Evaluation of the Effect of Chronic 94 GHz Exposure on Gene Expression in the Skin of Hairless Rats In Vivo

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Denis Habauzit[1*], Guillaume Nugue[2], Frédéric Bourbon[2], Catherine Martin[1], Flavia Del Vecchio[2], Séverine Maunoir-Regimbal[2], Thomas Poyot[2], Marco Valente[2], Rachid Jaoui[3], David Crouzier[2], Yves Le Dréan[1#], Jean Claude Debouzy[2#].

[1] Univ Rennes, Inserm, EHESP, IRSET (Institut de Recherche en Santé, Environnement et Travail) UMR_S 1085, F-35000 Rennes, France.

[2] IRBA (Institut de Recherche Biomédicale des Armées), F-91223 Brétigny, France

[3] Direction générale de l'armement (DGA/TA), F-31131 Balma, France

# These authors have participated equally.

***Mailing address for corresponding author:**

Dr Denis Habauzit. Transcription, Environment and Cancer Group, Institute for Research on Environmental and Occupational Health (IRSET), Inserm UMR1085, 9 avenue du Prof. Léon Bernard, 35043 Rennes Cedex, France. Tel: (33)2.23.23.70.34; E-mail: denis.habauzit@univ-rennes1.fr/habauzit.denis@gmail.com

Running Title: Effect of chronic 94 GHz exposure on rat skin gene expression

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Abstract

Millimeter waves (MMW) are broadband frequencies that have recently been used in several applications in wireless communications, medical devices, and nonlethal weapons (*i.e.,* the nonlethal weapon called Active Denial Systems, *ADS*, operating at 94-95 GHz, CW). However, little information is available on their potential effects on humans. These radiofrequencies are absorbed and stopped by the first layer of the skin. Therefore, our study aimed to evaluate the effects of 94 GHz on the gene expression of skin cells. Two rat populations consisting of 17 young animals and 14 adult ones were subjected to chronic long-term 94 GHz MMW exposure. Each group of animals was divided into exposed and sham subgroups. The two independent exposure experiments were conducted for 5 months with rats exposed 3 h per day for 3 days per week to an incident power density of 10 mW/cm², which corresponded to twice the ICNIRP limit of occupational exposure for humans. At the end of the experiment, skin explants were taken, and RNA was extracted. Then, the modifications to the whole gene expression profile were analyzed with a gene expression microarray. Without modification of the animal's temperature, long-term chronic 94 GHz-MMW exposure did not significantly modify the gene expression of the skin on either the young or adult rats.

Introduction

Radiofrequencies constitute a form of nonionizing radiation with a frequency range between 3 kHz and 300 GHz. These frequencies are now required for devices in common use, such as in cooking (induction table and microwave), telecommunications (mobile phone, Wi-Fi, radio, and radar), medicine (cancer radiofrequency ablation and MRI), and security (airport body scanner). Among these frequencies, millimeter waves (MMWs) are broadband frequencies between 30 and 300 GHz that are utilized in civilian or military applications, such as telecommunications, medical devices, security and weapon development. For example, nonlethal weapons such as the active denial system (ADS), which produces a strong heat sensation for people in the beam, operates at the 94 GHz band. The evolution of such military technologies involves the use of high output power, which increases the risk of inadvertent exposure of personnel. To date, very few studies have been published on the effects of such waves used in military applications on human health.

At these frequencies, the energy deposition on biological tissues penetrates into the first millimeter of the body surface (*1,2*), which implies that the exposure mainly concerns the epidermal and cutaneous regions of the skin. Therefore, the skin is the primary target of interest for the study of the potential biological effects of MMWs. Experiments conducted on human volunteers showed that very short exposure (3 s) at high power 94 GHz (1.8 W/cm²) was sufficient to increase the temperature at the surface of the skin (*3*). These acute high-power exposures lead to a sensation of pain or, when prolonged, to burns. It was also found that prolonged exposure at 94 GHz with high power intensity (75 mW/cm²) induces such extreme peripheral heat on rats that it could induce cardiovascular failure (*4,5*). It is important to note that these exposure conditions dramatically exceed the allowed limits for people. Reference levels for whole body exposure at millimeter frequencies are 5 mW/cm² for occupational exposure and 1 mW/cm² for the general public (*6*).

To gain insight into the biological effects of MMWs, several studies examined changes in global gene expression caused by exposure. For instance, the Millenbaugh group observed the modification of the mRNA content in rat skin tissue after exposure to 35 GHz at 75 mW/cm² (*7*). By using microarray analysis, these authors found 56 and 58 genes differentially expressed after 6 h and 24 h of exposure to MMWs, respectively. The majority of these genes were related to heat shock response, oxidative stress, inflammation, wound repair or tissue matrix recovery. A study carried out on human keratinocytes showed that MMW exposure at 20 mW/cm² led to an increase in temperature and to an important alteration of gene expression *in vitro* (*8*). However, this modification of the whole gene expression profile was mainly associated with the thermal effect of MMWs. Therefore, when the temperature was artificially maintained constant by decreasing the incubator temperature, no additional change was observed in whole gene expression profile after

MMW exposure compared to the sham (control) gene expression (*8*). This study is in agreement with others that suggest that, if care is taken to avoid MMW-associated thermal effects, acute low-power exposures have no notable effect on gene expression (*9–11*).

The purpose of the present study was to verify whether repeated and prolonged MMW exposure at low power can have an impact on gene expression in rat skin. Exposure was set to 94 GHz at an incident power density (IPD) of 10 mW/cm² to mimic the exposure of personnel handling ADS weapons.

Materials and methods

Animals

During this study, 2 exposure experiments were conducted on groups of 30 hairless male rats (CD Hairless rats, from Charles River Laboratories, Ecully, France). The rats were housed in pairs during the experiment and maintained in a temperature-controlled room (22°C) with an automatic 12 h light/dark cycle (06:00 18:00). Water and diet were given *ad libitum*. All animal procedures were approved by the Animal Care and Use Committee of the French Biomedical Research Institute (2019/13.0) and were in compliance with the guidelines of the European Union (directive 2010/63/EU and revisited Appendix A of European Treaty Series 123), implemented into French law (decree 2013–118) regulating animal experimentation. All efforts were made to reduce the number of animals used and animals were daily observed.

Exposure system, dosimetry and experimental setup

The 94 GHz generator (Figure 1A), which contains an EIK subsystem consisting of an EIK with 100 watt CW operation, a 94 GHz air-cooled EIK, an RF source with an integrated power supply, RF input and output components, and an integrated device cooling and protection system, was designed by the Communications & Power industries (CPI Europe Ltd., Walton-on-Thames, UK). This system was connected with a waveguide to a cornet antenna (Figure 1B) in an anechoic chamber in which the rats were exposed (Figure 1C). The temperature of the rats was monitored before and after exposure with a FLIR A320 IR camera (FLIR France, Issy-les-Moulineaux, France), and the temperature data were integrated with FLIR Quickreport software.

The dosimetry (Figure 2) was realized using the 262 W cornet antenna connected with circular waveguides in the TE11 mode (Flange W387) at 94 GHz. The simulation was performed with CST MicroWaveStudio software (Figure 2) in the FDTD resolution mode. The optimal distance, considering a gain of 26 dBi, to obtain a homogeneous exposure field was calculated. The distance between the antenna and the rats was 71 cm, creating an area of 1 m with a homogeneous beam of 100 W/m² (equivalent to 10 mW/cm², which is twice the ICNIRP limit for occupational exposure).

Animal exposure and sample recovery

Two exposure experiments were conducted. In the first experiment (E1), adult CD hairless male rats (average weight 521 ± 44 g, 14 weeks old) were used, and in the second experiment (E2), young CD hairless male rats (6 weeks old rat) that weighed an average of 301 ± 11 g at the beginning of the experiment were used. This choice of two age groups results on the question of the potential differential action of electromagnetic field betwenn ages. Younger animal are usually considered more sensitive toward environmental insults. For each experiment, the animals were divided into two distinct groups. The first group corresponded to the sham (control) (15 rats), and the second group was composed of rats (n = 15) exposed to MMWs at an incident power density of 10 mW/cm² for 3 h per day and 3 days per week for 5 months. Three groups of 5 rats were exposed or sham exposed while in the anechoic chamber shown in Figure 1C. The temperature in the anechoic room was maintained with air conditioning at a constant 20 ± 1°C level, and the temperature of each exposed or sham-exposed rat was monitored by the IR camera at 2 time points. The rectal temperature of each rat was also monitored before and after each exposure. Every week, each rat was weighed. During E2, one animal in each group died during the experiment. At the end of the exposure series, the rats were sacrificed by decapitation, and then, skin explants were extracted and conserved in RNAlater (Thermofischer) for use in the analysis.

RNA extraction and microarray

Rat skin biopsy samples were stored at -80°C in RNAlater (Thermo Fisher Scientific, Saint Aubin, France). Between 15 and 20 mg of the biopsy sample was first cut into small fragments and then mixed with Qiazol solution (Invitrogen, Thermo Fisher Scientific, Saint Aubin, France), and then, it was crushed with a micro pestle and homogenized at 6300 rpm with ceramic beads in a Precellys apparatus (Bertin Technology, France) for 5 cycles of 23 s with 2 min on ice between each cycle. The total homogenate containing the skin extract and 1 ml of Qiazol was mixed with 300 µl of chloroform

and then vortexed for 30 s and centrifuged for 15 min (14000 rpm, 4°C). Equal amounts of the aqueous phase were taken, mixed with 70% ethanol and transferred to a Qiagen column from an RNeasy lipid tissue kit (Qiagen, Hilden, Germany) for purification according to the manufacturer's instructions. Finally, the RNA was recovered and quantified on a Nanodrop 1000 spectrophotometer (Nanodrop Technology, Cambridge, UK), and the integrity of the RNA samples was assessed on a Bioanalyzer 2100 (Agilent Technologies, Les Ulis, France).

From the two sets of 30 animals exposed during E1 and E2, 15 and 17 animals were randomly selected, respectively, for the microarray experiments. The mRNA from the animals was analyzed using a one-color whole gene expression microarray with an Agilent SurePrint G3 Rat Gene Expression v2 8x60K microarray kit (Agilent Technologies, Les Ulis, France) according to the manufacturer's instructions. One sample in the E1 experiment was excluded due to low-quality feature extraction. Therefore, the data from the two experiments E1 (14 animals: 8 sham and 6 exposed) and E2 (17 animals: 8 sham and 9 exposed) were randomly analyzed with Genespring GX software (Agilent Technologies, Les Ulis, France). The fluorescence intensity of each microarray was log2-transformed, and the baseline was normalized by quantile. Finally, 30367 probes were detected on the microarrays. Each microarray was then filtered on the basis of the expression level (expression greater than 125). Finally, 15168 and 14853 unique probes were found for experiments 1 (E1) and 2 (E2), respectively. A total of 14721 probes were typically found for the two groups. The complete data set was deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo, GEO series accession numbers GSE132816, GSE132817 and GSE132818).

Statistics

The microarray analyses were performed with Genespring software (Agilent Technologies, Les Ulis, France). The probes were filtered by fold change (absolute fold change above 1.5) and then analyzed by two-tailed Mann-Whitney tests. The *P*-values were adjusted by controlling for the false discovery rate (FDR) with the Benjamini-Hochberg (BH) correction for multiple testing. Genes were considered differentially expressed if the adjusted *P*-value was below 0.05 with or without BH correction.

Results

Effect of MMW exposure on animal weight

The MMW exposure did not affect the body weight of the adult or young rats (Figure 3). The weight of the adult animals (E1) remained constant throughout the experiment, regardless of whether or not the animals were exposed. The weight of the young animals (E2) increased steadily until reaching a plateau that corresponded to the normal adult weight. The differences between the sham and exposed rats were not significant.

Rat temperature before and after each exposure.

Since rats have been exposed to twice the level of the recommended limit for humans, thermal effects were possibilities. Therefore, during exposure, the temperature of each rat was monitored by an IR camera. Figure 4A clearly shows that exposure significantly increased the temperature of the skin surface. The IR camera measurements show an average increase of 1.0±0.5°C for the adult animals (E1) and 0.5±0.5°C for the young rats (E2). Nevertheless, this clear increase in body surface temperature was not found for internal temperature levels (Figure 4B). The rectal temperature was assessed for each rat before and after each exposure. According to these internal measurements, the difference in temperature after exposure was only +0.2±0.5°C for the first experiment (E1) and -0.2±0.3°C for the second experiment (E2) (Figure 4B). The internal temperature of the Sham control group is modified in the same magnitude as the Exposed one (data not shown). These measurements clearly indicate that physiological compensation phenomena maintained the constant internal temperature during exposure.

Effect of low-intensity long-term exposure on gene expression in the skin

After normalization and filtration of the expression levels, 15168 (E1) and 14853 (E2) probes were selected. More than 97% of the detected probes were common to both experiments (E1 and E2). The first quality control test was conducted to verify that the 1°C increase observed on the skin surface did not induce tissue damage or a heat shock response. Genes from the heat shock protein family (HSP) and genes known to be involved in skin inflammation and tissue repair (*12*) were extracted based on the microarray data, and their overall expression was assessed. As shown in Figures 5 and 6, these genes were continuously expressed whether the animals had been exposed or not. Taken together, these observations suggest that the slight temperature increase due to exposure did not have an impact on the skin.

Then, we analyzed the differentially expressed genes between the two groups of animals (Sham E1 *vs* Sham E2). We observed with a MW test and a BH correction on the 15 210 common gene entities that 336 genes were differentially expressed between this two groups (Data not shown). Considering that we have difference in gene expression in the two groups we therefore analyzed data in each subgroup independently by filtering the microarray data by fold changes from each experiment. Approximately 1% of the detected probes (156 probes for E1 and 120 probes for E2) had a fold change greater than 1.5 between the sham and exposed group (Table I). The Mann-Whitney test with Benjamini-Hochberg (BH) correction was performed on this list of probes, and in both cases, no genes were shown to be differentially expressed between the sham and exposed groups. By removing the BH correction, 14 and 4 genes from the E1 and E2, respectively, were found to be differentially expressed between the two groups. These genes are described in detail in Table II. However, in the absence of the BH correction, some false positives for genes might represent contaminants among the sensitive genes listed. Since there seems to be little likelihood that a false positive would be found twice in the two independent analyses, we cross-referenced the lists of genes obtained from the two exposure experiments. There was no common gene between the two lists. This finding suggests that we did not detect strict exposure-sensitive genes that should have been differentially expressed in the 2 experiments. Therefore, we can conclude that long-term low-power 94 GHz exposure did not modify the gene expression in rat skin.

Discussion

Applications using high-power millimeter waves already exist, implying that people using these technologies may be inadvertently exposed. Several publications have studied some peculiar frequencies used in millimeter range such as at 30, 42, 60 GHz and now 94 GHz (*11,13–19*). All of these frequencies may be studied as the main difference in the physical action on skin is the penetration depth. Indeed these frequencies did not permit to reach the same skin layer (dermis, epidermis). For instance, at 30 GHz the penetration depth is near 0.9 mm and at 94 GHz, it is near 0,37 mm (*1*). To gain inside knowledge about the safety of these applications, this research aimed to evaluate the long-term chronic exposure of 94 GHz millimeter waves. The treatment chosen was midrange exposure, *i.e.,* for 3 h per day and 3 days a week at an IPD of 10 mW/cm². This condition

corresponds to a worst case scenario of exposure for personnel handling ADS weapons because this IPD is twice the limit recommended by the ICNIRP for workers (*6*). Moreover, to characterize the potential effect of these frequencies better, two different rat populations (young and old) were chosen.

Considering that little information is available on the impact of these waves on animals and humans, data on general physiological effects were monitored throughout the 5-month exposure period. First, the exposure did not significantly modify the body weight of the rats. The IPD used was low enough that it did not induce tissue damage or heat shock response. During the exposure, the skin surface temperature increased by 1°C on average, but this increase had no or very little impact on the internal temperature. Moreover histologic approach did not evidence any difference in the skin morphology (data not shown).

Then, we analyzed the gene expression to verify whether chronic exposure could have an impact on gene expression. The gene expression signature reflects the cell identity in a particular environmental context. Since the MMWs do not penetrate deeply into the body, we used skin as a model. The whole gene expression profile as analyzed by microarray was not modified by the MMW treatment, and only a few genes were observed to be modified when the statistical correction was not included in the analysis. Two independent exposure experiments were conducted, and no common gene was found to be differentially expressed in either experiment. This finding emphasizes a lack of reproducibility, which is likely explained by the fact that, in the absence of statistical correction, false positives may have emerged. Despite this risk of false positive, we chose to present the genes identified with this uncorrected statistical test. This in order to help researcher who would like to develop a meta-analysis on experiments performed in microarray. Moreover, no correlation was observed between these uncorrected differentially expressed genes and genes identified in previously published microarray experiments either in animal models (*7*) or in cells (*8,10,18)*. In parallel no modified genes were involved in membrane permeability, nor in protein involved in channels, although some recent MMW effects were found associated with cell membrane permeability in keratinocytes (20). We can therefore assume that, under the conditions of our study, MMW exposure had no impact on gene expression in the skin. These results are in agreement with those studies listed in the bibliography that show that acute and low-power MMW exposure does not dramatically change the whole gene expression profile in the primary cultures of human keratinocytes (*8,18,21*). Nevertheless the gene expression modification is not the only possible effect in chronic action of the MMW. Indeed, no information of epigenetic modifications or of DNA adducts are available in the literature. Moreover if some transitory effect are induced during the treatment time we cannot see it as the manipulation was design for an end point recovery.

In summary, our experiments did not reveal any deleterious effect on skin *in vivo* after chronic MMW exposure at power twice as high as that recommended by the ICNIRP for workers. Since skin is the main target of millimeter waves, these results are important in the context of risk assessment. However, we must not lose sight of the fact that our study was focused on the skin and that we did not investigate the possibility of a systemic response that activates nerve endings or induces secretion of factors into the blood circulation (*22,23*).

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Figure Legends

Figure 1: Exposure system: A) generator, B) anechoic chamber, C) horn antenna and exposed rats.

Figure 2: Dosimetry of the 94 GHz wave

Figure 3: Animal weight during chronic exposure. The body weight of each animal was measured every week (n=31) in each group (sham and exposed). E1 = first exposure experiment on adult rats. E2 = second exposure experiment on young animals. The data are expressed as the mean of the values ± standard deviation.

Figure 4: A) External temperature changes before exposure (initial T) and after exposure (final T) for the exposed groups of rats during the first (E1) and the second (E2) experiment. B) Internal temperature change before and after exposure in the exposed groups. The box plot presents a summary of the mean temperature of each animal throughout the chronic exposure period. The results are significant for P-value < 0.05 (*), p-value < 0.01 (**), or p-value < 0.001 (***) based on t-tests. NS = not significant.

Figure 5: Expression of Heat Shock Protein (HSP). The data are expressed as the means ± standard deviation.

Figure 6: Expression of selected genes involved in skin inflammation, wound repair and tissue remodeling. From Sass et al., 2017. (Data are expressed as the means ± standard deviation)

Table Legends

Table I: Microarray data on the two experiments of exposure.

Table II: Differentially expressed genes obtained by Mann-Whitney test without BH correction.

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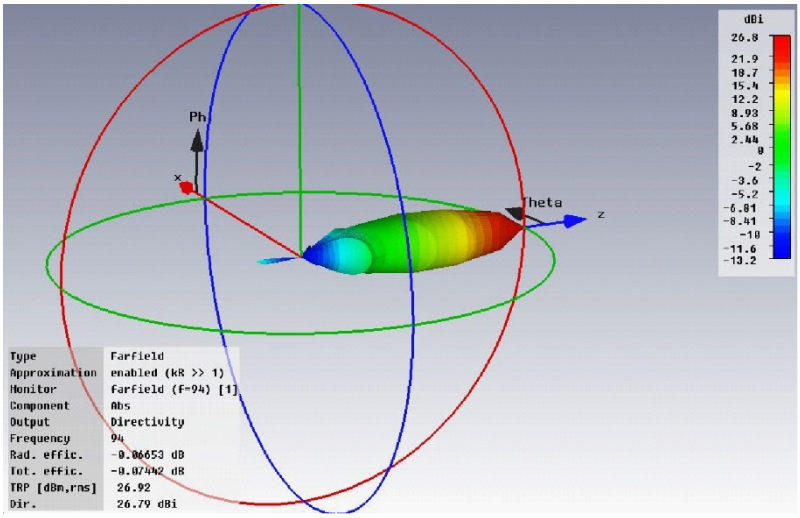

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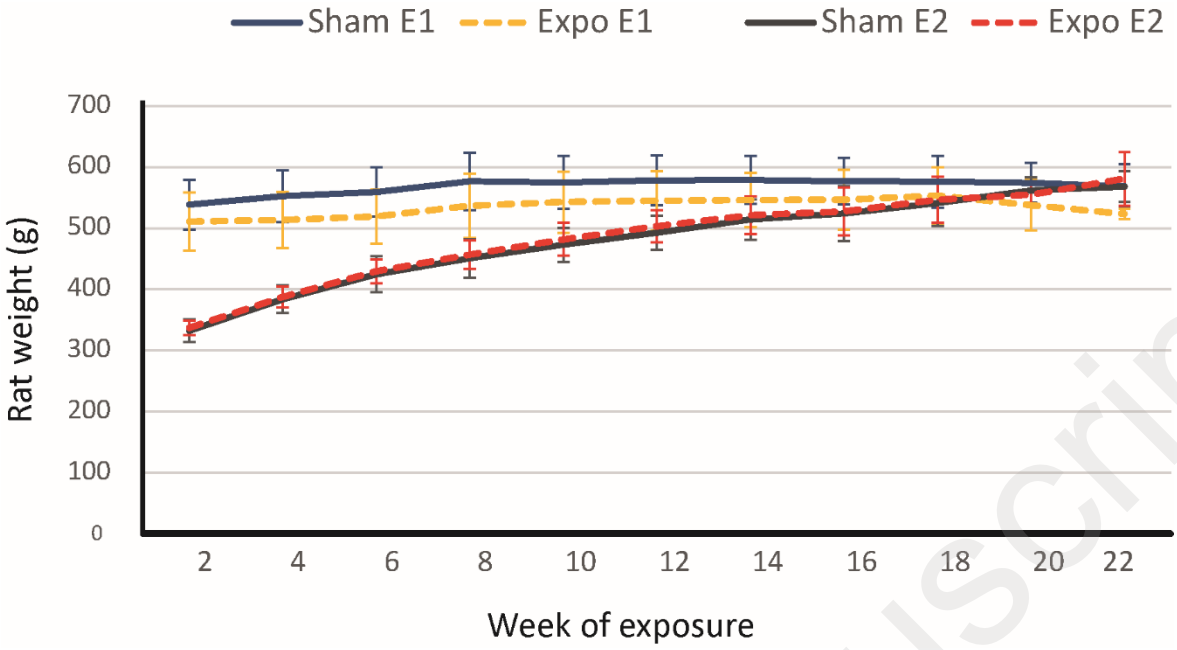

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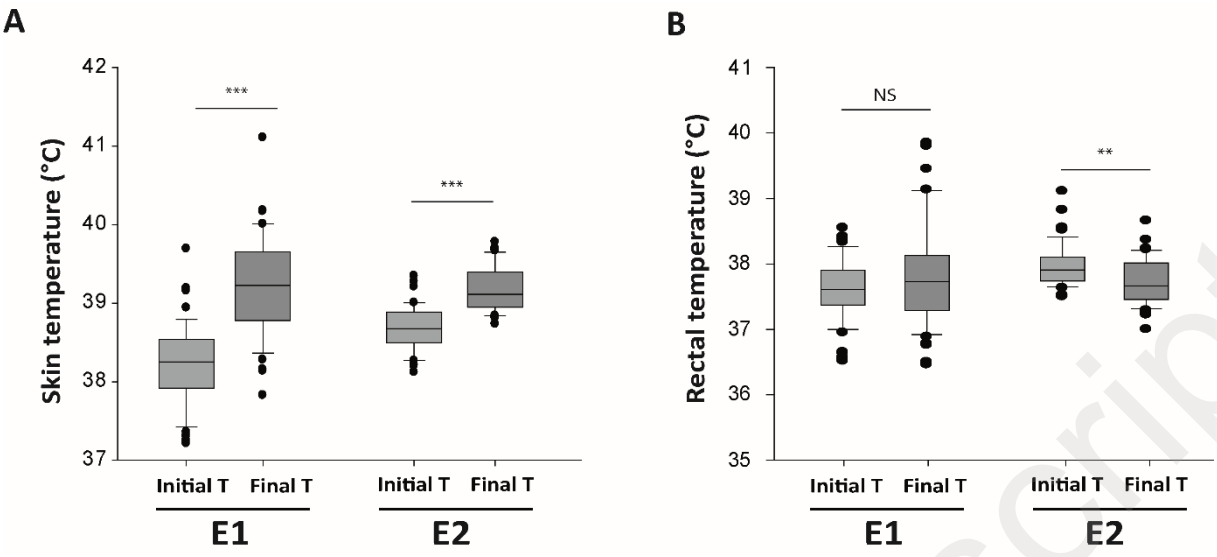

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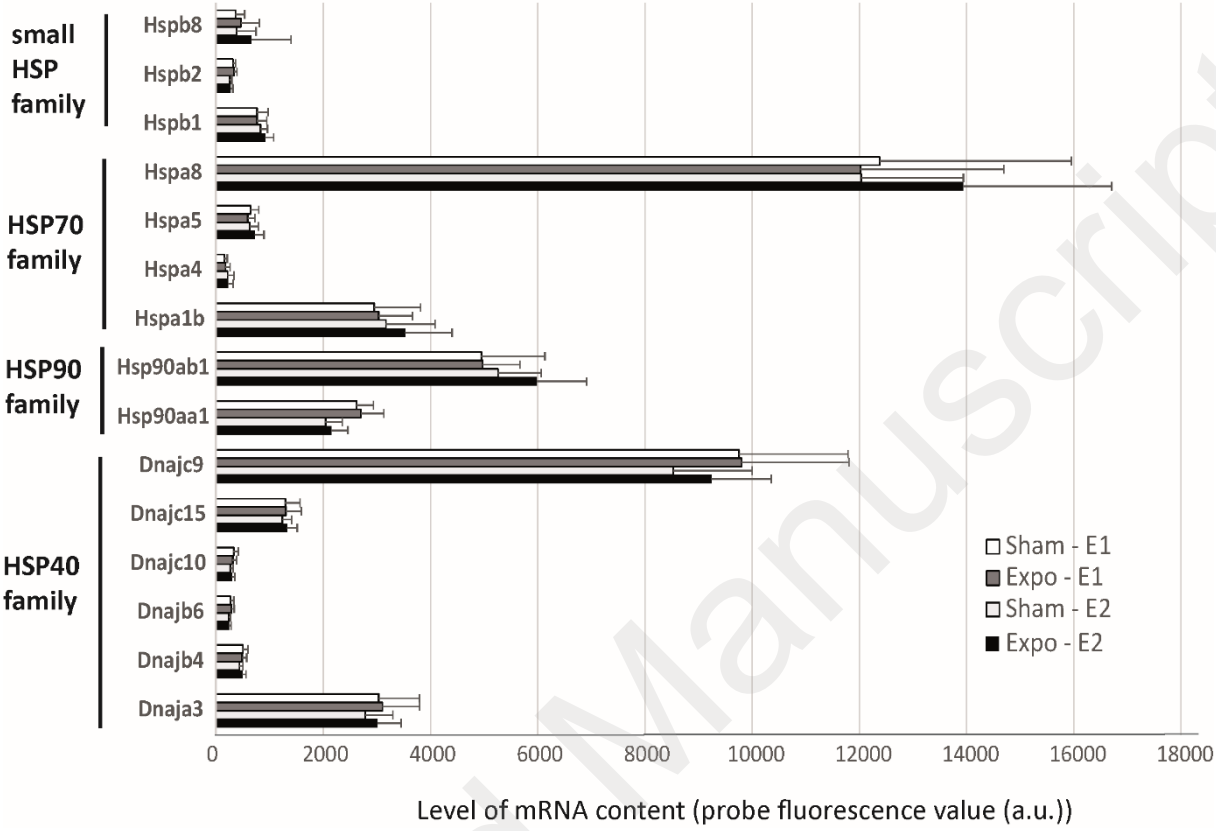

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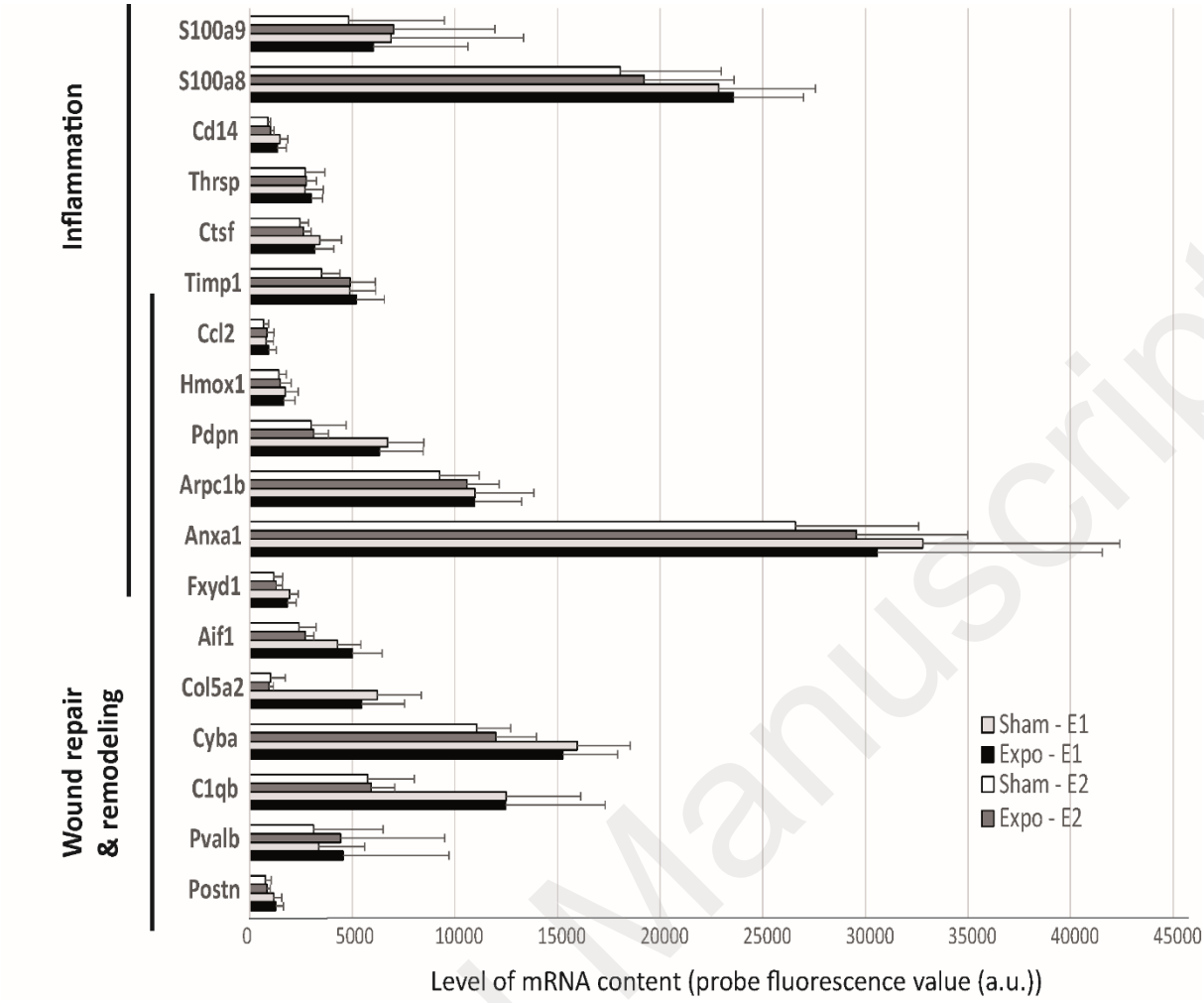

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Table I: Microarray data on the two experiments of exposure.

Experiment E1	Total of unique probes detected	Filtered fold change (more than 1.5) Numbers of probes		Mann-Whitney with BH correction	Mann-Whitney without BH correction	
Sham: 8 rats Exposed: 6 rats	15,168	156	Up: 107 Down: 49	0	14	Up: 11 Down: 3
Experiment E2	Total of unique probes detected	Filter on fold change (upper than 1.5) Numbers of probes		Mann-Whitney with BH correction	Mann-Whitney without BH correction	
Sham: 8 rats Exposed: 9 rats	14,853	120	Up: 112 Down: 8	0	4	Up: 3 Down: 1

Table II: Differentially expressed genes obtained by Mann-Whitney test without BH correction.

E1						
Probe Name	***P-value***	**Regulation**	**FC (abs)**	**Gene Symbol**	**GenBank Accession**	**Gene Name**
A_44_P142850	0.010	Up	1.66	Fam86a	NM_001106975	Family with sequence similarity 86, member A
A_64_P105342	0.039	Up	2.89	LOC102557336	XM_006223705	Keratin-associated protein 5-4-like
A_44_P555689	0.039	Up	1.59	LOC291863	NM_001013889	Carboxylesterase-like
A_64_P032683	0.039	Up	1.52	Mest	NM_001009617	Mesoderm specific transcript
A_42_P643349	0.039	Up	1.59	Myl4	NM_001109495	Myosin, light chain 4
A_44_P522461	0.028	Up	2.78	Olr1229	NM_001000444	Olfactory receptor 1229
A_44_P522461	0.039	Up	2.41	Vsig8	NM_001105972	V-set and immunoglobulin domain containing 8
A_64_P137704	0.028	Up	2.47	CCTGGTAACTGTAGGGGCCTTTTTAGCTAGCAAGTGA		
A_64_P063913	0.039	Up	2.43	CTGCTGTGCCCTTGAGTGCTGCCAGTGCAAGATATGA		
A_64_P095642	0.028	Up	2.63	TGCTGCAAACCCTGCTGCTGTCAGTCCAGCTGCTGCA		
A_44_P636423	0.028	Up	2.66	TGTTGCAAGCCCTGCTGCTGTCAGTCCAGCTGTTGTA		
A_64_P061524	0.020	Down	1.51	Prpg1	NM_172064	Proline-rich proteoglycan 1
A_64_P096589	0.014	Down	1.56	RGD1564482	NR_036617	RGD1564482
A_64_P117433	0.007	Down	1.51	Siglec10	NM_001106250	Sialic acid binding Ig-like lectin 10
E2						
Probe Name	***P-value***	**Regulation**	**FC (abs)**	**Gene Symbol**	**GenBank Accession**	**Gene Name**
A_64_P108389	0.021	Up	1.73	Plekhh1	NM_001108036	pleckstrin homology domain containing, family H (with MyTH4 domain) member 1
A_44_P288015	0.003	Up	2.10	Mrps10	NM_001008859	mitochondrial ribosomal protein S10
A_64_P086284	0.043	Up	2.30	Rptn	XM_001054767	repetin
A_42_P536702	0.016	Down	1.62	Sftpc	NM_017342	surfactant protein C