THE EFFECTS OF 2.45 GHZ RADIO FREQUENCY ENERGY ON NEUROLOGICAL TISSUE GENES USING AN UNRESTRAINED MURINE MODEL *IN VIVO*

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

The effects that radio frequency (RF) energy has on the body is currently an inconclusive and controversial topic. This is in part due to the differences and issues that can be found in previous studies. This thesis describes a study on the effect of continuous RF energy on the genome of *in vivo* mouse brain tissue for a duration of 31 days. To address the issues found in previous studies a new standardized procedure was followed. The genome of the brain tissue was quantified using RNA-seq and then analyzed using statistical combinations and empirical p-values. Transcripts with their respective p-values were uploaded into Integrity Pathway Analysis® to determine genes associated disease and function within the brain tissue. The results from this study provided evidence that supports RF energy induces changes in the genome. Additionally, the results provided evidence of the first reported case of a potential RF-controlled genetic transistor.

ACKNOWLEDGEMENTS

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DEDICATION

I would like to dedicate this work to my parents, Bill and Sandy Stevens, who raised and supported me to be able to reach where I am today. I would also like to dedicate this work to my wife, Shaina Stevens, and unborn child who have become my inspiration.

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LIST OF ABBREVIATIONS

RF.....Radio Frequency

IEEE.....Institute of Electrical and Electronics Engineers

WHO.....World Health Organization

SAR.....Specific Absorption Rate

EM.....Electromagnetic

qPCR.....Qualitative Polymerase Chain Reaction

IACUC.....Institutional Animal Care and Use Committee

GTAC.....Genome Technology Access Center

TPM.....Transcripts Per Million

RNA.....Ribonucleic Acid

INTRODUCTION

Radio frequency (RF) energy is used in wireless technologies and the devices that incorporate wireless technologies are becoming more common in today's society. As these devices increase in use, so has the concern for the effects that RF energy could have on the human body. Organizations such as Institute of Electrical and Electronics Engineers (IEEE) and the World Health Organization (WHO) have researched some of the effects of RF energy and have developed metrics for safe levels of RF energy [21], [22]. These safe levels were developed due the heating affect that RF energy can cause to tissues due to absorption of the RF energy above certain levels of power density. Research regarding the effects of RF energy within the safe levels has also been explored. To explore the effects of RF energy at these low levels many researchers observe the effects by looking at the changes in the genome. Even through the effects of these low levels of RF energy have been researched the results from those studies has been highly controversial and it is still inconclusive what the effects of RF energy may be. Reviews of research dealing with the effects of RF energy has been performed addressing the problems that previous studies have had [4], [5], [12], [15], [18], [19]. One of the issues that previous studies, as pointed out by the reviews, is that many of these studies have a variety of differences in the methods that they use in their studies due to a lack of a standardized procedure. Parts of a standardized procedure that vary between each study are: calibration of equipment, characterization of the rooms, in-vivo vs in-vitro, and restrained vs unrestrained. Also, the technologies used to observe changes in the genome also impact the way the results from previous studies are viewed. Lastly, another problem found are the issues found in previous work's statistical analysis.

Therefore, the purpose of this proposed research would be to address some of the current deficiencies found in the current research for the effects of RF energy on the genome. To do this we propose to investigate the effects 2.45GHz of continuous RF energy exposure on mice brain tissue, in-vivo, using a new standardized procedure. After a duration of 31 days of exposure the tissue will then be harvested and the RNA extracted to examine the genome by measuring the levels of expression of the mice using next generation sequencing technologies (RNA-seq). Statistical analysis of the data will then be done using combinations and empirical p values. Lastly, data was then mapped to their respective genes using QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) and further analyzed.

BACKGROUND

Overview

Radio frequency (RF) energy can be defined as energy that is in the frequency range of 3 kHz to 300 GHz [21]. The technologies that use RF energy can be found every day in our society. These technologies include products such as Wi-Fi, Bluetooth, LTE, GSM, microwaves, and many other devices. However, RF energy is also known to cause heating of tissue above certain RF power density levels. The levels of power density that are above the safe levels have been investigated by both IEEE and WHO. They each provide recommendations for safe levels of RF exposure which are dependent on the power density and/or specific absorption rate (SAR). WHO also goes on to explain some of the side effects and potential hazards of RF energy exceeding safe levels. Even though operating above-safe-levels of RF energy can be hazardous, it also has a few positive medical applications. An example of above-safe-levels RF energy is RF ablation, where specific tissue can be treated using the heating properties of RF energy. Additionally, RF energy is used in magnetic resonance imaging (MRI), where RF energy is used in short bursts to scan and image the body.

As for levels of RF energy within safe levels, it is still being widely debated as to what the effects, if any, RF energy has on the human body. To be able to quantify the effects that RF energy may have on the body researchers can observe changes in the genome. There have been many studies performed regarding the effects of RF energy and the focus of the previous research has been relatively broad. For example, some research has been performed focusing on the regulation of heat shock genes, general only seen with RF exposure above safe levels [4], [5], [12], [15], [19]. Another example is research into RF energy inducing changes in genes associated with cancer [4], [5], [12], [15], [19]. Additionally, there has also been research into RF energy and its influence on genes commonly connected to Alzheimer's [5], [15]. However,

the results from many of the previous studies are considered inconclusive and the effect of RF energy is still controversial [4], [5], [12], [14], [15], [19]. The reason for the previous research being inconclusive has been evaluated in many different reviews. These reviews summarize many previous studies and also point out the issues and differences in the methodologies and techniques used. For example, in a study performed by Lee et al. HL-60 cells were exposed to 2.45 GHz for 2 hours at a SAR of 10 W/kg. The cells were then analyzed using SAGE (Serial Analysis of Gene Expression) immediately after exposure and again 6 hours after exposure. It was then observed that a significant amount of genes were differentially expressed as compared to the sham group. However, as pointed out in the 2009 Review by McNamee et al. this study was only conducted from a single experiment with only one sham group and two experimental groups [12]. Without repeated studies, results from this experiment are still consider inconclusive. Issues also arise due to differences in methodologies used in previous studies. Four key experimental differences between previous studies are *in vivo* vs *in vitro*, exposure systems, genomic sequencing technologies, and the methods used for statistical analysis [4], [5], [12], [14], [15], [19].

In Vivo* vs *In Vitro

RF *in vitro* studies provide an advantage of being able to primarily focus on the effects of RF energy on specified protein or cell lines, such as HL-60 cells. A downside to *in vitro* testing are results which can be difficult to directly relate to its effects on the body as it does not provide cell-to-cell or system interaction. Alternatively, *in vivo* testing allows direct interpretation of the effect of RF energy in the body since it includes the cell-to-cell interactions. *In vivo* testing also allows testing for longer durations of exposure. However, *in vivo* testing may introduce stress on the animal, which can also impact the results of the studies.

Exposure Systems

Exposure systems can be categorized as either localized or whole body exposure and the exposure setups are separated into restrained or unrestrained studies [14]. In restrained exposure studies, generally in-vivo studies, the subject is placed into a setup to allow for little to no movement. The restrained exposure system is usually used in studies that are interested in localized exposure, to control for consistent exposure of RF energy, on a specific region or organ, with little to no exposure to the rest of the subject. An issue with many of the studies using localized exposure is only the effects on the organ or region of interest is studied, which ignores the potential system interactions that could be occurring. Additionally, when restraining subjects for localized exposure it also may induce regulation of genes associated with stress [2]. Restrained setups and whole body RF exposure to organs and tissues can also be used, however, stress-induced genes is still an issue. An unrestrained subject reduces inducing genes related to stress. Generally, a whole body exposure system is used with an unrestrained setup, as local exposure is near impossible. However, a downside to an unrestrained whole body exposure setup is that it may not provide consistent levels of exposure to individual regions or organs of interest.

Genome Sequencing

Another difference between studies is in the technologies used to quantify the genes. One technique that is currently used for genomic sequencing is polymerase chain reaction (PCR). This method of analysis is considered the gold standard among all of the current techniques due to it having the highest accuracy in quantifying the levels of expression within a sample [23]. One limitation that PCR possesses is that it is a low throughput technique, only capable of analyzing a handful of genes selected prior to analysis, leaving the levels of expression from others genes still unknown. [23]

Another technology that is commonly used is microarray. Unlike PCR, microarray is known as a high throughput technology providing the ability to observe and quantify a larger range of genes providing a more complete view of the response of the genome. The downside of using microarray is that it sacrifices accuracy in reading the levels of expression and potentially causing false positive results [17], [19]. A specific example of this was addressed by Sakurai et al. by comparing 23 genes that were considered significant by microarray analysis to PCR. The results from the comparison showed that PCR did not determine any of the 23 genes as significant.

Lastly, a more recent advancement in gene sequencing is next generation sequencing (NGS) technologies. One such example of a NGS technology is RNA-seq. Like microarray, RNA-seq is a high throughput method of quantifying the genome of a sample. However, unlike both PCR and microarray, it is possible to quantify thousands of genes without any prior knowledge of their sequence. A downside to RNA-seq is that it is not as accurate as qPCR, but it has a better accuracy as compared to microarray [11], [18], [20], [23].

Statistical Methods

Another area that previous studies differ and encounter issues is in their statistical analysis. Some previous studies commonly use methods of statistical analysis that incorporated parametric testing of the experimental data. Parametric tests are very good when applied in studies where the sample size is fairly large and the data have a normal distribution. The problems with these parametric tests are that they make underlying assumptions of the data. For example, using a parametric test, like a t-test, it assumes that the data has a normal distribution. However, such assumptions can lead to inaccuracies in the estimations of calculating which genes could be statistically significant if the data does not have a normal distribution [1]. Non-

parametric testing, on the other hand, includes statistical methods such as permutations and combinations. In these non-parametric tests a reference distribution is built using the experimental data, which avoids making the assumptions of a normal distribution and can provide more accurate results. However, the building of the distributions using non-parametric testing, such as permutations, can be computationally time consuming.

EXPERIMENTAL APPROACH

Selection of Species

Since this study focuses on the effects of RF energy in-vivo, BALB/c mice were used to explore the effects of RF energy on brain tissue. The reason for using BALB/c mice can be attributed to little genetic variability between animals, allowing for better comparison [25], [26]. The mice acquired for this study were from Jackson laboratory in Bar Harbor, Maine. Furthermore, the age of the mice used in this study were 6-9 weeks old. These mice were housed in Plexiglas mice cages with micro filer tops and bedding composed of Alpha-dri^{TM} paper.

Standardized Procedure

To address the issues found in previous studies a newly developed standardized procedure developed by Hansen et al [7] was used. The first step in Hansen et al's procedure was to calibrate the test equipment and calculate the uncertainty analysis to establish the potential error in calculating the SAR and the power density. Next, the room used for the experiment was characterized by analyzing the background RF environment. The equipment used to perform this analysis consisted of a biconical antenna on a wooden tripod which was connected to a spectrum analyzer via Agilent interconnects and two 2 foot long transmission lines. To scan for any potential interfering frequencies a spectrum analyzer recorded the RF power over a range of frequencies. These measurements were performed individually in the x, y, and z directions, each for a duration of 24 hours, by changing the orientation of the biconical antenna.

The last step in Hansen et al.'s procedure was the experimental setup. The parameters used for the experiment can be found in Table 1. To produce the desired continuous RF sinusoidal signal, a horn antenna was connected to a signal generator. Additionally, the horn antenna was placed 52 cm above a Plexiglas mouse cage. To monitor the power density of the

RF energy received by the mice during the study, a patch antenna was place next to the cage. To remove unwanted noise from potential reflections and external noise anechoic material was placed around the mouse cage. A diagram of the setup as well as a photograph of the setup used is shown in Figure 1 and Figure 2 respectively.

Table 1. Test Parameters.

No	Parameter	Value
1.	Frequency	2.45 GHz
2.	Power Density	1.434±0.159 mW/cm^2
3.	Specific Absorption Rate	0.3422±0.0003 W/kg (Average Whole Body)
4.	Signal Type	Continuous Sinusoid
5.	Time of Exposure	31 Days
6.	Number of test	11 Mice
7.	Number of control	11 Mice

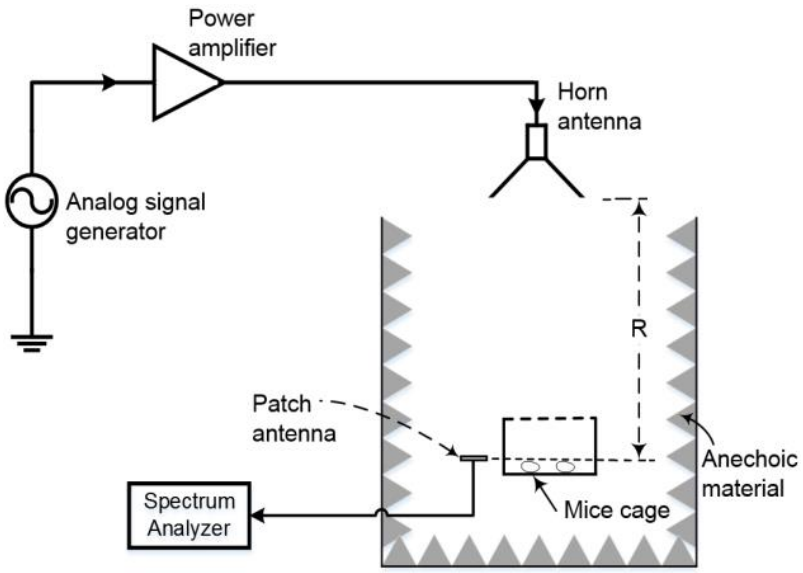

Figure 1 . Diagram of Experimental Setup. For shielding, anechoic material was placed around the cage. A patch antenna measured the power transmitted from the horn antenna which output a continuous sinusoidal signal at a power density of 1.434±0.159 mW/cm^2 [7].

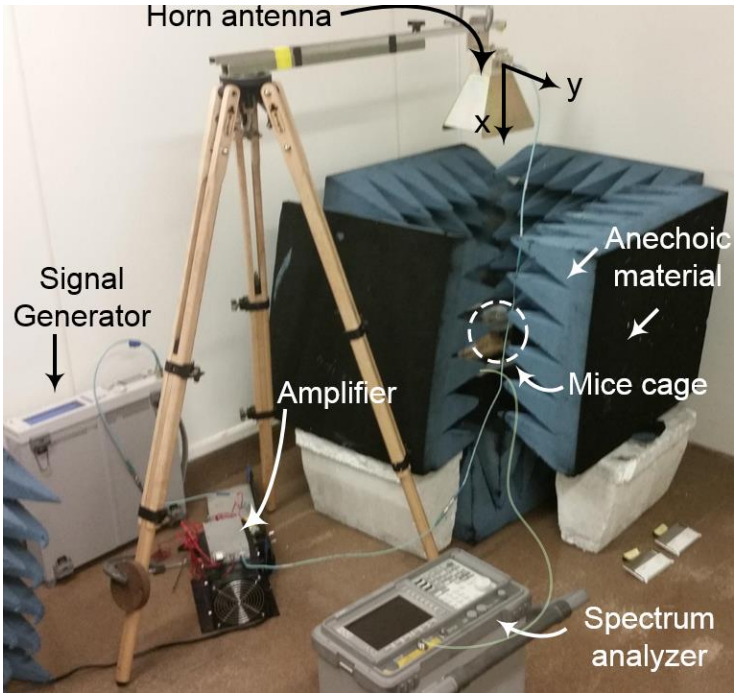

Figure 2. Photograph of Experimental Setup. The spectrum analyzer shown is connected to the patch antenna which was placed next to the mice cage and surrounded by anechoic material. The signal generator was connected through the amplifier to the horn antenna. The horn antenna was setup so that it was polarized in the z-direction.

Tissue Extraction

After a duration of 31 days of exposure the mice were transferred to a sterile environment where they were then euthanized using CO_2 asphyxiation following an approved IACCUC protocol [9]. To extract the tissue a procedure modified from "Necropsy of the Mouse" was followed and as shown in Figure 3 [3]. Briefly, an incision is made in the fur, allowing access to the skull, and then the top of the skull is cut away so that the brain tissue can be removed. After all the tissue was harvested from both the control and test groups they were frozen in liquid nitrogen they were stored in a freezer at -80˚C until they were ready for RNA-isolation.

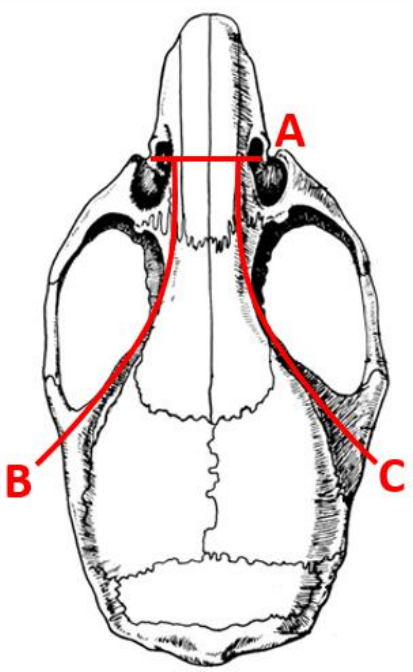

Figure 3. Brain Tissue Extraction. To access the brain three cuts in the skull must first be made. A. The first cut must be made between the nasal cavities. B, C. Then continuing from the cut in the nasal cavity, cuts must be made along the side of the skull to the base of the skull. After these cuts have been made the top of the skull can be removed to expose the brain for extraction. Drawing adapted from [24].

RNA Isolation

Before quantifying, the RNA had to first be isolated from the tissue through a process called Phenol-chloroform extraction. This method, as described by Ghosh S. et al [6], uses TRIzol reagent in a homogenized sample to separate the sample into three different phases. This process leaves RNA in the uppermost aqueous phase where the RNA is then removed and placed into a separate tube. To further purify the concentration of RNA, the samples underwent a process called alcohol precipitation. Lastly, to test the quality and purity of the samples the RNA samples were taken and screened with using Nanodrop™. Details of the output of Nanodrop™ and the required readings are shown in Figure 4.

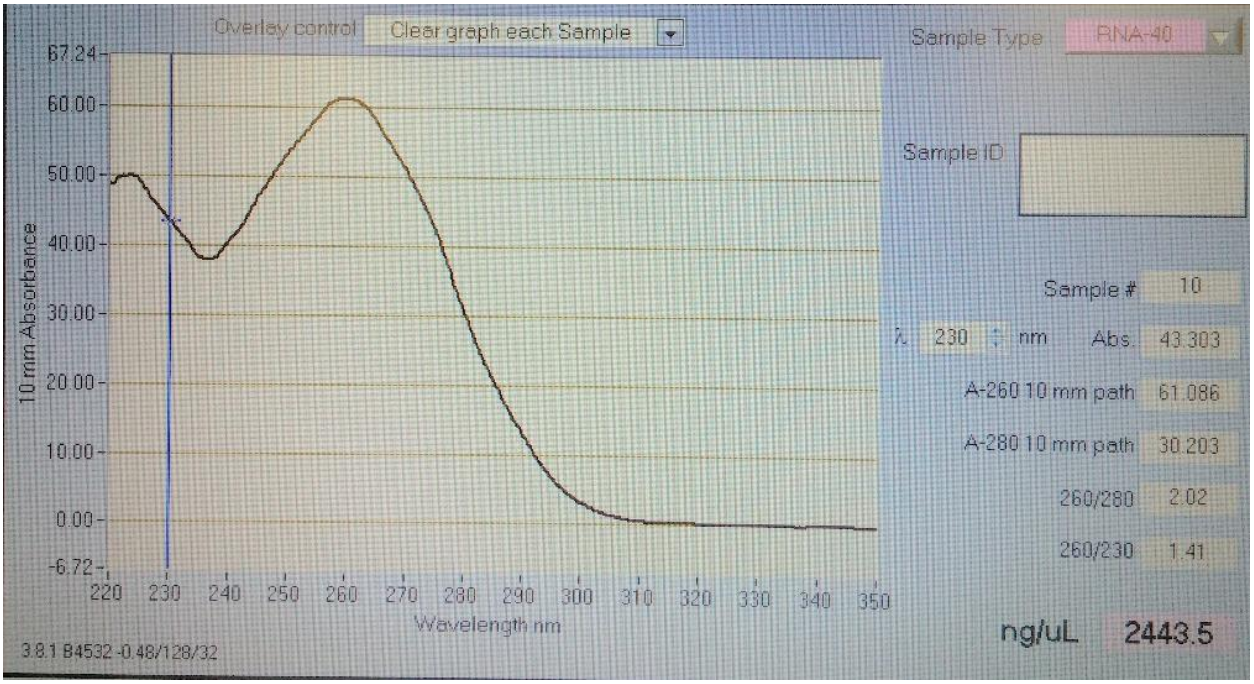

Figure 4. Nanodrop™ Spectrometry Reading. For RNA to be used for RNA-seq, the samples had to have a minimum concentration and 260/280 purity ratio greater than 200 ng/µL and 1.9 respectively.

Next Generation Sequencing

After the RNA was isolated it was sent to Genome Technology Access Center in the Department of Genetics at Washington University School of Medicine (GTAC) where RNA-seq was performed. RNA-seq is a next generation sequencing (NGS) technology that is used to quantify the levels of expression of RNA in each sample and output the results in terms of transcripts per million (TPM), as genes consist of multiple transcripts. RNA-seq was chosen due to its ability to quantify the levels of expression over the entire genome with accuracy better than other high throughput techniques, such as microarray.

Statistical Analysis

Once the TPM values are received from RNA-seq they must undergo statistical analysis so that significantly altered genes can be identified. Before statistical analysis is performed, transcripts were removed that showed zero levels or expression in both the test and control groups. These transcripts represent genes that are not expressed in the brain tissue and will not provide any significance in the results. Next, statistical equation 1 was created to represent the difference between the test and control groups for each transcript. Equation 1 can be defined as the mean of the test group minus the mean of the control group all divided by the overall mean of the test and control data for a given transcript.

$$stat_t = \frac{\bar{x}_{test} - \bar{x}_{control}}{\bar{x}_{test\ and\ control}} \quad (1)$$

Then to determine data that may be significant we used a non-parametric statistical test to avoid the assumptions of using predefine distributions that are made when using parametric tests. The non-parametric test that was used in this study was statistical combinations without replacement, as defined in equation 2. The statistical combinations was used to build a reference distribution for each individual transcript [10].

$$`\binom{n}{k} = \frac{n!}{k!(n-k)!} \quad (2)$$

Lastly, equation 3 was used to calculate the empirical p-values for each transcript using their respective distribution built from combinations as compared to the equation 1, all in relation to all possible combinations [8], [12]. Since the data was used to create its own distribution no assumptions of the data were made which increases the accuracy of the analysis. However, type one error was not controlled in this study.

$$P_{comb} = \frac{\sum_{1}^{\binom{n}{k}} (stat_c \geq stat_t)}{\binom{n}{k}} \quad (3)$$

RESULTS

To observe and represent the data received from the 7 control and 11 test mice, a statistical distribution was plotted as shown in Figure 5. Since the sample size of the control and test groups were not equivalent the statistical distribution does not demonstrate a normal distribution. For the distribution, the statistical values closest to 0.0 correspond to transcripts that expressed either little to no difference between the test and control group values. The extreme at +1.636 of the statistical distribution, however, corresponds to transcripts where the test group values are non-zero and the control groups values are zero. Similarly, the extreme at -2.571 corresponds to transcripts where the test group values are zero and the control group values are non-zero. For the values between 0.0 and the extremes are genes that were either up-regulated or down-regulated.

The data were also plotted, in Figure 6, showing the accumulation of the empirical p-values over the complete range of p-values. In this plot little to no change in gene expression would appear as a line with a slope of 1. For data that appears above this line it represents that data found from the study may be significant. Alternatively, if data were found at or below the unity line it would mean the data do not reflect any significant results. Additionally, this plot shows the percent of genes that are consider significant, having a p-value below 0.05. For a data set exhibiting no differential expression (a slope of 1), it is expected to see 5% of the transcripts being considered significant which is generally attributed to random error. However, in this study the data indicates that over 11% of the transcripts can be described as significant above the 5% random limit.

Lastly, the transcripts were mapped to their respective genes in IPA®. The disease and functions of the 11.36% affected genes with p-values less than 0.05, can be found in Table 2.

Table 2 also shows how many genes were found to be significantly expressed with respect to a specific disease or brain function. Genes that also exhibited an on/off attribute are in a column labeled “genes with extreme stat values.”

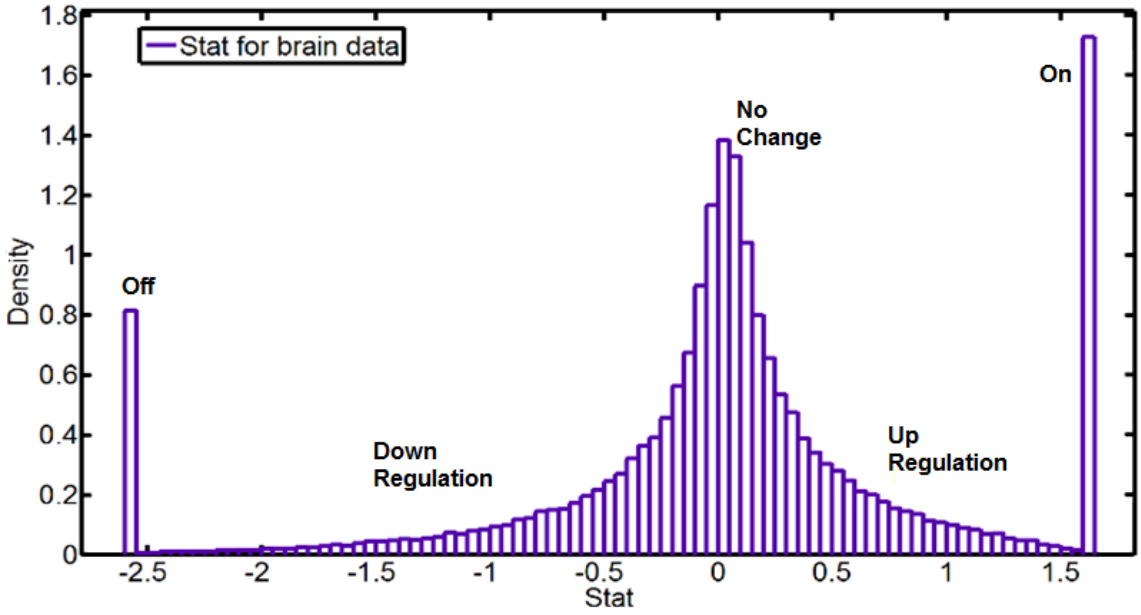

Figure 5. Distribution of Stat Values for Brain Data. The extremes of the figure are corresponding to where the test group transcript values are non-zero and the control groups transcript values are zero (+1.636) or vice versa (-2.571).

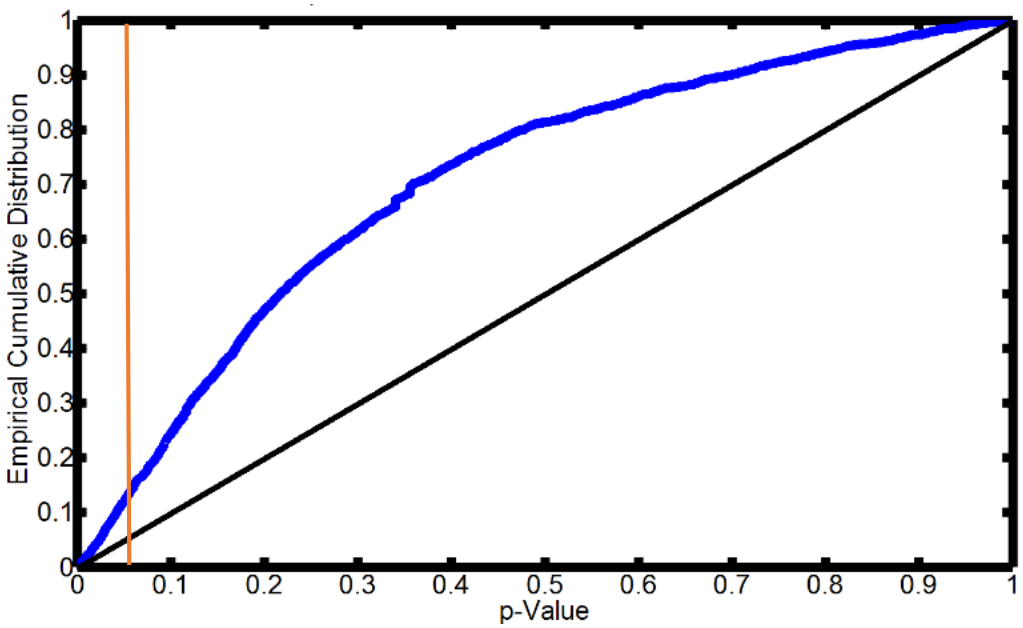

Figure 6. Empirical Cumulative Distribution for Brain Data. (Blue), empirical cumulative distribution for brain data. (Black), a reference line of where p-values are equivalent, which means no effect from RF energy exist.

Table 2. RNA Functions. This table represents the functions or genes that had a p-value of less than .05. The extremes column is attributed to the values that showed genes that were potentially turned on/off.

No	Disease or Function	Number of Genes with P-value <0.05	Number of Genes with Extreme stat Values
1	Development of central nervous system	51	9
2	Formation of brain	50	9
3	Quantity of cells	41	5
4	Quantity of neurons	35	5
5	Proliferation of cells	34	5
6	Proliferation of neuronal cells	24	2
7	Shape change of neurites	19	0
8	Morphology of rhombencephalon	19	2
9	Formation of rhombencephalon	19	3
10	Quantity of central nervous system cells	19	2
11	Morphology of cerebellum	18	2
12	Abnormal morphology of rhombencephalon	18	2
13	Behavior	18	2
14	Formation of cerebellum	17	3
15	Abnormal morphology of cerebellum	17	2
16	Quantity of brain cells	17	2
17	Growth of embryo	15	4
18	Growth of neurites	15	0

Table 2. RNA Functions (continued). This table represents the functions or genes that had a p-value of less than .05. The extremes column is attributed to the values that showed genes that were potentially turned on/off."

No	Disease or Function	Number of Genes with P-value <0.05	Number of Genes with Extreme stat Values
19	Growth of brain	14	4
20	Outgrowth of neurons	13	0
21	Growth of embryonic tissue	13	3
22	Outgrowth of neurites	12	0
23	Proliferation of brain cells	12	4
24	Abnormal morphology of cerebral cortex	11	2
25	Foliation of cerebellum	8	2
26	Shape change of axons	7	0
27	Length of neurons	7	0
28	Abnormal morphology of granule cell layer	7	1
29	Abnormal morphology of granule cells	7	1
30	Proliferation of embryonic cells	6	1
31	Quantity of Purkinje cells	6	0
32	Proliferations of granule cells	5	1
33	Proliferation of neuroblasts	4	0
34	Outgrowth of axons	4	0
35	Loss of dopaminergic neurons	4	1
36	Projection	4	1
37	Abnormal morphology of external granule cell layer	3	1
38	Lack of cerebellum	3	0
39	Abnormal morphology of pons	3	0
40	Neurodegeneration of axons	3	0
41	Formation of cerebral ventricles	3	0
42	Proliferation of granule cell precursors	3	0
43	Transport of synaptic vesicles	3	0
44	Development of subcommissural organ	2	0
45	Mislocalization of neurons	2	0
46	Phagocytosis by microglia	2	1
47	Quantity of macrophages	2	0
48	Quantity of selenium	2	1
49	Quantity of radial glial cells	2	1
50	Development of lateral cerebral ventricle	2	0
51	Long-term potentiation of entorhinal area	2	0
52	Morphogenesis of cerebellar cortex	2	1
53	Targeting of mossy fibers	2	0

DISCUSSION

As can be observed from the statistical distribution of the brain data in figure 5 the data mostly follows what is expected with the majority of the data centered around zero, which indicates the data showing little to no significant change. However, what is surprising to see are the quantities of genes that are found at each of the statistical distributions extremes at +1.636 and -2.571. The amount of transcripts being seen at these extremes provide the first indication that RF energy potentially caused significant changes and are being turned on/off.

Figure 6 also showed an indication that the RF energy induced a change in the genome. This can be supported by the empirical p-values found to be below 0.05 which was 11.36% of the transcripts. This value is much greater than the expected 5% random error that would be seen if there was no change in the levels of expression. However, some of these findings could be attributed due to type 1 error that was not controlled for in this study.

Another observation was that the data indicated the genes responded to RF energy similar to the operation of a transistor. A typical transistor allows a small flow of current to control a large current flow and in this case small exposure of RF energy causes large amounts of genetic flow. With a transistor there are different stages, which are the on, off, or the active/linear region. For example, genes in the test group were turned on by RF energy as these genes were not originally expressed in the control group. An example of genes being turned off would be genes that were originally being expressed in the control group but did not shown any expression in the test group. Lastly, genes showing significant up or down regulation in gene expression would be in the active/linear region. This overall characteristic is the first time genes have been observed to have the potential to operate as a RF-induced genetic transistor. As a RF-induced genetic

transistor it provides the potential to control targeted gene(s), such as those found in table 2, to either turn on, off, or modulate the gene expression to impact the body in a desired way.

Since this study is the first time RNA-seq was used to quantify the effects of RF energy on gene expression it is difficult to compare the finding to previous studies. In the studies where qPCR was used, many of which inconclusive, only a handful of genes can be compared, leaving many of the genes considered significant in this study still not being compared. Similarly, studies that used microarray and other high throughput techniques are difficult to compare since they were considered inconclusive due to insufficient number of samples used in the studies along with other issues. Even for studies that did produce more conclusive findings they did not have the same parameters as was used in this study. To be able to directly relate and compare this study to others, similar experiment parameters need to be used such as the frequency, power density, duration.

CONCLUSION

What was explored in this study was the effects of RF energy on the genome. To address issues found in previous research a newly standardized procedure was used and tissue was quantified using RNA-seq. The data was then analyzed of significant changes in the genome using statistical combinations and empirical p-values. The results of this study provided evidence that RF energy has the potential to induce genetic alterations. Additionally, the results of this study also showed the potential of the RF energy inducing some genes to exhibit states similar to a transistor. To verify the results future studies need to be held using the same standardized procedure and parameters to confirm the repeatability of the previous set of experiments. To improve accuracy of the statistical significance, future studies would also have an increased number in test and control groups. In addition, they should also include a comparison of significantly expressed genes from the result of RNA-seq to those of PCR to reconfirm accuracy and further support the findings. After significantly expressed genes have been identified, their interactions with RF energy can be used to determine their functions and effects on the body. If the results from this and future studies provide evidence that shows detrimental health effects would lead to the development of disease models and further standardizations on the use of RF energy. On the other hand, if this research was to provide evidenced of positive health benefits, such as the ability to turn on and of targeted genes, it could potentially introduce a new field of RF therapy.

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