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Molecular skin changes

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Abstract

Deliverable 6.2 is related to the evaluation of the skin immune-response and nociceptors activation after exposure to 5G frequency during the neonatal age.

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1 Introduction

The skin, as the body's largest organ, serves not only as a physical barrier but also as a dynamic, immune-responsive tissue that must constantly adapt to environmental stimuli, infections, and injuries. The complex interplay between neuromediators, high-affinity receptors, and regulatory proteases is central to maintaining this dynamic balance, ensuring both tissue integrity and proper regulation of inflammatory responses. The inflammatory process involves a complex interplay of immune cells, cytokines, chemokines and growth factors, which can ultimately drive genetic and epigenetic changes in skin cells, fostering a malignant transformation. Nociceptors and inflammation are closely linked, as nociceptors not only detect harmful stimuli but also play a key role in amplifying and perpetuating the inflammatory response. Inflammation can sensitize nociceptors, making them more responsive to stimuli, and in turn, nociceptor activation can enhance inflammatory processes.

In this context, understanding how 5G (FR2 27.5 GHz) exposure may act as a driver or modulator of the inflammatory skin response is crucial, given the widespread deployment of this technology. Evaluating the effects of the exposure during the neonatal age is critical for several reasons, because the neonatal period represents a time of significant physiological and developmental changes. The skin, nervous system, and immune system are highly dynamic during this early life stage, making neonates potentially more susceptible to external influences, including environmental stressors like 5G exposure.

2 Mouse exposure

To investigate the potential impact of 5G exposure on the skin's immune response and the activation of nociceptors, we exposed neonatal *Ptch1*^{+/-}/CD1 mice (n=71) and their wild-type (WT; n=67) counterparts (distributed between males and females in each experimental group) to whole-body exposure at 27.5 GHz for 23 hours per day, with 1 hour of stop dedicated to standard housing operations. All animals were exposed from postnatal (P) day 1 to P21, covering the period during which the first complete postnatal hair follicle cycle is completed. The 27.5 GHz frequency was delivered at two different incident power densities, i.e., 6.67 W/m² and 20 W/m², named Low-Power Density (LPD) and High-Power Density (HPD) experimental groups, respectively. A control group of age-matched SHAM-exposed mice was used for comparison housed under the same conditions. Note that the exposures were not conducted using blinded conditions, however, all the animals and samples were blinded prior to pathology and analysis.

3 Material and Methods

After exposure, skin samples were collected and either preserved in formalin-fixed paraffin-embedded (FFPE) blocks or snap-frozen for molecular analysis. To assess the skin immune-response, RNA obtained from snap-frozen skin dorsal samples was used to carry out a RT² Profiler

PCR Array (Gene GlobeID: PAMM-011Z, Qiagen Hilden, Germany). With this approach, the expression of 84 key genes central to the inflammatory processes (coding for cytokines, chemokines, interleukins, growth factors and small signalling proteins primarily secreted by immune cells that mediate inter- and intracellular signaling during immune responses) were analyzed. We performed RT² Profiler PCR Array on 3 pools of 3 animal each (biological replicates) for each sex and the statistical analysis of gene expression modulations was carried out with GeneGlobe's complimentary web-based analysis pipelines of Qiagen. Additionally, we analysed the activation of nociceptive neurons by measuring the gene expression levels of the *MrgprD* gene, a specific marker for a subpopulation of sensory neurons that exclusively innervate the epidermis, involved in the perception of itching and cutaneous pain and stress. Increased expression of *MrgprD* is known to enhance the excitability of cutaneous nociceptors and has been associated with chronic-induced neuropathic pain models. Finally, histological examinations of dorsal skin of exposed mice were carried out, and immunohistochemistry has been performed to validate the molecular findings.

4 Results: Skin immune-response

During the exposure, no significant changes in welfare or signs of distress and/or illness were observed in any of the exposed mice over the 21-days treatment period. At the end of the exposure, no significant differences in body weight were found between the SHAM, LPD, and HPD groups in both *Ptch1*^{+/-} and WT mice. Based on the evidence described above, and in the absence of significant differences between the two analysed genotypes, we proceeded with the molecular analysis of the skin, focusing solely on *Ptch1*^{+/-} mice. This decision was made with the consideration that this genotype could be more susceptible to the potential effects of 5G FR2 exposure. The results obtained from gene expression profiling, using targeted qPCR arrays on 84 key genes central to the inflammatory process, reveal distinct patterns of inflammatory gene expression between the two sexes. The heatmaps shown in Figure 1 illustrate the magnitude of gene expression, with colours ranging from green to red, in the comparison between the LPD and HPD experimental groups versus the SHAM group.

In detail, in *Ptch1*^{+/-} females, the RT² Profiler PCR Array revealed that out of 84 genes, 13 were significantly modulated, 5 of which were modulated both at low (LPD) and high-power density (HPD) and the other 8 genes were deregulated only at HPD. In *Ptch1*^{+/-} males, out of 84 genes, 21 were significantly modulated, 5 of which were modulated both in LPD and in HPD groups and the other 16 genes were deregulated only at HPD. For the selection of the most interesting genes that we found to be modulated in response to 5G FR2 exposure, we defined a threshold of 2-fold change and a *p* value ≤ 0.05. Therefore, we focused our attention on the inflammatory genes that were altered at both power densities and common to both sexes: *Ccl4*, *Cfs2* and *Tnfsf11*. The validation by qPCR was carried out both in *Ptch1*^{+/-} (n=71; 21 Sham, 25 LPD and 25 HPD distributed between two sexes) and was extended also in WT mice (3 animals for each experimental group).

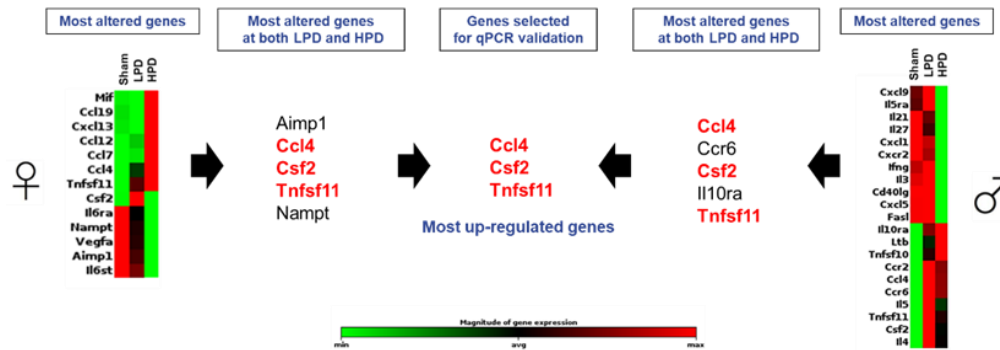


Figure 1. Heatmaps of gene expression of inflammatory genes deregulated in the comparison between LPD and HPD versus SHAM in *Ptch1*^{+/-} female and male mice, along with the pipeline used for selecting the most relevant altered genes for further validation.

According with data derived from array analysis, *Ccl4*, *Csf2* and *Tnfsf11* gene expression levels significantly increased both in *Ptch1*^{+/-} and WT mice, regardless of sexes and delivered power densities (Figure 2).

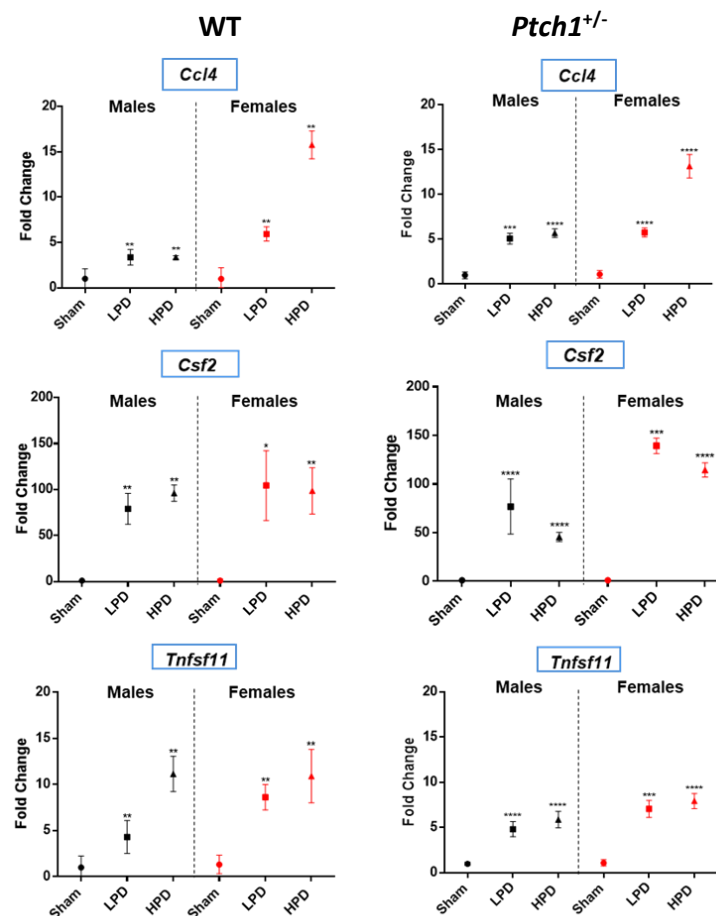


Figure 2. qPCR gene expression analysis of *Ccl4*, *Csf2* and *Tnfsf11* in *Ptch1*^{+/-} and WT mice skin. Results are expressed as fold-change of gene expression (mean ± SEM) in the comparison between LPD and HPD versus SHAM group. Mann-Whitney U test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Overall, molecular analysis carried out after exposure of mice at 27.5 GHz during the neonatal age showed a significant deregulation of the expression of genes involved in the inflammatory process, at both delivered power densities, revealing differences between males and females, regardless of genotype.

5 Results: Nociceptors activation

To address the second biological endpoint, we performed a gene expression analysis of *MrgprD* gene that codes for a receptor called "mas-related G protein-coupled receptor D" (*MrgprD*). This receptor is specifically associated with superficial skin nociceptors, involved in the perception of itching and cutaneous pain or stress. qPCR was conducted on dorsal skin samples of both *Ptch1*^{+/-} (n=71; 21 Sham, 25 LPD and 25 HPD distributed between two sexes) and WT (n=67; 25 Sham, 22 LPD and 20 HPD distributed between two sexes) mice. We observed a significant decrease of skin *MrgprD* gene expression level in WT group (Figure 3). In *Ptch1*^{+/-} mice, a similar trend was observed (bordering on statistical significance). Overall, no significant differences emerged in *MrgprD* gene expression level between male and female mice.

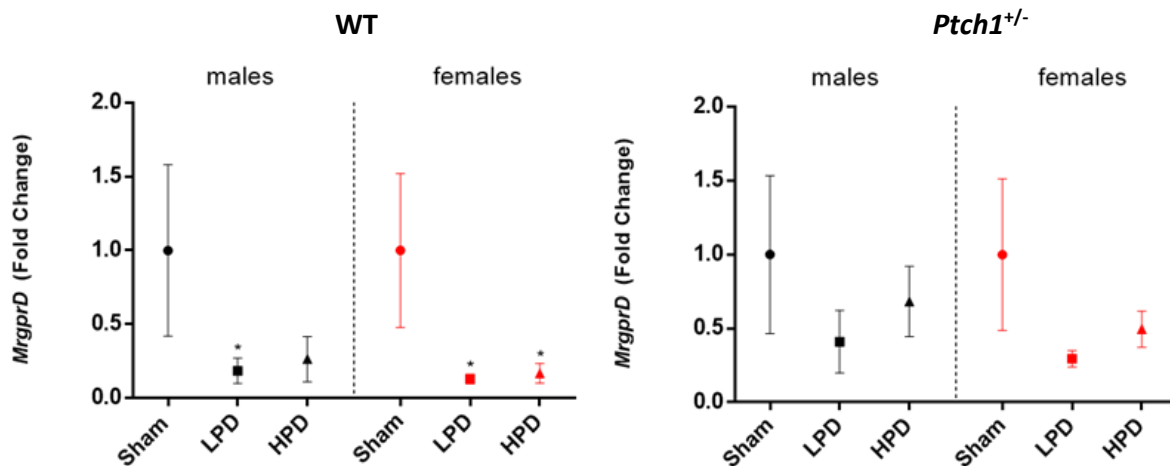


Figure 3. qPCR gene expression analysis of *MrgprD* in *Ptch1*^{+/-} and WT mice skin. Results are expressed as fold- change of gene expression (mean $\pm$ SEM) in the comparison between LPD and HPD versus SHAM group. Mann-Whitney U test, * $p < 0.05$.

Our results indicate that neonatal exposure to 5G (FR2 27.5 GHz) signals induced in the skin a down regulation of *MrgprD* gene expression. According to literature (Gour, N., & Dong, X. 2024; *The MRGPR family of receptors in immunity. Immunity*, 57(1), 28–39. <https://doi.org/10.1016/j.immuni.2023.12.012>), *MrgprD*⁺ nociceptors, acting as negative regulator of mast cell activity, control the skin homeostasis under physiological conditions. Our data confirm this relationship (decrease of *MrgprD* expression level and up-regulation of mast cell activation mediators: *Il4*, *Il13*, *Csf2*, Figure 1).

6 Conclusions

Overall, the molecular examination of the skin from animals exposed to 27.5 GHz during the neonatal period, regardless of genotype, reveals the activation of inflammatory processes, which are also positively influenced by the reduced activation of cutaneous nociceptors. It remains to be determined whether longer exposures (e.g., 100 days and 6 months; Task 6.4) may elicit this result or if the skin's inflammatory response is strictly age-dependent, with the neonatal period being particularly sensitive to external stressors.