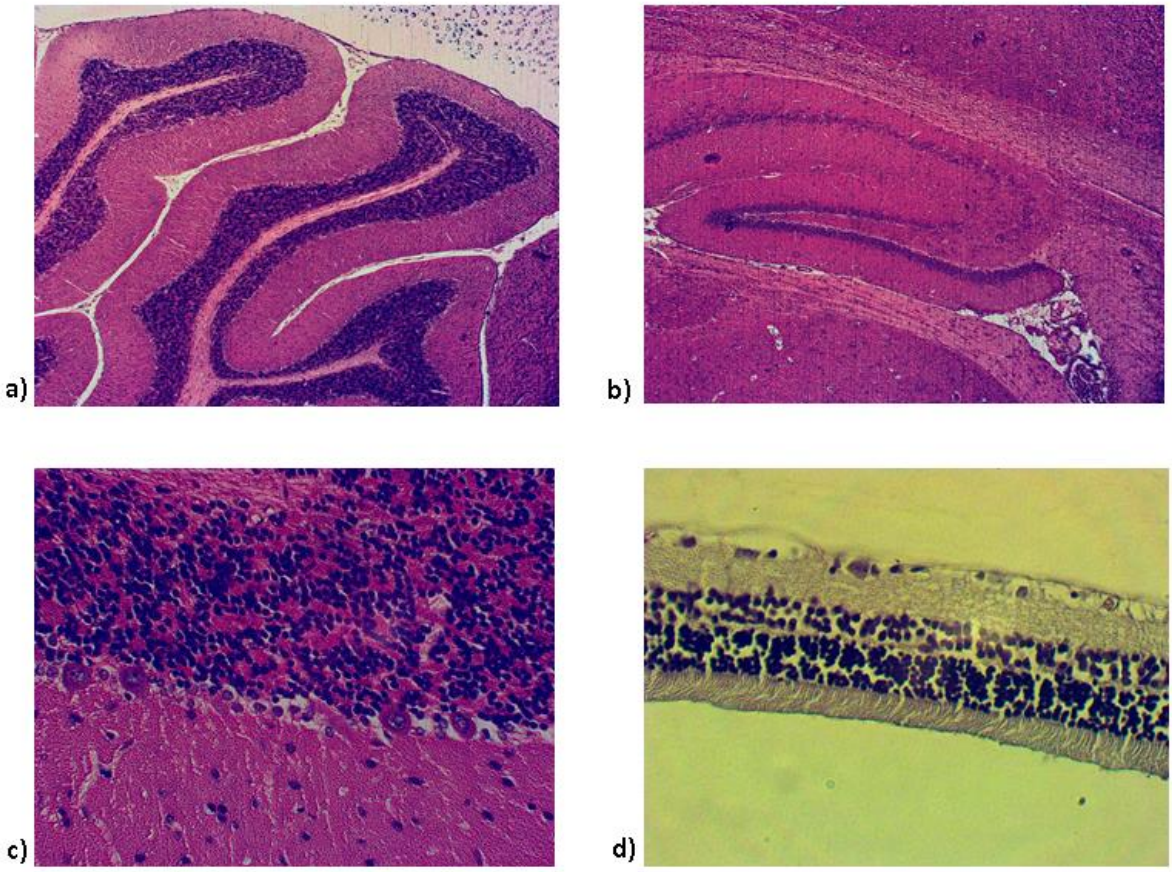

Figure 10 a-b) microscopic image at different regions of the rat brain; **c)** layer of neurons; **d)** microscopic image of rat retina;

The second histological method to analyze the degeneration of neurons due to proton exposure we used Fluoro-Jade B stain [11] on samples taken from control and irradiated rats. The protocol used has the following steps:

1. Xylene 1 (3 min)
2. Xylene 2 (3 min)
3. Xylene 3 (3 min)
4. Ethanol 80% (5min)

5. Ethanol 70% (2min)
6. dist. water (2 min)
7. 0,06% $KMnO_4$ (2min)
8. dist. water (2 min)
9. Fluoro-Jade B (0,01%) 30 min
10. dist. water 3 stage (1 min)
11. towel dry

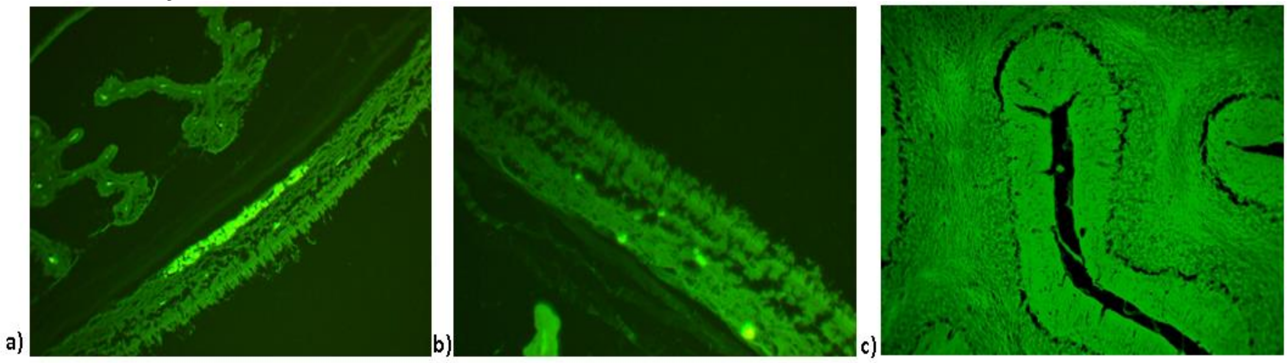

Figure 11 a-b) retina with degenerated neurons; **c)** fluorescence microscopy image of rat brain;

Figure 11 a and b shows areas with degenerate neurons. The presence of these areas may be due to proton irradiation but the presence of degenerated neurons in the retina was found in both rats groups: control and irradiated group. This suggests that a more likely cause is the age of the rats analyzed.

Conclusions

Analysis of behavioral test results indicated that there are no statistically significant differences between the control rats group and the irradiated group. This can be explained by the development of compensatory mechanisms which in long term repair the effects caused by proton irradiation. There is also a decrease in the locomotor activity, an increased level of anxiety and low interest in exploring new places in both groups of rats compared to young rats. Histological studies revealed the presence of degenerated neurons in certain areas but they cannot be correlated with the effects produced by proton exposure.

In the future it is important to consider tests with long term training of animals, identifying more complex behavioral functions and establishing correlations with morphological changes in the brain.

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References

[1] Frederico Kiffer, Marjan Boerma, Antino Allen, “Behavioral effects of space radiation: A comprehensive review of animal studies”, Life Sciences in Space Research 21, 1-21;
[2] Nelson G.A., “Space radiation and human exposures, a primer”. Radiat. Res. 185, 349-358;
[3] Drake B.G., “Human exploration of mars design reference architecture 5.0”, National Aeronautics and Space Administration 2009;
[4] Zeitlin C. et al., “Measurements of energetic particle radiation in transit to mars on the mars science laboratory”. Science 340, 1080-1084.
[5] Hassler D.M., et al., “Mars’ surface radiation environment measured with MSL’s curiosity rover”. Science 343, 1244797
[6] Robert M.J. Deacon, J. Nicholas P. Rawlins, “T-maze alternation in the rodent”, Nature Protocols 1, 2006.
[7] Tobias C. et al., 1955, University of California Radiation Lab report 3035: “Radiation Hypophysectomy with High-Energy Proton Beams”. https://digital.library.unt.edu/ark:/67531/metadc1017788/.
[8] Cruiso W.E., Sluyter Frans, Gerali R.T. “Ethogram of the mouse”. Behavioral Genetics of the Mouse, Cambridge University Press pp 17-22.
[9] Mann, Henry B, Whitney, Donald R, “On a Test of Whether one of Two Random Variables is Stochastically Larger than the Other”, Annals of Mathematical Statistics 18, 50-60;
[10] Titford M. “The long history of hematoxylin”, Biotechnic & Histochemistry 80, 73-80;
[11] Schmued LC, Hopkins KJ, “Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration” Brain Research 874, 123-130;