JOINT INSTITUTE FOR NUCLEAR RESEARCH
Laboratory of Radiation Biology

FINAL REPORT ON THE SUMMER STUDENT PROGRAM

One step forward to Mars.
A behavioral and histological study of the effects of proton irradiation on the brains and eyes of rodents.

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Content

Abstract

The purpose of this experimental study is to contribute to the understanding of the effects of proton irradiation before human exposure during the future interplanetary missions. Our research implies two groups of rodents, control group and proton irradiated group (Energy - 170 MeV, Dose - 5Gy). Their cognitive functions have been tested after 450 days from the irradiation with Open Field Test, T-maze Test, Morris Test etc. to see if there are any behavioral differences between the two groups (statistical data analysis – ANOVA, Mann-Whitney Test etc.). In order to be able to associate the changes that occurred in behavior to different structural and morphological changes in the animal's brain and eyes, we used different methods for staining (Hematoxylin and Eosin, Fluoro-Jade B) the tissue samples and afterwards we analyzed them using light and fluorescence microscopy. Among other studies, our behavioral and histological research on the effects of proton irradiation represents a starting point for finding suitable measures to reduce the danger that astronauts are subjected to, by highlighting the impact of this type of radiation on the central nervous system.

1.Introduction

The first expedition in space made by Yuri Gagarin in 1961 was the beginning of a new era of discoveries. Since then a manned mission to Mars has been the subject of scientific research, aerospace engineering and even of science fiction books and movies. In order to make this mission possible we must understand the hazards of charged-particle radiation associated with spaceflight before human exposure.

The biggest concern for astronauts' health is represented by the exposure to Galactic Cosmic Rays (GCR), which are composed of "approximately 86-91% protons, 8-13% helium nuclei and 1% heavy energetic nuclei (Z>2)" (Kiffer et al., 2019). These missions to Mars "will likely last 800-1100 days, of which approximately 500 days will be spent on the planet's surface" (Kiffer et al., 2019).

In low-earth orbit, astronauts are largely protected from exposure to charged particles due to the Earth's magnetic field, excepting the trapped particles within the Van Allen belts and those funneled into the South Atlantic anomaly. Since the current equipment can't provide 100% shielding, the dose (due to Galactic Cosmic Rays) was estimated around 25-50 cGy.

Our research aims to identify the changes that occurred in the behavior of rodents 450 days after proton irradiation in order to correlate them with the histological analysis. We used Open Field Test and T-Maze to assess their cognitive functions. The results obtained after different statistical tests indicate the development of compensatory mechanisms throughout time, after the cranial irradiation procedure. Microscopic analysis of brain and eye tissues is in progress and after the analysis of all the samples we will be able to make a comparison between the two groups. At the same time, we must consider the behavioral and morphological changes that occur due to age of the animals.

Moving forward, improvements in particle accelerators technologies will allow experiments with mixed fields in the future in order to find optimal solutions (new screening methods, choosing the most suitable astronauts etc.).

2. Methods and measurements

2.1. Study sample. Irradiation procedure

In this experiment we used 11 male rats that were 12 weeks old (3 months) at the time of irradiation, acquired in the Pushchino Laboratory for Animal Nursery. The animals have been separated with a month before irradiation in two groups: the control group consisting of 5 rats and the irradiated group consisting of 6 rats. The animals were kept on a standard diet with free access to water and food. 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, excepting the irradiation itself. The second group of rats (6) was irradiated with protons. The energy of the proton irradiation – 170 MeV was performed with the proton beam of the phasotron of the DLNP JINR. The total dose was 5Gy with a 1Gy/min dose rate. The particle flow on the path from the collimator was equal to 1,276×109 particles/cm^2. Dosimetric calibration of the observed ionization chamber TM30013 was made with the clinical dosimeter PTW UNIDOS-E. Only the animals' heads were exposed to the radiation. During the irradiation, the rodents were kept in plastic containers (without anesthesia) as in *Fig.1*.

Fig.1. The experimental arrangement used for rodent irradiation

2.2. Behavioral tests. Results

Behavioral testing is widely used and accepted as a part of irradiation effects evaluations. They assess motor responses to different stimuli and environments.

a) **Open Field Test** – A measure of the anxiety level

The Open Field Test was developed by Calvin S. Hall in 1932 in order to assay the locomotor (exploratory behavior) and emotional activity ("emotionality") of rodents (Hall and Bellachey, 1932). He used an open field (an arena with walls to avoid escape attempts) with dimensions around 2*2 m (7*7 foot) and he placed food in the center. Hall observed how animals slowly approached the center of the field with circular movements when there was food and a decreased curiosity and interest to explore the center in the absence of food. Even though this test implies many variables that are responsible for the observed behavior, both the quality and quantity of the activity can be measured. Nowadays the Open Field test is widely used in many science disciplines.

Besides being considered a 'standardized test', some parameters can vary (Belovicova et al., 2017).

The parameters of our experimental arrangement:

- Shape and design of the open field: circular with opaque walls, divided into smaller areas, including a center zone as in *Fig.2.*;
- Level of illumination: approx. 158 lx;
- Familiarity with the apparatus: single exposure after 450 days from the irradiation procedure;
- Total duration of testing: 6 minutes (this period is divided into two equal time intervals, the first 3 minutes are associated with the accommodation stage and the last 3 minutes with the habituation stage);
- Time of the day testing: afternoon (2 p.m.);
- Motivation: the animals had a standard diet with free access to water and food (they were not deprived of food/water); there was no food inside the open field area;
- Animal housing conditions before testing: the irradiated group (6 male rats) was kept in a cage and the control group (5 male rats) in another cage;
- There weren't shelters in the open field, only small holes as shown in *Fig.2.*;
- During the test we tried to eliminate all sources of stress for animals (noise, odors etc.);

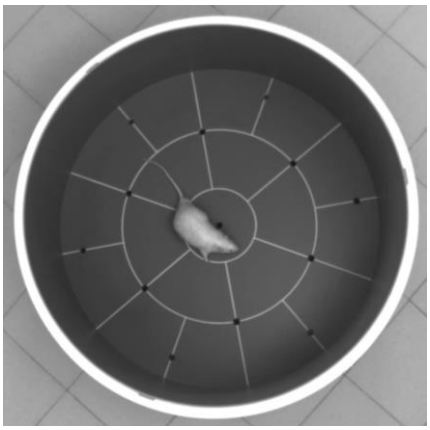

Fig.2. The experimental arrangement for Open Field Test

At the beginning of the test, the animal was placed in the central zone of the field *Fig.2.* A video camera recorded the rodent's activity for 6 minutes in order to analyze and process the information. Behavioral variables measured in the open field test were divided in two categories:

i. Emotional activity: defecation, urination, freezing, short and long grooming *(Fig.3.)*;
ii. Movement (motion): rearing *(Fig.3.)*, climbing walls *(Fig.3.)*, hole dipping and edge sniffing, crossing through the center area.

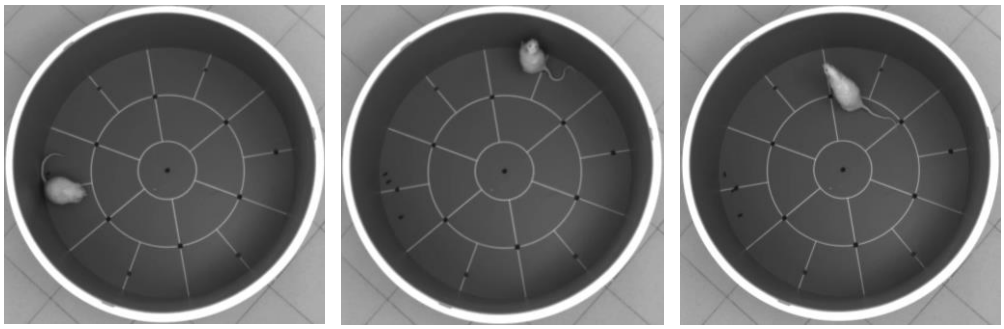

Fig.3. (from left to right) Grooming, Climbing walls, Rearing

Results

After watching the videos, all the activities listed above were counted and based on them we created 2 tables (*Table 1.* - for the first time period and *Table 2.* - for the last 3 minutes).

Table 1. Activities observed in the first stage of Open Field Test (time interval 0'-3')

Group	No.	Movement				Emotional activity				
		Rearing	Climbing walls	Hole dipping	Center Crossing	Short grooming	Long groom.	Freezing	Defecation	Urination
Control	1	-	1	-	-	-	5	7	5	-
	2	3	3	2	4	4	-	-	-	-
	3	3	7	1	1	4	1	3	1	-
	4	1	-	1	-	-	1	13	-	-
	5	-	-	-	-	1	1	9	-	-
Irradiated	1	-	-	1	2	2	1	-	-	2
	2	-	3	7	1	2	-	1	-	-
	3	-	-	3	-	5	1	-	2	-
	4	-	-	-	-	1	5	6	2	-
	5	-	2	1	-	1	-	8	3	-
	6	1	3	7	-	2	-	3	1	1

Table 2. Activities observed in the second stage of Open Field Test (time interval 3'-6')

Group	No.	Movement				Emotional activity				
		Rearing	Climbing walls	Hole dipping	Center Crossing	Short grooming	Long groom.	Freezing	Defecation	Urination
Control	1	-	4	3	-	3	6	7	3	-
	2	10	1	1	2	2	-	3	-	-
	3	-	4	3	-	1	-	4	-	-
	4	-	-	-	-	-	1	11	3	-
	5	-	-	-	-	2	-	10	2	-
Irradiated	1	-	3	2	-	3	1	8	1	-
	2	-	2	3	-	2	2	4	-	-
	3	5	10	1	-	-	2	-	1	-
	4	-	-	-	-	2	5	9	-	-
	5	-	6	3	-	2	1	4	-	-
	6	-	1	2	-	-	2	6	-	-

Considering the data from Table 1 and Table 2, the Mann-Whitney Test was performed in order to see if there are significant statistical differences in the behavior of the two groups. This is a nonparametric test used to compare two unpaired groups of data. "Mann-Whitney Test computes p values that test the null hypothesis that the two groups have the same distribution" (the resulting p value depends on the discrepancy between the mean ranks of the two groups). (GraphPad Statistics Guide) If the value of p is less or equal to 0.05, the obtained results are considered statistically significant.

In *Fig.4.* are shown the graphs obtained from the statistical data analysis from the first stage of the test (time interval 0'-3'). According to the values calculated for p, there are no significant statistical differences between the behavioral activities of the two groups of rats. The accommodation period has more emotional activity than movement in both cases (although we expected this behavior to be predominantly in the control group).

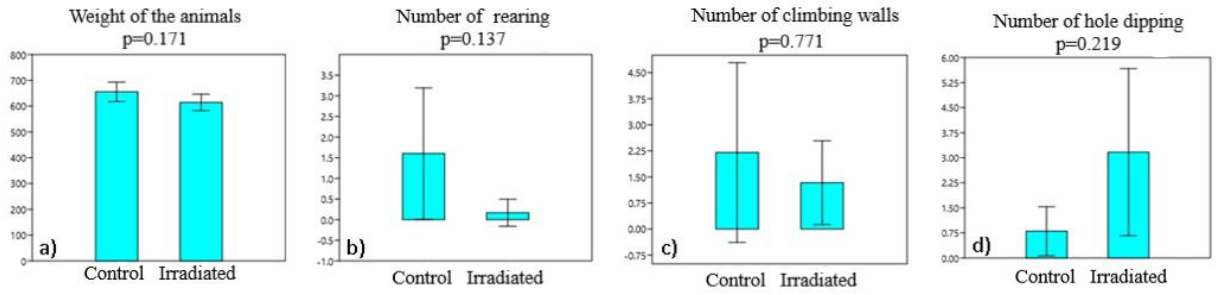

*Fig.4. Mann-Whitney Test's results for: **a)** Weight of the animals, **b)** Number of rearing, **c)** Number of climbing walls, **d)** Number of hole dipping;*

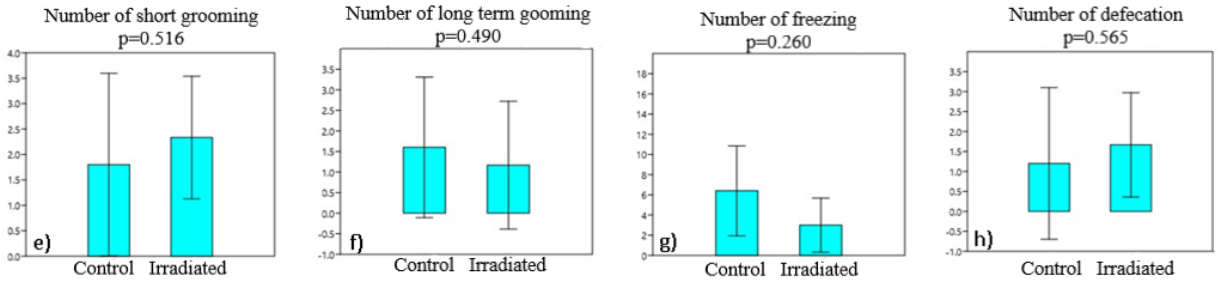

Fig.4. Mann-Whitney Test's results: ***e)*** *Number of short grooming,* ***f)*** *Number of long term grooming,* ***g)*** *Number of freezing,* ***h)*** *Number of defecation;*

The results of the Mann-Whitney Test for the second stage of the Open Field as pictured in *Fig.5* haven't revealed significant statistical differences between the behavioral activities of the control group and proton irradiated group.

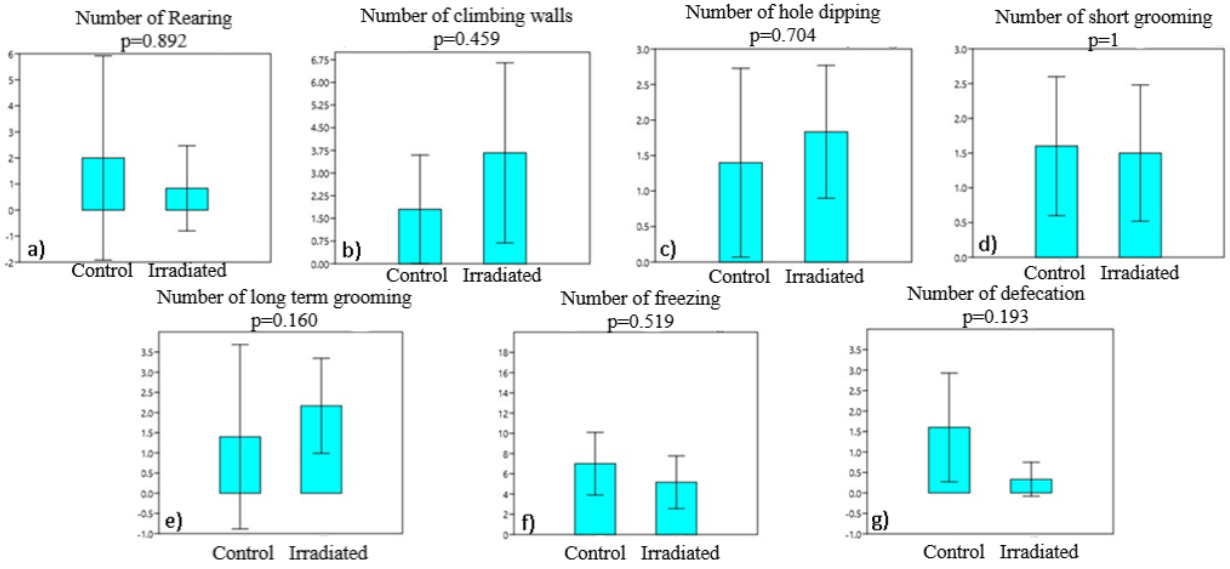

Fig.5. Mann-Whitney Test's results: ***a)*** *Number of rearing,* ***b)*** *Number of climbing walls,* ***c)*** *Number of hole dipping,* ***d)*** *Number of short grooming,* ***e)*** *Number of long term grooming,* ***f)*** *Number of freezing,* ***g****) Number of defecation;*

The reduced number of animals (11) used in this experiment, their size, their age (approx. one and a half years old), the time that has passed between the cranial irradiation procedure and the Open Field Test (450 days), but also the different personalities of the animals influenced the results and many behavioral changes were observed only in the form of trends. For example rat no. 3 from the irradiated group behaved as it was expected, in both stages he explored the field without signs of anxiety or fear, only grooming (memory refresh). The other rodents from the irradiated group had more affective activity, because above all, Open Field tests the animal's stress level.

The EthoVision XT software was used to track all the movements made by rodents inside the Open Field area (*Fig.6.*). A 'heat' map was also generated for each rat.

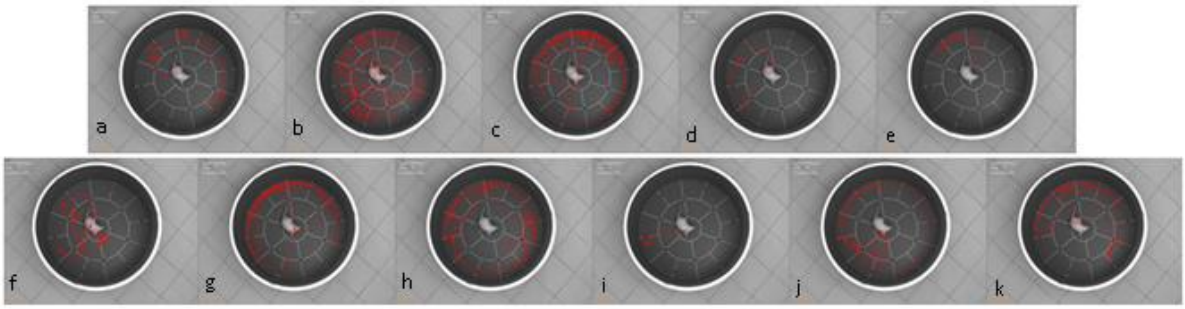

Fig.6. Recorded trace patterns for control group (a-e) and irradiated group (f-k)

Considering the measurements made by the software, the following variables were analyzed: the traveled distance and the velocity of the rats from both groups (*Fig.7.*)

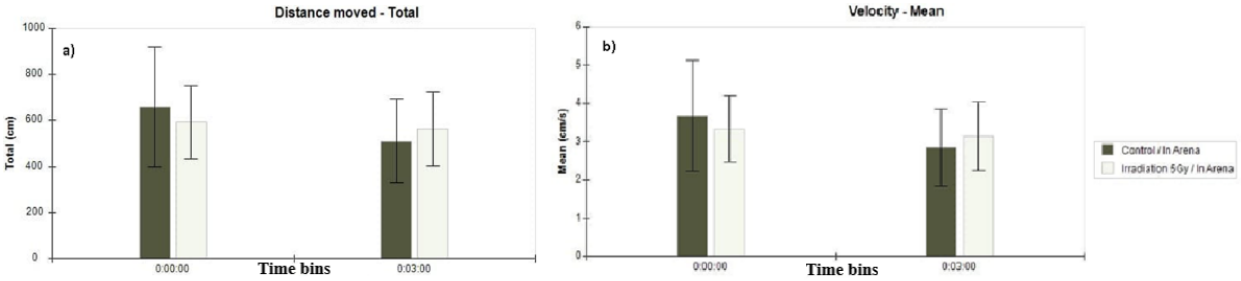

Fig.7. The statistical comparison of the: a) Total traveled distance; b) Velocity of the two groups in both stages;

Findings

The results obtained indicate the development of compensatory mechanisms throughout time, after the cranial irradiation procedure. These coping mechanisms are similar to normal behavioral reactions (locomotion, level of anxiety, research activity etc.). Compared to younger animals, our sample (due to age) has a decrease in the total number of reactions, which makes it difficult to find significant statistical differences. In the future, it is important to consider tests with long term training of animals (as Morris water maze[*]), to identify more complex behavioral functions and to establish correlations with morphological changes in the brain.

b) **T-Maze Test** – Spontaneous alternation

Spontaneous alternation is a measure of exploratory behavior and using the T-Maze test the cognitive ability of rodents can be assessed. The T-Maze is an apparatus in the form of letter T, placed horizontally as shown in *Fig.8. a-b.*

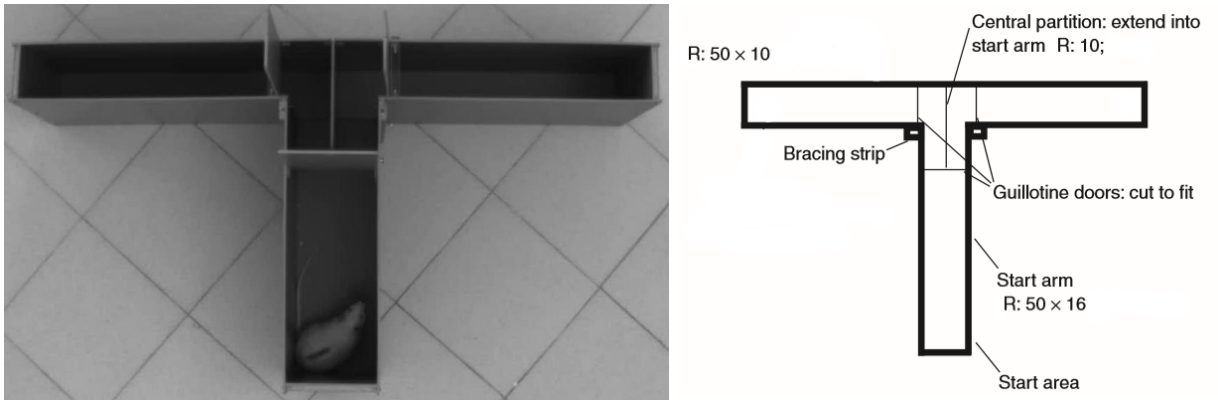

Fig.8. a) On the first T-Maze trial the rodent is placed in the stem of the T, b) T-Maze plan with construction details; Dimensions are for rats (in cm) (Deacon and Rawlins, 2006)

The parameters of our experimental arrangement:

- In *Fig.8. b)* is shown the construction plan of a T-maze for rats; walls are around 30 cm high.
- Our experiment was a free choice assay, without rewards after the trials and without food placed in the arms of the T-Maze.

[*]Morris water maze – Animals are placed in a large circular water-filled pool and are trained to find a hidden or missing platform, based on spatial cues. Additional testing can involve reversal training and testing, where animal must learn and find a new platform location. This maze assays spatial memory and cognitive flexibility, being directly correlated with the hippocampus (center of memory). Other tests that can be done and require long term training of animals are: Novel object recognition, Attentional set-shifting, Fear conditioning, Object in place, Temporal order etc. (Kiffer et al., 2019).

- In our experiment, on the first trial, the rat was placed in the starting arm (stem of the T) (*Fig.8.a*) with all the guillotine doors closed. The accommodation time on the starting area was 3 minutes.
- After 3 minutes, all the doors were opened and the rodent had another time interval of 3 minutes to make a decision and choose the right or the left arm of the maze.
- We considered that a decision was made only when the rat was with the whole body inside one of the two arms (it passed through the door of the chosen arm with his whole body). The chosen arm's door was closed and the rat had 1 minute to explore the new area.
- If the rodent chooses in the first trial an arm, the time spent in the chosen arm will be followed by a second trial respecting the steps mentioned above.
- If the rodent didn't choose an arm in 3 minutes after opening the doors the test would be considered a fail and no second trial will be performed that day.
- We recorded all trials in order to compare the time that both groups need to make a decision ("thought-speed").
- Our experiment took place in 3 days (11, 12 and 13 September) and the data obtained are in Table 3.

Results

Table 3. T-maze Test results (R – right, L – left)

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

Rodents possess a "strong tendency of alternating arm choices on successive trials" when they are placed in the T-Maze apparatus (Lalonde, 2002). There is a direct dependence between the willingness to explore new environments and the central nervous system's integrity (especially the hippocampus). The purpose of this experiment was to investigate the cognitive functions of the animals in order to compare and to discriminate the two groups involved.

If the animal does not fail the first trial, on the second one it has a choice of either repeating the same response or alternating. Analyzing the number of failures and the frequency of choice alternation (right-left or left-right) there are no significant statistical differences between the two groups. In our experiment there were different situations: some rats made quick decisions, other rats have chosen an arm in the last 20 seconds (one of them chose after 3 minutes), some animals couldn't decide which arm to choose (after they partially entered in both arms) and returned back in the starting point, some rodents wanted to choose an arm at the beginning but in the end they changed the decision, some animals were so anxious that they have 'frozen' after some attempts of exploring the area etc.. There are a lot of differences in their behavior, but unfortunately they cannot be supported by the results of the statistical tests performed. Even though only the rats from the irradiated group (due to hippocampal damage caused by the protons) were expected to make the same decision in successive trials, this behavior was observed in the control group.

Animals usually alternate at “levels significantly above chance” (Lalonde, 2002), highlighting their curiosity to discover and explore new environmental stimuli. Even though the T-Maze test isn’t as stressful as the Open Field test, spontaneous alternation is dependent on “optimal levels of anxiety”. The animal’s anxiety and emotional activity (defecation, urination, grooming and freezing) were correlated to the failed trials. The capacity of spontaneous alternation is related to spatial and short-term memory and also to the age of the subjects (decrease in spontaneous alternation rates as a function of aging). (Willig et al., 1987). The free trial T-Maze test is the most common method of evaluating the effects of lesions (produced in our case by the proton irradiation) on spontaneous alternation.

2.3. Histological studies

In order to correlate the behavioral activities observed during the performed tests (Open Field and T-Maze) with the changes that occurred in the morphology of the brain (as effects of proton irradiation), we did different histological investigations.

Samples

The rats from the both groups were beheaded with a special guillotine and after that the eyes and brains were removed. The organs (the eyes and the right hemisphere of the brain) were kept in a formalin solution (a solution of formaldehyde and methanol used especially as a preservative - fixation of the tissue) with a concentration of 10% for 24 hours (*Fig.9.*).

Fig.9. The specimen containers for the organs were filled with the formalin solution and placed on the ‘Orbital Shaker’ device

The following protocol was used in order to obtain tissue samples for the histological examination:

1. Distilled water (2 h);
2. Ethanol 70% (1 h);
3. Ethanol 80% (2 h for brain/1h for eyes);
4. Ethanol 96% (12 h for brain/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);

Fig.10. Dehydration procedure of different tissues

After dehydration with ethanol, we used chloroform because it degreases and increases tissue permeability followed by an intermediate medium (a mix between paraffin and chloroform), then the paraffin impregnation. To transfer the organs from one solution to another

one more easily and efficiently we made a small bandage bag for each organ (*Fig.10.*), including a label inside the bag in order to use only one container for each solution. There are differences in the protocol used for eyes and the one used for brains because these two tissues have different characteristics and dimensions. We fixed the organs with paraffin wax (a polycrystalline mixture of solid hydrocarbons produced during the refining of coal and mineral oils) as shown in *Fig.11.*

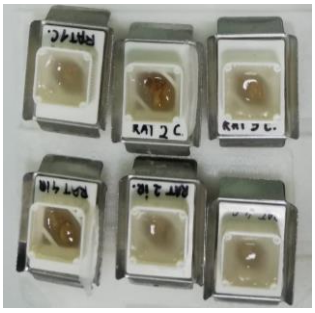

Fig.11. Paraffin fixed organs

Using the HM 340E semi-automated microtome produced by Thermo Scientific we cut slices of the paraffin fixed organs (*Fig.12. a*) at different depths and with different thickness. The slices obtained were placed in warm water for one minute *(Fig.12. b)* and then taken on glass lamellae (*Fig.12. c*). For every organ, around 20 samples were obtained.

Fig.12. a) The device used for cutting slices (HM 340E semi-automated microtone), b) The warm water device in which the slices were placed, c) The final samples

For our histological studies we used two different staining protocols. The first contrast method was represented by the ***Hematoxylin and Eosin*** stain:

1. Xylene (3 min);
2. Xylene (3 min);
3. Ethanol 96%;
4. Ethanol 80%;
5. Ethanol 60%;
6. Ethanol 40%;
7. Distilled water (2min);
8. Hematoxylin (2 min +);
9. Distilled water + NaOH;
10. Distilled water;
11. Eosin (1 min +);
12. Distilled water (1 min);
13. Ethanol 40%;
14. Ethanol 60%;
15. Ethanol 80%;

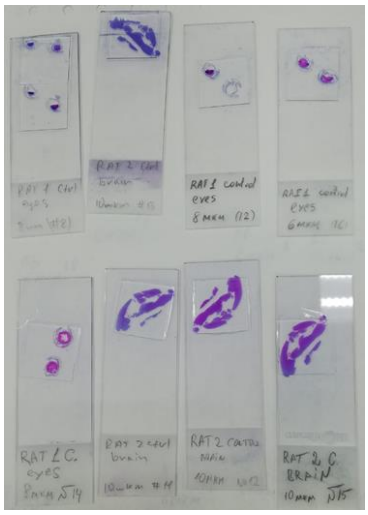

Fig.13. Hematoxylin and Eosin staining

16. Ethanol 90%;
17. Xylene (2 min);
18. Xylene (2 min);

The Hematoxylin and Eosin staining procedure follows a protocol in which:

- Xylene is used for removing the paraffin (dewaxing procedure);
- Hydration of the tissue was done by decreasing the concentration of the ethanol solutions;
- Hematoxylin is used to illustrate nuclear detail in cells (the depth of the blue coloration is related to the time spent in this solution);
- Distilled water was used to differentiate cellular components;
- Eosin distinguishes the cytoplasm (and colors it in different shades of pink depending on the connective tissue fibers) and nuclei of cells;
- Dehydration of the tissue was done by increasing the concentration of the ethanol solutions;
- Xylene was used for cleaning the samples;
- In the last step, Mount Quick glue was used for fixing the samples obtained on the lamellae (*Fig.13.*);

Using light microscopy we obtained images of the brain tissue and retina (Fig.14.).

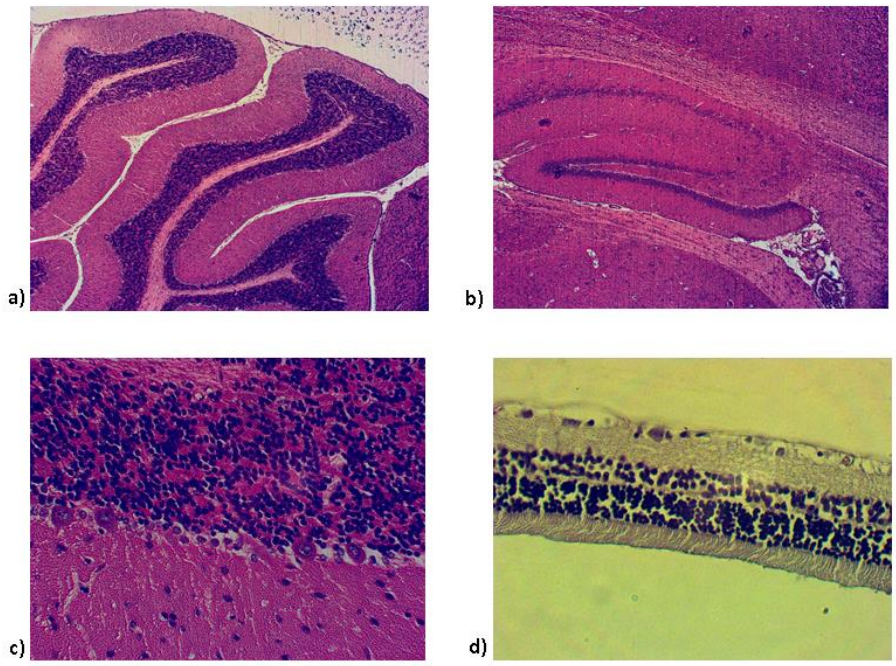

Fig.14. a), b) Hippocampus and other brain structures; c) Heterogeneous neuronal populations in the brain; d) Layers of the retina

After the analysis of all brain and eye tissues, we will be able to make a comparison between the samples obtained from the control and the irradiated group. At the same time, we must take into account the morphological changes that occur due to age of the animals.

Fluoro-Jade B stain is the second contrast method used by us in order to analyze the degeneration of neurons due to proton exposure. The following protocol was used:

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

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

The samples are kept in a dark place to preserve the fluorescence until the second day when we used Xylene - 3 min and fixed the slices with Mount Quick.

Fluoro-Jade B is an anionic fluorescein derivative with a specific affinity for degenerating neurons. This staining highlights the "fine neuronal processes including distal dendrites, axons and axon terminals" (Schmued LC, Hopkins KJ, 2000).

The images presented in Fig.15. were acquired with fluorescence microscopy. Different layers of retina have degenerating neurons as shown in Fig.15. a),b). These neurons were found in both groups and we cannot conclude that it was an effect of the proton irradiation (considering the age of the animals).

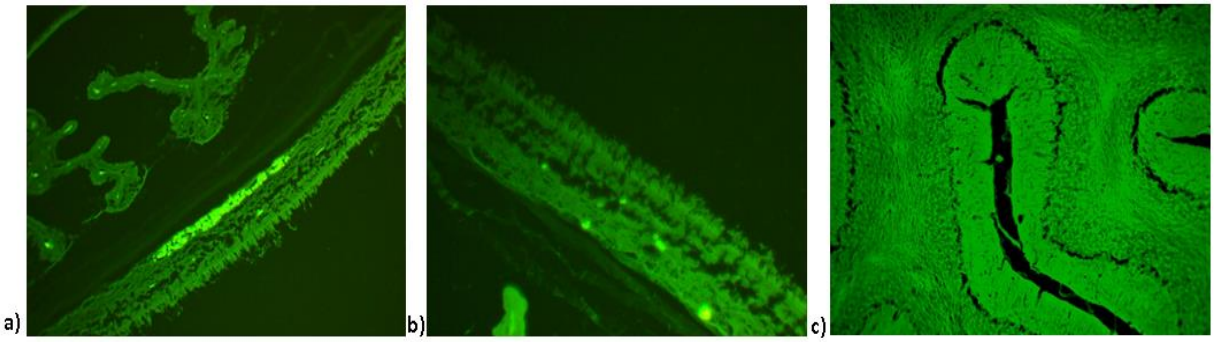

Fig.15. a),b) Degenerating neurons in different layers of the retina; c) Image of Fluoro-Jade B stained brain tissue

3. Conclusions

The results obtained from the analysis of the behavioral tests (Open Field test and T-Maze test) data haven't revealed significant statistical differences between the control group and the proton irradiated group. This fact indicates the development of compensatory mechanisms throughout time, after the cranial irradiation procedure. In long term (450 days), the effects of proton irradiation are diminished. Compared to younger animals, our sample has a decrease in the locomotor activity, low interest in exploring new environments and stimuli and the level of anxiety has increased. At this point the histological analysis has no relevant results regarding the effects of proton irradiation.

The future prospects of this study include the histological analysis of all samples and establishing correlations between the morphological changes that occurred in the brain and the observed behavioral activities.

In future experiments, it is important to consider mixed fields for irradiation (to simulate the real situation encountered by astronauts in spaceflights), tests with long term training of animals in order to identify more complex behavioral functions and to establish correlations with the morphological changes in the brain.

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