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Pilot Study of the Long-Term Effects of Radiofrequency Electromagnetic Radiation Exposure on the Mouse Brain

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Abstract: The increasing radiofrequency (RF) electromagnetic radiation pollution resulting from the development and use of technologies utilizing RF has sparked debate about the possible biological effects of said radiation. Of particular concern is the potential impact on the brain, due to the close proximity of communication devices to the head. The main aim of this study was to examine the effects of long-term exposure to RF on the brains of mice in a real-life scenario simulation compared to a laboratory setting. The animals were exposed continuously for 16 weeks to RF using a household Wi-Fi router and a laboratory device with a frequency of 2.45 GHz, and were compared to a sham-exposed group. Before and after exposure, the mice underwent behavioral tests (open-field test and Y-maze); at the end of the exposure period, the brain was harvested for histopathological analysis and assessment of DNA methylation levels. Long-term exposure of mice to 2.45 GHz RF radiation increased their locomotor activity, yet did not cause significant structural or morphological changes in their brains. Global DNA methylation was lower in exposed mice compared to sham mice. Further research is needed to understand the mechanisms behind these effects and to understand the potential effects of RF radiation on brain function.

Keywords: radiofrequency; electromagnetic radiation; behavioral tests; mice; brain; DNA methylation

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1. Introduction

The adoption of technologies that utilize electromagnetic fields has been growing rapidly, as reflected by the number of devices that use them. The most prevalent type of electromagnetic radiation is in the radiofrequency (RF) range. As the use of these technologies has increased, there has been much debate regarding the potential biological impacts of exposure to RF radiation. One particular concern related to the effects of RF is the central nervous system, given that the use of communication devices often involves close proximity or direct contact with the head.

The brain consists of various structures, including neuronal cell bodies, dendrites and axons (which can be myelinated or not and form either sparse branches or dense fiber bundles), the extracellular brain matrix, glial cells, blood vessels, and extracellular fluid [1,2]. Each of these components has distinctive electrical parameters, performs specific functions, has particular epigenetic patterns, and expresses different genes; thus, RF may potentially impact the brain in various ways, due to these diverse cellular characteristics.

RF electromagnetic radiation has been the subject of significant research due to its potential health effects, including the possibility of causing brain modifications (reviewed in [3]). There have been reports based on animal studies suggesting that exposure to RF radiation may have behavioral effects, such as changes in memory, hyperactivity, spatial learning, locomotor activity, grooming, passive avoidance, and anxiety-like behaviors. Moreover, there is some evidence these altered behavioral patterns might be determined by structural changes in the blood–brain barrier, hippocampus, cerebral cortex, glial cells, and neurotransmitter levels in different brain regions (reviewed in [4]). However, the evidence for these effects is not yet strong enough to be considered conclusive, and therefore more research is needed to fully understand the potential effects of RF on brain function and behavior.

The epigenome represents the complete set of chemical modifications of DNA and its associated proteins and regulates gene expression. Epigenetic modulation, such as DNA methylation, represents modifications in gene expression that are not caused by changes of the underlying DNA sequence. These modifications are dynamic, can be influenced by environmental stress [5–7] such as pollution [8,9] and may have long-lasting effects on gene expression, playing a role in the development of various diseases [10]. RF radiation pollution is generating concern [11], yet there is very little research on the potential for RF exposure to alter DNA methylation patterns, and even less about the brain.

Most studies that examine the biological effects of RF exposure use laboratory-based exposure systems, which may differ from environmental exposure to RF radiation in real-life settings. To our knowledge, there are no studies aiming to compare the effects of RF radiation exposure generated in a real-life scenario compared to laboratory settings. Thus, the aim of this pilot study was to evaluate behavioral changes and DNA methylation changes in the brain of mice exposed to RF radiation emitted in a real-life scenario—by a Wi-Fi router instead of a laboratory device.

2. Materials and Methods

2.1. Exposure System

The experimental setup aimed to accomplish two objectives: to determine the potential effects of long-term exposure to radiofrequency radiation on mice and to compare the biological responses to electromagnetic fields from real-life sources, specific to the ambient electromagnetic environment, to those generated in a laboratory setting. The exposure system, illustrated schematically in Figure 1, was composed of two 2.45 GHz whip antennas, each with a cylindrical reflector added to improve the directivity and focus the transmitted energy towards the exposed region, as well as a cylindrical enclosure holding the animals mounted above the antenna-reflector system. The 2.45 GHz frequency is used by some common wireless technologies and is typical for the IEEE 802.11 standard, which is the most commonly used standard for wireless communication.

The two antennas were each connected to a radiofrequency generator: one to a USRP 2900 generator (National Instruments, Austin, TX, USA) representing the laboratory source and the other to a Wi-Fi router device (WL-520GC from Asus, Taipei, Taiwan). The Wi-Fi router was connected by cable to the internet network and wirelessly to a computer accessing a webpage streaming large data packages (such as video), in order to achieve the maximum level of transmitted power. The router used the IEEE 802.11 standard, with the following parameters: 2.4 GHz frequency band, central frequency f = 2.45 GHz, OFDM modulation, and a maximum transmitted power of 71 mW. A typical transmission signal is shown in Figure 2a. However, signals transmitted by real-world sources have high variability, as they transmit the information contained within the video data package.

The laboratory source was connected through an amplifier to the antenna and configured in the GNU Radio programming environment to transmit a frequency-modulated signal with the same parameters as the other source (the Wi-Fi router), specifically the central frequency, frequency band, and power level. The key difference compared to the real-life scenario signal is that the transmission from the laboratory source does not vary its spectrum over time, as shown in Figure 2b.

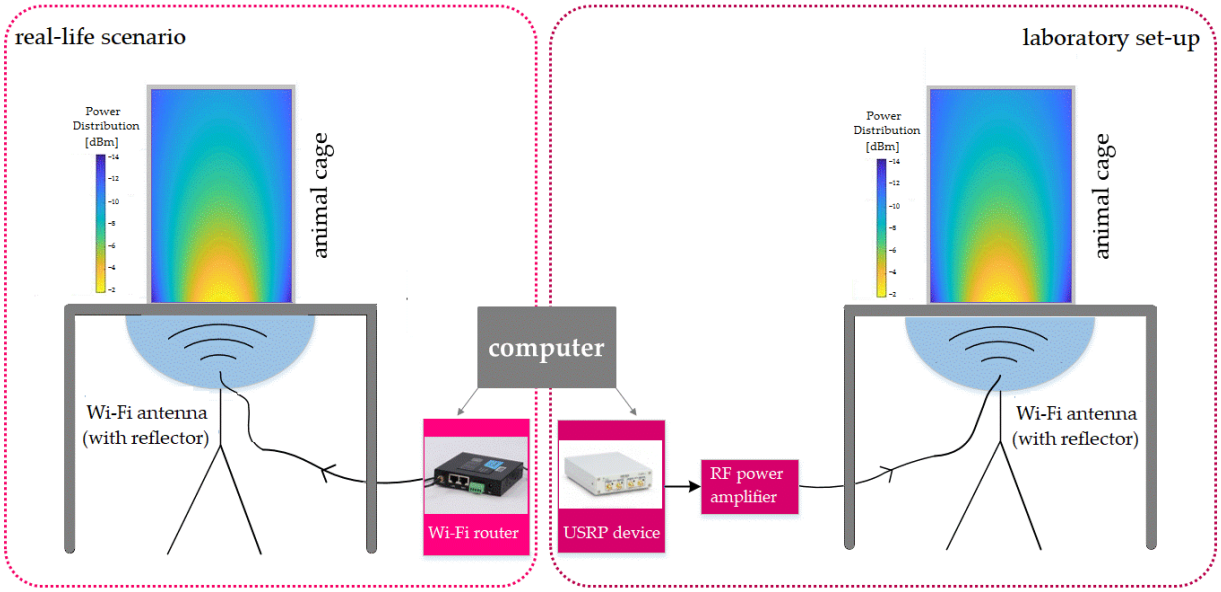


Figure 1. The experimental setup comprised two antenna systems each connected to a generator. (**a**) Wi-Fi router; (**b**) laboratory source.

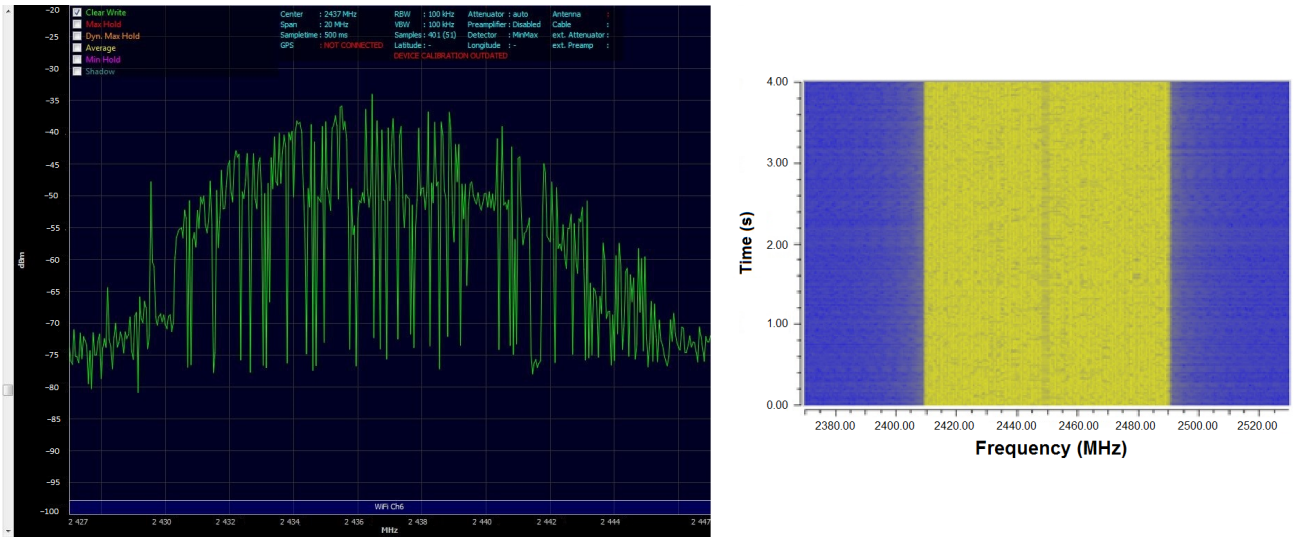


Figure 2. Spectrum of the signals transmitted by the two sources. (**a**) The Wi-Fi router transmission—one frequency sweep; (**b**) the laboratory source transmission variation in time (waterfall representation).

The specific absorption rate (SAR) was calculated by simulating a simplified layered mouse body model (Figure A2). During the exposure, the mice were able to move freely and were randomly subjected to different field intensities and different polarizations. The worst-case scenario resulted in a maximum local SAR of 17.86 mW/kg, as shown in Figure A3. A comprehensive description of the exposure systems and SAR calculation method can be found in Appendix A.

2.2. Animals

This study included a total of 30 healthy male BALB/c mice, weighing between 20 and 22 g, randomly selected from the "Cantacuzino" animal facility. The mice were provided with unlimited food pellets and tap water and were kept in a controlled environment with a 12/12 h light/dark cycle, a temperature of 20–24 °C, and a relative humidity of 55–65%.

After a period of adaptation lasting five days, the mice were randomly divided into a sham-exposed group (n = 10) and two experimental groups (n = 10 for each group) exposed to RF radiation emitted either by a household Wi-Fi router or a laboratory device (USRP). In order to simulate a normal, environmental exposure, mice within each group were housed together in cages and were able to move freely during the experiment. Each group was housed in a separate cylindrical glass cage with a diameter of 24 cm that was placed on top of an antenna system as depicted in Figure 1. The mice in the sham group were not exposed to RF radiation, while the mice in the RF radiation groups were continuously irradiated for 16 weeks. All experimental procedures involving animals were conducted in accordance with the European Guidelines for animal welfare (Directive 2010/63/EU) and approved by the Romanian National Sanitary Veterinary and Food Safety Authority (no. 19/12.08.2021). At the end of experimentation, all animals were humanely euthanized.

2.3. Behavioral Tests

All animals underwent behavioral tests at the beginning of the experiment to establish a baseline of their behavior and again at the end of the exposure period to evaluate any potential changes in behavior due to RF exposure. Locomotor activity, anxiety-related behavior, and the working memory of mice were assessed using the open-field test (OFT) and the Y-maze test. Briefly, OFT consists in placing the mice individually in one of the corners of a rectangular area (45 × 45 cm) of an open field setup (model LE800S + divider, Panlab, Barcelona, Spain) and allowing them to move freely for 10 min. The mice's movement paths were recorded by a Monochrome CAMDCBW USB camera connected to SMART V3.0 software platform (Panlab, Barcelona, Spain) video tracker and analysis software, and the information was digitally stored. The software then determined the activity parameters such as the distance traveled and the average speed and time spent in the center and border of the OFT arena. The Y-maze test setup (model LE849, Panlab, Barcelona, Spain) was used to analyze the working memory through the assessment of spontaneous alternation behavior using the same recording and analysis setup. An alternation triplet was defined as successive entries into three different arms on overlapping triplet sets. The percentage of alternation triplets was calculated as the ratio of actual to possible alternation (defined as the total number of arm entries − 2) × 100, using the following formula: % Alternation = (Number of alternations)/(Total arm entries − 2) × 100.

2.4. Tissue Preparation and DNA Isolation

The mice were sacrificed at the end of the experiment, and brain tissue was harvested. For histological analysis, one hemisphere of the brain was fixed in 10% formalin and processed for paraffin embedding. The FFPE brain-tissue samples were cut into sections of 4 µm thickness using a rotary microtome (Amos Scientific, Melbourne, Australia). The sections were deparaffinized in 3 successive xylene changes of 10 min each, rehydrated in graded ethanol series down to 70%, washed in water and stained with Mayer's hematoxylin (Bio-Optica, Milan, Italy). After treatment with lithium carbonate, the slides were differentiated with 0.5% HCl in a 70% ethanol solution and stained with an Eosin Y 1% aqueous solution (Bio-Optica, Milan, Italy). After dehydration by a series of ethanol solutions of increasing concentrations and clarification with xylene, all slides were mounted with coverslips using CV Mount (Leica Biosystems, Wetzlar, Germany) as a mounting medium. The hematoxylin-eosin (H&E) stained sections were analyzed by bright field microscopy (Zeiss LSM680) in blind by two individual researchers.

From the other hemisphere, a section spanning all brain structures weighing 20 mg was homogenized using a bead mill (SpeedMill, Analytik Jena, Thuringia, Germany) in 180 µL digestion buffer provided in the commercial kit used for DNA isolation (Invitrogen PureLink genomic DNA Mini Kit, Thermo Fisher Scientific, Waltham, MA, USA). The extraction proceeded using the manufacturer's instructions and the DNA quantity and purity were evaluated spectrophotometrically (NanoDrop™ One, Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Global DNA Methylation in the Brain

An enzyme-linked immunosorbent assay (ELISA) was used to measure the level of 5-methylcytosine in the brain using a commercial kit (Global DNA Methylation Assay Kit, 5 Methyl Cytosine Colorimetric, ab233486, Abcam, Cambridge, UK). To measure the methylated fraction of DNA, the DNA samples extracted from a section spanning all main brain structures were first adjusted to a concentration of 100 ng in 100 µL of binding buffer. A standard curve was also prepared according to the kit instructions. Both the standards and DNA samples were tested in duplicate. The capture and detection antibodies were used to detect the methylated DNA, which was then quantified using a colorimetric method by measuring the absorbance in a microplate spectrophotometer. The percentage of methylated DNA was proportional to the optical density (OD) measured. The OD values were converted to a 5 mC percentage by plotting them on the standard curve. The average 5 mC percentage per group was calculated and presented as the mean ± standard error of the mean (SEM).

Additionally, brain slides cut sagittally at 4 µm thickness from all animals were immunofluorescently stained for 5 mC. Briefly, sections were deparaffinized in 3 successive xylene changes of 10 min each, rehydrated in a graded ethanol series down to 70%, washed in PBS for 10 min, permeabilized with 0.1% Triton X-100 in 1× PBS (PBS-T) for 10 min at room temperature, and denatured for 30 min with freshly made 2 N hydrochloric acid (HCl) in 1× PBS in a 37 °C incubator. After denaturation, the sections were neutralized with 0.1 M Tris-HCl (pH 8.3) for 10 min, blocked with 5% normal goat serum in 0.1% PBS-T for 1 h at room temperature in a humidity chamber and then incubated overnight at 4 °C with the anti-5 mC primary monoclonal antibody (GT4111, Thermo Fisher Scientific, catalog no. MA5-31475) diluted 1:250 in blocking solution. The next day, the brain sections were washed with 0.1% PBS-T and then incubated with the secondary antibody (goat anti-mouse AF594, Thermo Fisher Scientific, catalog no. A11005) and diluted 1:500 in the blocking buffer. After the last wash, all slides were mounted with coverslips using antifade mounting medium (Leica Biosystems, Wetzlar, Germany). Slides were examined on a Zeiss LSM980 (Carl Zeiss, Jena, Germany) confocal microscope equipped with a 63×/1.4 plan-Apochromat oil differential interference contrast (DIC) objective lens using Zen Blue software. Cells in the isocortex of the mice were analyzed in order to quantify global DNA methylation. Photomicrographs were analyzed using ImageJ [12] and the 5 mC fluorescent signal was estimated by measuring the mean grey value for the regions of interest (ROI) comprising 50 cells from the isocortex of each animal. The average 5 mC per group was calculated and presented as the mean ± SEM.

2.6. Statistical Analysis

The statistical comparison between the three groups was analyzed using one-way ANOVA. Statistical analyses to compare the means of two groups were performed using a two-tailed *t*-test or paired sample *t*-test, as appropriate. Outliers were detected using Tukey Fence (k = 1.5) and the normality assumption was checked based on the Shapiro–Wilk test. These tests were conducted using an online calculator [13]. All data are presented as the mean ± standard error of the mean (SEM). A p value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Behavioral Tests

3.1.1. Locomotor Activity

In the OFT, the three mice groups displayed similar performances at the beginning of the experiment and did not differ in terms of mean (one-way ANOVA $p = 0.7312$). However, at the end of the experiment, the locomotor activity, quantified as total distance traveled measured in cm and average speed, differed significantly between the three groups (one-way ANOVA $p < 0.001$). Analyzed individually, both means of distance traveled and average speed of mice from groups exposed to RF emitted by the laboratory device and

the Wi-Fi router were significantly higher compared to sham-exposed mice (two-sample *t*-Test $p < 0.001$). Moreover, between the two RF-irradiated groups, the group exposed to the laboratory device exhibited higher locomotor activity compared to the group exposed to the Wi-Fi router (two-sample *t*-test $p = 0.049$). The results are presented in Figure 3.

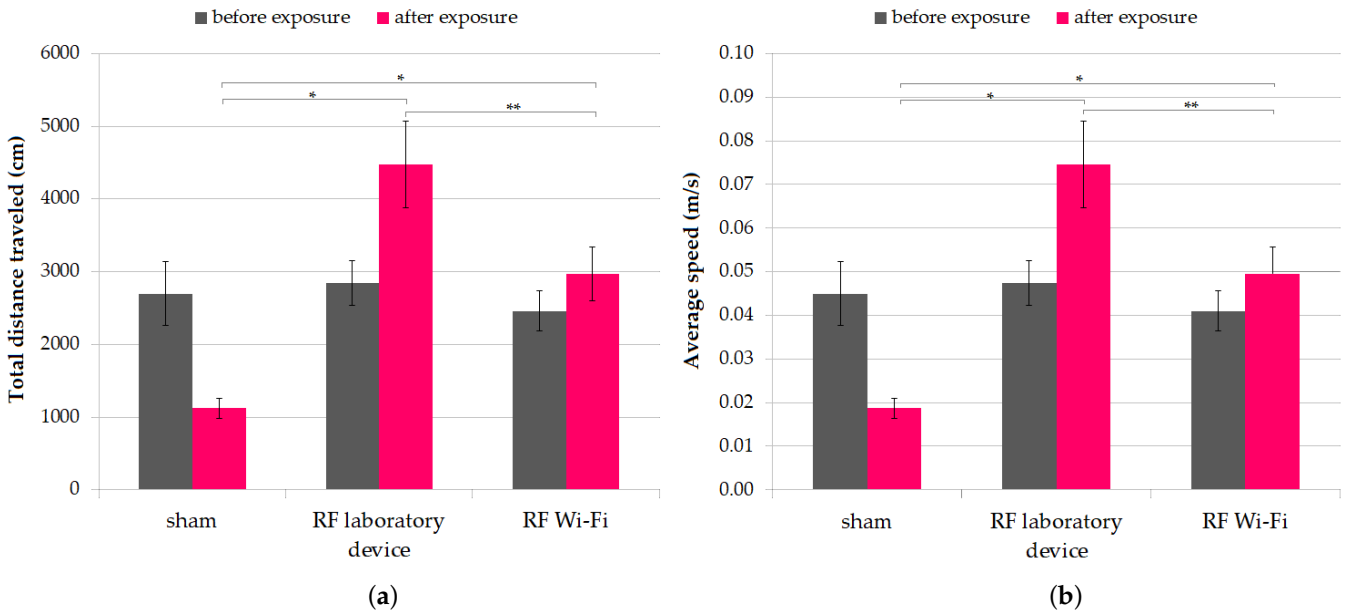


Figure 3. Locomotor activity of mice during the open field test (OFT). Data are represented as means and standard error of the mean (SEM). (**a**) total distance traveled measured in cm; (**b**) average speed. * $p < 0.001$, ** $p = 0.049$.

3.1.2. Anxiety-Related Behavior

Anxiety-related behavior was also assessed in the open-field test. In mice from all groups we observed a tendency to avoid the center of the field and to spend more time in the border. After the exposure, the percentage of time spent in the center of the OFT arena decreased slightly for mice in the Wi-Fi router RF-exposed group and in the sham group, while increasing in mice exposed to the laboratory-device-emitted RF (Figure 4). There was no significant difference in the time spent at the border vs. center between all groups, both before and after the exposure (one-way ANOVA $p > 0.05$).

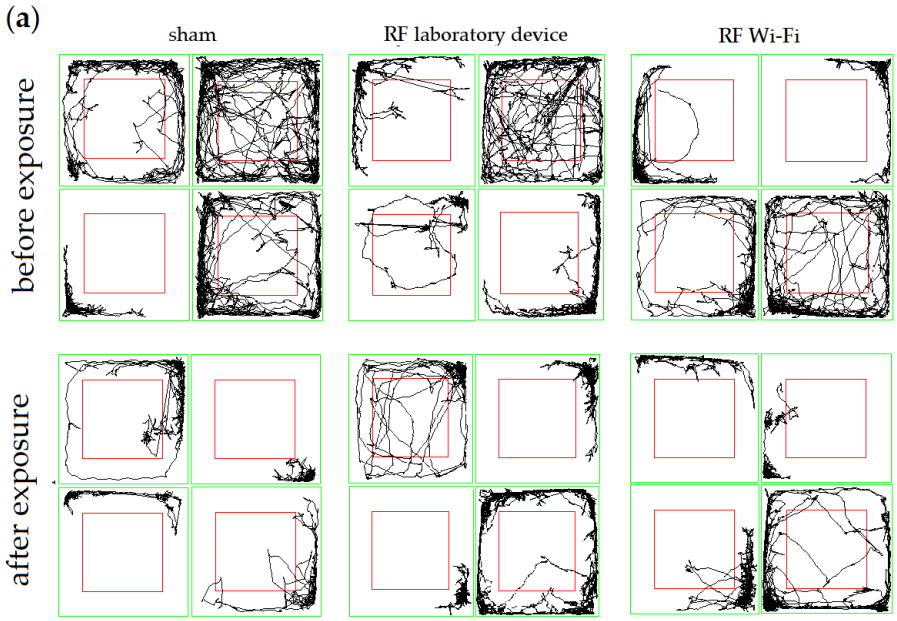
3.1.3. Working Memory

We evaluated the impact of RF radiation on working memory using the Y-maze test. After the completion of the experimental period, we observed a decrease in working memory in mice from all groups, without reaching statistical significance (paired *t*-test $p > 0.05$). Additionally, neither at the beginning nor at the end of the experiment did the working memory of mice exposed to RF radiation differ significantly between groups (Figure 5).

3.2. Histological Evaluation

Gross morphological analysis of H&E-stained sections did not reveal any significant changes in the analyzed regions, namely the cortex, hippocampus, and cerebellum. No degenerative changes, such as expansion of the ventricles or thinning of hippocampal or cortical cell layers, were identified in any of the animals. Additionally, no signs of focal or diffuse brain injuries such as contusions, lacerations or hemorrhage were observed.

Some dystrophic changes in ependymal cells, both at the level of the cerebellar cortex and especially at the level of the lateral ventricles, in the form of intracytoplasmic vacuolations, were observed in mice exposed to RF emitted by the laboratory device (Figure 6).



(b)

		sham group	RF group – laboratory device	RF group – Wi-Fi router
Before exposure	% time in the border	97.3 ±1.3	97.2±1.5	96.4±2.0
	% time in the center	2.7±1.3	2.8±1.5	3.6±2.0
After exposure	% time in the border	98.7±1.2	96.3±3.3	99.2±0.4
	% time in the center	1.3±1.2	3.7±3.3	0.8±0.4

Figure 4. Anxiety-related behavior of mice evaluated in the OFT. (**a**) Representative trajectory diagrams of mice during the open field test (OFT); the red square delimitates the center from the border area. (**b**) Time spent in the border and center during the open field test (OFT); data are represented as mean percent and standard error of the mean (SEM).

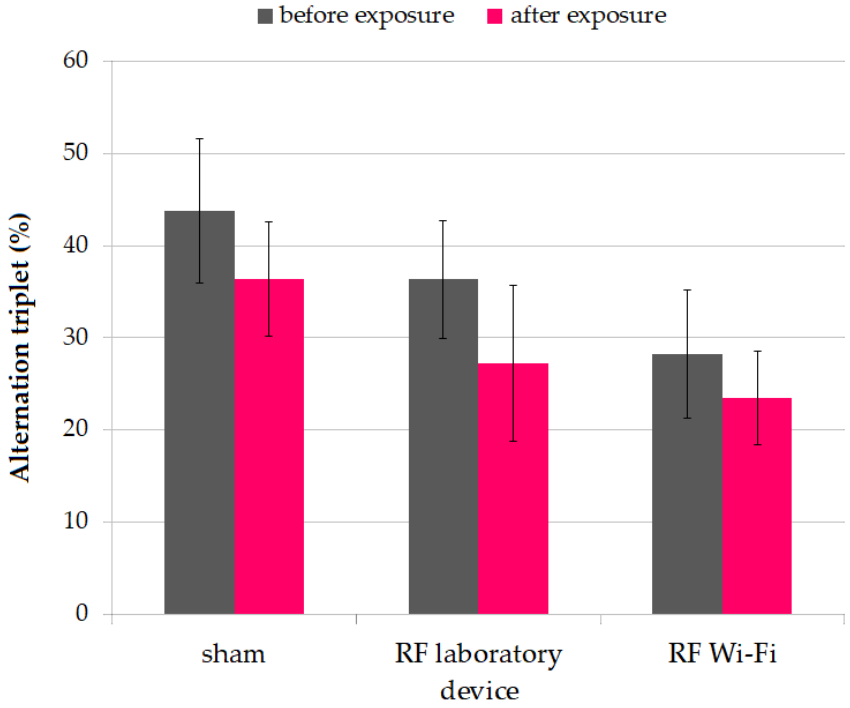


Figure 5. Alternation triplet percentage in mice during the Y-maze test. Data are represented as means and standard error of the mean (SEM).