Identification of vestibular organ-associated mRNA changes by acceleration stimulation in mice

Lee, Yujin

Department of Medicine
Graduate School
Yonsei University

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Advisor Kim, Sung Huhn

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This certifies that the Master's Thesis of Lee, Yujin is approved

Committee Chair **Kim, Yoon Jung**

Committee Member **Kim, Sung Huhn**

Committee Member **Lee, Hyun Jin**

Department of Medicine
Graduate School
Yonsei University
June 2025

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Last but not least, I dedicate this work to the most important person in my life, Legal Advocate Jaewook Jeon, whose boundless encouragement—along with his invaluable help with daily chores such as cleaning and laundry—made this thesis possible.

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ABSTRACT

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The vestibular organs of the inner ear play a critical role in maintaining body balance by detecting linear and angular acceleration and transmitting these signals to the central nervous system. However, there have been few attempts in inner ear research to investigate the state of the vestibular system in response to acceleration stimuli using RNA sequencing. In this study, we applied a 4G hypergravity stimulus to a mouse model under three different conditions: 48 hours, 2 weeks, and 2 weeks followed by a 1-week recovery period, and analyzed gene expression changes in the vestibular organs through RNA sequencing.

As a result, approximately 4,200 differentially expressed genes (DEGs) were identified and classified into three expression patterns. The 48-hour group showed gene expression patterns largely similar to the control group, whereas the 2-week group exhibited significant transcriptional changes. Notably, some of these changes persisted even after the 1-week recovery period.

Gene ontology analysis revealed that genes associated with calcium signaling and synaptic transmission were downregulated after long-term hypergravity exposure, while genes related to inflammatory responses, such as interferon-beta signaling, were upregulated. These findings suggest the presence of a persistent inflammatory response in the vestibular system.

In conclusion, the results indicate that 2-week hypergravity stimulation induces transcriptomic alterations in signal transmission and immune pathways within the vestibular system. This study may contribute to understanding the molecular mechanisms of vestibular dysfunction and offer insights into potential therapeutic approaches.

Key words : Vestibular organ, Acceleration stimulation, RNA sequencing

1. Introduction

The vestibular organ in the inner ear plays a critical role in maintaining the body balance by detecting linear and angular accelerations and transmitting these signals to the central nervous system [1]. Acceleration stimuli are detected by vestibular hair cells located in the macula of the saccule and utricle, as well as in the ampulla of the semicircular canals (SCC). Various ion channels, transporters, and exchangers distributed in these hair cells and surrounding epithelial cells are essential for signal transmission.

The vestibular system is plastic [2, 3] thus essential for adaptation to gravitational changes. Several studies have demonstrated that hypergravity can affect the vestibular system at the protein and functional levels. Iijima et al. reported that exposure to a 2G hypergravity environment led to upregulation of CREB and syntaxin proteins in the inner ear, suggesting that hypergravity may influence neurotransmission and synaptic plasticity in the vestibular organs [4]. Furthermore, a study using rotarod test showed that rats exposed to 2G for two weeks exhibited decreased cooperative locomotor ability, indicating impaired motor coordination, which are partly dependent on vestibular function [5]. Similarly, after 4 weeks of 2G exposure, mice required a longer duration to recover upright posture in righting reflex tests, suggesting reduced vestibular responsiveness [6]. Histological changes have also been observed. 2.5G exposure for 9 months resulted in morphological changes in hair cells and supporting cells of the vestibular sensory epithelium [7]. These studies indicate that hypergravity leads to changes at the protein, tissue, and functional levels within the vestibular system.

However, to date, there have been few attempts in the inner ear research to evaluate the molecular changes of the vestibular system in response to acceleration stimuli. In this study, we aim to analyze RNA sequencing data from mouse models subjected to hypergravity comparing to those that were not, by which we can investigate the differences in gene expression levels depending on the duration of the hypergravity challenge. Furthermore, based on this analysis, we can identify the genes and proteins that play critical roles in maintaining the human body balance against gravity changes or constant linear acceleration through the vestibular system. This research may provide insights into the potential application of these findings for the treatment and prevention of balance disorders as well as aerospace medicine.

2. Methods

2.1. Animals

Male C57BL/6 mice (Wild type, 8 weeks old, 18g) were purchased from KOATEC (n = 80) and housed under conditions equipped with a lighting automatically adjusted day-night cycle, a temperature control system, and equipment capable of monitoring vital signs. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei university (Approval No.2023-0171) and were conducted in accordance with the guidelines of the National Institutes of Health (NIH) and the Korean Animal Protection Act.

2.2. Experimental design

The study comprised four experimental groups: the control group, the 48-hour (48hrs) group, the 2-week (2wks) group, and the 2-week with a 1-week recovery (2wks–1wk) group. The control group underwent microdissection for vestibular organ extraction without acceleration stimuli, followed by RNA sequencing. The other three groups were exposed to the hypergravity acceleration (4G) stimulus but for varying durations: 48 hours, 14 days, and 14 days with an additional 7-day recovery period. RNA sequencing was then conducted for all groups, and the data were compared and analyzed.

Each group consisted of four mice, requiring a total of 16 mice per experimental cycle. Additionally, as a minimum of 500 ng of qualified RNA is necessary for a single bulk RNA sequencing experiment, the 3 – 4 biological replicates were included in each group. Therefore, at least 48 mice were required.

2.3. Hypergravity stimulation

Two mice were placed in each chamber of the accelerator and applied to 4G stimulation for 48 hours and 14 days. Hypergravity stimulation was applied using a custom-made centrifuge system equipped with a rotating arm, as previous study [8]. The motor controller was operated with a custom default program that provided predefined settings for rotational speed and duration, enabling consistent delivery of 4G stimulation. During rotation, the floor of each animal cage was

oriented perpendicular to the direction of centrifugal force, ensuring that hypergravity was applied vertically to the bodies. Mice had continuous access to food and water in each chamber during the stimulation. Room temperature was maintained at 24 ± 1 °C.

2.4. Microdissection

For each group, the vestibular organs of the inner ear were extracted within 1 hour after 4G stimulation. Mice were placed in a transparent chamber, and CO2 gas was maintained for 2 minutes to confirm respiratory and cardiac arrest. The euthanasia procedure was performed in accordance with the 2020 AVMA Guidelines for the Euthanasia of Animals. Following euthanasia in a CO2 chamber, the temporal bones of the mice were collected. Under a microscope, the membranous vestibular labyrinth—including the utricle, saccule, and semicircular canals—was carefully dissected using microdissection techniques.

2.5. RNA sequencing

A total of 13 samples from four experimental groups were used for RNA sequencing analysis. Dissected vestibular tissues were collected into Eppendorf tubes containing TRIzol reagent (Thermo Fisher Scientific) to prevent RNA degradation and were rapidly frozen in isopentane on dry ice, then stored at –80 °C. The stored samples were submitted to Macrogen Inc. (Seoul, Korea), where RNA extraction and RNA sequencing were performed. Total RNA was extracted and quantified using the RNA RiboGreen Assay Kit (Thermo Fisher Scientific). RNA integrity was assessed on the TapeStation 4200 system (Agilent Technologies) with High Sensitivity RNA ScreenTape, and samples with a RINe $\geq$ 7.0 were selected for library preparation. Sequencing libraries were constructed using a poly(A) selection protocol and sequenced on an Illumina platform to produce 150 bp paired-end reads. Raw reads were aligned to the Mus musculus reference genome (mm10) using HISAT2 (v2.1.0). Gene annotation was based on the NCBI_108. Transcript assembly and gene-level read count estimation were performed using StringTie (v2.1.3b).

Differential expression analysis was performed using the DESeq2 R package (v1.32.0). Raw read counts were normalized using the Relative Log Expression (RLE) method. Variance stabilizing transformation (VST) was applied to the normalized data for visualization and

clustering analyses, including correlation heatmap, principal component analysis (PCA). Among the 45,777 genes initially detected, those with a raw count of zero in any of the 13 samples were excluded, resulting in 20,139 genes retained for downstream analysis. Differentially expressed genes (DEGs) were defined as those with a $|\log_2$ fold change$| > 2$ and an adjusted p-value < 0.05.

3. Results

3.1. Overall gene expression similarity across groups

Based on RNA sequencing data, we analyzed the similarities and differences in gene expression profiles among the experimental groups. First, normalized values were used to assess correlations among the experimental groups (Figure 1A). Since the data did not follow a normal distribution, Spearman's rank correlation method was applied. The correlation coefficient between the control group and the 48-hour group was positive and close to 1, indicating that the gene expression patterns between these two groups were highly similar. Likewise, the 2wks group and the 2wks-1wk group also showed high similarity in their data. In contrast, the 2wks and 2wks-1wk groups displayed noticeable differences compared to the control and 48hrs groups. Additionally, correlation analysis within each group among three or four samples demonstrated strong consistency, further confirming the reliability of the data.

Principal component analysis (PCA) was performed, and the positions of each sample were visualized in a two-dimensional space using the first two principal components (Figure 1B). The closer the points are, the more similar the data, indicating group-wise clustering. Consistent with the findings in Figure 1A, samples within the same group exhibited similar distributions. Furthermore, the data from the Control and 48hrs groups were closely related, while the 2wks and 2wks-1wk groups also showed a high degree of similarity. These results suggest that short-term stimulation over 48 hours does not lead to significant changes in gene expression, whereas long-term stimulation over two weeks induces noticeable alterations.

Differential expression analysis was performed to compare the expression levels of 20,139 genes between the two groups, and the distribution of DEGs was analyzed (Figure 1C). The DEG distribution further confirmed that the Control and 48hrs groups exhibited similar patterns, and the

2wks and 2wks-1wk groups also showed a comparable trend.

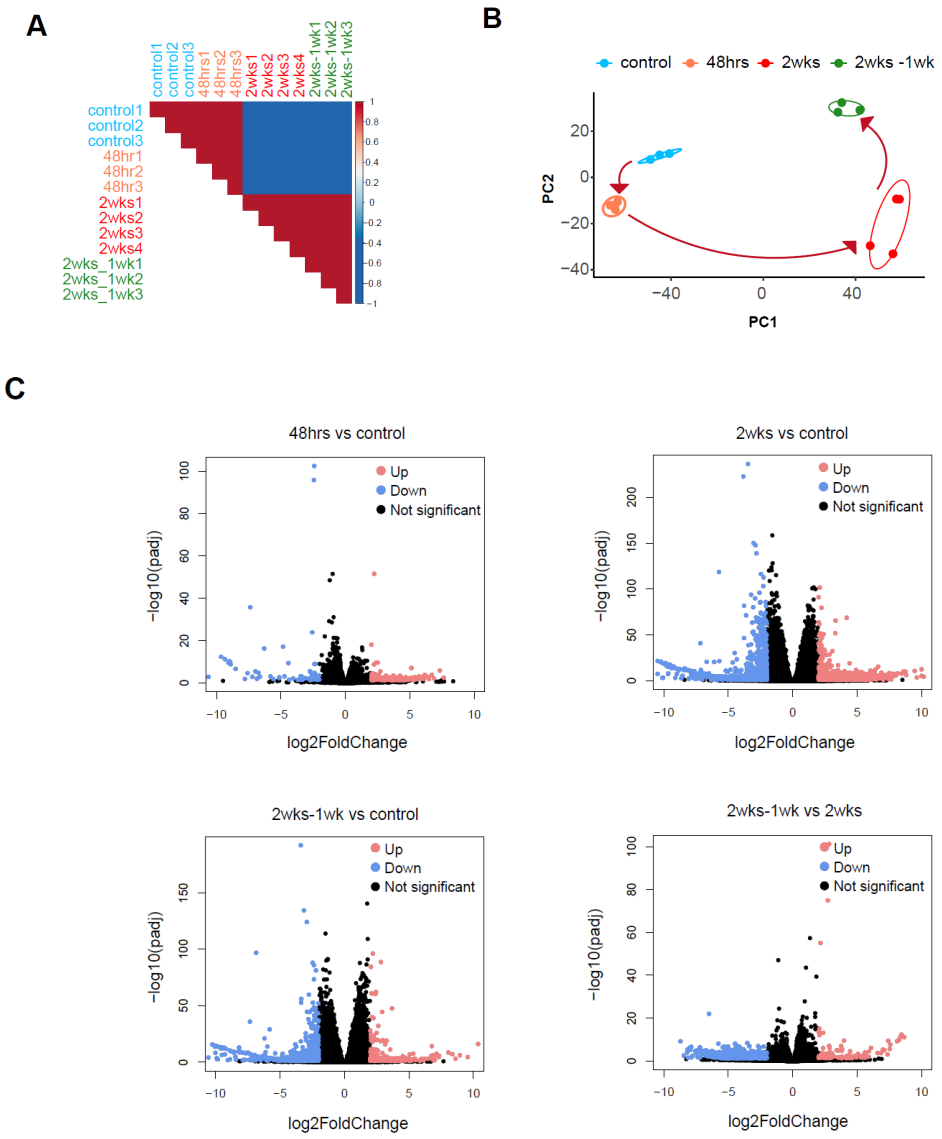

Figure 1. Correlation, Principal Component, and DEG Distribution Analyses. A. Correlation heatmap. The correlation coefficients are represented by colors on the right. Since the data did not follow a normal distribution, the Spearman method was used to calculate the correlations. **B.** The extracted genes were analyzed using principal component analysis (PCA), and the positions of each sample were visualized in two-dimensional space using the first two principal components. **C.** This Volcano plot visualizes the distribution of differentially expressed genes (DEGs) based on statistical

significance and expression changes. The x-axis represents the log2 fold change, indicating the relative difference in gene expression between the two compared groups. The y-axis represents the –log10 of the adjusted p-value, where higher values indicate greater statistical significance. DEGs were defined using the criteria of adjusted p-value < 0.05 and |log2 fold change| > 2, and they are highlighted in color accordingly.

3.2. Clustered DEG patterns and associated biological responses

As highlighted above, approximately 4,200 DEGs were identified and transformed into Z-scores through scaling to generate a heatmap (Figure 2A). This allowed for a comparative analysis of all DEGs, enabling clustering based on similar expression patterns, which were categorized into three groups: Pattern 1, Pattern 2, and Pattern 3. A comparison between the control and 48hrs groups revealed largely similar gene expression patterns. However, unlike the 48hrs group, the 2wks group exhibited distinct alterations in gene expression patterns compared to the control group. Notably, some genes did not return to their original expression levels even after a 1-week recovery period. These findings indicate that long-term exposure to hypergravity modulates gene expression in the vestibular system, with some changes persisting beyond the recovery period.

Gene ontology (GO) enrichment analysis was performed to identify the biological processes associated with the genes in each of the three patterns derived from the heatmap. The results were ranked in descending order based on statistical significance (Figure 2B).

Pattern-1 consists of 1,960 genes that are relatively highly expressed in the control and 48hrs groups but exhibit a marked decrease following 2wks of hypergravity exposure. This reduced expression persists even after the 1-week recovery period. As shown in Figure 2B, many of these genes are associated with biological processes such as Cellular response to calcium ion and Response to calcium ion. This suggests that while short-term hypergravity exposure may enhance calcium signaling, prolonged exposure likely suppresses the expression of genes involved in calcium signaling and neural activity. These findings imply that long-term hypergravity exposure diminishes signal transmission within the vestibular system and given that this suppression persists despite the recovery period, it may contribute to functional impairments such as postural instability. Among the genes related to the calcium ion response, *Trpm2* (a calcium channel subfamily member) and *Stx1b* (syntaxin 1B, protein involved in synaptic vesicle fusion) were identified. Previous studies have also reported that these genes are upregulated following short-term hypergravity exposure, further supporting this observation [4, 9].

In Pattern-2, 1,687 DEGs were identified, showing an increase in expression after 2 weeks followed by a decrease in the recovery group. Among these, genes associated with the *arachidonic acid metabolic process* and the *eicosanoid metabolic process* were significantly upregulated, suggesting the activation of inflammatory responses [10, 11]. Through these pathways, the production of bioactive lipid mediators such as prostaglandins (PGs), leukotrienes (LTs), and thromboxanes (TXs) is increased, which in turn induces inflammation. These findings imply that the 2-week stimulation triggered an acute inflammatory state at the molecular level.

Pattern-3 consists of 571 genes that exhibit increased expression in the 2wks and 2wks-1wk groups compared to the control. As indicated by the associated biological processes, such as *Response to interferon-beta* and *Cellular response to interferon-beta*, prolonged exposure to hypergravity appears to activate immune or inflammatory responses. Interestingly, in contrast to Pattern 2 where inflammatory gene expression decreased during the recovery phase, immune-related pathways such as interferon-beta response remained upregulated in Pattern 3, suggesting that the inflammatory response was not fully resolved. This persistent immune activation may reflect a shift toward a chronic or sustained inflammatory state, possibly contributing to prolonged tissue remodeling or immune surveillance. These findings suggest that long-term hypergravity exposure induces stress within the vestibular system, leading to a persistent inflammatory response that does not subside even after the recovery period, potentially contributing to chronic inflammation.

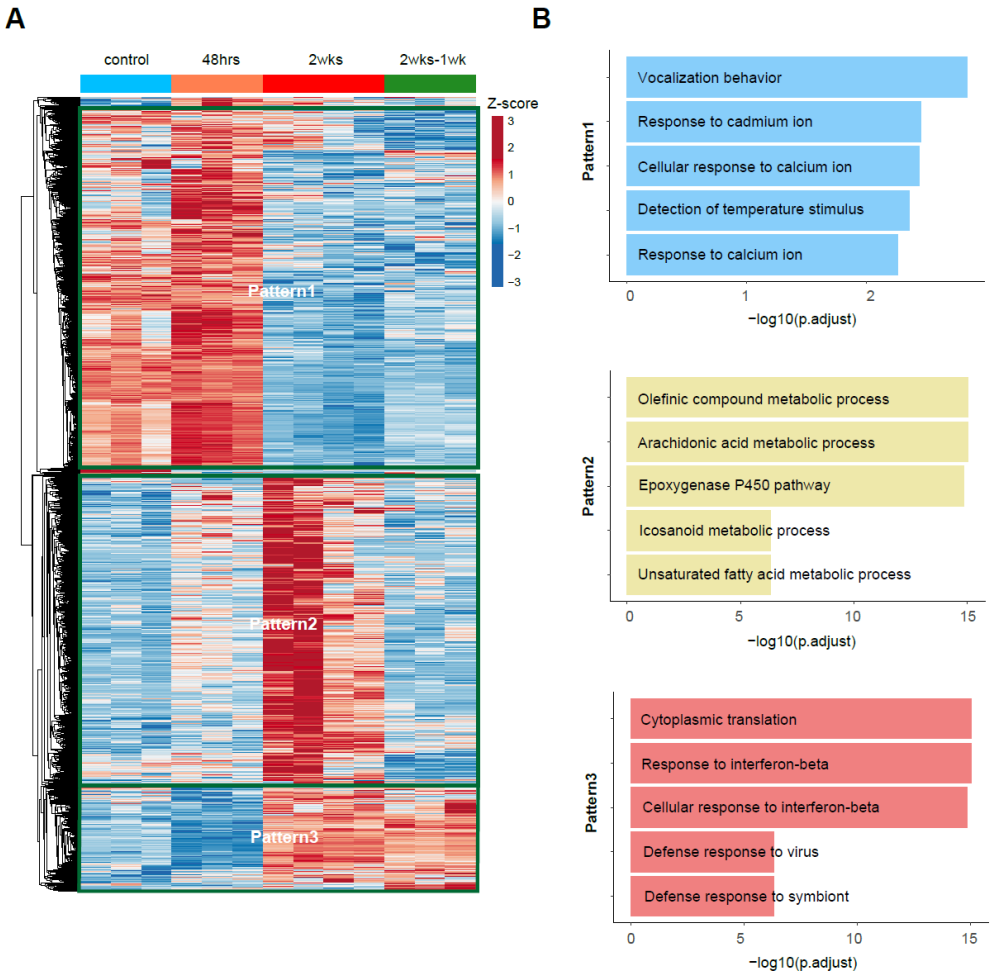

Figure 2. Heatmap-Based DEG Clustering and GO Analysis. A. Heatmap of DEGs across different time points (control, 48 hours, 2 weeks, and 2 weeks-1 week). Genes are clustered into three distinct expression patterns (Pattern1, Pattern2, and Pattern3) based on their Z-score. **B.** Gene Ontology (GO) enrichment analysis of each pattern. The x-axis represents the -log10(p.adjust) values indicating the statistical significance of the enriched terms.

3.3. Suppression of stereocilia maintenance genes following prolonged stimulation

To further investigate the biological relevance of the identified DEGs, we examined genes overlapping with hearing loss-related genes curated in the Mouse Genome Informatics (MGI)

database (The Jackson Laboratory). A total of 102 overlapping genes were identified and categorized into seven functional groups, including cochlear ion homeostasis, hair bundle development and functioning, among others. The scatter plots in Figure 3 visualize the normalized expression values of these 102 genes across different experimental groups. Genes positioned close to the diagonal indicate little to no difference in expression between the compared groups. In the comparison between the control and 48hrs group, most genes located near the diagonal, suggesting minimal gene expression changes following short-term hypergravity exposure (Figure 3A). In contrast, the comparison between the control and 2wks group revealed several genes with expression levels deviating significantly from the diagonal (Figure 3B). *Myo15, Loxhd1*, and *Strc* were among the genes that were downregulated by more than 2 on the $\log_2$ scale in the 2wks group compared to the control, all of which are known to be involved in hair bundle development and function. These findings, together with the heatmap and GO enrichment results, suggest that prolonged hypergravity exposure may suppress genes required for maintaining stereocilia morphology in hair cells, potentially as a secondary effect of increased inflammatory responses.

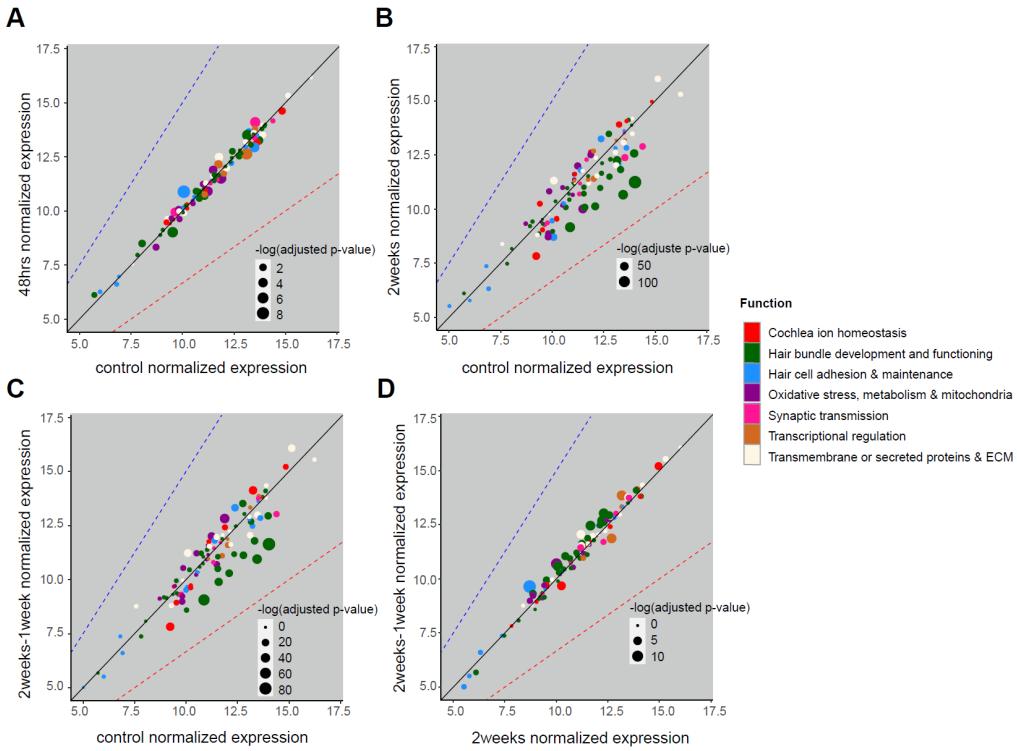

Figure 3. Scatter Plot Visualization. Scatter plot comparing gene expression levels across experimental conditions. The x-axis represents gene expression in the control group, while the y-axis represents gene expression in the experimental groups (48hrs, 2wks, and 2wks-1wk). Genes near the diagonal indicate minimal expression changes, whereas those deviating from the diagonal represent significantly altered expression patterns.

4. Discussion

This study analyzed the effects of 4G hypergravity-induced acceleration stimuli on gene expression in the vestibular system using RNA sequencing. Long-term exposure for 2 weeks led to substantial alterations in gene expression patterns. Notably, some genes did not return to their

original expression levels even after a 1-week recovery period, suggesting that prolonged hypergravity exposure may have chronic effects on vestibular function.

Prolonged hypergravity exposure induces a persistent inflammatory response in the vestibular system. GO enrichment analysis revealed a significant upregulation of inflammatory pathways, including the arachidonic acid metabolic process and eicosanoid metabolic process, which are known to generate pro-inflammatory lipid mediators such as prostaglandins and leukotrienes. Although some of these inflammatory genes returned to baseline during the recovery phase, other immune-related genes—particularly those involved in interferon-beta response and classified under Pattern 3—remained elevated even after recovery, suggesting sustained immune activation and possible chronic inflammation. Consistent with this, previous studies have demonstrated that alterations in gravitational environments may cause oxidative stress and inflammatory responses [9, 12]. Considering the previous report of hair cell morphological changes [7], which demonstrated a significant reduction in the apical surface area and cellular density of hair cells, along with the scatter plot results of this study, it is reasonable to suggest that chronic inflammation induced by hypergravity may contribute to structural damage in vestibular hair cells and ultimately lead to long-term balance disorders.

At the early phase of hypergravity exposure, genes associated with calcium signaling and synaptic activity, such as *Trpm2* and *Stx1b*, were upregulated. However, as the exposure continued, their expression levels declined markedly. This suggests that hypergravity initially induced transient increases in calcium influx and neuronal activity in the vestibular system. However, continued stimulation may impose mechanical acceleration stress on hair cells, leading to excessive depolarization and potentially triggering excitotoxicity or related cellular responses [13, 14]. This may have led to the activation of inflammatory mediators, potentially contributing to long-term impairment or structural damage in vestibular hair cells. Consistently, genes involved in calcium signaling and synaptic function also showed sustained downregulation, indicating prolonged disruption of neural transmission.

Upon analysis of gene expression patterns, *Fos, Jun*, and *Il4* were upregulated in the early phase in Pattern 1. *Fos* and *Jun* form the AP-1 transcription factor complex and are well known as early markers of acute stress responses [15, 16]. This may imply that the initial excessive Ca^{2+} signaling stimulation may have triggered an inflammatory response. In Pattern 2, inflammatory mediator genes such as *Il6*, *Lcn2*, *Mmp3*, and *Mpo* were significantly upregulated, reflecting

responses associated with acute phase inflammation and oxidative stress [17]. Furthermore, in Pattern 3, genes such as *Mmp9*, *Acp5*, and *Cybb* remained upregulated even during the recovery phase. This indicates that oxidative stress and chronic inflammatory responses persisted beyond the period of hypergravity exposure [18, 19] . These findings suggest that the initial Ca^{2+} overload induced by hypergravity exposure may have triggered a sequential transcription leading to inflammation and oxidative damage, ultimately resulting in long-term structural impairment of hair cells within the vestibular system.

However, unlike this study, a previous report [20] demonstrated that gene expression changes and balance impairments induced by hypergravity were fully restored after a one-week recovery period. This discrepancy may stem from differences in experimental parameters, including the intensity (2G vs. 4G) and duration (7 days vs. 14 days) of hypergravity exposure. These factors likely influence the degree of cellular stress and the capacity for molecular recovery, potentially explaining why some gene expression changes in the present study persisted even after the recovery phase. In our study, the sustained downregulation of synaptic transmission-related genes, despite the recovery period, suggests that long-term hypergravity exposure may induce irreversible modifications in calcium signaling pathways, potentially affecting neural plasticity and vestibular adaptation. Or, it is possible that even if irreversible changes occurred at the peripheral level, such as in the vestibular end organs, they may not have resulted in a complete functional loss. Consequently, central vestibular compensation could have preserved balance function, explaining the lack of observable dysfunction in previous studies.

This study is among the first to analyze gene expression changes in the vestibular system under hypergravity conditions using RNA sequencing. These findings demonstrate that long-term hypergravity exposure leads to the suppression of calcium signaling and synaptic transmission, sustained activation of inflammatory responses, and the potential long-term persistence of these changes. However, further validation at the protein level (e.g., immunohistochemistry, Western blot) is necessary to confirm the RNA sequencing results. In addition, future studies should assess whether the observed molecular alterations result in functional impairments by evaluating vestibulo-ocular reflexes (VOR) or visually evoked potentials (VEPs) in each group following hypergravity stimulation. This would allow for the evaluation of central nervous system adaptation or compensation. Additionally, direct observation of hair bundle structural changes through electron microscopy could provide further insight into the morphological effects of hypergravity.

Beyond vestibular disorder research, analyzing the adaptation process and underlying mechanisms of the vestibular system in hypergravity environments holds significant implications for space medicine, aerospace physiology, and broader gravitational biology studies.

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Abstract in Korean

과중력 가속도 자극 후 마우스 내이 전정기관 유전자 발현의 변화연구

내이의 전정기관은 선형 및 각가속도를 감지하여 그 신호를 중추신경계로 전달, 인체의 평형을 유지하는데 중요한 역할을 하고 있다. 그러나 현재 내이 연구에 있어서 가속도 자극을 가했을 때, 전정계의 상태를 RNA 시퀀싱을 이용하여 규명하려는 시도는 거의 없다. 이에 본 연구에서는 8주령 수컷 C57BL/6 마우스를 대상으로 4G 과중력 가속도 자극을 마우스모델에 각각 48시간, 2주, 2주 자극 후 1주 회복의 조건으로 적용한 뒤, 전정기관에서의 유전자 발현 변화를 RNA 시퀀싱을 통해 분석하였다.

총 20,139개의 유전자 중 약 4,200개의 유전자에서 유의미한 발현 변화가 관찰되었으며, 특히 2주 이상 장기간 자극을 받은 그룹에서 전정기관의 유전자 발현 패턴에 뚜렷한 변화가 확인되었다. 유전자 군집 분석을 통해 세 가지 주요 패턴을 확인하였으며, 그 중 Pattern-1은 칼슘 신호전달 및 시냅스 기능 관련 유전자들이 장기 자극 후 억제됨을 나타냈다. Pattern-2와 Pattern-3은 각각 급성 및 만성 염증 반응과 관련된 유전자 발현 증가를 특징으로 하였으며, 일부 면역 관련 유전자는 회복기 이후에도 지속적으로 활성화되어 만성 염증 상태로의 이행 가능성을 시사하였다.

또한, 청력 관련 유전자 중 stereocilia 유지에 관여하는 유전자들이 장기 자극 후 현저히 감소하였으며, 이는 구조적 손상 및 기능 저하로 이어질 수 있음을 보여준다.

본 연구는 2주간의 과중력 자극이 전정기관의 신호전달 및 면역반응 경로에 있어서 전사체 수준의 변화를 유도함을 보여주며, 향후 전정기능 장애의 기전을 이해하고 치료방안을 제시하는데 기여할 수 있을 것으로 기대된다.

핵심되는 말 : 전정기관, 과중력 자극, RNA 시퀀싱